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Sexual Behavior in Ovariectomized and Seasonally Anovulatory Pony Mares (*Equus caballus*)

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Ten ovariectomized (OVEX) and ten intact, but seasonally anovulatory (ANOV), pony mares were observed for sexual activity with five stallions, using a "harem group" social testing paradigm (two OVEX and two ANOV mares plus one stallion per group) for 15 consecutive daily tests lasting 20 min each. All mares in both conditions showed proceptive behavior in at least one test, all mares but one were mounted, and 14 of 20 mares received ejaculations. No statistical differences were found between the two conditions for any measure of proceptivity, copulatory activity, or days in estrus. The quality of estrus was judged to be equivalent to that displayed by periovulatory mares during their initial and terminal days of estrus, but less intense than that seen near ovulation. Mares in both groups were in estrus during approximately 60–70% of the tests and only 3 of the 20 mares were sexually refractory for more than five consecutive tests. Thus, the typical 2-week phase of sexual refractoriness seen in intact diestrous mares was absent in OVEX and ANOV mares, suggesting that the ovary plays a major role in actively suppressing estrous responses during the luteal phase of the cycle.

Steroids of gonadal origin have long been assumed necessary for the induction of estrous behavior in female mammals. Although Old World primates, such as the majority of rhesus and stumptail macaques, continue to show some copulatory behavior following ovariectomy (Goldfoot, Wiegand, and Scheffler, 1978; Baum, Everitt, Herbert, and Keverne, 1977), as is apparently also the case with a small percentage of rabbits (Yaschine, Mena, and Beyer, 1967), gonadectomized females of most mammalian species do not solicit coitus nor tolerate mount attempts unless administered exogenous steroids (Young, 1961; Goy and Goldfoot, 1975).

In this laboratory, estrous behavior has been noted in long-term ovariectomized mares (Garcia, published in Ginther, 1979). Estrous response has also been reported in mares whose ovaries were involuted during the nonbreeding season (Van Niekerk, 1967; Ginther, 1974; Webel, Franklin, Harland, and Dzuik, 1977). The present study was undertaken

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to investigate in more detail the sexual response of mares in which ovarian hormones are absent (ovariectomized mares) or minimal (seasonally anovulatory mares).

METHODS

Subjects

Five stallions, 10 ovariectomized (OVEX) mares, and 10 seasonally anovulatory (ANOV) mares were used. The ponies were of apparent mixed breeding and weighed between 160 and 250 kg. The stallions ranged in age from approximately 3 to 20 years and the mares from 5 to 15 years. Four of the stallions were intact and one was vasectomized. The sexual experience of the mares before ovariectomy was not known. Gonadectomy was performed 6 months to 2 years before the present project. Rectal palpation revealed no evidence of ovarian fragments in any of the OVEX mares. After the completion of behavioral testing, an OVEX mare which had shown consistently high levels of estrous behavior was sacrificed and the entire reproductive tract removed after gross inspection of the body cavity. The tract was examined and the ovarian pedicles excised for histology.

In a previous study of intact, ovulating mares (Asa, Goldfoot, and Ginther, 1979), estrous behavior was never observed in mares with follicles of less than 15 mm diameter. Day (1940) reported that diestrus was accompanied by 10- to 15-mm follicles in pony mares. Therefore, the 10 ANOV mares were assigned to the project only after rectal palpation revealed small ovaries with follicles of no more than 10 mm diameter.

Mares were pastured together outdoors and provisioned with hay. Stallions were stabled separately and fed hay and grain.

Behavioral Testing

The study was conducted for 15 consecutive days from January 9 to 23, 1978. Behavior tests were given for 2 days before the study began to familiarize the animals with the testing situation. Five harem groups, each consisting of two OVEX mares, two ANOV mares, and one stallion, were assembled daily. Assignment of animals to harems was random each day with the exception that stallions were sometimes chosen to insure that each mare was tested with each stallion at least one time. Each harem group was observed daily for 20-min periods in the early afternoon. The outdoor paddock used for testing was approximately 6 by 9.6 m, with a straw-covered concrete base.

Behavioral data were taken on an SSR event-recording keyboard (Stephenson and Roberts, 1977) which allowed rapid notation of the incidence, latency, duration, and sequence of behaviors onto magnetic tape for subsequent computer analysis. Behaviors reported included approach, follow, urination, tail raise near stallion (TRNS), walk away,

mount, intromission, and ejaculation, which are defined elsewhere (Asa, Goldfoot, and Ginther, 1979). Kick, which was not defined previously, was scored only when both hind feet were used to strike out forcefully.

A behavioral index of estrus was developed in an earlier study in which all observations were made for 1-hr periods (Asa *et al.*, 1979). That index has been modified here for 20-min tests and expanded to allow quantitative analysis of the behavior of mares in conditions less vigorous than full estrus. For this study mares were considered in full estrus when they had more than two occurrences of urination and ≥20 sec of tail raise near the stallion (TRNS) during the 20-min test. The category of weak estrus included mares with one of the following: one occurrence of urination plus ≥20 sec TRNS, two or more urinations but no TRNS, or no urination but ≥60 sec TRNS per 20-min test.

Statistical Analysis

Comparison of the OVEX and ANOV groups for the display of various proceptive and receptive measures of the mares as well as for copulatory activity of the stallions was made by *t* tests for independent samples. A paired *t* test was used to evaluate the effect of weak or full estrous conditions on the frequency of kicks or walk-aways per mount.

To investigate the possibility of changes in the expression of estrus as the study progressed, the 15 days were divided into three 5-day segments. Analysis of variance for repeated measures (Winer, 1962) was used, with means for each mare for each 5-day period used as the three repetitions. The relationship between frequencies of urination (an indicator of proceptivity) and kicks and walk-aways per mount (indicators of nonreceptivity) was calculated using the Pearson product-moment correlation and the *t* test for significance of a correlation.

RESULTS

All mares in both groups showed proceptive estrous signs on at least one of the test days, although the intensity of estrus was sometimes low. Estrus determinations for the OVEX group ranged from one mare that was only in weak estrus in one test to another that was in full estrus for all 15 tests. The OVEX group means, as shown in Table 1, were 7.7 (± 1.75 SEM) tests with full estrus and 2.9 (± 0.74 SEM) tests with weak estrus for the 15 tests. The ANOV group means were 4.8 (± 1.45 SEM) tests with full estrus and 3.9 (± 0.98 SEM) tests with weak estrus. The ANOV group had one mare that was only in weak estrus for one test, but another that was in full estrus for 14 tests.

That mares in both groups were attractive and receptive to the stallions was demonstrated by male approaches and by precopulatory investigation and stimulation similar to that displayed to periovulatory estrous mares (Asa *et al.*, 1979), as well as by the complete male ejaculatory pattern. All

TABLE 1
Comparison of Ovariectomized and Seasonally Anovulatory Mares for Mean Number of Tests with Estrus and Copulatory Activity

	Meal number of tests[a] per mare with					
	Weak estrus	Full estrus	Total	Mounts received	Intromissions received	Ejaculations received
Ovariectomized mares (n = 10)	2.9 ±0.74[b]	7.7 ±1.7	10.6 ±1.5	7.2 ±1.1	2.3 ± 0.52	1.8 ±0.47
Seasonally anovulatory mares (n = 10)	3.9 ±0.98	4.8 ±1.4	8.7 ±1.3	5.8 ±1.1	2.7 ±1.0	2.0 ±0.84

[a] Total number of tests = 15.
[b] $\bar{x}$ ± SEM.

mares, except one in the ANOV group, were mounted at least once and all but four ANOV and two OVEX mares received ejaculations. Most of the ejaculations (94.8%) occurred with full estrous mares, 5.1% with mares in weak estrus, and none with mares judged not to be in estrus.

No differences were found between the two groups of mares on any of the 15 behavioral endpoints (Tables 1, 2). The means for the two groups for incidence of estrus and selected measures of proceptivity, attractiveness, and copulatory activity are shown in Tables 1 and 2. The mean frequencies for two forms of negative behavior, walking away during a mount (OVEX 1.2 ± 0.1, ANOV 1.2 ± 0.2) or kicking at any time during a test in which a mount occurred (OVEX 3.6 ± 1.8, ANOV 3.1 ± 0.5) were not different for the two groups of mares. The proportion of kicks and

TABLE 2
Comparison of Ovariectomized and Seasonally Anovulatory Mares for Mean Frequency or Duration of Selected Measures of Proceptivity and Copulation

	Mean frequency or duration per mare per test						
	Tail raise near stallion (sec)	Female urination	Female approach plus follow	Male approach	Mount	Intromission	Ejaculation
Ovariectomized mares (n = 10)	278 ±51[a]	2.3 ±0.49	5.5 ±0.24	2.5 ±0.22	1.2 ±0.28	.26 ±0.07	.11 ±0.03
Seasonally anovulatory mares (n = 10)	209 ±57	1.7 ±0.62	3.7 ±0.52	2.4 ±0.21	1.3 ±0.34	.21 ±0.08	.13 ±0.13

[a] $\bar{x}$ ± SEM.

walk-aways was no higher for weak estrus than for full estrus in either group. The mean number of kicks, but not walk-aways, per mount was negatively correlated with the mean frequency of urination, a measure of estrous intensity, for both groups (OVEX: $r = -0.359$, $P < 0.005$; ANOV: $r = -0.423$, $P < 0.005$).

Only one mare was consistently in estrus throughout the 15 days of the study. The others displayed an irregular pattern of full, weak, or no estrus from day to day (Fig. 1). Comparison of means for estrus and copulatory activity among the first, second, and third 5-day segments of the study yielded no differences. There was a mean of 1.58 (± 0.07) mares in full estrus and 1.18 (± 0.03) in weak estrus per harem group per test (out of four mares/harem group), with medians of two in full and one in weak estrus per test. The identity of those females in estrus in each test varied from day to day.

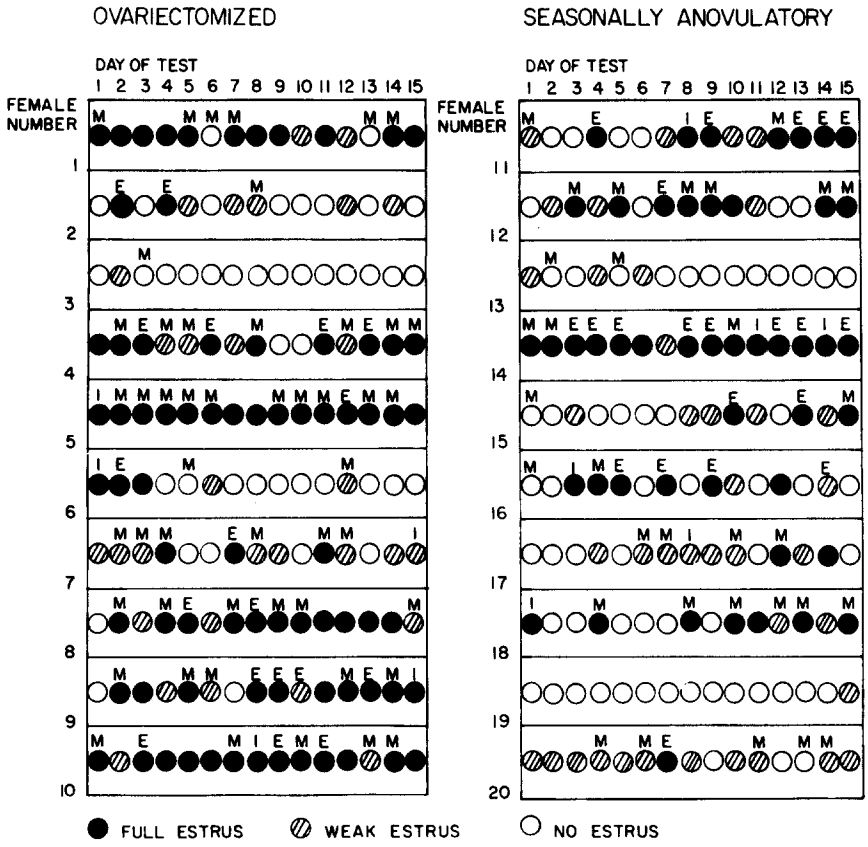


FIG. 1. Pattern of incidence of full, weak, or no estrus for 10 OVEX and 10 ANOV mares during 15 consecutive days of testing in harem groups. E, ejaculation; I, intromission; M, mount.

The five stallions displayed wide variability in copulatory performance, although mean number of mares in estrus per test was not different among stallions. In 15 days of testing, stallion 400 achieved only four ejaculations while stallion 100 had 12. The ratio of mounts per ejaculation was 3.25 for stallion 100, but 31.5 for the younger, less experienced stallion 200. The amount of time spent in precopulatory activity was reflected by latencies to first mount. These times ranged from a mean of 0.3 min for stallion 200 to 9.7 min for stallion 400. Stallion 100, the oldest, had the highest total number of ejaculations and the shortest latency to ejaculation while stallion 200, the youngest, had the most mounts and the longest ejaculation latency. For ejaculatory mounts, however, the mean interval from mount to ejaculation was not different among stallions.

Gross inspection of the body cavity and the reproductive tract of the sacrificed OVEX mare revealed no signs of ovarian-like tissue. Likewise, microscopic examination of the sectioned and stained ovarian pedicles disclosed no ovarian remnants or regrowth.

DISCUSSION

In this study both seasonally anovulatory mares and ovariectomized mares solicited copulation from stallions, were sexually attractive to them, and tolerated mounts, intromissions, and ejaculations. Such behavior in the pony under reduced hormone conditions is striking in light of the widely held belief that hormones of ovarian origin are exclusively responsible for displays of estrus in infraprimate mammals (Goy and Goldfoot, 1975; Leshner, 1978; Young, 1961). Nonetheless, it would be misleading to conclude that the pony is "gonadally emancipated," since the ovary is clearly involved in the regulation of estrous behavior during normal ovulatory cycles. Intact mares display very clear patterns of estrus for approximately 1 week out of every 3 throughout the spring and summer (Ginther, 1979). The week of behavioral estrus, with ovulation on about the fifth day, is followed by a period of marked sexual refractoriness during the 2-week diestrous phase, when mares respond to a stallion's approach and investigations with avoidance or threats (Asa *et al.*, 1979).

Comparisons of mares from this study to intact estrous and diestrous mares of a previous study suggest that the removal of the ovaries or the natural suppression of ovarian function during the winter months results in a chronic condition of moderate behavioral estrus, in which behavioral measures of attractivity, proceptivity, and receptivity are found with probabilities of at least 50% on any given test day. In contrast, estrus is seen on only about 33% of the days for intact mares during the ovulatory season (Asa *et al.*, 1979).

Proceptive components of estrus, such as the frequency of urinations, of following, or of standing near the stallion with the tail elevated were displayed with rates similar to those of intact mares during the initial and

terminal days of ovulatory estrus. Receptive elements of the response pattern were slightly reduced, however, in that proceptive OVEX and ANOV mares seemed to show more hard kicking and avoidance responses than intact estrous mares.

The intensity of estrus in ANOV and OVEX mares fluctuated in irregular patterns throughout the course of this study, perhaps due to the specific social composition of the groups formed on a given day, including individual preferences by mares for certain stallions, and dominance/subordination relationships among mares. Alternatively, these daily changes might have been related to changes of nonovarian-source steroids which could have been contributing to the phenomenon.

Only 3 of the 20 mares in this study displayed periods of sexual refractoriness for longer than 5 consecutive days, whereas half of the ponies never had more than a 2-day period in which estrous responses were not displayed. Thus, the absence of *cyclic* periods of nonreceptivity and nonproceptivity most accurately characterizes the behavioral changes following ovariectomy or seasonal ovarian quiescence in the mare. In contrast, a continual absence of estrous responses is typical of other ovariectomized or anovulatory infraprimate species.

These several observations and comparisons suggest the following three hypotheses. First, the luteal ovary appears to play a controlling role in the inhibition of estrous behavior, raising the possibility that progestational secretions of the corpus luteum provide the mechanism for periodic sexual inhibition during diestrus.

Second, although a nonhormonal mechanism might be responsible for estrous activity after ovariectomy, we hypothesize that the estrous behavior seen in this study was most likely supported by sex steroids, probably from the adrenals. Adrenal cortical mediation of sexual activity has been extensively considered in primate species (Everitt and Herbert, 1971; Everitt, Herbert, and Hamer, 1972; Goldfoot *et al.*, 1978; Baum, Slob, de Jong, and Westbroek, 1978). Hay and forage, which may contain phytoestrogens, present another potential source of steroids.

Third, since periovulatory estrus is more vigorous than that observed in OVEX and ANOV females, it is hypothesized that additional steroidal stimulation for these behavioral tendencies is supplied by the ovarian follicles in the days just prior to ovulation. Thus, it is likely that the follicular ovary of the mare, as in other species, increases the probability that copulation will occur during fertile phases of the cycle. However, the luteal ovary of the mare appears necessary to suppress estrus during the infertile phase of the cycle and therefore might be exhibiting a more potent inhibition of estrus than that thought to exist in most other infraprimate species (Feder, 1977).

The steroid condition resulting from ovariectomy represents an experimental manipulation which mares would normally never experience.

However, winter anestrus does occur naturally (Ginther, 1979), and 40–50% of mares in a typical free-ranging herd fail to deliver live young in a given season (Nelson, in press; Feist & McCullough, 1975). Thus, estrus might be displayed by some percentage of the nonpregnant, free-ranging mares during winter months.

The display of estrous behavior by mares at times during which conception is impossible might have functional significance to this species. The most commonly reported social unit of ponies and horses is of the harem type, with a stallion, several mares and their offspring (Feist and McCullough, 1976). Sexual activity outside of the breeding season, made possible by the absence of complete control of steroids from the ovaries over estrous behavior, could contribute to the maintenance of social bonds between the harem stallion and his mares. Further work directed to this possibility as well as toward the question of whether sex steroids from adrenal sources mediate these events is currently being investigated.

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Effect of stress induced by gathers and removals on reproductive success of feral horses

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Abstract Successful management of many species often relies on actions that involve intensive handling of individuals. Knowledge of how such handling may affect reproduction of a particular species is important and may be applicable to managing other species. We used pregnancy testing, field observation, Bureau of Land Management (BLM) records, and adopter surveys to determine effects of stress induced by gather and removal management practices on the reproductive success of feral horses in the Garfield Flat (GF) Herd Management Area (HMA) in Nevada. We analyzed pre-release confinement effect data from gathers conducted in August 1993 and January 1997 and data on the additional effect of removal in 1997. We used data for an ungathered population in the Granite Range HMA (GR) in Nevada as controls both years. Data from ungathered GR horses also were used as controls for habitat effects on reproduction. Granite Range and ungathered GF mares in 1997 had similar reproductive success rates (P=0.42). Pregnant removed mares in 1997 had less reproductive success than ungathered mares at GR (P=0.003) and GF (P=0.005). Gathered and released GF mares had less reproductive success than ungathered GF mares (P=0.05). The results suggest that minimizing time that mares are held prior to release will reduce fetal loss.

Key words abortion, horses, management, reproductive success, stress

In several studies, fetal loss in feral and domestic horses has been attributed to stress, often associated with frequent and extensive handling of pregnant mares (Mitchell 1971, Osborne 1975, Roberts 1986). Berger's (1983) study of feral horses in the wild found that forced copulation and social environment change after band takeovers in the Granite Range of Nevada caused abortions.

Most feral horse herds in the United States are managed intensively by the Bureau of Land Management (BLM, Boyles 1986). The Public Rangelands Improvement Act of 1978 authorized excess feral horse removal to preserve the quality of range habitat. Gathers and removals are the principal means to reduce populations to acceptable management levels (AML) for individual herd management areas (HMA; Boyles 1986). Each HMA is assessed by BLM range-condition specialists to determine stocking levels for livestock, feral horses, and game species. A 3- to 5-year interval between gathers and removals is necessary as many horse herds increase ≥20%/year (Wolfe 1980, Eberhardt et al. 1982, Berger 1986, Garrott and Taylor 1990). The removal magnitude depends on number of excess horses and the desired ending population size relative to the AML.

Gathers and removals may induce stress in several ways and for varying periods of time. Large-scale BLM gathers involve helicopter use to chase horses into a funnel trap leading to a corral. Trapped horses are individually restrained, aged, examined for physical

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condition, and segregated for removal or release as adult and juvenile males, lactating mares and foals, and nonlactating mares. *Ad libitum* food and water is provided in corrals to minimize nutritional stress.

Horses released after a gather typically have been held for 2 to 4 days at the capture site. Horses removed for adoption face longer periods of stress. From the capture site horses are trucked to a processing facility, often hundreds of km away. Here they are again individually restrained for blood sampling, aging, and freeze-branding. Some adoption occurs locally; however, many more horses are transported to satellite adoption centers across the country. Horses can spend months in confinement before adoption. We investigated the role that stress plays in reproductive failure following gathers and removals. We feel that the results of this study are valuable to BLM management and are applicable to managing large-bodied ungulates in general, when translocations involve extensive handling or long periods of confinement.

Study sites

For >20 years, BLM has actively managed feral horses at the Garfield Flat (GF) and Granite Range (GR) HMAs in the Great Basin by conducting periodic gathers and removals to control population size. Both areas are subject to cattle grazing. The vegetation at both study areas is predominantly mixed grass and shrubs at lower elevations. At higher elevations, pinyon pine (*Pinus monophylla*) and western juniper (*Juniperus occidentalis*) predominate at GF. Western juniper also occurs at higher elevations at GR with stands of quaking aspen (*Populus tremuloides*) at some springs. The 607-km^2 GF HMA, located in Mineral County in west-central Nevada, is bordered on the east, north, and west by the Garfield Hills and on the south by the Excelsior Mountains. The playa at GF is 1,700 m above sea level (asl), with the Garfield Hills and Excelsior Mountains each rising above 2,400 m asl (United States Department of Interior 1985). Water is available year-round from 4 springs and ephemerally from 4 small catchments, ≤100 m^2 each (Figure 1). The 411-km^2 GR HMA is located in Washoe and Lander counties in northwest Nevada in the Granite

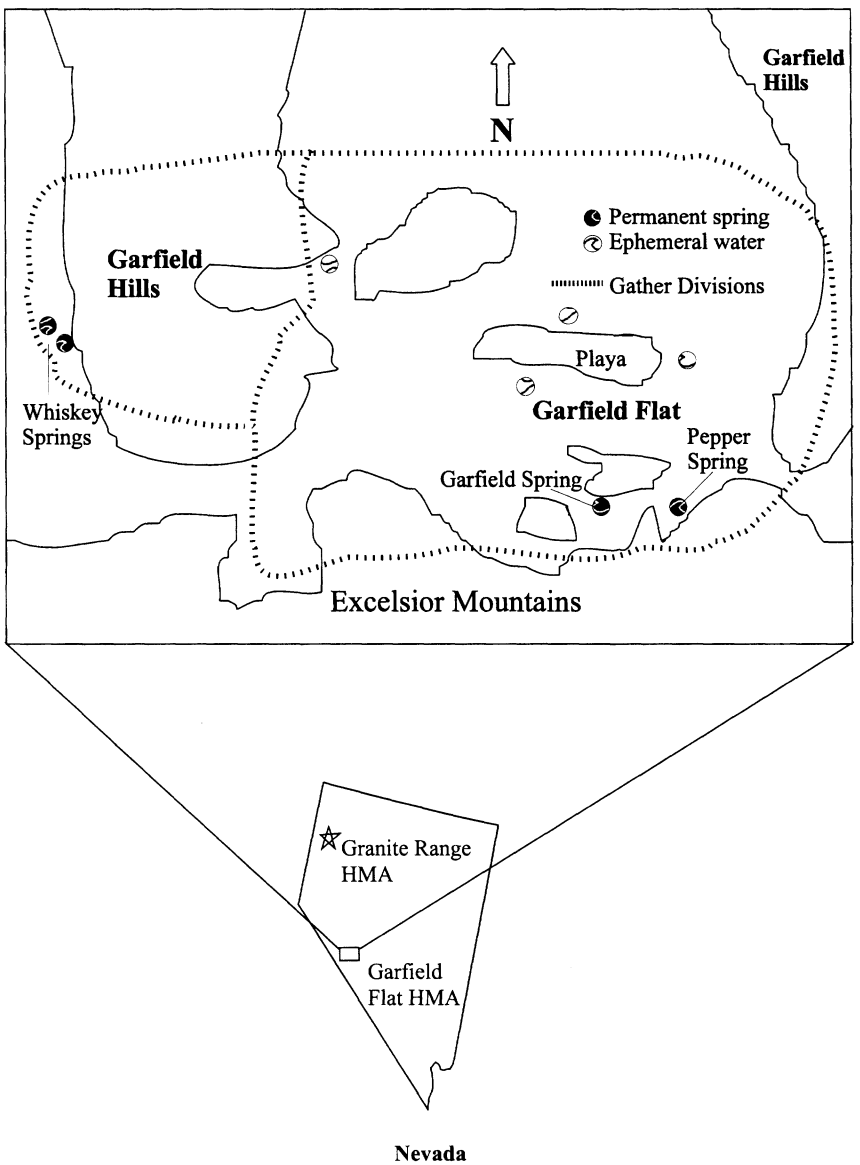


Figure 1. Locations of study sites and representation of the Garfield Flat Herd Management Area, including locations of available water and division of the HMA for the 1993 gather.



A mare being herded toward a squeeze chute for tissue collection and freeze-branding at the 1993 gather.

Range Mountains. Elevations range from 1,200 m asl in the surrounding lowlands to 2,735 m asl at Granite Peak (United States Department of Interior 1981). At this site, water is available year-round from numerous permanent springs and ephemeral ponds ranging from 50 to >400 m^2 in size.

Materials and methods

Gathers and removals

The first gather at GF was conducted in August 1993. The second occurred in January 1997. For both, a helicopter, drift fence, and funnel trap were used to capture horses. In the 1993 gather, resident horses in the eastern portion of the HMA were captured the first day and processed and released the second day. This procedure was repeated for horses in the western portion of the HMA on the following 2 days (Figure 1). Eighty-eight horses from the eastern area and 81 horses from the western area were captured, accounting for about 95% of the total HMA population. All horses gathered in 1993 were released except for 4 foals that were removed and one foal that was destroyed because of injuries. In 1997, horses were captured from the entire HMA over a 2-day period. Processing started immediately and continued for 2 days post-capture, culminating with the release of horses not shipped for adoption. The released horses were held 3 or 4 days, depending on capture order. Horses removed for adoption were trucked on days 3 and 4 to the Palomino Valley Center (PVC), a BLM adoption and processing center 246 km from the study site. Horses from the 1997 gather were examined and freeze-branded at PVC 2 weeks after the conclusion of the gather.

Horses appeared to be stressed at gathers, exhibiting agitated behavior. Confinement kept horses from different bands, groups of ≥1 adult female and any offspring attended by ≥1 adult male, at much closer quarters than we observed on the range. Mares too close to an unrelated foal were often threatened or kicked by the foal's mother. In contrast, we found that intra-band female–female aggression in the field was infrequent and limited to mild threats. Band spacing in the field generally precludes aggressive behavior. Inter-band aggression was usually observed between band stallions that often traveled >100 m to interact. Some bands were permanently disrupted after the release.

Tissue collection

In January 1997, we collected jugular blood samples at the gather site and at PVC in evacuated tubes from horses that would be ≥2 years old the following spring. We separated the serum component on site with a portable centrifuge (5,000 × g for 5 minutes) and stored the samples on ice for transport. We found that centrifugation at greater gravity than applied by Wolfe et al. (1989) efficiently separated the serum in less time. At the lab, we pipetted ≥1 ml serum/sample into individually labeled 1.5-ml microcentrifuge tubes and stored them at −80°C until samples could be assayed. Serum progesterone was determined using a solid phase radioimmunoassay kit (Coat-a-Count, kit #TKP6, Diagnostic Products Corporation, Los Angeles, Calif.; Wolfe et al. 1989). The coefficients of variation for progesterone were 8.1% for intra-assay and 9.7% for inter-assay results. Because nonpregnant mares generally do not cycle during the winter, we classified mares that had titers of ≥2 ng/ml serum progesterone as pregnant (Plotka et al. 1988).

Field methods

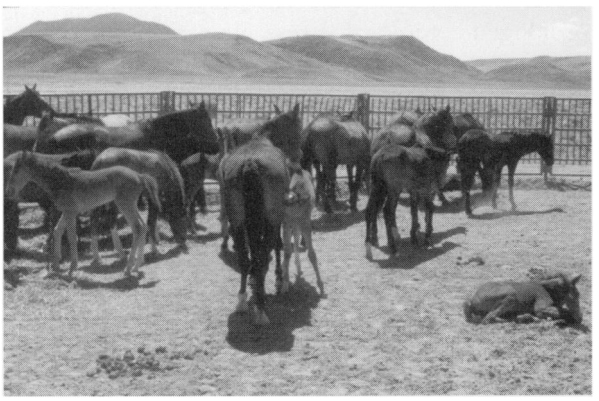
All released horses from both gathers at Garfield Flat were photographed and left-hip freeze-branded with unique numbers. Age (American Association of Equine Practitioners 1981), sex, and distinguishing marks were noted to aid in field identification. In February 1993 and November 1995 the BLM conducted gathers and removals at the GR HMA, releasing 359 and 123 freeze-branded horses, respectively. Identifying marks also were recorded for many of these horses.

We conducted intensive ground surveys of the GF and GR horses from March through August of

1994 and 1997 to assess reproductive success. Using 80-mm, 20X60-power spotting scopes, we identified horses by number and, when necessary, unique markings. To have reasonable confidence of accurately assessing reproductive success or failure, we only used data on mares that were sighted prior to, during, and after the prime foaling season, April to July. These sightings allowed us to assess whether an animal was visibly pregnant and whether a viable foal was produced. Mares classified as pregnant that were not accompanied by a foal by the end of August were scored as reproductive failures. We verified the foaling success or failure of all GF mares released after the 1997 gather. All successful pregnancies at both study sites were detected pre-partum in that surveyed mares seen with foals had been classified correctly as pregnant prior to foaling. Some of these mares at both sites were classified pregnant but were unaccompanied by foals at the end of the season. These reproductive failures of visibly pregnant mares may have included losses to mountain lions (*Puma concolor*) or coyotes (*Canis latrans*), even though we found no direct evidence of predation.

We assessed reproductive success for GR horses in 1994 and 1997 to serve as unmanipulated controls for the GF horses. The GR horses were gathered February 1993 and November 1995, early enough that there should have been no negative impacts on foaling in 1994 and 1997.

We collected GF removed mare reproductive success data in 1997 from BLM adoption records, adopter questionnaire, and telephone interviews with adopters. Unable to separate early foal death from fetal abortion in the field population, we included one stillborn, one death during birth, and the death after 1 day of a premature foal as evidence of reproductive failure for removed horses. In this tally, we also included 3 cases of pregnant removed mares that died prior to adoption. The BLM determined that the cause of these deaths was "poor condition" that could have been influenced by prolonged stress.



Segregated bands of lactating mares and foals at the 1993 gather.

Data analysis

Foaling success is a dichotomous event suitable for 2-by-2 contingency table analysis for pairwise comparisons. We used the 1-tailed Conditional Binomial Exact Test (CBET, Rice 1988) for these analyses, expecting that increased periods of stress would result in lesser foaling rates. This test is robust with moderate to small sample sizes and occasional small cell values.

The tests of differences in 1997 foaling success between gathered Garfield Flat mares and ungathered GR and GF mares are not equal comparisons, as the last 2 classes of mares were not tested for pregnancy. It is unlikely that all of the ungathered GR and GF mares assessed for foaling success were pregnant. Accordingly, this makes the comparisons of foaling success conservative tests.

Results

At GF, 45 (67.2%) of 67 marked mares foaled in 1994. These data represented all but 2 of the mares released in 1993 that would be ≥2 years old the following year. At GR, 20 (71.4%) of the 28 marked mares we surveyed foaled during the 1994 field season. Gathered and released GF mares had a foaling rate little different from GR mares (CBET: $P=0.43$). The minimum age of marked GR mares in 1994 was 4. Garfield Flat mares ≥4 years old ($n=34$) foaled at a greater rate (77.4%) than GR but did not differ significantly (CBET: $P=0.30$).

At the 1997 gather, released horses were held somewhat longer than in 1993, 3 to 4 days depending upon capture order. Removed horses were held ≥21 days before adoption. During the 14-day period prior to blood sampling at PVC, there were 15 to 18 spontaneous miscarriages as evidenced by shed fetuses (Sharon Kipping, PVC Manager, personal communication). One mare died that had a well-developed fetus *in utero*. Two pregnant mares died at interim locations before being adopted.

We pregnancy-tested 21 mares that were released at GF in 1997 and found 18 (85.7%) pregnant. Ten of these mares (55.6%) foaled. Of the mares removed in 1997 (66 tested, 1 autopsied), 43 (64.2%)

were pregnant. We verified the reproduction of 37 pregnant mares with only 17 (45.9%) producing viable foals. We also collected resighting data to determine reproductive success of 17 ungathered mares at GF in 1997. Fourteen (82.4%) produced foals. That same season we identified 54 mares at GR and found that 40 (74.1%) produced foals.



Two bands in the field, summer 1996.

At GF in 1997, stress duration for the 3 classes of mares was 0 days for ungathered ($n = 17$), 3.5 days for gathered and released ($n = 18$), and 21 days for removed mares ($n = 37$). Pairwise comparisons of the 3 classes of mares showed that pregnant mares exposed to longer periods of stress had lesser reproductive success. The greatest difference in reproductive success was between ungathered and removed mares (CBET: $P = 0.005$). There also was a significant difference between ungathered and released mares (CBET: $P = 0.05$). There was, however, no significant difference between released and removed mares (CBET: $P = 0.26$).

The reproductive rates of GR and the ungathered GF horses did not differ (CBET: $P = 0.42$). Granite Range horses had a greater reproductive rate than the pregnant released mares at GF, but the difference was not significant (CBET: $P = 0.08$). As expected, GR horses had greater reproductive success than pregnant mares removed from GF (CBET: $P = 0.003$).

Discussion

The effect of gather-and-removal-induced stress on pregnant feral horses is reduction in foaling success, sensitive to the duration of the stressor. The GF horses that were removed for adoption were transported long distances and subjected to repeated handling. Long-distance transportation has been linked to abortions in 2-year-old domestic mares (Roberts 1986). Mitchell (1971) and Osborne (1975) associated abortions in yearling and 2-year-old domestic mares with extensive handling. Generally, high pregnancy rates for yearlings and 2-year-olds followed by fetal losses have been reported for domestic (Mitchell and Allen 1975) and feral horses (Lucas et al. 1991). The reproductive losses for GF mares, however, were spread across age classes.

Stress, whether acute or chronic, stimulates the release of cortisol and other adrenocorticoids in mammals (Yates and Urquhart 1962). For example, in studies of gray squirrels (*Sciurus carolinensis*, Guthrie et al. 1967) and snowshoe hares (*Lepus americanus*, Boonstra et al.1998), trapping stress elevated serum corticosteroids. Snowshoe hares produced smaller litters during the decline phase of their 10-year cycles because of chronic stress imposed by elevated predation (Boonstra et al. 1998). Decline in serum progesterone has been linked to acute stress (van Niekerk and Morgenthal 1982). Serum progesterone is critical to pregnancy maintenance (Ball 1993). It likely is that severe and chronic reduction of serum progesterone due to prolonged removal stress was responsible for spontaneous abortions at PVC and overall decrease in reproductive success. An examination of serum cortisol and progesterone levels in response to gathers and removals would provide a definitive causal relationship between stress and reduced reproductive success.

Band takeover is another possible source of stress for feral mares. Berger (1983) reported instances of harassment and forced copulation in the Granite Range by stallions that had taken over bands. The stress of forced copulation coupled with changes in social structure caused induced abortions in mares

less than 6 months pregnant (Berger 1983). During our 6 seasons of field work, we observed many cases of harassment but only one forced copulation. The sole forced copulation was performed by a resident band stallion upon an unreceptive yearling mare. Most harassment incidents, however, were performed by bachelor males directed at harem mares. We saw no cases of harassment or forced copulation by stallions that acquired new bands. Further study is necessary to better evaluate the potential effects of forced copulation, harassment, and change in social structure on reproduction.

Removals from managed herds reduce the number of young horses ≤ 5 years old. Interestingly, this has the effect of temporarily increasing the proportion of the more reproductively successful horses in the population (Garrott et al. 1991). Seemingly, this works at cross purposes to desired population sizes at or below AML. To counter this effect, horses of all ages or a greater proportion of prime-age horses should be removed. Additionally, timing of removals can be exploited to extend the period of reduced population size. Gathers conducted in September or October, after the foaling season (Garrott et al. 1991), would reduce the next year's reproduction by interrupting early-stage pregnancies at a time when successful re-impregnation is unlikely. Feral horses are protected federally, and the BLM is charged with maintaining the general health and well-being of those horses under its jurisdiction (Boyles 1986). Such pregnancy disruptions would achieve a temporary reduction in population growth without large impacts on mare condition due to the small resource investment in early-stage fetuses.

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Induction and Immunohistology of Autoimmune Ovarian Disease in Cynomolgus Macaques (*Macaca fascicularis*)

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Autoimmune ovarian disease (AOD) is a probable cause of human premature ovarian failure, and a potential complication of contraceptive vaccines based on ovarian antigens. The diagnosis depends on detection of noninfectious ovarian inflammation (oophoritis) and serum antibody to ovarian and placental antigens. Mechanisms underlying AOD have been investigated in mice but not in primates. Herein, we report induction of AOD in primates, and compare the immunopathology between monkey and murine AOD. Four cynomolgus macaques immunized with monkey or human zona pellucida 3 peptide (pZP3) in adjuvant, developed T-cell responses to the immunizing peptide and produced antibody that bound to native zona pellucida *in vivo*. Immunostaining of ovaries from pZP3-immunized macaques showed numerous clusters of T cells co-localized with major histocompatibility complex II-positive macrophages in the ovarian interstitium. Such foci were not detected in untreated or adjuvant-treated control monkeys. This finding is comparable to murine pZP3-induced AOD. However, unlike murine AOD in which numerous granulomatous lesions are detected, severe granulomatous inflammation was detected in only one of three monkeys with abnormal immunohistology. Similar to mice with pZP3-induced AOD, the immunized monkeys retained normal ovarian function. The results are discussed in the context of complications of ZP-based human immunocontraceptive vaccines and case reports of human autoimmune oophoritis. *(Am J Pathol 2002, 160:141–149)*

Autoimmune ovarian disease (AOD) is considered to be a cause of human premature ovarian failure (POF).[1–3] However, the immunological basis of POF remains poorly understood. This is in part because early POF is clinically silent and advanced stages of the disease lack ovarian inflammation (oophoritis), a diagnostic feature of autoimmune etiology.[4] The strongest evidence for human AOD is the finding of oophoritis in a subset of patients with POF,[4,5] as well as the co-existence of ovarian inflammation with autoimmune adrenal disease or autoimmune polyglandular syndrome.[6] The diagnosis of immune POF is based on detection of circulating autoantibodies to ovarian or placental antigens. However, there is currently no consensus on the antigen specificity of these antibodies or the clinical assay system for their detection. Although serum autoantibodies to ovarian 17α-hydroxylase and side-chain cleavage enzyme of steroid-producing cells have been characterized, they are detected only in POF patients associated with autoimmune polyglandular syndrome type I or type II.[7,8]

Studies on rodent models of AOD have added some insights into the pathology and mechanisms of immune-mediated ovarian injury. Neonatal thymectomy in susceptible mouse strains causes ovarian inflammation and autoantibodies resulting in ovarian atrophy and ovarian dysfunction.[9] This model has been used to understand regulation of autoimmune disease in general and AOD in particular.[10,11] The second rodent model of AOD is elicited by immunization with a self-peptide of murine zona pellucida (ZP)3 (330 to 342).[12] Murine pZP3 immunization causes activation of autoreactive T cells and generation of anti-ZP autoantibody. The autoreactive T cells mediate autoimmune oophoritis characterized by focal or diffuse inflammation in the ovarian interstitium and organized monocytic granulomata. Although oophoritis alone does not affect ovarian function, a high level of ZP autoantibody reduces fertility in female mice.[13] On the other hand, combined ZP antibody and T-cell-mediated inflammation are required for the induction of ovarian atrophy and infertility.[14] Chimeric peptides that contain foreign T-cell epitope and native ZP3 B-cell epitope induce ZP antibody without inducing ovarian inflammation, and they are potential candidate antigens for contraceptive vaccine development.[15]

In primates, long-term immunization with zona pellucida proteins for >1 year resulted in antibodies to zona pellucida with contraceptive effect. However, the infertility was irreversible and the monkeys developed POF.[16,17]

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The ovaries from immunized animals show few oocytes or developing follicles but increased numbers of aberrant granulosa cell nests. Ovarian inflammation was not reported in the atrophic ovaries of animals after long-term immunization. To investigate AOD as a basis for POF and a complication of ZP vaccination, we have developed a primate model of AOD. In this study, we demonstrate that cynomolgus macaques are indeed susceptible to AOD using human and macaque homologues of murine pZP3 (330 to 342). The resulting ovarian histopathology is compared to murine AOD, and with the reported findings in human AOD.

Materials and Methods

Peptides

The ZP3 peptides from different species used in this study are as follows: human 328 to 341 [GTPSHSRRQPH-VMS pZP3(hu)]; macaque 328 to 341 [GTPSHSRRQPH-VVS pZP3(mac)], and mouse 330 to 342 [NSSSSQFQIH-GPR pZP3 (mou)].[18] The peptides were synthesized in an automated peptide synthesizer (Beckman Instruments, Fullerton, CA) using F-moc chemistry. Deprotection was achieved by 20% piperidine in dimethyl formamide, and the peptides were chemically cleaved from the resin with 85% trifluoroacetic acid. The peptides were purified by HPLC eluting as a single peak on PRLP-S columns (Waters, Milford, MA) and were of >90% purity.

Monkeys: Immunization, Cyclicity, Surgery, Sacrifice

All animal housing and procedures were performed in accordance with the National Institutes of Health guidelines. Adult female cynomolgus macaques (*Macaca fascicularis*) were anesthetized with ketamine hydrochloride (10 to 20 mg/kg) for immunization and bleeding. Experimental groups included immunization with peptides [pZP3(mac) or pZP3(hu): two animals per group] or controls (untreated or adjuvant-treated: two animals per group). Immunizations were done on days 0, 14, and 28 intramuscularly with 50 nmol/L of peptide and 0.1 mg of nor-muramylated dipeptide (a gift from Dr. Vernon Stevens, Ohio State University, Columbus, OH) in phosphate-buffered saline (PBS) emulsified in equal volume of squalene:arlacel A (4:1, v:v; Sigma Chemical Co., St. Louis, MO). Animals were given booster immunizations at times indicated. Blood collected from the femoral vein was used for serum antibody analyses, T-cell responses, and circulating steroid levels. All of the animals were monitored daily for menstrual bleeding. Ovaries were surgically removed under general anesthesia through a midline abdominal incision. To study ovarian immunopathology at multiple time points, the first ovary was removed between 1.5 to 6 months and the second between 11 to 16.5 months. Each ovary was cut into 4 to 5 pieces collected in buffered formalin for histopathology. One piece was snap-frozen in liquid nitrogen for detection of ZP-bound IgG. The animals were euthanized at the end of the study and organs collected for histopathological analysis.

Mice: Immunization and Sacrifice

(C57BL/6 X A/J)F1 females were obtained from the National Cancer Institute, Bethesda, MD and housed as per National Institutes of Health guidelines. As previously described, for induction of AOD, mice were anesthetized by intraperitoneal injection of tribromoethanol (0.5 mg/mouse) and immunized subcutaneously with 50 nmol/L of pZP3(mou) emulsified in CFA (*Mycobacterium* tuberculosis H37RA strain, 0.16 mg/mouse) (Difco Laboratories, Detroit, MI) and sacrificed 14 days later.[12] Control mice were injected with CFA alone. One ovary was collected in Bouin's fixative for histopathological grading of AOD and the other ovary was collected in 4% paraformaldehyde in PBS (w/v), pH 7.4, for immunohistochemical staining.

Detection of Antibody to Peptides by Enzyme-Linked Immunosorbent Assay

Serum IgG to pZP3(hu) or pZP3(mac) were detected by enzyme-linked immunosorbent assay.[18] Ninety-six well plates (Corning Glass Works, Corning, NY) were coated overnight at 4°C with 25 nmol/L of peptide in 0.1 mol/L bicarbonate buffer, pH 9.0. All subsequent incubations were performed for 2 hours at room temperature. The plates were washed with 0.05% Tween 20 in PBS, pH 7.4, and blocked with 3% bovine serum albumin (BSA) in PBS. The plates were incubated with serial dilutions of monkey sera. The plates were then washed and incubated with horseradish peroxidase-labeled goat anti-monkey IgG (Nordic Immunological Laboratories, Capistrano Beach, CA) (1:3000 dilution). After washing, the reaction was developed by *o*-phenylene diamine (0.5 mg/ml; Sigma Chemical Co.) with freshly added 0.06% H_2O_2 in 0.1 mol/L citrate buffer, pH 5.0. The color intensity was read at 490 nmol/L by an enzyme-linked immunosorbent assay reader (Molecular Devices, Menlo Park, CA). The end-point titer was calculated as dilution of serum giving an absorbance of 2 SD above the background reading using preimmune monkey serum.

Detection of Anti-ZP Antibody by Direct and Indirect Immunofluorescence

In vivo binding of ZP antibody was detected by direct immunofluorescence. Frozen monkey ovary sections from peptide-immunized macaques or adjuvant-treated controls were fixed in 95% ethanol for 10 minutes and rinsed with PBS. The sections were then blocked with normal goat serum (1:10 v/v) diluted in PBS with 3% BSA for 20 minutes in a humid chamber at room temperature. After rinsing with PBS, the bound antibody was detected by fluorescein isothiocyanate conjugated to anti-monkey IgG (1:100; Nordic Immunological Laboratories) for 30 minutes. The slides were rinsed and mounted in Vectashield medium (Vector Laboratories, Burlingame, CA).

Antibody to zona pellucida in serum was detected by indirect immunofluorescence. The procedure followed was same as above with some differences. Five μm sections of snap-frozen normal monkey ovary were fixed in 95% ethanol, blocked with normal goat serum in PBS with 3% BSA (1:10 v:v). The sections were then incubated with immune serum or adjuvant control serum diluted in 3% BSA in PBS for 1 hour. IgG antibody bound to ZP was detected by fluorescein isothiocyanate conjugated to anti-monkey IgG (Nordic Immunological Laboratories).

Lymphocyte Proliferative Responses in Monkeys

Estimation of T-cell proliferative responses was done as previously described.[18] Monkey peripheral blood was collected in heparinized tubes and centrifuged to separate supernatant plasma. The cell pellet was resuspended in sterile PBS, layered over Histopaque 1083 (Sigma), and centrifuged at 400 $\times$ *g* for 20 minutes. Mononuclear cells at the interphase were collected, washed, and resuspended at 2 $\times$ 10^6/ml in Dulbecco's minimum essential medium supplemented with 1% sodium pyruvate, 1% of 200 mmol/L glutamine, 1% nonessential amino acids, 5 $\times$ 10^{-5} mol/L β-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal calf serum. The assay was set up in triplicates in which cells (0.1 ml/well) were cultured with an equal volume of peptide (0 to 30 μmol/L) in 96-well flat-bottom plates for 4 days at 37°C in 5% CO_2. [^{3}H]-thymidine (0.5 μCi/well; Dupont NEN Products, Boston, MA) was added to each well 16 hours before harvesting the cells (Skatron Instruments, Sterling, VA), and the cell-associated thymidine measured by scintillation counting (Beckman Instruments). Results are presented as stimulation index (experimental counts per minute with peptide/background counts per minute without peptide). The monkeys Babs and Harriette gave high background counts when cultured in media with 10% fetal calf serum. For these animals, fetal calf serum was substituted with 10% autologus heat-inactivated plasma.

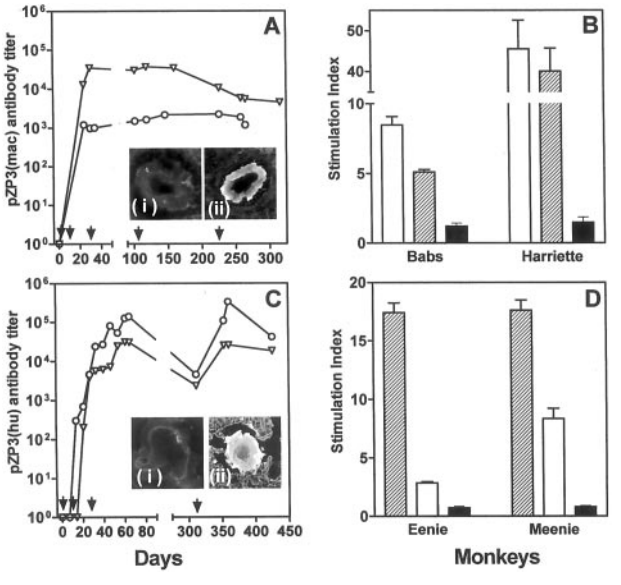


Figure 1. Immune response in female macaques immunized with pZP3(mac) (**A** and **B**) or pZP3(hu) (**C** and **D**). Serum antibody titers to the immunizing peptide were detected by enzyme-linked immunosorbent assay (**A** and **C**). **Arrows** indicate time of primary and booster immunizations. **A:** Antibody titers to pZP3(mac) [Babs (**circles**) and Harriette (**triangles**)]. Serum antibody from peptide-immunized Harriette recognized macaque zona pellucida (**inset Aii**) whereas sera from adjuvant-treated controls did not (**inset Ai**). In lymphocyte proliferation assays (**B** and **D**) animals immunized with pZP3(mac) (**B**) respond to the immunizing macaque peptide (**open bars**) and its human homologue (**hatched bars**) but not to the mouse peptide (**solid bars**). **C:** Antibody titers to pZP3(hu) [Meenie (**circles**) and Eenie (**triangles**)]. Direct immunofluorescence showed antibody bound to ZP (**inset Cii**) in Meenie but not in an adjuvant-treated macaque (**inset Ci**). In lymphocyte proliferative assays animals immunized with pZP3(hu) (**D**) respond to the immunizing peptide (**hatched bars**) and its homologous macaque peptide (**open bars**) but not to the dissimilar mouse peptide (**solid bars**). Data show stimulation index (±SEM) at 30 μmol/L concentration for each peptide. **C** is reprinted with permission from the Society of Reproduction, Inc. (Biol Reprod 1997, 56:767).

Immunohistology: Monkey and Mouse Ovaries

Serial sections (5 μm) were cut through the entire formalin-fixed, paraffin-embedded, monkey ovaries. Every 10th section was stained with hematoxylin and eosin. Adjacent sections were stained with the following antibodies at indicated dilutions. Polyclonal rabbit antibody to human CD3 and mouse monoclonal antibodies to HLA (CR3/43), CD20 (L26), CD 68 (KP1), and myeloid/histiocyte antigen (Mac 387) obtained from DAKO Corporation, Carpinteria, CA. A rabbit polyclonal antibody to HLA DR (a gift from Dr. S. M. Fu, University of Virginia, Charlottesville, VA)

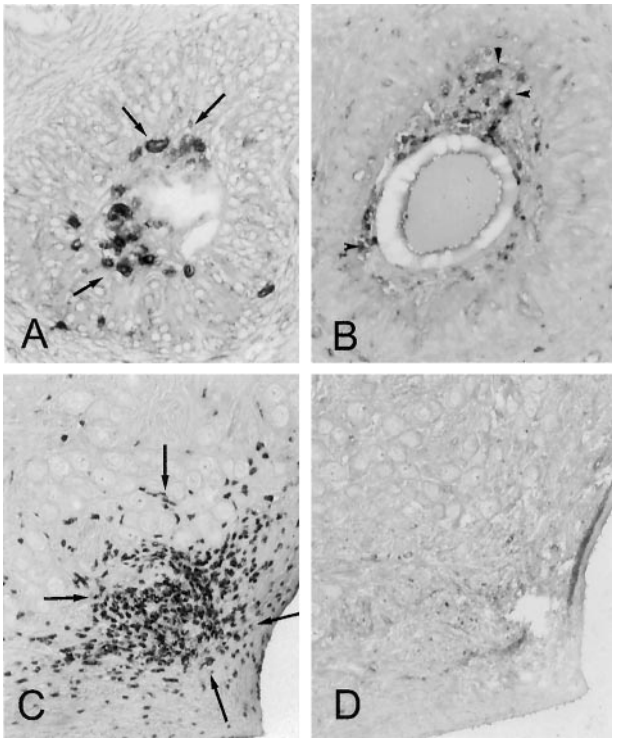
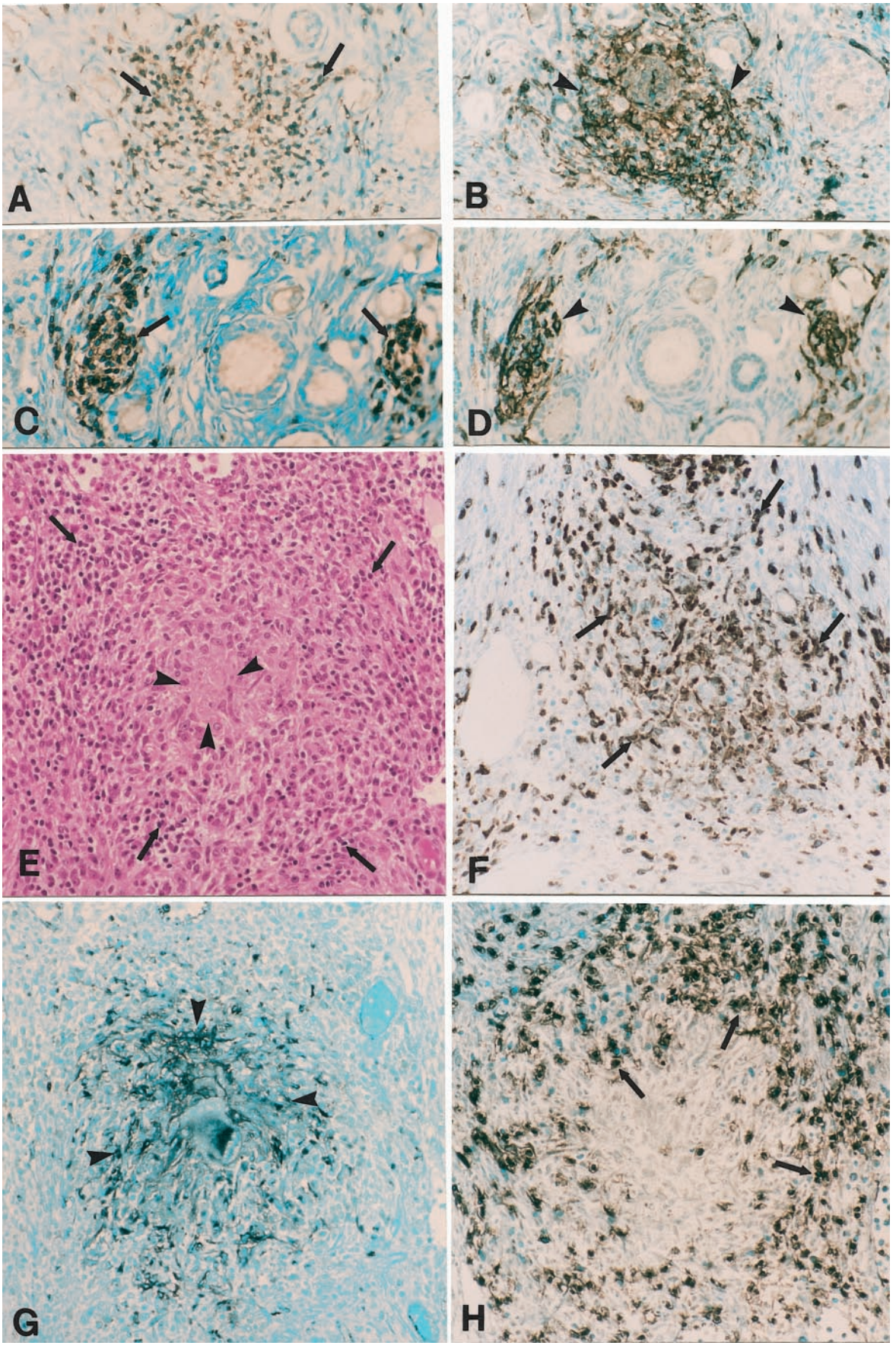


Figure 2. Immunoperoxidase staining for CD3+ T cells (**arrows**) and MHC II+ macrophages (**arrowheads**) in control macaque ovaries. **A** and **B:** Atretic follicles in adjacent sections have invading CD3+ T cells (**A**) along with MHC II-expressing cells (**B**). **C** and **D:** Adjacent sections of ovarian cortex reveal a cluster of CD3+ T cells (**C**) but absence of MHC II+ cells (**D**). Original magnifications, ×400.


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was also used in some of the studies. Optimal dilutions of primary antibody (1:20 for rabbit anti-HLA polyclonal; 1:50 for L26, Mac387, CR3/43; and 1:100 for anti-CD3 and KP1) were determined using control monkey spleen sections.

The tissues were deparaffinized in xylene, transferred to absolute alcohol, rehydrated with decreasing grades of alcohol, and rinsed in PBS. Endogenous peroxidase was inactivated with 0.6% hydrogen peroxide in methanol PBS (4:1, v:v). Because some antibodies (CD3, CD68, and Mac 387) did not recognize their cognate antigens, unmasking of epitopes was performed by digestion with pepsin (4 mg/ml) in 0.1% HCl at 37°C for 7 minutes. For HLA monoclonal and polyclonal antibodies, antigen retrieval was done by heating slides in 10 mmol/L of citrate buffer, pH 6.0, for 20 minutes in a microwave. Evaporated fluid was replaced with water every 4 minutes. The slides were allowed to cool to room temperature before use. Sections were rinsed in PBS after antigen retrieval and blocked with normal goat or rabbit serum followed by primary antibody diluted in 3% BSA PBS. After washing, the slides were incubated with biotinylated goat anti-mouse or anti-rabbit Ig (Vector Laboratories) (1:100 dilution). Detection of bound antibody was done by avidin-biotinylated enzyme complex (Vectastain ABC kit; Vector Laboratories) and visualized by incubation with diaminobenzidine substrate (Biogenex Corp., San Ramon, CA). The sections were counterstained with methylene blue, rinsed with water, and dehydrated with ascending grades of alcohol. The slides were then rinsed with xylene and mounted with Cytoseal60 (Stephens Scientific, Kalamazoo, MI). Monkey spleen sections concomitantly processed were positive controls and ovaries without primary antibody served as negative controls.

Immunochemical staining of mouse ovaries was done as previously described.[13] Briefly, 5-μm sections from mouse ovaries fixed in 4% paraformaldehyde were incubated with primary antibody to CD5 (Lyt1 53-7.313), major histocompatibility complex (MHC) class II (M5/114.15.2), B220 (RA3-3A1/61), or macrophage (F4/80). After washing in PBS, the sections were incubated with biotinylated rabbit anti-rat antibody. Bound antibody was visualized by the ABC detection system followed by diaminobenzidine and counterstained with methylene blue as described above.

Steroid Assays

For analysis of ovarian function, monkeys were bled every 3 days for 1 to 3 consecutive months at two phases of the study, with the first early phase starting 1 to 2 months before immunization until 2 months after immunization and the second late phase between 3 to 11 months when the antibody titers were high. Endocrine function was monitored by estimation of circulating steroid hormone levels. One hundred μl of serum was used in competitive radioimmunoassays for progesterone and estradiol estimations, as per the manufacturer's instructions (ICN Pharmaceuticals, Costa Mesa, CA, for progesterone and Diagnostic Products Corporation, Los Angeles, CA, for estradiol). The detection limit for progesterone was 0.27 ng/ml with an interassay coefficient of variation (CV) of 11.7% and intra-assay CV of 8.9%. For estradiol, the sensitivity limit was 27 pg/ml with an interassay and intra-assay CV being 7.4% and 4.5%, respectively.

Results

ZP3 Peptide from Macaque or Human Elicits an Autoimmune Response in Female Cynomolgus Macaques

Two normal, cycling, adult female cynomolgus macaques were immunized with 50 nmol/L of pZP3(mac) in a squalene arlacel A emulsion. Both animals (Babs and Harriette) had detectable serum anti-peptide antibody on day 24 after immunization (Figure 1A). The titers were maintained at 10^3 to 10^5 by subsequent booster immunizations until sacrifice. Their serum IgG reacted with macaque ZP by indirect immunofluorescence (Figure 1A, inset). Antibody bound to ovarian ZP was also detected by direct immunofluorescence at time of sacrifice (data not shown). Thus pZP3(mac) includes a native B-cell epitope. The antibody detected was of the IgG isotype suggesting a helper T-cell response and the presence of a T-cell epitope in pZP3(mac). T-cell proliferative response was detected *in vitro* by days 28 to 32 in both animals (data not shown). The T-cell proliferative response could also be recalled 10 days after the final booster immunization (Figure 1B).

Two macaques immunized with a homologous human ZP3 peptide [pZP3(hu)] were also studied for AOD. Therefore, we evaluated the immunological cross-reactivity between the two peptides. The human ZP3 protein has 94% homology with the monkey ZP3, and the peptides used in this study differ at only a single amino acid residue (340V to M).[19] As previously reported, macaques (Eenie and Meenie) were immunized with pZP3(hu) in adjuvant.[18] Antibody to pZP3(hu) of the IgG isotype was detected in the serum and titers sustained at 10^4 to 10^5 for >400 days by repeated boosting (Figure 1C). Antibody bound to ovarian ZP was detected by direct immunofluorescence at the time of sacrifice (Figure 1C, inset). Circulating antibody to ZP was also detectable by indirect immunofluorescence (data not shown). T cells from pZP3(hu)-immunized animals proliferated strongly in response to the immunogen and cross-reacted to a lesser extent with the homologous pZP3(mac) but not at all with pZP3(mou) (Figure 1D). These experiments demonstrate

Figure 3. Ovarian inflammation in pZP3(mac)- and pZP3(hu)-immunized macaques. **A–D:** Adjacent sections of a typical primate AOD lesion consisted of clusters of CD3+ T cells (**A**, **arrows**) and MHC II + cells (**B**, **arrowheads**). **C** and **D:** Adjacent sections of another typical primate AOD lesion stained for CD3 (**C**) and MHC II (**D**). Adjacent sections of a large granuloma in the ovary of Eenie that was immunized with pZP3(hu) in adjuvant. **E:** Typical granulomatous inflammation with eosinophilic center (**arrowheads**) surrounded by cuff of lymphocytes (**arrows**) (H&E). The granuloma contains many macrophage (Mac 387+) (**F**), a central core of MHC II-positive cells (**G**), and a rim of CD3+ T cells (**H**). Original magnifications, ×400.

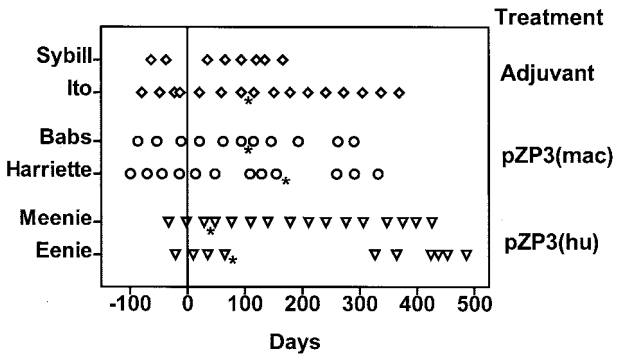


Figure 4. Ovarian cyclicity in adjuvant-immunized (Sybill, Ito), pZP3(mac)-immunized (Harriette, Babs), and pZP3(hu)-immunized (Meenie, Eenie) macaques monitored until sacrifice. Each point indicates time of menstrual bleeding. Time of first immunization is indicated as day 0. **Asterisk** denotes surgical removal of one ovary.

that the macaque and human peptides cross-react at the B-cell and T-cell levels even in outbred macaques. Our subsequent investigations showed that immunization with these immunologically cross-reactive peptides have similar effects on the ovary and on ovarian function.

pZP3(mac) and pZP3(hu) Immunization Is Associated with Ovarian Infiltration of T Cells and Activated Macrophages without Detectable Oophoritis

The ovary has many leukocytes that play a critical role in normal ovarian function including follicular atresia, ovulation, and involution of the corpus luteum.[20–22] Thus we first examined the distribution of T cells and macrophages in the ovaries from two untreated and two adjuvant-treated cycling adult macaques. As described in Materials and Methods, serial sections were cut through the entire ovary for each animal, and every 10th section was studied histologically. Adjacent sections were immunostained with antibodies to CD3, MHC II, macrophages (CD68, Mac 387) and B cells. As reported in humans,[23] T cells and macrophages expressing MHC II were detected in normal or adjuvant-treated monkey ovaries. In addition, atretic follicles and corpora lutea were infiltrated by small numbers of T cells and MHC II-positive macrophages (Figure 2, A and B). Occasional clusters of T cells were also located in the ovarian cortex and medulla (Figure 2C). However, these T-cell clusters did not contain any detectable MHC II-positive macrophages (Figure 2D).

In contrast, a unique pattern of cellular infiltration was detected in three of four pZP3-immunized monkeys. In these lesions, the T cells were found to co-localize with MHC II-expressing cells (Figure 3; A to D). These clusters of inflammatory cells were found in the interstitial or perifollicular locations. Because these typical leukocyte infiltrates were detected in pZP3-immunized monkeys but not in monkeys injected with adjuvant alone, they were considered to be pathologically relevant. Although macrophages (Mac 387 or CD68 positives) were detected in these T-cell- and MHC II-positive cellular infiltrates, they did not account for all of the MHC II staining in the inflammatory foci (data not shown). The additional MHC II-expressing cells may represent ovarian dendritic cells or granulosa cells of adjacent follicles, as described in human AOD.[24]

Interestingly, despite the impressive cellular infiltrates detected immunohistologically, inflammatory leukocytes were not visualized histologically on adjacent sections of the same ovaries. This was true for formalin-fixed or the Bouin's-fixed tissues. An exception was the ovary of Eenie, which was studied at 3 months after immunization with pZP3(hu) and adjuvant. This ovary contained several foci of granulomatous inflammation characterized by a central eosinophilic area surrounded by a cuff of lymphocytes (Figure 3E). Immunostaining of adjacent sections showed invasion of macrophages in the region (Figure 3F). The granuloma showed characteristic features of central MHC II-expressing cells surrounded by a ring of CD3-positive T lymphocytes (Figure 3, G and H). Histopathological evaluation of other organs collected at sacrifice failed to show any detectable inflammation.

Ovarian Function Is Retained in the Presence of Oophoritis

Menstrual bleeding in the pZP3-immunized and adjuvant-treated animals was monitored. The cycles in all of the animals except Eenie (the animal with granulomatous oophoritis) were found to be regular and comparable to adjuvant-treated controls (Figure 4). Eenie stopped menstruating for 7 months after ovariectomy after which menstrual bleeding resumed spontaneously. Sera were collected at two phases of the study to monitor endocrine function as described in Materials and Methods. The mean peak estradiol and progesterone levels studied early (when antibody titers were low) and late (when ZP antibody titers remained high) were comparable in all of

Table 1. Peak Serum Estradiol and Progesterone in Macaques Immunized with ZP3 Peptides

	Early phase		Late phase	
Animal	Peak estradiol (pg/ml)	Peak progesterone (ng/ml)	Peak estradiol (pg/ml)	Peak progesterone (ng/ml)
Babs	127.3 ± 79.5* (3)†	37.1 ± 13.5 (3)	56.9 ± 14.9 (2)	40.0 ± 31.82 (2)
Harriette	61.8 ± 37.5 (3)	24.6 ± 6.2 (3)	64.9 ± 44.2 (3)	21.9 ± 3.18 (2)
Meenie	145.7 ± 26.5 (3)	21.1 ± 5.5 (3)	84.3 ± 50.5 (2)	27.7 ± 7.4 (2)
Eenie‡	31.4 ± 11.7 (2)	26.9 (1)	119.6 (1)	15.7 (1)

*Data are mean ± SD of peak values in sera.
†No of peaks studied.
‡Includes only those cycles where menstrual bleeding was detected.

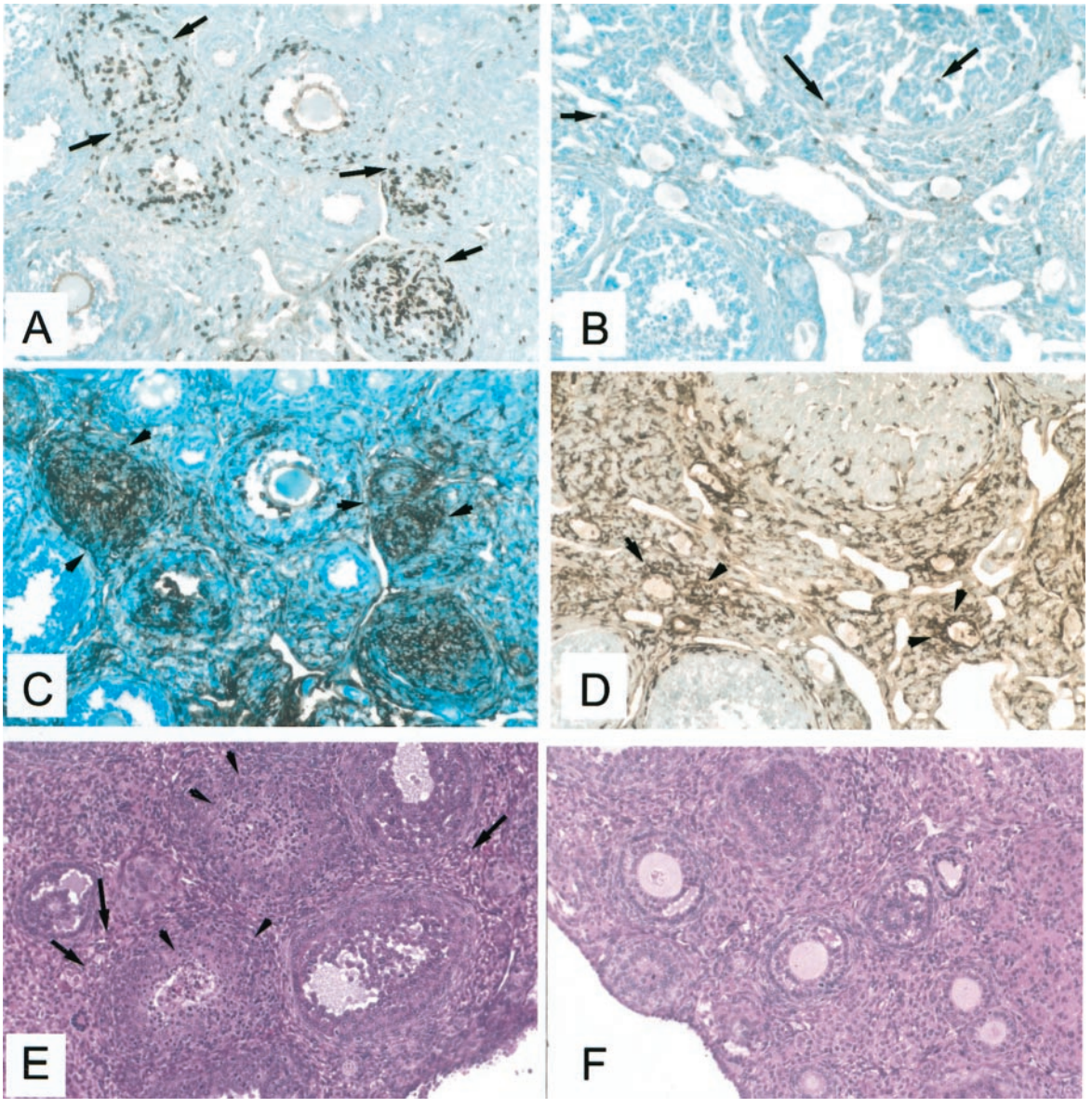


Figure 5. Immunohistopathology of murine AOD. **A**, **C**, and **E:** Ovary of a mouse immunized with pZP3(mou) in CFA. Adjacent sections showing clusters of cells T cells (CD5+) (**A**, **arrows**) that co-localize with intensely MHC II+ cells (**C**, **arrowheads**). The opposite ovary stained with H&E (**E**) shows lymphocytic infiltration in the interstitial regions (**arrows**) and the ovarian follicles (**arrowheads**). **B**, **D**, and **F:** Ovary of an adjuvant-treated mouse showing a few T (CD5+) cells (**B**) and an adjacent section (**D**) with many MHC II+ macrophages in the interstitium and the corpus luteum. The opposite ovary (**F**) stained with H&E appears completely normal.

the animals except Eenie (Table 1). Although the data collected is during a limited period of study, the pattern showed functioning ovaries with an estradiol peak preceding the progesterone peak (data not shown). Steroid analysis after ovariectomy in Eenie showed loss of cyclicity with estradiol <20 pg/ml and peak progesterone reaching only 2.3 ng/ml.

Comparative Histology and Immunopathology of Murine ZP3 Peptide-Induced AOD

AOD is induced by injection of pZP3(mou) in adult B6AF1 female mice,[12] but a detailed analysis of the immunopathology of the oophoritic lesion has not been described. To compare murine and macaque AOD, we evaluated mouse ovaries for the distribution of T cells (CD5), macrophages (F4/80), MHC II, and B cells (B220).

Immunostaining of the ovarian inflammatory infiltrates in murine AOD and the primate AOD are similar. In murine AOD, the infiltrating lymphocytes were predominantly T cells (Figure 5A), with only occasional B cells (data not shown). Like the lesion in macaque AOD, T cells were found to form clusters with F4/80-positive macrophages that expressed MHC II (Figures 5, A and C). Macrophages were normally present in large numbers in the normal mouse ovary. Adjuvant treatment resulted in the increase in ovarian macrophages that expressed MHC class II (Figure 5D), but there was no increase in T-cell infiltration (Figure 5B).

In contrast to macaque ovaries, histological evidence of oophoritis was readily detected in murine AOD. The lesions consisted of inflammation in the interstitium and some involvement of developing follicles (Figure 5E). The inflammation was focal or diffuse, frequently appearing as monocytic granulomata and in some cases, multinucleated giant cells. No inflammatory lesions were seen in animals immunized with adjuvant alone (Figure 5F).

Discussion

Human AOD is characterized by a wide spectrum of histopathological changes.[3–6] Lonsdale and colleagues[25] have summarized a comprehensive list of histologically confirmed reports of human autoimmune oophoritis. The inflammation may involve ovarian follicular structures or interstitial regions. The interstitial inflammation may be restricted to the hilar region, perifollicular regions, or may extend diffusely throughout the ovary. The wide range of inflammatory lesions is associated with varying clinical presentation from asymptomatic oophoritis, menstrual disturbances, to POF and infertility. Lymphocytes and plasma cells are the most common infiltrating cells. The distributions of inflammatory cells in normal human ovary defined by immunohistology serve as a baseline for identification of pathological infiltrates.[23] However, immunohistological studies on the ovarian disease are limited to three published reports on four patients.[25–27] They have identified T cells as the dominant infiltrating cells. Some macrophages were also detected in the infiltrates. Local production of proinflammatory cytokines such as interferon-γ by infiltrating T cells has been implicated in the aberrant expression of MHC II on granulosa cells at the site of inflammation.[24] These features are similar to our findings in the macaque and mouse ZP-induced AOD. Although typical granulomatous oophoritis resembling the inflammation seen in Eenie, has been reported in patients their immunohistological findings were not studied.[5] B cells and plasma cells are identified in the human AOD as well as murine AOD induced by neonatal thymectomy (preliminary data) but are rarely found in monkey or murine AOD induced by pZP3. Another type of human oophoritis contains mainly eosinophils,[28] and similar pathology has been elicited in mice by adoptive transfer of Th2 (interleukin-4 and interleukin-5 producing), pZP3-specific T cells (P Alard and KSK Tung, unpublished), and in pinworm-infected neonatal mice injected with pZP3(mou) without adjuvant.[29]

Immunization with zona pellucida proteins and peptides has also been investigated as immunocontraceptive vaccine. Vaccines based on the whole porcine zona pellucida proteins have been applied effectively to control feral horse and elephant populations.[30,31] As a potential vaccine for human population control, primates (marmoset, baboons, squirrel monkeys, and macaques) have been immunized with heterologous zona pellucida proteins. Although this approach resulted in infertility,[16,17,32] varying extents of irreversible ovarian dysfunction were also observed. Antibody to ZP was postulated to interfere with junctional complexes between granulosa cells and oocytes leading to the loss of developing follicles, and accelerated recruitment of the primordial follicles. However, other studies on squirrel monkeys and bonnet monkeys immunized with porcine ZP proteins with alum and muramyl di-peptide (MDP) adjuvants were found to develop high levels of circulating ZP antibodies. These studies showed a reversible contraceptive effect without any loss of ovarian function.[33,34] These contradictory data suggest that the high titers of ZP antibody alone are not sufficient to explain irreversible loss of ovarian function after ZP protein immunization in primates. Because no obvious inflammation was detected, autoimmune oophoritis was not considered a basis for ovarian failure in ZP-immunized primates.

The ZP3 peptide with well-defined T- and B-cell epitopes capable of eliciting a restricted immune response has been used to dissect the mechanism of AOD[12] in the mouse model. Transfer of ZP3 peptide-specific T cells into naïve recipient mice resulted in granulomatous oophoritis and enhanced ovarian expression of interleukin-1, tumor necrosis factor-α, and interferon-γ. However, the ovarian function of cell recipients was normal and the mice remained fertile.[13] Antibody to ZP3 also does not cause any ovarian pathology. These results indicate that T-cell-mediated ovarian inflammation alone or ZP antibody alone does not affect ovarian function. However, co-transfer of pathogenic T cells and ZP antibody targets the inflammation into developing follicles leading to their destruction and the development of ovarian atrophy.[14] Thus combined action of both antibody and proinflammatory T cells is required for severe ovarian injury leading to POF. In this study we show that ZP3 peptide immunization in primates can elicit a T-cell response and cause ovarian immunopathology that is similar to murine AOD. These data suggest a role for T cells in macaque AOD. It will be important to determine in the future whether the combined effects of antibody and proinflammatory T cells are responsible for the long-term ovarian changes in monkeys injected with ZP3.

Acknowledgments

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became pregnant. The records prior to treatment showed that in 24 doe-years, 34 fawns were born (1.4 fawns/doe-year). In contrast, during 54 doe-years posttreatment with SpayVac®, only one fawn was born (0.019 fawns/doe-year). SpayVac has also been proven to provide multi-year efficacy with a single dose in Fallow Deer (*Dama dama*), White-tailed Deer (*Odocoileus virginianus*), horses (*Equus caballus*), and Gray Seals (*Halichoerus grypus*).

Field Evaluation of the Immunocontraceptive, GonaCon-B, in Free-ranging Horses (*Equus caballus*) at Theodore Roosevelt National Park

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Unchecked free-ranging horse populations can have adverse effects on native plant communities and ecosystem processes, and may lead to conflict regarding appropriate resource allocation between sympatric grazing ungulate species. In response, many land management agencies have reduced free-ranging horse populations through periodic roundups and adoption or sale of excess animals. These methods are time and resource intensive, and can result in injuries to animals and humans. Consequently, more efficient, effective, and humane approaches are desired. One alternative is controlling the fertility of female horses through immunocontraception. We are currently in the final year of a 3-year study at Theodore Roosevelt National Park (ND) to evaluate the effects of the gonadotropin-releasing hormone vaccine, GonaCon-B, on fertility, behavior, and injection site reactions in female horses. In October 2009, park horses were captured via helicopter round-up. All mature mares and band sires were retained to avoid disrupting existing herd dynamics, whereas foals, yearlings, and most satellite males were removed. Mares were blocked according to age and pregnancy status and randomly assigned to a treatment ($n = 29$; 2.0 ml GonaCon-B) or control ($n = 28$, 2.0 ml 0.9% NaCl) group. During March and July 2010 there was no difference between treatment groups in the number of foals observed with mares ($P = 0.65$). During the same time period in 2011 treated mares were three times less likely to have a foal by side than control mares (odds ratio 0.11–1.01, 95% CI). Estimated foaling rate in 2011 was higher ($P = 0.04$) in control mares (0.74, 0.56–0.88) than treatment mares (0.48, 0.31–0.66). Between 25 and 280 days posttreatment nearly 80% of treated mares had visible swelling at the site of injection and a purulent draining abscess was observed in one animal. By 380 days posttreatment approximately half of these swellings were no longer visually detectable. No treatment effects were observed on mare activity budgets, and sociosexual behavior data are currently being analyzed. Our preliminary examination of the data indicate that vaccination with GonaCon-B during gestation does not affect foaling success, decreases fertility for one year posttreatment, is associated with injection site swelling, and does not affect general activity budgets in free-ranging female horses.

Effects of Gonadotropin-releasing Hormone Vaccination (GonaCon-B) on Fertility in Free-ranging Female Rocky Mountain Elk (*Cervus elaphus nelsoni*)

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Fertility control is one method, among a suite of management tools, which may assist in mitigating conflicts associated with locally overabundant wildlife. Rocky Mountain elk (*Cervus elaphus nelsoni*) are resilient, adaptable cervids that can have ecological as well as socio-political impacts on the coupled human-natural environment. We evaluated the safety and efficacy of the gonadotropin-releasing hormone vaccine, GonaCon-B, in free-ranging elk. In January 2008, 120 mature (>2 yr) female elk were captured in Rocky Mountain National Park (CO) and randomly assigned to treatment ($n = 60$; 1.5 ml GonaCon-B) or control ($n = 60$; 1.5 ml 0.9% NaCl) groups. All injections were given with hand-held syringes in the biceps muscle. Location of the injection was recorded in reference to anatomic landmarks. Transrectal palpation and pregnancy specific protein B analyses were used to diagnose pregnancy at the time of application. During the following three winters females were recaptured and euthanized in conjunction with park management culling operations (2009, $n = 20$; 2010, $n = 25$; 2011, $n = 34$). At necropsy pregnancy status was determined. Tissues were collected and preserved from organs associated with the hypothalamic-pituitary-gonadal (HPG) axis. Injection sites were relocated, muscle tissue collected, and microbial culture performed when gross evidence of inflammation was present. There were no differences in pregnancy rates between groups at the time of treatment ($P = 0.558$). In the following three years saline administered control females ($n = 36$) had a mean pregnancy rate of 0.83 (0.71 - 0.95, 95% CI). Pregnancy rate in the treatment group was 0.0 (0.0 - 0.22, $P = 0.0001$) year one, 0.33 (0.12 - 0.61, $P = 0.05$) year two, and 0.65 (0.43 - 0.83, $P = 0.17$) year three. Study animals showed no evidence of lameness or external swelling at the injection site prior to euthanasia; however, at the time of necropsy all females in the treatment group had pyogranulomatous inflammation at the site of injection.

Gonadotropin-releasing hormone vaccine (GonaCon-Equine) suppresses fertility in free-ranging horses (Equus caballus): Limitations and side effects of treatment

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In many areas of the western United States, overabundant and rapidly expanding feral horse (Equus caballus) populations pose a significant challenge for natural resource managers. There is wide-spread concern that unregulated feral horse populations are severely altering or degrading native plant communities. Controlling the fertility of female horses offers a potential strategy for limiting the growth of overabundant populations.

We evaluated the long-term effectiveness of GonaCon-Equine vaccine both as primary immunization and subsequent reimmunization four years later to suppress fertility in free-ranging female horses at Theodore Roosevelt National Park, North Dakota. In the fall of 2009, horses were gathered via helicopter and 57 adult mares were randomly assigned to either a treatment (n = 29; 2.0 mg GnRH conjugate + 2.0 mL adjuvant) or control (n =28; 2.0 ml 0.9% saline) group. Females in both groups received an intramuscular injection in the gluteal musculature by hand-injection. Four years later, in 2013, horses were similarly gathered and revaccinated with the same doses. We determined the effectiveness, duration, and reversibility of vaccination with GonaCon on reproduction by comparing foaling rates of treated and control mares during 1 March to 31 December, 2009-2016. In conjunction with foaling observations, we also evaluated potential side effects of GonaCon on pregnancy, neonatal health and survival, injection site reactions, daily activity patterns and socio-sexual behaviors.

Foaling proportions in GonaCon-vaccinated mares, following the primary vaccination were lower than for control mares for the second (2011) (44.8% (13/29) vs 70.3% (19/27) (P= 0.047) and third (2012) (53.6% (15/28) vs 77.8% (21/27) (P = 0.053) post-treatment foaling seasons but were absent the fourth (2013) (73.1% (19/26) vs 69.2% (18/26) (P = 0.50) post-treatment season, demonstrating reversibility of the vaccine. However, the effectiveness of a single immunization, measured as the percent decrease in foaling when compared with control mares, was low to modest; estimated at 36.3% and 31.1%, in years 2 and 3, respectively. In contrast, revaccination with GonaCon, four years post-primary treatment was 100% (0/25) effective in suppressing fertility, whereas mean foaling rate of control mares was 84% (21/25) (95% CI = 65.3-93.6%) in 2015 and 2016. The effectiveness of revaccination persisted, although slightly diminished, during the second year (2016) at 80.9% (16% (4/25) foaling rate; 95% CI = 6.4-34.6).

With the exception of non-debilitating inflammation at the vaccine injection site, no other adverse effects were observed. These data indicate that GonaCon-Equine can be effective in suppressing fertility in free-ranging horses when both a primary immunization and booster are administered. Ongoing research will inform the optimum revaccination schedule for effective contraception.

PLOS ONE

RESEARCH ARTICLE

Reimmunization increases contraceptive effectiveness of gonadotropin-releasing hormone vaccine (GonaCon-Equine) in free-ranging horses (*Equus caballus*): Limitations and side effects

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Abstract

Wildlife and humans are increasingly competing for resources worldwide, and a diverse, innovative, and effective set of management tools is needed. Controlling abundance of wildlife species that are simultaneously protected, abundant, competitive for resources, and in conflict with some stakeholders but beloved by others, is a daunting challenge. Free-ranging horses (*Equus caballus*) present such a conundrum and managers struggle for effective tools for regulating their abundance. Controlling reproduction of female horses presents a potential alternative. During 2009–2017, we determined the long-term effectiveness of GnRH vaccine (GonaCon-Equine) both as a single immunization and subsequent reimmunization on reproduction and side effects in free-ranging horses. At a scheduled management roundup in 2009, we randomly assigned 57 adult mares to either a GonaCon-Equine treatment group ($n = 29$) or a saline control group ($n = 28$). In a second roundup in 2013, we administered a booster vaccination to these same mares. We used annual ground observations to estimate foaling proportions, social behaviors, body condition, and injection site reactions. We found this vaccine to be safe for pregnant females and neonates, with no overt deleterious behavioral side effects during the breeding season. The proportion of treated mares that foaled following a single vaccination was lower than that for control mares for the second ($P = 0.03$) and third ($P = 0.08$) post-treatment foaling seasons but was similar ($P = 0.67$) to untreated mares for the fourth season, demonstrating reversibility of the primary vaccine treatment. After two vaccinations, however, the proportion of females giving birth was lower ($P < 0.001$) than that for control mares for three consecutive years and ranged from 0.0–0.16. The only detectable adverse side effect of vaccination was intramuscular

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swelling at the vaccination site. Regardless of vaccine treatment (primary/secondary), approximately 62% (34/55) of immunized mares revealed a visible reaction at the vaccine injection site. However, none of these mares displayed any evidence of lameness, altered gait or abnormal range of movement throughout the 8 years they were observed in this study. Our research suggests that practical application of this vaccine in feral horses will require an initial inoculation that may provide only modest suppression of fertility followed by reimmunization that together could result in greater reduction in population growth rates over time.

Introduction

Anthropogenic disturbance of landscapes and natural resources is pervasive across much of the earth, resulting in increased conflict between humans and wildlife and a need for effective resource management [1]. Humans indeed have tried to control animal abundance in some capacity for over 13,000 years [2]. Regulating abundance of wild animals using fertility control or contraception is a relatively new development, emerging only 50 years ago [3]. Such tools are appealing to wildlife managers and stakeholders because they present a non-lethal solution for regulating abundance when species pose a risk to human interests and safety, and when wildlife densities are high enough to disrupt ecosystem function [4,5].

Feral horses (*Equus caballus*) present perhaps one of the most unique wildlife management problems worldwide. Humans have spent centuries propagating and dispersing domestic horses to every continent except Antarctica over the last several centuries, only to have inadvertently created expansive feral populations that now compete with humans, wildlife, and domestic animals for resources [6]. The unique relationship between humans and horses has resulted in a precarious dichotomy, with the struggle for relief from conflict and resource competition challenged by a mutualistic societal view where feral horses are perceived as part of our social environment. This struggle is elevated in the United States, where federal law (*P*. L. 92–195, as amended) provides protection for feral horses and burros (*Equus asinus*) on large expanses of public land, and establishes guidance for their management as a wildland species [7].

Current methods of population control for free-ranging horses in the U.S. involve periodic removals and adoption or sale of surplus animals, or maintaining excess animals in long-term holding facilities which are expensive, resource intensive, and unsustainable [8]. Clearly, more efficient, cost effective, and humane approaches to reducing feral horse densities on public lands are needed. Controlling the fertility of female horses offers a potential complementary or alternative strategy for limiting the growth of some populations [9].

A promising immunological approach to contraception in feral horses and other wild ungulate species involves immunization against gonadotropin- releasing hormone (GnRH), a small neuropeptide that performs an obligatory role in mammalian reproduction [10]. When conjugated to a highly immunogenic carrier protein and combined with a potent adjuvant, GnRH vaccination actively stimulates a persistent immune response resulting in prolonged antibody production against endogenous GnRH. These antibodies induce transient infertility by binding to GnRH, thus preventing attachment to receptors on pituitary gonadotropes, suppression of gonadotropin release, and ultimately ovulation in females [11, 12]. As anti-GnRH antibodies decline over time, the availability of endogenous GnRH increases and treated animals generally regain normal fertility [13–17].

The GnRH-based contraceptive agent known as GonaCon-Equine (National Wildlife Research Center, Fort Collins, CO, USA; [18] is registered by the United States Environmental Protection Agency as a restricted-use pesticide for contraception of adult female feral horses and burros. A single immunization with this or earlier versions of this vaccine (more generally referred to as GonaCon) have been shown to induce extended infertility ($\geq$ 2 yr) in numerous wild ungulate species including captive and free-ranging elk (*Cervus elaphus*) [15–17] white-tailed deer (*Odocoileus virginianus*) [18–20], bison (*Bison bison* [21]), and feral horses [22– 24]. However, multiple years of infertility are only experienced in a fraction of vaccinated animals. In free-ranging elk, for example, there was approximately a 90% treatment effect the first year after vaccination but this declined to 50% by the second year; with no measurable effect by year three [16]. Similar declines in effectiveness have been reported for captive feral horses treated with the same vaccine [22].

Booster vaccinations generally result in a more profound and longer-lasting antibody production due to the anamnestic (cell memory) response [25]. Traditional veterinary vaccinology suggests that non-replicating vaccines most often require two initial doses 2–6 weeks apart followed by booster vaccinations every 1–3 years [26]. Repeat immunizations using a variety of GnRH vaccines in domestic horses improves contraceptive efficacy and suppress behavioral and physiological estrus [27–29]) However, these GnRH vaccines differ from GonaCon-Equine in that they incorporate different protein carrier molecules and adjuvants, and are formulated for short duration ($<$ 1 yr.) effectiveness. They are also administered on a more traditional vaccination schedule with a primary set of immunizations followed by periodic boosters.

Other forms of wildlife fertility control vaccines have adopted comparable initial and booster recommendations [30–32]. However, this intensive vaccination schedule places significant logistical barriers on application in free-ranging animals. GonaCon vaccine is formulated with highly immunostimulating mycobacteria as a component of the adjuvant. This may prolong the initial and subsequent booster vaccination windows for optimum efficacy as initial antibody concentrations are maximal 2–12 months post-primary vaccination [15]. GonaCon vaccine is one of the rare exceptions among animal vaccines in that the formulation initiates high antibody titers that remain elevated in some individuals after a single-injection ; however, little research has been conducted to evaluate booster doses of this vaccine in any free-ranging wild ungulate [17, 24] or domestic species [33]. While a single immunization against GnRH may be preferred from a practical perspective, there may be a more optimal vaccination schedule that balances the need for minimizing animal handling or contact while maximizing vaccine effectiveness. Thus, it's imperative to investigate the safety and long-term effectiveness of repeat vaccination and to evaluate its potential to limit fertility in this long-lived and perennially pregnant species.

In female wild ungulates, adverse side effects following a single immunization against GnRH appear to be minimal. Evaluation of biological side effects has been reported for numerous wild ungulate species including white-tailed deer [13, 34], elk [15, 16, 35], feral pigs [36], bison [21], and free-ranging horses [17, 24]. A summary of results from these investigations indicate that GonaCon is reversible, safe for use in pregnant females, does not significantly change social behaviors [37] or negatively affect neonatal development, survival, or maturation [15, 35]. No adverse effects of vaccination have been shown to be related to general health, body condition, blood chemistry parameters, or hematology of treated animals. The most apparent pathological side effect has been the development and persistence of non-debilitating granulomatous and often purulent inflammation at the site of injection. In all studies, where post-mortem examinations have been conducted, injection-site lesions were pervasive, but in some species, such as white-tailed deer and elk, they were not apparent antemortem. Likewise,

in cases where injection-site reactions have been documented, no clinical evidence of lameness, impaired mobility, or depression, have been reported [13, 15–17, 21, 24, 34, 35].

While documentation of contraceptive efficacy and side effects of GonaCon have been described for a variety of wild ungulates, similar evidence for feral horses is limited. To our knowledge, only two long-term ($\geq$ 3 years) empirical investigations have been conducted using GonaCon-Equine. These include a clinical trial with captive feral mares [22] and the other with free-ranging mares in a natural environment [23]. In the study with free-ranging horses, vaccination significantly reduced foaling rates of treated females, however, effectiveness was inconsistent over time and was substantially lower than that reported for captive feral mares treated with the same vaccine [22]. Furthermore, neither of these studies integrated revaccination as a strategy to increase vaccine efficacy. Lastly, these inquiries provide little quantitative evidence of the reversibility of the effects of this vaccine, the presence or absence of adverse side effects related to inoculation of pregnant mares, and neither examined the potential for increased side effects with reimmunization.

Knowledge of the effects of GonaCon-Equine on equid fetal health, neonatal survival, and body condition is largely anecdotal, whereas injections site reactions to booster immunization and the efficacy of revaccination are limited to two investigations [24, 33]. Clearly, additional research is needed to further define the long-term therapeutic effectiveness and contraindications of this potential technology before resource managers can make informed decisions regarding its practical application for stabilizing the growth rate of free-ranging feral horse populations.

Consequently, the fundamental objectives of this investigation were: 1) to determine the duration, effectiveness, and reversibility of both a single immunization and subsequent reimmunization against GnRH in suppressing reproductive rates of free-ranging mares in a natural environment, 2) to determine the safety and adverse side effects (if any) in free-ranging mares including assessment of general health, body condition, effects on current pregnancy, injection site reactions, and neonatal health and survival and, 3) to compare the effects of a single vaccination against GnRH on time budgets and social behaviors [37] to similar behaviors following reimmunization. Based on evidence from prior studies with feral horses and other wildlife species, we predicted ($H_{1:}$) that a single vaccination against GnRH would suppress fertility for multiple years with decreasing effectiveness over time but would not result in permanent infertility. Furthermore, we surmised ($H_{2:}$) that the anamnestic immune response to revaccination would be more effective and longer lasting in suppressing fertility than the initial immunization alone. Moreover, we reasoned ($H_{3:}$) that except for localized inflammatory reactions at the injection site, we would not observe other adverse side effects (i.e. lameness, detrimental effects on existing pregnancy, neonatal health and survival, body condition, behavioral changes). Apart from determination of return to normal fertility of treated mares, these objectives and hypotheses were addressed and accomplished in this investigation.

Materials and methods

Study area

We conducted this research in the South Unit of Theodore Roosevelt National Park (THRO), USA) (45˚ 55'N/103˚ 31'W). This unit is located near the town of Medora in southwestern North Dakota and encompasses approximately 19,000 ha of native vegetation. The landscape is topographically diverse and consists of eroded badlands with gullies and ravines separated by relatively large upland plateaus and small erosion-resistant buttes capped by scoria. Elevation ranges from 683 m to 870 m. Its continental climate is characterized by short, arid summers (mean temperature 21^0 C) and long, cold winters (mean temperature -12^0 C) [38].

Precipitation is irregular in amount and distribution with a long-term annual mean of 38 cm with most of this falling as rain showers from April to June [39].

Vegetation is primarily mixed-grass prairie dominated by needle-and -thread grass (*Hesperostipa comata)*, western wheatgrass (*Pascopyrum smithii)*, threadleaf sedge (*Carex filifolia)*, blue gramma (*Boutelous gracilis)*, and little blue-stem (*Schizachyrium scoparium)*. Cottonwood (*Populus deltoides)* gallery forests occur along perennial water courses while hardwood stands of green ash (*Fraxinus pennsylvanica)* and chokecherry (*Prunus virginiana)* dominate the upland drainages. Dense stands of Rocky Mountain juniper (*Juniper scopulorum*) are common on steep north-facing slopes [40].

Besides feral horses, sympatric wild ungulate species include bison, elk, mule deer (*Odocoileus hemionus)*, white-tailed deer, and pronghorn (*Antilocapra americana)*. Horses and bison are confined to the South Unit of the Park by a 1.8–2.4 m woven-wire boundary fence. Currently, horse numbers are controlled through periodic live capture and removal of select individuals. Free-ranging horses at THRO are classified by the National Park Service (NPS) as "feral livestock" and managed as a "historical demonstration herd". The most recent estimate of population size (2017) is 150–175 horses and the Park has set a management goal for this herd at approximately 50–90 animals.

The social structure of this population consists of 14–16 social groups (bands) that include a single dominant stallion, subdominant stallions, and 1–5 adult mares, yearlings, and foals of both sexes. Males greater than 1 year of age that have not acquired a band are usually found in ephemeral bachelor groups of 3–6 individuals. These bands are non-territorial and are spatially distributed across the South Unit primarily east of the Little Missouri River. All horses are known by unique coloration and markings and have been previously identified and assigned individual identifiers by managers. Photographs of each animal from birth to adulthood assist in the identification of individuals. Age, reproductive history, and genealogy data for each animal has been maintained since 1993.

In spring/summer 2009, we collected pre-treatment data on all mares and bands within THRO. The purpose of this effort was: 1) to determine the sample size and sampling intensity required to achieve acceptable statistical power ($\geq$ 80%) to detect fixed differences ($\geq$ 50%) in foaling proportions of experimental groups, 2) to assess unknown logistical limitations of locating and identifying specific study mares within bands of horses, and 3) to train field technicians to observation protocols, and collect pre-treatment time budget and social behavioral data.

Experimental animals and treatments

Primary vaccination (2009–2013). During a scheduled management roundup at THRO (18–23 October 2009), 160 horses were guided by helicopter into permanent corrals and handling facilities. An attempt was made to capture the entire population to maximize sample sizes for this research project and to remove excess horses to meet desired herd management objectives. A total of 57 adult mares (2–17 years of age) and associated foals, and band stallions, were captured, identified, treated, and retained in the Park for this experiment. Using a randomized complete block design, we established two experimental groups consisting of a GonaCon-Equine treatment group (n = 29) and a saline control group (n = 28). Mares were paired (blocked) based on age and pregnancy status such that animals within a block were as similar as possible. Within each block, individual mares were then randomly assigned to either a control or treatment group.

Equine veterinarians and a reproductive specialist, blinded to treatment status, assessed the general health, body condition, pregnancy status, and approximate gestational stage of each

mare. We determined pregnancy status and gestational age by transrectal palpation and ultrasonography of the reproductive tract [41]. We collected whole blood (up to 50 mL) via jugular venipuncture (BD Vacutainer SST; Becton Dickinson and Co., Franklin Lakes, NJ) then centrifuged these samples at the capture site, and temporarily stored serum in cryovials at -20^0 C. We later transferred frozen serum on dry ice to Fort Collins, Colorado, where it was stored at -80^0 C. We also assessed serum for exposure to common pathogens known to cause abortions in horses (e.g. equine herpesvirus-1, equine infectious anemia, equine viral arteritis and contagious equine metritis) that could confound the interpretation of treatment-induced infertility [42].

We applied treatments while mares were restrained in a squeeze chute. Females in the treatment group received an intramuscular injection in the lower left gluteal musculature, by hand-held syringe (18-gauge, 3.8 cm needle) containing GonaCon-Equine (2.0 mg GnRH conjugate + adjuvant; 2.0 mL). The vaccine contained multiple synthetic copies of GnRH coupled to a large immunogenic carrier protein (Blue Carrier; Biosonda, Santiago, Chile) that was combined with a water-in-oil adjuvant containing killed *Mycobacterium avium* ssp. *avium* (AdjuVac, National Wildlife Research Center) [18]. Mares in the control group were injected in a similar manner, with an equal volume of physiologic saline solution (0.9% NaCl; 2.0 mL). We chose to inject the vaccine into the gluteus muscle (~ 15 cm distal to the point of the hip) rather than the neck because of greater safety for hand-injection, enhanced detection of potential injection site reactions under field conditions, and the preferred location for potential remote dart delivery of the vaccine.

Secondary vaccination (2013–2017). Four years later, during 23–25 September 2013, we similarly rounded up the entire THRO horse population and moved and handled them through existing corrals and chute systems to remove excess animals from the Park. Given this unique opportunity and endorsement from the Park, we retained all available mares previously immunized and control mares, retreated them, assessed pregnancy status, and determined body condition using techniques identical to those applied at the 2009 roundup. Two mares in the control group and 4 mares in the treatment group died between 2009–2013 and therefore, were not available for this experiment. We attributed these mortalities to malnutrition, dystocia, broken appendage, and unknown causes not related to treatments. The one exception in our 2013 protocol was that we injected the booster vaccination into the opposite (right) hip from where the primary (left hip) vaccination was previously administered. This provided the opportunity to simultaneously evaluate injection site reactions related to both immunizations. Treatment mares again received 2.0 mL GonaCon-Equine and control mares 2.0 mL saline.

Field measurements

Using 2–3 trained technicians and occasional equally trained volunteers, we conducted field measurements and observations consistently from year to year. Prior to field observations, technicians were provided with photographic images of individual horses and required to recognize them by band association, natural markings, and pelage coloration. They were also trained or had previous experience in identifying prepartum characteristics of pregnancy (e.g., enlarged abdomen, mammary gland development, waxing teats, behavior, etc.), as well as, body condition scoring, and the appearance and classification of injection site reactions to the vaccine. We collected all data from ground surveys (foot, vehicle, horseback) using binoculars and spotting scopes. Although technicians were unaware of treatment assignments of individual mares, the presence of injection site reactions in several GonaCon-treated mares could have revealed their treatment designation.

Reproduction. We predicted that pregnant females inoculated with GnRH at the fall gathers of 2009 and 2013 would give birth to a healthy foal the following spring (2010 or 2014) and presumably be infertile during subsequent breeding seasons. Thus, the effects of the primary or booster vaccinations on reproduction (foaling proportions) would not be observed until the 2011 and 2015 foaling seasons, respectively. These are the first breeding seasons that a treatment or retreatment effect on mare fertility could be detected when using foaling observations to assess successful contraception by the vaccine.

We determined the effectiveness, duration of effects, and reversibility of the primary and booster vaccinations on reproduction by comparing foaling proportions of treated and control mares during 1 March to 31 December 2009–2017. We chose to use the term vaccine "effectiveness" rather than "efficacy" because it more realistically represents how GonaCon-Equine affects fertility under more natural field conditions compared to a controlled clinical trial [43, 44]. We defined vaccine effectiveness (VE) as the proportional reduction in annual foaling (F = number of mares with a foal/ total number of mares in a treatment group) between control and treated mares. Vaccine effectiveness is equivalent to relative risk reduction (RRR) in medical statistics and was calculated from the risk ratio $\left(\mathrm{RR} = \frac{\mathrm{F}_{Trt}}{\mathrm{F}_{Con}}\right)$ where F_{Con} = foaling proportion of the control mares, and F_{Trt} = the foaling proportion of the treated mares. Risk ratio was calculated using the *fmsb* package in program R [45–47] and we then solved for VE as follows:

$$\mathrm{VE} = \frac{\mathrm{F}_{Con} - \mathrm{F}_{Trt}}{\mathrm{F}_{Con}} = 1 - \frac{\mathrm{F}_{Trt}}{\mathrm{F}_{Con}} = 1 - \mathrm{RR}$$

Each year of the study, we estimated annual foaling proportions by locating all bands to identify individual mares and determine the presence or absence of foals. During the intensive sampling period (1 March–1 August), we attempted to observe 95% or greater of all experimental mares and foals (when present) at least weekly and 100% of them every two weeks, then opportunistically until 31 December. We did not attempt to assess contraceptive effect based on visual characteristics of pregnancy but did use these criteria to prioritize weekly observations of individual mares. Instead, we defined foaling as a parturition event or neonatal foal by side, as detected by direct observation. We matched foals with dams through observations of nursing and repeated close association during feeding, bedding, and traveling [48, 49]. We collected neonatal data at first sighting of a foal and estimated date of birth by observing the foal's level of activity, presence of an umbilicus, and elapsed time since the dam was last observed pregnant [50]. We photographed and estimated the age of each new foal when first observed, recorded its sex, general health (vigorous, average, poor), markings, and band association, and gave it a unique identifier; then entered these observations into a herd database. Finally, we assessed the utility of using foaling proportions as a proxy for pregnancy proportions by comparing pregnancy proportions determined at the time of each gather in 2009 and 2013 to foaling proportions observed in 2010 and 2014.

Side effects

Behavioral. We repeated thee behavioral measurements with the same treatment groups of mares that were previously conducted during an earlier phase of this project [37]. We proposed that, if greater contraceptive effectiveness after reimmunization against GnRH was achieved, it would potentially provide a larger and more statistically powerful sample size of contracepted animals in which to detect behavioral changes related to this vaccine (if they occurred).

We completed an intensive behavioral study during an earlier phase of this larger GnRH study during 2009–2010 [37]. To make these analyses directly comparable to that prior study, we followed the same behavioral sampling design as that described previously [50]. Briefly, this included blocking observations into three daylight time periods (08:00–12:00 h, 12:01–16:00 h, and 16:01–20:00 h) with observations conducted during the primary breeding season, 1 March– 1 August 2014. Each observation session included collection of a 20-min instantaneous scan sample of time budgets at 1 min intervals for each adult band member (≥1 year old), and all-occurrence data collection for social interactions [37].

Primary behavior categories included feeding, resting, locomotion, maintenance, and social behaviors [51]. Social behavior data included herding, reproduction, agonism, harem-tending, and harem-social behavior, and were collected at all occurrences throughout the observation sessions. Harem-social behavior was not collected through all-occurrence sampling in our previous study; however, it was collected during the scan sample in the previous study and was worthy of further consideration here. We defined this category as interactions between two individuals that did not meet the definition of the other all-occurrence behaviors (e.g. allogrooming and non-reproductive olfactory investigation).

We observed all horses from the nearest distance that did not elicit attention to the presence of the observer, typically 50–200 m. All observations were conducted using a 15–45 × 600 mm spotting scope or 10 × 42 mm binoculars when the distance between horses and observers was too far to allow unassisted detailed observation. We observed each band of horses weekly or bi-weekly in conjunction with other field assessments.

Physiological. Concurrently with foaling and behavior observations, we evaluated and compared potential adverse side-effects of treatment on injection-site reactions, body condition, success of existing pregnancy, and neonatal survival in treated and control mares. We made assessments of these potential side-effects monthly during the primary foaling season and opportunistically for the remainder of the year. We observed each mare for the presence or absence of visible lesions, swellings, or discharge at the injection site. In addition, we documented evidence of lameness (e.g. limping, gait alteration, reluctance to stand or bear weight on a limb), as well as behavioral depression, muscle tremors, or other systemic reactions that could be related to the vaccine treatment. We classified injection-site reactions according to the following criteria: 1) **abscess**–an open sore usually with fluid drainage or discharge, 2) **swelling**–a raised area of tissue of variable size and shape with no visible fluid drainage, 3) **lameness**–any abnormal range of movement or stiffness in the leg where the vaccine injection was delivered, 4) **none**–no observable reaction [52]. These categories were not mutually exclusive with respect to a single observation and both sides of the animal were observed, when possible. For these observations, we approached as near as possible to individual horses (≤ 50 m) and assessed and photographed each injection-site reaction for later evaluation. At the same time, we visually evaluated body condition of each mare and scored condition as previously described [53]. We evaluated the success of the existing pregnancy by comparing foaling proportions between treated and control groups in 2010 and 2014. We measured neonatal survival as the proportion of foals surviving to 14 days of age and post-natal survival to 200 days.

Statistical analysis

Reproduction. Yearly foaling data are reported as the proportion of mares observed with a foal in each group. We used asymptotic approximation to the binomial distribution to compute 95% confidence intervals for these proportions using package *binom* in program R [45, 47]. We used a risk ratio analysis ($\alpha = 0.05$) to compare all observed annual proportions between treatment groups. We used the same method to evaluate the success of the existing

pregnancy between groups during the first foaling season post-vaccination (2010 and 2014). All comparisons between treatment groups were made within a single year and without multiple testing corrections.

Behavior. We used the same statistical approach for the analyses in 2014 as that used in 2010 [37]. We modeled the frequency of each behavior using mixed-effects linear regression, where individual female identity and sampling time (time of day) were included as random effects on the intercept term of each model. This accounted for variation that may have been present among individuals who were sampled repeatedly, though not always equally over time, and for temporal variation in behavior when samples were not equally collected across all times of the day. Time budget behaviors sampled at 1-min intervals were aggregated into proportion of time spent per behavior to calculate an independent measure of behavior per observation session. We used the *lme4* package of R version 3.1.2 (The R Foundation for Statistical Computing 2014) and SYSTAT 12.02.00 (SYSTAT Software, Inc. 2007) to calculate descriptive statistics and obtain mixed-effects model estimates using restricted maximum likelihood [54]. Separate models were fitted for each time budget behavior with the fixed effects of treatment group (treated or control), foal presence (dependent foal $<$ 1 year of age present with the female, or no foal present with the female), female age, and band size. In the previous study, we considered band fidelity (number of times a female moved bands within a year), but data were too homogeneous to consider that factor in 2014: only 8 horses moved bands at all (4 treated/4 saline) and five of those moved collectively to a different stallion.

Physiological. We used descriptive statistics (arithmetic means with ± 95% CI) to compare, occurrence of lesions at the injection site and 1-tailed Fisher's exact test ($\alpha = 0.05$, 1df) to compare foal survival proportions of treated females to that of controls. We used normal binomial distributions to compute confidence limits for the differences between proportions using Jeffrey's interval for small sample sizes [55]. Effects on body condition scores were examined using generalized linear models in the *lmer* package in program R [56]. We employed random effects for year and individuals and then compared this nested model to full models which added the effect of either treatment or foaling using an ANOVA.

This research was approved by the Institutional Animal Care and Use Committees of the National Park Service (NPS) (Permit Numbers: MWR_THRO_Baker_Horse_2013.A3, MWR_THRO_Baker_Horse_2015.A3) and Colorado State University (IACUC Protocol No. 17-7651A). This study was conducted in accordance with good laboratory practices (GLP) and oversight from United States Department of Agriculture/National Wildlife Research Center (No.QA1647). All data collections were conducted after obtaining a scientific collection permit issued by Theodore Roosevelt National Park (THRO-2010-SCI-0010). All work, other than animal handling and vaccination at the two feral horse roundups, was observational. Every effort was made to prevent and minimize disruption of natural band dynamics and individual horse behavior and well-being during handling and treatment application.

Results

The statistical process used to select experimental mares for this investigation resulted in two treatment groups that were relatively homogeneous in age, body condition, body mass, and pregnancy status [S1 Table]. Results of pregnancy assessment indicated that most mares were pregnant at the 2009 (0.86 (49/57), 95% CI = 0.74–0.93) and 2013 (0.90 (46/51), 95% CI = 0.79–0.96) roundups, thus providing sufficient opportunity to evaluate and compare the safety and potential side effects of vaccine treatment on pregnancy and neonatal survival.

Transrectal ultrasonography revealed that the fetuses of most pregnant females were approximately 120+ days old at the roundup and that most had descended over the pelvic rim

preventing a more accurate assessment of gestational age at treatment application [57]. To provide a more precise estimate, we used an estimated gestation period for horses of 342 days [58] and the approximate foaling date (± 5 days) of each mare in 2010 and 2014 and then back-calculated to the date of treatment application at the 2009 (18–23 October) and 2013 (23–25 September) roundups. Using these calculations, we estimated mean gestational age at vaccine inoculation in 2009 to be 162 days (95% CI = 150–175) for treated mares and 154 days (95% CI = 138–170 days) for control mares. For 2013, we used the same calculation and projected that, on average, females were reimmunized against GnRH at approximately 129 days (95% CI = 105–151 days) of gestation and saline-treated control mares at 132 days (95% CI = 119–144 days).

Following the 2009 and 2013 roundups and release, experimental mares distributed themselves among 16–19 individual bands. At least one treated or control mare was present in all bands during 2010–2017. Likewise, the composition of adult mares in each band, as well as the band stallions, remained relatively stable during this period. By the end of the 2017 foaling season, 14% (4/29) of treated mares and 11% (3/28) of control mares had died of various causes (e.g., malnutrition, broken appendage, dystocia, unknown causes). Except for these mares and one vaccinated mare that was not re-captured at the 2013 gather, all others were observed for foaling and other field measurements for all eight years of this investigation.

We met our sampling objective by observing more than 95% of all mares weekly (and sometimes more often) from 1 March to 1 August each year of the study. It is possible that some foals were born and died without being detected but given the intensity of the sampling observations, we feel that this was highly unlikely. Observations during the remainder of the year and following winter were less intense and more opportunistic depending upon available personnel, weather, and road conditions. During this time, mortality of foals was more likely to have gone undetected.

Vaccine effectiveness

Primary vaccination (2009–2013). Mean foaling proportions of treated (0.62 (18/29) 95% CI = 0.44–0.79) and control (0.68 (19/28) 95% CI = 0.50–0.85) mares during the 2009 pre-treatment foaling season were not different ($P = 0.65$) indicating that prior to contraception, treatment groups exhibited equal fertility [S1 Table]. Further evidence was provided by individual mares at the 2009 gather and primary vaccine inoculation. The proportion of treated (0.86 (25/29), 95% CI = 0.71–0.95 and control (0.85 (24/28), 95% CI = 0.70–0.95) mares determined to be pregnant, via transrectal ultrasonography, were not different ($P = 0.63$) [S1 Table, Fig 1]. This provided an opportunity to compare the effects of GonaCon-Equine vaccination on the existing pregnancy of treated mares and neonatal health and survival to that of untreated control mares. Foaling proportions of treated (0.68 (19/28) 95% CI = 0.50–0.85) and control (0.64 (18/28), 95% CI = 0.46–0.82) mares during 2010 were not different ($P = 0.78$) (Fig 1). Births occurred from early March to early September with 97% (35/36) observed during the first four months of the foaling season (1 March to 1 June). Average foaling dates in 2010 for treated and control mares were 5 May (95% CI = 22 April–18 May) and 10 May (95% CI = 25 April–25 May), respectively. No foal was detected for 12 mares (6 treated: 6 control) that were determined to be pregnant at the 2009 gather. None of these mares showed evidence of pregnancy during the intensive foaling period or for the remainder of the year. We surmised that most of these foals were either aborted or died as neonates between the periods from 20 October 2009 (gather) to 1 March 2010 (beginning of foaling observations). Regardless of timing or cause of death, the proportion of mares that foaled in

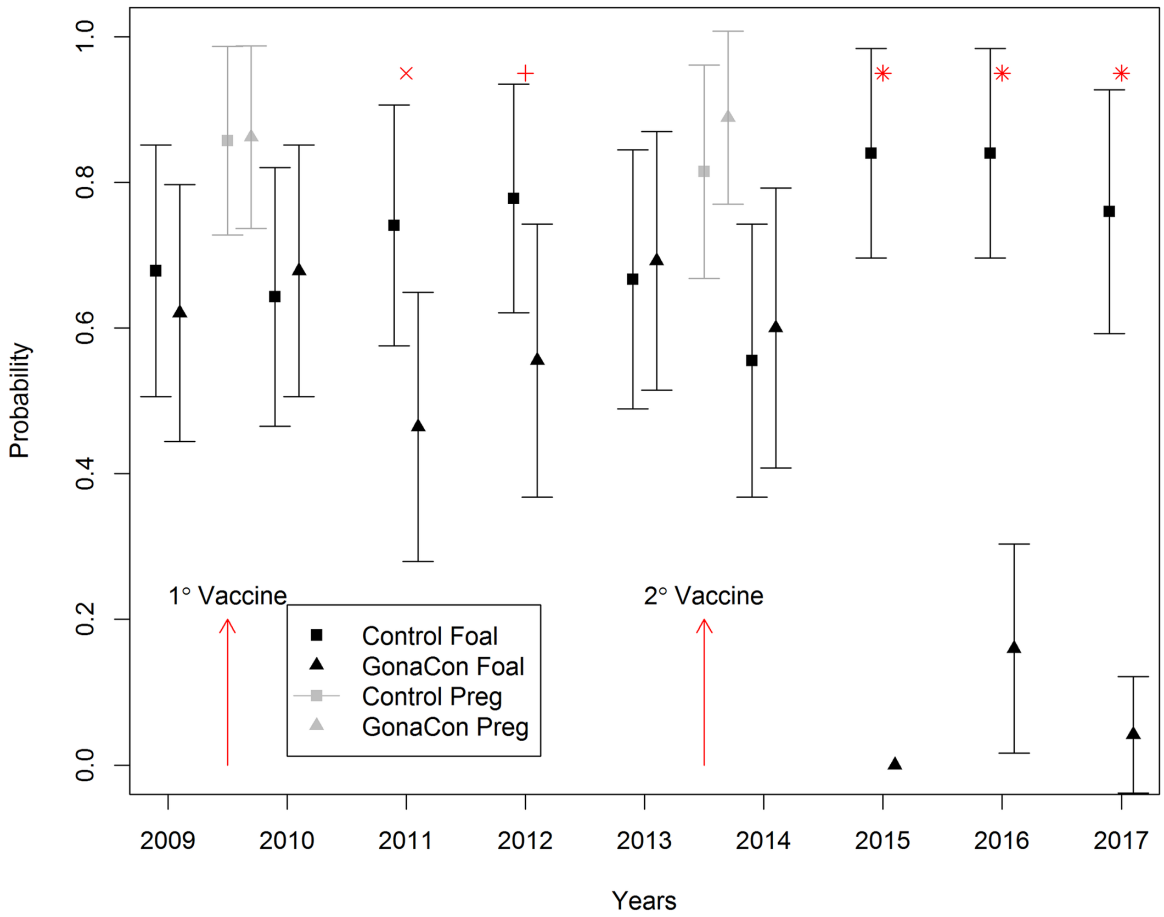


Fig 1. Comparative probability of foaling and pregnancy for treatment and control groups of free-ranging feral horses (*Equus caballus)* mares selected for this experiment. Mares were treated with a primary vaccination of GonaCon-Equine in October 2009 and then reimmunized with the same vaccine in September 2013 at scheduled gathers at Theodore Roosevelt National Park, North Dakota, USA. GonaCon vaccinations occurred at the time points represented by the red arrows. Symbols correspond to observed p-values for relative risk comparisons between treatment groups within years (p-value between 0.05 and 0.1 = +, for < 0.05 = x, and for $< 1x10^{-05}$ = *).

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2010 underestimated the proportion of mares that were determined to be pregnant at the 2009 gather by 24% for treated mares and 21% for control mares.

Estimated age of all foals at first observation was 2.4 days (95% CI = 1.7–3.1days). Most neonates (97%), from both experimental groups, were classified as vigorous and in good to excellent condition when first observed. Neonatal survival rate from parturition to 14 days of age was estimated to be 0.95 (18/19, 95% CI = 0.75–0.99) for foals born to GonaCon-treated females and 0.88 (16/18, 95% CI = 0.64–0.98) for foals born to control mares ($P = 0.54$). After 14 days of age, post-neonatal survival rates (14–200 da) averaged 0.97 (30/31, 95% CI = 0.84–0.99) and were similar for both experimental groups ($P = 0.57$). These results support our prediction (H_3) that inoculation with GonaCon-Equine vaccine, during approximately the second trimester of pregnancy, does not affect the existing pregnancy of treated females or neonatal health and survival.

The proportion of treated mares that foaled (13/28) following a single vaccination was lower than that for control mares (19/26) for the second (2011) ($P = 0.04$) and third (15/27 vs 21/27) (2012) ($P = 0.08$) post-treatment foaling seasons but was similar (18/26 vs (18/27)

($P = 0.67$) to control mares for the fourth (2013) season, demonstrating reversibility of the primary vaccine treatment (Fig 1). Even though we observed a significant reduction in foaling proportions between treated and control mares during 2011 and a declining effect in 2012, therapeutic effectiveness and relative risk reduction estimates were low to modest and estimated to be 0.37 (95% CI = 0.01–0.60) and 0.28 (95% CI = -0.06–0.51), respectively (Table 1). These findings lend support to our hypotheses *(H_1)* that a single vaccination with GonaCon-Equine is reversible and suppresses fertility for multiple years post-treatment in a portion of treated animals but with diminished effectiveness over time.

Secondary vaccination (2013–2017). At the scheduled gather in October 2013, we extended our evaluation of GonaCon-Equine by assessing the effects of revaccination on fertility and safety in these same experimental mares treated four years after the primary vaccination. Evidence of similar fertility for individual mares was demonstrated at the 2013 gather, where pregnancy proportions of treated (0.92 (23/25), 95% CI = 0.75–0.98) and control (0.88 (23/26), 95% CI = 0.71–0.96) mares were similar ($P = 0.86$) [S1 Table]. Except for one treated and one control mare, all others had conceived and given birth to at least one foal during 2009–2013. For the 2013 foaling season, foaling proportions of treated (0.69 (18/26), 95% CI = 0.51–0.87) and control (0.66 (18/27) 95% CI = 0.49–0.84) mares were not different ($P = 0.84$) providing additional evidence that treatments groups were of equal fertility prior to reimmunization (Fig 1).

Like 2010, mean foaling proportions during the first post-treatment foaling season (2014) were not different ($P = 0.74$) between treated (0.60 (15/25), 95% CI = 0.41–0.79) and control (0.56 (15/27), 95% CI = 0.37–0.74) mares (Fig 1) supporting similar observations in 2010 that revaccination could be applied to pregnant mares, during mid-gestation, without risk to the existing pregnancy. Foaling date distribution was comparable to that observed in 2010 following the primary vaccination. Average foaling date for treated mares was estimated to be 27 April (95% CI = 5 April– 20 May) and 19 April (95% CI = 6 April– 2 May) for controls. No foal was observed for 15 mares (8 treated: 7 control) that were determined to be pregnant at the 2013 gather. Like 2010 estimates, foaling proportions underestimated pregnancy proportions determined at the 2013 gather for both treated and control mares by approximately 30% and 34%, respectively (Fig 1). These data, together with similar observations in 2010, support the inference that foaling proportions are not an accurate proxy for pregnancy proportions but provide a limited but practicable field measurement for determining contraceptive

Table 1. Comparative relative risk reduction (RRR), 95% confidence intervals, and p-values associated with differences in foaling proportions between GonaCon-treated and control mares during 2009–2017.

Year	Relative Risk Reduction	95% Confidence Interval		p-value
	(RRR)	Lower	Upper	
2009	0.0852	0.3757	-0.3402	0.6500
2010	-0.0555	0.2750	-0.5301	0.7797
2011	**0.3732**	**0.6028**	**0.0109**	**0.0381***
2012	0.2857	0.5178	-0.0581	0.0861
2013	-0.0384	0.2826	-0.5032	0.8430
2014	-0.08	0.3216	-0.7194	0.7482
2015	**1**	**1**	**NA**	**2.57E-09***
2016	0.8095	0.9236	0.5247	1.94E-06*
2017	0.9451	0.9920	0.6217	4.15E-07*

*Significant p-values (<0.05).

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effectiveness. Average foal age at first observation across both treatment and control groups was 2.6 (95% CI = 1.5–3.3) days. Nearly all foals born to revaccinated and control mares were classified as vigorous and found to be in good to excellent condition when first observed.

Neonatal survival rate to 14 days of age for foals born to revaccinated mares was 0.87 (13/15), 95% CI = 0.62–0.96 and 0.93 (14/15), 95% CI = 0.70–0.98) for foals born to control mares ($P = 0.49$). After 14 days of age, post-neonatal survival rates were 0.80 (12/15), 95% CI = 0.55–0.92) for revaccinated mares and 0.73 (11/15), 95% CI = 0.48–0.89) for control mares ($P = 0.55$). These results reflect similar findings following a primary vaccination with GonaCon-Equine and reinforces the deduction (H_3) that reimmunization is safe for treatment of pregnant females and does not affect neonatal or post-neonatal health or survival when applied at approximately mid-gestation.

Unlike results from the single vaccination trial, we observed, not only highly significant reduction in foaling proportions between treated and control mares following reimmunization but also a remarkably effective contraceptive response. Except for the first foaling season following treatment application, (2014) in which the vaccine was not expected to have an effect ($P = 0.75$), foaling proportions in reimmunized mares were lower ($P < 0.001$) than that for control mares for all subsequent years (2015–2017) (Fig 1). This was particularly evident for the second post-treatment foaling season (2015) when none 0.00 (0/25), 95% CI = 0.0) of the reimmunized mares produced a foal while the proportion of control mares foaling was estimated to be 0.84 (21/25, 95% CI = 0.69–0.98). During the third post-treatment foaling season (2016), four treated mares produced a foal resulting in a foaling proportion of 0.16 (4/25), 95% CI = 0.01–0.30) while the proportion of control mares foaling was identical to that observed in 2015 (Fig 1). These foals were determined to be vigorous and in good to excellent condition at birth, however, two of these foals, born in September, were not observed the following spring and were categorized as post-natal mortalities and presumed to have died during winter (2016/2017).

In 2017, no additional treated mares produced a foal or showed evidence of pregnancy. However, one of the treated mares that had foaled in 2016 died of apparent natural causes (age-related malnutrition) during 2017 and two other revaccinated mares that had foaled in 2016 failed to produce a foal that year resulting in a foaling proportion of 0.041 (1/24), 95% CI = 0.03–0.12) (Fig 1). The foaling proportion for mares in the control group (2017) was 0.84 (21/25, 95% CI = 0.69–0.98) and higher ($P < 0.001$) than that for GonaCon-treated mares (Fig 1). It should be noted that the apparent decrease in foaling proportions in GonaCon-treated mares from 2016–2017 and resulting increase in vaccine effectiveness (Table 1) is likely due to the inherent error associated with the small sample size ($n = 4$) of mares in this treatment group that regained fertility. Overall, there was both a substantial decrease in foaling proportions (Fig 1) and an exceedingly high level of effectiveness (Table 1) for treated mares compared to controls for 3 years post-revaccination (2015–2017) ($P < 0.001$). Thus, fertility measurements during 2015–2017 support our prediction (H_2) that revaccination with GonaCon-Equine would be more effective in suppressing foaling proportions in treated females compared to controls than a single immunization (Fig 1, Table 1).

Side effects

Behavioral. We collected behavioral data on 73 feral horses (22 males, 25 treated females, 26 saline females) for 218.3 h in 2014. The median age of observed stallions was 12 years (range = 9–19 years), median age of observed control females was 8 years (range = 7–20), median age of observed treated females was 9 years (range = 7–22), and median band size was 8 horses (range = 2–14). There were no differences detected between treatment groups in any

time budget behavior category (Table 2). As band size increased, feeding decreased 1.24% (95% CI = 0.48–2.00) per additional horse in the band. Likewise, locomotion increased 0.20% (95% CI = 0.07–0.33) and maintenance decreased 0.10% (95% CI = 0.01–0.19) per additional horse in the band.

Foal presence influenced locomotion, with barren females moving 1.24% (95% C I = 0.40–2.08) more than females with dependents. Foal presence also influenced the social behavior component of time budgets, with barren females interacting with others 2.74% (95% CI = 0.59–4.90) more than females with dependents.

Variance among individuals had little influence on any of the behaviors modeled (Table 2). Variance was also minimal between time periods of observation; however, there were some significant differences in amount of activity by time of day. An estimated 6.88% (95% C I = -0.73–14.5) more feeding occurred in the 1601–2000 h time-period than did earlier in the day, and this was reciprocated by an estimated 3.33% (95% CI = 1.09–7.79) less resting, 0.34% (95% CI = 0.15–0.82) less maintenance, and 1.30% (95% CI = 0.51–3.11) less social behavior during the same period.

There were no differences detected between treatment groups in herding, reproduction, or agonism, but treatment group did influence harem-social behavior. Observed instances of harem-tending behavior provided too few data to model. Because these social behaviors were not as dependent on other broad categories as is the case with compositional time budgets [51], we re-estimated the social behavior models with only treatment and supported effects to allow for clearer interpretation of the results.

Stallions initiated harem-social behavior 13.9% (95% CI = 3.25–24.68) less toward control females than toward treated females. Though all harem-social records were analyzed as a group, it should be noted that 55.8% of the 308 harem-social events were sub-categorized as allogrooming. While the significant difference between treatment groups was detected, the variance among individuals for this behavior was near zero (Table 2).

Physiological. No study mares exhibited antibody titers to any of the infectious diseases that were surveyed for (i.e., equine herpesvirus-1, equine infectious anemia, equine viral arteritis and contagious equine metritis) thus eliminating this factor as a potential cause of infertility in GonaCon-treated females.

No control mares, treated with saline, showed any evidence of injection site reactions. Swelling and discharge were never observed in this group. Likewise, these mares showed no evidence of lameness or gait abnormalities in either hind limb. Consistent with our hypothesis (H_3), approximately 72% of treated mares (21/29) displayed a visible reaction at the site of injection after a single vaccination with GonaCon-Equine (S1 Photo). A single mare developed a draining abscess after the initial vaccination. These lesions were persistent over multiple years. At the time of the 2013 roundup and revaccination, 81% (21/26) of vaccinated mares continued to have palpable swelling at the original site of vaccine injection.

Like initial vaccination reactions, during the first-year post-revaccination, approximately 50% (13/26) of mares continued to show swelling on the left hip at the site of the 2009 injection and 50% developed a reaction on the right hip at the site of revaccination in 2013. Two of these new reactions were draining abscesses. Yet again, injection site reactions were persistent with approximately half of the mares with swellings at one or both injection sites, 3 years after revaccination. None of the GonaCon-treated mares displayed any evidence of lameness, altered gait or abnormal range of movement throughout the 8 years they were observed.

While body condition varied between individuals and study years, it did not vary between treatment groups ($P = 0.14$) over the course of the study. Likewise, there was no effect of presence of a foal on body condition ($P = 0.16$). Average body condition ranged from 3.7–4.9

Table 2. Treatment and supported effects in a mixed-effects linear regression of feral horse (*Equus caballus*) time budget behaviors (e.g. feeding, resting, locomotion, maintenance, social) and all-occurrence social behaviors (e.g. herding, reproduction, agonism, harem- social) at Theodore Roosevelt National Park, USA. Variance for the random effects of time of day (j) and individual horse identity (k) are shown as σ_j^2 and σ_k^2.

Behavior	Effect	*t*	*P*	Difference	95% confidence limit		σ_j^2	σ_k^2
					Lower	Upper		
Feeding	Treatment	-0.125	0.900				0.004	0.003
	Band Size	-3.193	0.001	-0.012	-0.020	-0.005		
Resting	Treatment	0.590	0.555				0.001	0.001
Locomotion	Treatment	-0.143	0.886				<0.001	<0.001
	Band Size	3.047	0.002	0.002	0.001	0.003		
	Foal Presence	2.900	0.004	0.012	0.004	0.021		
Maintenance	Treatment	-1.193	0.233				<0.001	<0.001
	Band Size	-2.238	0.025	-0.001	-0.002	-0.001		
Social	Treatment	-0.037	0.970				<0.001	0.001
	Foal Presence	2.499	0.013	0.027	0.006	0.049		
Herding	Treatment	-0.909	0.368				0.009	<0.001
Reproduction	Treatment	1.555	0.159				<0.001	<0.001
Agonism	Treatment	0.669	0.528				<0.001	0.048
Harem-social	Treatment	2.620	0.012	0.140	0.033	0.247	0.007	<0.001

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(moderately thin to moderate body condition) for all study animals over the 8 years that mares were observed. Individual body condition scores ranged from 1–7.

Discussion

Reproduction

This study demonstrated that a single vaccination against GnRH, using GonaCon-Equine, administered during mid-gestation, was safe, initiated short duration (2 yrs.) infertility in some mares, and was reversible, but was minimally effective in reducing fertility of treated females compared to controls. For two foaling seasons following vaccine treatment, we observed statistically significant reductions (28–38%) in foaling proportions of treated versus control mares but no effect by the third-year post-treatment, thus confirming the reversibility of the vaccine.

These results parallel similar findings from other experimental evaluations of GonaCon-Equine reported for captive and free-ranging mares. In a comparable study in Nevada with feral horses in a natural environment, GonaCon-Equine reduced foaling proportions by an average of 33% over a 3-year period but, like our study, contraception was only modestly effective over this period [23]. In contrast, contraceptive effectiveness of captive mares treated with GonaCon was greater and longer lasting ($\geq$ 4yrs) than either of these studies [22]. The disparity between captive and free-ranging animals in contraceptive response to GonaCon vaccine is not limited to feral horses but has also been observed between captive and free-ranging white-tailed deer [14, 18, 20] and elk [15, 16, 19]. Although these investigations did not suggest a definitive causation for these differences, they all pointed to suppressed and less persistent GnRH antibody concentrations in free-ranging ungulates compared to their captive counterparts suggesting a relatively compromised or weakened immune response to the vaccine that resulted in reduced contraceptive effectiveness.

It is widely acknowledged that differences in vaccine effectiveness can be attributed to increased environmental stressors (i.e., nutritional status, injuries, parasite load, pathogen exposure, and social dynamics) that can inhibit a more vigorous immune response in free-

ranging animals in a natural environment [59, 60]. It follows that while efficacy trials with captive animals can provide an important first approximation of vaccine safety and performance under controlled conditions; they may offer only limited inference to free-ranging animals that are not buffered against natural stressors that may decrease immune response and vaccine effectiveness. Regardless of the factor(s) contributing to the limited effectiveness of GonaCon in free-ranging animals, it appears that the immune response from a single vaccination does not consistently provide multiple years of infertility in all or even a high proportion of these animals.

In comparison to a single inoculation with GonaCon-Equine, the effect of reimmunization on foaling proportions was highly significant which allowed clear differentiation between treated and control mares for multiple breeding seasons. Compared to a single vaccination, reimmunization of mares in this study resulted in a much higher (58%) average vaccine effectiveness (range = 0.80–0.94) than the single vaccination for a 3-year period (2015–2017). Likewise, this level of effectiveness following reimmunization was on average higher than that previously reported for free-ranging mares treated with a single application with GonaCon-Equine [23] and 32% above what was reported for captive mares treated with the same vaccine formulation [22]. These results support the conclusion that a booster immunization with GonaCon-Equine can provide a highly effective, multi-year suppression of fertility in free-ranging horses and these results may be consistent in other animal species, as well.

It is fundamental knowledge that a secondary response to a vaccine generally results in a more rapid production of antibodies that are produced in greater amounts and over a longer time compared to the primary vaccination [25]. Repeat immunizations using a variety of GnRH vaccines in domestic horses have been shown to improve contraceptive efficacy. However, unlike commercially available short duration vaccines ($<$ 1 yr.) developed for domestic horses [29, 61], GonaCon-Equine is formulated by combining a non-biodegradable oil in water-based emulsion and an optimum concentration of immunostimulatory killed mycobacteria to form a depot usually deep in muscle tissue. This depot injection is thought to allow for a slow release and prolonged stimulation so that the formulation can act for much longer periods of time (years) than is possible with standard injections (months). This effect is thought to be responsible for the extended antibody response of 3–4 years in vaccinated deer [14, 18, 20, 62], elk [15, 16], and horses [22].

While this response was not unexpected, the magnitude and duration of effectiveness of GonaCon-Equine following revaccination, even 4 years after the initial vaccination, is salient and relevant to the management of fertility in free-ranging horses. First, it demonstrates that a booster vaccination can stimulate a highly effective immune response that can result in multiple years ($\geq$ 3 yrs.) of contraception. Second, it provides an initial reference point for defining the optimum revaccination schedule required for long-term reproductive management of female horses in a natural environment. And finally, it supports the consideration that while a single application may be preferred from a practical management perspective, GonaCon-Equine is more effective, in free-ranging horses, if repeat vaccinations are delivered on a periodic basis. While initial results are encouraging, additional research is needed to complete the objectives of this study including: 1) to define the duration of effective contraception post-revaccination, 2) to determine if long-term or permanent infertility is a possible outcome, and 3) to assess if return to fertility (if it occurs) results in altered birth phenology of treated mares. We will investigate these questions over the next three years of this study. Additionally, there may be a more optimal revaccination schedule which allows for altered duration of effectiveness or is more conducive to management schedules.

Side effects

After revaccinated in October 2013, time budget and social behaviors of mares in spring/summer of 2014 were comparable to those observed during the same period in 2010, following the initial treatment in October 2009. We found no evidence of differences in frequency or intensity of social behaviors including estrous behavior associated with treatment. Both treatment and control groups displayed few estrus behaviors in either 2010 [37] or during 2014. Behaviors associated with estrus were observed only 17 times in treated and 57 times in control mares out of 1148 observed social behavior events. This supports our earlier findings that pregnant mares rarely show overt estrous-related behaviors and similarly GonaCon-Equine treated mares only occasionally display these behaviors, although each for different reasons. Once a mare is pregnant, progesterone likely subverts much of the estrous type behavior that would generally be displayed with high estrogen levels, and only occasionally do domestic horses display and stand for mounting when pregnant [63]. Relatively small amounts of estrogen are secreted as follicles develop and then regress. In the absence of progesterone, relatively small amounts of estrogen are likely sufficient to induce erratic estrous behavior as was observed in these mares. However, the small amounts of estrogen were likely insufficient to induce an LH surge and subsequent ovulation.

Regardless of the underlying endocrinology associated with these behaviors, vaccinated and control mares both displayed social interactions that maintained herd structure; herding, tending, and defending behaviors from the stallion; and social hierarchies. The only meaningful factor that influenced the amount of time spent in social behaviors (e.g. allo-grooming, herding and tending) was the presence or absence of a foal. Mares with foals spent more time alone with the foal than those without off-spring, which is to be expected given their social and nutrient requirements during the neonatal and post-natal periods [50]. It is possible that long-term absence of foals could influence social behavior on a longitudinal scale, but additional studies are needed to investigate such phenomena on an appropriate time scale.

Other techniques for reducing the fertility of free-ranging species, such as vaccination with the native porcine zona pellucida vaccine (PZP) and tubal ligation, maintain the competency of the endocrine aspects of fertility. This can lead to unintended consequences with repeated estrous cycling in polyestrous species. In fact, in a population of white-tailed deer, where most reproductive females had received tubal ligations, fawning was negligible; however, there was more than a 700% increase in the number mature males attracted to the area occupied by a high number of estrous cycling females [64]. Similarly, PZP vaccination has extended the length and intensity of breeding seasons in horses [49, 65–68], deer [69, 70], and elk [71]. GonaCon-Equine may avoid these inadvertent consequences by functionally inducing mimicry of pregnancy in females which continues to be an important part of the social structure of the group but does not invite intense adverse breeding behaviors.

Researchers have generally hypothesized that by alleviating the energetic demands of gestation and lactation, contracepted females will attain improved body condition over pregnant females that require additional food resources to produce and rear an offspring. However, for free-ranging large ungulates, empirical evidence supporting [72] or refuting [73–75] this prediction is limited and equivocal. In this investigation, contracepted mares that experienced no gestation and lactation did not exhibit improved body condition over mares that successfully reproduced. Individual mares in each experimental group, attained an average BCS of 5.0 (moderate) or better, which has been reported to be the minimally optimal level of stored fat necessary to achieve maximum reproductive efficiency during pregnancy and lactation [53, 76]. These levels of body condition were reflected in the high proportion of pregnant mares (0.85–0.92) observed in each treatment group at the management roundups in 2009 and 2013.

We acknowledge that our sampling intensity and/or sensitivity of our ocular index to body condition may not have enabled us to detect fine-scale differences between experimental groups. However, we conducted these evaluations during time periods when differences in body condition between pregnant and non-pregnant (GonaCon-treated) females should have been the greatest. Namely, during early spring (March) when fats deposits are depleted over winter and during April–August when the energetic demands of late gestation and lactation are increasing.

The body condition of an animal is dependent on a balance between energy intake and expenditure. When intake is not sufficient to meet energy requirements for various activities (i.e. maintenance, growth, activity, gestation, lactation, etc.), fat reserves and eventually lean body tissue will be lost. The fact that pregnant and lactating mares in this study were in similar body condition to that of contracepted ones suggest that food is unlikely a limiting factor for free-ranging horses at THRO. This is primarily due to the conservative management of multiple species of ungulates and their food resources [77–79]. The consequence of this approach is that only under the most extreme climatic conditions, such as prolonged drought, will forage be limiting to herbivores at THRO, regardless of reproductive status.

The only detectable adverse side effect of vaccination was intramuscular swelling at the vaccination site. Mares treated with GonaCon-Equine consistently showed evidence of inflammatory reactions at the injection site. While we never observed lameness associated with this reaction, several mares revealed draining abscesses within one-year post-vaccination. This is consistent with results for other wild ungulates treated with the same or similar GonaCon vaccines [13, 15, 34]. Given the designed highly inflammatory nature of both the adjuvant, which contains killed mycobacteria and non-biodegradable oil, as well as, the foreign protein carrier molecule, these types of reactions are predictable. In fact, they are likely necessary for optimum vaccine efficacy [80]. It is impossible to assess the total impact of these lesions on animal welfare; however, in this investigation, these did not have a measurable effect on body condition, locomotion, or social behaviors. Therefore, until additional research suggests otherwise, we conclude that the presence of injection site lesions following GonCon vaccination do not pose a serious contraindication associated with the application of this vaccine, and there appear to be minimal long-term effects on individual animal welfare.

Conclusions

Controlling abundance of wildlife species that are simultaneously protected, abundant, competitive for resources, and in conflict with some stakeholders is a formidable challenge for resource managers. We demonstrated that the GnRH vaccine, GonaCon-Equine, could be an effective immunocontraceptive for free-ranging feral horses, particularly when the primary vaccination is followed by reimmunization four years later. This vaccine was shown to be safe for pregnant females and neonates and did not result in deleterious behavioral side effects during the foaling/breeding season. The only adverse reactions to vaccination were non-debilitating inflammatory responses at injection sites. One noteworthy implication has emerged regarding long-term management of free-ranging horse populations using GonaCon-Equine vaccine: effective management and development of population models will need to incorporate repeat immunizations of this vaccine to optimize management strategies aimed at stabilizing the growth rate of feral horse populations. Our research suggests that practical application of this vaccine in feral horses will require an initial inoculation that may provide only modest suppression of fertility followed by reimmunization over time that together could result in greater reduction in population growth rates. Future research will begin to define the most effective revaccination schedule with GonaCon-Equine for suppressing reproductive rates in

free-ranging horses, the duration of effectiveness, and the return to fertility following treatment. Moreover, applying GonaCon-Equine to control the growth of feral horse populations will require that resource managers choose specific tactics for treating animals. Choices must be made on the number and age to treat and the frequency of treatment needed to maintain the desired population age structure and genetic diversity. Decisions on the most beneficial tactics will depend on overarching management goals and long-term objectives for the population.

Supporting information

S1 Table. Comparative metrics and pregnancy proportions, by age class, for treatment and control groups of free-ranging mares selected for this experiment.
(PDF)

S1 Photo. An Intramuscular swelling (raised area of tissue) observed in a Theodore Roosevelt National Park horse, 9 months after hand-injection with GonaCon-Equine vaccine.
(TIF)

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Immunization against gonadotropin-releasing hormone in dairy cattle: Antibody titers, ovarian function, hormonal levels, and reversibility

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ABSTRACT

Suppression of cyclic activity in cattle is often desired in alpine farming and for feedlot cattle not intended for breeding. A cattle-specific anti-GnRH vaccination (Bopriva, Zoetis Australia Ltd., West Ryde, Australia) is approved for use in heifers and bulls in New Zealand, Australia, Mexico, Brazil, Argentina, Turkey, and Peru. Eleven healthy, cyclic Swiss Fleckvieh cows were included in the study and vaccinated twice with Bopriva 4 wk apart. Injection site, rectal body temperature, and heart and respiratory rates were recorded before and 3 d following each vaccination. Blood samples were taken weekly for progesterone and estrogen analysis and to determine GnRH antibody titer. Ovaries were examined weekly, using ultrasound to count the number of follicles and identify the presence of a corpus luteum. Thirty weeks after the first vaccination, the cows were subjected to a controlled internal drug-releasing device-based Select-Synch treatment. The GnRH antibody titers increased after the second vaccination and peaked 2 wk later. Estrogen levels were not influenced by vaccination, and progesterone level decreased in 7 of 11 cows up to 3 wk after the second vaccination and remained low for 10 to 15 wk following the second vaccination. The number of class I follicles (diameter $\leq$5 mm) was not influenced by vaccination, whereas the number of class II follicles (diameter 6–9 mm) decreased between 7 and 16 wk after the first vaccination. Class III follicles (diameter >9 mm) were totally absent during this period in most cows. The median period until recurrence of class III follicles was 78 d from the day of the second vaccination (95% confidence interval: 60–92 d). After vaccination, all cows showed swelling and pain at the injection site, and these reactions subsided within 2 wk. Body temperature and heart and respiratory rates increased after the first and second vaccinations and returned to normal values within 2 d of each vaccination. The cows in our study were not observed to display estrus behavior until 30 wk after the first vaccination. Therefore, a Select-Synch protocol was initiated at that time. Ten cows became pregnant after the first insemination (the remaining cow was reinseminated once until confirmed pregnancy). Bopriva induced a reliable and reversible suppression of reproductive cyclicity for more than 2 mo. The best practical predictor for the length of the anestrus period was the absence of class III follicles.

Key words: anti-gonadotropin releasing hormone (anti-GnRH), gonadotropin releasing hormone (GnRH), immunization, antibody titer, anestrus

INTRODUCTION

Estrus inhibition is frequently requested by livestock owners. Preventing recurrent estrus is desired for alpine farming and for feedlot cattle not intended for breeding. These cows, when untreated, cause disturbance in the herd during estrus and, additionally, are often pregnant at slaughter. In contrast to surgical methods (ovariectomy; Hobson and Hansel, 1972; Bleul et al., 2005), mechanical contraception methods (foreign objects in the uterus; Turin et al., 1997), or permanent administration of progesterone derivatives (Burns et al., 1993; Jacobsen et al., 1995; Denicola et al., 1997; Petta et al., 1998), immunological techniques represent an animal-friendly method for controlling populations (Miller et al., 2000; Curtis et al., 2002). Immunization against GnRH has been used to suppress sexual behavior and to prevent the development of secondary sexual characteristics in males (Finnerty et al., 1998; Cook et al., 2000; Thompson, 2000; D'Occhio et al., 2001; Aïssat et al., 2002; Janett et al., 2003, 2009b, 2012a; Turkstra et al., 2005; Theubet et al., 2010) as

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well as in females (Dalin et al., 2002; Imboden et al., 2006; Elhay et al., 2007; Janett et al., 2009a). In heifers, administration of a human chorionic gonadotropin or LH vaccine (Johnson et al., 1988), as well as a GnRH vaccine (Johnson et al., 1988; Adams and Adams, 1990; Prendiville et al., 1995; Bell et al., 1997; Stevens et al., 2005), has been tested. It could be demonstrated that antibody titers, ovarian function, progesterone concentration, and uterus weight differed significantly after immunization with a recombinant GnRH antigen from nonimmunized heifers (Geary et al., 2006). Note that these studies all used different presentations of the GnRH antigen as well as different adjuvants. Immunization with GnRH prevented the growth of follicles >5 mm in diameter and induced anestrus. However, as in mature mares (Dalin et al., 2002; Imboden et al., 2006), the effect of the vaccine varied individually in terms of duration and behavior (Prendiville et al., 1995; Crowe et al., 2001). No information is available in the literature regarding reversibility to estrus in cows after vaccination against GnRH.

The aim of the present study was to evaluate the effect of an active immunization against GnRH (Bopriva, Zoetis Australia Limited, West Ryde, Australia) on GnRH antibody titer, plasma progesterone and estrogen concentrations, and ovarian activity. Of particular interest were the duration and reversibility of the vaccination-induced anestrus. We hypothesized that the vaccine would increase GnRH antibody titer, decrease the plasma concentrations of progesterone and estrogen, and reduce the number of follicles >5 mm in diameter and therefore induce a phase of anestrus of unknown length. Once pregnancy was confirmed, cows were terminated from the study.

MATERIALS AND METHODS

Animals and Inclusion and Exclusion Criteria

Eleven lactating Swiss Fleckvieh cows (cows A through M), from second to sixth lactation (median = third lactation) and 88 to 201 d postpartum (median = 137 d) were included in the study. Inclusion criteria were regular cyclicity before first treatment and uterine health. A uterine swab was taken in diestrus for microbiological and cytological analysis. Results of microbiological samples had to be negative on bacterial culture, and less than 5% neutrophils (as a proportion of total cells) had to be counted to demonstrate uterine health, according to Gilbert et al. (2005). Exclusion criteria were a history of preceding dystocia or caesarian section, acute or chronic endometritis, cystic ovarian disease, or general diseases (e.g., chronic mastitis, claw diseases).

Animal Care and Housing

The cows were housed in tie-stalls on straw bedding. From May to October, the cows had daily access to pasture. During winter months, they had access to a courtyard 3 times a week. They were fed an individually calculated ratio of hay and concentrate determined on the basis of individual milk yield, had free access to water, and were milked twice daily.

Treatment with GnRH Vaccine

The treatment consisted of 2 vaccinations (initial and booster) with the anti-GnRH vaccine Bopriva (Zoetis Australia Ltd.) 4 wk apart (d 0 and 30). Bopriva contains an analog of GnRH linked to a carrier protein combined with a synthetic aqueous adjuvant (400 μg of GnRH-protein-conjugate per mL) and is registered with claims to suppress testosterone blood levels in postpubertal bulls and estrus behavior in postpubertal heifers. The dosage used for both the initial and the booster vaccination was 400 μg of GnRH-protein-conjugate (1 mL of Bopriva). All injections were administered subcutaneously on the right side of the neck.

All animal experimentation was performed with permission and in accordance with Swiss law (Nr. BE 29/11).

Clinical Evaluation and Side Effects

Clinical evaluation included the parameters of body temperature, heart and respiratory rates, and daily milk production. The injection site was meticulously inspected for signs of tissue reaction. Cows were examined directly before each vaccination (d 0) and daily on the following 3 d or until all parameters examined had returned to normal. Estrus behavior observation was conducted daily after feeding on pasture or in the courtyard.

Blood Sampling, GnRH Antibody Titer, and Hormones

In all animals, blood was sampled weekly starting 3 wk before vaccination until pregnancy was confirmed. The blood samples were collected by venipuncture from the coccygeal vein into heparinized tubes and centrifuged (4,000 × *g*, 10 min), and plasma was then stored at −18°C for later analysis.

GnRH Antibody Titers. Antibody titers of GnRH were determined by dissociation-enhanced lanthanide fluorescence immunoassay (**DELFIA**; PerkinElmer Pty Ltd., Glen Waverly, Australia) and results were expressed as relative light units (**RLU**). Briefly, 384-well,

streptavidin-coated plates (PerkinElmer Pty Ltd.) were coated for 1 h at room temperature with 1 μg/mL of biotinylated modified GnRH peptide in DELFIA buffer (50 m*M* Tris-HCl, 0.9% NaCl, 0.05% Tween 20, 20 μ*M* EDTA, 0.2% ovalbumin). Plates were washed and then incubated for 1 h at room temperature with 50-μL aliquots of cattle samples serially diluted to 1:800 in DELFIA buffer. Unbound plasma and antibodies were removed by washing, and bound antibody was detected by incubating plates for a further 1 h with 50 μL of europium-labeled protein G (PerkinElmer Pty Ltd.). After washing off excess europium-labeled protein G, DELFIA Enhancement Solution (PerkinElmer Pty Ltd.) was added to all wells to dissociate bound lanthanide from the antigen, forming a highly fluorescent chelate. After 10 min, the intensity of fluorescence was measured by excitation at 340 nm and emission readings at 615 nm using a time-resolved fluorometer (Envision 2102 Multireader, PerkinElmer Pty Ltd.). Pooled nonvaccinated cattle samples served as a negative control. Unknown samples were compared with serial dilutions of a standard positive reference immune cattle sample. Calculation of a standard curve and reading of unknown samples were performed using the WorkOut 2.5 software (Dazdaq Solutions Ltd., East Sussex, UK). Intra- and interassay coefficients of variation (**CV**) were 6.7 and 8.5%, respectively. Serology titers showed a 120-fold range from 3,850 RLU for negative samples to 495,000 titers units for immune samples, with 11,500 RLU being taken as the lower cut-off differentiating positive from negative samples. The lowest detection level of GnRH antibody titer was 3,850 RLU.

Hormone Analysis. Progesterone concentration was measured using RIA (Immunotech, Beckman Coulter, Sinsheim, Germany). The intraassay CV was 5.3% and the interassay CV was 7.7%, determined by using a bovine serum pool measured 30 times within a test routine and within 25 assay runs. The analytical sensitivity was 0.05 ng/mL and the measurement range was 0.05 to 50 ng/mL. The assay was performed according to the manufacturer's instructions. Total estrogen concentration was measured after cleavage of glucoronidated and sulfated estrogen by using 0.2% β-glucoronidase-acrylsulfatase (Merck, Darmstadt, Germany) for 2 h at 37°C in sodium actetate buffer (Sigma Aldrich, pH 4.8). Then, steroid hormones were extracted from plasma (300 μL) by adding 3 mL of ether (30% butylmethylether, 70% benzene). Then, a direct enzyme-immunoassay on microtiter plates using a secondary antibody-coating technique and horseradish peroxidase as the enzyme label were used to measure total estrogen concentration. The enzyme-immunoassay was previously described by Meyer et al. (1990) and Prakash et al. (1987). The used antiserum was raised against 17-estradiol hemisuccinate, and 17-estradiol hemisuccinate horseradish peroxidase was used as steroid-enzyme conjugate (purchased from Technical University of Munich, Freising-Weihenstephan, Germany). The intra- and interassay CV were calculated by repeated measurements of bovine plasma samples. The minimal detectable concentration was 8 pg/mL. The recovery was 84.6 to 96.2%, the intraassay CV was 8.5% and the interassay CV was 17.4%.

Gynecological Examination, Insemination, and Pregnancy

The cows were examined weekly by rectal palpation until they were inseminated. Using ultrasonography (Aquila Pro Vet, Esaote Piemedical, Köln, Germany), the number of follicles was recorded and assigned to 1 of 3 categories, according to the classification of Moreira et al. (2000): class I follicles (≤5 mm in diameter), class II follicles (6–9 mm in diameter), and class III follicles (>9 mm and <2 cm in diameter) (Vanholder et al., 2006). The presence of a corpus luteum (**CL**) was also noted. Thirty weeks after the first vaccination, all cows were treated with a Select-Synch protocol and inseminated. The Select-Synch protocol started with an injection of 20 μg of buserelin i.m. (Receptal, MSD Animal Health GmbH, Lucerne, Switzerland) and an intravaginal progesterone-releasing device containing 1.9 g of progesterone (Eazi-Breed CIDR B, Zoetis, Zurich, Switzerland). Six days later, the cows were injected with 500 μg of cloprostenol i.m. (Estrumate, MSD Animal Health GmbH), and the CIDR was removed the next day. Cows were inseminated 48 and 72 h after CIDR removal and received 20 μg of buserelin i.m. with the first insemination. Pregnancy diagnosis was performed 35 d after the first insemination. Cows not pregnant were further treated individually. Cows with confirmed pregnancies were slaughtered.

Statistical Analysis

Metric variables were described with means, medians, standard deviations, and ranges. Categorical data were analyzed by using contingency tables. We applied nonparametric methods, as the rank correlation coefficient for the relationship of variables that were at least ordinal, and nonparametric confidence intervals. The cow was the statistical independent unit; we observed dependent data within each cow. Because the number of cows was small, we could not apply simple statistical methods, and more sophisticated methods were not appropriate. Hence, *P*-values (e.g., of the rank correlation or χ^2 test of the contingency tables) should be interpreted only as an explorative indication. Only

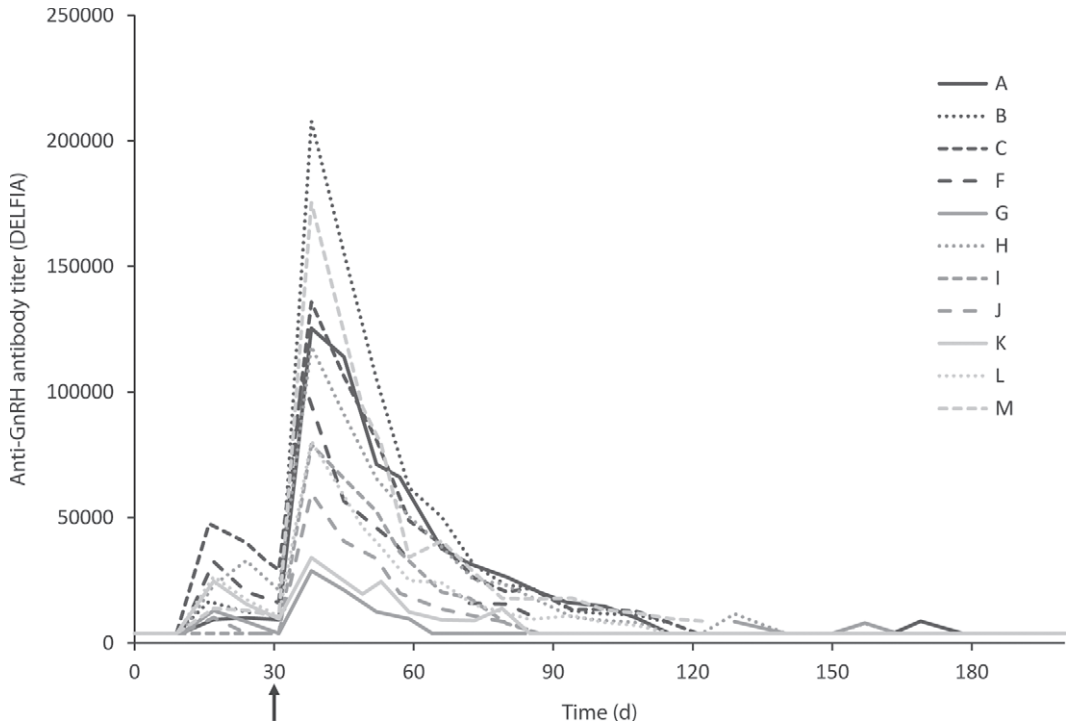


Figure 1. Gonadotropin-releasing hormone antibody titers of 11 cows (A through M) after 2 vaccinations (d 0 and d 30, arrow) with anti-GnRH vaccine (Bopriva, Zoetis Australia Ltd., West Ryde, Australia). Peak values are shown 6 wk after the first vaccination. DELFIA = dissociation-enhanced lanthanide fluorescence immunoassay.

when the cow was used as statistical unit were the *P*-values of test and nonparametric confidence intervals appropriate and exact. We used SAS software (SAS Institute Inc., Cary, NC) and Statxact software (Cytel Inc., Cambridge, MA) for the statistical analyses.

RESULTS

GnRH Antibody Titers

Antibody titers began to increase 2 to 3 wk after the first vaccination but increased significantly after the second vaccination and reached peak values at approximately 6 wk after the first vaccination (Figure 1). Titers decreased to basal values approximately 12 to 17 wk after the first vaccination in all cows. In 7 of 11 cows, the titers remained at basal levels for the rest of the study. In 4 cows, titers increased slightly from basal values at wk 18 and 22 (cows G and H, respectively) and at wk 24 and 25 (cows A and F, respectively). The GnRH antibody levels were below the detection limit at the time of insemination in all cows. A large variation in the maximum titer was seen in individual vaccinated cows, ranging between 21,174 units (cow G) and 207,830 units (cow B). A titer exceeding 3,850 units (detection limit) was found from 17 d (95% CI: 16.5–17) to 97.5 d (95% CI: 83–111.5) after the first vaccination.

Plasma Progesterone and Estrogen Concentration

The maximal plasma progesterone concentration measured during the entire study was 49 ng/mL and the minimal value was 0.1 ng/mL (Figure 2). The maximal plasma total estrogen concentration measured during the study was 102.0 pg/mL and the minimal value was the detection limit of 8 pg/mL.

At the time of the first vaccination, 6 cows (A, B, F, G, L, M) showed plasma progesterone concentrations >3 ng/mL, 4 cows (H, I, J, K) had values <1 ng/mL, and cow C had a concentration of 1.8 ng/mL. The individual plasma progesterone level fluctuated greatly in the first weeks after vaccination. In 7 cows (A, B, C, G, H, K, M), plasma progesterone decreased to a basal level 3 to 7 wk after the first vaccination (i.e., up to 3 wk after the booster) until wk 14 to 19. After this time, an increase in plasma progesterone concentration was apparent in 6 cows (A, C, G, H, K, M). One cow (F) showed an increase in plasma progesterone concentration from wk 5 that remained high until wk 16. Cows I and J showed a plasma progesterone concentration >5 ng/mL in wk

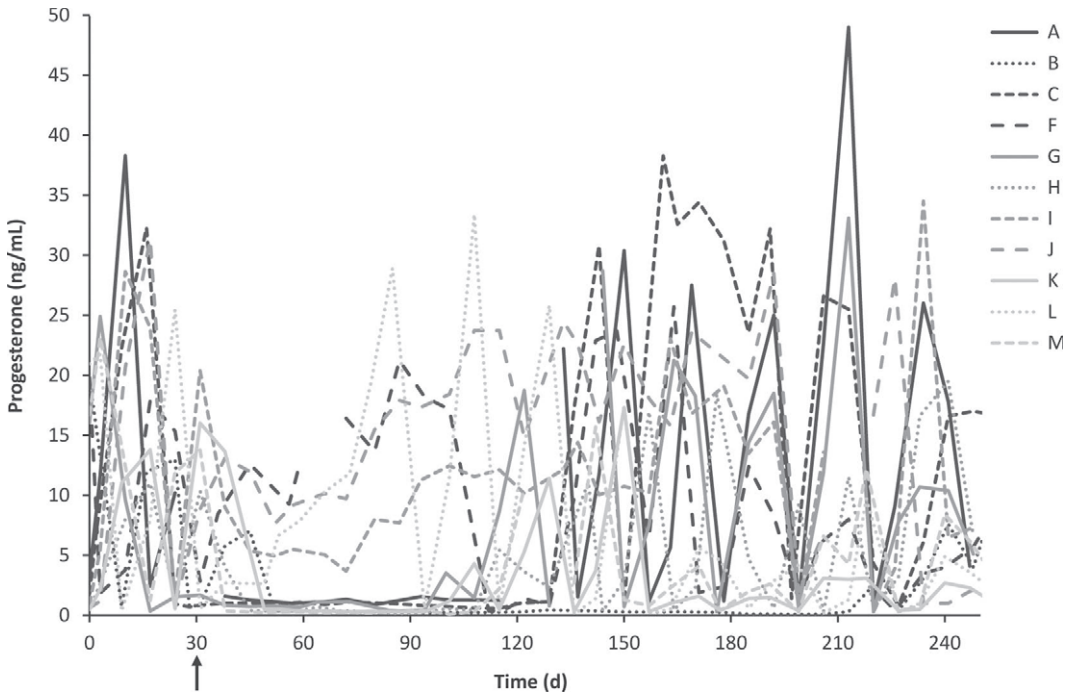


Figure 2. Plasma progesterone values of 11 cows (A through M) after 2 vaccinations (d 0 and d 30, arrow) with anti-GnRH vaccine (Bopriva, Zoetis Australia Ltd., West Ryde, Australia).

5, which increased above 10 and 15 ng/mL until wk 28 and 17, respectively. The plasma progesterone level of cow I returned to a fluctuating pattern after wk 28. In cow J, progesterone levels between 15 and 25 ng/mL were measured during wk 17 and 28. Cow L showed a continuous increase of the plasma progesterone level from wk 5 to 12 and reached a peak value of 28.9 ng/mL. The level of plasma progesterone concentration was positively correlated with the presence of a CL (Spearman r = 0.75; $P < 0.0001$).

Seven cows (A, B, C, F, H, I, J) showed fluctuating values of total plasma estrogen concentration during the study (data not shown), with peak values of 33.1 to 58.3 pg/mL, and minimal values below the detection limit. Cows K, L, and M showed fluctuating values until wk 11 after the first vaccination and then the values decreased below the detection limit until insemination, except for a very slight increase for cows L and M in wk 15 and 19, respectively. Cow G showed fluctuating values of total plasma estrogen concentration throughout the study, with very high peak values (102.0 pg/mL at wk 13).

Ovarian Ultrasonography

The number of class I follicles did not vary for 30 wk after the first immunization (Figure 3), whereas the number of class II follicles decreased between wk 7 and 17, although they were still present in some cows. Class III follicles were completely absent from wk 7 (±2 wk) to wk 16 (±3 wk) after the first vaccination (i.e., 3 wk after the booster) in all the cows except for cow G (Figure 4 a,b).

At the time of the first vaccination, a CL was present in 70% (8/11) of the cows. Four of the 11 cows developed a persistent CL over a maximum of 166 d after the second vaccination.

Figure 5 illustrates the correlation between GnRH antibody titers (d 17–97.5) and number of class III follicles; fewer class III follicles were present when GnRH antibody titers were high ($P < 0.0001$; phi coefficient = −0.461).

Pregnancy Diagnosis

Ten of 11 cows were confirmed pregnant 35 d after insemination following the Select-Synch protocol; 1 cow (J) was not pregnant at this time. In cow J, a CL on the right ovary and some class II follicles on both ovaries were observed, and the uterus showed no pathological findings. The cow was injected with 500 μg of cloprostenol i.m. (Estrumate, MSD Animal Health GmbH) on d 39 postinsemination and with 20 μg of buserelin i.m. (Receptal, MSD Animal Health GmbH) on d 41. Inseminations were performed on d 41 and 42 and she was confirmed pregnant 35 d later.

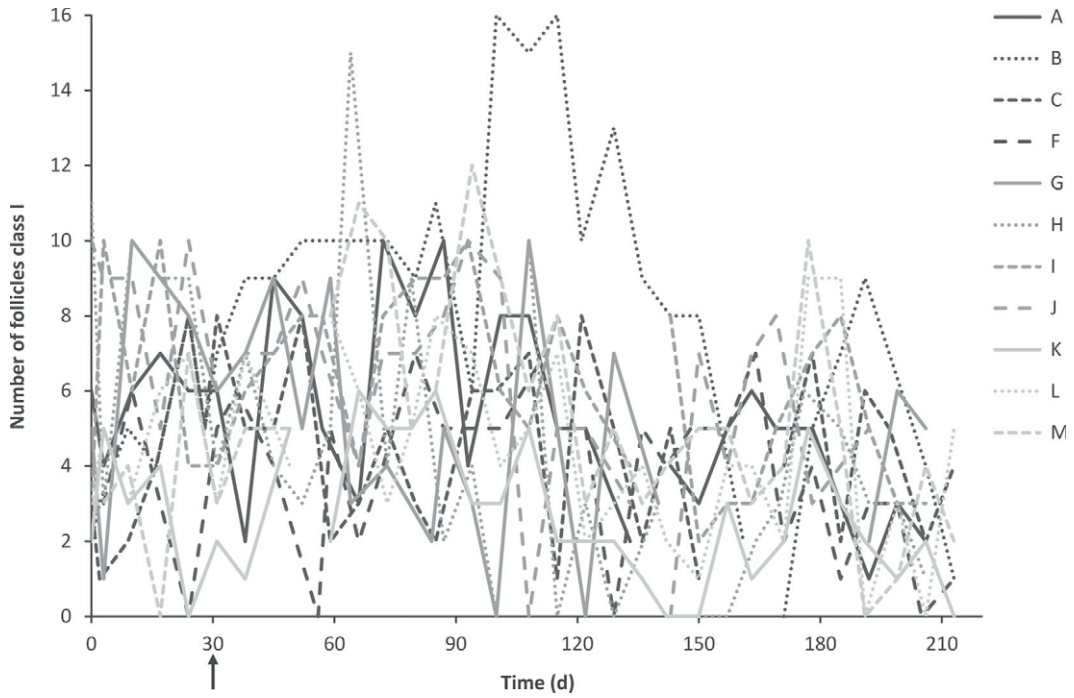


Figure 3. Number of class I follicles of 11 cows (A through M) after 2 vaccinations (d 0 and d 30, arrow) with anti-GnRH vaccine (Bopriva, Zoetis Australia Ltd., West Ryde, Australia).

Clinical Evaluation and Adverse Effects

No estrus behavior was observed in the GnRH-vaccinated cows even after anti-GnRH titers reached basal values and although class III follicles and the presence of a CL on the ovaries indicated resumption of reproductive cyclicity.

All cows treated with Bopriva showed a swelling at the injection site (varying in severity) and moderate to substantial local pain. In all animals, the local reactions subsided within 2 to 4 wk. A transient increase in rectal body temperature was apparent 24 h after the first (median; 25% and 75% quartiles: 39.2°C; 39.1°C, 40.3°C) and the second vaccination (39.5°C; 38.8°C, 40.4°C; Table 1). Heart rate (beats per min) increased after the first (88; 84, 100) and the second (88; 80, 90) vaccinations and returned to normal within 48 h after vaccination. Respiratory rate (breaths/min) increased after the first (34; 36, 56) and the second (32; 40, 48) vaccinations and returned to normal within 48 h postvaccination. No signs of apathy, reduced food intake, or decrease in milk production were observed. In 1 cow (J), a hydrometra was diagnosed 28 wk after the first vaccination and was successfully treated with 500 μg of cloprostenol i.m. (Estrumate, MSD Animal Health GmbH).

DISCUSSION

All cows showed similar patterns of GnRH antibody production after immunization, with a rapid onset of immune response and a peak 2 to 3 wk after the second vaccination. However, the maximal values of individual anti-GnRH antibodies varied greatly, due to the multistep involvements required for the immune system to establish antibodies to exposed antigens, as well as the individual variation in terms of the condition of the animals, their nutritional state, and a strong genetic component for major histocompatibility complex (Fluri et al., 2006).

Antibody titers >3,850 (detection limit) were found between 17 and 97.5 d after the first vaccination. This duration of effect is consistent with results in pubertal bull calves (Theubet et al., 2010; Janett et al., 2012a) but shorter than that observed in prepubertal bull calves (Janett et al., 2012b). The longer duration seen in prepubertal bull calves is most likely due to the vaccine resulting in a delay in the onset of puberty.

As expected, the number of class I follicles was not influenced by vaccination. Class I follicles in cattle are independent of gonadotropin (Campbell et al., 1995). The number of class II follicles was partly reduced, but class II follicles did not completely disappear after the second vaccination. In heifers, follicles with a diameter >5 mm are suppressed after vaccination against GnRH (Prendiville et al., 1995; Crowe et al., 2001). In our study, class III follicles were completely absent in 10 of 11 cows between 7 and 16 wk following 2 vaccinations of Bopriva. Cow G exhibited a low immune response to Bopriva as reflected in low GnRH antibody titers

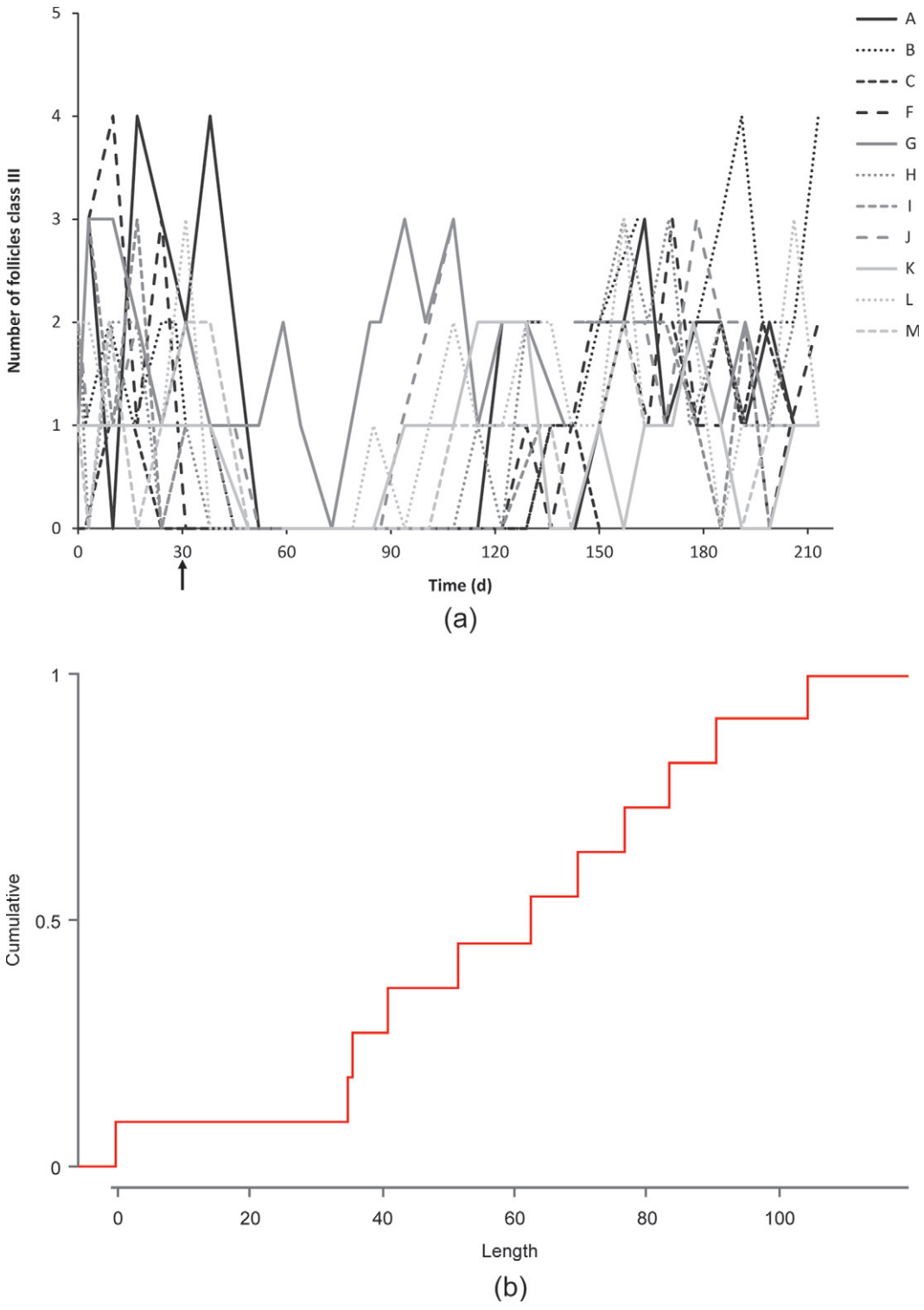


Figure 4. (a) Number of class III follicles of 11 cows (A through M) after 2 vaccinations (d 0 and d 30, arrow) with anti-GnRH vaccine (Bopriva, Zoetis Australia Ltd., West Ryde, Australia). These follicles were completely absent from wk 7 (±2 wk) to wk 16 (±3 wk) for all the cows except for cow G. (b) Survival analysis of class III follicles (empirical distribution, function of length in days of absence of class III follicles until class III follicles are present again). Color version available in the online PDF.

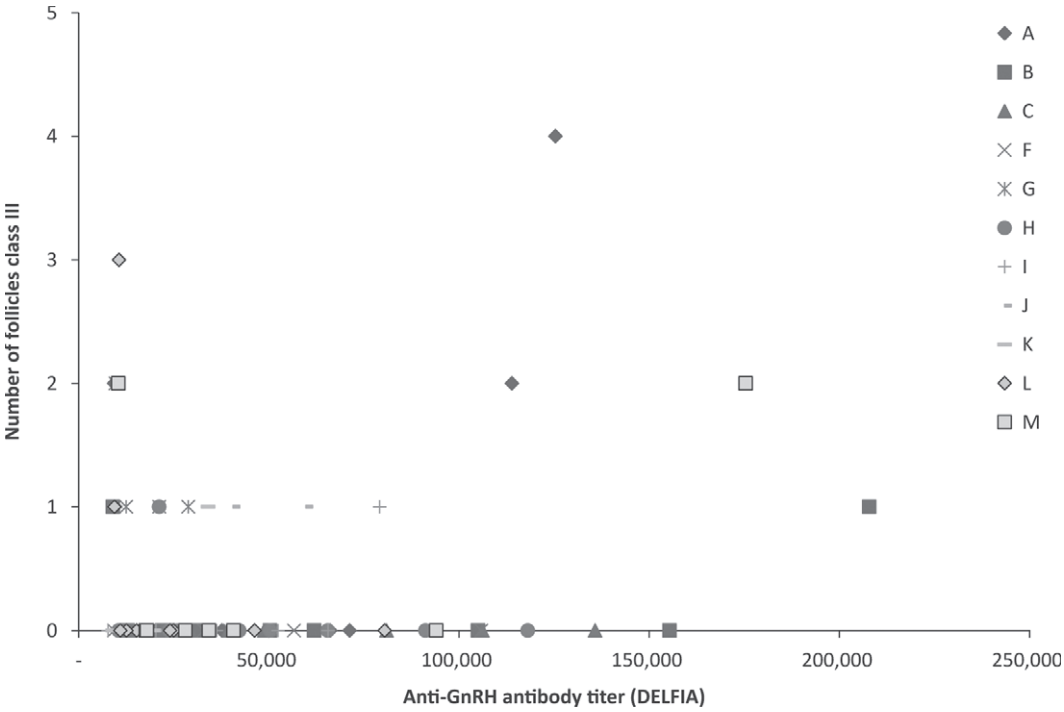


Figure 5. Association of class III follicles with GnRH antibody titer. DELFIA = dissociation-enhanced lanthanide fluorescence immunoassay.

Table 1. Temperature, heart rate, and respiration before vaccination (d 0) and on the 3 d following the first vaccination (1) with anti-GnRH vaccine (Bopriva, Zoetis Australia Ltd., West Ryde, Australia) and the second vaccination (2)

Trait	25% quartile	Median	75% quartile
Temperature (°C)			
d 0 (1)	38.1	38.4	38.4
d 1 (1)	39.1	39.2	40.3
d 2 (1)	38.2	38.4	38.6
d 3 (1)	38.1	38.5	38.7
d 0 (2)	38.5	38.6	38.8
d 1 (2)	38.8	39.5	40.4
d 2 (2)	38.1	38.7	38.8
d 3 (2)	38.1	38.3	38.5
Heart rate (beats/min)			
d 0 (1)	68	80	84
d 1 (1)	84	88	100
d 2 (1)	73	80	85
d 3 (1)	68	80	88
d 0 (2)	72	84	90
d 1 (2)	80	88	90
d 2 (2)	80	84	90
d 3 (2)	74	80	84
Respiratory rate (breaths/min)			
d 0 (1)	30	32	36
d 1 (1)	36	44	56
d 2 (1)	32	38	46
d 3 (1)	36	40	46
d 0 (2)	24	36	44
d 1 (2)	32	40	48
d 2 (2)	30	32	52
d 3 (2)	28	35	42

and, subsequently, large follicle development was not suppressed. Earlier studies in cattle suggest that recruitment of follicles is likely to be FSH-dependent and that selection and growth of follicles above 7 to 9 mm in diameter is LH-dependent (Gong et al., 1996; Bao et al., 1997).

Plasma progesterone (e.g., estrus values vs. diestrus values) at the time of the first vaccination did not affect later values of plasma progesterone concentration during the study. Despite the production of GnRH antibodies, 4 cows showed a CL and a plasma progesterone concentration >1 ng/mL. Earlier studies in heifers and ewes (Hixon et al., 1975; Hughes et al., 1987) suggested that estradiol production from follicles could play a role in luteolysis; therefore, the suppression of follicle development could prolong the lifespan of a CL (Okuda et al., 2002). The lack of class III follicles might explain why no luteolysis occurred in some cows. In our study, the total plasma estrogen concentration was not affected by the production of GnRH antibodies. A good correlation exists between total estrogen and estradiol-17β (Mondal et al., 2006). Other studies in mares (Dalin et al., 2002; Imboden et al., 2006) and heifers (Prendiville et al., 1995) described a reduced estradiol-17β level after GnRH vaccination. Dalin et al. (2002) indicated that in mares, the estrus cycle stage at first immunization might have an influence on hormonal levels. Furthermore, in the study of Dalin et al. (2002), ovarian atrophy in mares did not correlate with cycle blockade. Among all parameters evaluated in our study, the suppression of class III follicles was the most reliable and easily detectable parameter for clinicians to predict the efficiency of the vaccine. Large follicles are almost always present in healthy cyclic cows because follicle growth occurs in waves (Lucy et al., 1992). In this study, the class III follicles were absent from wk 7 to 16 after the primary vaccination and cows entered anestrus approximately 3 wk after the booster injection. An important finding of the current study was that the immunization against GnRH was entirely reversible as shown by the return to basal levels of the GnRH antibody titer and with successful insemination of all the cows. These results confirm that Bopriva can be used in a safe manner in cows, as already seen in bull calves (Theubet et al., 2010; Janett et al., 2012a,b). In our study, the general condition and milk yield of cows were not disturbed by the vaccine, despite a short-term increase in rectal body temperature and a local reaction at the site of vaccination (data not shown). One noteworthy adverse effect was the hydrometra developed by cow J. This was the only cow needing 2 inseminations to become pregnant. The authors did not find any other case of hydrometra published in conjunction with the use of GnRH immunization. It is unclear whether it occurred causally or as a coincidence.

In addition to seasonal breeding systems, temporary immunization against GnRH could be used in seasonal alpine pasturing systems. Cows not pregnant during the seasonal alpine pasturing disturb the herd because of recurring estrus behavior. This is especially accentuated in the Swiss Hérens breed, which is raised for fighting and selected for dominance abilities (Plusquellec and Bouissou, 2001). Suppression of estrus during alpine pasturing is highly valued but, with the exception of intravaginal progesterone devices, currently no pharmaceuticals are permitted in Switzerland for production animals. Immunization against GnRH could be considered an animal-friendly solution for these cows and producers.

The cows in our study were not observed to be displaying estrus behavior until wk 30, when the Select-Synch protocol was started. In future studies, we plan to evaluate estrus behavior and return to cyclicity of cows in herds that include a bull. Alternatively to using a bull, heat detection patches could be used. Furthermore, a large clinical field study is planned, in which we will evaluate the influence of cycle stage at first and second vaccinations on characteristics of hormones and behavior.

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Evaluation of Somatic and Reproductive Immunotoxic Effects of the Porcine Zona Pellucida Vaccination

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ABSTRACT Immunological, immunocytochemical and fertility analyses were performed to determine the potential toxic side effects of porcine zona pellucida (pZP) vaccinations on target animals, including horses and dogs. The study was designed to determine the effect of antibodies, raised against highly purified pZP, on somatic tissues. Immunocytochemical studies performed with fixed tissues showed that rabbit anti-pZP antiserum did not crossreact with brain, heart, lung, kidney, liver, bladder, stomach, small intestine, large intestine, muscle, skin, spleen, pancreas, or lymph node of either the dog or horse. To determine the effect or oral intake on nontarget animals, female rabbits were fed a contraceptive vaccine containing pZP glycoproteins and the synthetic trehalose dicorynomycolate in drakeol (S-TDCM) adjuvant. Enzyme-linked immunoadsorbent assay (LISA) analyses showed that rabbits fed with the adjuvanted pZP proteins did not develop circulating anti-pZP IgG antibodies that crossreacted with pZP. Furthermore, fertility studies performed on rabbits fed with adjuvanted pZP revealed no significant differences in the number of embryos or stage of the embryos produced between the treated and control animals. Results of these studies suggest that the pZP vaccine delivered to dogs or horses in field studies have no recognizable somatic tissue effects. Moreover, there were no side effects on nontarget animals should they eat the vaccine. This substantiates field trials results about the safety of the pZP immunocontraceptive vaccine. *J. Exp. Zool. 286:641–646, 2000.* © 2000 Wiley-Liss, Inc.

The zona pellucida (ZP) is an extracellular matrix that surrounds the oocyte of all mammalian species. The ZP is composed of several families of acidic glycoproteins that exhibit biological and immunobiochemical variation among various species (Dunbar et al., ’91). These glycoproteins have been assigned several different roles, of which species-specific sperm–egg interaction has been most investigated (Shur, ’81; Shur and Hall, ’82; Florman and Wassarman, ’85; Bleil et al., ’88; Miller and Ax, ’90; Yurewicz et al., ’91, ’93). If the mechanism of interaction between the zona pellucida and sperm could be interrupted, this would be a nonhormonal method of contraception.

Total porcine zona pellucida (pZP) has been the most common ZP antigen used so far for immunocontraception because of its immunological antigenicity and crossreactivity with many mammalian species (Sacco ’77; Trounson et al., ’80; Hasegawa et al., ’85; Kirkpatrick et al., ’96; Fayrer-Hosken et al., ’97b; Barber et al, ‘98) and the availability of pig ovaries in relatively large amounts. Immunization with pZP has resulted in immunocontraception in many animal model systems (Tsunoda and Chang, ’76; Gwatkin et al., ’77; Gulyas et al., ’83; Sacco et al., ’83; Bamezai et al., ’86; Sacco, ’87; Dunbar, ’89; Kirkpatrick et al., ’92a; Willis et al., ’94). In most of these cases, pZP induced a loss of fertility while blood levels of the antibodies, usually IgG, remained high. Alterations in fertility have been recorded for monkeys (Sacco et al., ’83), horses (Liu et al., ’89), and multiple zoological species (Kirkpatrick et al., ’96).

Although some side effects of the pZP vaccine have been reported, the precise mechanisms of action are unclear. The anti-pZP antibodies have been shown to affect the cyclicity, ovarian structure, and follicular development in dogs (Mahi-Brown et al., ’82) and primates (Lou et al., ’96; Garza et al., ’98). In dogs, immunization of bitches with an uncharacterized pZP preparation led to

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long-term changes in estrous cycles and aberrant steroid profiles (Mahi-Brown et al., '85). Baboons immunized with pZP showed greatly reduced number of antral follicles and a drop in the blood levels of estrogen (Dunbar et al., '89). Kirkpatrick et al. ('92b) inoculated 10 feral mares with pZP and monitored fertility effects. After 5 consecutive years of treatment, urinary estrogen levels were shown to become elevated as in estrus. However, the estral phase was never followed by a luteal or progesterone phase. Generally, in most species there are no clinically detectable side effects with short-term immunocontraception.

In our previous studies on equine immunocontraception (Willis et al., '94), we have shown the efficacy of using the new adjuvant, synthetic trehalose dicorynomycolate in Drakeol (S-TDCM was kindly provided by Dr. Terry Ulrick, RIBI Immunochem Research Inc., Billings, MT), with the pZP. Our results demonstrated a 12–24 month increase in anti-pZP titers, which were associated with infertility. In addition, our laboratory has reported an immunocytochemical study on ovarian tissues from the dog, cat, horse, and elephant (Barber et al., '98). These results revealed that the anti-pZP antibodies crossreacted with ovarian tissues, specifically the zona pellucida. A practical concern for field applications of contraceptive programs would be the somatic tissue effects on target animals and fate of stray pZP darts, particularly if the expelled adjuvanted-pZP is ingested by a nontarget species. This study was carried out to assess immunocytochemistry of somatic tissues in efforts to evaluate the vaccine's safety for dogs and horses. In addition, the possibility of fertility effects of orally fed, adjuvanted pZP vaccine, in rabbits (nontarget species) was investigated.

MATERIALS AND METHODS

Preparation of the pZP antigen

Porcine ovaries were collected from an abattoir and stored frozen at –20°C until processed. Briefly, the ovaries were minced and the oocytes were isolated by sequentially sieving through nylon meshes of decreasing pore sizes. The oocytes were homogenized in a glass homogenizer, and the solubilized zonae pellucidae glycoproteins were obtained by heating the solution at 73°C for 20 min. This was followed by centrifuging at 10,000*g* for 1 hr to precipitate unwanted proteins. The purified pZP protein in the supernatant was separated and lyophilized. Protein estimations were performed according to Peterson ('76).

Immunohistochemistry of equine and canine formalin fixed somatic tissues

Fresh somatic tissue from euthanized dogs and horses were obtained at necropsy from Department of Pathology, College of Veterinary Medicine, University of Georgia. Fourteen different somatic tissues were taken from both canine and equine carcasses. Ovaries, used for positive control sections, were obtained from spayed dogs from local area veterinary clinics, and horse ovaries were obtained from horses euthanized for nonreproductive reasons. Small blocks (1 cm^3) of tissue were fixed in neutral buffered formalin immediately after being removed from the animal and then embedded in paraffin. Three-micrometer-thick sections were cut with a microtome and adhered to slides.

Sections were heated in a 60°C oven for 20 min followed by two washes with xylene in order to remove all paraffin. The sections were then rehydrated through an ethanol series followed by incubation in a 9:1 methanol/H_2O_2 solution for 30 min to remove endogenous peroxidase. Nonspecific binding sites on the sections were blocked by incubating sections in 5% BSA dissolved in Tris buffered saline (TBS) containing 0.05% Tween 20 (TBST) overnight. Rabbits that had been vaccinated with highly purified pZP provided the source of the anti-pZP IgG. The serum was diluted 1:1000 with TBST and 200 µl were applied to the tissue sections and incubated for 1 hr in a wet chamber. Negative control sections were treated with a similarly diluted normal rabbit serum. Sections were washed twice with TBS after incubation with primary antibody followed by incubation with 200 µl of goat anti-rabbit IgG biotin conjugate at a 1:1000 dilution for 30 min in a wet chamber. After incubation, the sections were washed twice with TBS and then treated with Extravidin peroxidase (1:100 in TBST, Sigma Chemical, St. Louis, MO) for 30 min in a wet chamber. Tissue sections were washed twice with TBS and treated with diaminobenzidine (DAB) for 2 min. Color reaction was stopped with running distilled water followed by counterstaining with Mayer's hematoxylin. Sections were then dehydrated through an ethanol series and permanently mounted. Ovarian sections from both dog and horse were used as the positive control. These sections were treated as the stated above somatic tissues. Observations were made with a Zeiss Axioscope microscope at 40 and 100× magnification.

Additional canine stomach tissues sections were

treated with alkaline phosphatase enzyme and nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in 100 mM Tris buffer as the substrate in the place of horseradish peroxidase/DAB.

Oral administration of pZP

A 0.5 ml sample of blood for pretreatment serum antibody levels was collected from the marginal ear vein of each rabbit prior to dosing. The vaccine was prepared by mixing one part of S-TDCM with nine parts of phosphate buffered saline (PBS) containing 0.4% Tween-80 and pZP. The solution was mixed in a glass grinder with a Teflon pestle and then passed 10 times through a 25-gauge needle fitted to a 10 ml syringe. The white emulsion was introduced into the oral cavity of the rabbit with the open end of a French tom cat catheter. A treatment group of four adult New Zealand white female rabbits was fed with 400 μg of pZP per rabbit, whereas the control group of four adult New Zealand white females received the same volume of emulsion without the pZP.

Embryo production and collection

Four days after feeding the pZP, the rabbits were superovulated by injecting each rabbit with 75 IU of eCG intramuscularly. Seventy-two hours later, each rabbit was mated one to three times with a proven male and then given 150 IU human chorionic gonadotropin (hCG) intravenously. Blood samples were again collected for determining the possible presence of anti-pZP antibodies in the serum. The rabbits were killed by cervical dislocation 24-hr later. The embryos from the treated and control rabbits were flushed from the oviducts with warm PBS and immediately observed and evaluated for stage of cell division.

pZP antibody estimation in orally dosed rabbit

Anti-pZP antibody in the serum was estimated by a standard enzyme-linked immunoadsorbent assay (ELISA). Microtitration plates (Immulon-4, Dynatech Laboratories, Inc., Chantilly, VA) were coated with solubilized isolated pZP 92 μg/well) in carbonate buffer (100 mM sodium carbonate-bicarbonate, pH 9.0) and incubated at 4°C overnight. All subsequent incubations were carried out at room temperature. The wells were washed with TBS and blocked with 5% BSA for 24 hr. Triplicates of the preimmune serum and the anti-pZP serum were assayed at a dilution of 1:1,000. After incubating with 50 μl of the primary antibody for 2 hr, the plates were washed three times with TBS. Then a second antibody, alkaline phosphatase labeled goat anti-rabbit IgG, was added and incubated for 2 hr. The plates were then washed with TBS and the enzyme activity detected by adding *p*-nitrophenyl phosphate in bicarbonate buffer. After 30 min the reaction was stopped by adding 50 μl 3 M sodium hydroxide. The absorbance was read in an ELISA plate reader at 405 and 492 nm.

RESULTS

Immunohistochemistry of equine and canine fixed somatic tissues

The cross-reactivity of rabbit anti-pZP serum with canine and equine somatic tissues was determined in formalin fixed, paraffin embedded sections of brain, heart, lung, kidney, liver, bladder, stomach, small intestine, large intestine, muscle, skin, spleen, pancreas, and lymph node. For each tissue tested, with the exception of canine stomach tissue, there was no observable staining as compared to controls. In the canine stomach, the Parietal cells' intracytoplasmic granules did show staining with the peroxidase/DAB system in both treatment and control. But on subsequent treatment the cells' contents were negative for staining with alkaline phosphatase/NBT-BCIP enzyme system.

Oral administration of pZP

The sera collected from the four rabbits fed with pZP and the four control rabbits were tested for the presence of anti-pZP antibodies by the ELISA method. Several dilutions of the sera ranging form 1:10 to 1:1,000 were assayed. At each of the dilutions tested, the absorbance readings were not significantly different from those of the serum control rabbits. The readings in both treatment and control groups were the same as the control wells (i.e., without the primary antibody or the preimmune serum).

Effects of embryo production

The oviductal embryos from superovulated rabbits orally fed with pZP and the control rabbits were successfully collected. The embryos ranged from the one-cell to the six-cell stages (Table 1). In both the control and treatment groups, maximum number of embryos were observed at the four-cell stage, which is the appropriate stage 24 hr postbreeding. For each of the stages examined, the number of embryos in the treatment rabbits was not significantly different ($P < 0.05$) from the

TABLE 1. Effects of orally ingested pzp on fertility in rabbits[1]

No. of cells in the embryo[2]	Control	Treatment
1 cell	5[a]	6[a]
2 cells	4[b]	4[b]
4 cells	44[c]	49[c]
6 cells	25[d]	26[d]

[1]Like superscripts on the same row indicate no statistical differences.
[2]The number of cells in embryos recovered following superovulation and 24 hr after breeding.

number of the control rabbits as determined by a pooled *t*-test (Table 1).

DISCUSSION

The potential toxic effects of vaccination with pZP in a mammal include ovarian follicle damage, somatic organ damage, inhibition of circulating hormones, induction of neoplasia, or autoimmune disease. Immunocytochemical results from formalin fixed, paraffin-embedded somatic tissue showed that, in light microscopy, anti-pZP antibodies do not bind to brain, heart, lung, kidney, liver, bladder, stomach, small intestine, large intestine, muscle, skin, spleen, pancreas, and lymph node of either the dog or horse. False initial positive staining of the canine parietal cells with the peroxidase enzyme system was most likely caused by the highly acidic nature of the parietal cells themselves. Parietal cells are located in gastric mucosa of the stomach, which produce gastric juice consisting of hydrochloric acid and intrinsic factor. These cells produce HCl at a pH of 0.8. This is 3 million times the hydrogen ion concentration of arterial blood (Berglindh, '84; Malinowska and Sachs, '84). However, the false positive results were clarified by the alkaline phosphatase enzyme system, which demonstrated that there was no binding to the parietal cells by the pZP antibodies. These results, compared with the positive control ovarian sections that show that rabbit anti-pZP antibodies crossreact with ovarian tissue, clearly support the follicular specificity of anti-pZP antibodies. From the immunocytochemical data and the field vaccination trials in horses (Kirkpatrick et al., '96) and elephants (Fayrer-Hosken et al., '97a), it is clear that the systemic effects of the pZP vaccination are minimal. Furthermore, preliminary results on Western blots of equine luteinizing hormone and equine chorionic gonadotropin with anti-pZP antibodies show no crossreaction with the glycohormones (Fayrer-Hosken, unpublished results).

Although the vaccine is systemically safe, what are the effects on reproductive physiology? In field studies, horses vaccinated for more than three consecutive years with pZP and Freund's adjuvant (Kirkpatrick et al., '92b) showed some signs of decreased luteal activity defined by depressed progesterone levels. Studies of dogs showed long-term alterations in estrus cycles and abnormal steroid profiles (Mahi-Brown, et al., '85). In many other species, vaccination with various pZP preparations has caused erosion of the follicle pool, leading to infertility (Sehgal et al., '89; Upadhyay et al., '89; Grootenhuis et al., '96; Paterson et al., '96). Although these effects are acceptable in some forms of population control in which the animal should be sterilized (e.g., feral dogs and cats), this is unacceptable in wildlife conservation and ecology (Fayrer-Hosken et al., '97b). However, the possible deleterious effects on follicle populations in the immunocontraception of wildlife can be avoided by limiting the number of years of sequential pZP vaccination, or vaccinating annually, but in different regions of the ecosystem.

A remaining problem of population control with pZP would be the potential effect on the fetus. The primary consequence would be reduction or loss of fertility in female offspring. Anti-pZP antibodies are passed through the placenta and colostrum (Sacco et al., '81) in female mice immunized with pZP. However, the potential target of anti-pZP antibody effects, the pZP, is not developed in the fetal horse ovary (Deanesly, '75). Hence, the potential degradation of follicular reserved in the fetuses is extremely insignificant. Moreover, offspring of dams vaccinated in utero have been shown to be fertile (Kirkpatrick et al., '96).

In most wildlife immunocontraception studies, pZP is delivered remotely. Currently the primary modes of remote delivery are darts (Kirkpatrick et al., '96) and biobullets (Willis et al., '94). A practical consideration of the delivery vehicles is what happens to the contents if they miss their target. Adjuvanted pZP would be liberated, and one possible outcome would be the ingestion of the material by nontarget species. To test the effect of this eventuality, rabbits were dosed with a full dose of adjuvanted pZP. This single oral dose did not induce detectable titers in the female rabbits. In addition, there was no inhibition of fertility as reflected in the number and stage of embryos. From this data, the likelihood of there being any deleterious effects on reproduction from oral ingestion is nil. Thus, in a field situation of primary problem of uncollected darts would be mechanical.

Another concern with immunocontraception is

its effects on behavior and social structure, especially in herd animals like elephants, horses, and deer. This most extensive observations have been conducted in feral horse herds whose populations have been controlled by immunocontraception via pZP vaccination. Kirkpatrick and colleagues observed harem group fidelity and social hierarchy in mares and found no differences in these as compared with normal nonvaccinated mares (Kirkpatrick et al., '95). More investigation needs to be conducted on the behavior altering effects of pZP inoculation in other species as well as comparison of these effects, or the lack thereof, with the behavior effects of other forms of population control such as culling and relocation of animals.

The safety results of this study, along with other studies conducted on the practicality of zona pellucida proteins as an immunocontraceptive, provides encouragement to proceed with anti-pZP vaccination strategies. Based on the individual species under study, the vaccine delivery mechanism provides ecological control of populations.

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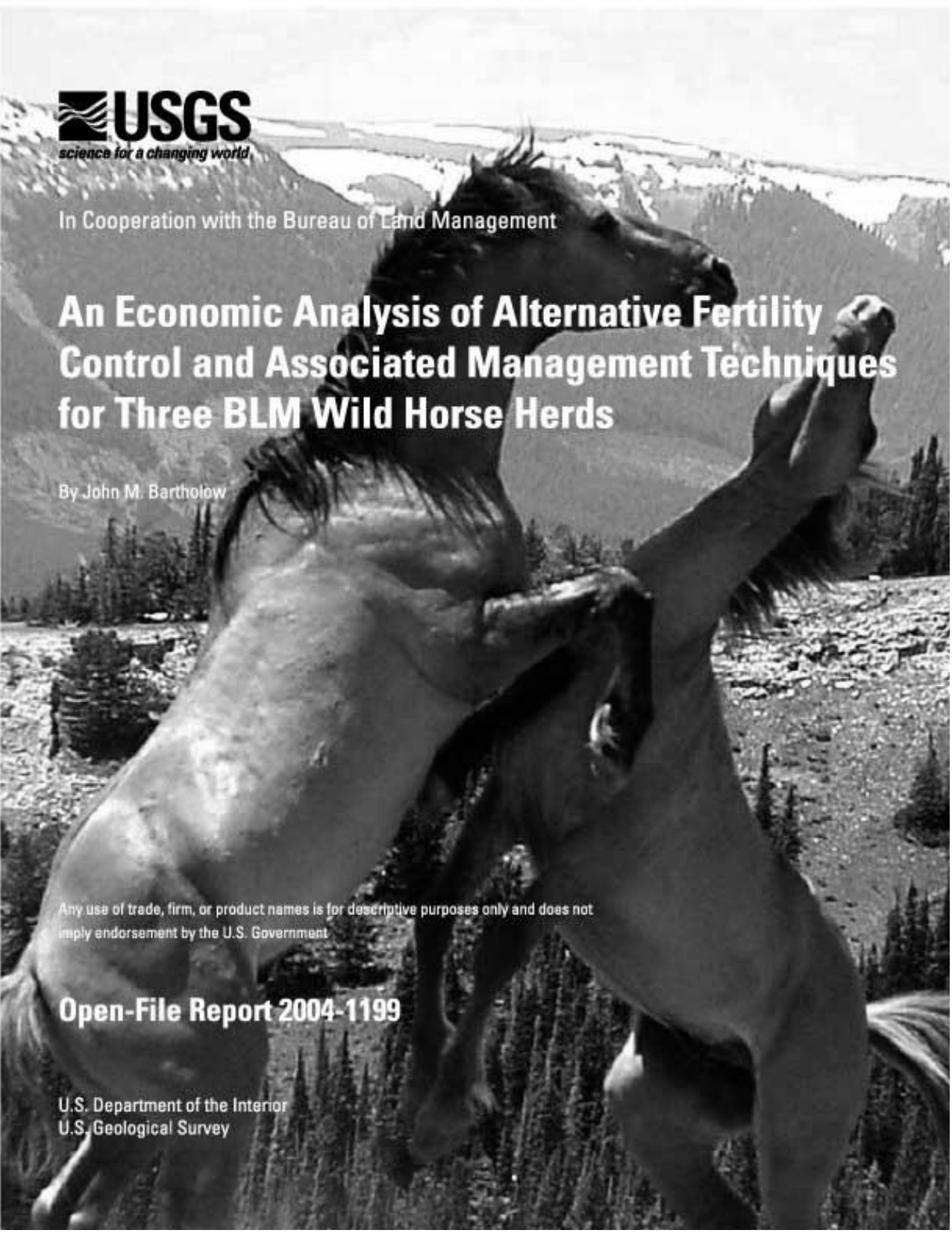
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An Economic Analysis of Alternative Fertility Control and Associated Management Techniques for Three BLM Wild Horse Herds

By John M. Bartholow

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An Economic Analysis of Alternative Fertility Control and Associated Management Techniques for Three BLM Wild Horse Herds

By John M. Bartholow

Executive Summary

Contemporary cost projections were computed for several alternative strategies that could be used by BLM to manage three wild horse populations. The alternatives included existing gather and selective removal methods, combined with potential contraceptive applications of varying duration and other potentially useful management techniques. Costs were projected for a 20-year economic life using the Jenkins wild horse population model and cost estimates from BLM that reflect state-by-state per horse removal, adoption, long-term holding, and contraceptive application expenses. Important findings include:

- Application of currently available 2-year contraceptives appears capable of reducing variable operating costs for wild horse populations by about 21% on average.
- Application of 3-year contraceptives, when fully tested and available, may be capable of reducing variable operating costs by about 27% on average.
- Combining contraceptives with modest changes to herd sex ratio (e.g., 55-60% males) can trim existing costs by about 31%.
- All savings are predicted to increase when contraception is applied in conjunction with the proposed removal policy that targets horses age zero to four, instead of zero to five.
- Reductions in herd size result in greater predicted variation in annual operating expenses for each herd, especially below about 200 animals, but are always at least ±20%.
- Because the horse program's variable operating costs only make up about one half of the total program costs (which include fixed and sunk costs), even with aggressive contraceptive management, total program costs could only be reduced by about 17%. This would still save about $7.7 million per year.
- None of the contraceptive options examined eliminated the need for long-term holding facilities over the 20-year period simulated, but the number of horses held may be reduced by about 23% with aggressive contraceptive treatment.
- Cost estimates are most sensitive to adoption age and per day holding costs.
- There are opportunities to improve both the population modeling software and the modeling processes used in assembling Herd Management Area environmental assessments.

Introduction

Managing wild horses (*Equus caballus*) as dictated by the Wild Free-roaming Horse and Burro Act of 1971 (as amended) has proven to be costly. Legislation has restricted options used to manage populations; sanctuary and adoption alternatives have been expensive and less than fully effective (U.S. General Accounting Office, 1990; Conover, 2002). Herds continue to grow, often at high rates (6% to 25% or more; Eberhardt and others, 1982; Wolfe, 1986). Budgetary limitations demand tough decisions on how to best minimize expenditures yet still meet legislated goals to maintain a "thriving natural ecological balance" and preserve existing multiple-use relationships, including rangeland health (U.S. General Accounting Office, 1990). Fertility control appears to be one option worth evaluating for cost effectiveness. It is viewed as humane (Reiter and others, 1999), safe and reversible (Kirkpatrick and Turner, 2002), and has been shown to successfully complement other methods of population control for wild horses (Garrott, 1991; Garrott and others, 1992; Gross, 2000; Hobbs and others, 2000).

BLM has maintained a database for state-by-state herd monitoring, census, selective removal, preparation and holding, adoption, compliance check, and other program costs. These new data have been used to compute estimates similar to those from Garrott and others (1992) for the early 1990s. In this paper, I update cost estimates using 2004 management expenses and currently available contraceptive technology for three specific BLM-managed wild horse populations. The three herds were chosen by BLM based on availability of data suitable for population modeling, and because they represented three different western states, different habitats, and herd sizes that characterize a majority of the managed populations.

Objectives

An economic analysis – with proposal costs weighed against anticipated benefits – is appropriate to examine differences between alternative courses of action where limited funds are invested in the public interest, i.e., is one investment more desirable than another? However, BLM's goal is herd management area reductions to clearly established appropriate management levels (AMLs), not to bring in revenue. This simplifies the objective to one of cost-avoidance. Alternatives explored include: (1) the status quo of selective removal, adoption, and sanctuary; (2) the frequency of gathers and how efficient they are in rounding up animals, (3) status quo plus several alternative contraceptive application scenarios, specifically the duration of the contraceptive agent, and (4) other potential management techniques, such as sex ratio manipulation through age- and sex-specific removal decisions.

It has been argued that contraceptive programs must be tailored for individual herds because small differences in reproductive biology and inherent population growth rates can have large influences on population dynamics (Kirkpatrick and Turner, 1986; Garrott, 1991), and because herd-specific data vary in quality (Wolfe, 1986). In addition, BLM tracks wild horse expenditures on a state-by-state basis for planning purposes because costs differ between states. Therefore this analysis has secondary objectives to examine multiple horse populations and perform appropriate sensitivity analyses to address the range of variability and flexibility inherent in herd management decision-making.

Specific Alternatives Analyzed

To address the issue of cost minimization in setting management guidelines for wild horse populations, several questions emerge as key within the confines of existing or proposed BLM policy guidelines:

- How often should horses be removed and/or treated with contraceptives?
- What sex and age horses are best to remove and/or treat with contraceptives?
- What other findings may be inferred from examination of simulation results (e.g., benefit: cost ratio for gather efficiency, general behavior of the HMA models)?
- Do the answers to the above questions depend strongly on the characteristics of individual herds or their locale?

Potentially, it would be easy to generate so many combinations of treatment alternatives that the results would prove cumbersome and would shift the focus away from valuable generalizations. Therefore, this analysis has concentrated on the following scenarios to help answer the questions posed above:

- Baseline Scenario – Existing "baseline" conditions as reflected in current gather policy (U.S. BLM, 2002). This is a regular 4-year gather with age-specific removal rates meant to mimic those shown in Figure 1. Age classes are removed in successive *tiers* as necessary to achieve the designated appropriate management level (AML), with age 0-5 horses removed first, followed by age 10+, and finally by age 6-9 animals.
- Alternative Baseline – Modification of the Baseline Scenario being considered as an update to the existing selective removal policy. Much of the existing selective removal process has yet to be fully institutionalized and may change to something like this scenario, which is the same as Baseline Scenario except Tier 1 includes age classes 0-4, Tier 2 remains 10+, and Tier 3 is 5-9. Contrasting with Figure 1, in the Alternative Baseline Scenario, 70 percent of Tier 3, if removed, is directed to the adoption pool, and 23% of age 4 animals end up in long-term holding.
- Gather Frequency Scenario G_i, where *i* represents regular gather interval in years (e.g., 2, 4, 6, or 8 years)
- Contraceptive Scenario C_d, where *d* represents duration of contraceptive in years (e.g., 2, or 3 years), defined more precisely with percent effectiveness in the first and subsequent years as shown in Table 1. Scenario 2-year-a represents values that have been used in some BLM Environmental Assessments, where Scenario 2-year-b represents values actually measured for one herd. However, scenario 2-year-c represents the most up-to-date estimates available. Contraceptives are assumed to be applied to all mares returned to the range. Note that it is considered to be the case, and the Jenkins model assumes, that if the vaccine does not produce infertility in the first year for a given mare, it would never be effective in subsequent years until retreatment.
- Sex Ratio Scenario S_m, where *m* represents the long-term sex ratio of males to females resulting from the selective removal process (e.g., 55% male to 45% female). Note that these sex ratios will usually not be given consistently. This is because the sex ratio is generated by long-term changes to sex- and age-class specific removal rates and could not be precisely predicted by specifying population model inputs.

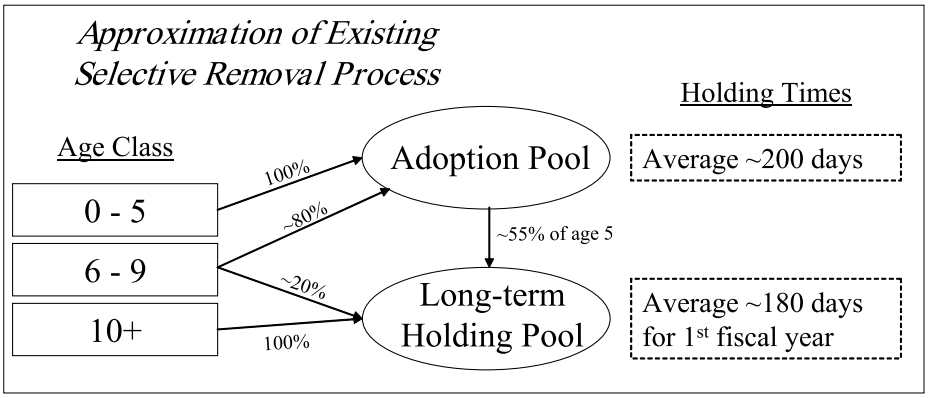


Figure 1. Approximation of existing selective removal policy (Baseline Scenario). Values are taken from Gather Policy & Selective Removal Criteria for Wild Horses, Instructional Memorandum No. 2002-095 (2/13/2002) and information provided by Ron Hall, BLM/NV (9/23/03).

Table 1. Annual effectiveness of existing and potential contraceptive treatments.

Nominal Duration	Effectiveness Year 1 (%)	Effectiveness Year 2 (%)	Effectiveness Year 3 (%)	Effectiveness Year 4 (%)	Effectiveness Year 5 (%)
2-year-a	80	50	0	0	0
2-year-b[1]	94	82	34	0	0
2-year-c[1]	94	82	68	0	0
3-year[2]	95	85	75	33	0

[1]Values taken from draft BLM Instruction Memorandum by Ron Hall (2003, personal communication 2004) reflecting results from the Clan Alpine (NV) HMA. 2-year-b represents a low range estimate and 2-year-c represents a high range estimate.
[2]Hypothetical values liberally extrapolated from 2-year efficacy rates.

- Gather Efficiency Scenario $E_{\%}$, where % represents percentage point change in 'normal' gather efficiency (i.e., if the given gather efficiency were 75%, a –5 would indicate 70% and a +10 would indicate 85%).
- As appropriate, combinations of the above scenarios have been considered. For example, 3-year Contraceptive/Sex ratio-55 would mean the combination of a 3-year contraceptive duration and 55 male:45 female sex ratio. Implicitly, all unspecified parameters would be the same as the baseline case.

Study Areas

The three wild horse populations chosen by BLM for this analysis were the Challis Herd Management Area (HMA) managed out of Challis Idaho; the McCullough Peaks HMA near Cody, Wyoming; and the Little Book Cliffs HMA close to Grand Junction Colorado. As will be seen later, costs and demographics for these three herds capture a large portion of the variability inherent in state-by-state cost differences.

The Challis herd has been managed exclusively by 'gate cut' gathers. Gate cut is not a representative sampling technique because it is dependent on the ease of capture and handling characteristics of corralled horses. Gate cut techniques likely over represent mares, foals, and younger horses in the gathered sample. In 2002, the herd size was estimated at approximately 271 horses with an annual growth rate of about 17%. Foals are included in the AML that is set to 185 animals post-removal. It is estimated that this herd has a foal sex ratio of 58 males:42 females.

The Little Book Cliffs herd is located about eight miles northeast of Grand Junction, Colorado, on 30,000 acres of public land. Many of these horses are descended from escaped domestic stock, but some may be of Spanish origin. This herd of about 150 horses receives periodic gathers and is already participating in an ongoing contraceptive research trial. All yearlings and 2-year-olds, half of those age 3 to 15, and all mares older than 15 that were captured at the 2002 roundup and returned to the range were primed with contraceptive agent porcine zona pellucida (PZP). Even though the Little Book Cliffs HMA is a herd currently undergoing research, this analysis is using it solely as a proxy for other populations with similar demographics. The simulations that explored this herd's response to contraceptive treatment were not meant to emulate the research protocols currently being applied.

The McCullough Peaks HMA is located about 20 miles east of Cody, Wyoming, covering 110,000 acres of open sagebrush prairie and badlands. The current wild horse population in the McCullough Peaks area is about 400 animals, with the AML set between 70 and 140 animals, not including foals. This herd, too, was chosen by BLM and USGS for additional study pertaining to fertility control, population census techniques, and so forth, but no contraceptives have yet been applied. Unlike the other two herds, McCullough Peaks requires Tier 2 aged horses to be removed to achieve its appropriate management level, and may be required by law to do so.

Methods

The analysis of each herd was completed using five main steps. There was nothing fundamentally difficult about this process except the rather rigorous bookkeeping involved.

Step 1. Organize Jenkins Model Input Data and Parameters for Each HMA

Data representative of each of the three HMAs were compiled and organized in a fashion suitable for the Jenkins wild horse population model (Jenkins, 2002). Much of the vital background and operational philosophy for the Jenkins model is given in Appendix A. Suffice it to say that the model attempts to mimic the on-the-ground gather, selective removal, and contraceptive application processes faithfully while including variability in both annual environmental conditions and individual animals. The model requires inputs describing what is known or estimated for survival and reproductive rates, as well as how removals and contraception may be handled. For the Challis HMA, data and modeling parameters were adapted from U.S. BLM Challis Field Office (2002), a task simplified because modeling for this HMA had already been assembled for the Jenkins model and I assumed it to be correct. Existing computer input files (or equivalent) were used for the other two populations, supplemented by information provided by the herd managers. Important demographic parameters and other data on the three herds are provided in Table 2. Note that for two of the herds, Challis and McCullough Peaks, the survival and foaling rates used were borrowed from other better-studied populations assumed to be similar to their counterparts. Complete listings for the baseline data set for the three herds are given in Appendix B.

Step 2. Exercise the Jenkins Model for Each Scenario

The Jenkins model was employed for each herd. Each scenario was run as a separate simulation using model input parameters to describe the various management actions that might be taken, contraceptive effectiveness, and so on. Like any model, there are deviations from reality, some addressed in Appendix D. Nonetheless, for this analysis I have assumed that the Jenkins model provides a reasonably accurate portrayal of possible futures important in choosing one cost minimization strategy over another.

Step 3. Estimate Dollar Value for Each Management Cost Component

Dollar values were estimated for each of the main gathering, treating, and selective removal expenditures, along with associated costs related to wild horse management. Most dollar figures were taken from the state-specific costs listed in Table 3. These costs represent FY 2004 values, but are assumed to increase 3% annually regardless of geographic area to parallel the inflation rate BLM uses for planning. Removal costs include all expenses of gathering and transport to adoption or holding facilities, averaged across all removed horses. Preparation and holding costs include freeze branding and required vaccinations. Adoption costs are largely administrative with follow-up compliance checks (site visits to adopted horses).

Costs for multi-year contraceptives are given in Table 4. The range of costs given for the various duration time-release pellets reflects their production method. Higher cost pellets are currently more reliable (and that cost was used in this analysis), but BLM's goal is to reduce the cost to below the lower cost values, on the order of $120 per applied dose. (Note: These cost estimates may not be applicable for herds undergoing research where a variety of protocols may be tried.) Several other potential costs were also considered in the analysis. It was assumed that the minimum gather cost was $10,000. This comes into play only if the number of animals removed times the appropriate per horse removal cost would be below $10,000. A $5,000 per year HMA census flight cost was applied for non-gather years to assess contraceptive treatment effectiveness and routine monitoring per the recommendation of Ron Hall (2003), and was applied only for contraceptive scenarios.

Table 2. Key demographic elements and other information for the three HMAs considered in this analysis. These values were set to generally mimic an existing or proposed Environmental Assessment (EA) for each HMA.

	Challis	Little Book Cliffs	McCullough Peaks
Cost state	ID	CO	WY
Initial sex ratio (% male)	46	46	46
Sex ratio at birth (% male)	58	50	57
Age 0-9 female survival (geometric mean %)	98	95	95
Age 0-9 male survival (geometric mean %)	93	97	95
Average foaling rate age 2-9 (%)	79	65	71
Gather trigger (# of horses)	253	150	140
Gather efficiency (%)	75	80	90
AML (# of horses)	185	90	100
AML includes foals?	Yes	No	No
AML requires Tier 2 removal	No	No	Yes
Released mares treated for contraceptive alternatives by age	0-1 year: 100%[1] 2-9 year: 50% 10+: 100%	0-4 year: 100%[1] 5-9 year: 75% 10+: 100%	0-4 year: 100%[1] 5-9 year: 75% 10+: 100%

[1]Note: After this report was finalized, a reviewer noted that no foals would ever be given contraceptives. This is true. However, a careful review of simulation results revealed that eliminating contraceptives for foals would make only a small difference for the three populations studied. The reason for this is that most gathered foals are removed for adoption instead of being released back to the herd and the foaling rate for yearlings and 2-year old mares is low compared with older aged mares. Should this analysis ever be updated, the models should incorporate this change.

Table 3. Summary of variable cost estimates for BLM wild horse management across states. Unusually high costs in each column may arise when they are averaged over a small number of horses. Cost estimates were taken from budget planning spreadsheet supplied by Linda Coates-Markle, BLM/MT (4/29/2003) as interpreted by Don Glenn, BLM/DC (6/17/2003) and Lili Thomas, BLM/NV (9/22/2003). NA means not applicable.

Management Office	Removal Cost (/horse; $)	Prep & Holding Cost (/horse/day; $)	Adoption Cost (/horse; $)	Compliance Check (/horse; $)	Comments
Arizona	345	2.80	318	50	
California	211	3.13	305	59	
Colorado	433	3.04	291	60	Little Book Cliffs
Eastern States	NA	7.66	361	46	
Idaho	285	2.10	396	18	Challis
Montana	450	13.99	500	150	
Nevada	460	3.11	510	66	
New Mexico	433	3.15	362	81	
Oregon	360	3.35	300	50	
Utah	434	4.72	367	50	
Wyoming	300	5.21	760	70	McCullough Peaks

Table 4. Estimated cost range for current 2-year contraceptive materials. Estimates derived from information supplied by Ron Hall (BLM/NV) and Linda Coates-Markle (BLM/MT) 9/23-30/2003.

Component	Cost Range
Primer (PZP and Freunds complete adjuvant)	$21.50
Time release booster doses (PZP and QA-21 adjuvant)	
1-month pellet	$28 – $37
3-month pellet	$31 – $40
12-month pellet	$65 – $95
Application	$20
TOTAL	$165.50 – $213.50

Since some scenarios call for contraceptives of longer duration, I received an estimate for applications having greater longevity. This estimate is given in Table 5 along with the more reliable estimate for the 2-year agent. These are the dollar costs used in this analysis.

Table 5. Estimated per horse costs for existing 2-year and hypothetical 3-year contraceptive materials and application used in this analysis. Cost for the 3-year agent is composed of the total cost of a 2-year agent plus additional 12-month time-release pellets (from Table 2). Estimates derived from Linda Coates-Markle (BLM/MT) 9/30/2003.

Contraceptive Duration	Estimated Cost per Horse
2 years	$214
3 years	$309

Step 4. Estimate Dollar Costs from Simulated Scenarios

The results of the Jenkins model simulations were summarized and converted to dollar expenses over a 20-year planning horizon. Twenty years was chosen because it is long enough (five complete 4-year gather cycles) to 1) reduce the uncertainty inherent in estimates of the initial age and sex structure, and 2) reveal most of the effects of variation in sex and age structure that would result over time from the variety of treatment options. Tallying the total expenditures required all cost estimates previously described, including which ages were eligible for adoption and how long adoptable and unadoptable horses are held (see Figure 1). All unadoptable horses (those that have not been successfully placed after three adoption attempts) were assumed to remain in holding facilities for the remainder of their natural life, estimated to be 25 years. In other words, the economic model became its own population model, in a manner of speaking, because it kept track of horses in life-long holding facilities. Results were summarized by software that computed the mean number of horses gathered, removed, and treated by sex and age class for each year of the 20-year simulations, along with average annual costs. In addition, the cost summarization step computed the likely annual variation in costs that would be expected as a result of the variability inherent in the Jenkins model. This step is explained in more detail in Appendix B.

Step 5. Conduct Sensitivity Analysis

The Jenkins simulation model captures environmental and demographic variability, but the uncertainty in cost estimates for the various management options remained to be explored. To accomplish this, a sensitivity analysis was performed for the three populations to see where opportunities for cost cutting might lie and which factors contribute most to the bottom line.

Results

A full suite of results for the Challis HMA is given in Table 6 and Figures 2-6. In general, the results confirm that a four-year gather cycle has been a good management decision without contraceptive intervention. Waiting longer between gathers has the potential to significantly increase annual costs because population sizes compound so rapidly (~10-20% annually). Interestingly, Figure 2 shows that there is not much cost difference between a 2- versus a 4-year gather cycle, or between a 6- versus an 8-year cycle, but a large difference between the 4-year and 6-year cycles.

Annual costs are far less sensitive to gather efficiency (Figure 3), at least in the small increments examined here, but are nicely responsive to seemingly modest changes in sex ratio (Figure 4). Removing fewer males and increasing the sex ratio of animals remaining on the range from about 51% to 57% male can dramatically reduce costs.

Contraceptive treatments are shown to be cost effective. Contraceptives alone (Figure 5) can reduce management costs for the Challis herd up to about $15,000 per year. The best estimate of 2-year efficacy (Figure 5, 2c) rivals the results from a 3-year agent. When combined with alterations to herd sex ratio (Figure 6), these treatments appear capable of reducing costs to about 65-70% of baseline.

Interestingly, the Alternative Baseline Scenario alone is not predicted to generate a significant cost savings for the Challis herd. This appears to be because the foaling rate for 5-year olds is so much higher than for 4-year olds, and though this alternative does reduce the total number of horses held in long-term facilities, the population growth rate is slightly higher so the number of horses gathered and removed must also be higher. However, as noted above, combinations of contraceptive treatment and sex ratio manipulation (e.g., 3-year contraceptives/Sex ratio-56% male and Alternative Baseline/3-year contraceptive/Sex ratio-56%) have the potential to reduce average annual costs by about 25-35%. This is an important conclusion.

Results for Little Book Cliffs and McCullough Peaks are given in Tables 7 and 8, respectively. I have not repeated the alternatives for gather frequency or efficiency because they provided no additional insight.

Aggregate results were examined to see what other information they provide. Figure 7 shows the relationship between the variability in annual costs and herd population size developed from a variety of scenarios examined. The apparent trend appears to be an L-shaped function of population size; at population sizes below about 200 animals, the variability in annual costs increases dramatically. This is probably an effect engendered by the demographic stochasticity inherent in the Jenkins model, i.e., random events applied to small numbers of animals foster greater variability in the results, with variation in population size translating into variation in expected annual costs. However, the results may simply be an artifact of dealing with three herds, one of which is smaller than, and has different parameters than, the other two.

Table 6. Summary of results for scenarios of the Challis HMA.

Scenario	Average Annual Cost ($)	Percent of Baseline Cost (%)	Median Annual Growth Rate (%)
Baseline	86,677	100.0	19.8
Alternative Baseline	85,814	99.0	20.4
2-year contraceptive-a	83,095	95.9	17.8
2-year contraceptive-b	75,415	87.0	16.1
2-year contraceptive-c	73,478	84.7	15.6
3-year contraceptive	72,156	83.2	14.9
2-year contraceptive-a/ Sex ratio-56	67,442	77.8	12.9
2-year contraceptive-c/ Sex ratio-56	60,687	70.0	11.7
3-year contraceptive-a/ Sex ratio-56	59,900	77.0	11.0
Alternative Baseline/3-year contraceptive/Sex ratio-56	57,136	65.9	11.4
2-year gather cycle	83,765	96.6	19.9
6-year gather cycle	145,474	167.8	20.8
8-year gather cycle	149,745	172.8	20.4
-5% gather efficiency	89,899	103.7	19.9
+5% gather efficiency	86,667	99.9	20.0
+10% gather efficiency	84,178	97.1	20.0
Sex ratio = 53.1% males	83,209	96.0	18.1
Sex ratio = 55.1% males	78,569	90.7	16.6
Sex ratio = 57.3% males	68,619	79.2	14.5

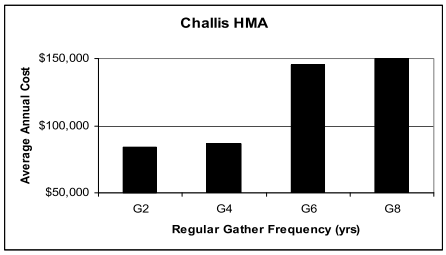


Figure 2. Annualized cost over a 20-year period for four gather frequencies for the Challis HMA (G-2, 4, 6, and 8 years, respectively).

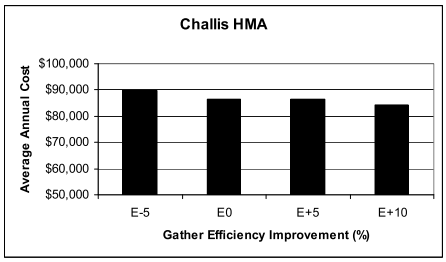


Figure 3. Annualized cost over a 20-year period for four gather efficiencies for the Challis HMA. Challis has an estimated baseline efficiency of 75%, so these scenarios represent 70, 75, 80, and 85% efficiencies, respectively.

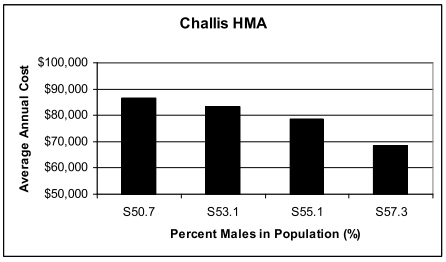


Figure 4. Annualized cost over a 20-year period for four resulting sex ratios for the Challis HMA (S-51, 53, 55, and 57% male, respectively).

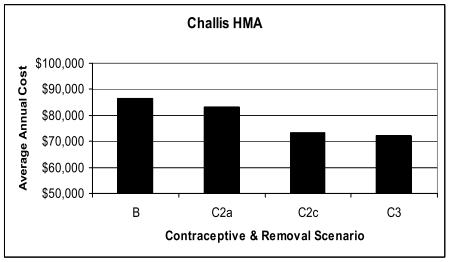


Figure 5. Annualized cost over a 20-year period for three contraceptive scenarios (2a,. 2c, and 30-year) compared to the baseline (B) for the Challis HMA.

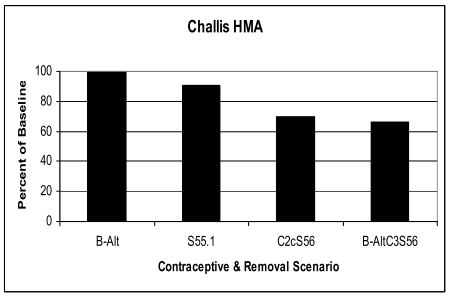


Figure 6. Percent of Baseline cost over a 20-year period for four scenarios: alternative baseline (B-Alt), 55.1% male, 2c with 56% male, and B-Alt with 3-year contraceptive and 55% males, respectively.

Table 7. Summary of results for scenarios of the Little Book Cliffs HMA.

Scenario	Average Annual Cost ($)	Percent of Baseline Cost (%)	Median Annual Growth Rate (%)
Baseline	50,766	100.0	12.9
Alternative Baseline	42,264	83.3	13.6
2-year contraceptive-a	46,230	91.1	9.6
2-year contraceptive-b	46,307	91.2	8.1
2-year contraceptive-c	37,596	74.1	6.9
3-year contraceptive	36,111	71.1	6.2
Sex ratio = 51.8% males	50,573	99.6	11.7
Sex ratio = 53.3% males	50,469	99.4	10.8
Sex ratio = 55.0% males	46,444	91.5	9.4
2-year contraceptive-a/ Sex ratio = 54% males	42,181	83.1	8.3
2-year contraceptive-c/ Sex ratio = 53% males	33,079	65.2	5.2
3-year contraceptive/ Sex ratio = 53% males	31,511	62.1	4.7
Alternative Baseline/ 3-year contraceptive/ Sex ratio = 53% males	28,128	55.4	5.1

Table 8. Summary of results for scenarios of the McCullough Peaks HMA.

Scenario	Average Annual Cost ($)	Percent of Baseline Cost (%)	Median Annual Growth Rate (%)
Baseline	16,8214	100.0	17.5
Alternative Baseline	150,836	89.7	17.5
2-year contraceptive-a	151,430	90.0	14.1
2-year contraceptive-b	137,295	81.6	11.2
2-year contraceptive-c	133,562	79.4	10.6
3-year contraceptive	109,197	64.9	10.0
Sex ratio = 60% males	157,759	93.8	14.5
Sex ratio = 61% males	145,534	86.5	12.2
Sex ratio = 63% males	138,669	82.4	10.0
2-year contraceptive-a/ Sex ratio = 63% males	130,136	77.4	8.4
2-year contraceptive-c/ Sex ratio = 64% males	118,241	70.3	6.3
3-year contraceptive/ Sex ratio = 63% males	118,497	70.4	5.9
Alternative Baseline/ 3-year contraceptive/ Sex ratio = 62% males	108,792	64.7	6.0

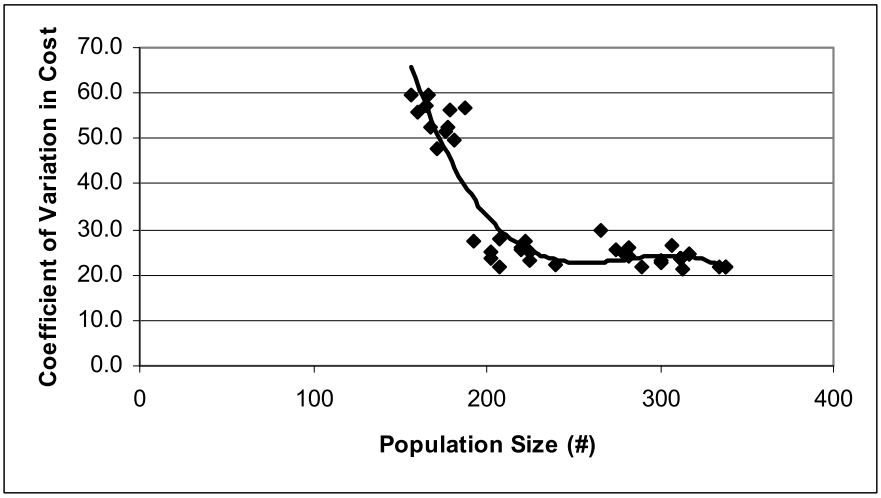


Figure 7. Apparent relationship between the variability in annual costs and herd population size developed from a variety of scenarios for the three populations examined. Trend line is 3rd order polynomial.

Figure 8 shows the relationship between the mean annual growth rate and percent of baseline scenario costs for the three populations combined. As growth rate declines through the application of various management and contraceptive options, the likelihood of reducing costs also declines. The variability (scatter) in results arises from the individual herd demographics and model-induced random variability. However, it seems apparent that when growth rates decline below about 10% annually, there is essentially a guarantee that costs will decline. Between 10 and 20% growth, large variability remains.

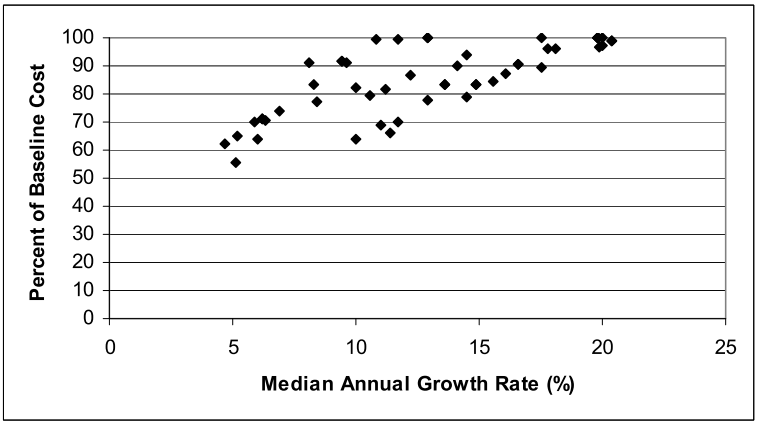


Figure 8. Relationship between mean annual growth rate and percent of the Baseline Scenario costs developed across all three populations examined.

Sensitivity Analysis for Cost Components and Related Factors

A basic sensitivity analysis was completed for the various elements that contribute to the cost estimates. This analysis tests how sensitive bottom line costs are to small changes in each of the contributing factors. The chart below (Figure 9) was generated by changing each cost and management factor ±10% and taking the ratio of the resulting cost fluctuation to the base cost of the 2-year Contraceptive Scenario-b for the Challis herd. Age class values were incremented up and down one age class – the minimum possible. (Because these age increments more closely represent a 20% change, their results have been divided by two to more closely reflect their relative impact.)

The results unsurprisingly indicate that the maximum age usually considered mostly adoptable (5 years) is the single largest influence on total costs. In other words, if this age were really four or six instead of five, there would be a large change in costs. Adoption age is followed by the average per day holding cost (*$/Unadoptable/day* = $5.21). Then comes a group of factors that have roughly equal sensitivity: the percent of age 5 horses that are adoptable (very much related to the maximum adoptable age), the number of days that horses are held prior to adoption (200 days), and the costs of adoption themselves. Costs related to contraceptives, treatment and off-year censuses, contribute little to the sensitivity, indicating that their cost is trivial compared with other management expenses. Four parameters have little or no effect: minimum gather cost, the maximum possible age of adoptable horses (10 years), the percentage of horses between age 5 and 10 (called *% of mid-ages* in Figure 9) that are adoptable (20%), and days unadoptable horses are held during their first year of removal (180 days). The two parameters dealing with age classes (*max age adoptable* and *% of mid-ages*) made no difference because none of these older aged animals needed to be removed to achieve the stated AMLs for the Challis herd.

Results of this form of sensitivity analysis for the other two herds were similar, but with some important differences. The holding cost (*$/Unadoptable/day)* was more important than the adoption age for the McCullough Peaks herd. This may not be surprising considering the high holding costs for this HMA given in Table 3. Likewise, the costs associated with adoption were somewhat less important for the Little Book Cliffs herd than the other two, again reflecting this herd's favorable adoption-related expenses.

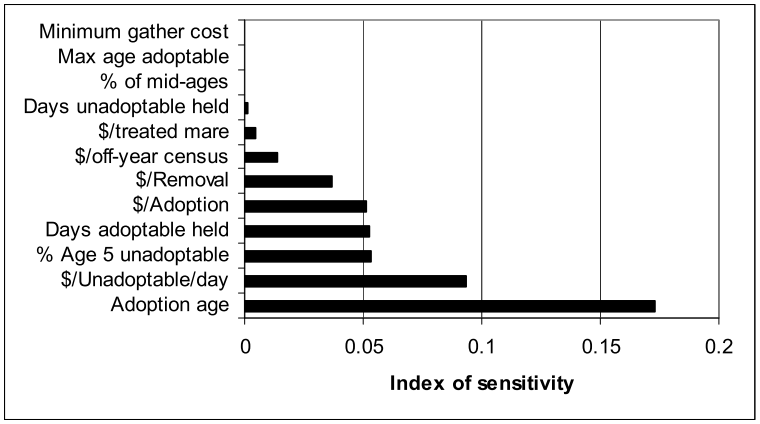


Figure 9. First order sensitivity analysis for management costs and other attributes for the Challis herd relative to the 2-year Contraceptive Scenario-b. The impact of adoption age has been divided by a factor of two to better represent its true relationship with the other factors.

Conclusions and Discussion

If all economists were laid end to end, they would not reach a conclusion.
– George Bernard Shaw

Results of this analysis have shown that when contraceptives are added to the gather-removal program, they are more cost effective than current or anticipated removal strategies that have no contraceptive component. When averaged over the variable costs anticipated with a regular 4-year gather (Figure 10), and depending on the herd and the aggressiveness of treatment, cost savings could range from 4 to 46%, averaging approximately 25%.

Populations controlled solely by gathers decline dramatically with each gather but increase rapidly between gathers. In contrast, populations controlled by contraceptives in conjunction with removals increase less rapidly. If sex ratio is also managed to increase males, even greater stability can result. Regardless of the management option, program costs always parallel population growth (Figure 10), agreeing with the findings of Gross (2000) and Garrott and others (1992).

A 4-year gather frequency appeared to be quite well matched with the demographic effects (and dollar costs) associated with 2-year contraceptive agents. However, if a viable 4-year agent becomes available, it would be wise to revisit the issue of gather frequency as it seems plausible that increasing the interval to five or more years may have economic benefits.

Comparison with Findings of Others

Garrott and others (1992) explored the impacts of alternative contraceptive management on wild horses in the early 1990s. They separated costs for wild horse management into three basic components: contract gather costs, contraceptive purchase and per mare application (including manufacture, implantation drugs and supplies, and

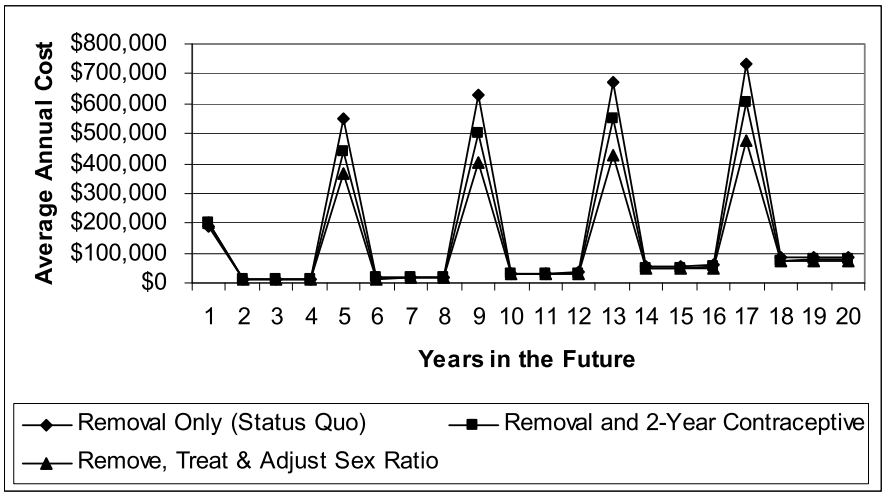


Figure 10. Example illustrating how periodic management expenses grow due to population growth, annual cost inflation, and holding of sanctuary horses for three possible scenarios.

veterinary services), and removal costs. They assumed all contraceptive applications would be associated with gathering as opposed to aerial darting for safety and logistic reasons. Adoption placement costs (including transportation, brand inspection, vaccinations and disease testing, branding, wrangler fees, age determination, administration, facility maintenance, and per day feeding) were more difficult to estimate. They broke costs down by age of horse since some age classes were considered adoptable while others were considered unadoptable, each having their own per horse cost. Like this analysis, Garrott and others (1992) simulated a variety of different herds, and considered more contraceptive alternatives than examined here. Their results were comparable, indicating that most variable program expenses are not related to contraceptive treatment per se, but rather remain associated with adoption program costs.

Others have pointed out that the cost-efficiency of contraceptive treatment for a species as long lived and with as high a reproductive rate as wild horses is extremely dependent on herd-specific demographics and management constraints (Garrott, 1991; Hobbs and others, 2000), and that contraception must be used in conjunction with an ongoing removal program (Gross, 2000). This example confirms that the current 22-month contraceptive agent within a 4-year gather cycle (2-year Contraceptive Scenario-c) can make an important difference in variable costs associated with management when gather efficiencies are typical (e.g., only 80%) and natural rates of increase are relatively high (Garrott, 1995). However, when combined with modest changes to the demographic structure of the population, such as altering the sex ratio to only a slight male bias, contraceptives may offer the possibility of far more substantive cost reductions.

It should be noted, however, that none of the contraceptive options considered in this analysis eliminate the need for long-term holding facilities. Collectively, the number of additional horses added to long-term holding is reduced by 23% over the simulated 20-year period with aggressive contraceptive treatment, yet only one of the three HMAs examined (McCullough Peaks) actually showed a decreasing number of horses added through the 20 years. The other two populations continued to increase the horses added to holding through time, albeit at rates 18-36% below the baseline scenario.

Putting the Results in Perspective

This analysis has explored methods of reducing costs through a combination of contraceptive and other potential management techniques. However, costs tabulated in the Results section should be considered *variable* costs because they arise from the variation in herd population dynamics and management strategy. In other words, the dollar values presented thus far are comparative variable costs that will accrue due to impending management decisions.

BLM also incurs certain *fixed* costs associated with the wild horse program that include all overhead (Washington and regional offices, many HMA monitoring costs, holding facility maintenance, etc.). The fixed costs could be assumed to continue more or less unchanged regardless of the specific gather and selective removal process, with the knowledge that some of these fixed costs, like annual monitoring, aerial censusing, and new or revised AML establishment, are subject to annual budgetary constraints. Portions of the fixed costs are 'sunk' costs that support the 20,000 wild horses currently residing in holding facilities. Like all fixed costs, these sunk costs have been ignored so far in this analysis because their presence (obligation) is the result of past decisions. Table 9 presents a summary of nationwide costs, both fixed and variable, for BLM's wild horse program.

As one can see, the variable costs represent slightly less than half (46%) of the aggregate costs. Results presented above have indicated that it might be reasonable to expect about a 38% annual decrease in variable costs if the most aggressive management methods were to be implemented. If these savings accrue solely to the variable side of the ledger, and if fixed/sunk costs remain the largest component of the overall wild horse program, even if variable costs were reduced by 38%, aggregate program costs would likely decrease by only 17%. Though it is probably best to couch the results of this analysis in relative terms, assuming that 17% is approximately correct for all herds combined, this would amount to about a $7.7 million annual savings in variable program costs in 2004 dollars. Significant changes to gather and selective removal procedures could alter the fixed cost structure (specifically labor and facility costs) such as completely closing an existing facility, further reducing costs. Sunk costs would be expected to decline through time if the number of unadoptable horses declines. These potential changes to overall program costs have not been considered in this analysis.

Table 9. Summary of nationwide fixed and variable cost estimates for BLM's wild horse program for FY2004. Cost estimates were summarized from budget planning spreadsheet supplied by Linda Coates-Markle, BLM/MT (4/29/2003) as interpreted by Don Glen, BLM/DC (6/17/2003) and Lili Thomas, BLM/NV (9/22/2003).

Cost Type	Factor	Expense	Total	Percent (%)
Fixed				
	Annual Monitoring	$219,912		
	Annual Censusing	$369,590		
	AML Establishment	$56,635		
	Labor	$8,862,197		
	Non-unit cost	$3,513,962		
	Long Term Holding Facilities	$6,387,500		
	==========	=========		
	Total Fixed Costs	$23,801,893		54
Variable				
	Selective Removal	$5,331,471		
	Prep & Holding	$12,046,381		
	Adoption	$2,713,550		
	Compliance Checks	$279,150		
	==========	=========		
	Total Variable Costs	$20,370,552		46
Fixed + Variable			$44,172,445	100

Uncertainty Inherent in Results

It is important to try to describe each of several major forms of uncertainty inherent in this analysis. *First* data quality for individual herd status (age and sex composition, fertility and survival rates, etc.) can vary widely. We must acknowledge that these horse populations are difficult and expensive to monitor, and our estimates of initial conditions and the various vital parameters are inaccurate (or indeed borrowed from other, better studied populations), but we must start with the information as best as it is currently understood. Fortunately, these data may be improved through time.

Second is forecasting. Population births and deaths are subject to the whims of animal and range conditions, predation, and other poorly understood population regulation mechanisms. In essence, the nature of the Jenkins population model used in this analysis automatically accounts for much of the uncertainty in initial conditions and lack of predictability of forecasts. As we have seen, this variability, as reflected in the coefficient of variation of annual costs, appears to be in the neighborhood of 20-30% annually for each HMA simulated. It may take many years to improve our estimates of this forecast uncertainty.

Third, there is the uncertainty in the BLM-derived estimates of expenses for each of the several cost components: gathering, selective removal, adoption, holding, contraceptive treatment, and population censusing. Costs vary through time and with the vagaries of local conditions. The cost summarization software automatically accounts for the uncertainty passed to it from the population model, but does not automatically incorporate uncertainty in the cost estimates, though this has been addressed to some degree through sensitivity analysis. Further, since this cost analysis has focused on variable program expenses, overhead costs have been treated as fixed costs and, along with 'sunk' costs (those costs originating from past decisions) such as the existing sanctuary inventory, have been summarized only to put the variable costs in perspective. There are obvious uncertainties in fixed costs that hinge on the practical life of all physical facilities (e.g., adoption corrals, fencing, etc.) and it is reasonable to assume that the 20-year time frame used in this analysis is long enough that new contraceptive techniques or agents may be available that change the complexion of the results presented here.

There is a bright side to some of this uncertainty. The computed coefficients of variation for costs are meant to be relevant for single populations. When considering the variation in costs that might arise program-wide,

it is reasonable to assume that the overall variance would be reduced. This is largely because gather schedules would presumably be staggered, but also because some populations would be increasing while others would be decreasing, with the 'average' expected to be less erratic, much like the behavior of a 'balanced' portfolio. One should never discount region-wide oscillations that might be due to widespread drought or other climatic oscillations (El Niño) that may provide broadly favorable or unfavorable habitat conditions over large geographic areas like the Intermountain West. On average though, variable program costs would not be expected to fluctuate more than about ±20% across all herds at the same time.

Research and Management Recommendations for BLM's Consideration

Sex Ratio Manipulation

Many of the environmental assessments that I reviewed, as well as some additional published literature (Berger, 1986), have recommended maintaining populations with 'natural' sex ratios that tend toward females, e.g., 40 males:60 females (e.g., U.S. BLM Worland Field Office 2000). Since sex ratio measured in the field results from the combined effects of sex ratio at birth and relative male:female survival and removal rates that vary from herd to herd, I believe that it may be problematic to characterize a 'natural' sex ratio, especially for a feral species undergoing active management.

In any event, this analysis has assumed no *a priori* constraint on the sex ratio resulting from any given management plan, and even though examining how changes to each HMA's sex ratio influenced cost was not the primary goal of this analysis, simulations revealed that only modest changes in herd sex structure could rival cost savings inherent in available contraceptive application techniques. When both contraceptives and sex ratio manipulation were combined, cost savings could be even more significant. Herds that have small groups of bachelor males that are difficult to gather may lend themselves easily to this paradigm, while other herds that have many small harems may prove more difficult and costly to manage favoring males. Further, there may be potential genetic concerns regarding sex ratios that favor males. Nonetheless, any implicit or explicit BLM policy recommending sex ratios inclined toward females should be reexamined in light of the results presented here.

Balancing Range Conditions with Population Size

The 20-year simulations used in this analysis often showed that any given management strategy might take several years to achieve relatively stable demographic characteristics for each herd. For example, Figure 11 is taken from one Jenkins model simulation for McCullough Peaks. This plot illustrates that the population size approaches stability after a few gather cycles. Recall that McCullough Peaks was simulated by removing animals from only Tiers 1 and 2. It may have reached its AML more quickly if Tier Three animals had also been immediately removed. However, the apparent delay in population reduction has management implications. One might be tempted to adjust the management strategy after only one or two gather cycles if the population did not appear to be responding (or management costs declining) as fast as considered desirable. Although removal decisions must always be made aided by good data and concrete management objectives, the results shown here also argue for patience in population management for these long-lived animals. In particular, a management option that removes only age 0-4 horses without any other age classes removed may result in a somewhat higher number of horses on the range initially, yet produce a satisfactory reduction over the long term. If the range is capable of sustaining a larger number of horses for several years, this more patient strategy could significantly help reduce long-term management and holding costs.

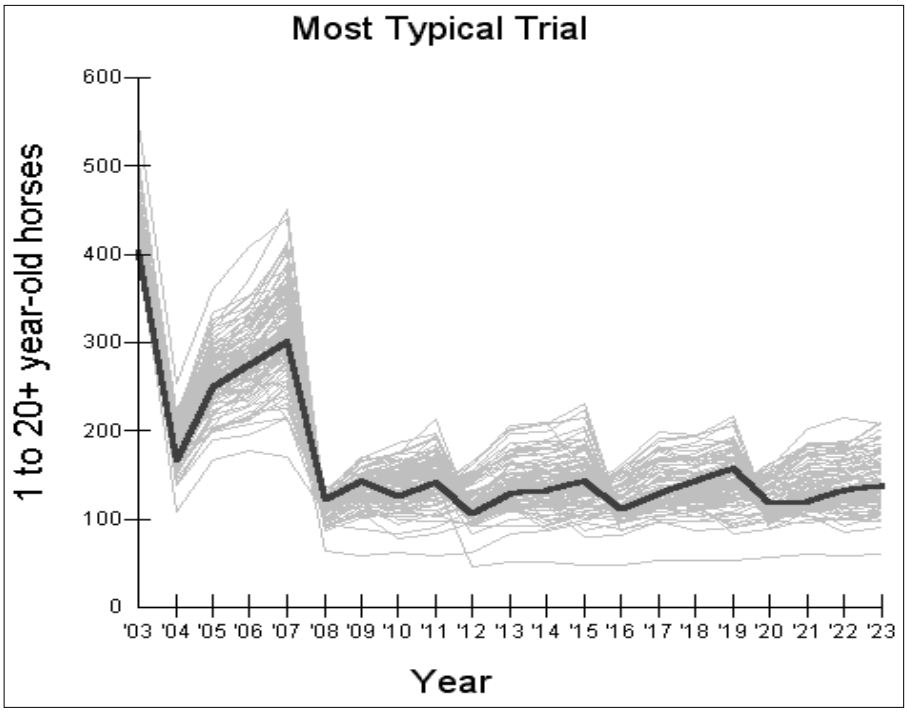


Figure 11. Example Jenkins model output for the McCullough Peaks HMA showing the total after-gather population size (minus foals since foals are not included in this AML) through a 20-year simulation. The light lines trace the 100 individual simulation trials and the heavy line traces the "most typical" result.

Population Modeling Software Improvements

Though modeling each wild horse herd with the Jenkins model is established BLM policy (U.S. BLM 2002), not all herds currently have such models. Even if the models have been assembled for a specific herd, these models may not necessarily reflect established selective removal guidelines, but instead be tailored toward previous removal practices. This analysis was not intended to be a critical assessment of the existing HMA models, but simply used what was available for the three herds examined here.

I have mentioned several areas where the Jenkins model might be improved to more faithfully represent the specific gather, selective removal, and contraceptive practices currently being used or contemplated as management decisions are fine-tuned (see Appendix D). I must admit that I do not have a good feel for whether modifications would significantly change the relative standing of the various scenarios examined here. My assumption is that simulation results would indeed differ, but that these differences would not actually change decisions based on those results, the ultimate form of sensitivity analysis. Nonetheless, it will be worthwhile to continually monitor where the model deviates from reality and test or upgrade it in response to perceived problems or improved data quality from research results.

Tom Hobbs and colleagues at Colorado State University are developing another individual-based modeling package. This software will contain a mathematical routine that will have the capability to suggest an 'optimum' gather, selective removal, and contraceptive management program for various herds, something that this current cost

analysis addressed only in a limited trial-and-error manner. When fully developed, the new software could be applied side-by-side with the Jenkins model to identify areas where each product might be improved.

Population Modeling Process Improvements

Results given in this analysis have seconded the findings of Garrott (1991) and Kirkpatrick and Turner (1986) that specific contraceptive prescriptions can depend strongly on individual herd population dynamics. Drawing on a large experience in big game population modeling in the West, I believe there will be a need to have one or more 'experts' to consistently apply whatever population model is used to evaluate HMA environmental assessments. Data gathering for each herd can be unique with subtle data quality issues. Modeling software, though ostensibly easy to use once you are familiar with its nuances, can produce very different results depending on exactly how the model structure is interpreted and applied for each herd. Though it will always be a good idea to have the herd managers closely involved in population modeling, such modeling will never be a day-to-day activity for them. These individuals often move from position to position, and applying the software will often receive only minimal attention. Filtering the HMA models through a specialist could 1) provide a level of quality control that may be necessary both in making cost effective long-term management decisions that can more easily respond to changing management directives, and 2) stand up to increasing scrutiny in our ever more sophisticated and litigious society. BLM should consider hiring an individual who can uniformly apply the appropriate population modeling software across the various herds, and assist and advise all of the herd managers in the uniform application of techniques. Further, it might be wise to encourage publication of modeling results in peer-reviewed journals to provide a solid foundation for scientific credibility.

Revised Cost Accounting

Because of the way BLM has tabulated removal costs, the base cost of gathering is averaged across all horses removed. Any management activity, like contraceptives, that results in an overall reduction of the number of horses that need to be removed to achieve the stated AML would likely end up with a higher cost per animal removed because all relatively invariant gather costs would be averaged across fewer horses. If, for example, gathering were used solely for a contraceptive treatment so effective that no horses were ever removed, the cost of the gather would not be accountable with BLM's current cost estimation procedure, at least as I have interpreted it. This is the reason why I included an estimate of the minimum gather cost.

Fortunately, sensitivity analysis has indicated that this is not likely to dramatically influence the results for many populations. Nonetheless, BLM may wish to partition total gather costs into two categories, one that would represent the base cost for a gather (to get the crew and equipment in place, construct fences and traps, permits, supplies, etc.) and specific per horse removal costs for marking, any veterinary services, and transport. This would support a cost accounting that more easily scales downward to reduced removal levels, a desirable target for the horse program.

Acknowledgments

This report could not have happened had it not been for many helping hands. Linda Coates-Markle (BLM) and Francis Singer (USGS) helped define the objectives, establish useful contacts, supplied interesting insights, and reviewed progress. Don Glenn, Ron Hall, and Lili Thomas (BLM) coached me on the details of BLM's cost estimates and how their existing processes worked. Tricia Hatle (BLM) and Linda Zeigenfuss (USGS), with help from Jim Dollerschell (BLM) and Gerald Thygerson (BLM retired), supplied Jenkins' HMA models or equivalent for the McCullough Peaks and Little Book Cliffs populations. Linda Zeigenfuss also helped tirelessly with numerous other data gathering and related activities. Stephen Jenkins (U. Nevada Reno) was generous with his time in coaching me in the use of his population modeling software, WinEquus. John Turner (Medical College of Ohio) supplied cost estimates for longer-term contraceptive treatments under development. Jason Ransom supplied

background information on two of the herds examined. Linda Zeigenfuss, Steve Jenkins, Francis Singer, Linda Coates-Markle and Al Kane (USDA) all provided excellent comments on the first draft of this document. Finally, I must acknowledge that this report would have been unnecessary if we had been able to devise a successful abstinence program for wild horses.

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Appendix A. Using the Jenkins' WinEquus Software

General Description

A description of the Jenkins modeling software taken from selected portions of the WinEquus 'Help file' (Copyright 2002 by Jenkins and Veech, Version 1.4, April 2002 – used with permission) follows:

Overview

This is a population model for feral horses, designed to help you evaluate various management plans that might be considered for a particular area. The model uses data on average survival probabilities and foaling rates of horses to project population growth for up to 20 years. The model accounts for year-to-year variation in these demographic parameters by using a randomization process to select survival probabilities and foaling rates for each age class from a distribution of values based on these averages. This aspect of population dynamics is called environmental stochasticity, and reflects the fact that future environmental conditions that may affect horse populations can't be known in advance. Therefore each trial with the model will give a different pattern of population growth. Some trials may include mostly 'good' years, when the population grows rapidly; other trials may include a series of several 'bad' years in succession. The stochastic approach to population modeling uses repeated trials to project a range of possible population trajectories over a period of years, which is more realistic than predicting a single specific trajectory.

The model incorporates both selective removal and fertility treatment as management strategies. A simulation may include no management, selective removal, fertility treatment, or both removal and fertility treatment. You can specify many different options for these management strategies such as the schedule of gathers for removal or fertility treatment, the threshold population size which triggers a gather, the target population size following a removal, the ages and sexes of horses to be removed, and the effectiveness of fertility treatment.

To run the program, you must supply an initial age distribution (or have the program calculate one for you), annual survival probabilities for each age-sex class of horses, foaling rates for each age class of females, and the sex ratio at birth. Sample data are available for all of these parameters. You must also specify basic management options.

Population Data: Age-Sex Distribution

An important point about the initial age-sex distribution is that it is NOT necessarily the starting population for each of the trials in a simulation. This is because the program assumes that the initial age-sex distribution supplied on this form or calculated from a population size that you enter is not an exact and complete count of the population. For example, if you enter an initial population size of 100 based on an aerial survey, this is really an estimate of the population, not a census. Furthermore, it is likely to be an underestimate, because some horse will be missed in the survey. Therefore, the program uses an average sighting probability of about 90% (Garrott and others, 1991) to "scale-up" the initial population estimate to a starting population size for use in each trial. This is done by a random process, so the starting population sizes are different for all trials.

Contraceptive Efficacy

A set of parameters on the management options form called Percent Effectiveness of Fertility Control interacts with the contraception parameters to determine the actual reduction in reproduction in the population. For example, if effectiveness for the first year = 80% and 50% of released mares of each age class are treated, then only 40% of released mares in the population (80% x 50%) are actually infertile for the first year.

Contraceptive Parameters

Because of the way immunocontraceptives work (Turner and others, 1997), sterility in the first year really means that an effectively treated female will not produce a foal in the second foaling season after being treated. Mating in horses typically follows fairly soon after foaling. If gathers for treatment occur following mating, as the model assumes, then the female may already be pregnant and the immunocontraceptive won't cause her to abort the foal she is carrying. Instead, it will prevent fertilization during the mating season after this foal is born the following spring or summer, so will prevent foaling the spring or summer after that.

It may seem contradictory to remove 100% of 0 to 5 year-old horses [as an example] and at the same time treat 100% of 0 to 5 year-old mares. But remember that the contraception parameters are percentages of *released* mares to be treated. If the gather stops when the population has been reduced to the target level, then under these conditions no treated 0 to 5 year-old mares will be released. But it may be desirable to continue the gather after the population has been reduced to the target level, and treat and release extra females that are brought in. If your choice … is to continue the gather to treat females, and the removal and contraception parameters are as stated in the beginning of this paragraph, then until the target is reached all 0 to 5 year-old females (as well as males) will be removed as they are brought in. The gather will continue after the target is reached, and additional 0 to 5 year-old females gathered will be treated and released.

Environmental Stochasticity

For any natural population, mortality and reproduction vary from year to year due to unpredictable variation in weather and other environmental factors. This model mimics such *environmental stochasticity* by using a random process to increase or decrease survival probabilities and foaling rates from average values for each year of a simulation trial. Each trial uses a different sequence of random values, so gives different results for population growth. Looking at the range of final population sizes in many such trials will give you an indication of the range of possible outcomes of population growth in an uncertain environment.

How variable are annual survival probabilities and foaling rates for wild horses? The longest study reporting such data was done at Pryor Mountain, Montana by Garrott and Taylor (1990). Based on 11 years of data at this site, survival probability of foals and adults combined was greater than 98% in 6 years, between 90 and 98% in 3 years, 87% in 1 year, and only 49% in 1 year of severe winter weather. These values clearly aren't normally distributed, but can be approximated by a logistic distribution. This pattern of low mortality in most years but markedly higher mortality in occasional years of bad weather was also reported by Berger (1986) for a site in northwestern Nevada. Therefore, environmental stochasticity in this model is simulated by drawing random values from logistic distributions.

Because year-to-year variation in weather is likely to affect foals and adults similarly, this model makes foal and adult survival perfectly correlated. This means that when survival probability of foals is high, so is survival probability of adults, and vice versa. By contrast, you may adjust the correlation between survival probabilities and foaling rates to any value between -1 and +1. The default correlation is 0 based on the Pryor Mountain data and the assumption that most mortality occurs in winter and winter weather is not highly correlated with foaling-season weather.

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WinEquus produces a variety of tables and graphs of simulation results. For this economic analysis, the WinEquus simulation results were saved to a text file that was subsequently read by a special-purpose program (see Appendix C) to calculate expected economic costs (and their variability) over a 20-year period.

Setting Jenkins Model Simulations for Economic Analysis

Following the lead of U.S. BLM Challis Field Office (2002) and the values or settings the Jenkins' WinEquus model uses by default if no other choice is made, the following 'switches' were used in all HMA model simulations. All other management switches or options varied depending on the specifics of each alternative evaluated and have been described in the main text.

- Simulations were run for 20 years (producing 21 years of simulation output) with 100 trials each (100 trials is the default)
- Gathering for removal occurred at regular 4-year intervals
- When fertility control was used:
    - Gathers for fertility control occurred regardless of population size
    - Gathers continued after removals to treat additional females to be released (default if the above condition is true. Note, however, that the percentage of females actually treated by age class depends on other model input.
- Scaling factors for annual variation:
    - survival probabilities = 1.00 (default)
    - foaling rates = 1.00 (default)
- Correlation between annual variation in survival probabilities and foaling rates = 0.00 (default)
- Initial population size is inexact but smoothed (default)
- Foal survival is not density dependent (default)
- Minimum age of sanctuary-bound horses: Not applicable (default)

The general procedure for each HMA model would be to 1) load the three relevant input files [initial sex and age structure, survival probabilities by sex and age class, and foaling rates by mare age class along with sex ratio of foals] making any change to the total estimated population size as necessary; 2) define each management option that consists of six parts: selective removal rates by sex and age class, contraceptive options, the 'gather when population exceeds' trigger, the 'reduce population to' level, whether or not foals are included in the AML, and percent of the population that can be gathered; 3) run the simulation model for 20 years using 100 trials each; 4)

examine the simulation results to make sure that AML was achieved; 5) save the results to a uniquely named text file designating each scenario; 6) record the median growth rate associated with that simulation; and 7) proceed to the cost estimation program.

Appendix B. Listings of Jenkins Model Parameters for the Three HMAs Analyzed

The following three tables present the basic parameters used in Jenkins' model simulations. Note, however, that the values in the columns titled "Initial Base Population" for males and females are actually randomly sampled values that assume that the user-specified model inputs are approximately 90% of the 'true' value. In addition, the log files incorrectly report that gathers do not continue after removals to treat additional females (Steve Jenkins, personal communication). This error has been corrected in these three tables.

Note: After this report was finalized, a reviewer noted that no foals would ever be given contraceptives. This is true. However, a careful review of simulation results revealed that eliminating contraceptives for foals would make only a small difference for the three populations studied. The reason for this is that most gathered foals are removed for adoption instead of being released back to the herd and the foaling rate for yearlings and 2-year old mares is low compared with older aged mares. Should this analysis ever be updated, the models should incorporate this change.

Table B.1. Challis Jenkins model log file for 2-year Contraceptive Scenario-a. Survival and foaling rates were borrowed from the Garfield Flat herd for BLM's modeling.

Age Class	Initial Base Population		Survival Probabilities		Foaling Rates	Percentages for Removals		Percentages for Fertility Treatment
	Females	Males	Females	Males		Females	Males	
foal	25	25	0.919	0.877	0.00	100%	100%	100%
1	12	10	0.996	0.950	0.00	100%	100%	100%
2	32	36	0.994	0.949	0.52	100%	100%	50%
3	16	16	0.993	0.947	0.67	100%	100%	50%
4	16	11	0.990	0.945	0.76	100%	100%	50%
5	9	10	0.988	0.942	0.89	100%	100%	50%
6	11	7	0.985	0.939	0.76	0%	0%	50%
7	10	5	0.981	0.936	0.90	0%	0%	50%
8	8	5	0.976	0.931	0.88	0%	0%	50%
9	3	5	0.971	0.926	0.91	0%	0%	50%
10-14	12	11	0.947	0.903	0.81	0%	0%	100%
15-19	9	4	0.870	0.830	0.82	0%	0%	100%
20+	3	2	0.591	0.564	0.75	0%	0%	100%

Sex ratio at birth: 58% males
Scaling factors for annual variation: survival probabilities = 1.00, foaling rates = 1.00
Correlation between annual variation in survival probabilities and foaling rates = 0.00

Management by removals and fertility control
Starting year is 2003
Gathering occurs at regular interval of 4 years
Initial gather year is 2003
Gathers for fertility treatment occur regardless of population size.
Gathers continue after removals to treat additional females.
Threshold population size for gathers is 253.
Target population size following removals is 185.
Foals are included in AML.
Percent of population that can be gathered = 75%.
Percent effectiveness of fertility control: year 1 is 80%, year 2 is 50%, year 3 is 0%, year 4 is 0%, year 5 is 0%.

Table B.2. Little Book Cliffs Jenkins model log file for 2-year Contraceptive Scenario-a. Survival and foaling data were not borrowed from any other herd.

Age Class	Initial Base Population		Survival Probabilities		Foaling Rates	Percentages for Removals		Percentages for Fertility Treatment
	Females	Males	Females	Males		Females	Males	
foal	11	11	0.919	0.936	0.00	100%	100%	100%
1	13	13	0.983	0.962	0.00	100%	100%	100%
2	7	9	0.935	0.948	0.04	100%	100%	100%
3	5	5	1.000	0.963	0.59	100%	100%	100%
4	6	8	0.968	0.957	0.58	100%	100%	100%
5	4	6	0.976	0.971	0.75	100%	100%	75%
6	4	4	0.985	0.962	0.83	0%	0%	75%
7	3	5	0.938	0.979	0.74	0%	0%	75%
8	3	3	0.879	1.000	0.81	0%	0%	75%
9	2	5	0.944	1.000	0.82	0%	0%	75%
10-14	8	9	0.970	0.983	0.64	0%	0%	100%
15-19	2	3	0.980	0.001	0.74	0%	0%	100%
20+	1	1	0.794	0.001	0.00	0%	0%	100%

Sex ratio at birth: 50% males
Scaling factors for annual variation: survival probabilities = 1.00, foaling rates = 1.00
Correlation between annual variation in survival probabilities and foaling rates = 0.00

Management by removals and fertility control
Starting year is 2003
Gathering occurs at regular interval of 4 years
Initial gather year is 2003
Gathers for fertility treatment occur regardless of population size.
Gathers continue after removals to treat additional females.
Threshold population size for gathers is 150.
Target population size following removals is 90.
Foals are excluded from AML.
Percent of population that can be gathered = 80%.
Percent effectiveness of fertility control: year 1 is 80%, year 2 is 50%, year 3 is 0%, year 4 is 0%, year 5 is 0%.

Table B.3. McCullough Peaks Jenkins model log file for 2-year Contraceptive Scenario-a. Survival and foaling rates applied by BLM were borrowed from the Granite Range herd.

Age Class	Initial Base Population		Survival Probabilities		Foaling Rates	Percentages for Removals		Percentages for Fertility Treatment
	Females	Males	Females	Males		Females	Males	
foal	20	5	0.917	0.917	0.00	100%	100%	100%
1	12	3	0.969	0.969	0.00	100%	100%	100%
2	8	8	0.951	0.951	0.35	100%	100%	100%
3	8	3	0.951	0.951	0.40	100%	100%	100%
4	10	5	0.951	0.951	0.65	100%	100%	100%
5	12	2	0.951	0.951	0.75	100%	100%	75%
6	33	5	0.951	0.951	0.85	0%	0%	75%
7	33	11	0.951	0.951	0.90	0%	0%	75%
8	30	12	0.951	0.951	0.90	0%	0%	75%
9	31	20	0.951	0.951	0.90	0%	0%	75%
10-14	29	54	0.951	0.951	0.85	0%	0%	100%
15-19	19	49	0.951	0.951	0.70	0%	0%	100%
20+	11	39	0.951	0.951	0.70	0%	0%	100%

Sex ratio at birth: 57% males
Scaling factors for annual variation: survival probabilities = 1.00, foaling rates = 1.00
Correlation between annual variation in survival probabilities and foaling rates = 0.00

Management by removals and fertility control
Starting year is 2003
Gathering occurs at regular interval of 4 years
Initial gather year is 2003
Gathers for fertility treatment occur regardless of population size.
Gathers continue after removals to treat additional females.
Threshold population size for gathers is 140.
Target population size following removals is 100.
Foals are excluded from AML.
Percent of population that can be gathered = 90%.
Percent effectiveness of fertility control: year 1 is 80%, year 2 is 50%, year 3 is 0%, year 4 is 0%, year 5 is 0%.

Appendix C. Program to Estimate Economic Costs from WinEquus Simulation Results

As mentioned in Appendix A, the Jenkins model was used to simulate the individual populations' alternative futures and the simulation results were written to a text file. A Microsoft VisualBasic program was constructed to read these results and calculate average yearly costs as well as overall average costs for a 20-year period. User-specified input to this program (Figure C.1) includes cost estimates for the individual components of the variable costs for each state.

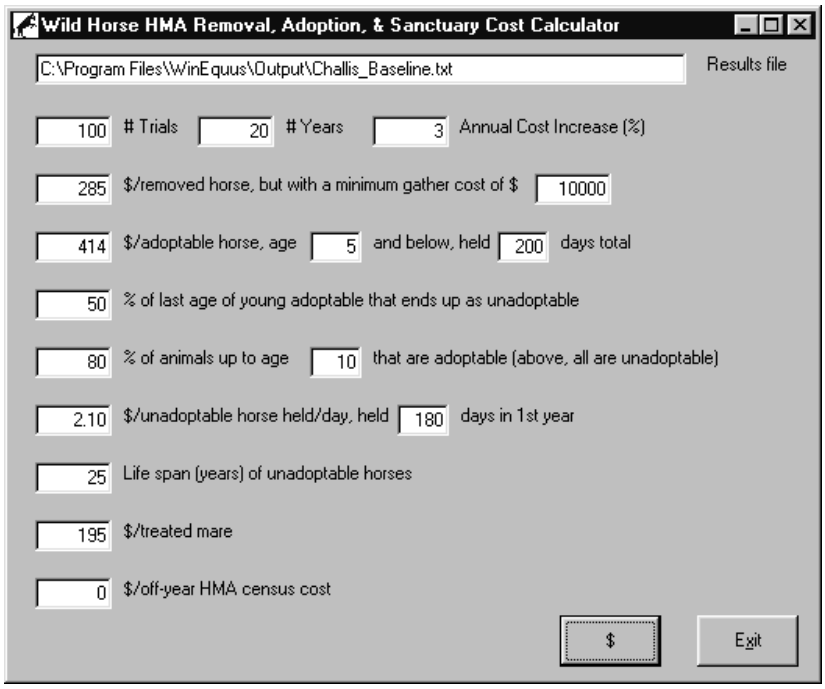


Figure C.1. Input costs and parameters for companion program to estimate costs of each specific simulation run with the Jenkins modeling software.

The number of *trials* and number of *years* are set to match the Jenkins model set-up. The number of trials and number of years captures both the variability inherent in stochastic simulation model and any population adjustments in age and sex structure that occur over about one horse life span. The *annual cost increase* adjusts all future expenditures for the rate of inflation. The *$/removed horse* reflects the cost of gathering and removal averaged across all removed horses. *Minimum gather cost* is just what it says, i.e., even if the number of gathered horses is small, there would be a minimum cost just to have a gather. The *$/adoptable horse* reflects the combined cost of adoption and compliance checks (Table 3). All horses up to the first age listed are assumed to be adoptable, except for the *% of last age of young adoptable that ends up as unadoptable*. In other words, a certain percentage of the oldest age class of adoptable animals (50% of age 5 animals in the above figure) is considered unadoptable. Adoptable animals are held for the first number of *days* listed prior to adoption. A *% of animals up to age* xx are also considered adoptable. Unadoptable animals accrue a cost of *$/unadoptable horse held/day*, are held *days in 1st*

year, and 365 days thereafter through their *life span*. Note that adoptable horses also accrue the same holding cost for the days they are held prior to adoption. Contraceptive application is reflected in the *$/treated mare* cost estimate. *$/off-year HMA census cost* reflects any additional costs involved with a contraceptive program in non-gather years (typically years 2 through 4), such as flight costs to assess treatment effectiveness and perform other routine monitoring (Hall, 2003). This last item would be zero except for scenarios involving contraceptive treatment.

The program reads the simulation results, averaging the costs for each year over the number of trials for which the software was run, and then summarizes the results across all simulation years. The output from this program looks like that shown in Table C.1.

Table C.1. Example output from cost estimator program for Challis 2-year Contraceptive Scenario-a.

```
C:\Program Files\WinEquus\Output\Challis_C2a.prn  11/21/2003  3:06:40 PM
 Trials = 100   Years = 20   Inflation % = 3
 $/Removed horse = 285 , with minimum gather cost = 10000
 $/Adoption = 414 up to age 5 and held 200 days
 55 % of last 'fully' adoptable age diverted to unadoptable
 80 % of ages up to 10 that are adoptable
 $/Unadoptable/Day = 2.1 held 180 days the 1st year
 Life span (yrs) = 25        $/Treated mare = 214
 $/Off-year census = 5000 (Include for treatment scenarios only!)

Year     Expense    ±_CV  PopSize SexRat | Gather Treat Remove Adopt UnAdopt | Held  Die
-------------------------------------------------------------------------------------
   1    $137,325   15.6%      305  0.467 |    218    29    120   114       6 |    6    0
   2      $9,934   11.3%      238  0.478 |      0     0      0     0       0 |    6    0
   3     $10,232   11.3%      269  0.488 |      0     0      0     0       0 |    6    0
   4     $10,539   11.3%      318  0.497 |      0     0      0     0       0 |    6    0
   5    $256,159   19.3%      390  0.508 |    278    19    197   194       3 |    9    0
   6     $14,202   12.5%      242  0.487 |      0     0      0     0       0 |    9    0
   7     $14,629   12.5%      264  0.492 |      0     0      0     0       0 |    9    0
   8     $15,067   12.5%      316  0.505 |      0     0      0     0       0 |    9    0
   9    $276,073   26.4%      377  0.509 |    267    20    187   181       5 |   15    0
  10     $21,295   13.2%      239  0.487 |      0     0      0     0       0 |   15    0
  11     $21,934   13.2%      260  0.493 |      0     0      0     0       0 |   15    0
  12     $22,592   13.2%      301  0.505 |      0     0      0     0       0 |   15    0
  13    $300,125   27.8%      364  0.514 |    262    25    176   170       6 |   21    0
  14     $30,463   13.2%      233  0.500 |      0     0      0     0       0 |   21    0
  15     $31,377   13.2%      255  0.508 |      0     0      0     0       0 |   21    0
  16     $32,318   13.2%      299  0.519 |      0     0      0     0       0 |   21    0
  17    $330,713   26.3%      354  0.526 |    255    22    168   163       5 |   26    0
  18     $41,064   12.4%      228  0.512 |      0     0      0     0       0 |   26    0
  19     $42,296   12.4%      248  0.517 |      0     0      0     0       0 |   26    0
  20     $43,565   12.4%      291  0.526 |      0     0      0     0       0 |   26    0
-------------------------------------------------------------------------------------
Mean     $83,095   21.6%      290  0.502 |    256    23    169   164       5 |   15    0

          71.0% for Adoptions
          19.2% for Holdings
           9.8% for Treatment
```

The economic model output contains the name of the Jenkins model simulation results file and echoes the input values used. *Expenses* are inflation-adjusted values and *CV* is the coefficient of variation, i.e., the percent that expenses might be expected to vary annually given the variability reflected in the stochastic population model. The CV value is calculated as one standard deviation from the mean value for the year divided by that mean value. The remaining values listed (population size, sex ratio, number gathered, number treated, number removed, number adopted, number unadoptable, number held, and number dying) also represent annual averages, rounded to the nearest animal. The *mean* values listed near the bottom are averages across the number of years, except for those associated solely with gathering (population size, sex ratio, number gathered, number treated, number removed, number adopted, number unadoptable), which are averaged across the number of gathers. Finally, the program

provides the percentage of the mean annual expense attributable to the total cost of adoptions, long-term holding, and contraceptive treatment.

Scanning the values listed in Table C.1 above, one can usually see how the population is adjusting through time to the management strategy implemented in the population modeling software. It is also a useful way to assess whether the selective removal rates specified in the Jenkins model have been effective in reaching the specified herd-specific AML – if foals are included in the AML.

Appendix D. Caveats to this Analysis

In conducting any sort of analysis that pretends to forecast the future, there are numerous caveats that should be mentioned. It is the author's considered opinion that none of the items listed below should be regarded as truly significant in interpreting the main conclusions of this analysis; they merely serve to document and inform any subsequent analysis, and point to opportunities for additional research.

Practical ecological and management problems such as preservation and regulation of wild horse populations can be addressed with quantitative tools such as models. A model is nothing more than a mathematical caricature of the real world. Models can be excellent tools for developing a better understanding of the way things work and the expected effects of certain management interventions, but they typically do not -- indeed cannot -- fully capture all the possible variability inherent in the system under study. Models are therefore compromises between available data and complete understanding (Akcakaya and others, 1997).

There are several particular areas where the Jenkins model may not fully capture biological processes or current management practices. One deviation from reality is the model's view that horses gathered and removed approximate a sample of the population. As one reviewer pointed out, gathers are not likely to be a random sample, but instead probably over represent mares, foals, and younger horses due to their relative 'gatherability'. Small, scattered groups of bachelor males are likely underrepresented in the gather because they can be elusive. The full ramifications of this *lack* of bias in the model are unknown and need further work. Another limitation deals with the application of specified sex- and age-specific removal 'rates'. This is a two-pronged issue. Removal rates can be set too low to actually achieve the target AML and, because of the model's implementation, it can be difficult to tell whether or not the AML has actually been achieved under all conditions simulated, especially if foals are not included in the AML. Related to this, the current selective removal policy's three-tiered age removal guidelines can be difficult to emulate with the existing Jenkins software because the specified removal rates are applied as if the animals are being run through a random removal process. That is, each animal is handled in a random order and either removed or returned to the field. The software does not, as an example, first remove all Tier One animals, followed by Tier Two and then Three only as necessary. One can approximate the three-tiered policy using a trial-and-error approach, but this can be tedious and cannot be changed during a multi-year simulation period. Fortunately, if the software can reach AML solely by removing Tier 1 animals – as seemed to be the case for two of populations studied here, all is well. In the case of McCullough Peaks, Tier Two removals were needed, and even then it took several years to truly achieve its AML (see previous Figure 11). Herds with significant numbers of older aged animals might pose additional modeling problems. Finally, there is a small lack of fidelity in the Jenkins model's representation of age classes. The last three age classes in the software are aggregates (10-14, 15-19, and 20+). I assumed that unadoptable horses in these age categories were 12, 17, and 22 years old, respectively, when calculating how long they would remain in long-term holding.

Because of randomness inherent in Jenkins model simulations, running exactly the same scenario a second time is guaranteed to produce slightly different results. Changing a parameter and re-running the software will also produce different results both because of chance phenomena in the model and the parameter change itself. This has implications when interpreting the results, occasionally making it difficult to unilaterally assign effects to causes, especially when counter-intuitive or questionable predictions arise from small input changes. This phenomenon could perhaps be minimized if more simulation trials were used, but it also mimics difficulties with real-world experiments.

It would be prudent to address whether the three populations examined here are truly representative of horse herds larger than those simulated. It is my opinion that the estimates of relative cost savings of contraceptive alternatives are not likely to change much for larger herds. I say this because these dynamics are fundamentally governed by the relative ratio of survival and foaling rates that, in combination with removal strategy, controls the effective population growth rate. However, I have not actually tried to confirm these conclusions by simulating larger populations.

Additional caveats to this analysis include: (1) I have not accounted for any potential compensatory reproductive stimulus after a contraceptive has worn off (Garrott and Taylor, 1990) or any increase in longevity of treated mares (Garrott and others, 1991). (2) This analysis did not consider any effects on genetic integrity,

cumulative risks to population persistence, or herd social/behavioral effects. It was assumed that these considerations were already reflected in the establishment of each herd's AML. However, the particular method of achieving an AML (such as using fertility control) can influence the minimum projected population size, an important factor in ensuring genetic integrity. (3) This analysis did not factor in any density dependent survival or reproduction because it was assumed that each population's AML was well below estimates of range carrying capacity. (4) There are some inaccuracies inherent in the cost-accounting model when percentages are applied to a small number of removed animals, such as to divide an age class into adoptable or unadoptable animals. (5) There are other small deviations from reality inherent in the cost accounting model. In the existing baseline situation (as depicted in Figure 1), slightly more males than females are moved from the adoption pool to the long-term holding pool due to difficulty of handling or other problems that tend to make these males more unadoptable (see Godfrey and Lawson 1986). (6) This analysis was not intended to cover other generalities of managing populations with contraceptives such as those provided by Garrott and Siniff (1992), Gross (2000) or Hobbs and others (2000).

Economic analysis is not a substitute for judgment. Judgment will continue to play a role in assessing values, uncertainties, and elements that do not lend themselves to quantification. Economic analysis is but one component of the whole decision making process. Economic analysis assumes rational behavior, which can be both a strength and a weakness. There are likely to be many other intangibles: socio-political acceptability (Boyles, 1986; Berger 1986; Berger, 1991), legality, income-distributional effects, and potential environmental aspects. I have not done a full-blown *net benefit to society* analysis. For example, I have not considered costs of decreased grazing by domestic livestock and wildlife on horse range, potential increased siltation of streams if horse populations were higher, maintenance of fences and other improvements, nor the 'subsidy' that may be received by some individuals participating in the adoption program (Godfrey, 1979). In addition, I have not included the costs of research into contraceptive application and follow-up, a necessary component of any large-scale undertaking.

Management and Conservation Article

Economic Benefit of Fertility Control in Wild Horse Populations

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ABSTRACT I projected costs for several contraceptive treatments that could be used by the Bureau of Land Management (BLM) to manage 4 wild horse (*Equus caballus*) populations. Potential management alternatives included existing roundup and selective removal methods combined with contraceptives of different duration and effectiveness. I projected costs for a 20-year economic life using the WinEquus© wild horse population model and state-by-state cost estimates reflecting BLM's operational expenses. Findings revealed that 1) currently available 2-year contraceptives in most situations are capable of reducing variable operating costs by 15%, 2) experimental 3-year contraceptives may be capable of reducing costs by 18%, and 3) combining contraceptives with modest changes to herd sex ratio (e.g., 55–60% M) could trim costs by 30%. Predicted savings can increase when contraception is applied in conjunction with a removal policy that targets horses aged 0–4 years instead of 0–5 years. However, reductions in herd size result in greater variation in annual operating expenses. Because the horse program's variable operating costs make up about half of the total program costs (which include other fixed costs), contraceptive application and management can only reduce total costs by 14%, saving about $6.1 million per year. None of the contraceptive options I examined eliminated the need for long-term holding facilities over the 20-year period simulated, but the number of horses held may be reduced by about 17% with contraceptive treatment. Cost estimates were most sensitive to the oldest age adoptable and per-day holding costs. The BLM will experience significant cost savings as carefully designed contraceptive programs become widespread in the wild horse herds it manages. (JOURNAL OF WILDLIFE MANAGEMENT 71(8):2811–2819; 2007)

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KEY WORDS Bureau of Land Management, contraceptive, *Equus caballus*, feral horses, modeling, porcine zona pellucida (PZP), reproduction, sex ratio.

Recent management of wild horses in the western United States has been governed by the Wild Free-roaming Horses and Burros Act of 1971 (Public Law 92–195), as amended. Management has typically been achieved by the Bureau of Land Management (BLM) by rounding up each herd about every 4 years and removing selected animals from the range to attain herd-specific population goals known as appropriate management levels (AMLs). Under 2003 BLM policies and guidelines, young horses (age 0–5 yr), which have a higher probability of being adopted, are removed first to achieve the AML. If necessary, older horses (≥10 yr) compose a second tier of removals, followed by horses aged 6–9 (tier 3). All young horses and most middle-aged horses that have been removed enter a pool from which private adoptions are encouraged. All other horses removed to achieve population goals are maintained in long-term holding facilities for the remainder of their natural lives (Fig. 1).

Management of these herds has been costly. Legislation has restricted the options that can be used to manage populations; sanctuary and adoption alternatives have been expensive and less than fully effective because of the long-term care requirements (U.S. General Accounting Office 1990, Conover 2002), and herds continue to grow, often at high rates (6–25% or more annually; Eberhardt et al. 1982, Wolfe 1986). Budgetary limitations demand tough decisions on how best to minimize expenditures yet still meet legislated goals to maintain a "thriving natural ecological balance" and preserve existing multiple uses including rangeland health (U.S. General Accounting Office 1990:9). Fertility control has been proposed as one option but has not been thoroughly evaluated for expected cost-effectiveness. Fertility control is generally viewed as humane (Reiter et al. 1999), is safe and reversible (Kirkpatrick and Turner 2002), and has been shown to successfully complement other methods of population control for wild horses (Garrott 1991, Garrott et al. 1992, Gross 2000, Hobbs et al. 2000).

The BLM maintains a state-by-state database on the costs of herd monitoring, population estimates, selective removal, preparation and holding, adoption, compliance checking, and other program activities. With this database, I estimated costs of several management alternatives using 2004 management expenses for available or reasonably foreseeable contraceptive technology for 4 BLM-managed wild horse populations in a manner similar to that of Garrott et al. (1992). My specific objective was to identify the expected effectiveness and costs for a limited set of contraceptive options in the face of variable, state-by-state management expenses representing BLM's 2003 management policies.

STUDY AREA

The 4 wild horse populations chosen by BLM for this analysis were the Challis Herd Management Area (HMA) near Challis, Idaho, USA; the McCullough Peaks HMA near Cody, Wyoming, USA; the Pryor Mountain Wild Horse Range north of Lovell, Wyoming, USA; and the Little Book Cliffs HMA near Grand Junction, Colorado, USA. Each herd had a different management history, discrete population goals, and unique demographic attributes with accompanying data reliability issues.

The Challis herd occupied a large area (323,749 ha) of

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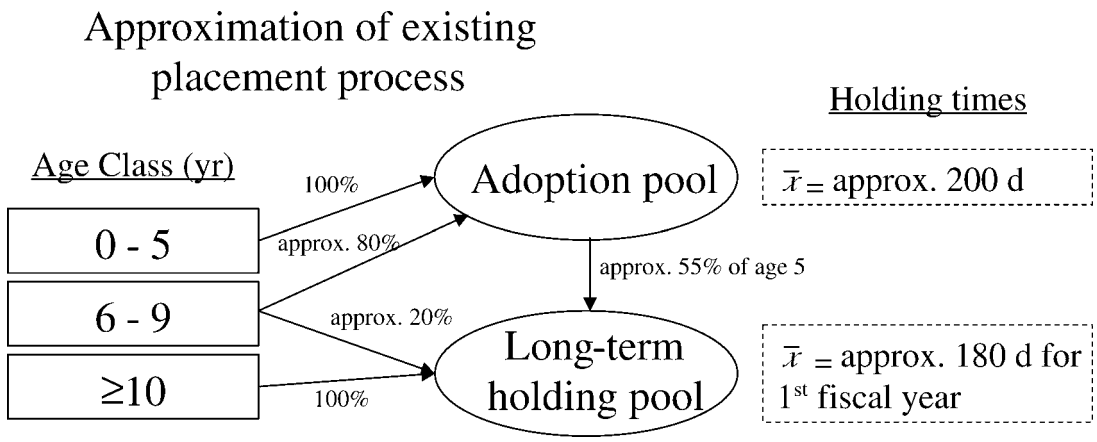


Figure 1. Approximation of existing selective removal and placement policy (baseline scenario) for the Bureau of Land Management's wild horse program in the western United States. For example, 100% of any 0-year-old to 5-year-old horses removed from the range to achieve the target population size are placed in the adoption pool. I obtained values from Bureau of Land Management instructional guidance representative of 2003 procedures.

mostly public land that included many livestock grazing allotments. In 2002, the herd size was estimated at approximately 271 horses, growing at about 17% annually. Rangeland and aquatic system health goals dictated a postremoval population goal of 185 animals, including young-of-the-year.

The Little Book Cliffs herd was located on 12,140 ha of public land. This herd of about 150 horses was rounded up periodically and had been the subject of ongoing contraceptive research. All yearlings and 2-year-old females, half of those aged 3–15 years, and all females >15 years that were captured at the 2002 roundup and returned to the range were primed with the contraceptive agent porcine zona pellucida (PZP). Though the Little Book Cliffs HMA was undergoing research during my study, I used it as a proxy for other populations with similar demographics. Simulations were not meant to emulate research protocols currently being applied.

The McCullough Peaks HMA occupied 44,515 ha of open sagebrush (*Artemisia tridentata*) prairie and badlands. The wild horse population was about 150 animals, with the AML set between 70 animals and 140 animals, excluding young-of-the-year. Contraceptives had only recently been applied to this herd. Unlike the other herds, McCullough Peaks had more older-age horses (tier 2) removed to achieve its AML.

The Pryor Mountain Wild Horse Range occupied 15,378 ha of multi-jurisdictional lands along the border of Montana and Wyoming. This mostly arid range supported 100–200 freely roaming wild horses. Harsh winters and abundant predators likely limited the number of older animals and contributed to variable, intermittently high young-of-the-year mortality. Conflicting perspectives stimulated research on several issues germane to management of this herd, including vegetation dynamics and population genetics (Singer and Schoenecker 2000). Fertility control research began on the Pryor Mountain herd in 2001, with contraceptives applied via remote darting after an initial roundup. Darting, which delivered an initial primer and annual booster, can take place over time, ultimately reaching 100% of the females targeted for contraceptives. Though BLM had managed this herd somewhat differently from the others, I included it in this analysis because of its unique demography. For tractability, I treated the Pryor Mountain herd in a manner parallel to the other 3 herds and assigned it management costs representative of Montana because these costs most closely represent the Pryor-specific program costs estimated by BLM.

METHODS

Population Model

I extracted data for each herd from BLM records, supplemented as necessary with information provided by herd managers (Table 1). I then conducted simulations using WinEquus© (Version 1.4; Jenkins 2002) for each herd. This sex- and age-based model mimics roundup, selective removal, and contraceptive applications against a backdrop of random variability in both annual environmental conditions and individual animal mortality and reproductive status. The model requires an initial age and sex distribution, age-specific survival and foaling rates, young-of-the-year sex ratio, and rules governing how removals and contraception alternatives are simulated. Each random model trial results in a different pattern of population growth, at times producing runs of good and bad years.

I ran each scenario as a separate stochastic simulation using model input parameters that control the various assumptions or management actions that might be taken. I ran all simulations for 20 years and repeated them for 100 trials each (Fig. 2). I chose the model's default simulation assumptions: 1) there is no correlation between annual variation in survival probabilities and foaling rates, 2) initial population size and structure are treated as inexact (slightly variable in each trial) and smoothed by the model, and 3) young-of-the-year survival is density independent. I simulated each scenario by the appropriate combination of 6 model parameters: selective removal rates by sex and age class, efficacy of contraceptive agent (if applied), threshold population size triggering a roundup, target population size

Table 1. Key demographic and other information for the 4 wild horse herd management areas I assessed in the western United States. Values are representative of 2004 data.

Attribute	Herd management area			
	Challis	Little Book Cliffs	McCullough Peaks	Pryor Mountain
Cost state	ID	CO	WY	MT
Initial sex ratio (% M)	46	46	46	53
YOY[a] sex ratio (% M)	58	50	57	51
Age 0–9 yr F survival (geometric $\bar{x}$ %)	98	95	95	89
Age 0–9 yr M survival (geometric $\bar{x}$ %)	93	97	95	87
$\bar{x}$ foaling rate age 2–9 yr (%)	79	65	71	62
Age at which foaling rate first exceeds 50% (yr)	2	3	4	4
Baseline annualized growth rate (%)	20	13	17	1
Roundup trigger (no. of horses)	253	150	140	150
Roundup efficiency (%)	75	80	90	70
AML[b] (no. of horses)	185	90	100	100
AML includes YOY?	Yes	No	No	No
Requires tier 2 (age ≥10 yr) removals to achieve AML	No	No	Yes	No
Proportion of released F treated with contraceptives by age group (applied only for contraceptive scenarios)	YOY: 0% 1 yr: 100% 2–9 yr: 50% ≥10 yr: 100%	YOY: 0% 1–4 yr: 100% 5–9 yr: 75% ≥10 yr: 100%	YOY: 0% 1–4 yr: 100% 5–9 yr: 75% ≥10 yr: 100%	YOY: 0% 1–2 yr: 100% 3–9 yr: 0% 10–14 yr: 50% ≥15 yr: 100%

[a] YOY = young-of-the-yr.
[b] AML = appropriate management level.

following removal, whether young-of-the-year were included in the AML, and percentage of the population that can be rounded up. The WinEquus model calculated growth rates as the geometric average of year-to-year changes in population size, excluding any management removals.

Specific Alternatives Analyzed

Population management alternatives that I explored using WinEquus included 1) the status quo of selective removal, adoption, and sanctuary; 2) changing the frequency of roundups and how efficient roundups are; 3) status quo plus a variety of alternative contraceptive application scenarios, specifically varying the duration of the contraceptive agent; and 4) sex ratio manipulation through age- and sex-specific removal rates. I modeled the following limited set of scenarios:

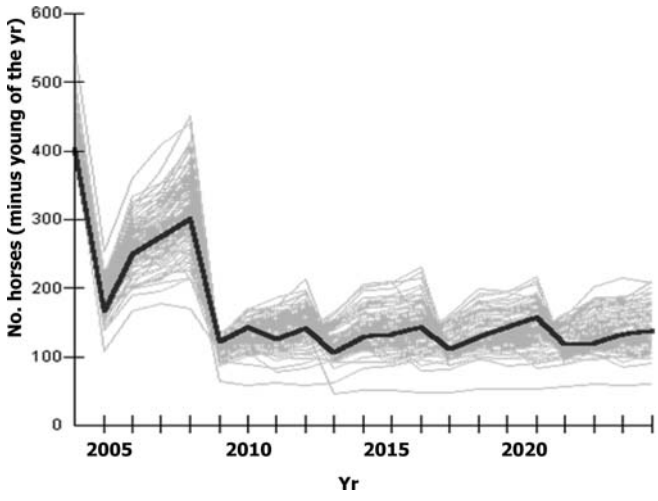


Figure 2. Example WinEquus model output for the McCullough Peaks Herd Management Area in Wyoming showing the total postroundup population size of wild horses (minus young-of-the-yr, because they are not included in the target population size) through a 20-year simulation beginning in 2004. Light lines trace the 100 individual simulation trials and the heavy line traces the median (most typical) result.

1. *Baseline.*—Management conditions as reflected in BLM's recent roundup policy, including a regular 4-year roundup cycle with age-specific placement rates (Fig. 1). Age classes are removed in successive age-specific tiers to achieve the designated AML for each herd, with 0-year-old to 5-year-old horses removed first, followed by ≥10 year olds, and finally 6-year-old to 9-year-old animals.
2. *Alternative baseline.*—An alternative to the Baseline Scenario being considered by BLM in which the first tier of removals targets age classes 0–4 years, tier 2 remains ≥10 years old, and tier 3 is 5–9 years old. In the Alternative Baseline Scenario, 70% of tier 3, if removed, is directed to the adoption pool and 23% of 4-year-old animals end up in long-term holding, in contrast with Figure 1.
3. *Roundup frequency.*—Scenarios representing regular intervals of every 2 years, 6 years, or 8 years.
4. *Contraceptive.*—Scenarios representing either a 2-year or 3-year contraceptive agent defined more precisely with percent effectiveness in the first and subsequent years (Table 2). I assumed that if a contraceptive proved ineffective in the first year for a given female, it would never be effective in subsequent years until re-treatment.
5. *Sex ratio.*—Scenarios representing reductions in the percentage of males removed across all targeted age classes. Normally, if an age class is subject to removals, a herd-specific percentage of animals of both sexes is targeted for that removal. In these scenarios, I reduced male removals by 10%, 20%, and 30%, resulting in more females removed to satisfy herd size goals.
6. *Roundup efficiency.*—Scenarios representing changes in

Table 2. Estimated treatment cost and annual effectiveness of existing 2-year and potential 3-year contraceptive treatments I used in analysis of 4 wild horse herds in the western United States. Costs include both contraceptive materials and application. Effectiveness in this context refers to the percentage of treated females that do not give birth. All values are representative of 2004.

Nominal duration (yr)	Cost/F ($)	Effectiveness yr 1 (%)	Effectiveness yr 2 (%)	Effectiveness yr 3 (%)	Effectiveness yr 4+ (%)
2[a]	214	94	82	34	0
3[b]	309	95	85	75	0

[a] Effectiveness values reflect preliminary results from the Clan Alpine Horse Management Area in NV (R. Hall, Bureau of Land Management, personal communication).

[b] I extrapolated hypothetical effectiveness values from 2-yr effectiveness rates. Cost of 3-yr agent is cost of 2-yr agent plus additional 12-month time-release pellets.

the estimated efficiencies for the 4 herds. I varied the nominal roundup efficiency by −5%, +5%, and +10%.

7. I also considered a limited set of combinations of the above scenarios.

Estimating Dollar Values

I estimated costs for each of the main expenses (i.e., roundup, treating, and selective removal) along with other miscellaneous management expenses from the state-specific costs compiled by the BLM (Table 3). These costs represented 2004 values, but I assumed they increased 3% annually regardless of geographic area to approximate inflation. Removal costs included all expenses of roundup and transport to adoption or holding facilities, averaged across all removed horses. Preparation and holding costs included operational and labor expenses for freeze-marking, health care, required vaccinations, feed, and facility maintenance. Adoption costs were largely administrative, including follow-up site visits to adopted horses to assess compliance with adoption policies.

Multiyear contraceptive costs I used in this analysis were provided by the BLM (L. Coates-Markle, BLM, personal communication). The BLM gave a range of costs associated with the 2-year contraceptive. Though costs are likely to decline in the future, I chose the more expensive estimate provided, $214 per horse, to be conservative.

I also considered several other costs in the analysis. I assumed a minimum roundup cost of $15,000 for the Pryor Mountain HMA and $10,000 for the other 3 herds, reflecting higher costs for the Pryor herd. This minimum applies only if the number of animals removed times the appropriate per horse removal cost is below the minimum. For each contraceptive scenario, I also assumed a flight cost of $5,000/year/HMA (R. Hall, BLM, personal communication) to estimate contraception effectiveness.

I summarized the results of WinEquus simulations and converted them to dollar expenses over a 20-year planning horizon. I chose 20 years because it is long enough (5 complete 4-yr roundup cycles) to reduce the uncertainty inherent in estimates of the initial age and sex structure and to reveal most of the effects of change in sex and age structure that would result over time from the variety of treatment options simulated. To tally the total expenditures, I summed all costs previously described, including which age groups were eligible for adoption and how long adoptable and unadoptable horses were projected to be held (Fig. 1). I assumed all unadoptable horses would remain in holding facilities for the remainder of their natural lives, estimated to be 25 years. I summarized cost estimates by computing the mean number of horses rounded up, removed, and treated by sex and age class for each year of the 20-year simulations, along with average annual costs and the coefficient of variation in annual costs.

First-Order Sensitivity Analysis

The WinEquus simulation model incorporates environmental and demographic stochasticity, but the uncertainty in cost estimates and sensitivity of other management options was unknown. To examine this, I performed a first-order sensitivity analysis for the 4 populations to see where opportunities for cost cutting might be found and which factors contributed most to the estimated costs. I conducted

Table 3. Variable cost estimates I obtained from the Bureau of Land Management pertaining to wild horse management across the United States, representative of 2004 expenses. Atypically high costs in any column may arise where totals are averaged over a few horses. NA = not applicable.

Management area	Removal cost ($/horse)	Preparation and holding cost ($/horse/d)	Adoption cost ($/horse)	Compliance check ($/horse)
AZ	345	2.80	318	50
CA	211	3.13	305	59
CO	433	3.04	291	60
Eastern states	NA	7.66	361	46
ID	285	2.10	396	18
MT	450	13.99	500	150
NV	460	3.11	510	66
NM	433	3.15	362	81
OR	360	3.35	300	50
UT	434	4.72	367	50
WY	300	5.21	760	70

Table 4. Results of 20-year removal and contraceptive scenarios forecast for the 4 wild horse herd management areas I examined in the western United States. Forecasts begin in, and use values representative of, 2004. RU = Roundup, RU effic. = roundup efficiency.

	Herd management area							
	Challis (baseline cost = $86,418)		Little Book Cliffs (baseline cost = $48,744)		McCullough Peaks (baseline cost = $165,464)		Pryor Mountain (baseline cost = $14,971)	
Scenario	% of baseline cost	Median annual growth rate (%)	% of baseline cost	Median annual growth rate (%)	% of baseline cost	Median annual growth rate (%)	% of baseline cost	Median annual growth rate (%)
Remove only								
Baseline	100	20.0	100	12.6	100	17.1	100	1.0
Alternative baseline	102	20.0	98	12.6	99	17.2	111	1.0
−10% M removal	94	18.2	95	11.6	94	14.9	96	1.5
−20% M removal	88	16.8	89	10.4	91	12.4	77	0.3
−30% M removal	82	15.1	90	9.8	83	9.9	76	0.6
Remove and treat								
2-yr treatment	88	16.1	84	7.9	86	11.9	108	−0.3
3-yr treatment	85	15.5	81	7.5	80	10.5	109	−0.9
2-yr treatment, 2-yr RU	74	11.2	45	2.2	68	6.6	112	−1.7
2-yr treatment, 6-yr RU	152	18.5	125	10.0	159	13.8	119	0.1
2-yr treatment, 8-yr RU	156	19.0	121	10.8	165	14.4	106	0.1
2-yr treatment, −5% RU effic.	94	17.0	86	8.7	89	12.4	122	0.7
2-yr treatment, +5% RU effic.	87	15.9	77	7.1	81	11.1	132	0.8
2-yr treatment, +10% RU effic.	83	15.3	77	6.8	78	10.4	124	−0.5
2-yr treatment, −30% M removal	73	12.0	73	6.3	74	7.2	104	−0.4
3-yr treatment, −30% M removal	71	15.5	72	5.9	70	6.4	99	−0.9
Alternative baseline, 3-yr treatment, −30% M removal	73	11.8	68	5.9	69	6.1	69	−0.9

the sensitivity analysis by varying each cost (e.g., cost/adopted horse) by ±20% and each age class category ±1 year class, one parameter at a time, and then calculating the ratio of the resulting cost fluctuation to the base cost of the 2-year contraceptive scenario.

RESULTS

The alternative baseline scenario proved to be more expensive than the baseline scenario for the Challis and Pryor Mountain herds, 102% and 111%, respectively (Table 4). For these 2 herds, the alternative baseline resulted in an average of 1–2 additional horses held in long-term facilities with attendant costs. Costs were reduced for the Little Book Cliffs and McCullough Peaks herds under the alternative baseline, 98% and 99%, respectively (Table 4).

In the remove-only scenarios, adjusting the herds' sex ratio by removing fewer males was always successful at reducing management costs, with the −30% male removal scenarios resulting in 10–24% cost reductions on average and corresponding reductions in median annual growth rate depending on the herd. Resultant sex ratios for these most extreme simulations were generally in the range of 52–57% males as opposed to baseline conditions that were 46% males for 3 of the herds. The McCullough Peaks HMA was an exception, because the sex ratio increased over time to 65% males (Table 4).

Either a 2-year or 3-year contraceptive application was effective in reducing management costs by 12–20% for 3 of the herds; the Pryor Mountain HMA was an exception because of its comparatively slow growth rate and higher costs. In fact, as simulated, the contraceptive scenarios reduced the Pryor Mountain growth rate below zero. None of the herds would benefit by delaying the regular 4-year roundup frequency to 6 years or 8 years in combination with a 2-year contraceptive, although reducing the period to 2 years could reduce costs 26–55% in all but the Pryor Mountain herd because of its low growth rate and high costs compared with the other herds. A 2-year roundup cycle combined with contraceptive treatment precludes reproduction by even more of the youngest females.

Annual costs were sensitive to roundup efficiency, although increasing the efficiency 5–10% only resulted in decreasing costs by about 10%. The Pryor Mountain herd remained a notable exception. The estimated coefficient of variation in average annual costs differed among the herds. McCullough Peaks and Challis had the lowest variability in costs, averaging 25%. Little Book Cliffs variability was higher at 56%, and Pryor Mountain annual variability was almost 100%.

Finally, the 3-year contraceptive application combined with either adjusting the sex ratio or modifying the age classes targeted for the adoption program were the most cost-effective treatments across all herds, with reductions up to 29% for Challis, 32% for Little Book Cliffs, 31% for McCullough Peaks, and 31% for Pryor Mountain. Though not shown in Table 4, the more effective a scenario was at reducing costs, the smaller the average annual population size over all simulation trials. However, the consequence of smaller population sizes was typically an increase in the coefficient of variation in annual costs. In other words, the smaller the population, the more inherent variability in annual growth rates (a function of the stochasticity in

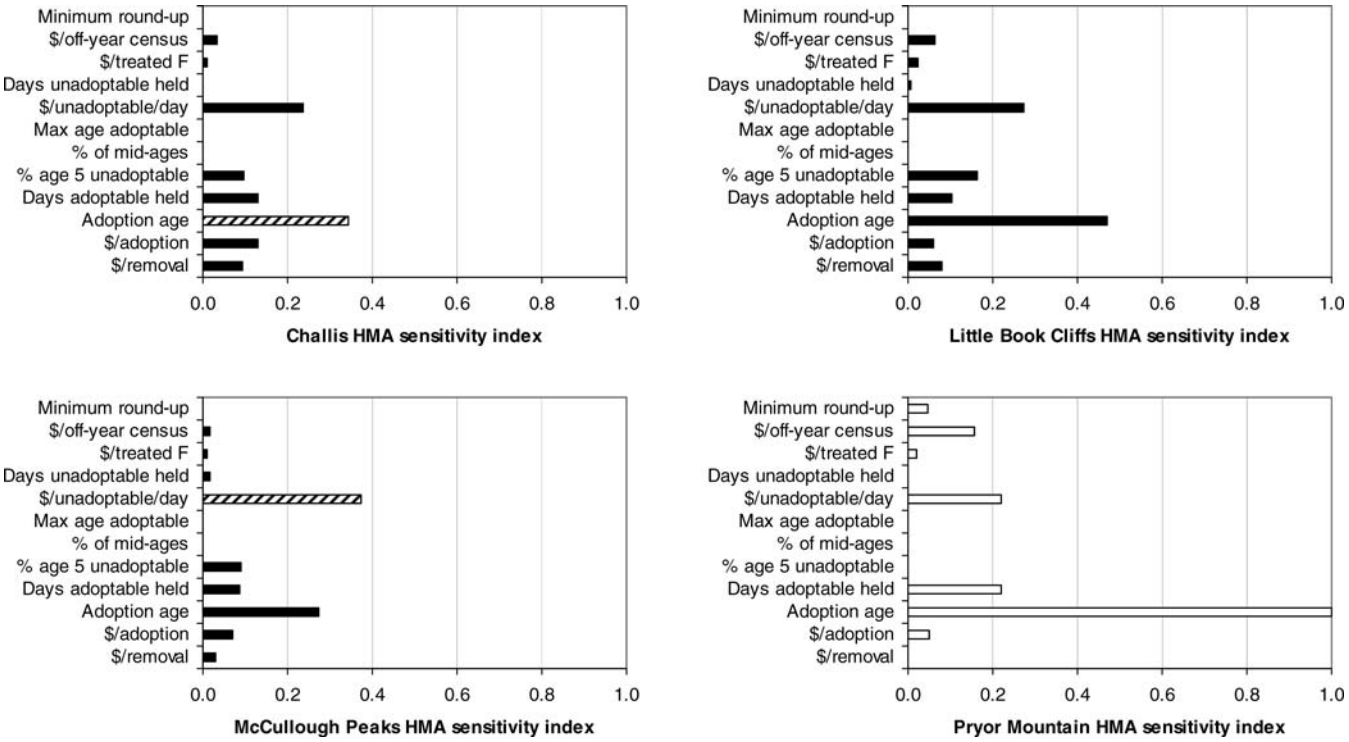


Figure 3. First-order sensitivity analysis for management costs and other attributes for the 4 wild horse herd management areas (HMA) in the western United States relative to the 2-year contraceptive scenario. Hatched bars indicate potential to be more cost-effective than the baseline alternative using data indicative of 2004 costs. Empty bars for Pryor Mountain signify that all contraceptive alternatives can be more expensive than the baseline within the sensitivity range examined.

WinEquus) and the greater the expected fluctuation in management costs. This trend was more evident in the alternative roundup interval and alternative efficiency of roundup scenarios than in the sex ratio adjustment or contraceptive alternatives.

The sensitivity analysis modeled how responsive bottom-line costs were to changes in each contributing factor (Fig. 3) relative to the 2-year contraceptive. The maximum age considered adoptable (5 yr) generally had the largest influence on total costs, indicating that if BLM were successful in placing more 6-year-old animals, costs could be reduced substantially. Adoption age was followed by the average per-day holding cost, a factor that actually exceeded maximum age adoptable for the McCullough Peaks herd because of its high holding costs (Table 3). Other factors (e.g., % of 5-yr-old horses that are unadoptable, no. of d that horses are held prior to adoption, and actual cost of adoption) had approximately equal and low sensitivity indices relative to the other factors. Costs related to contraceptive treatment, minimum roundup costs, and costs of off-year population estimates were insensitive because they were inconsequential compared with other management expenses.

In summary, contraceptive treatment added to the status quo roundup and removal program was predicted to be cost-effective for wild horse herds except for those herds with low growth rates. Depending on the herd and the effectiveness of treatment, operational cost savings projected ranged from 12% to 20% compared with baseline estimates. When combined with other actions that adjust age- and sex-specific removal rates, cost savings could increase to around 30% for all herds, even those like the slowly growing Pryor Mountain herd. Populations controlled solely by roundup declined markedly with each roundup (Fig. 2) but typically increased rapidly between them. In contrast, modeled populations controlled by contraceptives in conjunction with removals grew much less rapidly. Increasing the male sex ratio further reduced the growth rate, resulting in greater stability.

DISCUSSION

Contraceptive Effectiveness

All contraceptive options I simulated were effective at reducing costs for at least 3 of the herds studied. However, it should be noted that none of the contraceptive options I considered were projected to eliminate the need for long-term holding facilities. In total, the number of horses added to long-term holding was reduced by 17% over the simulated 20-year period with the most effective contraceptive treatment, yet only the McCullough Peaks HMA actually showed a decreasing number of horses held through the 20 years. Little Book Cliffs and Challis continued to increase the horses added to holding through time, albeit at rates 6–36% below the baseline scenario. Instead, contraceptive treatment would likely reduce the number of horses that must be removed in total, with attendant cost reductions in the number of adoptions and total holding costs. Overall, proportionate costs were predicted to shift

Table 5. Nationwide fixed and variable cost estimates for the Bureau of Land Management's wild horse program, 2004.

Cost type	Factor	Expense ($)	Total ($)	% of total
Fixed				
	Annual monitoring	219,912		
	Annual population estimates	369,590		
	AML[a] establishment	56,635		
	Labor	8,862,197		
	Non-unit cost	3,513,962		
	Long-term holding facilities	6,387,500		
	Total fixed costs	23,801,893		54
Variable				
	Selective removal	5,331,471		
	Preparation and holding	12,046,381		
	Adoption	2,713,550		
	Compliance checks	279,150		
	Total variable costs	20,370,552		46
Fixed + variable			44,172,445	100

[a] AML = appropriate management levels.

from 73% related to adoption and 27% related to holding in the baseline case to 56% for adoption, 30% for holding, and 14% for contraceptive treatment in the most effective contraceptive scenarios.

Sex Ratio Manipulation

Some published literature (Berger 1986), as well as many unpublished wild horse environmental assessments, recommend maintaining populations with natural sex ratios inclined towards females (e.g., 40 M:60 F). Because sex ratio measured in the field results from the combined effects of young-of-the-year sex ratio, relative male:female survival, and removal rates that vary from herd to herd, it may be problematic to characterize a natural sex ratio, especially for a species undergoing such active management. My analysis assumed no a priori constraint on sex ratio resulting from management. Herds that have small groups of bachelor males that are difficult to round up may lend themselves easily to such a management strategy, whereas other herds that have many small harems may prove more difficult and costly to manage favoring males. Although I did not assess any potential genetic or behavioral ramifications of management favoring a male-dominated herd structure, it is clear that changing sex ratios could be significant in determining program costs.

Balancing Range Conditions with Population Size

The 20-year simulations typically showed that any management strategy might take several roundup cycles to achieve relatively stable demographics. For example, take the McCullough Peaks herd I examined (Fig. 2). I simulated this herd by removing animals from both tiers 1 and 2. A herd manager might be tempted to adjust the management strategy by also removing tier 3 animals after only 1 or 2 roundup cycles if the population (and costs) did not appear to fall as quickly as expected (i.e., if the population trajectory were near the upper extent of some of the traces on the graph). Although removal decisions must always be made using the best available data and be guided by management objectives, the results shown here argue for patience in population management for these long-lived ungulates. In particular, a management option that removes only 0-year-old to 4-year-old horses may result in a larger number of horses on the range initially, yet achieve the desired herd reduction goal over the long term. If the range is capable of sustaining a larger number of horses for several years, a patient approach could significantly reduce long-term management and holding costs.

Program-Wide Costs

My analysis explored methods of reducing costs through a combination of contraceptive and other potential management techniques. However, the costs I tabulated should be considered variable costs because they arise from variation in herd population dynamics and management strategies.

The BLM also incurs certain fixed costs associated with the wild horse program's overhead (e.g., WA and regional offices, many HMA monitoring costs, holding facility maintenance, support for the 20,000 wild horses currently residing in holding facilities). The fixed costs might be assumed to continue essentially unchanged regardless of the specific roundup and selective removal process, with the knowledge that some fixed costs, like annual monitoring, aerial population estimation, and new or revised AML establishment, are subject to annual budgetary constraints. These fixed costs have been ignored so far because they are the result of past decisions.

The BLM provided nationwide costs, both fixed and variable, for its wild horse program (Table 5). Variable costs represent slightly less than half (46%) of the aggregate costs. Results from the WinEquus model indicated that one might expect an annual decrease in variable costs of about 30% if the most effective management methods were implemented (Table 4). If these savings accrued solely to the variable side of the ledger, and if fixed costs remained stable, aggregate program costs would likely decrease by only about 14%. Characterizing the results of this analysis in relative terms, assuming that a 30% reduction is approximately correct for all herds combined, would amount to a $6.1

million annual savings in variable program costs in 2004 dollars.

Fixed costs might be expected to decline through time if the number of unadoptable horses declines. Other changes to roundup and selective removal procedures, such as completely closing an existing sanctuary facility, could alter the fixed-cost structure (specifically labor and facility costs), further reducing costs. I did not consider such potential changes to overall program costs in this analysis.

Comparison with Findings of Others

Garrott et al. (1992) explored the impacts of alternative contraceptive management on wild horses in the early 1990s. Similar to this analysis, Garrott et al. (1992) simulated a variety of different herds and considered more contraceptive alternatives than those examined here. Their results were comparable to mine, indicating that most variable program expenses are not related to contraceptive treatment per se, but instead are associated with adoption program costs. Similarly, Garrott (1995) showed that a 2-year contraceptive agent applied on a 4-year roundup cycle can make a large difference in variable costs associated with management when roundup efficiencies are around 80% and natural rates of increase are comparatively high. In my analysis, program costs always paralleled population growth regardless of the management option; this is consistent with the findings of Gross (2000) and Garrott et al. (1992).

Others have pointed out that the cost-efficiency of contraceptive treatment for a long-lived and highly productive species is extremely dependent on herd-specific demographics, management constraints, and data quality (Kirkpatrick and Turner 1986, Wolfe 1986, Garrott 1991, Hobbs et al. 2000) and that contraception must be used in conjunction with an ongoing removal program (Gross 2000). Although I made no attempt to optimize a specific management alternative for any of the 4 herds I examined, this analysis confirms that contraceptive programs must be tailored for individual herds, given differences in reproductive biology and inherent population growth rates.

Analysis Uncertainty

These simulations contain several forms of uncertainty. First, estimates of initial conditions and vital parameters are likely inaccurate, and in some cases I borrowed values for one herd from better studied herds assumed to be similar. Second, uncertainty due to range conditions, predation, and other poorly understood regulatory mechanisms is accounted for by WinEquus but results in the predicted variation in annual herd management costs of 20–30%. Such high variability likely means that it is difficult to distinguish precisely among many cost estimates. Although it would be possible to run more model trials to better estimate central tendencies, doing so would only result in a reduction of statistical uncertainty rather than actual uncertainty. Third, the BLM-derived estimates of expenses for each of the several cost components are uncertain. Costs vary through time and with the vagaries of local conditions. Further, new contraceptive techniques or agents will likely become available within 20 years. If, for example, a contraceptive were perfected that could extend the nominal efficacy well beyond 3 years, projected cost savings would mount accordingly. However, it seems likely that the program-wide cost variance would be less than the 20–30% herd level variance because some herds would be increasing whereas others would be decreasing. Nonetheless, it would be wise to consider the possibility of region-wide synchronous oscillations accompanying climatic variation that might provide favorable or unfavorable habitat conditions throughout the Intermountain West.

Analysis Limitations

Practical ecological and management problems can be addressed with models, but models are merely mathematical abstractions of the real world. Models can help develop a better understanding of the way things work and predict outcomes of specific management interventions, but they never capture the full variability and complexity inherent in the real world. WinEquus may not always faithfully represent management practices used in some herds. The model assumes that horses captured and removed approximate a random sample of the herd structure, but in reality, depending on the size of the herd, the number of animals that need to be removed, and other factors that effect roundup efficiency, females and younger animals may be overrepresented in the roundup.

As always, additional caveats extend beyond the modeling domain. This analysis did not account for any potential compensatory reproductive stimulus after $\geq$1 year of being spared from the energetic demands of pregnancy and lactation (Garrott and Taylor 1990) or any potential increase in longevity of treated adult females (Garrott et al. 1991), nor did I consider any effects on genetic integrity, cumulative risks to population persistence, or any herd social–behavioral effects. I assumed that these considerations were already reflected in the establishment of each herd's AML. This analysis did not factor in any density-dependent survival or reproduction because I assumed that each population's AML is set well below levels that would normally impact survival or population growth. For any given horse population, there are many intangibles such as the public acceptability of BLM management activities (Berger 1986, 1991; Boyles 1986) and other potential environmental ramifications. For example, I did not consider costs of decreased or increased grazing by domestic livestock and wildlife on range quality, potential decreased siltation of streams if horse populations were lower, or any secondary effects associated with the adoption program (Godfrey 1979).

MANAGEMENT IMPLICATIONS

Using the most recently available demographic and cost data for 4 widely dispersed wild horse herds, this analysis has shown that WinEquus is capable of discriminating the economic implications of a variety of management prescriptions and that prudent, patient contraceptive applications are likely to result in cost savings up to 30% for

BLM's wild horse management program. Model results clearly illustrate that the most significant cost savings accrue only when 2-year or 3-year contraceptives are combined with removals that explicitly raise the proportion of males above 50%. As herd demographic data and contraceptive techniques improve, or accepted BLM management guidance evolves, additional modeling can be used to tailor management strategies for each herd, potentially resulting in even greater cost savings.

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IgG4/7 responses correlate with contraception in mares vaccinated with SpayVac

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ABSTRACT

SpayVac® is an immunocontraceptive vaccine based on porcine zona pellucida (pZP) antigens and uses a patented liposome formulation (VacciMax™ or DepoVax®). It has delivered single-dose, long-lasting (4–10 years) immunocontraception in several species. Previous studies have demonstrated a positive correlation between levels of pZP antibodies produced and contraceptive effect; however, individual mares that were consistently infertile did not necessarily have the highest antibody titers. The objective of this study was to identify potential differences in specific immunoglobulin G (IgG) isotype responses among mares treated with SpayVac (VacciMax formulation) to improve our understanding of vaccine efficacy and potential management applications. We analyzed serum samples collected 1, 2 and 4 years post-vaccination from mares in another study that were continuously infertile or had foaled at least once during the 4-year period ($n = 14$ each). Additional samples from the continuously infertile mares were collected 5 years post-vaccination. A fluorescent bead-based assay was used to distinguish IgG isotype responses against pZP. IgG1 antibodies were generally higher in the infertile compared to the fertile mares, but only IgG4/7 antibodies were significantly higher in infertile mares during years 1 and 2 post-vaccination ($p < 0.05$). Interestingly, IgG4/7 isotype levels were significantly higher during year 5 compared to year 4 in the continuously infertile mares ($p < 0.02$). SpayVac's ability to preferentially stimulate IgG4/7 antibodies may contribute to its long-term immunocontraceptive efficacy, and measuring IgG4/7 isotypes may help differentiate effectively contracepted mares from those that may need additional vaccination.

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1. Introduction

The Bureau of Land Management (BLM) is responsible for managing feral horse (*Equus caballus*) and burro (*E. asinus*) populations on public lands, and horse numbers are currently estimated to be around 75,000 [pers. comm. Dean Bolstad, 7/17/17]). This far exceeds the appropriate management level of 26,715 [1]; and maintaining horses in off-range facilities costs the BLM millions of dollars annually [2]. Immunocontraceptive vaccines have the greatest potential to help BLM maintain horse populations at appropriate management levels; however, vaccines must have multi-year efficacy to be both technically feasible and cost-effective.

Two main classes of contraceptive vaccines have been developed based on gonadotropin-releasing hormone (GnRH) and porcine zona pellucida (pZP) antigens. It is presumed that pZP immunocontraception has fewer side effects compared to GnRH vaccines (like GonaCon™), because GnRH receptors are located in a variety of tissues in addition to reproductive organs, including the nervous system [3], bladder [4], and heart [5]. Antibody occupation of ZP receptors is thought to disrupt sperm binding [6–8], which normally initiates the acrosome reaction and subsequent fertilization [9]. ZonaStat-H is a pZP vaccine currently used in horses; however, it requires a booster 3–4 weeks after the initial inoculation and subsequent annual boosters to maintain contraceptive efficacy [10]. PZP-22 uses time-release pellets to obviate the need for boosters; however, it has a reported efficacy of up to 22 months [11], which is still not ideal for BLM [12].

SpayVac® is a pZP vaccine that uses a unique liposome technology and has delivered single-dose, long-lasting (4–10 years)

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immunocontraception in a variety of species including fallow deer (*Dama dama*) [13], grey seals (*Halichoerus grypus*) [14], and horses [15]. SpayVac comes in two formulations: VacciMax™ is a water-in-oil emulsion, and DepoVax® is first lyophilized and then components are resuspended in oil prior to injection. A study by Killian et al. [15] demonstrated pregnancy rates of 0%, 17%, 17% and 17%, respectively, 1–4 years after treatment of mares with a single SpayVac VacciMax dose (400 μg pZP; n = 12) administered in the neck compared to 75%, 75%, 88% and 100% for untreated mares. Bechert et al. [16] demonstrated that 3–4 months post-vaccination with SpayVac VacciMax or DepoVax (200 μg pZP) administered in the neck, 93% of treated mares (n = 14) ceased cycling as evidenced by significantly lower serum concentrations of progesterone ($p < 0.025$) and smaller ovaries with fewer follicles ($p < 0.001$), while other organ systems were unaffected. The U.S. Geological Survey (USGS), and the U.S. Department of Agriculture, Animal and Plant Health Inspection Service initiated field trials with SpayVac in mares (200 μg pZP in DepoVax [n = 30] and VacciMax [n = 30] SpayVac formulations administered in the rump) and found that pregnancy rates for the VacciMax treatment group were significantly lower ($p < 0.001$) compared to controls for 3 years following the single injection [12].

However, results from this last study were not as robust as expected, given earlier results from both Bechert et al. [16] and Killian et al. [15]. For example, the fertility rate for the SpayVac VacciMax group (n = 30) was 13%, 47%, and 43% compared to 100%, 98%, and 100% in controls for years 1, 2 and 3 post-vaccination, respectively. The relationship between antibody titer, measured by a pZP ELISA, and fertility was also not clear, because individual mares that were consistently infertile did not necessarily have the highest antibody titers [12]. Previous studies have demonstrated a positive correlation between levels of pZP antibodies produced and contraceptive effect [10,17], and similarly in the Roelle et al. study [12], antibody titers within 50–60% of the positive reference serum appeared to effectively contracept 90–95% of the mares.

Wagner [18] reviewed different horse immunoglobulin (Ig) antibody classes (e.g., IgG, IgM, IgA, IgE) as well as IgG isotypes, which are more numerous in horses (IgG1-IgG7) compared to other mammalian species [19]. IgG is the predominant antibody class in equine serum and the major component of humoral immunity. After stimulation with specific antigens, B-cells are activated and, with the help of T-cells, develop into antibody producing plasma cells. After successful antigen stimulation or vaccination, long-lived plasma cells produce large amounts of high-affinity IgG antibodies. These antibodies, depending on their immunoglobulin subclass or isotype, can neutralize antigens or pathogens. The latter is a key function of many protective vaccines, and it has been suggested that "different IgG isotypes should be considered in developing therapeutic antibodies" [20].

The SpayVac VacciMax formulation, when administered to rabbits, created a significantly greater antibody response compared to that produced by alum-adjuvant vaccines, and this enhanced response was correlated with an increased number of antigen-specific plasma cells in the bone marrow [21]. SpayVac may similarly stimulate increased production of plasma cells in mares. Various mechanisms potentially responsible for maintenance of pZP antibody titers in mares are discussed in Bechert et al. [16], but the role different IgG isotypes may play with respect to SpayVac has not been considered.

Lewis et al. [22] analyzed different effector functions of the seven equine IgG isotypes, including complement activation, IgG-receptor binding and affinity to different bacterial cell-wall proteins. Their work suggested that equine IgG1, IgG4 and IgG7 isotypes are most capable of mediating all of these effector functions and most likely have a better neutralizing capacity compared to IgG3 and IgG5. The remaining two equine IgG2 and IgG6 isotypes are only weak immune mediators. Some of the objectives of the Roelle et al. [12] study were to: 1) evaluate the contraceptive efficacy of SpayVac over several breeding seasons, and 2) determine serum pZP antibody titers. The objective of this study was to identify potential differences in specific IgG isotype responses among mares treated with SpayVac VacciMax in the Roelle study and improve our understanding of vaccine efficacy and potential management applications.

2. Materials and methods

2.1. Vaccine preparation

Immunovaccine, Inc. (IMV; Halifax, Nova Scotia, Canada) obtained frozen pig ovaries from Sioux-Preme Packing Company (Sioux Center, IA), isolated pZP, and prepared the vaccine as previously described by Brown et al. [23]. Purified pZP was incorporated in phosphate buffered saline (PBS; pH 7.4) at a final concentration of 200 μg per dose. Lipids containing lecithin and cholesterol at a ratio of 10:1 (0.2 g lecithin and 0.02 g cholesterol/dose; Lipoid, Newark, NJ) were added to the pZP solution to form multilamellar liposomes. Modified Freund's Adjuvant (MFA; Calbiochem, La Jolla, CA) was added to half of the prepared pZP-liposome mixture to form a water-in-oil (aqueous) emulsion (1:1, v/v; 1 mL/dose). The second half of the prepared pZP-liposome mixture was lyophilized (non-aqueous) and then reconstituted with MFA for a final deliverable dose volume of 0.5 ml. Vehicle control vaccines were produced as described above without the addition of the pZP antigen (0.5 mL/dose). All vaccines and control doses were shipped to USGS for the Roelle et al. study in individual syringes kept frozen on dry ice.

2.2. Animals

The captive breeding trial was initiated in 2011 at a BLM wild horse and burro adoption facility near Pauls Valley, OK [12]. The study's protocol was approved by the USGS Animal Care and Use Committee in Fort Collins, CO. Mares were maintained in 12-ha pastures and fed bermudagrass (*Cynodon* sp.) *ad libitum* and alfalfa hay at approximately 6.4 kg/animal/day. Protein/fat supplements and trace mineralized salt blocks were also provided, and annual prophylactic vaccines and anthelmintics were administered by BLM personnel (see Roelle et al. [12] for details).

Ninety mares (4–7 years of age) were randomly assigned to one of three experimental groups (SpayVac VacciMax vaccine, SpayVac DepoVax vaccine, and control; n = 30 each), and mixed treatment groups were maintained on three separate pastures. On 30 March 2011, all injections were administered by hand in the left rump during restraint in a padded hydraulic squeeze chute. Mares had opportunities to breed with stallions starting in June 2011 (approximately 2 months after vaccination), with a new stallion being introduced every 2 weeks on a rotation schedule that allowed each stallion to rest at least 2 weeks between sessions.

As described by Roelle et al. [12], 10-mL blood samples were collected from each mare at the time of vaccination (in March) as well as several other time points. All samples were used to determine pZP antibody titers, and blood samples collected in December 2011, and January and November 2013 were also analyzed to quantify pregnant mare's serum gonadotropin (PMSG) and estrone sulfate. Mares were considered to be pregnant if any one of the following three criteria were positive: 1) elevated hormone levels (PMSG >600 mIU/mL or estrone sulfate >20 ng/mL), 2) positive transrectal ultrasound or palpation exams, or 3) observations of nursing foals (mares were observed in each pasture for at least 2 h per day 3 days per week).

2.3. Samples

The fertility rates in the control group (n = 30) were 100% and 96.7% for year one and two, respectively, compared to 16.7% in the SpayVac DepoVax treated group (n = 30) during the first year ($p < 0.001$) and 75.9% during the second year (n = 29) [12]. Because the performance of this vaccine during the second year was so poor, BLM decided to drop the DepoVax treatment group from the study. In contrast, the fertility rate in the SpayVac VacciMax treated mares was 13.3%, 46.7%, and 43.3% for 2012, 2013 and 2014, respectively (n = 30 each year), compared to 100%, 96.7%, and 100% for the control group ($p < 0.001$ [12]). Half of the mares vaccinated with SpayVac VacciMax in 2011 were still infertile 4 years later (15 of 30). We analyzed serum samples collected 1, 2 and 4 years post-vaccination (7 March 2012, 6 March 2013, 3 February 2015) from mares that were continuously infertile or had foaled at least once during the 4-year period (14 complete sample sets from each group were available for comparative analysis in our study). We also analyzed serum collected on 24 February 2016 from 14 of 15 continuously infertile mares.

2.4. Laboratory analyses

2.4.1. Determination of pZP antibody titers by ELISA

IMV technicians determined pZP antibody titers as a percentage of a positive reference serum using an enzyme-linked immunosorbent assay as described by Roelle et al. [12]. Briefly, pZP antigen (1 μg/mL, prepared in-house) was used to coat 96-well polystyrene microtiter plates (Bio-Rad, Hercules, CA) prior to incubation overnight at 4° C. Plates were washed with a Tris wash buffer (Tris-buffered saline/Tween-20) and blocked with 3% gelatin (Bio-Rad) for 30 min at 37° C. Following another wash, a standard horse serum sample as well as unknown samples were added to the wells prior to incubation at 4° C overnight. Unknown and positive reference serum samples were diluted to 1/25,000 and each sample was added to the plates in triplicate. Plates were washed and a secondary antibody (Protein G [Calbiochem] conjugated to alkaline phosphatase enzyme) was added to each well at a dilution of 1/1000 prior to incubation for 1 h at 37° C. Plates were washed, a substrate solution containing 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was added at a concentration of 1 mg/mL, and plates were then incubated for 1 h at 37° C. A microtiter plate reader (ASYS Hitech GmbH, Eugendorf, Austria) measured the absorbance of each well at a wavelength of 405 nm. Absorbance values from the triplicate wells were averaged for each sample and expressed as a percentage of the average absorbance of the positive reference sample. The positive reference sample was considered to have a titer of 100% and came from a female previously vaccinated with SpayVac in a separate study and determined to have a strong, pZP-specific antibody response.

2.4.2. Determination of pZP antibody values by a fluorescent bead assay

Purified pZP antigen (from IMV) was coupled to fluorescent bead 33 (Microspheres; Luminex Corporation, Austin, TX) as previously described in detail by Wagner and Freer [18]. In brief, 5×10^6 activated beads were used for coupling of 300 μg pZP antigen. After coupling, the beads were re-suspended in blocking buffer (PBS with 1% [w/v] BSA and 0.05% [w/v] sodium azide) and incubated for 30 min. The beads were washed three times in PBS with 0.1% (w/v) BSA, 0.02% (v/v) Tween 20 and 0.05% (w/v) sodium azide (PBS-T), counted and stored in the dark at 2–8 °C.

For the assay, all serum samples were diluted at 1:100 in blocking buffer immediately before the assay was started. Five control serum samples from healthy mares not vaccinated with SpayVac were included as controls in each assay run. Wells containing blocking buffer were included as background controls. Using a Biotek ELx50 plate washer (Biotek Instruments Inc., Winooski, VT), PBST (PBS with 0.02% [v/v] Tween 20) was dispensed to all Millipore Multiscreen HTS plate wells (Millipore, Danvers, MA), and aspirated after soaking the plate for 2 min. Then 50 μl of the diluted serum samples, control sera, or blocking buffer were added to the plate wells. Beads coupled with pZP were vortexed and sonicated for 20 s, and 50 μl of bead solution containing 5×10^3 pZP beads were added to each well. The plates were covered to protect the beads from light and were incubated at room temperature with shaking for 30 min. Plates were then washed five times using the Biotek ELx50 plate washer. For total Ig detection, 50 μl of a biotinylated goat anti-horse IgG (H + L) antibody (Jackson Immunoresearch Laboratories, West Grove, PA) was added to each plate at a 1:5000 dilution in blocking buffer. The plates were incubated and washed again as previously described. Subsequently, 50 μl of streptavidin-phycoerythrin (PE; Invitrogen, Carlsbad, CA) was added to each well at a dilution of 1:100 in blocking buffer. Plates were incubated for another 30 min as described above. Finally, the plates were washed by adding 100 μl of blocking buffer to each well, and then the plates were covered and placed on a shaker for 15 min to re-suspend the beads. The assay was then analyzed in a Luminex 200 instrument (Bio-Rad Laboratories, Inc., Philadelphia, PA) using BioPlex Manager 6.1 software (Bio-Rad, Hercules, CA). The data were reported as median fluorescent intensities (MFI).

2.4.3. Anti-pZP IgG isotype detection

For IgG isotype detection, the fluorescent bead-based assay setup was conducted as described above, but detection antibodies used were different. Instead of biotinylated goat anti-horse IgG (H + L) antibody measuring total IgG, biotinylated monoclonal antibodies to IgG1 (CVS45), IgG4/7 (CVS39), IgG5 (clone 416), or IgG6 (clone 267) were added to the plates. All four IgG detection antibodies were used at previously determined optimal dilutions of 1:500 (IgG1), 1:1000 (IgG4/7), or 1:100 (IgG5 and IgG6). Monoclonal antibodies against equine IgG1 and IgG4/7 were described by Sheoran et al. [24], and antibodies to IgG5 and IgG6 were described by Keggan et al. [25].

2.5. Statistical analysis

D'Agostino and Pearson normality tests indicated that most antibody values were not normally distributed. All anti-pZP antibody measurements were thus compared by repeated-measures ANOVAs with Sidak post tests between the fertile and infertile groups. For comparing anti-pZP antibody values between years 4 and 5 in the infertile group, a Wilcoxon matched-pairs test was used. *P*-values <0.05 were considered significant. The statistical analyses were performed and the graphs were created using GraphPad Prism 6 for Mac OS X, version 6f.

3. Results

3.1. Fertility

Of the 15 SpayVac VacciMax treated mares that remained infertile for 4 years post-vaccination in the Roelle et al. study [12], 12 were still infertile 5 years post-vaccination (February 2016) and 3 had become pregnant. The 15 other SpayVac VacciMax treated mares that had conceived or foaled at least once during the initial 4 years post-vaccination were no longer being monitored.

3.2. Antibodies against pZP as measured by ELISA and fluorescent bead assay

The pZP antibody titers for the first 4 years post-vaccination from the Roelle et al. study [12] are illustrated in Fig. 1A. Serum pZP antibody values obtained by ELISA showed a trend towards increased pZP antibodies in the infertile group during the first year, which did not reach significance. The same serum samples from years 1, 2 and 4 were measured in the newly developed pZP fluorescent bead-based assay (Fig. 1B; $n = 14$ per group). Similar to the ELISA assay, the bead-based assay was initially used to analyze all serum antibodies against pZP independent of their isotype. By analyzing the sera in the bead-based assay, anti-pZP antibodies were clearly elevated in infertile mares in years 1 and 2 (both $p < 0.001$), and still showed a trend towards increased antibody values in the infertile group in year 4 post-vaccination (Fig. 1B).

3.3. IgG isotype responses against pZP

The fluorescent bead assay was then used to distinguish IgG1, IgG4/7, IgG5, and IgG6 isotype responses against pZP (Fig. 2). IgG1 antibodies showed a trend towards higher anti-pZP IgG1 values in the infertile compared to the fertile mares; however, the differences did not reach significance at any of the three observation time points (Fig. 2A). IgG4/7 responses were most similar to the total anti-pZP antibody response (Fig. 1B) and resulted in increased anti-pZP IgG4/7 values in years 1 and 2 ($p < 0.05$) post-vaccination. During year 4, anti-pZP IgG4/7 antibodies in infertile mares still had a trend towards higher antibody values compared to the fertile mares, but this did not reach statistical difference anymore (Fig. 2B). Interestingly, IgG4/7 isotype levels were significantly higher during year 5 compared to year 4 in the continuously infertile mares ($p = 0.0166$; $n = 14$; Fig. 3). In contrast, anti-pZP IgG5 and IgG6 values were low overall in both groups (Fig. 2C and D).

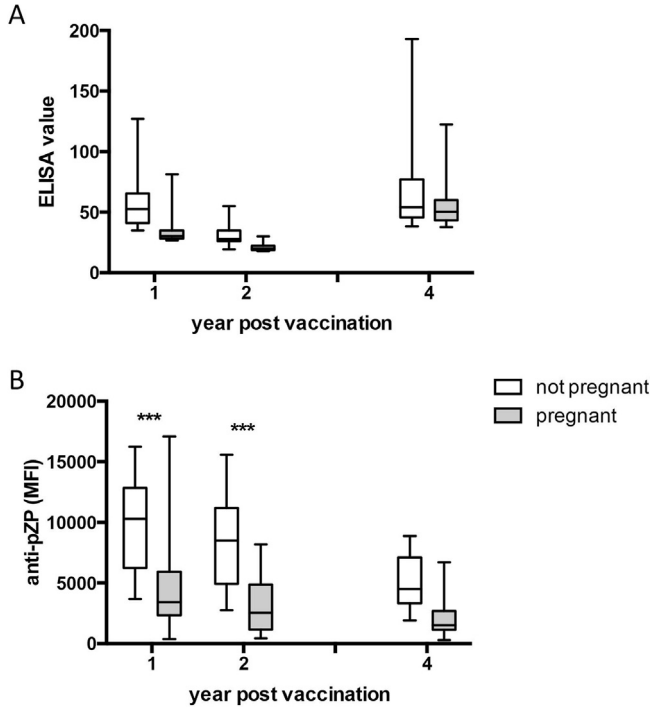


Fig. 1. Comparison of ELISA (A) and fluorescent bead-based assay (B) to measure serum pZP antibodies in mares 1, 2, and 4 years post-vaccination with a single dose of SpayVac VacciMax ($n = 14$ each) depicted as median, 25 and 75 percentiles, and ranges (extended bars). Asterisks indicate significant differences between antibody values in the fertile and infertile groups (p-value *** <0.001).

4. Discussion

To optimize application of SpayVac to free-ranging herds of horses, a better understanding of how the vaccine affects the immune system and results in contraception would be helpful. A variety of internal physiologic factors can influence an animal's ability to mount an immune response (e.g., age, pregnancy, nutrition, and other environmental factors) [26]. The primary mediators of adaptive immune function are B and T lymphocytes as well as antibodies; however, dendritic cells (DCs) control T-cell activation [27], and regulate antigen-specific immunity [28]. DCs arise primarily from the bone marrow and serve as an interface between the body and its environment by patrolling the dermis and epidermis (e.g., Langerhans cells), and mucosal membranes [29]. They can initiate T-effector cell, cytolytic, or suppressive immune responses, as well as production of cytokines like IL-12 [30]. “Dendritic cells act as nature's adjuvants for regulating antigen-specific immunity … and elicit T-cell memory, a critical goal of vaccination” [28]. Immature DCs take up antigen, and after this, or exposure to pro-inflammatory cytokines (e.g., TNFα and IL-1β), they transform into mature DCs. Maturation involves a significant cytoplasmic reorganization that brings MHC class II loaded with antigenic peptides to the cell surface, chemokine receptors remodel to facilitate movement to lymphoid organs, and membrane folds expand as “dendritic” processes in lymph nodes to increase opportunities for T-cell interaction [30,31]. Key to DCs' effectiveness is their movement into lymph nodes after maturation. The main lymph node homing chemokine receptor (CCR7) needs to be expressed by DCs for successful migration through peripheral lymph vessels [32]. There are also other mediators of DC migration to lymph nodes, including CCR8, which facilitates the migration of monocyte- (but not skin-derived) DCs to lymph nodes [33].

Dendritic cells form clusters with antigen-specific T-cells in lymph nodes to promote the immune response [27]. DCs present antigenic peptides to T-cells via MHC class I and II complexes. Activated T-cells can subsequently induce B-cells to proliferate and mature into memory B-cells and plasma cells, which produce large amounts of antibodies. Cytokines produced by T-cells also directly affect B-cells to enhance Ig secretion and isotype switching [30]. T follicular helper (T_{FH}) CD4 cells in lymph nodes are required for production of antibodies by plasma cells; however, *circulating* (T_{FH}) CD4 cells were recently identified, and it was proposed that these cells might further facilitate immune memory by seeding new germinal cell reactions in other lymphoid tissues [34].

Vaccine efficacy can be improved by enhancing antigen capture and processing by immature DCs, DC maturation, and migration to the lymph nodes [28]. Adjuvants can help condition the site of injection by causing tissue inflammation, which enhances DC migration to lymph nodes [32], and a failure of DCs to migrate to lymph nodes can exacerbate local immune responses because DCs release pro-inflammatory mediators and attract other leukocytes [35]. The liposomes in SpayVac may themselves serve as immunological adjuvants [36]. VacciMax created a significantly greater humoral response in rabbits compared to that produced by alum-adjuvant vaccines, and this response was correlated with an increased number of antigen-specific plasma cells in the bone marrow [21]. Plasma cells originate in lymph nodes, enter the bloodstream, and travel to the bone marrow where they can form a stable long-lived population of fully differentiated cells, especially after reactivation of memory B-cells during a secondary immune response [37]. Both Bechert et al. [16] and Roelle et al. [12] reported

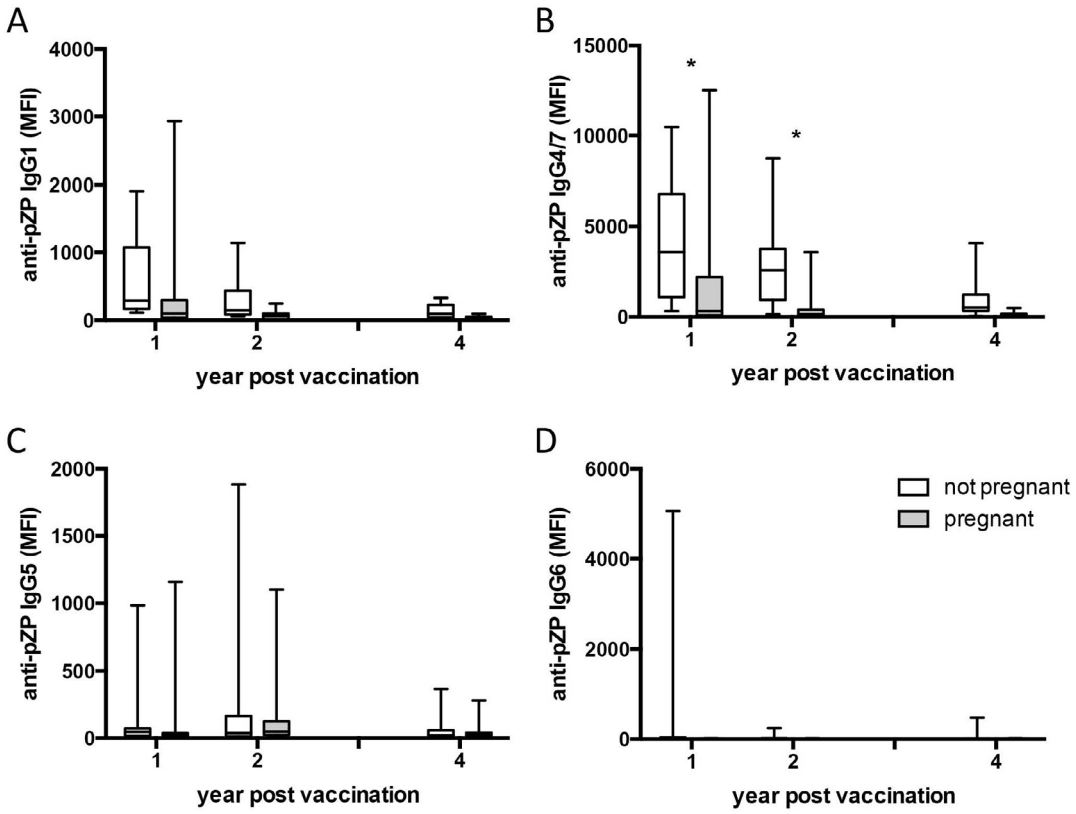


Fig. 2. Anti-pZP IgG isotypes in serum samples collected 1–4 years post-vaccination with SpayVac VacciMax, evaluated by different fluorescent bead-based assays for IgG1 (A), IgG4/7 (B), IgG5 (C), and IgG6 (D). *P*-values (* <0.05) show differences between fertile and infertile groups (n = 14 each).

vacillating reactions at the SpayVac injection site over a period of months, and this could be initiating the secondary immune response.

A variety of cells have been shown to support plasma cell populations in the bone marrow, including eosinophils [38] and most recently regulatory T cells [39]. Furthermore, regulatory T-cells modulate the activation of DCs [40], and DCs are required for regulatory T-cell homeostasis [41]. DCs are organized into perivascular clusters in the bone marrow, which include mature B lymphocytes (e.g., terminally differentiated B-cells or long-lived plasma cells) and T lymphocytes (e.g., $CD4^+$ and $CD8^+$ T cells); and removal of these bone marrow stroma cells resulted in a significant loss of mature B-cells [42]. pZP antibody titers remained consistently elevated for 7 years in African elephants (*Loxodonta africana*) given a single injection of SpayVac [43], and grey seals vaccinated with a single-dose of SpayVac [14] maintained high titers for at least 10 years (Robert Brown, personal communication). SpayVac formulations seem to support maturation and longevity of dendritic and plasma cells in bone marrow.

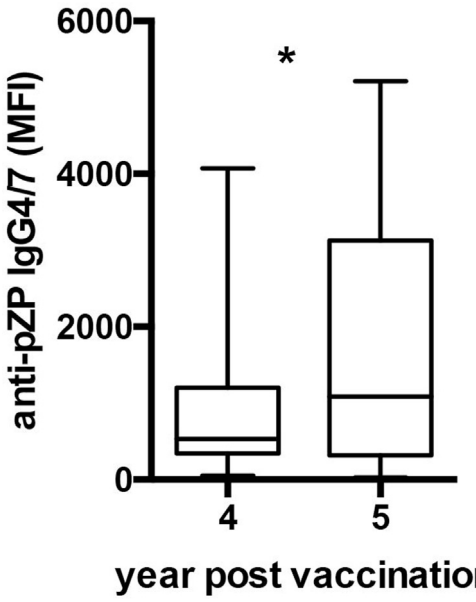


Fig. 3. Anti-pZP IgG4/7 isotypes values in serum of the infertile mares in years 4 and 5 post-vaccination (n = 14 each) with SpayVac VacciMax (p = 0.0166).

Antibody occupation of ZP receptors is thought to disrupt sperm binding [6–8], which normally initiates the acrosome reaction and subsequent fertilization [9]. However, the ZP is also involved in intercellular communication between the oocyte and surrounding granulosa cells [44]. Bechert et al. [16] and subsequently Joone et al. [45] reported significantly smaller ovaries and fewer follicles in pZP-vaccinated mares; and absence of estrous cyclicity likely contributes to infertility. This may be due to a non-specific immune response to an ovarian cellular component in the vaccine [46], or immune-mediated disruption of folliculogenesis [47]. Contamination with a non-ZP ovarian protein is possible (reviewed by Bechert and Fraker [44]) and supported by the finding that recombinant ZP vaccines do not have the same contraceptive effect [48,49]. Species-specific differences in response to pZP vaccination are reviewed by Joone et al. [49], and are likely due to differences in ZP structure and glycosylation [50]. Adverse effects on ovarian function have been described in a number of species receiving pZP vaccinations,

including hamsters (*Mesocricetus auratus*) [51], dogs (*Canis lupus familiaris*) [52], rabbits (*Oryctolagus cuniculus*) [53], white-tailed deer (*Odocoileus virginianus*) [54], sheep (*Ovis aries*) [46], and primates (*Macaca radiate*) [55]. Nonetheless, pZP antibody titers above a certain threshold level are clearly linked to infertility in a variety of species [10,17].

Equine IgG1, IgG4, and IgG7 isotypes are capable of mediating all of these effector functions and most likely have a better neutralizing capacity compared to the remaining four equine IgGs [22]. IgG1 and IgG4/7 antibodies are typically involved in responses to viral infection [56–59] or intracellular bacteria [3,60], while IgG3 and IgG5 isotypes are induced after nematode infection [61], tetanus vaccination or during allergic responses of the horse [62,63]. In this study, mares that were continuously infertile post-vaccination had higher levels of IgG4/7 compared to mares that foaled at least once. Although the exact mechanisms of the immune response against pZP antigen still needs to be unraveled, it likely induced a Th1 type immune response leading to the development of mainly IgG1 and long-lasting IgG4/7 antibodies as previously described for some other vaccines [64]. It is not clear why IgG4/7 antibody values increased in the infertile group during year 5 compared to year 4. It is possible that the length of time samples were in storage or multiple thaw/freeze cycles led to an artificial decrease in sera pZP antibody concentrations prior to year 5.

The fluorescent bead-based assay clearly enhanced the analytical sensitivity and linear detection range of pZP antibody detection compared to the ELISA, resulting in more pronounced differences in IgG concentrations between fertile and infertile SpayVac-treated mares. The advantages of fluorescent bead-based assays have also been reported previously for other analytes [18,65–67]. The bead-based assay also allowed for antibody isotyping, which identified anti-pZP IgG4/7 as the primary equine isotypes mediating the long-term contraceptive immune response. This assay could eventually be used as a management tool to identify effectively contracepted mares based on established cut-off values for anti-pZP IgG4/7.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Wild Horse and Burro Program
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Cover Photographs – Wild horses in Utah and burros in Nevada.

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ACRONYMS

- ❒ AML – Appropriate Management Level
- ❒ APHIS – Animal Plant Health Inspection Service (Department of Agriculture)
- ❒ AUM – Animal Unit Month
- ❒ AU – Animal Unit
- ❒ BLM – Bureau of Land Management (Department of Interior)
- ❒ CFR – Code of Federal Regulations
- ❒ DNA – Determination of NEPA Adequacy
- ❒ DR – Decision Record
- ❒ EA – Environmental Assessment
- ❒ EIS – Environmental Impact Statement
- ❒ ESI – Ecological Site Inventory
- ❒ FLPMA – Federal Land Policy and Management Act of 1976
- ❒ FONSI – Finding of No Significant Impact
- ❒ HA – Herd Area
- ❒ HMA – Herd Management Area
- ❒ HMAP – Herd Management Area Plan
- ❒ IBLA – Interior Board of Land Appeals
- ❒ IUD – Intrauterine Device
- ❒ LUP – Land Use Plan
- ❒ MFP – Management Framework Plan
- ❒ MLRA – Major Land Resource Area (NRCS Ecological Site Descriptions)
- ❒ MUD – Multiple Use Decision
- ❒ NEPA – National Environmental Policy Act of 1969
- ❒ NPO – National Program Office (Reno, Nevada)
- ❒ NRCS – Natural Resources Conservation Service
- ❒ PZP – Porcine Zona Pellucida
- ❒ RMP – Resource Management Plan
- ❒ ROD – Record of Decision
- ❒ SOPs – Standard Operating Procedures
- ❒ TES – Threatened, Endangered or Sensitive Species
- ❒ TNEB – Thriving Natural Ecological Balance
- ❒ WFRHBA – Wild Free-Roaming Horses and Burros Act of 1971 (as amended)
- ❒ WH&B – Wild Free-Roaming Horses and Burros
- ❒ WHT – U.S. Forest Service Wild Horse Territory
- ❒ WO – Washington Office (Headquarters)
- ❒ WSA – Wilderness Study Area
- ❒ USC – United States Code
- ❒ USFS – United States Forest Service (Department of Agriculture)
- ❒ USGS – United States Geological Service (Department of Interior)

CHAPTER 1—INTRODUCTION

GENERAL

The Bureau of Land Management (BLM) is responsible for the protection, management and control of wild free-roaming horses and burros (WH&B). Under the Wild Free-Roaming Horses and Burros Act (WFRHBA), WH&B are considered an integral part of the national system of public lands in the areas where they were found in 1971. The BLM's goal is to manage healthy WH&B populations on healthy rangelands. To achieve this goal, the BLM designates Herd Management Areas (HMAs) for the long-term maintenance of WH&B herds and collects data about the animals and their habitat. The BLM also prescribes management to assure WH&B populations are in balance with other uses of the public lands and that a thriving natural ecological balance (TNEB) is achieved and maintained. Activities are carried out with the objective of maintaining free-roaming behavior and at the minimum feasible level of management necessary to attain the objectives identified in approved land use plans (LUPs) and Herd Management Area Plans (HMAPs).

1.1 PURPOSE

This handbook describes the authorities, objectives, policies and procedures that guide the management of WH&B on the public lands administered by the BLM.

1.2 OBJECTIVE

The objective of this handbook is to provide guidance for the protection, management and control of WH&B in accordance with the 1971 WFRHBA, as amended, and the implementing regulations in 43 Code of Federal Regulations (CFR) 4700.

CHAPTER 2—LAND USE PLANNING

GENERAL

Section 202(a) of the 1976 Federal Land Policy and Management Act (FLPMA) requires the Secretary to develop, maintain, and when appropriate revise LUPs that provide (by tracts or areas) for the use of the public lands. The responsible BLM official shall follow the established LUP procedures in 43 CFR 1600, associated BLM manual sections and policy for fulfilling the planning requirements prescribed in the statute.

LUPs are the basis for every on-the-ground management decision that the BLM makes. LUPs establish goals and objectives (desired outcomes), identify the management actions needed to achieve the desired outcomes, and identify allowable uses of the public lands.

2.1 LAND USE PLAN DECISIONS

LUP planning requirements for the WH&B program are applicable to all BLM LUP documents, including Management Framework Plans (MFPs) and Resource Management Plans (RMPs).

2.1.1 Comparability Consideration

Under 43 CFR 4700.0-6(b), WH&B shall be considered comparably with other resource values in the formulation of LUPs. This means WH&B are to be considered in the same manner as other resource values (e.g., cultural, historic, scenic, rangelands, timber, and minerals). WH&B are a resource value, as opposed to a land use (e.g., livestock grazing or timber harvest).

2.1.2 Herd Areas

Herd areas (HAs) are limited to areas of the public lands identified as habitat used by WH&B at the time that the WFRHBA passed (December 15, 1971). When preparing a LUP, identify the HAs (in whole or in part) which will not be managed as HMAs and explain the reasons they will not be managed for WH&B.

HA boundaries can be adjusted through a LUP when the current boundary does not correctly portray where WH&B were found in 1971 based on well-documented historical data. Existing herd areas where all animals were privately-owned (claimed during the claiming period) shall be dropped from HA status in a LUP.

Under 43 CFR 4710.2 and to assure transparency regarding the disposition of HAs, the authorized officer shall permanently maintain a record regarding the location of all HA boundaries and explanations of any changes in field office (FO) files.

2.1.3 Herd Management Areas

HMAs shall be designated in those HAs within which WH&B can be maintained over the long term in LUPs. For each HMA designated within the planning area, the LUP should identify the following:

- The HA(s) that contain the HMA.

- The initial and estimated herd size that could be managed while still preserving and maintaining a TNEB and multiple-use relationships for that area.

- The guidelines and criteria for adjusting herd size.

LUPs should also identify:

- The HMAs to be managed for non-reproducing wild horses to aid in controlling on the range population numbers and the criteria for their selection (16 United States Code (USC) § 1333(b)(1)). See Chapter 4 (Population Management). Examples of criteria that could be used to select HMAs for management of non-reproducing wild horses include: no special or unique herd characteristics, low ecologic condition, limited public land water, and reliance on private water.

- The management tools to control population size within AML and to extend (reduce) gather frequency.

2.1.4 Herd Areas Not Designated as HMAs

Where appropriate, the LUP may include decisions not to manage WH&B in all or a part of an HA. An example is intermingled and unfenced private lands within HAs where the landowners are unwilling to make them available for WH&B use, or the animals present at that time were later found to be claimed domestic horses (or burros). Another example would be where essential habitat components (forage, water, cover and space) are unavailable or insufficient to sustain healthy WH&B and healthy rangelands over the long term.

2.1.5 Changes to HA or HMA Boundaries

Decisions to change HA boundaries, to designate HMAs for the maintenance of WH&B, or to remove all or a portion of an area's designation as an HMA must be made through a LUP amendment, revision or new RMP (43 CFR 4710.1 and H-1601-1: Land Use Planning Handbook).

HMA boundaries may be changed within HAs through the LUP process to facilitate WH&B management or mitigate unacceptable impacts to other resources.

An area may lose its designation as an HMA when WH&B cause unacceptable impacts to other resource values, or conditions change and one or more of the four essential habitat components are not present in sufficient quantities to sustain WH&B use over the long term. Similarly, if conditions change, all or part of an HA may be reconsidered for designation as an HMA through LUP. See Chapter 3 (Habitat Management).

2.1.6 Wild Horse and/or Burro Ranges

An HMA may be considered for designation as a WH&B Range to be managed principally, but not necessarily exclusively, for WH&B. Criteria for consideration as a WH&B Range should include the presence of one or more of the following: unique herd characteristics, outstanding viewing opportunities, unique landscape, or significant historical or cultural features (H-1601-1: Appendix D). The authorized officer, currently only the Director or Assistant Director (AD) (refer to BLM Manual 1203: Delegation of Authority), may establish a WH&B Range after a full assessment of the impact on other resources through the LUP process.

2.2 GRAZING AUTHORIZATIONS WITHIN HMAs

Domestic horses and burros may not graze under permits or leases within HMA boundaries (43 CFR 4710.5(b)). If necessary to provide habitat for WH&B, to implement herd management actions, or to protect WH&B from disease, harassment or injury, the authorized officer may close areas of the public lands to grazing use by all or a particular kind of livestock (43 CFR 4710.5(a)).

Closure to livestock grazing may be temporary or permanent (43 CFR 4710.5(c)). Prior to issuing a final decision to permanently close an area to livestock grazing use, a LUP amendment should be completed. Completion of site-specific environmental analysis and issuance of a proposed and final decision pursuant to 43 CFR 4160 (2005) is also required.

2.3 OTHER PERMITTED ACTIVITIES

2.3.1 Commercial Recreation Use

Proposals for commercial recreational use of the public lands should be evaluated through the appropriate BLM permitting process. Permits should stipulate the safeguards necessary to protect the health and welfare of WH&B, particularly before, during and immediately following the peak foaling period (e.g., generally March – June). See Chapter 4 (4.4.4).

Proposals for motor vehicle racing, air racing, or other potentially intrusive activities have potential to harass or harm WH&B. Permit authorizations should contain provisions to minimize impacts to WH&B from these activities. Should adequate safeguards prove impractical, permits should not be approved (refer to BLM Manual Section 2930 and H-2930-1: Recreation Permit Administration).

2.3.2 Energy and Minerals Exploration and Development

The health and welfare of WH&B should be considered during project planning for hard-rock mining or oil and gas exploration and development; non-mineral sales such as gravel; or wind or solar energy development proposals. Specific mitigation measures will be identified through a site-specific environmental analysis and decision process in accordance with the 1969 National Environmental Policy Act (NEPA). Mitigation measures will be implemented through applicable permits.

2.4 LAND USE PLAN GOALS AND OBJECTIVES

LUPs normally contain general habitat and population management goals and objectives. LUPs may also include the management requirements or actions necessary to make progress toward attainment of Land Health Standards and to preserve and maintain a TNEB and multiple-use relationship on the public lands.

2.4.1 Habitat and Population Management

Habitat or population management and monitoring objectives regarding the management of a specific HMA or complex of HMAs are normally identified in a Herd Management Area Plan (HMAP) rather than a LUP.

2.5 IMPLEMENTATION DECISIONS

Implementation decisions make progress toward achieving LUP goals and objectives and may include:

- Establishing or adjusting appropriate management levels (AMLs) based on monitoring and evaluation, including the population range within which the herd size will be allowed to fluctuate.
- Identifying and setting objectives for herd composition, animal characteristics and habitat development needs (e.g., HMAPs).
- Specific habitat improvement projects (e.g., construction, modification, or removal of fences; water development construction/reconstruction or removal; or re-vegetation projects).
- Site-specific population management actions (e.g., decisions to gather/remove excess WH&B, apply fertility control, or adjust age or sex ratios).

2.5.1 Appropriate Management Level (AML) Adjustments

AML may be adjusted (either up or down) through a site-specific environmental analysis and decision process (NEPA). An analysis under NEPA is also required to establish a population range (upper and lower limit) for AMLs initially established as a single number. Development of a LUP amendment or revision is not generally required.

2.5.1.1 AMLs Established in LUPs

- When AML is established in a LUP, follow the process outlined in the LUP to adjust AML.

- When the LUP does not outline a process for AML adjustment, the LUP may need to be amended or revised to adjust AML.

2.5.1.2 AMLs Established through Implementation Decisions

See Chapter 4 (4.2.2).

2.5.2 Herd Management Area Plans

Herd Management Area Plans (HMAPs) are prepared under 43 CFR 4710.3-1. HMAPs establish short- and long-term management and monitoring objectives for a specific WH&B herd and its habitat. HMAPs also identify the actions to be taken to accomplish herd and habitat management objectives. An HMAP assists the authorized officer in tracking progress toward achieving LUP goals.

HMAPs tier to and must be in conformance with the applicable LUP. If the proposed management strategy is not consistent with the LUP, then the LUP should be amended, or the proposal should be modified or rejected.

HMAPs may be prepared for a single HMA or a complex of adjacent HMAs where animal interchange occurs. When two or more jurisdictions have management responsibility for portions of a single HMA or an HMA complex, BLM will designate one field office with the lead responsibility for development of the HMAP and management of the HMA or complex. When a WH&B herd is dependent on the habitat within a BLM HMA and an adjacent U.S. Forest Service (USFS) Wild Horse Territory (WHT), the agencies should jointly determine lead responsibility for development of the HMAP and management of the herd and habitat.

2.6 NOTICES, CONSULTATIONS AND HEARINGS

2.6.1 Agency Consultation and Public Involvement

See BLM Manual Section 4710.25.

2.6.2 Hearings

See BLM Manual Section 4740.3.

CHAPTER 3—HABITAT MANAGEMENT

GENERAL

Habitat for WH&B is composed of four essential components: forage, water, cover, and space. These components must be present within the HMA in sufficient amounts to sustain healthy WH&B populations and healthy rangelands over the long term. If they are not present in sufficient amounts, the authorized officer should consider amending or revising the LUP to remove the area's designation as an HMA. If the decision is made to return a designated HMA to HA status, the total population of WH&B should then be gathered and removed. See BLM Manual Section 4710.3.

A recurring pattern of WH&B movement out of the HMA to access forage, water, or thermal or hiding cover is an indication that year-long WH&B use cannot be sustained. If one or more of the key habitat components is missing, the HMA should be considered as unsuitable for year-long use. In these situations, the authorized officer should consider removing the area's designation as an HMA through LUP. An exception would be two or more HMAs which adjoin and are managed as a complex of HMAs, or HMAs which adjoin USFS WHTs that can be managed as a complex.

3.1 FORAGE (VEGETATION)

Forage (vegetation) is one of the essential components of WH&B habitat. The authorized officer should determine whether vegetation provides sustainable forage (and cover) for the animals. Vegetation should be managed within each HMA in a manner that achieves and maintains a TNEB and assures significant progress is made toward achieving the Standards for Land Health and other site-specific or landscape-level objectives.

3.2 WATER

An adequate year-round quantity and quality of water must be present in the HMA to sustain WH&B numbers within AML. If baseline information concerning access to and availability of water does not exist, then a public land water inventory should be conducted.

If privately-owned water is essential to sustaining WH&B populations within HMAs, cooperative agreements with the owners or acquisition of water rights should be considered. If agreement cannot be achieved with private water owners, or if public land water cannot be developed and maintained, one of the following should occur:

- AML should be adjusted based on the available public land water within the HMA boundary.

- The authorized officer should amend or revise the LUP to remove the area's designation as an HMA.

3.3 COVER (VEGETATION) AND SPACE

The terrain and vegetation are needed to provide WH&B with escape (hiding) cover and shelter from the prevailing weather. (Vegetation also provides sustainable forage. See 3.1 above). WH&B require sufficient space to allow the herd to move freely between water and forage within seasonal habitats. Cover and space are interrelated. If the HMA has barriers preventing free movement of WH&B throughout the HMA or between forage and water, it would not have sufficient cover and space. Barriers can be natural (e.g., rock rims, rivers) or human-induced (e.g., fences, highways).

3.4 HABITAT IMPROVEMENT PROJECTS

3.4.1 Nonstructural Improvements

Nonstructural improvement projects such as seeding, prescribed fire, emergency fire rehabilitation or shrub and tree removal have potential to increase the forage available for WH&B use and to improve habitat conditions. They may also cause grazing animals to concentrate their use within the project area. As a result, it may be necessary to close these areas to grazing until vegetation management objectives are achieved.

Before making the decision to exclude an area from grazing use, the authorized officer should first take a hard look at alternatives to fencing. These alternatives could include:

- Reduce WH&B populations to the AML lower limit.
- Reduce WH&B to a number below the AML lower limit (based on the available forage and water).
- One of the above, in combination with closing the area to domestic livestock use (in whole or in part).
- Use of mineral supplements to modify distribution.
- Restrict or eliminate access to selected water sources.

If the authorized officer determines fence construction is necessary, the fences should be designed to maintain WH&B access to critical water sources. Fences should then be removed once objectives have been achieved.

3.4.2 Structural Improvements

Construct and maintain structural improvement projects (e.g., fences, cattle guards, or water developments) in a manner that protects the wild, free-roaming nature of WH&B and provides for normal herd distribution and movement as well as genetic interchange.

While some projects might increase the forage, cover, or water available for WH&B, others might negatively impact individual animals or entire populations:

- Fences and cattle guards might restrict seasonal WH&B movement or use of critical escape (hiding) or thermal cover, key spring-summer-fall-winter use areas, or critical water sources.

- Water development projects might encourage use outside the HMA or concentrate use in sensitive areas.

Existing projects that negatively impact WH&B should be considered for removal or modification through coordinated activity plans. Proposed projects should be analyzed and mitigation proposed to minimize adverse impacts to WH&B where possible.

3.4.2.1 Existing Fences and Cattle Guards

Existing fences and cattle guards with negative impacts to WH&B should be reviewed to determine:

- If the project is still needed; or
- Whether the project can be modified to minimize impacts to WH&B.

Possible fence or cattle guard modifications could include:

- Make fences open-ended allowing WH&B movement around the ends.
- Construct large gates or sections of "let-down" fence, or consider the use of electric take-down fence where feasible, to allow movement when fences are not needed for livestock control.
- Weld rebar strips between cattle guard grates; or
- Replace the cattle guard with a gate, if possible.

3.4.2.2 Proposed Fences and Cattle Guards

Fencing within an HMA should be done only after the impacts are carefully analyzed through the NEPA process. If fences and cattle guards are essential for proper resource management, they should be constructed with particular attention to location and design. Avoid locating fences across migration routes, to prevent WH&B (or livestock) concentration, or the unintended trapping or death of WH&B.

3.4.1.3 Existing Water Developments

Consistent with resource management objectives, existing projects may be modified to provide WH&B with access to water through one or more of the following methods:

- Piping water to a trough away from the source.
- Piping water to a trough outside an exclosure.
- Retaining a portion of the source outside the exclosure.

3.4.1.4 Proposed Water Developments

Water sources may be excluded from use by WH&B to protect the water source, the associated riparian area, and to maintain or improve the quality and quantity of water. Where possible, projects should be designed to provide WH&B with access to water as described in 3.4.1.3 above.

Fences constructed in proximity to major WH&B water sources and smaller riparian pastures or exclosures should be constructed with a top rail composed of pipe or wooden poles. The top rails provide a visual barrier to prevent WH&B from entering the exclosure and becoming trapped. Project design may also consider the use of specialized gates (e.g., finger gates) to allow any WH&B entering an exclosure to exit safely.

Reservoirs and similar water sources should be designed and maintained with gently sloping (rather than steep) sides to avoid trapping foals.

Where WH&B are, or may become, dependent upon mechanically provided water, the authorized officer shall assure that alternative sources of known and accessible water are available to WH&B in the event of a system failure of a well, storage tank, pipeline, trough, or float device.

Actions to assure water is available to WH&B may include:

- Entering into Cooperative Range Improvement Agreements (Form 4120-6) whose terms and conditions require water to be made available to WH&B and wildlife.

- Using WH&B program funding (in whole or part) for construction and/or maintenance of a well, spring development, catchment, pond, or other permanent water improvement providing WH&B with access to water on public land.

- Acquiring the necessary water rights in order to provide and maintain access to water sources. Refer to BLM Manual 7250 (Water Rights).

CHAPTER 4—POPULATION MANAGEMENT

GENERAL

Under the 1971 WFRHBA, WH&B are to be managed in a manner designed to achieve and maintain a thriving natural ecological balance and protect the range from the deterioration associated with WH&B overpopulation.

4.1 MANAGEMENT REQUIREMENTS

4.1.1 Self-Sustaining

WH&B shall be managed as self-sustaining populations of healthy animals in balance with other uses and the productive capacity of their habitat. Some selected HMAs may be managed for non-reproducing wild horses to aid in controlling on-the-range population numbers (see 4.5.4).

4.1.2 Free-Roaming Behavior

In accordance with 43 CFR 4700.0-6(c), management activities affecting WH&B shall be undertaken with the goal of maintaining free-roaming behavior.

4.1.3 Constraints on Management

Management of WH&B on the public lands is limited to herd areas (HAs), consistent with the WFRHBA (16 USC § 1339) which states: “Nothing in this Act shall be construed to authorize the Secretary to relocate wild free-roaming horses or burros to areas of the public lands where they do not presently exist.”

Consistent with 43 CFR 4710.3-1, herd management areas (HMAs) shall be established for the maintenance of WH&B herds. In delineating each HMA, the authorized officer shall consider the appropriate management level for the herd, the habitat requirements of the animals, and the relationships with other uses of the public and adjacent private lands, and the constraints contained in § 4710.4.

4.1.4 Minimum Feasible Level of Management

As required in 43 CFR 4710.4, management shall be at the minimum level necessary to attain the objectives identified in approved LUPs and HMAPs.

1. Limit population management actions by:

    a. Establishing AML as a population range within which herd size will be allowed to fluctuate.

    b. Evaluating HMAs that require frequent emergency or nuisance removals due to inadequate habitat (e.g., limited availability of forage or water) for the possible removal of the area’s designation as an HMA through LUP.

2. It is not consistent with management at the minimal level to provide supplemental feed or rely on water developments that require frequent maintenance. It may, however, be appropriate to provide water in temporary emergency situations.

4.1.5 Thriving Natural Ecological Balance (TNEB)

Consistent with 43 CFR 4700.0-6, WH&B shall be managed in balance with other uses and the productive capacity of their habitat (i.e., WH&B will be managed to achieve and maintain a thriving natural ecological balance (TNEB) and multiple use relationships on the public lands).

The WFRHBA requires the BLM to manage horses in a manner that is designed to achieve and maintain a thriving natural ecological balance on the public lands (16 USC § 1333(a)). *See also Animal Protection Institute of America*, 109 IBLA 112, 115 (1989) ("…the 'benchmark test' for determining the suitable number of wild horses on the public range is 'thriving natural ecological balance'…") (*Dahl v. Clark*, 600 F. Supp. 585, 594 (D. Nev. 1984)).

To achieve a TNEB on the public lands, WH&B should be managed in a manner that assures significant progress is made toward achieving the Land Health Standards for upland vegetation and riparian plant communities, watershed function, and habitat quality for animal populations, as well as other site-specific or landscape-level objectives, including those necessary to protect and manage Threatened, Endangered, and Sensitive Species (TES). WH&B herd health is promoted by achieving and maintaining TNEB.

4.2 ESTABLISHING THE APPROPRIATE MANAGEMENT LEVEL

4.2.1 AML Definition

The appropriate management level (AML) shall be expressed as a population range within which WH&B can be managed for the long term. AMLs previously established as a single number will be modified to include an upper and lower limit. For reporting purposes, the upper limit of the AML range will be used.

AML applies to the number of adult wild horses or burros to be managed within the population and does not include current year's foals. All WH&B one year of age and older are considered adults (a foal is considered one year of age on January 1 of the year following its birth).

The AML upper limit shall be established as the maximum number of WH&B which results in a TNEB and avoids a deterioration of the range. This number should be below the number that would cause rangeland damage (refer to *Animal Protection Institute of America v. Nevada BLM*, 118 IBLA 63, 75, (1991)).

The AML lower limit shall normally be established at a number that allows the population to grow (at the annual population growth rate) to the upper limit over a 4-5 year period, without any interim gathers to remove excess WH&B. Some HMAs may require more frequent removals to maintain population size within AML. For HMAs that require more frequent gathers, the authorized officer should consider management options which would either extend the gather

cycle or broaden the AML range; amend or revise the LUP to remove the area's designation as an HMA; or manage the HMA for non-reproducing wild horses.

4.2.2 Establishing or Adjusting AML

An interdisciplinary and site-specific environmental analysis and decision process (NEPA) with public involvement is required to establish or adjust AML.

4.2.2.1 Establishing AML

When establishing AML, the analysis shall include an in-depth evaluation of intensive monitoring data or land health assessment. Intensive monitoring data shall include studies of grazing utilization, range ecological condition and trend, actual use, and climate (weather) data. Population inventory, use patterns and animal distribution should also be considered. A minimum of three to five years of data is preferred. Progress toward attainment of other site-specific and landscape-level management objectives should also be considered. See Appendix 3 (AML Establishment and Adjustment).

4.2.2.2 Evaluation and Adjustment of AML

In-depth AML evaluations should be completed when review of resource monitoring and population inventory data indicates the AML may no longer be appropriate. The following should be considered when evaluating AML:

- Changes in environmental conditions which may have occurred since the AML was established. Changing environmental conditions could include drought, wildfires, noxious weed infestations, effect of varying numbers of WH&B on forage utilization or range ecological condition/trend, an increase or decrease in the available forage, changes in livestock management, etc.

- The presence of any newly listed Threatened, Endangered or Sensitive Species (TES).

- Any additional resource monitoring, population inventory or other relevant data collected since AML was established.

For additional information refer to Appendix 3 (AML Establishment and Adjustment) and BLM Manual Section 4720.3.

4.2.3 WH&B Animal Unit and Animal Unit Month

Wild horses, one year of age or older, count as one (1) Animal Unit (AU) and burros one year of age and older count as 0.5 AU. One Animal Unit Month (AUM) is the amount of forage necessary to sustain one adult horse or two adult burros for one month (or approximately 800 pounds of air dried forage).

4.2.4 Forage Allocations for WHB

The amount of forage available to allocate to WH&B shall be determined through in-depth evaluation of resource monitoring data and following a site-specific environmental analysis and decision process. Forage for WH&B (AUMs) is allocated based on the AML upper limit.

4.3 DETERMINATION OF EXCESS

Before issuing a decision to gather and remove animals, the authorized officer shall first determine whether excess WH&B are present and require immediate removal. In making this determination, the authorized officer shall analyze grazing utilization and distribution, trend in range ecological condition, actual use, climate (weather) data, current population inventory, wild horses and burros located outside the HMA in areas not designated for their long-term maintenance and other factors such as the results of land health assessments which demonstrate removal is needed to restore or maintain the range in a TNEB.

The term "excess animals" is defined as those animals which must be removed from an area in order to preserve and maintain a thriving natural ecological balance and multiple-use relationship in that area (16 USC § 1332(f)(2)). This definition underscores the need to remove excess animals before damage to the range begins to occur.

4.4 PARAMETERS FOR POPULATION MANAGEMENT

4.4.1 Age Structure and Sex Ratio

Age structure and sex ratio influence herd health, social interactions, and population growth rates of reproducing WH&B herds. A herd's age structure and sex ratio may have been altered by previous selective removal policies or natural mortality and should be estimated from the best available data (e.g., capture records, preparation records, and final gather reports). Most populations will have representatives from each age class, and rapidly growing populations will have a greater proportion of younger WH&B. WH&B populations will produce roughly equal numbers of males and females over time.

In order to maintain the desired age structure and sex ratios, consider retaining male and female animals from each age group (0-4, 5-9, 10-15, 15+ years of age) following a removal operation. If specific age structure and sex ratio objectives are not established for an HMA or complex of HMAs, a desired post-gather age structure and sex ratio should be established during the pre-gather planning and analysis process. These objectives should be consistent with the selective removal criteria outlined in BLM Manual Section 4720.33 and the AML lower limit.

The authorized officer should consider alternatives which would manage WH&B herds for a sex ratio with a female component of less than or equal to 50 percent, to reduce population growth rates and extend the gather cycle. Opportunities to manage sex ratios as a tool to slow population growth rates are discussed later in this chapter.

4.4.2 Selective Removal

Selective removal and release of animals back to the range should be conducted to achieve AML, and the age structure and sex ratio objectives established for the specific HMA or complex of HMAs. In the absence of specific age structure or sex ratio objectives for the HMA or complex, see BLM Manual Section 4720.33.

4.4.3 Population Growth Rates

Population growth rates represent the net difference between births and deaths over time. Population growth rates can be highly variable and influenced by habitat quality, forage production, water availability and other factors. Data from recent gathers and population estimates derived from aerial surveys are used to determine population growth rates. The annual growth rate from Year 1 to Year 2 is calculated as follows:

Population on 2/28 (Year 2) – Population on 2/28 (Year 1)
÷ Population on 2/28 (Year 1) ×100 = Percent Annual Growth Rate

The same formula can be used to calculate growth over a period of several years (e.g., between aerial population surveys which are normally conducted at 3-4 year intervals).

Significant variation from historical growth rates may indicate that either immigration into the HMA or migration out of the HMA is occurring.

4.4.4 Foaling Period

Foaling period can be documented for each population (individual HMA or HMA complex) through direct observation. The capture of wild horses by using a helicopter to herd the animals is prohibited during the foaling period, which is defined as six weeks on either side of the peak of foaling to assure that young foals are mature enough to be able to remain with their band during gather activities. This period is generally March 1 to June 30 for most wild horse herds. Helicopters may be used year-round in the removal of burros. See BLM Manual Section 4740.1.

The capture of wild horses and burros by using bait (e.g., food, water, salt or sexual attraction) to lure animals into a trap may be used year-round.

Population surveys or distribution flights involving fixed-wing aircraft may be conducted during the foaling period. The use of helicopters for these activities during this time should be evaluated on a case-by-case basis.

4.4.5 Gathers and Gather Cycles

The gather cycle is the interval between gathers. Gather frequency is a function of the AML range, population growth rates and other environmental factors. Once AML is achieved, gathers to remove excess WH&B should be planned to occur at intervals of approximately 4 to 5 years to maintain population size within AML.

4.4.6 Genetic Diversity

Reproducing WH&B herd health is dependent, in part, on maintaining desirable genetic diversity (avoiding inbreeding depression).

4.4.6.1 Baseline Genetic Diversity

Baseline genetic diversity will be determined for all WH&B herds. Once a baseline is established, additional samples will be collected to reassess genetic diversity every other gather (e.g., every 6-10 years). If testing indicates diversity is less than desired, the herd should be reassessed more frequently (e.g., every gather).

In the past, genetic diversity was assessed by evaluating blood samples from the herd. Hair samples are now used to assess genetic diversity. Procedures for collecting and processing WH&B hair samples are described in Appendix 1 (Genetics Data and Hair Sample Collection Instructions). A report assessing genetic diversity is developed for each set of samples from an HMA.

Baseline data can be compared between adjacent HMAs to determine if the WH&B herd is isolated, or if genetic material is being exchanged between reproducing herds as part of a larger population of WH&B. Movement of WH&B from one HMA to another may enhance genetic diversity.

4.4.6.2 Interpreting Genetics Data

Measures of individual animal and population-wide genetic diversity are based on the number of individuals sampled within each HMA or complex. The most important measure of genetic diversity is Observed Heterozygosity (Ho). The observed heterozygosity is a measure of how much diversity is found, on average, within individual animals in a WH&B herd and is insensitive to sample size, although the larger the sample, the more robust the estimate.

Population-wide measures for the presence of genetic variants and the effective number of alleles provide information about changes in a herd's diversity, now and in the future. Other measures provide a comparison of the herd's genetic similarity to domestic horse breed types. All measures are compared to values derived from domestic horse breeds and to WH&B herds which have already been sampled.

Values below the mean for feral populations are an indication that the WH&B herd may have diversity issues. Herds with observed heterozygosity values that are one standard deviation below the mean are considered at critical risk. For DNA-based (hair) samples this value is 0.66, and the value is 0.31 for blood samples.

Few herds sampled to date show values below the feral mean. Those with values below the feral mean tend to be herds with small population size, which are the most at-risk for inbreeding. There is no specific diversity value that is a clear indicator of imminent inbreeding risk. The lower the genetic variation, the greater the risks for inbreeding-related impacts to the WH&B

herd. Mitigation to address or resolve genetic diversity concerns should be considered using the recommendations in the genetics report as a starting point.

4.4.6.3 Herd Size

A minimum population size of 50 effective breeding animals (i.e., a total population size of about 150-200 animals) is currently recommended to maintain an acceptable level of genetic diversity within reproducing WH&B populations (Cothran, 2009). This number is required to keep the rate of loss of genetic variation at 1 percent per generation. Animal interchange between adjacent HMAs with smaller population sizes may reduce the need for maintaining populations of this size within each individual HMA. Research has not yet established a recommended minimum breeding herd size for burros.

4.4.6.4 Management Actions

If the recommended minimum wild horse herd size cannot be maintained due to habitat limitations (e.g., insufficient forage, water, cover and/or space) or other resource management considerations (e.g., T&E species), a number of options may be considered as part of an appropriate site-specific NEPA analysis to mitigate genetic concerns:

- Maximize the number of breeding age wild horses (6-10 years) within the herd.
- Adjust the sex ratio in favor of males to increase the number of harems and effective breeding males.
- Introduce 1-2 young mares every generation (about 10 years), from other herds living in similar environments.

If wild horse herd size in small, isolated HMAs is so low that mitigation is not feasible, consideration should be given to managing the HMA for non-reproducing wild horses or to removing the area's designation as an HMA through LUP.

4.4.7 Herd Social Structure

Some management actions (e.g., sex ratio adjustments to favor stallions) may affect social structure and herd interactions (e.g., band size).

4.4.7.1 Wild Horses

The social structure of most wild horse herds consists of breeding and bachelor bands. Breeding bands or harems usually consist of a dominant stallion, lead or dominant mare(s), a group of breeding mares, and associated foals and yearlings. Most wild horses breed and foal in the spring of the year. Bachelor bands consist of various aged males that either have not yet established their own harem or have lost their mares. The composition of bachelor bands varies considerably throughout the year. In most breeding bands, male foals leave the band at 1 or 2 years of age and generally join bachelor bands.

Although wild horse bands tend to use the same habitat areas (home ranges) from year to year, they are not territorial and do not defend preferred habitat areas. As a result, bands often graze

and water near each other, and there may be movement of mares between bands. This, in combination with the periodic displacement of the dominant stallion and removal or death of other horses, results in a very fluid social structure in most herds. These factors are beneficial in enhancing genetic diversity.

4.4.7.2 Wild Burros

Wild burros do not form breeding bands. There are no strong individual bonds other than jenny-foal relationships. Wild burros present themselves as single animals, all-male groups, all-female groups, jenny-foal groups, or mixed groups. All of the groups are variable and their composition may change at any time. This loose social structure, where all animals are potential breeding partners, maximizes genetic diversity in small or dispersed burro populations.

Some of the older jacks establish a breeding territory but do not prevent other males from entering this area unless there is an estrous female present. It is common for males to roam freely throughout their habitat and breed upon encountering an estrous female. Large male groups may form in the vicinity of an estrous female, and it is normal for the jenny to have multiple breeding partners.

In dispersed populations in a desert environment, breeding efficiency increases as the population densities increase. As daily temperatures increase and water availability decreases, more and more animals will gather around the remaining available water sources. These areas become important areas for maximizing breeding efficiency. This temporary or seasonal increase in population density increases the chance for males to encounter estrous females. Thus, although breeding occurs year-round, increased breeding and foaling may occur during this period of time.

4.5 POPULATION CONTROLS

The authorized officer may consider a number of population control methods to achieve and maintain WH&B population size within AML.

4.5.1 Natural Population Controls

The rate at which WH&B herd sizes naturally increase or decrease is affected by a number of factors, including the nutritional value of the forage consumed, weather, disease, and predation. Experience gained in managing WH&B and other large herbivores indicates that soil, vegetation, and water resources are almost always severely damaged before these factors negatively affect population growth rates. Human intervention may be necessary when these factors, acting alone or in combination, do not exert sufficient influence to maintain WH&B population size within AML.

4.5.2 Gather and Removal

When the authorized officer has determined that excess WH&B exist, gathers to capture and remove the animals immediately or as soon as possible are required. For additional information, refer to BLM Manual Section 4720 (Removal) and 43 CFR 4720.1, 4740.1 and 2.

4.5.3 Reduce Population Growth Rates

During gather or herd management area planning, the authorized officer should consider a range of alternatives to reduce population growth rates and extend the gather cycle for all wild horse herds with annual growth rates greater than or equal to 5 percent. Alternatives may include but are not limited to: use of fertility control, adjustments in the sex ratio in favor of males, a combination of fertility control and sex ratio adjustment, and management of selected HMAs for non-reproducing wild horses. Additional management alternatives (tools) may be considered in the future, pending further research (see Chapter 8).

4.5.3.1 Use of Fertility Control as a Tool to Slow Population Growth Rates

a. Use of Porcine Zona Pellucida (PZP). There are two forms of the conventional PZP agent:

- The 1-year agent, delivered as a liquid primer injection and follow-up booster one month later; additional boosters must be injected annually by hand or by darting to continue treatment.

- The 22-month agent that includes the same primer shot as the one-year agent as well as a second injection of three time-release pellets (1-, 3- and 12-month pellets) to booster the vaccine over a 12-month period of time.

Foaling rates of 6 percent in Year One, 14 percent in Year Two and 32 percent in Year Three following treatment compared with 54 percent foaling in untreated controls have been reported for PZP-22 (Turner, 2007). Maximum effectiveness is not achieved unless mares are treated during a 3-4 month window prior to foaling. Research has shown that the best time to apply PZP is during the winter gather season (i.e., November-February).

The use of PZP, under an investigational exemption held by the Humane Society of the United States, requires treated mares to be physically marked (freeze branded) or readily identifiable in order to be compliant with FDA requirements. Application is limited to individuals specifically trained to handle, mix, and administer the product. Post-treatment monitoring in accordance with the Standard Operating Procedures (SOPs) is required.

As part of an appropriate environmental analysis, the authorized officer will analyze the use of the 22-month PZP vaccine in all wild horse herds, particularly those where: (1) the annual herd growth rate is greater than 5 percent and (2) the post-gather herd size is 50 animals or greater. Fertility control will be most effective when treatment of 50-90 percent of all breeding-age mares within the herd is possible using application in conjunction with gathers or remote delivery (darting). Our current understanding is that to maximize treatment effects, at least 90 percent of all mares should be treated.

The authorized officer should apply the 22-month PZP vaccine to all release mares when the NEPA analysis supports its use. In herds where sex ratio adjustments are made, fertility control may be implemented in combination with sex ratio adjustments to further reduce population growth rates.

b. Increased Use of PZP. One option to slow population growth rates (and reduce the number of excess wild horses removed) would be to gather selected HMAs and apply PZP-22 every 2 years. Because PZP does not totally eliminate reproduction, some excess horses may need to be removed from treated herds over time. Implementation should generally be limited to HMAs that have had high gather efficiencies (i.e., a high percentage of the actual population is captured). Because 70-90 percent of the breeding-age mares may need to be treated to effectively reduce population growth rates, 80-100 percent of the actual population may need to be captured.

c. Remote Application of PZP. Remote application of the 1-year formulation of PZP is problematic, as it is very difficult to approach most wild horses closely enough to allow darting (i.e., follow-up treatment). Remote application of PZP-22 is not possible at the present time since the pellets must be administered by hand injection.

   Remote application of the 1-year PZP agent may be considered for herds where individual horses can be identified (consistent with FDA requirements) and are approachable. Remote application is limited to individuals specifically trained to administer the product by darting.

4.5.3.2 Adjust Male/Female Sex Ratios

The authorized officer should consider alternatives which would manage WH&B herds for a sex ratio with a female component of less than or equal to 50 percent, as this reduces the population growth rate and extends the gather cycle. See Chapter 4 (4.4.1).

Adjusting sex ratios to favor males is another possible management tool which should be considered when the suppression of herd growth rate is desired. This management option should be considered in HMAs and complexes where the low end of AML is greater than 150 animals. Implementation of sex ratio adjustments is most feasible during maintenance gathers (4-5 years after AML is achieved). Sex ratio adjustments may be accomplished by shifting the overall sex ratio to favor males by (1) releasing greater numbers of stallions post-gather or (2) releasing geldings back to their home range following castration. Adjusting the sex ratio so that males comprise 60-70 percent of the adult herd could be considered.

Herd dynamics may change somewhat with adjustments in sex ratios. An increase in the proportion of stallions may have a greater impact when water resources are limited and bands are more concentrated.

Though additional information is needed, geldings are likely to have fewer impacts on the herd's social structure than would an increase in the proportion of stallions. Based on anecdotal observations, geldings released back to their home range: (1) tend to remain near where they were released (with adequate forage and water), (2) form small bachelor groups rather than join

with a reproducing band, (3) maintain better body condition than the herd average because they are sexually inactive, (4) live longer in comparison to sexually active horses, and (5) were easy to recapture (many have been recaptured and released several times).

Post-treatment monitoring should be conducted to observe behavior of individual animals and the herd during the first breeding season following treatment. Monitoring should be designed to determine whether bachelor stallions or geldings interfere with breeding harems, and whether there is increased competition for forage or water. Monitoring should also be designed to determine if the bachelor geldings form bands or intermix with the breeding population and whether band size changes with a greater proportion of stallions. This monitoring information will be used to determine if sex ratio adjustment is an effective population management technique that should be continued.

If post-treatment monitoring indicates that initial introductions of geldings or a greater proportion of stallions have resulted in negative impacts to breeding harems, mitigation could include removal of the additional stallions or geldings in whole or in part, no further introductions of stallions or geldings during future gathers, or release of a larger proportion of mares during the next gather. The need for post-treatment monitoring will decrease as the effectiveness of sex ratio adjustments as a tool to slow population growth rates is determined.

4.5.4 Manage Selected HMAs for Non-Reproducing Wild Horses

Under the WFRHBA (16 USC § 1333(b)(1)), the authorized officer may determine whether AML should be achieved by removal of excess animals, or if options such as sterilization or natural population controls should be implemented. Consistent with this authority, some selected HMAs may be managed for non-reproducing wild horses to aid in controlling on the range population numbers.

4.5.4.1 Manage HMAs for Non-Reproducing Wild Horses

LUPs should identify the HMAs to be managed for non-reproducing wild horses and the criteria for their selection. Completion of additional site-specific environmental analysis, issuance of a decision, and providing opportunity for administrative review under 43 CFR Part 4.21 may also be necessary.

Actual on-the-ground implementation would be influenced by gather efficiency and it may take several gathers to work toward an HMA with non-reproducing wild horses. Animals would be gathered to the extent possible and sterilized for return to the range, or removed. A safe, effective and humane means to sterilize stallions is castration, but a safe, effective and humane means to sterilize females has not yet been perfected. Therefore, initial efforts should focus on returning sterilized males to the HMA of origin. Sterilized males from HMAs with similar environments may be added as long as population size remains within AML. Care should be taken to ensure they are introduced and located near other animals in areas with good water and forage.

4.5.4.2 Manage Reproducing and Non-Reproducing Wild Horses in Selected HMAs

In selected HMAs with large AMLs, management of both reproducing and non-reproducing wild horses could be considered. As an example, non-reproducing wild horses could be geldings released back to their home range HMA following castration. Post-treatment monitoring and/or mitigation would be conducted as discussed in 4.5.3.2 above.

4.6 PRIORITIES FOR GATHER AND REMOVAL

When removal of excess WH&B from the range is necessary, gathers shall be prioritized in the following order:

- Emergencies
- Court Orders
- Nuisance animals (public health and safety)
- Impacts to threatened, endangered, or sensitive species (TES)
- WH&B located outside the HMA in areas not designated for their long-term maintenance
- Landowner request
- To achieve and maintain population size within AML
- Coordinate gathers across State, District and/ or Field Office boundaries and conduct gathers jointly whenever possible to improve gather efficiency and implementation of other population control measures such as application of fertility control and sex ratio adjustments.

4.7 NATIONAL GATHER SCHEDULE

A National Gather Schedule is developed annually based on the need to remove excess WH&B to achieve or maintain AML and the available funding and facility space. Situations that may require adjustments to the National Gather Schedule are: (1) Emergencies and (2) Escalating Problems.

4.7.1 Escalating Problems

Escalating problems are defined as conditions that deteriorate over time. The key indicator is a decline in the amount of forage or water available for WH&B use, which result in negative impacts to animal condition and rangeland health. Causal factors are normally drought or animal numbers in excess of AML. These situations can be detected in advance and are managed through the normal planning process. See BLM Manual Section 4720.21.

4.7.2 Emergencies

Emergencies generally are unexpected events that threaten the health and welfare of a WH&B population and/or their habitat. Examples of emergencies include fire, insect infestation, disease, or other events of a catastrophic and unanticipated nature. Immediate action is normally required. For some emergency actions, it may be appropriate to use alternative means or

procedures to comply with NEPA (see BLM NEPA Handbook, H-1790-1: National Environmental Policy Act Handbook, Chapter 2.3 Emergency Actions). If timeframes permit, a gather plan environmental assessment should be prepared prior to WH&B removal. If this is not possible, emergency actions should be documented and a report prepared after resolution of the problem. Public notification can be addressed through a press release. See BLM Manual Section 4720.22.

4.8 POPULATION MODELING

Version 3.2 of the Winn Equus population model developed by Dr. Steven Jenkins will be used during gather or herd management area planning to analyze and compare the effects of proposed wild horse management. The model is not designed for use on burros.

The model's primary purpose is to analyze and compare the effects of the proposed action (and alternatives) on population size, average population growth rate, and average removal number. Possible management alternatives or strategies may include, but are not limited to:

- Proposals to gather/remove excess animals, with or without fertility control.
- Proposals involving various gather cycles.
- Proposals to make adjustments in sex ratios.
- Proposals to manage all or a portion of a herd as a non-reproducing population.
- Proposals for gate-cut or selective removal gather.
- No Action.

Another objective of the modeling is to identify whether any of the alternatives would be likely to "crash" the population based on a number of stochastic factors (varying environmental conditions).

Use of the Win Equus population model requires the following:

- **Gather Planning:** Run the model for 10 years and 100 trials; select and display the most typical trial.
- **Herd Management Area Planning:** Run the model for 20 years and 100 trials; select and display the most typical trial.
- **Adjustment of Sex Ratios:** Select 100 percent gather for mares and adjust (decrease) the gather percentage for studs to achieve the desired post-gather sex ratio.
- **Results Analysis:** Drop the highest trial and the lowest trial and analyze and compare the remaining trials.

4.9 HUMANE DESTRUCTION

The BLM authorized officer will euthanize or authorize the euthanasia of a wild horse or burro when any of the following conditions exist:

1. Displays a hopeless prognosis for life.

2. Is affected by a chronic or incurable disease, injury, lameness or serious physical defect (includes severe tooth loss or wear, club feet, and other severe acquired or congenital abnormalities).

3. Would require continuous treatment for the relief of pain and suffering in a domestic setting.

4. Is incapable of maintaining a Henneke body condition score greater than or equal to 3, in its present environment.

5. Has an acute or chronic illness, injury, physical condition or lameness that would not allow the animal to live and interact with other horses, keep up with its peers or maintain an acceptable quality of life consistently or for the foreseeable future.

6. A State or Federal animal health official orders the humane destruction of the animal(s) as a disease control measure.

7. Exhibits dangerous characteristics beyond those inherently associated with the wild characteristics of wild horses and burros.

    a. Dangerous animals are defined as those horses or burros that are unusually aggressive and pose an unacceptable risk of injury to humans or other animals.

    b. Unusually dangerous horses or burros possess characteristics that are not desirable to breed into the wild herd.

    c. It is also reasonable to conclude that an average adopter could not humanely care for the animal.

CHAPTER 5—INVENTORY AND MONITORING

GENERAL

The authorized officer is required to identify, plan, collect and analyze the resource monitoring data necessary to prepare resource management plans, plan amendments, gather plans, herd management area plans or other associated environmental documents through which WH&B management decisions are made.

Inventory (monitoring) shall be completed in order to determine:

- If an overpopulation of WH&B exists and action is needed to remove the excess animals.
- WH&B AMLs; and,
- If AMLs should be achieved by removal of the excess animals or other appropriate means.

Monitoring data is needed to support AML establishment and decisions to remove excess WH&B. Various rulings from the Interior Board of Land Appeals (IBLA) underscore the need to base WH&B management decisions on the results of monitoring.

5.1 HABITAT MONITORING

The primary purpose of habitat monitoring is to collect the resource data necessary to:

- Make a determination of excess animals (i.e., support the need to gather and remove excess wild horses or burros).
- Establish or adjust AML.
- Develop or revise HMAPs.
- Evaluate conformance with Land Health Standards, LUP goals and objectives, or other site-specific or landscape-level objectives.

Collection of habitat monitoring data should be coordinated with other resource programs (e.g., range, watershed, wildlife) to maximize efficiency and minimize duplication.

Appendix 2 (Habitat Monitoring References) provides a list of BLM Technical References that can assist in planning for, and collection and evaluation of monitoring data. BLM Technical References can be found at: http://www.blm.gov/nstc/library/techref.htm

5.1.1 Annual Monitoring

Annual monitoring objectives include:

- Determine whether or not forage and water is adequate to support the animals in a healthy condition through the remainder of the year.
- Assess and evaluate animal condition.

To achieve these objectives, monitoring efforts should focus on the following:

- Evaluate current year's forage production and water flows.
- Evaluate/measure use, map patterns of use and monitor seasonal distribution/movement.
- Evaluate WH&B body condition.

Data collection to accomplish the above could include:

1. Photographs and Field Inspection Notes. Document observations on current year's growing conditions (average, below average or above average precipitation and soil moisture); plant phenology (are plants maturing earlier or later than normal); and forage production and water flows (average, below normal, above normal). When taking photographs, label each photograph with the date and the location to facilitate re-photographing the area in the future.

2. Use Mapping. Map utilization of current year's growth. Where possible, document forage utilization by WH&B in rest pastures or prior to livestock use. Where separation of use by type of animal is not possible, map total utilization at the end of the season. Use the information collected to identify and establish key areas, determine distribution, and seasonal use areas. If mapping utilization of the HMA on an annual basis isn't possible, focus monitoring on measuring utilization at key areas used by WH&B within the HMA on an annual and continuing basis.

3. Utilization. Estimate the proportion of annual forage production used by herbivores (WH&B, domestic livestock, wildlife, and insects).

4. Residual Vegetation or Stubble Height, Woody Species Use or Streambank Alteration. Measuring stubble height, use on woody species, and the degree of streambank utilization occurring annually (especially in rest pastures or prior to livestock use) may also be helpful in documenting resource impacts associated with WH&B use.

5. Grazing Use Records. Summarize the actual grazing use (animal unit months of forage) by livestock, WH&B, and wildlife by unit or pasture for the year.

6. Weather Data. Document information on temperature, precipitation, and growing conditions.

7. Animal Condition. Using the Henneke Body Condition Scoring (BCS) system, document the number of animals seen, where they were observed, and their BCS class. Document average group size (e.g., animals are concentrating in large groups or scattered, small groups, evidence of lameness, or other possible animal health concerns). This information may be used to assist in the evaluation of TNEB within the HMA.

When collected, data should be filed in the 4710-Herd Management case file and cross-referenced to the applicable rangeland management or other resource monitoring files.

5.1.2 Long-Term Monitoring

The objective of long-term monitoring is to determine whether management is resulting in significant progress toward attaining Land Health Standards, other applicable site-specific or landscape-level objectives, or changes in range ecological condition and trend (up, stable or downward). Data are typically collected by an interdisciplinary team of resource specialists in preparation for a formal and detailed evaluation of current management and its effect on resource conditions.

5.1.2.1 Ecological Sites

Rangeland landscapes are divided into ecological sites for the purposes of inventory, evaluation and management. Each site is defined and described by the Natural Resources Conservation Service (NRCS) based on climate, geology, soils, vegetation and other environmental factors. An ecological site is also described based on its ability to produce and support a characteristic plant community (i.e., kind, amount, and proportion of natural vegetation). Ecological site descriptions can be used to evaluate current management and to identify the potential effects in range ecological conditions or trends that could be expected from proposed changes in WH&B management. Ecological site descriptions for each Major Land Resource Area (MLRA) are available from NRCS. If an area has not yet been inventoried by NRCS, an interdisciplinary team of resource specialists will use the best available data to identify the ecological sites in the HMA.

5.1.2.2 Riparian Areas

Properly functioning riparian areas produce abundant forage, important habitats for fish and wildlife, and improve water quality and quantity. Functional-at-risk riparian areas have one or more attributes which place them at risk of deterioration, while nonfunctional riparian areas are unable to capture sediment, enhance infiltration, recharge aquifers or dissipate high energy flows. Assessments of proper functioning condition can be used to identify issues, establish objectives, and propose and evaluate changes in WH&B management.

5.1.2.3 Key Areas

Key areas are selected to monitor changes in range ecological condition or trend across representative areas within the HMA or complex which may result from current WH&B management. Key areas may be selected to represent a particular plant community or a specific ecological site. They should also be selected to represent ecological conditions and trends, utilization or use patterns, seasonal distribution, and resource production within the HMA or complex.

Long-term monitoring within key areas may include measurements of frequency, production, and cover (canopy cover, foliar cover, ground cover and basal cover). Plots are generally read at intervals of 5-10 to 15-20 years.

5.1.2.4 Key Species

Key plant species are selected for forage utilization studies. Key species are important forage species within plant communities that can indicate changes in resource conditions resulting from WH&B management. More than one key species may be selected, depending on management objectives.

Key forage species are used to indicate the allowable degree of forage utilization on a key area which promotes attainment of attaining vegetation management objectives. They may be identified during land use planning, or result from habitat management objectives established during site-specific planning efforts such as herd management area planning.

5.1.3 Integrated (Interdisciplinary) Resource Monitoring Plans

Development of integrated (interdisciplinary) resource monitoring plans can help assure that the data needed to support WH&B management decisions is collected in an effective and efficient manner. Resource monitoring plans should identify the following:

- **Who** (The resource specialty responsible for data collection, i.e., WH&B specialist, range specialist, etc.).
- **What** (Data to be collected).
- **When** (Time of year data should be collected and how often).
- **Where** (Location(s) for data collection, i.e., location and documentation of key areas).
- **Why (**The specific monitoring objective).
- **How** (Methods to be used).

Also refer to Appendix 4 (Table 4.2).

5.2 POPULATION MONITORING

An accurate and current assessment of WH&B health and condition, as well as population size, growth rate, and distribution, is needed for proper WH&B management. At a minimum, population surveys should be conducted every 2 years whenever possible, and within 6-12 months prior to establishing the need to gather and remove excess WH&B.

5.2.1 Population Estimation

WH&B population size should be estimated primarily by aerial survey. See BLM Manual Section 4710.45.

1. Aerial surveys will be conducted and documented using standardized sampling methods and procedures. Survey methods (types of aircraft and techniques) should utilize the most effective and cost-efficient techniques for the specific HMA or complex.

2. To permit comparison with prior data and detect population trends, aerial surveys should be conducted during the same time of the year, using the same or similar type of equipment, methodology, and personnel.

3. The following data should be should be recorded during aerial surveys:

   - HMA name and number
   - State/District or Field Office
   - Survey date
   - Observer(s) name
   - Weather conditions
   - Type of aircraft used
   - Altitude
   - Flight time
   - Time of day
   - Survey method (direct count, simultaneous double-count, mark-resight, etc.)
   - Flight path, number and location of animals seen in GPS/GIS data format
   - Number of adults, number of foals
   - Basic herd health and condition information
   - Basic information about range, forage or water conditions, wildlife, or livestock use, etc.

4. In areas where gather efficiencies have been historically low or WH&B range widely, a post-gather survey should be conducted in the 6-12 months following gather completion to obtain a more accurate estimate of the number of WH&B left on the range.

5. Monitoring WH&B distribution is critical in identifying seasonal use areas, determining movement patterns and identifying areas for habitat monitoring. While seasonal distribution may vary over the years, movement is normally related to the abundance and quality of the forage, climate, weather patterns, and availability of water.

6. The HMA monitoring file shall contain a permanent record of data collected during the aerial surveys and the results should be entered into the Wild Horse and Burro Program System (WHBPS) and the local GIS data base.

5.2.2 Herd Condition and Health Monitoring During Gathers

The condition class of individual horses should be evaluated and documented during gathers using the Henneke Body Condition Scoring System (refer to H-4760-1: Compliance Handbook).

1. Data should also be collected on age structure and sex ratio. This information can be used to evaluate the effects of the management actions on herd health and condition.

2. Estimate the number of WH&B left on the range (number remaining ungathered) following each gather.

3. Other data that may be collected during gather operations include: parasite load, disease (from blood samples), percentage of pregnant mares (through analysis of blood or fecal samples), and/or the effects of fertility control.

4. The incidence of albinism, parrot mouth, club feet, severely crooked legs or other physical deformities should also be documented and the subject animals removed from the population when possible. The incidence of these traits should be minimized within individual herds over time.

CHAPTER 6—HERD MANAGEMENT AREA PLANNING

GENERAL

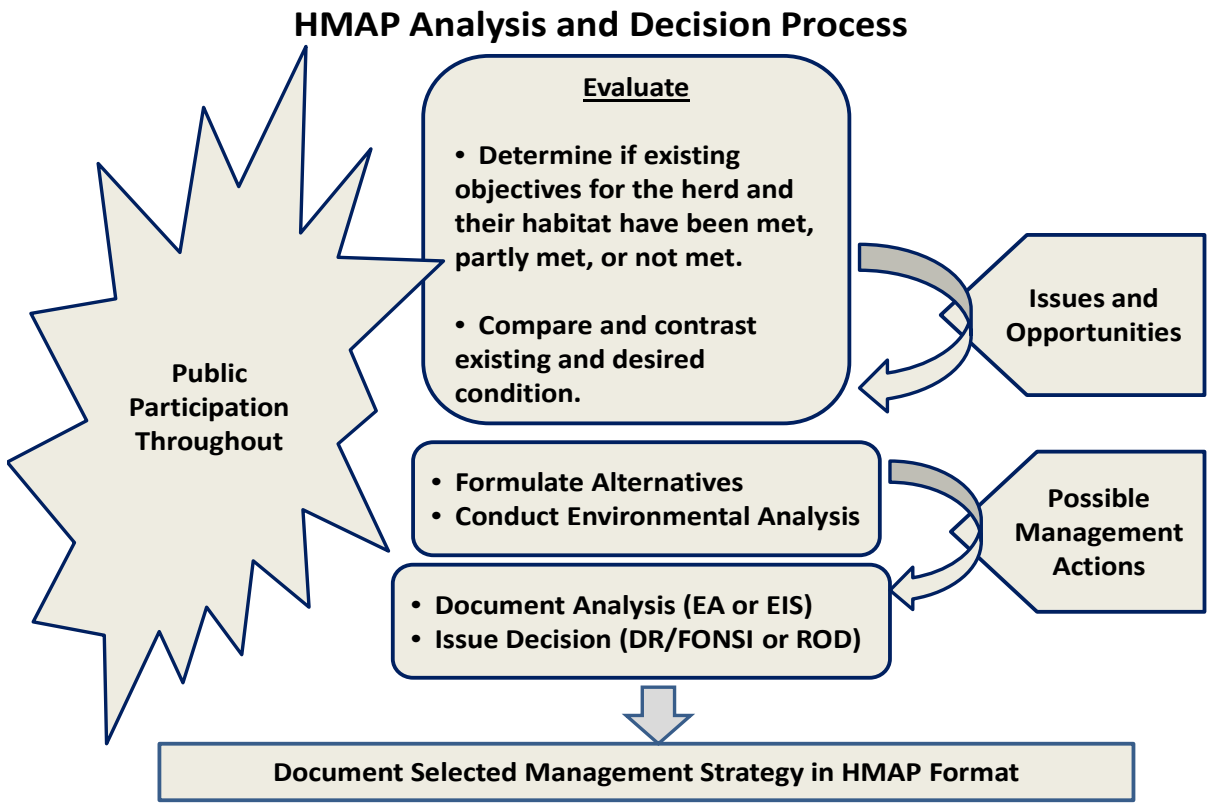
Herd Management Area Plans (HMAPs) identify and set objectives for WH&B herds and their habitat. HMAPs are prepared with public involvement through a site-specific environmental analysis and decision process (NEPA). During the NEPA process, the environmental impacts associated with a range of alternative management strategies for the WH&B herd and its habitat is analyzed.

The approved HMAP outlines the selected management actions, together with the management and monitoring objectives which, when implemented, would make progress toward achieving land health standards, LUP goals/objectives, and other relevant objectives.

6.1 HMAP ANALYSIS AND DECISION PROCESS

An overview of the HMAP analysis process is provided in Figure 6.1. Also refer to Appendix 4 (Herd Management Area Planning) for additional information.

Figure 6.1
Overview of the HMAP Analysis and Decision Process



6.1.1 Evaluate Current Management

The first step in the HMAP analysis process should generally be to evaluate existing management. At the conclusion of the management evaluation, a report will be prepared and made available to the public for a 30-day review and comment period (public scoping). To facilitate public review, the document will be posted on the state, district or field office web page.[1] The management evaluation report is not a decision subject to administrative appeal.[2] Rather, the report documents the key issues identified as a result of the management evaluation. A range of the possible management alternatives (Proposed Action and Alternatives) that would address or resolve the identified issues may also be identified. The range of alternative may be expanded as a result of scoping comments.

Key steps in the management evaluation process include:

1. Review existing goals and objectives for the herd and its habitat and determine whether these have been met, partly met, or not met. Incorporate a summary of the rationale supporting BLM's determination in the evaluation report.

2. Compare and contrast the existing and desired condition for the herd and its habitat. Refer to the information in Chapter 3 (Habitat Management) and Chapter 4 (Population Management) as a starting point for the analysis.

3. Document the gaps (or differences) between the current and desired conditions. These differences represent the issues to be addressed and resolved (i.e., the purpose and need for action).

4. Identify the possible management actions that could be implemented in response to the identified issues.

5. Formulate a range of alternative management strategies for the herd and its habitat from the list of possible management actions (Proposed Action and Alternatives). Each alternative considered should meet the purpose and need for action and respond to the identified issues in whole or in part.

6. Finalize the management evaluation report and provide it to the public for a 30-day review and comment period (i.e., public scoping).

6.1.2 Conduct and Document the Site-Specific Environmental Analysis

As a next step, conduct and document the interdisciplinary, site-specific analysis of potential impacts that could result from implementation of the No Action (generally defined as "Continue

[1] If an Environmental Impact Statement (EIS) is prepared, scoping will be conducted following issuance of a Federal Register Notice.

[2] Once the HMAP decision is made, the Evaluation Report is part of the administrative record for that decision.

Existing Management" for HMAP development and analysis), the Proposed Action, and other action alternatives. Refer to H-1790-1: National Environmental Policy Act Handbook.

Though it may be appropriate in some instances to prepare an Environmental Impact Statement (EIS), normally an environmental assessment (EA) is prepared to document and analyze an HMAP decision. The section below provides guidance about how an HMAP EA may be structured.

6.1.2.1 Preparing the HMAP Environmental Assessment (EA)

1. Introduction. Limit the information included in the "Introduction" section of the EA to the minimum needed to allow the reader to understand the existing situation. Include a brief description of the HMA and its location, and the AML (and when/how it was established). Describe the purpose and need for the Proposed HMAP. Identify the relevant issues, summarize the results of public scoping, and document how the BLM used the comments in preparing the preliminary environmental assessment. Tier the analysis to applicable decisions in the LUP.

2. Proposed Action and Alternatives. Each alternative considered in detail in the EA shall meet the purpose and need for action and respond to the identified issues to varying degrees. The range of alternatives considered will include a No Action Alternative (i.e., Continue Existing Management) and the Proposed Action (Proposed HMAP). Other alternatives should be developed in response to the purpose and need and the identified issues, as appropriate.

   Each alternative will describe the strategy to be used to manage the herd and its habitat over the long-term, together with the associated management, monitoring and implementation objectives. In formulating the alternative management strategies, various management tools may be considered to protect, control and manage WH&B populations and their habitat. For example, consider differing gather seasons or methods, various tools to slow population growth, or various habitat improvement tools, projects or techniques.

3. Management Objectives and Actions. Management objectives and actions may differ by alternative. This section of the EA would summarize the objectives and actions specific to each alternative.

   For each alternative specify: (a) the proposed habitat and population management objectives, (b) the associated management actions (e.g. implementation objectives), and (c) the proposed monitoring objectives. These objectives and actions should conform to and be consistent with LUP goals.

   a. SMART Objectives. Well-written objectives should be specific, measurable, achievable/attainable, reasonable/relevant and trackable within a specified timeframe (SMART):

1) Specific: Specifies what and where.
2) Measurable: Identifies the amount of change expected; desired change can be measured or observed.
3) Achievable/Attainable: Realistic for the specific site conditions.
4) Reasonable/Relevant: Limited in scope, within the BLM's control and influence, measures results (not activities).
5) Trackable/Timeframe: Identifies where the activity will occur, short- and long-range targets, as well as interim steps and a plan to monitor progress.

b. Habitat Objectives. Habitat objectives may involve vegetation, trend or key area objectives for upland vegetation or riparian plant communities within the HMA or complex or objectives to maintain or improve the wild, free-roaming behavior of the population. Site-specific objectives to improve habitat conditions (e.g., forage or water) may also be established.

Examples of SMART Objective(s)

- ❒ Improve riparian condition from functioning at-risk (FAR) in 1998 to properly functioning condition (PFC) by decreasing utilization from heavy to moderate within a ½ mile circumference of Bird Spring.
- ❒ Adjust sex ratios from 40 percent studs and 60 percent mares in 2010 to 60 percent studs and 40 percent mares by 2014.

c. Population Objectives. These objectives would establish a framework for management of the WH&B herd over the longer term. Objectives could include when and how AML would be adjusted in the future, or when and how the population within the HMA would be gathered. Among other population management parameters, objectives may be identified for desired age structure and sex ratio, animal condition, phenotype, genetic diversity, population growth rate, or selective removal criteria. These objectives may be based on historical attributes or other management considerations.

d. Monitoring Objectives. As necessary, identify a monitoring objective that would measure the progress made toward achieving proposed habitat or population management objectives.

e. Implementation Objectives (Management Actions). Implementation objectives are management actions that, when implemented, are expected to make progress toward attaining the proposed habitat or population management objectives. These could include application of fertility control, adjustment of sex ratios to favor males, or other actions to slow population growth, management actions to assure genetic diversity or achieve the desired sex ratio or age structure for the WH&B herd, or range improvement projects.

4. Description of the Affected Environment and Environmental Impacts

   a. General Description of the Affected Environment. This section of the EA should include a concise general description of the HMA or HMA complex. Voluminous material may be included in the EA appendix. Maps, tables, charts or graphs may also be useful in summarizing or displaying relevant information. Information could include:

      1) General information about the HMA or complex's size, location, acres of public land or other land ownership, general description of topography, elevation, climate, and dominant vegetation.

      2) A summary of the WH&B herd's history using best available data. This could include the probable origin of the WH&B population, identification of the general type of WH&B historically occupying the HMA, any interchange between this herd and adjacent HMAs, known genetic diversity (observed heterozygosity or Ho) and any introductions of WH&B from another HMA.

      3) A brief discussion of any historic adjustments that have been made in the HA/HMA boundary, and the name and date of the decision document(s) used to initiate those adjustments. Maps or descriptions of HA/HMA boundaries may also be included.

      4) Initial forage allocations together with the existing AML range. The name and date of any decision document(s) used to establish or adjust AML along with a brief description of the causal factors leading to the change.

      5) Any population and habitat management actions or events that have impacted herd size including scheduled, emergency or nuisance animal gathers, application of fertility control, and the occurrence of catastrophic events such as extended drought or wildfire.

      6) A concise list of applicable LUP decisions that affect the HA/HMA, including any constraints on WH&B distribution and management (voluminous information can be included in an appendix to the EA).

      7) A list of applicable Land Health Standards (these may be included in an appendix to the EA or an internet address can be provided if they are available on the internet).

      8) Brief descriptions of other resource values and uses relative to the management of WH&B, as appropriate. This could include: authorized and/or actual livestock use, TES species and their habitat, Wilderness, Wilderness Study Areas (WSAs), etc.

b. Description of the Affected Environment and Environmental Impacts. For each element of the human environment that has the potential to be affected by the Proposed Action or alternatives, describe the existing situation (affected environment). Also describe the direct and indirect impacts to these resources that could result from implementation of the Proposed Action and Alternatives.

5. Cumulative Impacts. The National Environmental Policy Act (NEPA) regulations define cumulative impacts as impacts on the environment that result from the incremental impact of the Proposed Action when added to other past, present, and reasonably foreseeable future actions, regardless of what agency or person undertakes such actions (40 CFR 1508.7). Cumulative impacts can result from individually minor but collectively significant actions taking place over a period of time.

   In assessing the cumulative impacts associated with preparation of a proposed HMAP, first define the area of potential effect—cumulative impacts will be evaluated within the area of potential effect.

   Next define the issues and resource values identified during scoping that are of major importance (in accordance with the 1994 BLM *Guidelines for Assessing and Documenting Cumulative Impacts*, the cumulative impacts analysis should be focused on only those issues and resource values identified during scoping that are of major importance).

6. Public Review and Comment. The HMAP environmental assessment shall be made available to the public for a 30-day review and comment period by posting on the state/district or field office web page. See H-1790-1 for instructions if an EIS has been prepared.

7. Finalize the HMAP EA. Soliciting public comments obligates reviewers to give fair consideration to the input received. Summarize the results of public review and comment and document how the BLM used the comments in finalizing the environmental assessment.

6.1.2.2 Prepare the Decision Record and Finding of No Significant Impact (FONSI) or an EIS and Record of Decision (ROD)

Based on the analysis of potential environmental consequences, the authorized officer will determine whether to prepare an EIS or issue a Decision Record (DR) and "Finding of No Significant Impact" (FONSI). A FONSI documents why implementation of the selected alternative will not result in environmental impacts that significantly affect the quality of the human environment. The DR identifies the authorized officer's final decision and is a separate and subsequent document to the FONSI. See H-1790-1 for additional information.

If an EIS has been prepared, due to the potential for significant environmental impacts, the authorized officer's final decision will be documented in a Record of Decision (ROD).

6.1.2.3 Prepare the HMAP

As a final step and to facilitate on-the-ground implementation, document the selected management strategy, together with the habitat and population management, monitoring and implementation objectives in HMAP format. The HMAP shall be included as an attachment to the authorized officer's final decision.

The approved HMAP should include the following components:

- **Introduction.** Briefly introduce or summarize the relevant background information (repetition of detailed information incorporated in the NEPA document is not required in the approved HMAP).

- **Management Strategy.** Summarize the selected management strategy as outlined in the EA together with the site-specific habitat and population objectives and management actions established to accomplish the objectives.

- **Monitoring and Evaluation Plan** (refer to 6.1.2.3.1).

- **Tracking Log/Project Implementation Schedule** (refer to 6.1.2.3.2).

6.1.2.3.1 Resource Monitoring Plan/Schedule

The HMAP shall include a resource monitoring plan and schedule that will be used to facilitate the timely and effective completion of habitat and population monitoring. The plan should identify the specific monitoring item, the methodology to be used, the resource specialty needed to complete the monitoring (e.g., field office WH&B specialist, rangeland management specialist, wildlife biologist, etc.), where and when monitoring should occur, as well as any actions to be taken to adjust or adapt management as needed and appropriate pending monitoring results. The plan should include both habitat and population monitoring components. See Appendix 4 (Table 4.2).

6.1.2.3.2 Tracking Log/Project Implementation Schedule

The HMAP will include a tracking log and project implementation schedule to facilitate and monitor plan implementation. The tracking log may be developed in a tabular format, and will list the selected habitat and population management actions (what), the entity responsible for implementation (e.g. who -- BLM, USFS, volunteer groups, etc), and define where, when, and how often the action is to be completed. Columns to document the date the action was completed and/or implemented and for any remarks (e.g. project numbers from BLM's Rangeland Improvement Project system, etc) should also be included. See Appendix 4 (Table 4.3).

6.2 HMAP MONITORING AND EVALUATION

Resource (habitat and population) monitoring data should be analyzed periodically to determine whether minor adjustments in management are needed (adaptive management). Periodic evaluations are conducted to assure HMAP implementation is on track and to gauge progress toward achieving the selected habitat and population management and other relevant objectives. Refer to the process outlined in 6.1.1.

The goals for HMAP monitoring and evaluation are twofold: (1) to track implementation of the management actions/decisions outlined in the HMAP (implementation monitoring); and (2) to collect the data/information necessary to evaluate the effectiveness of those decisions (effectiveness monitoring).

1. Implementation Monitoring. Implementation monitoring tracks implementation of the selected management actions. It answers the question "Did we do it?" To assure selected management actions are being implemented in a timely manner, annual review is recommended.

2. Effectiveness Monitoring/HMAP Evaluation. Plan evaluation is the process of periodically reviewing the HMAP to determine if it is effective in making progress toward or accomplishing the approved habitat and population management objectives (plan evaluation typically occurs at intervals of 5, 10 or 15-20 years). Effectiveness monitoring answers the question "Did it work?"

   The HMAP shall also be evaluated to determine: (1) whether management goals and objectives are still appropriate or need to be revised, and (2) whether progress is being made toward achieving the goals and objectives, or additional management actions are needed (adaptive management). Evaluation of the HMAP could also be completed in conjunction with land health assessment.

   Evaluations that highlight the need for substantial changes may require consultation with interested public as well as appropriate NEPA analysis and documentation prior to implementation.

6.3 4710 CASE FILE

A 4710-Herd Management Area file shall be established for individual HMAs or complexes. The case file should include (but is not limited to) the following:

- A copy of the HMAP.
- HMA and HA maps.
- The associated NEPA and BLM decision documents (or a cross-reference to those documents).
- Any correspondence pertaining to HMA management.
- Aerial survey results.

- Field inspection reports and monitoring data (or a cross-reference to the applicable rangeland management or other case files).
- Information on habitat improvement projects (or a cross-reference to the applicable rangeland management or other case files).
- Final gather reports.
- Fertility control reports, if applicable.
- Results from periodic HMAP evaluations.

Additional 4710 case files may be created if the number and type of monitoring studies is large and varied (e.g. 4710-1, 4710-2, etc).

Relevant data and information should also be entered in a timely manner into WHBPS, as appropriate.

HA/HMA maps and a record of the HA/HMA history should be included in the 4710 HMA file. However, the HA and HMA boundaries delineated in the State GIS data base and provided to the National Coordinator for upload into the National Data Set serve as the official record. Any future changes to HA or HMA boundaries should be documented in the State GIS data base as well as in the HMAP file.

CHAPTER 7—DECISIONS AND APPEALS

7.1 DECISIONS

Implementation decisions form the basis for approval of on-the-ground management actions. When implemented, these actions further the incremental progress toward achieving LUP goals and objectives, Standards for Land Health, or other site-specific habitat or population management objectives. If proposed management is not in conformance with the LUP, the proposal shall be modified or rejected, or the LUP shall be amended or revised.

WH&B implementation decisions can include:

- Establishing or adjusting AML
- Developing HMAPs
- Population management actions
- Habitat improvement projects

Program-specific procedures must be followed when issuing a decision. WH&B implementation decisions can be made in a variety of ways and administrative review (appeal) requirements may vary based on the decisions made (Figure 7.1).

Figure 7.1
Decisions and Appeals Process

An Overview

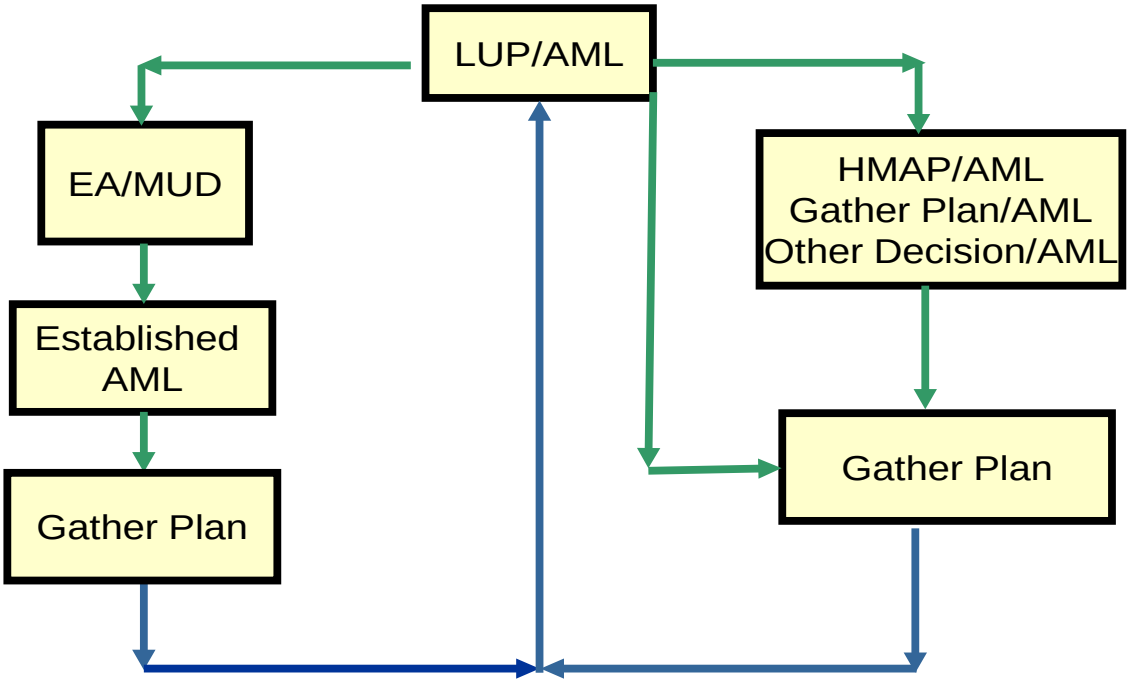
Authority	43 CFR 4770.3(c)	43 CFR Part 4.21
Decision Type	Decisions to Gather/Remove Excess WH&B	AML, HMAP or Project Decisions
Effective Date	• *Emergency Removal Decisions:* make effective upon issuance. • *TNEB Removal Decisions:* make effective on a date specified in the decision *(*e.g., 31-76 days prior to proposed gather start).	Effective the day after the appeal period expires (i.e., 31 days) unless a petition for stay is filed.
Administrative Review Timeframe	Appeals and petitions for stay must be filed within 30 days of the date of the decision.	
Implementation Timeframe	Unless a petition for stay is granted, the gather and removal may proceed as outlined in the authorized officer's decision.	The authorized officer's decision may be implemented immediately if the Appeals Board either denies the petition for stay or fails to act on it within 45 calendar days of the expiration of the time for filing a notice of appeal.

* AML decisions issued as MUDs are heard by a OHA/ALJ and are not implementable until OHA has ruled.

7.1.1 AML Decisions

AML decisions determine the maximum number of WH&B to be managed in the HMA that results in a TNEB and avoids a deterioration of the range. Decisions to establish or adjust AML or establish a population range if the AML was initially set as a single number, can be made in a number of ways (refer to Figure 7.2).

Figure 7.2
AML Decisions



AML decisions are generally implementation decisions as compared to LUP decisions. All AML decisions must meet the following criteria:

1. AML is set based on in-depth analysis of site-specific resource monitoring data including grazing utilization, trend in range condition, actual use and climatic factors (i.e., AML is not set based on administrative convenience or single surveys); and

2. Guidelines and criteria for adjusting herd size are included. Refer to *Dahl v. Clark*, 600 F. Supp. 585 (D. Nev. 1984); *Animal Protection Institute*, 117 IBLA, 208, 219 (1990); and *Animal Protection Institute*, 109 IBLA 112, 118 (1989).

AML decisions may also be made through:

- Issuance of WH&B (single-use) decisions.

- Issuance of multiple-use decisions (MUDs, which allocate forage for domestic livestock, wildlife and WH&B).
- As a part of HMAP decisions.
- As part of gather/removal plan decisions.

AML is not generally established or adjusted as part of the gather planning (NEPA) process due to the in-depth and complex nature of the analysis required. Complexity also increases if allocation of the available forage for use by wildlife, livestock and WH&B will be made concurrently (e.g., MUDs).

If the authorized officer elects to formally review AML as part of the same environmental document which evaluates the proposed removal, the AML decision should be separated from the gather/removal decision. This may be accomplished by issuing separate Decision Records:

1. Gather/removal decision.
2. AML decision.

Separation of the two decisions is desirable because there are different rules governing the timing and appeals process for those decisions:

- Under 43 CFR 4770.3(c), the authorized officer can make decisions to remove wild horses or burros from public or private lands effective upon issuance or on a date specified in the decision, where removal is needed to preserve or maintain a thriving ecological balance and maintain a multiple use relationship.

- The authorized officer does not have similar authority with respect to issuing AML decisions, which, if no petition for stay is filed, become effective on the day after the expiration of the appeals period. See 43 CFR § 4.21(a)(2).

7.1.2 Gather/Removal Decisions

Prior to removing WH&B from public lands, the authorized officer must make a determination, based on current information, that excess animals are present and their removal is necessary to restore a TNEB and multiple-use relationship. BLM's authority to remove excess animals from public lands is found in 16 USC § 1333(b)(2) of the WFRHBA (as amended). This provision requires the BLM to immediately remove the animals upon determination that excess WH&B exist, so as to achieve appropriate management levels, restore a thriving natural ecological balance, and protect the range from the deterioration associated with the overpopulation.

In making the determination that excess WH&B are present and require immediate removal, the authorized officer will analyze current information including grazing utilization and distribution, trend in range ecological condition, actual use, climate (weather) data, current population inventory, WH&B located outside the HMA in areas not designated for their long-term maintenance and other factors which demonstrate removal is needed to restore or maintain the range. Justifying a removal based on nothing more than the established AML is not acceptable.

7.1.2.1 Environmental Analysis (Gather Plans)

The following tools can be used to focus the discussion during the site-specific environmental analysis conducted for Gather Plans:

1. Limit the analysis to that needed to determine the potential impacts associated with the Proposed Action and Alternatives, including No Action. Refer to H-1790-1 for a detailed discussion of the appropriate components of a NEPA analysis.

2. Tier the analysis to LUPs, HMAPs, or other relevant decision documents, as appropriate.

3. Incorporate previous analysis or decisions by reference (e.g., reference to previous AML decisions, HA/HMA boundary decisions, etc), as appropriate.

These tools allow reference to the information without the need to either include the documents in whole or in part, or to restate the information in voluminous detail.

7.1.2.2 Decision Record (Gather Plan)

Unless an emergency situation exists, gather/removal decisions shall be issued 31-76 days prior to the proposed gather start to provide an opportunity for administrative review of the authorized officer's decision to be completed. The Decision Record (DR) for Gather Plans that are documented in an environmental assessment should:

1. Summarize the substantive comments received and describe how BLM used these comments to finalize the environmental assessment. See 7.2 for additional information.

2. In accordance with 43 CFR 4770.3(c):

   - The authorized officer will issue gather decisions effective upon a date established in the decision in situations where removal is required by applicable law, or is necessary to preserve or maintain a thriving natural ecological balance and multiple use relationship.

   - When an emergency situation exists, and timeframes permit, a Gather Plan EA should be prepared and the decision should be made effective upon issuance. See BLM Manual Section 4720.36.

3. Cite the regulatory authority upon which the decision is based.

4. Explain the timeframe and procedures for filing an appeal.

5. Describe the procedures for requesting a stay of the action under 43 CFR 4.21.

7.1.3 Other Management Decisions

Other management decisions may include:

- Approval of HMAPs that establish site-specific population or habitat objectives or actions.
- Project-specific decisions (i.e., water development construction or reconstruction, etc.).

These decisions are made following site-specific environmental analysis (NEPA) with public involvement and are subject to administrative review (appeal) under 43 CFR 4.21.

7.1.4 Actions Excluded from Further NEPA Documentation

Certain actions may be categorically excluded from NEPA analysis (refer to H-1790-1, Chapter 4, Categorical Exclusions). Other actions may have already been adequately analyzed through NEPA and a Determination of NEPA Adequacy (DNA) may be prepared.

Before conducting a new NEPA analysis for a proposed Gather Plan, existing NEPA documentation should be reviewed to determine if it is adequate (i.e., review previous Gather Plan EAs together with the associated Decision Records and Findings of No Significant Impact). Changes in numbers of WH&B since the previous gather that result in changes in forage utilization, use patterns, and/or ecological conditions and trends, or changing environmental conditions such as drought, wildfire, noxious weed infestations, and others, may require that a new NEPA analysis be conducted.

If the existing NEPA documentation appears to be adequate, consult with the Office of the Solicitor to determine whether the issuance of a DNA may be appropriate.

7.2 PUBLIC INVOLVEMENT

The authorized officer may conduct public scoping. See H-1790-1, Chapter 6.3.

The authorized officer will provide the public 30 days to review and comment on the NEPA document, typically an Environmental Assessment that documents and analyzes the environmental effects of the BLM's Proposed Action.

The authorized officer shall make Gather Plan EAs and DNAs available to interested individuals, groups, and agencies for a 30-day review and comment period, except when an emergency situation exists. The NEPA document(s) identified in the DNA (e.g., the EA and Finding of No Significant Impact (FONSI)) will also be made available to the public for information.

The authorized officer should consider substantive comments and summarize how they were addressed in the NEPA document or DNA for the Gather Plan. This summary should be presented in the NEPA document, the DNA, or the decision document. See H-1790-1, Chapter 6.9 for additional information.

Possible options for responding to substantive comments include:

- Modifying one or more of the alternatives.
- Developing and evaluating additional alternatives.
- Supplementing, improving, or modifying the analysis.
- Making factual corrections.
- Explaining why the comments do not warrant further agency response, citing cases, authorities or reasons to support the BLM's position.

When an EIS has been prepared, follow the guidance provided in H-1790-1, Chapter 9.

7.3 APPEALS (ADMINISTRATIVE REVIEW)

Any party who is adversely affected by a final decision of the authorized officer may file an appeal and request a stay of the action. WH&B decisions are subject to administrative review (appeal) and remedies under the provisions of 43 CFR 4.21, 4.410, and 4770.3.

As a general matter, WH&B decisions will not become effective during the time in which a person adversely affected may file a notice of appeal. Unless a petition for a stay pending appeal is filed together with a timely notice of appeal, these decisions will become effective on the day after the expiration of the time during which a person adversely affected may file a notice of appeal (generally 31 days from the decision date). If a petition for stay pending appeal is filed together with a timely notice of appeal, a decision will become effective immediately if:

- The stay request is denied; or,
- The Interior Board of Land Appeals fails to act on the petition for a stay within 45 days from the date of the decision.

Under certain circumstances, however, the authorized officer may make decisions to gather/remove excess wild horses or burros effective upon issuance or on a date specified in the decision. See 43 CFR 4770.3(c).

Under the authority provided in 43 CFR 4770.3 (c), the authorized officer may make decisions to gather/remove excess wild horses or burros effective upon issuance or on a date specified in the decision.

When issued under this authority, the gather may be implemented unless a stay is granted or a Federal Court enjoins the gather.

7.3.1 AML Decisions

AML decisions are subject to administrative review:

1. When administrative review of multiple-use decisions (MUD) is requested, BLM may ask to combine the wild horse decision with the grazing decision for review by an administrative law judge (ALJ) in the Office of Hearings and Appeals (OHA).

2. Wild horse decisions are subject to administrative review by the Interior Board of Land Appeals (IBLA) in accordance with 43 CFR 4.21 and 4.410.

7.3.2 Gather/Removal Decisions

The authorized officer has the authority to make decisions to gather and remove excess WH&B effective upon issuance or on a date specified in the decision when removal is court ordered or is needed to preserve a thriving natural ecological balance and multiple use relationship.

Any party who is adversely affected by the authorized officer's final decision may file an appeal and/or request a stay of the action with the Interior Board of Land Appeals (IBLA). The appeal and/or request for stay must be filed within 30 days from the date of the decision and in accordance with the regulations found at 43 CFR 4.411(a).

7.3.3 HMAP or Other Management Decisions

AML, HMAP, or other management decisions are subject to administrative review through the IBLA under provisions of 43 CFR 4.21 and 4.410. Under this authority, decisions **cannot** be implemented effective upon issuance or on a date specified in the decision.

A notice of appeal must be filed in the office of the authorized officer within 30 days from the date of the decision. See 43 CFR 4.411.

CHAPTER 8—RESEARCH

GENERAL

Direction to conduct research is contained in the WFRHBA, 16 U.S.C. § 1333(b)(2)(C)(3), and the Strategic Plan for Management of Wild Horses and Burros on Public Lands (1992). In late 2000, the Midcontinent Ecological Science Center of the U. S. Geological Survey (USGS) was charged with developing a strategic research plan for the management of WH&B. The purpose of the strategic planning process was to:

- Review past progress and identify problems that could be addressed with research.
- Set broad research goals central to the BLM's mission for WH&B management.
- Establish specific, time-bound, measurable research goals, and strategies to achieve them.
- Evaluate the progress towards those goals at set time periods, and to readjust the planning as needed.

There had been no previous prioritization of the BLM's WH&B research and management needs, and no effort to develop a strategy for fulfilling those needs within a specified time period. The strategic planning process was designed to fill this void.

8.1 STRATEGIC RESEARCH PLAN

The Strategic Research Plan for Wild Horse and Burro Management identified research priorities to include health and handling, fertility control, population estimation and modeling, genetic conservation, habitat assessment and setting population goals. The components of fertility control and population estimation have been implemented and research is being conducted. Genetic diversity of WH&B herds is being evaluated. Other research projects may be initiated as needed to support the management of WH&B. Research results will be used to improve management practices within the WH&B program.

8.1.1 Development

The Strategic Research Plan was a joint effort between the USGS, the BLM, the Animal and Plant Health Inspection Service (APHIS), and the U.S. Department of Agriculture (USDA). The plan was developed over a period of 2 years with input from 39 subject area experts representing 11 universities, 3 Federal agencies (BLM, USGS, APHIS), and two State wildlife agencies.

The USGS took the lead role in planning and coordinating meetings of the expert committees and in drafting the Strategic Plan based on committee and agency input. Assisting in this effort were the BLM's National WH&B Research Coordinator and equine health experts from APHIS.

Principal input from the USGS was focused on topics of contraception, aerial population estimation, population modeling, and genetics. Principal input from APHIS was focused on topics of disease and animal health monitoring and surveillance.

Advice and input has also come from BLM including managers and specialists assigned to the WH&B program, the National WH&B Advisory Board, and WH&B staff in the Washington Office. Advice and input was also provided by the BLM Director's Science Advisory Committee, and from seven topic-specific advisory panels that were convened by the USGS.

8.2 RESEARCH REQUESTS

A National Research Advisory Team exists to review research proposals, monitor research project progress, update the Strategy as necessary, and provide recommendations to the WH&B Division Chief. The Research Advisory Team has been formed with equal participation by the BLM (BLM's National WH&B Research Coordinator), the USGS, and APHIS.

All requests for research and/or research proposals pertaining to the Wild Horse and Burro program, whether generated from the field or through field-related contacts, are to be directed to the Research Advisory Team for review. This Team is also available to assist field offices with the development of research proposals. Proposal format and submission should be coordinated through the National WH&B Research Coordinator. Proposals will be reviewed on an as-needed basis and recommendations for funding will be made directly to the WH&B Division Chief.

8.3 RESEARCH PROJECTS

8.3.1 Fertility Control

An effective, safe, and cost-effective fertility control agent is an essential tool in the management of wild horses. A Fertility Control Field Trial Plan has been developed and field research is underway for two components of the Plan: (1) Individual-Based Trials and (2) Population-Based Trials. Over the last decade, BLM research efforts in fertility control have been focused on PZP (Porcine zona pellucida) and the development of time-release pellets to extend the effectiveness of PZP. PZP has proven effective in reducing foaling rates, the population impacts of which are presently being determined.

8.3.2 Other Possible Fertility Control Tools

Other possible fertility control tools that could potentially be considered in the future include:

- Spaying mares.
- Vasectomizing studs.
- Use of IUDs.
- Use of GonaCon™.
- Use of SpayVac™.
- Use of other fertility control agents or sterilants.

8.3.2.1 Spaying (Mares)

Spaying mares involves major abdominal surgery, is risky, and requires good post-operative care. Spaying mares could be considered in the future if safe, effective and humane surgical methods and post-operative care procedures can be perfected for use on wild horses.

8.3.2.2 Vasectomies (Stallions)

Performing vasectomies on stallions is not a widely practiced procedure within veterinary medicine. Post-vasectomy, it is expected that studs would retain their stud-like behavior. By contrast, gelding studs (castration) is a routine veterinary procedure in both domestic and wild horses. However, geldings lose their stud-like behavior after a few months.

Vasectomized studs would be expected to continue to cover mares and keep them in a harem, but eventually most mares would be bred by an intact stallion. If this continued over several estrous cycles it could lead to extended foaling seasons and potentially an increase in foal mortality. Research shows that at 15-33 percent of foals are sired by non-harem stallions, making it unlikely that fertility control focused on males would be effective in slowing population growth (Bowling and Touchberry 1990, Kaseda and Kahlil 1996, Asa 1999). Further research is needed to perfect a safe technique for performing vasectomies in stallions and demonstrate whether this approach will reduce population growth rates.

8.3.2.3 Use of IUDs

Pilot studies using coil-type intrauterine devices (IUDs) and glass balls or marbles as IUDs have failed to demonstrate a long-lasting effect on conception in mares. In both instances, mares "slipped" the devices and became pregnant soon thereafter. The application of IUDs is further complicated by the difficulties associated with identifying a time window for application when mares are not pregnant.

8.3.2.4 Use of GonaCon™

GonaCon™ is an experimental fertility control vaccine that is being developed for potential use as a management tool for deer. Tests of the GnRH vaccine are ongoing in several States and countries, involving a wide range of wildlife and feral species, including horses. A research study conducted with estray horses in Nevada suggests GonaCon™ will reduce foaling rates for 1 year following treatment. At present, the effectiveness of GonaCon™ as a fertility control agent beyond one year appears similar to or less than that of PZP-22, suggesting limited potential for use of the product to reduce population growth rates over a longer period of time.

8.3.2.5 Use of SpayVac™

SpayVac™ is an experimental fertility control vaccine using PZP antigens and a novel liposome technology. It is easy to handle and administer. A single vaccination with SpayVac™ has maintained a high level of contraception throughout a 4-year study with estray horses in Nevada. However, there is currently no regulatory approval for the management or investigational use of

SpayVac™ through the EPA or FDA, and the product is not currently commercially available. SpayVac™ may have potential for use as an effective, longer-lasting fertility control agent in the future and could offer an alternative to spaying mares. However, additional research would be needed before it could be used on a population-management basis.

8.3.3 Population Estimation and Setting Population Goals

Aerial surveys rarely detect all WH&B within an HMA. As a result, population estimates must be developed using correction factors to account for WH&B not identified during the census. At present, research is being conducted on two techniques (simultaneous double-count and photo mark-resight) that will allow use of such correction factors.

8.3.4 Other Research

In the future, research may be conducted in other areas identified as research priorities, depending on need and funding. When other research needs are identified or research projects are proposed, they will be reviewed and evaluated by the National WH&B Research Advisory Team and recommendations will be made to the WH&B Division Chief.

GLOSSARY OF TERMS

Activity Plan – Examples of activity plans include Herd Management Area Plans (HMAPs) and Allotment Management Plans (AMPs). These plans lead to implementation decisions that usually describe multiple projects or management actions and apply best management practices to make progress toward attainment of Land Use Plan (LUP), Land Health Standards, or other resource objectives.

Adaptive Management – WH&B management is adjusted as indicated based on the results of monitoring and evaluation.

Adult Wild Horse – WH&B 1 year of age and older are considered adults. A foal is considered 1 year of age on January 1 of the year following its birth.

Adult Breeding Population - Wild horses within a population that are 1 year of age and older.

Aerial Survey – A method of counting WH&B using an aircraft. Aerial survey allows the BLM to obtain estimates of WH&B population size with associated confidence intervals.

Animal Unit (AU) - Wild horses 1 year of age and older count as 1 AU and burros 1 year of age and older count as 0.5 AU.

Animal Unit Month (AUM) – The amount of forage (approximately 800 pounds of air dried forage) necessary to sustain one adult horse or two burros for one month.

Appropriate Management Level (AML) - The number of adult horses or burros (expressed as a range with an upper and lower limit) to be managed within an HMA. Forage for WH&B (AUMs) is allocated based on the AML upper limit.

AML Range – The number of adult WH&B within which herd size will be allowed to fluctuate.

AML Upper Limit – The maximum number of WH&B that results in a thriving natural ecological balance (TNEB) and avoids a deterioration of the range. This number should be somewhere below the number that would cause damage to the range (refer to 118 IBLA 75).

AML Lower Limit – The number that allows the population to grow to the AML upper limit over 4-5 years, without the need for gathers to remove excess WH&B in the interim.

Authorized Officer – Any employee of the BLM to whom authority has been delegated to perform the duties described in this Handbook (refer to BLM Manual 1203 for a further explanation of delegation of authority).

Census – A complete count of the total WH&B population within an area. Because aerial surveys do not usually detect all WH&B within an HMA, a true census is usually not possible. Better population estimates are developed using correction factors to account for WH&B not seen during the survey (i.e., sightability correction factors).

Considered Comparably - During the LUP process, WH&B are considered in the same manner as other resource values (e.g., cultural, historic, scenic, rangelands, timber, and minerals).

Emergency – An unexpected event that threatens the health and welfare of a WH&B population and/or its habitat. Examples include fire, insect infestation, disease or other events of a catastrophic and unanticipated nature.

Escalating Problems - Conditions that deteriorate over time. The key indicators are a decline in the amount of forage or water available for WH&B use, with negative impacts to rangeland health and, ultimately, animal condition. Causal factors are normally drought and/or animal numbers in excess of AML. These situations can be detected in advance and are managed through the normal planning process.

Evaluation (LUP Evaluation) – The process of reviewing the land use plan and periodic plan monitoring reports to determine whether the decisions and NEPA analysis are still valid and whether the plan is being implemented.

Evaluation (HMAP Evaluation) – Periodic review of herd management area plans (HMAPs) to determine if the selected management practices were implemented as planned (answers the question "Did we do it?") and whether or not the plan is effective in making progress toward established habitat and population management objectives (answers the question "Did it work?").

Excess Animals – Wild, free-roaming horses or burros which have been removed or which must be removed from in order to preserve and maintain a thriving natural ecological balance and multiple-use relationship in an area.

Fertility Control – A tool to decrease fertility and which, when implemented, reduces (slows) population growth rates and extends the gather cycle.

Free-Roaming – WH&B are able to move without restriction by fences or other barriers within a HMA.

Goal – A broad statement of a desired outcome.

Genetic Diversity – The absence of inbreeding depression as monitored through an established baseline and periodic reassessment. This represents an expression of the genetic health of an individual animal or the population.

Herd (Reproducing) – One or more stallions and their mares and foals.

Herd Area (HA) - Geographic areas of the public lands identified as habitat used by WH&B at the time the WFRHBA was enacted (12/15/1971).

Herd Management Area (HMA) – May be established in those HAs within which WH&B can be managed for the long term. HMAs are designated through the LUP process for the

maintenance of WH&B herds. In delineating each HMA, the authorized officer shall consider the appropriate management level (AML) for the herd, the habitat requirements of the animals, the relationships with other uses of the public and adjacent private lands, and the constraints contained in 43 CFR 4710.4.

Herd Management Area Plan (HMAP) – An HMAP establishes management actions and short- and long-term management and monitoring objectives for a specific WH&B herd and its habitat. HMAPs assist the authorized officer in tracking progress toward achieving LUP goals. They are prepared under authority of 43 CFR 4710.3-1. An HMAP can be prepared for an individual HMA or a complex of HMAs.

Implementation Decision – Decisions that make progress or take action toward achieving LUP goals and objectives.

Implementation Plan – Refer to Activity Plan above.

Land Use Plan (LUP) – LUPs provide (by tracts or areas) for the use of public lands. LUPs are prepared in accordance with established land use planning procedures in 43 CFR 1600 and pursuant to FLPMA. They establish goals and objectives (desired outcomes), identify the management actions needed to achieve the desired outcomes, and identify the allowable uses of the public lands.

Manage Principally but not Exclusively – An HMA may be considered for designation as a wild horse or burro range when there is significant public value present, such as unique characteristics in a herd or an outstanding opportunity for public viewing. These HMAs may be managed principally, but not exclusively, for wild horse or burro herds.

Minimal Feasible Level of Management - The minimum number of habitat or population management tools or actions necessary to attain the objectives identified in approved LUPs and HMAPs for a HMA or HMA complex.

Monitoring – The process of collecting and analyzing the data necessary to evaluate existing management and determine whether progress is being made toward attaining established habitat and population management goals and objectives.

Non-Reproducing Wild Horses – An HMA composed, in whole or in part, of sterilized wild horses (either stallions or mares) to aid in controlling on the range population numbers.

Objective(s) – A description of the desired outcome of a management decision. Objectives are specific, measurable, achievable/attainable, reasonable/relevant, and progress can be quantified within established timeframes for achievement (SMART).

Plan Monitoring – The process of tracking land use plan implementation, including collecting and assessing the data or information necessary to evaluate the effectiveness of land use planning decisions.

Population Growth Rates – Represent the net effect of births and deaths in any given year. The BLM reports annual population numbers as of February 28th.

Range – The amount of land necessary to sustain an existing herd or herds of wild free-roaming horses and burros, which does not exceed their known territorial limits.

Resource Management Plan – see LUP above.

Self-Sustaining – The ability of reproducing herds of wild horses and burros to maintain themselves in a healthy condition and to produce healthy foals.

Standards for Land Health – Standards are statements of physical and biological condition or degree of function required for healthy sustainable rangelands. Achieving or making significant progress towards these functions and conditions is required of all uses of public lands. Historical data, when available, should be utilized when assessing standards.

Standard Operating Procedures – The Bureau follows standardized methods of operation which have been developed over time to assure the safe, effective, and humane handling and treatment of WH&B.

Thriving Natural Ecological Balance (TNEB) – WH&B are managed in a manner that assures significant progress is made toward achieving the Land Health Standards for upland vegetation and riparian plant communities, watershed function, and habitat quality for animal populations, as well as other site-specific or landscape-level objectives, including those necessary to protect and manage Threatened, Endangered, and Sensitive Species.

Utilization – The portion of annual forage production that has been consumed by herbivores (WH&B, domestic livestock, wildlife, and insects). The term is also used to refer to the pattern of such use.

WH&B Range – A HMA which meets the special criteria defined in H-1601-1, Land Use Planning Handbook. These include unique herd characteristics, viewing opportunities, unique landscape, significant public demand or other reasons. WH&B Ranges are managed principally, but not necessarily exclusively, for WH&B.

Wild Free-Roaming Horses and Burros – All unbranded and unclaimed horses and burros that use public lands within 10 contiguous Western States as all or part of their habitat, or that have been removed from these lands by the authorized officer, or have been born of wild horses or burros in authorized BLM facilities, but have not lost their status under the WFRHBA (16 USC § 1332 (f)).

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Kaseda, Y., and A.M. Khalil. 1996. Harem size and reproductive success of stallions in Misaki feral horses. Applied Animal Behaviour Science 47:163–173.

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Turner, J.W., Jr., I.K.M. Liu, D.R. Flanagan, A.T. Rutberg, and J.K. Kirkpatrick. 2007. Immunocontraception in wild horses: one inoculation provides two years of infertility. Journal of Wildlife Management 71(2):662–667.

APPENDIX 1 - Genetics Data and Hair Sample Collection Instructions

Analysis of DNA to determine genetic diversity of wild horse and burro (WH&B) herds is currently being done using hair samples rather than blood samples. Unless there is a previously recognized concern regarding low genetic diversity in a particular herd, it is not necessary to collect genetic information at every gather. Typical herds should be sampled every other gather. A new baseline does not need to be established through hair analysis if blood analysis has already been completed. Please follow the instructions below for collecting hair samples. Call BLM's National WH&B Research Coordinator if you have any questions.

While it is preferable to sample release horses, you may sample removed horses if necessary. In complexes or HMAs where separate breeding populations are suspected, WH&B in each distinct population should be sampled separately. Do not mix samples from different horses or different breeding populations. The minimum sample size is 25 animals or 25 percent of the post-gather population, not to exceed 100 animals per population. Samples should be collected from males and females in the same approximate ratio as the population. Animals of any age class may be sampled. Burros should be sampled in the same manner as horses.

1. You will need one plain white paper envelope (a white #10 business envelope works best) for each horse. DO NOT use plastic or zip-lock bags and DO NOT use plastic coated envelopes or envelopes with windows in them.



2. Hair samples must be obtained by pulling the hair out by the root NOT cutting or shaving it off the horse. This is because the DNA is in the root follicle not the hair itself. Mane hair will work, but for foals or young horses you may need to obtain tail hair. Please submit about 30 hairs per animal. A bundle of 30 hairs is about the diameter of a pencil.



The easiest way to pull a good sample is to grasp a bundle of hair and wrap it around a clean mane comb or hoof pick. Holding the bundle close to the neck, pull **straight out** firmly. Foal hair is more brittle and tends to break off. If you are having trouble getting hair with the root attached try obtaining a tail hair sample instead.



(James Sturdivant – The Jockey Club, modified with permission)

3. Check that you have the hair roots or hair bulbs attached to the hair at the base. They look, and feel like little bumps on the end of each hair.

Keep the hair in a loose bundle pointed in one direction or twist it together and place it in an envelope. You can cut off excess hair and leave only a few inches with the hair root attached to put in the envelope if that is easier.

4. Seal the envelope and **write the sample number on the envelope.**

Also write the sample number, color, sex and age of the horse on the data collection sheet. If animals cannot be aged in years, at a minimum record adult, yearling or foal. Be sure to keep stray hairs out of the comb and off your clothes so they don't contaminate the next sample.

Please NOTE: it is best to sample when the hair is dry. If you need to sample when it is raining or the horses are wet, then DO use zip-lock bags for each sample AND keep the samples cool not frozen (refrigerate, then ship the samples with cold packs).

Please fill out the top of the form completely, including the HMA name, HMA number and the date the sample was collected.

Within 3 days after hair collection, send a copy of the data collection form to:

Attn: Research Coordinator, National Program Office-Reno

FEDEX the samples, to arrive on a weekday not the weekend **with the Data Collection Sheet to Dr. Gus Cothran, address below:**

Dr. E. Gus Cothran
VIBS, CVM
TAMU4458
Texas A&M University
College Station, TX 77843
Phone (979) 845-0229

For questions or comments, contact: **National Program Office @ 775-861-6583**.

HAIR SAMPLE GENETICS DATA COLLECTION SHEET
WILD HORSE AND BURRO HERD GENETICS EVALUATION

PAGE _____ OF _____

HMA: _____ HMA #: _____

DATE: _____

TRAP/SUBGROUP: _____

Field Office:_____ STATE:_____

BLM CONTACT PERSON: _____

PHONE: _____

ADDRESS:
_____

EMAIL: _____

SAMPLED HORSES WERE (circle) RELEASED or REMOVED

	SAMPLE #	COLOR	SEX	AGE	COMMENTS
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					

15					
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25					

APPENDIX 2 - Habitat Monitoring References

BLM Technical References can be found at http://www.blm.gov/nstc/library/techref.htm
A list of references which may be helpful follows:

1. Biological Soil Crusts: Ecology and Management, TR-1730-2, Interagency, 2001.

2. Inventory and Monitoring, Ecological Site Inventory, TR-1737-7, BLM, 2001.

3. Measuring and Monitoring Plant Populations, TR-1730-1, 1998

4. National Range Handbook, Handbook, H-4410-01, 1990.

5. Rangeland Inventory and Monitoring: Selected Bibliography of remote Sensing Applications, TR-4400-9, BLM, 1986)

6. Rangeland Health Standards, Handbook 4180, BLM, 2001.

7. Interpreting Indicators for Rangeland Health, Version 4, TR 1734-6, Interagency, 2005.

8. Rangeland Monitoring and Evaluation, TR-4400-1, BLM 1988.

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11. Rangeland Inventory and Monitoring: Supplemental Studies, TR-4400-5, BLM, 1992.

12. Rangeland Monitoring: Analysis, Interpretation, and Evaluation, TR-4400-7, BLM, 1984.

13. Riparian Area Management, Grazing Management for Riparian Areas, TR-1337-4, BLM, 1989.

14. Riparian Area Management, Management Techniques for Riparian Areas, TR-1737-6, BLM, 1992

15. Riparian Area Management, Process for Assessing Proper Functioning Condition, TR-1737-9, Interagency, 1990.

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18. Riparian Area management, A Guide to Managing, Restoring, and Conserving Springs in the Western Unites States, TR-1737-17, BLM, 2001.

19. Riparian Area Management, Riparian Wetland Soils, TR-1737-19, Interagency, 2003.

20. Statistical Considerations in Rangeland Monitoring. (See TR 4700-8, 1986)

21. Sampling Vegetation Attributes, TR-1734-4, 1996.

22. Utilization Studies and Residual Measurements, TR-1734-3, Interagency, 1996.

APPENDIX 3 - AML Establishment and Adjustment Process

AML decisions determine the number of WH&B to be managed within an HMA or complex of HMAs. AML is expressed as a population range with an upper and lower limit. The AML upper limit is the number of WH&B which results in a TNEB and avoids a deterioration of the range. The AML lower limit is normally set at a number that allows the population to grow to the upper limit over a 4-5 year period, without any interim gathers to remove excess WH&B. See Chapter 4.

A multi-tiered analysis process should be used to establish and adjust the appropriate management level (AML) of WH&B.

- ❒ **Tier One**: determine whether the four essential habitat components (forage, water, cover and space) are present in sufficient amounts to sustain healthy WH&B populations and healthy rangelands over the long-term.

- ❒ **Tier Two**: determine the amount of sustainable forage available for WH&B use.

- ❒ **Tier Three**: determine whether or not the projected WH&B herd size is sufficient to maintain genetically diverse WH&B populations (i.e., avoid inbreeding depression).

Should the Tier One analysis determine that one or more of the essential habitat components is insufficient to maintain a healthy WH&B population and healthy rangelands; the authorized officer should consider amending or revising the LUP to remove the area's designation as an HMA.

Tier 1

Determine if the four essential habitat components (forage, water, cover, and space) are present in sufficient amounts to sustain healthy WH&B populations and healthy rangelands over the long-term. In making this determination, the most limiting factor(s) within the HMA should be considered. In some HMAs, the most limiting factor may be:

- The water available for WH&B use.
- The limited habitat available for WH&B use on either the summer or winter range, in HMAs with seasonal ranges.
- The naturally occurring, low productive capability of the dominant ecological sites.
- The low ecological status of key WH&B use areas.
- Low annual rainfall or extended periods of drought.

The essential habitat components must be located on public lands within the HMA boundary. If forage or water located on private lands within the HMA is needed to maintain healthy WH&B populations, a written agreement with the private landowner allowing use by WH&B is required. In the absence of private landowner agreement, the forage and water on private lands is not available for use by WH&B and may not be included when establishing or adjusting AML.

Forage

The amount of forage available for WH&B use is expressed in animal unit months (AUMs). One AUM is defined as the amount of forage necessary to sustain one adult horse or two adult burros for one month (or approximately 800 pounds of air dry forage). Twelve AUMs of forage are needed to support one wild horse or two adult burros for one year.

As a **starting point**, the amount of sustainable forage available for WH&B use may be determined based on the results of utilization monitoring and use pattern mapping for the years evaluated. A determination of forage availability should not be based on the results of only one year's monitoring data. A minimum of three to five years of monitoring data is preferred. This is because forage production can vary substantially from year to year based on the timing and amount of precipitation received, among other factors. Under the same level of grazing, use pattern mapping may indicate light to moderate utilization during above normal precipitation years, yet indicate heavy or severe utilization during below normal precipitation years.

The nature of the forage (i.e., perennial vs. annual) should also be considered. While annual forage may be available to support WH&B use during years with normal or above normal precipitation, it may be greatly reduced during below average precipitation years. As a result, annual forage is not typically used to support or justify WH&B numbers within a HMA. Within perennial ranges, the production of perennial species may also be greatly reduced during drought, and the production of annual grasses and forbs may be largely absent.

The flexibility to remove WH&B in below average forage years, or to move the animals to another area, can be constrained by funding, contractor capability, facility space, or animal behavior. To prevent range damage or adverse impacts to animal health, the upper limit of AML for WH&B should be established in consideration of the most limiting forage (or water) production years.

Other Considerations

1. Situations in which the Land Health Standards are achieved but population inventory indicates more WH&B are present than expected. These situations may indicate additional forage is present to support use by higher numbers of WH&B, wildlife, or livestock, consistent with LUP guidance.

2. Situations in which deteriorated land health conditions exist but population inventory indicates the number of WH&B is the same or lower than expected. These situations may indicate there is less forage available to support the existing use by WH&B, wildlife, or livestock, and that a reduction in the allowable use may be needed, consistent with LUP guidance.

The number of WH&B which can be sustained based on the available forage is determined in **Tier Two.** To determine if there is sufficient forage to sustain long-term WH&B:

- Analyze utilization data, use pattern mapping, and/or production, ecological site condition (ESI), trend, frequency, precipitation (weather), and indicators of land health.

- Determine the AUMs of actual use by WH&B for each of the evaluation years. (Calculate the AUMs of actual WH&B use based on population estimates derived from aerial surveys. You can also project actual use by WH&B using previous population estimates together with herd's average annual population growth rate.)
- Identify key WH&B use areas (distribution). Calculate carrying capacity on the basis of the key use areas (primary range).

Water

The amount of water available for WH&B use is generally based on public, natural waters (i.e., water occurring on private lands is not considered unless a written agreement with the private landowner is obtained). Water availability during drought conditions is also considered. Sufficient water for WH&B must be available during drought to achieve and maintain a thriving natural ecological balance and multiple use relationship on the public lands.

In determining the amount of water available for WH&B use, a thorough inventory of the available public, natural water resources is needed. Developed and man-made waters should also be inventoried if they are available for WH&B use (i.e., BLM holds the necessary water rights and WH&B access to the water can be depended upon over the long-term). The water resources inventory should include the name, location, and flow (in gallons per minute or cubic feet per second). Wild horses require a minimum of 10 gallons of available water per animal per day (15 gallons per day is not unusual during the hot, dry summer months) while burros require about 5 gallons available per animal per day. The analysis of available water should also be based on the most limiting season of the year (i.e., generally summer when flows are reduced).

Cover and Space

A recurring pattern of WH&B movement out of the HMA to access forage, water, or thermal or hiding cover is an indication the HMA cannot sustain year-long WH&B use. If one or more of the key habitat components is missing, the HMA should be considered as unsuitable for year-long use by WH&B. In these situations, the authorized officer should consider amending or revising the LUP to remove the area's designation as an HMA. An exception would be two or more HMAs which adjoin and are managed as a complex of HMAs, or HMAs which adjoin Forest Service wild horse territories (WHT) that can be managed as a complex.

The results of the **Tier One** analysis may be summarized in a simple table (see example Table 3.1 below). The table should be followed by a detailed description of the analysis and the rationale used in making the determination that a key habitat component either is (or is not) sufficient to support healthy WH&B populations and healthy rangelands over the long-term.

Table 3.1
Sufficient Forage, Water, Cover and Space

HMA	Forage		Water		Cover		Space	
	Sufficient	Insufficient	Sufficient	Insufficient	Sufficient	Insufficient	Sufficient	Insufficient
HMA 1	X			X	X		X	
HMA 2		X		X	X		X	
HMA 3	X			X	X		X	

Tier 2

Determine the AUMs of sustainable forage available for WH&B use. Based on the desired level of utilization, propose an AML for WH&B (expressed as a range with an upper and lower limit).

Forage availability should be determined based on in-depth analysis of rangeland monitoring data. As a first step, monitoring data should be reviewed to determine if Land Health Standards or other site-specific vegetation management objectives are being met. If standards and objectives are being met, WH&B population estimates are then examined to determine the range in number of WH&B using the HMA during the evaluation years. The upper values are used to establish AML when no land health issues occur.

In HMAs where Land Health Standards or other objectives are not being achieved, WH&B use should be examined to determine if this level of use has contributed to or is the causal factor for the standards or objectives not being met (or only partly met). When standards or objectives are not being met and WH&B use is a contributing (or causal) factor, AML is proposed based on the estimated number of WH&B present relative to the level of forage utilization that is occurring (i.e., AML would be established at a number below that which has contributed to the standards or objectives not being met). The need for frequent emergency removals of WH&B due to lack of forage and/or water or the emigration of WH&B out of the HMA due to population size or concentration levels may also be a consideration in proposing AML for WH&B.

The sustainable forage (carrying capacity) available for WH&B use within a HMA is determined pending detailed analysis of utilization data and use pattern mapping for all users. For each evaluation year, determine the following: (1) weighted average utilization, (2) potential carrying capacity, and (3) the proposed carrying capacity. An example for the Mojave Desert ecosystem follows.

Example – Determining Sustainable Forage Available for WH&B Use (Mojave Desert Ecosystem)

The following example can be used to assist with establishing and/or adjusting AML for WH&B use. To determine if there is sufficient forage to sustain long-term WH&B use, analysis of utilization data, use pattern mapping, and/or production, ecological site condition, trend, frequency or other indicators of rangeland health is necessary.

Selection of Allowable Use Criteria

The recommended allowable utilization by all herbivores under year-round use within the Mojave Desert ecosystem is 25-35 percent of the current year's forage production.

WH&B use will be limited to 25 percent of current year's production on key vegetation species as measured at seed dissemination for these species.

Rationale: Within the Mojave Desert ecosystem, allowable use levels of 25 percent are recommended for areas in poor ecological condition or for areas grazed during the growing season. Allowable use levels of 35 percent are recommended for areas in good ecological condition or for areas grazed during the dormant season. Allowable use is established at 25 percent for this HMA due to year-round grazing by WH&B as well as a number of areas in poor ecological condition.

Determine Weighted Average Forage Utilization

Deriving a weighted average utilization from use pattern maps generally provides a more representative picture of the forage use that has occurred throughout the HMA and leads to a more accurate estimate of carrying capacity. To derive a weighted average utilization for the HMA, determine the number of acres within the HMA which received moderate, heavy or severe utilization for each evaluation year.[3] Refer to BLM Technical Reference 4400-7 for additional information.

The weighted average use is calculated by multiplying the number of acres by the mid-point of the utilization category (example: moderate use is classified as 41-60 percent, therefore, 50 percent is the mid-point used in the calculation).

Data used in the example carrying capacity analysis outlined below includes: utilization data, use pattern mapping data, actual livestock use information, and WH&B population estimates.

[3] To protect rangeland health over the long-term, consider WH&B use only on the primary range when deriving a weighted average utilization. Primary range is defined as the areas which animals (WH&B) prefer to use when management is limited (refer to Glossary of Terms Used in Range Management, 4th Edition, Published by Society for Range Management, 1998). Secondary range is defined as range which is not ordinarily used until the primary range has been overused. Therefore, areas receiving slight or light use or that are unused should not be considered when deriving a weighted average utilization.

Weighted Average Utilization =

(Moderate use acres*50 percent) + (Heavy use acres*70 percent) + (Severe use acres*90 percent)

Analyze the total acres of moderate, heavy, and severe use.

1995 $\frac{(19639*50\text{ percent}) + (34351*70\text{ percent})}{53{,}990}$ = 63 percent

1996 $\frac{(8023*50\text{ percent}) + (48957*70\text{ percent}) + (9885*90\text{ percent})}{66{,}865}$ = 71 percent

1997 $\frac{(3081*50\text{ percent}) + (22888*70\text{ percent})}{25{,}969}$ = 68 percent

1999 $\frac{(5627*50\text{ percent}) + (3696*70\text{ percent})}{9{,}323}$ = 58 percent

2000 $\frac{(4344*50\text{ percent}) + (4386*70\text{ percent})}{8{,}730}$ = 60 percent

2002 $\frac{(5627*50\text{ percent}) + (3696*70\text{ percent})}{9{,}323}$ = 58 percent

2004 $\frac{(4395*50\text{ percent}) + (7933*70\text{ percent})}{12{,}328}$ = 63 percent

The example below demonstrates the impact of variable precipitation (and forage production) on actual forage utilization and the desired carrying capacity.

Potential Carrying Capacity =

Average use in AUMs (of cattle or horse use)*25 percent Allowable Use Objective
Weighted Average Utilization

Year	Average Use (AUMs)		Effective Precipitation
1995	3000 AUMs*25 percent / 63 percent	= 1190 AUMs	Above Average
1996	600 AUMs*25 percent / 71 percent	= 211 AUMs	Severe Drought
1997	444 AUMs*25 percent / 68 percent	= 163 AUMs	Severe Drought
1999	600 AUMs*25 percent / 58 percent	= 259 AUMs	Severe Drought
2000	1440 AUMs*25 percent / 60 percent	= 600 AUMs	Slightly Below Average
2002	1632 AUMs*25 percent / 58 percent	= 703 AUMs	Average
2004	3576 AUMs*25 percent / 63 percent	= 1419 AUMs	Well Above Average

Proposed Carrying Capacity =

In the example above, the carrying capacity would be set in consideration of the driest years in order to prevent damage to the range or adverse impacts to animal health.

211 (1996) +163 (1997) +259 (1999) +600 (2000) +703AUMs (2002) = 387 AUMs
5 (# years of data)

Proposed AML =

387 AUMs (Desired Carrying Capacity) = 32 cattle or wild horses
12 months

Or:

$$\frac{\text{387 AUMs (Desired Carrying Capacity)}}{\text{6 months}} = 65 \text{ burros}$$

***Discussion*:**

- Allocation of the forage between WH&B, wildlife and domestic livestock would be made after site-specific NEPA analysis is completed.

- In the example above, the upper limit of the AML range for wild horses was calculated by dividing the proposed carrying capacity for the HMA (expressed in AUMs) by 12 months.

- The upper limit of the AML range for burros was calculated by dividing the proposed carrying capacity for the HMA by 6 months (an adult burro is equivalent to only 0.5 AU).

- The lower limit of the AML range would be determined based on the herd's average annual growth rate (i.e., the lower limit will normally be set at a number that allows the herd to grow to the upper limit over a 4-5 year period without the need for gathers to remove excess WH&B in the interim).

Tier 3

Determine whether or not the WH&B herd size proposed in Tier Two is sufficient to maintain genetically diverse WH&B populations (i.e., avoid inbreeding depression). To avoid inbreeding depression in wild horse populations, a minimum herd size of 50 effective breeding animals (a total population size of about 150-200 animals) is recommended. An effective breeding size for burro herds has not yet been determined.

If the herd size proposed in Tier Two is not sufficient to maintain genetically diverse WH&B populations, determine if there is WH&B interchange between the HMA and other adjacent HMAs or WHTs and whether this interchange would be sufficient to maintain genetic diversity (avoid inbreeding depression). Genetic diversity baseline or monitoring information can be used to evaluate whether WH&B interchange between HMAs is occurring.

If the proposed herd size is less than 150 animals and the HMA is isolated with limited potential for WH&B egress/ingress, possible management actions which could be considered as part of an appropriate site-specific NEPA analysis could include:

1. Removing the area's designation as an HMA through LUP and returning it to HA status.
2. Maximizing the number of breeding age WH&B in the herd (animals age 6-10 years).
3. Adjusting the sex ratio to favor males to encourage formation of additional breeding harems.
4. Introducing 1-2 young mares from outside the HMA every generation (about every 10 years). Introduced animals should come from herds living in similar environmental conditions.

Documentation

The results of the multi-tiered analysis should be documented in an HMA (AML) Evaluation Report. The completed report should be provided to the affected and interested public for a 30-day review and comment period (e.g., public scoping). The management evaluation report is not a decision subject to administrative appeal. However, once the decision is made, the Evaluation Report is part of the administrative record for that decision.

Following public scoping, a site-specific environmental analysis should be completed to analyze the environmental impacts associated with the Proposed AML and any alternatives. As part of the environmental analysis, summarize the results of public scoping and document how BLM used the comments in preparing the environmental assessment (or EIS).

Based on the analysis of potential environmental impacts, the authorized officer will determine whether to prepare an Environmental Impact Statement (EIS) or issue a Decision Record and Finding of No Significant Impact (FONSI). A FONSI documents why implementation of the selected AML will not result in environmental impacts that significantly affect the quality of the human environment. Or, if an EIS has been prepared, the authorized officer's decision will be documented in a Record of Decision.

Administrative Review

AML decisions are subject to administrative review (appeal) pursuant to 43 CFR Part 4.

APPENDIX 4 - Herd Management Area Planning

Herd management area plans (HMAPs) establish the management actions and the short and long-term management and monitoring objectives for WH&B herds and their habitat. Once established with public involvement through site-specific NEPA analysis and decision, the objectives guide management of the HMA or complex over the life of the plan.

Management Evaluation

Typically, the first step in the HMAP analysis process should be to evaluate existing management. The existing vs. the desired condition of the both the herd and its habitat are examined during the evaluation. Refer to Figures 4.1 and 4.2.

Figure 4.1
Habitat Management Evaluation

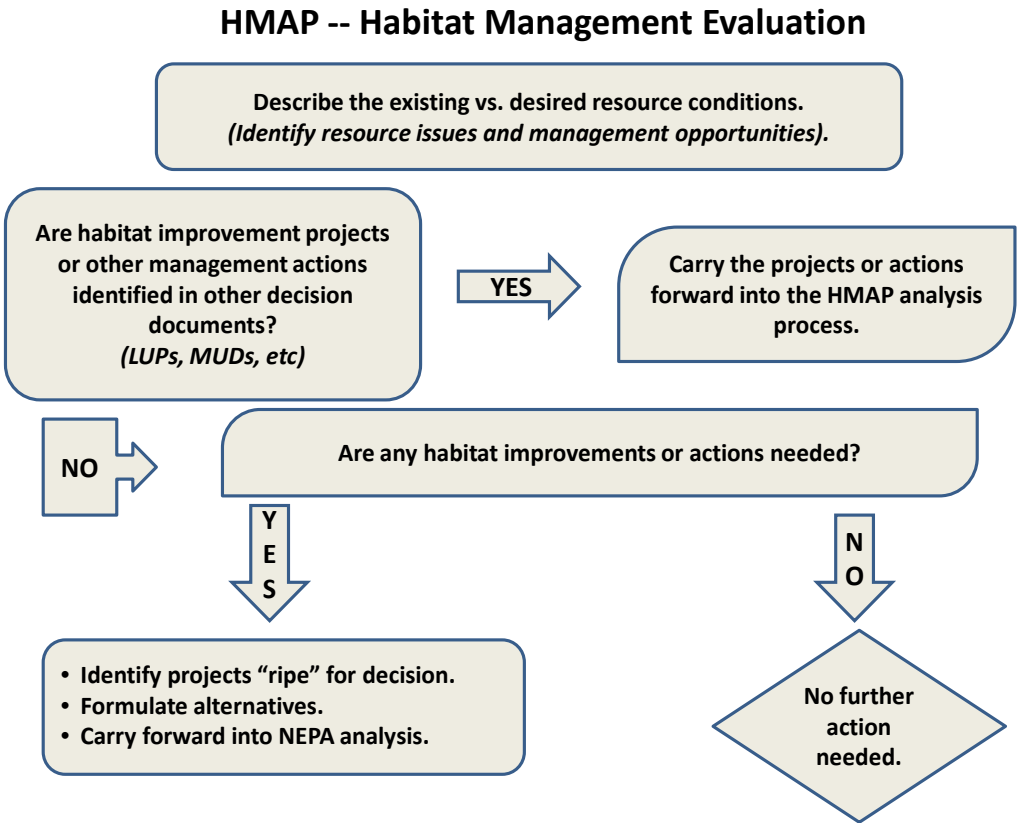
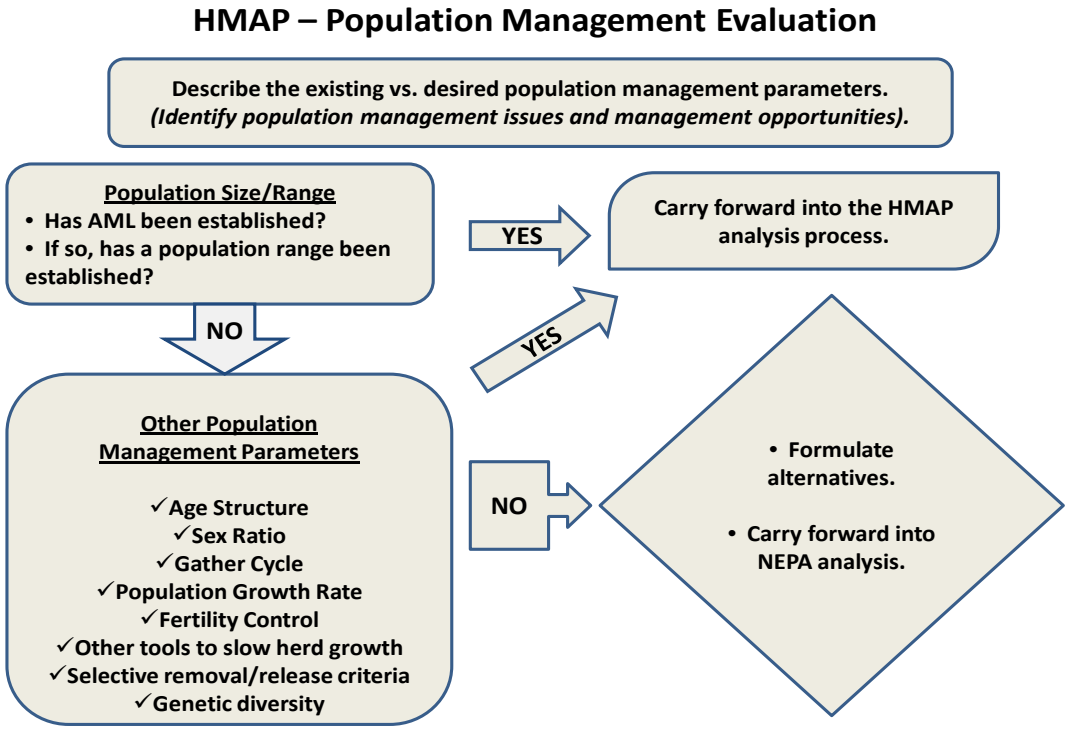


Figure 4.2
Population Management Evaluation



At the conclusion of the management evaluation, a report is prepared and provided to the public for a 30-day review and comment period (public scoping). The management evaluation report is not a decision subject to administrative appeal. Rather, the report documents the key issues identified as a result of the management evaluation. A range of the possible management alternatives (Proposed Action and Alternatives) which could address/resolve the identified issues may also be identified.

NEPA Analysis and Decision
The next step in the HMAP analysis process would be to analyze the potential environmental consequences which could result from implementation of the Proposed Action or Alternatives through completion of a site-specific environmental analysis (NEPA).

To prepare a concise environmental assessment (EA), site-specific management, monitoring and implementation objectives can be displayed in a tabular format within the environmental assessment. Refer to the example which follows.

Example – Documentation of the No Action Alternative in the HMAP EA

Alternative One: No Action Alternative, Continue Existing Management
Under Alternative 1 (No Action), the HMA would continue to be managed as a range of 150-250 wild horses as follows:

- The sex ratio of the animals released back to the range following future gathers would be about 34 percent males and 64 percent females.
- Existing monitoring, including utilization, forage condition, water availability, animal health and periodic population estimates and sampling for genetic diversity (inbreeding) would occur.
- AML would be adjusted, as needed, based on in-depth analysis of resource monitoring data.
- Fertility control would be applied to mares released back to the range following future gathers.

Table 4.1
No Action Alternative (Continue Existing Management) in HMAP Format

Management Objective(s)	Monitoring Objective(s)	Implementation Objective(s)
A. Control Population Numbers Manage the wild horse population within the established AML range to protect the range from the deterioration associated with overpopulation.	Aerially estimate populations a minimum of once every 3-4 years. Determine population number and average annual herd growth rate.	Schedule gathers to remove excess wild horses when (1) the population reaches or exceeds the AML upper limit (about every 3 years), or (2) when forage utilization exceeds 50 percent of current year's growth in ½-2/3 of the key areas; or (3) when animal health/condition is at risk.
B. Assure Land Health Limit utilization by all herbivores to 50 percent of the current year's above ground primary production for key grasses and 45 percent for key shrubs and forbs.	• Locate key monitoring areas within the HMA. • Establish baseline trend studies using the frequency sampling procedures outlined in the (cite applicable BLM Technical Reference or Handbook). • Measure utilization at key areas and complete use pattern mapping annually.	Re-adjust AML or identify management actions to address resolve land health issues, as needed/appropriate, based on results of land health monitoring.

Based on the analysis of potential environmental consequences, the authorized officer will determine whether to prepare an EIS or issue a Decision Record and FONSI.

HMAP
As a final step and to facilitate on-the-ground implementation, the selected management strategy (selected alternative), together with the habitat and population management, monitoring and implementation objectives are documented in HMAP format. The HMAP is included as an attachment to the authorized officer's final decision.

HMAP Format

1. Introduction. Briefly summarize the relevant background information.

2. Selected Management Alternative. Briefly summarize the selected management strategy/alternative as outlined in the EA, together with the site-specific habitat and population management objectives and management actions.

3. Monitoring and Evaluation Plan.

4. Project Tracking and Implementation Schedule.

The Monitoring Plan and Tracking Log/Project Implementation Schedule may also be prepared in a tabular format. See the examples which follow.

Table 4.2
Example -- Monitoring Plan

Item	How	Who	When	Actions to Take (Adaptive Management)
Population Management Monitoring				
Manage wild horse populations within the established AML range.	Aerial survey flights following established protocols. Simultaneous double count method or other approved protocol pending population estimation research results & recommendation.	FO WH&B Specialist	Aerial population estimates a minimum of every 3 years, i.e., 2010, 2013, etc.	Schedule gathers to remove excess wild horses when (1) the population reaches or exceeds the AML upper limit (about every 3 years), or (2) when forage utilization exceeds 50 percent of current year's growth in ½-2/3 of the key areas; or (3) when animal health/condition is at risk.
Habitat Management Monitoring				
Limit utilization by all herbivores to 50 percent of the current year's above ground primary production for key grasses and 45 percent for key shrubs and forbs.	Measure utilization at key areas and complete use pattern mapping annually.	FO WH&B Specialist	Annually (October)	Re-adjust AML or identify management actions to address resolve rangeland health issues, as needed/appropriate, based on results of rangeland health monitoring.

Table 4.3
Example – Tracking Log/Project Implementation Schedule

Description	Who	Where	When	Completed	Remarks
Population Management Actions					
Schedule gathers to remove excess wild horses when the population reaches/exceeds AML, utilization exceeds allowable limits, or animal health/condition is at risk.	BLM	(Name) HMA	About every 3 years (2010, 2013, etc)		
Habitat Management Actions					
Reconstruct Cactus Spring water development.	Friends of Spring Snails	T. 44 N., R 12 W, SE,SE Section 2	2009	2010	RIP No. XXXXXX

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WILD HORSE AND BURRO COMPREHENSIVE ANIMAL WELFARE PROGRAM

PIM 2021-002
Permanent Instruction Memorandum

December 18, 2020

In Reply Refer To:
4700 (260) P

EMS TRANSMISSION 12/18/2020
Permanent Instruction Memorandum No. 2020- 002

To: All Headquarters Officials and Field Office Officials

Attn: State and Center Directors

From: Assistant Director, Resources and Planning

Subject: Wild Horse and Burro Comprehensive Animal Welfare Program

Program Area: Wild Horse and Burro Program

Purpose: The purpose of this Permanent Instruction Memorandum (PIM) is to re-affirm the Bureau of Land Management's (BLM's) longstanding commitment to ensure the humane treatment of wild horses and burros (WHB) in all on-range and off-range management activities through the Comprehensive Animal Welfare Program (CAWP). The humane treatment of wild horses and burros outlined in this policy reiterates the BLM's longstanding practices but also provides additional clarification to BLM employees. This PIM consolidates current humane practices, incorporates the existing Standard Operating Procedures (SOPs) outlined in Attachments 1 and 2 to ensure humane care and handling of the animals, and increases transparency concerning the humane treatment of wild horses and burros.

Policy/Action: The BLM is committed to continue protecting animal welfare and providing humane care and treatment to WHBs both on and off the range. Consistent with long-standing policies and practices, the handling and treatment provided by the BLM, its contractors, and its partners is based on compassion and concern for the animals' well-being at all times. Animals are managed and cared for to optimize animal condition, health, and safety.

All state, district, and field offices will continue to comply with the WHB CAWP policy within their jurisdiction at all times. The WHB CAWP policy includes:

1. CAWP Standards for Wild Horse and Burro Gathers (Attachment 1): These standards include requirements for trap and temporary holding facility design, capture and handling, transportation, and care after capture. The standards are also incorporated into helicopter gather contracts as specifications for performance.
2. Training for gather operations: All BLM staff associated with gather operations (incident commanders, contracting officer representatives, project inspectors, public affairs officials, and law enforcement officers) and contractor crews are required to complete online training annually for CAWP – Gathers (BLM-TC-4700-13), which is available through DOI Talent.
3. CAWP Standards for Off-Range Corral Facilities, Transportation, and Adoption/Sale Events (Attachment 2): This includes requirements for holding of animals in off-range corrals, transportation, and adoptions/sales events. These SOPs are incorporated into off-range corral contracts as specifications for performance.
4. Training for the Care, Handling, and Transportation of Wild Horses and Burros at off-range corrals and adoption/sales events: All personnel, including contractors and partners involved in the care, handling, and transportation of wild horses and burros at off-range

VIEW ALL POLICIES

ATTACHMENTS

COMPREHENSIVE ANIMAL WELFARE PROGRAM FOR WILD HORSE AND BURRO GATHERS (PDF / 409 KB)
CAWP STANDARDS OFF RANGE CORRAL TRANSPORT ADOPTION SALE EVENTS (PDF / 364 KB)

FISCAL YEAR

2021

corrals and adoption/sale events are required to complete online training annually. Development of this training will begin following issuance of this PIM.

5. CAWP Standards and SOPs (to be developed) for animals on the range, in off-range pastures, and public off-range pastures: All personnel, contractors, and partners involved in management of WHBs on the range, in off-range pastures, or public off-range pastures are required to complete online training annually following the development of these guidelines and SOPs.

The purpose of this PIM is to continue to ensure that the humane care and treatment of WHBs remains the priority for the BLM, its contractors, and its partners. The BLM will continue to use the best available science, husbandry, and handling practices applicable for WHBs. Whenever possible, the BLM will make improvements to its practices while also meeting planned objectives in accordance with current BLM policy, SOPs, as well as contractual and cooperative agreement requirements. The CAWP will be reviewed periodically by WHB program leadership and modified as necessary to strengthen public trust, transparency, and effectiveness in assuring the humane care and treatment of the WHBs.

The authorized officer is the primary party responsible for immediately addressing any actions that are inconsistent with the CAWP. Consistent with applicable BLM delegation of authority manuals, the authorized officer may delegate responsibility to an alternate individual. The authorized officer should promptly notify the employees, contractors, and partners of actions that are not compliant with the CAWP and correct all issues immediately. It is the responsibility of all individuals engaged in actions throughout the WHB program to implement all aspects of the CAWP.

The WHB CAWP Coordinator will lead the implementation, development, and modification of all aspects of the CAWP, including those developed to date as well as future components of the CAWP.

Timeframe: This policy is effective immediately.

Budget Impact: This PIM codifies long-standing policy, practice, and guidance to include mandatory annual training and reporting requirements for BLM personnel, contractors, and partners associated with gather operations. The cost for the required training is approximately $100 per person per year. This PIM also formalizes SOPs for off-range holding, adoptions, sales, and transportation of excess WHB. When fully developed, the CAWP program implementation, oversight, data compilation, and reporting requirements will require an additional 12 to 18 work months per year.

Background: Public Law-195, Wild Free-Roaming Horses and Burros Act of 1971 (as amended) and 43 CFR 4700.0-2 provide the authority for a CAWP for WHB.

This CAWP policy provides a comprehensive approach to long-standing policies and practices within the BLM WHB program. Despite these policies and practices, the BLM has been criticized by some animal protection organizations on its handling and care of wild horses and burros, especially during the gathering activities and transitioning of the horses from public lands to off-range facilities. Some of the criticisms include concerns as to the transportation conditions of the horses, facility design, environmental conditions, handling practices, and nutrition. Similar to policies adopted by other organizations in agricultural and animal research industries, this PIM establishes a framework to ensure there are programs in place to verify humane care and handling of animals. It provides transparency on how the animals are assured humane care and proper handling. This framework seeks to confirm that the WHB program is conducted in a humane and caring manner.

The CAWP for WHB consolidates and highlights the BLM policies, procedures, and ongoing commitment to protect animal welfare; provides training for employees, contractors, and partners on animal care and handling, and implements assessment tools to evaluate adherence to the CAWP SOP by the agency, contractors, and partners.

Manual/Handbook Sections Affected: This PIM affects policies in BLM Manual/Handbooks H-4700-1 “Wild Horse and Burro Management” and H-4750-2 “Adoption of Wild Horses and Burros”.

Coordination: This PIM was coordinated among HQ-100, HQ-200, HQ-260, HQ-600, Deputy State Directors, WHB State Leads, APHIS Veterinarian, WHB Facility Managers, and WHB Specialists.

Contact: If you have any questions, please contact the Wild Horse and Burro Division Chief, at 866-468-7826 or wildhorse@blm.gov.

Signed by:	Authenticated By:
David Jenkins	Ambyr Fowler
Assistant Director	Division of Regulatory Affairs (HQ-630)

Resources and Planning

2 Attachments

1. - CAWP Gather Standards (20 pp)
2. - Comprehensive Animal Welfare Program Standards for Off-Range Corrals, Transportation and Adoption/Sale Events (24 pp)

ATTACHMENT 1: COMPREHENSIVE ANIMAL WELFARE PROGRAM FOR WILD HORSE AND BURRO GATHERS

STANDARDS

Developed by

The Bureau of Land Management
Wild Horse and Burro Program

in collaboration with

Carolyn L. Stull, PhD
Kathryn E. Holcomb, PhD
University of California, Davis
School of Veterinary Medicine

June 30, 2015

WELFARE ASSESSMENT STANDARDS for GATHERS

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STANDARDS

Standard Definitions

Major Standard: Impacts the health or welfare of WH&Bs. Relates to an alterable equipment or facility standard or procedure. Appropriate wording is "must," "unacceptable," "prohibited."

Minor Standard: unlikely to affect WH&Bs health or welfare or involves an uncontrollable situation. Appropriate wording is "should."

Lead COR = Lead Contracting Officer's Representative

COR = Contracting Officer's Representative

PI = Project Inspector

WH&Bs = Wild horses and burros

I. FACILITY DESIGN

A. Trap Site and Temporary Holding Facility

1. The trap site and temporary holding facility must be constructed of stout materials and must be maintained in proper working condition, including gates that swing freely and latch or tie easily. (**major**)
2. The trap site should be moved close to WH&B locations whenever possible to minimize the distance the animals need to travel.(minor)
3. If jute is hung on the fence posts of an existing wire fence in the trap wing, the wire should be either be rolled up or let down for the entire length of the jute in such a way that minimizes the possibility of entanglement by WH&Bs unless otherwise approved by the Lead COR/COR/PI. (minor)
4. Fence panels in pens and alleys must be not less than 6 feet high for horses, 5 feet high for burros, and the bottom rail must not be more than 12 inches from ground level. (**major**)

5. The temporary holding facility must have a sufficient number of pens available to sort WH&Bs according to gender, age, number, temperament, or physical condition. (**major**)
    a. All pens must be assembled with capability for expansion. (**major**)
    b. Alternate pens must be made available for the following: (**major**)
        1) WH&Bs that are weak or debilitated
        2) Mares/jennies with dependent foals
    c. WH&Bs in pens at the temporary holding facility should be maintained at a proper stocking density such that when at rest all WH&Bs occupy no more than half the pen area. (minor)
6. An appropriate chute designed for restraining WH&Bs must be available for necessary procedures at the temporary holding facility. This does not apply to bait trapping operations unless directed by the Lead COR/COR/PI. (**major**)
7. There must be no holes, gaps or openings, protruding surfaces, or sharp edges present in fence panels or other structures that may cause escape or possible injury. (**major**)
8. Padding must be installed on the overhead bars of all gates and chutes used in single file alleys. (**major**)
9. Hinged, self-latching gates must be used in all pens and alleys except for entry gates into the trap, which may be secured with tie ropes. (**major**)
10. Finger gates (one-way funnel gates) used in bait trapping must be constructed of materials approved by the Lead COR/COR/PI. Finger gates must not be constructed of materials that have sharp ends that may cause injuries to WH&Bs, such as "T" posts, sharpened willows, etc. (**major**)
11. Water must be provided at a minimum rate of ten gallons per 1000 pound animal per day, adjusted accordingly for larger or smaller horses, burros and foals, and environmental conditions, with each trough placed in a separate location of the pen (i.e. troughs at opposite ends of the pen). Water must be refilled at least every morning and evening. (**major**)
12. The design of pens at the trap site and temporary holding facility should be constructed with rounded corners. (minor)

13. All gates and panels in the animal holding and handling pens and alleys of the trap site must be covered with materials such as plywood, snow fence, tarps, burlap, etc. approximately 48" in height to provide a visual barrier for the animals. All materials must be secured in place.(**major**)

    These guidelines apply:

    a. For exterior fences, material covering panels and gates must extend from the top of the panel or gate toward the ground.(**major**)
    b. For alleys and small internal handling pens, material covering panels and gates should extend from no more than 12 inches below the top of the panel or gate toward the ground to facilitate visibility of animals and the use of flags and paddles during sorting. (minor)
    c. The initial capture pen may be left uncovered as necessary to encourage animals to enter the first pen of the trap. (minor)

14. Non-essential personnel and equipment must be located to minimize disturbance of WH&Bs. (**major**)
15. Trash, debris, and reflective or noisy objects should be eliminated from the trap site and temporary holding facility. (minor)

B. Loading and Unloading Areas

1. Facilities in areas for loading and unloading WH&Bs at the trap site or temporary holding facility must be maintained in a safe and proper working condition, including gates that swing freely and latch or tie easily. (**major**)
2. The side panels of the loading chute must be a minimum of 6 feet high and fully covered with materials such as plywood or metal without holes that may cause injury. (**major**)
3. There must be no holes, gaps or openings, protruding surfaces, or sharp edges present in fence panels or other structures that may cause escape or possible injury. (**major**)
4. All gates and doors must open and close easily and latch securely. (**major**)

5. Loading and unloading ramps must have a non-slip surface and be maintained in a safe and proper working condition to prevent slips and falls. Examples of non-slip flooring would include, but not be limited to, rubber mats, sand, shavings, and steel reinforcement rods built into ramp. There must be no holes in the flooring or items that can cause an animal to trip. (**major**)
6. Trailers must be properly aligned with loading and unloading chutes and panels such that no gaps exist between the chute/panel and floor or sides of the trailer creating a situation where a WH&B could injure itself. (**major**)
7. Stock trailers should be positioned for loading or unloading such that there is no more than 12" clearance between the ground and floor of the trailer for burros and 18" for horses. (minor)

II. CAPTURE TECHNIQUE

A. Capture Techniques

1. WH&Bs gathered on a routine basis for removal or return to range must be captured by the following approved procedures under direction of the Lead COR/COR/PI. (**major**)
   a. Helicopter
   b. Bait trapping
2. WH&Bs must not be captured by snares or net gunning. (**major**)
3. Chemical immobilization must only be used for capture under exceptional circumstances and under the direct supervision of an on-site veterinarian experienced with the technique. (**major**)

B. Helicopter Drive Trapping

1. The helicopter must be operated using pressure and release methods to herd the animals in a desired direction and should not repeatedly evoke erratic behavior in the WH&Bs causing injury or exhaustion. Animals must not be pursued to a point of exhaustion; the on-site veterinarian must examine WH&Bs for signs of exhaustion. (**major**)

2. The rate of movement and distance the animals travel must not exceed limitations set by the Lead COR/COR/PI who will consider terrain, physical barriers, access limitations, weather, condition of the animals, urgency of the operation (animals facing drought, starvation, fire, etc.) and other factors. (**major**)
    a. WH&Bs that are weak or debilitated must be identified by BLM staff or the contractors. Appropriate gather and handling methods should be used according to the direction of the Lead COR/COR/PI. (**major**)
    b. The appropriate herding distance and rate of movement must be determined on a case-by-case basis considering the weakest or smallest animal in the group (e.g., foals, pregnant mares, or horses that are weakened by body condition, age, or poor health) and the range and environmental conditions present. (**major**)
    c. Rate of movement and distance travelled must not result in exhaustion at the trap site, with the exception of animals requiring capture that have an existing severely compromised condition prior to gather. Where compromised animals cannot be left on the range or where doing so would only serve to prolong their suffering, euthanasia will be performed in accordance with BLM policy. (**major**)
3. WH&Bs must not be pursued repeatedly by the helicopter such that the rate of movement and distance travelled exceeds the limitation set by the Lead COR/COR/PI. Abandoning the pursuit or alternative capture methods may be considered by the Lead COR/COR/PI in these cases. (**major**)
4. When WH&Bs are herded through a fence line en route to the trap, the Lead COR/COR/PI must be notified by the contractor. The Lead COR/COR/PI must determine the appropriate width of the opening that the fence is let down to allow for safe passage through the opening. The Lead COR/COR/PI must decide if existing fence lines require marking to increase visibility to WH&Bs. (**major**)
5. The helicopter must not come into physical contact with any WH&B. The physical contact of any WH&B by helicopter must be documented by Lead COR/COR/PI along with the circumstances. (**major**)
6. WH&Bs may escape or evade the gather site while being moved by the helicopter. If there are mare/dependent foal pairs in a group being brought to a trap and half of an identified pair is thought to have evaded capture, multiple attempts by helicopter may

be used to bring the missing half of the pair to the trap or to facilitate capture by roping. In these instances, animal condition and fatigue must be evaluated by the Lead COR/COR/PI or on-site veterinarian on a case-by-case basis to determine the number of attempts that can be made to capture an animal.(**major**)

7. Horse captures must not be conducted when ambient temperature at the trap site is below 10ºF or above 95ºF without approval of the Lead COR/COR/PI. Burro captures must not be conducted when ambient temperature is below 10ºF or above 100ºF without approval of the Lead COR/COR/PI. The Lead COR/COR/PI will not approve captures when the ambient temperature exceeds 105 ºF. (**major**)

C. Roping

1. The roping of any WH&B must be approved prior to the procedure by the Lead COR/COR/PI. (**major**).
2. The roping of any WH&B must be documented by the Lead COR/COR/PI along with the circumstances. WH&Bs may be roped under circumstances which include but are not limited to the following: reunite a mare or jenny and her dependent foal; capture nuisance, injured or sick WH&Bs or those that require euthanasia; environmental reasons such as deep snow or traps that cannot be set up due to location or environmentally sensitive designation; and public and animal safety or legal mandates for removal. (**major**)
3. Ropers should dally the rope to their saddle horn such that animals can be brought to a stop as slowly as possible and must not tie the rope hard and fast to the saddle so as to intentionally jerk animals off their feet. (**major**)
4. WH&Bs that are roped and tied down in recumbency must be continuously observed and monitored by an attendant at a maximum of 100 feet from the animal. (**major**)
5. WH&Bs that are roped and tied down in recumbency must be untied within 30 minutes. (**major**)
6. If the animal is tied down within the wings of the trap, helicopter drive trapping within the wings will cease until the tied-down animal is removed. (**major**)
7. Sleds, slide boards, or slip sheets must be placed underneath the animal's body to move and/or load recumbent WH&Bs. (**major**)

8. Halters and ropes tied to a WH&B may be used to roll, turn, position or load a recumbent animal, but a WH&B must not be dragged across the ground by a halter or rope attached to its body while in a recumbent position. (**major**)
9. Animals captured by roping must be evaluated by the on-site/on-call veterinarian within four hours after capture, marked for identification at the trap site, and be re-evaluated periodically as deemed necessary by the on-site/on-call veterinarian. (**major**)

D. Bait Trapping

1. WH&Bs may be lured into a temporary trap using bait (feed, mineral supplement, water) or sexual attractants (mares/jennies in heat) with the following requirements:
   a. The period of time water sources other than in the trap site are inaccessible must not adversely affect the wellbeing of WH&Bs, wildlife or livestock, as determined by the Lead COR/COR/PI. (**major**)
   b. Unattended traps must not be left unobserved for more than 12 hours. (**major**)
   c. Mares/jennies and their dependent foals must not be separated unless for safe transport. (**major**)
   d. WH&Bs held for more than 12 hours must be provided with accessible clean water at a minimum rate of ten gallons per 1000 pound animal per day, adjusted accordingly for larger or smaller horses, burros and foals and environmental conditions. (**major**)
   e. WH&Bs held for more than 12 hours must be provided good quality hay at a minimum rate of 20 pounds per 1000 pound adult animal per day, adjusted accordingly for larger or smaller horses, burros and foals. (**major**)
      1) Hay must not contain poisonous weeds, debris, or toxic substances. (**major**)
      2) Hay placement must allow all WH&Bs to eat simultaneously. (**major**)

III. WILD HORSE AND BURRO CARE

A. Veterinarian

1. On-site veterinary support must be provided for all helicopter gathers and on-site or on-call support must be provided for bait trapping. (**major**)

2. Veterinary support must be under the direction of the Lead COR/COR/PI. The on-site/on-call veterinarian will provide consultation on matters related to WH&B health, handling, welfare, and euthanasia at the request of the Lead COR/COR/PI. All decisions regarding medical treatment or euthanasia will be made by the on-site Lead COR/COR/PI. (**major**)

B. Care

1. Feeding and Watering
    a. Adult WH&Bs held in traps or temporary holding pens for longer than 12 hours must be fed every morning and evening with water available at all times other than when animals are being sorted or worked. (**major)**
    b. Water must be provided at a minimum rate of ten gallons per 1000 pound animal per day, adjusted accordingly for larger or smaller horses, burros and foals, and environmental conditions, with each trough placed in a separate location of the pen (i.e. troughs at opposite ends of the pen). . (**major**)
    c. Good quality hay must be fed at a minimum rate of 20 pounds per 1000 pound adult animal per day, adjusted accordingly for larger or smaller horses, burros and foals. (**major**)
        i. Hay must not contain poisonous weeds or toxic substances. (**majo**r)
        ii. Hay placement must allow all WH&Bs to eat simultaneously. (**major**)
    d. When water or feed deprivation conditions exist on the range prior to the gather, the Lead COR/COR/PI should adjust the watering and feeding arrangements in consultation with the onsite veterinarian as necessary to provide for the needs of the animals. (minor)
2. Dust abatement
    a. Dust abatement by spraying the ground with water must be employed when necessary at the trap site and temporary holding facility. (**major**)

3. Trap Site
    a. Dependent foals or weak/debilitated animals must be separated from other WH&Bs at the trap site to avoid injuries during transportation to the temporary holding facility. Separation of dependent foals from mares must not exceed four hours unless the Lead COR/COR/PI authorizes a longer time or a decision is made to wean the foals. (**major**)
4. Temporary Holding Facility
    a. All WH&Bs in confinement must be observed at least once daily to identify sick or injured WH&Bs and ensure adequate food and water. (**major**)
    b. Foals must be reunited with their mares/jennies at the temporary holding facility within four hours of capture unless the Lead COR/COR/PI authorizes a longer time or foals are old enough to be weaned during the gather. (**major**)
    c. Non-ambulatory WH&Bs must be located in a pen separate from the general population and must be examined by the BLM horse specialist and/or on-call or on-site veterinarian as soon as possible, no more than four hours after recumbency is observed. Unless otherwise directed by a veterinarian, hay and water must be accessible to an animal within six hours after recumbency.(**major**)
    d. Alternate pens must be made available for the following: (**major**)
        1) WH&Bs that are weak or debilitated
        2) Mares/jennies with dependent foals
    e. Aggressive WH&Bs causing serious injury to other animals should be identified and relocated into alternate pens when possible. (minor)
    f. WH&Bs in pens at the temporary holding facility should be maintained at a proper stocking density such that when at rest all WH&Bs occupy no more than half the pen area. (minor)

C. Biosecurity

1. Health records for all saddle and pilot horses used on WH&B gathers must be provided to the Lead COR/COR/PI prior to joining a gather, including: (**major**)
   a. Certificate of Veterinary Inspection (Health Certificate, within 30 days).
   b. Proof of:
      1) A negative test for equine infectious anemia (Coggins or EIA ELISA test) within 12 months.
      2) Vaccination for tetanus, eastern and western equine encephalomyelitis, West Nile virus, equine herpes virus, influenza, *Streptococcus equi*, and rabies within 12 months.
2. Saddle horses, pilot horses and mares used for bait trapping lures must not be removed from the gather operation (such as for an equestrian event) and allowed to return unless they have been observed to be free from signs of infectious disease for a period of at least three weeks and a new Certificate of Veterinary Examination is obtained after three weeks and prior to returning to the gather. (**major**)
3. WH&Bs, saddle horses, and pilot horses showing signs of infectious disease must be examined by the on-site/on-call veterinarian. (**major**)
   a. Any saddle or pilot horses showing signs of infectious disease (fever, nasal discharge, or illness) must be removed from service and isolated from other animals on the gather until such time as the horse is free from signs of infectious disease and approved by the on-site/on-call veterinarian to return to the gather. (**major**)
   b. Groups of WH&Bs showing signs of infectious disease should not be mixed with groups of healthy WH&Bs at the temporary holding facility, or during transport. (minor)
4. Horses not involved with gather operations should remain at least 300 yards from WH&Bs, saddle horses, and pilot horses being actively used on a gather. (minor)

IV. HANDLING

A. Willful Acts of Abuse

1. Hitting, kicking, striking, or beating any WH&B in an abusive manner is prohibited. (**major**)
2. Dragging a recumbent WH&B without a sled, slide board or slip sheet is prohibited. Ropes used for moving the recumbent animal must be attached to the sled, slide board or slip sheet unless being loaded as specified in Section II. C. 8. (**major**)
3. There should be no deliberate driving of WH&Bs into other animals, closed gates, panels, or other equipment. (minor)
4. There should be no deliberate slamming of gates and doors on WH&Bs. (minor)
5. There should be no excessive noise (e.g., constant yelling) or sudden activity causing WH&Bs to become unnecessarily flighty, disturbed or agitated. (minor)

B. General Handling

1. All sorting, loading or unloading of WH&Bs during gathers must be performed during daylight hours except when unforeseen circumstances develop and the Lead COR/CO/PI approves the use of supplemental light. (**major**)
2. WH&Bs should be handled to enter runways or chutes in a forward direction. (minor)
3. WH&Bs should not remain in single-file alleyways, runways, or chutes longer than 30 minutes. (minor)
4. Equipment except for helicopters should be operated and located in a manner to minimize flighty behavior . (minor)

C. Handling Aids

1. Handling aids such as flags and shaker paddles must be the primary tools for driving and moving WH&Bs during handling and transport procedures. Contact of the flag or paddle end of primary handling aids with a WH&B is allowed. Ropes looped around the hindquarters may be used from horseback or on foot to assist in moving an animal forward or during loading. (**major**)

2. Electric prods must not be used routinely as a driving aid or handling tool. Electric prods may be used in limited circumstances only if the following guidelines are followed:
    a. Electric prods must only be a commercially available make and model that uses DC battery power and batteries should be fully charged at all times. (**major**)
    b. The electric prod device must never be disguised or concealed. (**major**)
    c. Electric prods must only be used after three attempts using other handling aids (flag, shaker paddle, voice or body position) have been tried unsuccessfully to move the WH&Bs. (**major**)
    d. Electric prods must only be picked up when intended to deliver a stimulus; these devices must not be constantly carried by the handlers. (**major**)
    e. Space in front of an animal must be available to move the WH&B forward prior to application of the electric prod. (**major**)
    f. Electric prods must never be applied to the face, genitals, anus, or underside of the tail of a WH&B. (**major**)
    g. Electric prods must not be applied to any one WH&B more than three times during a procedure (e.g., sorting, loading) except in extreme cases with approval of the Lead COR/COR/PI. Each exception must be approved at the time by the Lead COR/COR/PI. (**majo**r)
    h. Any electric prod use that may be necessary must be documented daily by the Lead COR/COR/PI including time of day, circumstances, handler, location (trap site or temporary holding facility), and any injuries (to WH&B or human). (**major**)

V. TRANSPORTATION

A. General

1. All sorting, loading, or unloading of WH&Bs during gathers must be performed during daylight hours except when unforeseen circumstances develop and the Lead COR/CO/PI approves the use of supplemental light. (**major**)

2. WH&Bs identified for removal should be shipped from the temporary holding facility to a BLM facility within 48 hours. (minor)
    a. Shipping delays for animals that are being held for release to range or potential on-site adoption must be approved by the Lead COR/COR/PI. (**major**)
3. Shipping should occur in the following order of priority; 1) debilitated animals, 2) pairs, 3) weanlings, 4) dry mares and 5) studs. (minor)
4. Planned
5. transport time to the BLM preparation facility from the trap site or temporary holding facility must not exceed 10 hours. (**major**)
6. WH&Bs should not wait in stock trailers and/or semi-trailers at a standstill for more than a combined period of three hours during the entire journey. (minor)

B. Vehicles

1. Straight-deck trailers and stock trailers must be used for transporting WH&Bs. (**major**)
    a. Two-tiered or double deck trailers are prohibited. (**major**)
    b. Transport vehicles for WH&Bs must have a covered roof or overhead bars containing them such that WH&Bs cannot escape. (**major**)
2. WH&Bs must have adequate headroom during loading and unloading and must be able to maintain a normal posture with all four feet on the floor during transport without contacting the roof or overhead bars. (**major**)
3. The width and height of all gates and doors must allow WH&Bs to move through freely. (**major**)
4. All gates and doors must open and close easily and be able to be secured in a closed position. (**major**)
5. The rear door(s) of the trailers must be capable of opening the full width of the trailer. (**major**)
6. Loading and unloading ramps must have a non-slip surface and be maintained in proper working condition to prevent slips and falls. (**major**)

7. Transport vehicles more than 18 feet and less than 40 feet in length must have a minimum of one partition gate providing two compartments; transport vehicles 40 feet or longer must have at least two partition gates to provide a minimum of three compartments. (**major**)
8. All partitions and panels inside of trailers must be free of sharp edges or holes that could cause injury to WH&Bs. (**major**)
9. The inner lining of all trailers must be strong enough to withstand failure by kicking that would lead to injuries. (**major**)
10. Partition gates in transport vehicles should be used to distribute the load into compartments during travel. (minor)
11. Surfaces and floors of trailers must be cleaned of dirt, manure and other organic matter prior to the beginning of a gather. (**major**)

C. Care of WH&Bs during Transport Procedures

1. WH&Bs that are loaded and transported from the temporary holding facility to the BLM preparation facility must be fit to endure travel. (**major**)
    a. WH&Bs that are non-ambulatory, blind in both eyes, or severely injured must not be loaded and shipped unless it is to receive immediate veterinary care or euthanasia. (**major**)
    b. WH&Bs that are weak or debilitated must not be transported without approval of the Lead COR/COR/PI in consultation with the on-site veterinarian. Appropriate actions for their care during transport must be taken according to direction of the Lead COR/COR/PI. (**major**)
2. WH&Bs should be sorted prior to transport to ensure compatibility and minimize aggressive behavior that may cause injury. (minor)
3. Trailers must be loaded using the minimum space allowance in all compartments as follows: (**major**)
    a. 12 square feet per adult horse.
    b. 6.0 square feet per dependent horse foal.
    c. 8.0 square feet per adult burro.
    d. 4.0 square feet per dependent burro foal.

4. The Lead COR/COR/PI in consultation with the receiving Facility Manager must document any WH&B that is recumbent or dead upon arrival at the destination. (**major**)
    a. Non-ambulatory or recumbent WH&Bs must be evaluated on the trailer and either euthanized or removed from the trailers using a sled, slide board or slip sheet. (**major**)
5. Saddle horses must not be transported in the same compartment with WH&Bs. (**major**)

VI. EUTHANASIA OR DEATH

A. Euthanasia Procedure during Gather Operations

1. An authorized, properly trained, and experienced person as well as a firearm appropriate for the circumstances must be available at all times during gather operations. When the travel time between the trap site and temporary holding facility exceeds one hour or if radio or cellular communication is not reliable, provisions for euthanasia must be in place at both the trap site and temporary holding facility during the gather operation. (**major**)
2. Euthanasia must be performed according to American Veterinary Medical Association euthanasia guidelines (2013) using methods of gunshot or injection of an approved euthanasia agent. (**major**)
3. The decision to euthanize and method of euthanasia must be directed by the Authorized Officer or their Authorized Representative(s) that include but are not limited to the Lead COR/COR/PI who must be on site and may consult with the on-site/on-call veterinarian. (**major**)
4. Photos needed to document an animal's condition should be taken prior to the animal being euthanized. No photos of animals that have been euthanized should be taken. An exception is when a veterinarian or the Lead COR/COR/PI may want to document certain findings discovered during a postmortem examination or necropsy. (minor)
5. Any WH&B that dies or is euthanized must be documented by the Lead COR/COR/PI including time of day, circumstances, euthanasia method, location, a

description of the age, gender, and color of the animal and the reason the animal was euthanized. (**major**)

6. The on-site/on-call veterinarian should review the history and conduct a postmortem physical examination of any WH&B that dies or is euthanized during the gather operation. A necropsy should be performed whenever feasible if the cause of death is unknown. (minor)

B. Carcass Disposal

1. The Lead COR/COR/PI must ensure that appropriate equipment is available for the timely disposal of carcasses when necessary on the range, at the trap site, and temporary holding facility. (**major**)
2. Disposal of carcasses must be in accordance with state and local laws. (**major**)
3. WH&Bs euthanized with a barbiturate euthanasia agent must be buried or otherwise disposed of properly. (**major**)
4. Carcasses left on the range should not be placed in washes or riparian areas where future runoff may carry debris into ponds or waterways. Trenches or holes for buried animals should be dug so the bottom of the hole is at least 6 feet above the water table and 4-6 feet of level earth covers the top of the carcass with additional dirt mounded on top where possible. (minor)

CAWP

REQUIRED DOCUMENTATION AND RESPONSIBILITIES OF LEAD COR/COR/PI

Required Documentation

Section	Documentation
II.B.5	Helicopter contact with any WH&B.
II.C.2	Roping of any WH&B.
III.B.3.a and III.B.4.b	Reason for allowing longer than four hours to reunite foals with mares/jennies. Does not apply if foals are being weaned.
III.C.1	Health status of all saddle and pilot horses.
IV.C.2.h	All uses of electric prod.
V.C.4	Any WH&B that is recumbent or dead upon arrival at destination following transport.
VI.A.5	Any WH&B that dies or is euthanized during gather operation.

Responsibilities

Section	Responsibility
I.A.10	Approve materials used in construction of finger gates in bait trapping
II.A.1	Direct gather procedures using approved gather technique.
II.B. 2	Determine rate of movement and distance limitations for WH&B helicopter gather.
II.B.2.a	Direct appropriate gather/handling methods for weak or debilitated WH&B.
II.B.3	Determine whether to abandon pursuit or use other capture method in order to avoid repeated pursuit of WH&B.
II.B.4	Determine width and need for visibility marking when using opening in fence en route to trap.
II.B.6	Determine number of attempts that can be made to capture the missing half of a mare/foal pair that has become separated.
II.B.7	Determine whether to proceed with gather when ambient temperature is outside the range of 10°F to 95°F for horses or 10°F to 100°F for burros.
II.C.1	Approve roping of any WH&B.
II.D.1.a	Determine period of time that water outside a bait trap is inaccessible such that wellbeing of WH&Bs, wildlife, or livestock is not adversely affected.
III.A.2	Direct and consult with on-site/on-call veterinarian on any matters related to WH&B health, handling, welfare and euthanasia.

III.B.1.e	Adjust feed/water as necessary, in consultation with onsite/on call veterinarian, to provide for needs of animals when water or feed deprivation conditions exist on range.
III.B.4.c	Determine provision of water and hay to non-ambulatory animals.
IV.C.2.g	Approve use of electric prod more than three times, for exceptional cases only.
V.A.1	Approve sorting, loading, or unloading at night with use of supplemental light.
V.A.2.a	Approve shipping delays of greater than 48 hours from temporary holding facility to BLM facility.
V.C.1.b	Approve of transport and care during transport for weak or debilitated WH&B.
VI.A.3	Direct decision regarding euthanasia and method of euthanasia for any WH&B; may consult with on-site/on-call veterinarian.
VI.B.1	Ensure that appropriate equipment is available for carcass disposal.

COMPREHENSIVE ANIMAL WELFARE PROGRAM

STANDARDS

OFF-RANGE CORRAL FACILITIES

TRANSPORTATION

ADOPTION EVENTS

Developed by

The Bureau of Land Management
Wild Horse and Burro Program

in collaboration with

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PREFACE

The Bureau of Land Management (BLM) estimates that 58,150 wild horses and burros (WH&Bs) are roaming on BLM-managed rangelands in 10 Western states, based on the latest data available, compiled as of March 1, 2015. Wild horses and burros have virtually no natural predators and their herd sizes can double about every four years. As a result, the agency must remove thousands of animals from the range each year to control herd sizes.

The Bureau of Land Management manages many off-range corral facilities throughout the U.S. The primary mission of the off-range corral facilities is to act as a holding and preparation facility that accepts WH&Bs from gather/removal operations and prepares them for adoption to the public or placement into off-range pastures. As of November 2015, there are 16,182 WH&Bs in off-range corral facilities.

The design of each facility is organized to handle large numbers of WH&Bs with pens, corrals, alleys, and loading/unloading areas that facilitate animal movement. There are multiple pens and corrals to sort animals for compatibility and care upon arrival to the facility. Each pen or corral offers all animals continuous water accessibility and quality feed on a daily basis.

The BLM strives to place horses removed from the range into private homes that provide quality care. Horses placed in off-range corral facilities are made available to the public for adoption or sale at adoption events held throughout the U.S. and through the BLM's Adopt or Sales Program (http://www.blm.gov/wo/st/en/prog/whbprogram/adoption_program/schedule.html). The off-range corral facility transitions WH&Bs to hay diets, performs necessary vaccinations and deworming procedures, provides hoof care and may train WH&Bs. Other WH&Bs, due to age, temperament, or other factors, may not be adopted or sold and subsequently are placed in off-range pastures. These facilities are designed to provide unadoptable wild horses with humane life-long care in a natural setting off the public rangelands. There are approximately 46,016 WH&Bs being cared for in off-range pastures (November 2015). All WH&Bs residing in both off-range corral facilities and off-range pastures, like those roaming Western public rangelands, are protected by the BLM under the 1971 Wild Free-Roaming Horses and Burros Act, as amended.

The BLM has been criticized by the public and animal protection organizations on their handling and care of wild horses and burros especially during the gathering activities and transitioning of the horses from public lands to off-range facilities. Some of the issues include the transportation conditions of the horses, facility design, environmental conditions, handling practices, and nutrition. Agricultural and research animal industries have developed assessment or auditing programs to verify humane care and handling of their industry's animals. These industries are performing both internal and 3rd-party audits for animal welfare to assure their stakeholders and provide transparency on how these animals are provided care and proper handling. Assessment programs based on standards have multifaceted benefits in providing education to employees and assurance to societal entities that their programs are responsible for properly handling and providing care for the animals. An assessment welfare program reported routinely to the public will increase transparency concerning the humane treatment of BLM's WH&Bs, as recommended by the 2008 US Government Accountability Office.

STANDARDS

Deficiency Definitions

Major Deficiency: impacts welfare of WH&Bs; usually is a procedure. Appropriate wording is "must," "unacceptable," "prohibited."

Minor Deficiency: a reporting deficiency (lack of, not enough detail) or involves an uncontrollable situation. Appropriate wording is "should."

WH&Bs = Wild horses and burros

SECTION 1.

OFF-RANGE CORRAL FACILITY STANDARDS

I. FACILITY PERSONNEL

A. Facility management establishes and implements standards of care, treatment, and handling of WH&Bs, and communicates expectations to all personnel to ensure the humane care and treatment of all WH&Bs at the facility. (**major**)

B. The facility must have employees with training, skills, and experience to observe, move, and handle the WH&Bs on the facility. (**major**)

C. The facility must have personnel that can properly maintain the working chute systems and facility infrastructure to provide for the safe housing, movement, and processing of the WH&Bs. (**major**)

D. The facility should be staffed by appropriate office staff for record maintenance and recording. (minor)

II. FACILITY DESIGN

A. Facilities must be operated in a manner to provide a safe, clean and supportive environment for all WH&Bs. (major)

B. WH&Bs must be allowed to exist in an undisturbed environment unless being prepared for health procedures, identification, adoption events, or transportation. (major)
C. All WH&Bs must have adequate space to move freely within a pen enclosure. (major)
D. The facility must have a sufficient number of pens available to sort WH&Bs according to sex, age, temperament, health status, or physical condition as needed. (major)
E. Facility fences, gates, alleys, tubs, and working chutes must be constructed of stout materials and must be maintained in proper working condition. (major)
F. Fences in pens, alleys, and working chute systems must be not less than 6 feet high for horses, 5 feet high for burros, and the bottom rail must not be more than 12 inches from ground level. (major)
G. Fences must be of stout design and be maintained in proper condition with no holes, gaps, or sharp edges which could result in WH&Bs being injured. (major)
H. All WH&Bs within a pen must have adequate dry space to rest. (major)
I. Ground surfaces in pens must be maintained to promote drainage, reduce wet ground conditions, and allow for routine manure removal. (major)
J. Watering systems in WH&B holding pens must provide unlimited access to clean water appropriate for livestock at all times. (major)
K. Feeding areas must be accessible to all WH&Bs in the enclosure and maintained as a dry area without excessive manure accumulation. (major)
L. An appropriate squeeze chute maintained in proper working order must be available for safely restraining WH&Bs for necessary procedures at the facility including hoof trimming. (major)
M. Facilities must provide access to shade and shelter (wind breaks) in pens designated for compromised animals needing special care (i.e., injured or weak animals). Additional provisions for shade and shelter (wind breaks) will be evaluated and determined by managers as appropriate for their region, the function of their facility and the condition of the animals under their care. (major)

N. Shelter structures should be designed or constructed to reduce the risk for injury of the WH&Bs and maintained to avoid excessive manure and mud. (minor)

III. RECEIVING WILD HORSES AND BURROS

A. Loading and Unloading Facilities

1. Facilities in areas for loading and unloading WH&Bs must be maintained in a safe and proper working condition, including gates and doors that swing freely and latch or tie easily as designed. (**major**)
2. The side panels of the loading chute must be a minimum of 6 feet high and covered with materials such as plywood or metal without holes that may cause injury. (**major**)
3. There must be no holes, gaps or openings, protruding surfaces, or sharp edges present in fence panels or other structures that may cause escape or possible injury. (**major**)
4. Loading and unloading ramps must have a non-slip surface and be maintained in a safe and proper working condition to prevent slips and falls. Examples of non-slip floors would include, but not be limited to, rubber mats, sand, shavings, and steel reinforcement rods or rough surfaces built into the ramp. There must be no holes in flooring or items that cause an animal to trip. (**major**)
5. Trailers must be properly aligned with loading and unloading chutes and panels with no gaps between the chute/panel and floor or sides of the trailer creating a situation where a WH&B could injure itself. (**major**)
6. Stock trailers should be positioned for loading and unloading such that there is no more than 12" clearance between the ground and floor of the trailer for burros and 18" for horses. (minor)

B. Receiving Procedures

1. All unloading of WH&Bs must be performed during daylight hours except when unforeseen circumstances develop and the Authorized Officer approves of the use of supplemental light. (**major**)

2. At the time of unloading, WH&Bs must be inspected by facility personnel to look for signs of infectious disease, sickness or injury and take appropriate steps if these conditions are observed. (**major**)
3. Any WH&B needing immediate veterinary attention must be identified and separated as necessary for examination, and a consultation with a veterinarian must be conducted within 4 hours. (**major**)
4. An authorized, properly trained, and experienced person, as well as euthanasia equipment and supplies, must be available immediately following unloading in the event that the emergency euthanasia of an animal is required. Euthanasia must be done in compliance with BLM Euthanasia policy. (**major**)
5. The receiving Authorized Officer must document any WH&B that is recumbent or dead upon arrival at the destination. (**major**)
6. Reporting of animals received from gather operations or from another BLM facility must be done in compliance with BLM policy. (**major**)
7. WH&Bs received from a gather must remain segregated from resident animals at the facility until a negative test for Equine Infectious Anemia (EIA) can be confirmed. (**major**)
8. Newly arrived WH&Bs must immediately be provided hay and water upon unloading. (**major**)
9. When WH&Bs are received from gather operations or from another facility, the receiving facility should unload them in a manner to minimize stress. (minor)
10. WH&Bs should be penned with WH&Bs of like age, sex, and temperament. (minor)
11. Newly arrived WH&Bs should be observed for signs that they may have difficulty transitioning to hay in a domestic setting. (minor)

IV. CARE OF WILD HORSE AND BURROS

A. Veterinarian

1. Routine presence by an on-site or on-call veterinarian must be provided at each facility with records of those visits maintained at the facility. (**major**)
2. A veterinarian must be available to collect and submit blood samples for Equine Infectious Anemia (EIA) testing and rabies immunization. (**major**)
3. Health care protocols must be in accordance with Program guidelines and accepted by the facility veterinarian for preventive health care procedures (vaccinations, deworming, hoof trimming, freeze marking). Medical treatments will be prescribed by or performed under the supervision of a veterinarian. Surgical procedures will only be performed by a veterinarian. (**major**)
4. WH&B deaths in a facility should be discussed with the facility veterinarian to review the probable causes of death. (minor)
5. If multiple animals die due to a related cause or unknown cause(s), a post-mortem examination or necropsy should be performed by the facility's on-call or on-site veterinarian to identify the cause of death where possible. Laboratory testing should be performed when possible to aid in determining the cause of death or related underlying factors that may be present when these are not readily apparent during the gross examination. (minor)
6. If a series of unexplained deaths occurs, facility quarantine measures must be considered with input from the attending veterinarian until the cause has been determined or when indicated until adequate bio-security measures are in place to prevent the spread of disease. Coordination with State and/or Federal Animal Health Officials in consultation with the attending veterinarian must be done if this occurs. (**major**)

B. Bio-Security

1. Authorized Officers must consult with the on-site or on-call veterinarian(s) to establish and review biosecurity and health care decisions. (**major**)

2. Newly arrived WH&Bs from gather operations must not be co-mingled with resident animals at the facility. (**major**)
3. Pens should be provided to segregate sick, infectious, injured, or weak WH&Bs from other healthy individuals in the facility when possible. (minor)
4. Hazardous or infectious biological waste (i.e., sharps, needles, blood samples, etc.) must be properly disposed in labeled containers. (**major**)
5. Working chute and tub systems should be routinely cleaned and disinfected to reduce the risk of disease transmission, especially after moving contagious WH&Bs through the system. (minor)

C. Feed and Water

1. Quality hay must be provided daily to WH&Bs in the amount of 2-3% of their body weight per day. **(major)**
2. Fresh, clean water appropriate for livestock must be accessible to WH&Bs located in holding pens at all times. (**major**)
3. Water troughs and watering systems must be checked daily to ensure they are clean and operating correctly. (**major**)
4. Feeding sites must allow all WH&Bs within a pen simultaneous access to hay. (**major**)
5. Newly arrived WH&Bs from gather operations may be fed grass hay and transitioned to alfalfa. (minor)
6. Salt and/or mineral blocks should be provided in holding pens at all times. (minor)
7. Supplemental feeds should be provided to address the nutritional needs of the WH&Bs, if needed. (minor)

D. Preparation Procedures

1. Facilities must identify individual WH&Bs and maintain inventories in accordance with BLM policy. This includes unmarked WH&Bs as well as those provided a freeze-mark. **(major)**
2. Facilities must conduct Equine Infectious Anemia (EIA) testing and apply freeze-marks within 30 days of receiving WH&Bs, unless directed by the facility veterinarian when age or physical condition requires a delay. (**major**)

3. WH&Bs receiving a freeze-mark must be recorded on the animal preparation record using the approved Signalment Key. (**major**)
4. Foals born in the facility should be freeze-marked no earlier than 3 months of age. (minor)
5. WH&Bs must be evaluated daily by facility personnel to identify animals in poor body condition, poor hoof condition, injured, or in need of veterinary evaluation/treatment, and/or supplemental feeding. (**major**)
6. Facilities must adhere to the current BLM vaccination policy. (**major**)
7. Foals must be tested for EIA and vaccinated when weaned or at 6 months of age. (**major**)
8. Hoof trimming must be performed twice per year at minimum or as necessary to maintain hooves in a proper condition. (**major**)
9. Castration of stallions and jack burros must be performed by a veterinarian using general anesthesia. If a surgical plane of anesthesia is not reached initially animals must be re-dosed or the procedure postponed until the problem can be resolved. (**major**)
10. Stallions and jack burros should be castrated as soon as approved by the on-site veterinarian for the procedure in accordance with BLM policy. (minor)
11. WH&Bs being prepared for freeze-marking, blood collection, vaccination, hoof trimming, sorting, and other care treatments should be handled to minimize stress and injuries (minor).

E. Euthanasia Procedures

1. The decision to euthanize and method of euthanasia must be directed by the Authorized Officer(s) who may consult with the on-site/on-call veterinarian. (**major)**
2. Euthanasia must be done in compliance with BLM Euthanasia policy. (**major**)
3. An authorized, properly trained, and experienced person, as well as euthanasia equipment and supplies, must be available at off-range corral facilities in the event that the emergency euthanasia of an animal is required. (**major**)

4. Any WH&B that dies or is euthanized must be documented including the circumstances, freeze brand ID if present, a description of the age, sex, color of the animal and the reason the animal was euthanized. A death record must be entered into the WHBPS. (**major**)
5. If an animal is euthanized due to an unknown cause, a post-mortem examination or necropsy should be performed by the facility's on-call or on-site veterinarian to identify the cause of death where possible. Laboratory testing should be performed when possible to aid in determining the cause of death or related underlying factors that may be present when these are not readily apparent during the gross examination. (minor)

F. Carcass Disposal

1. The Authorized Officer must ensure that appropriate procedures are in place for the timely disposal of carcasses when necessary at off-range corral facilities. (**major**)
2. Disposal of carcasses must be in accordance with applicable state and local laws. (**major**)

V. HANDLING WILD HORSES AND BURROS

A. Willful Acts of Abuse

1. Hitting, kicking, or beating any WH&Bs in an abusive manner is prohibited. **(major)**
2. Dragging a recumbent WH&Bs without a sled, side board or slip sheet is prohibited. Ropes used for moving the recumbent animal must be attached to the sled, slide board, or slip sheet. (**major**)
3. There should be no deliberate driving of WH&Bs into other animals, closed gates, panels, or other equipment. (minor)
4. There should be no deliberate slamming of gates and doors on WH&Bs. (minor)
5. There should be no excessive noise (e.g., constant yelling) or sudden activity causing WH&Bs to become unnecessarily flighty, disturbed or agitated. (minor)

B. General Handling

1. All sorting, loading or unloading of WH&Bs must be performed during daylight hours except when unforeseen circumstances develop and the Authorized Officer approves of the use of supplemental light. (**major**)
2. Halters and ropes tied to a WH&B may be used to roll, turn, position, or load a recumbent animal, but a WH&B must not be dragged across the ground by a halter or rope attached to its body while in a recumbent position. (**major**)
3. WH&Bs should be moved into and out of pens in a manner that will minimize stress and injury. (minor)
4. When possible, WH&Bs should be allowed to move at their own pace to new pens or sorting/handling locations. (minor)
5. WH&Bs should be handled to enter runways or chutes in a forward direction. (minor)
6. Any unattended WH&Bs or a single WH&B should not remain in single-file alleyways, runways or chutes longer than 30 minutes. (minor)
7. No equipment should be operated in such a manner as to cause flighty behavior or injury to WH&Bs. (minor)

B. Handling Aids

1. Handling aids such as flags and shaker paddles must be the primary tools for driving and moving WH&Bs during handling and transport procedures. Contact of the flag or paddle end of primary handling aids with a WH&B is allowed. Ropes looped around the hindquarters may be used from horseback or on foot to assist in moving an animal forward or during loading. **(major)**
2. Electric prods must not be used routinely as a driving aid or handling tool. Electric prods may be used in limited circumstances only if the following guidelines are followed:
    a. Electric prods must only be a commercially available make and model that uses DC battery power with batteries fully charged at all times. (**major**)
    b. The electric prod device must never be disguised or concealed. (**major**)

c. Electric prods must only be used after three attempts using other handling aids (flag, shaker paddle, voice or body position) have been tried unsuccessfully to move the WH&Bs. (**major**)
d. Electric prods must only be picked up when intended to deliver a stimulus; these devices are not constantly carried by the handlers. (**major**)
e. Space in front of an animal should be available for the WH&B to move forward prior to application of the electric prod. (**major**)
f. Electric prods must never be applied to the face, genitals, anus, or underside of the tail of a WH&B. (**major**)
g. Electric prods must not be applied to any one WH&B more than three times during a procedure (e.g., sorting, loading) except in extreme cases with the approval of the Authorized Officer. Each exception must be approved at the time by the Authorized Officer. (**major**)

SECTION 2.

TRANSPORTATION STANDARDS TO ANOTHER OFF-RANGE CORRAL FACILITY, OFF-RANGE PASTURE, ECO-SANCTUARY OR ADOPTION EVENT

I. LOADING AND UNLOADING FACILITIES

A. Facilities in areas for loading and unloading WH&Bs must be maintained in a safe and proper working condition, including gates and doors that swing freely and latch or tie easily as designed. (**major**)

B. The side panels of the loading chute must be a minimum of 6 feet high and covered with materials such as plywood or metal without holes that may cause injury. (**major**)

C. There must be no holes, gaps or openings, protruding surfaces, or sharp edges present in fence panels or other structures that may allow escape or cause possible injury. (**major**)

D. Loading and unloading ramps must have a non-slip surface and be maintained in a safe and proper working condition to prevent slips and falls. Examples of non-slip floors would include, but not be limited to, rubber mats, sand, shavings, and steel reinforcement rods or rough surfaces built into the ramp. There must be no holes in flooring or items that cause an animal to trip. (**major**)

E. Trailers must be properly aligned with loading and unloading chutes and panels with no gaps between the chute/panel and floor or sides of the trailer creating a situation where a WH&B could injure itself. (**major**)

F. Stock trailers should be positioned for loading and unloading such that there is no more than 12” clearance between the ground and floor of the trailer for burros and 18” for horses. (minor)

II.CARE OF WILD HORSES AND BURROS DURING TRANSPORT PREPARATION PROCEDURES

A. WH&Bs being transferred to another off-range corral facility, off-range pasture, eco-sanctuary or adoption event must have a current negative EIA test, be in a state of health appropriate for a Certificate of Veterinary Inspection (CVI), and be accompanied by appropriate paperwork as required by the laws of the receiving state. **(major)**

B. WH&Bs being transferred to another off-range corral facility, off-range pasture, eco-sanctuary or adoption event must be fit to endure the travel. (**major**)

    1. WH&Bs that are non-ambulatory, blind in both eyes, or severely injured must not be loaded and shipped unless it is to receive immediate veterinary care or euthanasia (**major**)

    2. WH&Bs that are weak or debilitated must not be transported without the approval of Authorized Officer in consultation with the on-site veterinarian. Appropriate actions for their care during transport must be taken according to the Authorized Officer. (**major**)

C. WH&Bs being transferred to another off-range corral facility, off-range pasture, eco-sanctuary, or adoption event must be sorted prior to shipping to ensure compatibility and minimize aggressive behavior that may cause injury during transport. (**major**)

D. Except for exceptional circumstances including but not limited to trap site adoptions, WH&Bs being offered for adoption should have received primary and booster vaccinations as well as deworming medications prior to adoption. (minor)

II. VEHICLES

A. Straight deck trailers or stock trailers must be used for transporting WH&Bs to another off-range corral facility, off-range pasture, eco-sanctuary or adoption event. **(major)**

    1. Two-tiered or double deck trailers are prohibited. **(major)**
    2. Transport vehicles for WH&Bs must have a covered roof containing them such that WH&Bs cannot escape. **(major)**

B. WH&Bs must have adequate headroom during loading and unloading and must be able to maintain a normal posture with all four feet on the floor during transport without contacting the roof. **(major)**

C. The width and height of all gates and doors must allow WH&Bs to move through freely. **(major)**

D. All gates and doors must open and close easily and be able to be secured in a closed position. **(major)**

E. Loading and unloading ramps of vehicles must have a non-slip surface and be maintained in a safe and proper working condition to prevent slips and falls. Examples of non-slip flooring would include, but not be limited to, rubber mats, sand, shavings, and steel reinforcement rods built into ramp. There must be no holes in the flooring or items that can cause an animal to trip. **(major)**

F. Transport vehicles more than 18 feet and less than 40 feet in length must have a minimum of one partition gate providing two compartments; transport vehicles 40 feet or longer must have at least two partition gates to provide a minimum of three compartments. **(major)**

G. Partition gates in transport vehicles should be used to distribute the load into compartments during travel. (minor)

H. All partitions and panels inside of trailers must be free of sharp edges or holes that could cause injury to WH&Bs. **(major)**

I. The inner lining of all trailers must be strong enough to withstand failure by kicking that would lead to injuries. **(major)**

J. Surfaces and floors of trailers must be cleaned of dirt, manure and other organic matter prior to the beginning of a shipping event, and must have non-skid material on the trailer floor, such as, wood shavings, rubber non-skid mats etc. **(major)**

III. TRANSPORT PROCEDURES

A. WH&Bs must be loaded at the following rates: **(major)**

1. 12 square feet per adult horse.
2. 6.0 square feet per dependent horse foal.
3. 8.0 square feet per adult burro or yearling horse.

4. 4.0 square feet per dependent burro foal

B. Planned drive time from off-range corral facility to another off-range corral facility, off-range pasture, eco-sanctuary, or adoption must not exceed 24 hours without unloading. (**major**)

C. Trucking companies or haulers must be provided directions and contact phone numbers for the facility or adoption location. **(major**)

D. A transportation event that is longer than 24 hours will require unloading and a minimum rest period of 8 hours with access to hay and water prior to being re-loaded and sent to the final destination. **(major**)

E. While in transit, WH&Bs must be observed by the transport driver a minimum of once every 8 hours. **(major)**

F. Non-ambulatory or recumbent WH&Bs must be evaluated on the trailer and either euthanized or removed from the trailers using a sled, slide board or slip sheet. (**major**)

G. WH&Bs should not be allowed to remain standing on straight-deck and stock trailers while not in transport for a combined period of greater than three (3) hours. (minor)

IV. RECEIVING PROCEDURES

See Section 1. Off-range Corral Standards, Part III. B. 1-5 and 8-11.

SECTION 3.

ADOPTION EVENT STANDARDS

I. ADOPTION EVENT PERSONNEL

A. BLM establishes and implements standards of care, treatment, and handling of WH&Bs, and communicates expectations to all personnel to ensure the humane care and treatment of all WH&Bs at the adoption event. **(major)**

B. The adoption event must have employees with training, skills, and experience to observe, move, and handle the WH&Bs at the event. **(major)**

C. The adoption event must have maintenance staff that can properly maintain the facilities to provide for the safe housing, movement, and processing of the WH&Bs. **(major)**

II. SATELLITE FACILITY DESIGN

A. All satellite facilities must be operated in a manner to provide a safe, clean and supportive environment for all WH&Bs. **(major)**

B. The satellite facility must have a sufficient number of pens available to sort WH&Bs according to sex, temperament, or physical condition as needed. (**major**)

C. The satellite facility's fences, gates, alleys, and working chutes must be constructed of stout materials and must be maintained in proper working condition. (**major**)

D. Fences in adoption pens, alleys, and working chute systems must not be less than 6 feet high for horses and 5 feet high for burros. (**major**)

E. Adoption pen fences must be of stout design and must be maintained in proper condition with no holes, gaps, or sharp edges that could result in WH&Bs being injured. (**major**)

F. Watering systems in WH&B adoption holding pens must provide unlimited access to clean water appropriate for livestock at all times. (**major**)

G. Adoption pen feeding areas must be accessible to all WH&Bs in the pen at the same time. (**major**)

H. Ground surfaces in adoption pens should promote drainage to reduce wet ground conditions. (minor)

I. WH&Bs in pens at the satellite facility should be maintained at a stocking density such that when at rest all WH&Bs occupy no more than half the pen area. (minor)

III. CARE OF WILD HORSES AND BURROS RECEIVED AT SATELLITE FACILITIES

A. Provisions to provide care by a veterinarian in a timely manner on-site, on call, or by transporting an animal to a veterinarian must be made in advance**. (major)**

B. Provisions for medical care (treatments including euthanasia if necessary) at adoption events will be made in accordance with BLM policy and carried out as described above for preparation facilities (Section 1. Off-range Corral Facility Standards, Part IV. A. 1-6). (**major**)

C. WH&Bs must be sorted upon arrival at adoption events into pens with animals of appropriate age and temperament such that animals can promptly begin a period of rest, eating, and drinking with limited disturbance. **(major)**

D. All WH&Bs must have adequate space to move freely within an adoption facility pen. **(major)**

E. WH&Bs, to the greatest extent possible, must be allowed to exist in an undisturbed environment after arriving at an adoption facility until the adoption event begins. **(major)**

F. Quality hay must be provided to WH&Bs in the amount of 2-3% of their body weight per day upon unloading following transport and daily during the adoption event. **(major)**

G. WH&Bs at adoption events may be supplemented with feed or water supplements to address the stress of transport. (minor)

H. WH&Bs will be sorted during the adoption event in a manner to reduce stress and opportunity for injury. (minor)

I. WH&Bs transported for more than 12 hours to an adoption event should be allowed a rest period of 5 hours or more prior to formal viewing by the adopting public. (minor)

IV. EUTHANASIA PROCEDURES AT SATELLITE FACILITIES

See Section 1. Off-range Corral Facility Standards, Part IV. E. 1-4.

V. CARCASS DISPOSAL AT SATELLITE FACILITIES

See Section 1. Off-range Corral Facility Standards, Part IV. F. 1-2.

VI. HANDLING WILD HORSES AND BURROS AT SATELLITE FACILITIES

A. Willful Actus of Abuse

See Section 1. Off-range Corral Facility Standards, Part V. A. 1-5

B. General Handling

See Section 1. Off-range Corral Facility Standards, Part V. B. 1-7

C. Handling Aids

See Section 1. Off-range Corral Facility Standards, Part V. C. 1-2

REQUIRED DOCUMENTATION AND RESPONSIBILITIES OF AUTHORIZED OFFICERS

REQUIRED DOCUMENTATION

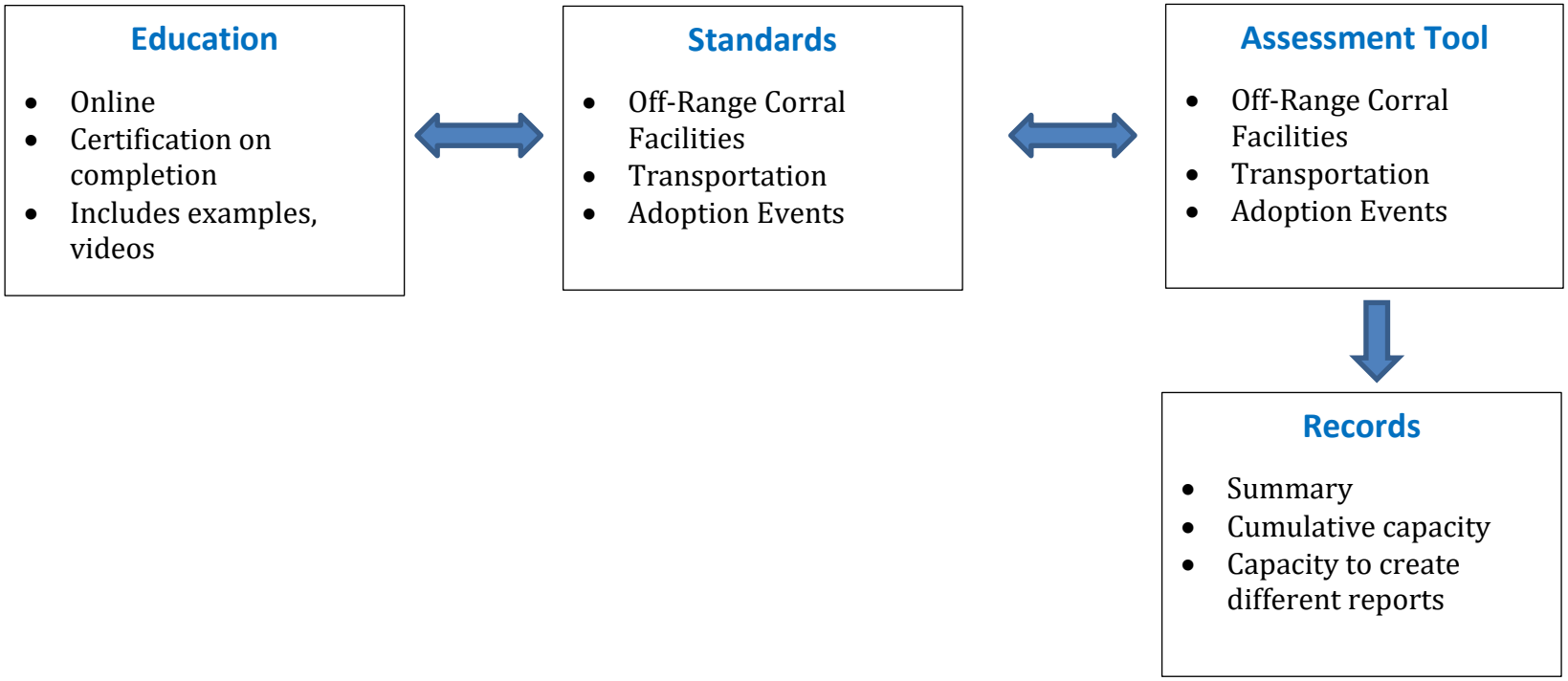
STANDARD	DOCUMENTATION
Section 1	**Off-Range Corral Facility Standards**
Part III. B. 5	Any WH&B that is recumbent or dead upon arrival at the destination
Part IV. A. 1	Record of visits by on-site/on-call veterinarian
Part IV. E. 4	Any WH&B that dies or is euthanized at facility, including the circumstances, freeze brand ID if present, a description of the age, sex, color of the animal and the reason the animal was euthanized. A death record must be entered into the WHBPS.
Section 2	**Transportation Standards**
Part V	Refer to Section 1. Off-range Corral Facility Standards, Part III. B. 5, as above.
Section 3	**Adoption Event Standards**
Part IV	Refer to Section 1. Off-range Corral Facility Standards, Part IV. E. 4, as above

RESPONSIBILITIES

STANDARD	RESPONSIBILITY
Section 1	**Off-Range Corral Facility Standards**
Parts III. B. 1 & V.B.1	Approve use of supplemental lighting when sorting, loading, or unloading WH&Bs
Part III. B. 5	Document any WH&B that is recumbent or dead upon arrival at the destination
Part IV. B. 1	Consult with on-site/on-call veterinarian to establish and review biosecurity and health care decisions
Part IV. E. 1	Direct decision regarding euthanasia and method of euthanasia for any WH&B; may consult with on-site/on-call veterinarian
Part IV. F. 1	Ensure that appropriate procedures are in place for carcass disposal
Part V. C. 2. g	Approve use of electric prod more than three times, for exceptional cases only

STANDARD	RESPONSIBILITY
Section 2	**Transportation Standards**
Part II. D	Approve of transport and care during transport for weak or debilitated WH&B
Section 3	**Adoption Event Standards**
Part VI. B	Refer to Section 1. Off-range Corral Facility Standard, Part IV.B.1, above.
Part VI. C	Refer to Section 1. Off-range Corral Facility Standard, Part V.C.2.g, above.

PROPOSED CAWP COMPONENTS FOR OFF-RANGE CORRAL FACILITIES, TRANSPORTATION, AND ADOPTION EVENTS



BioOne RESEARCH EVOLVED

EFFECTS OF A GONADOTROPIN-RELEASING HORMONE VACCINE ON OVARIAN CYCLICITY AND UTERINE MORPHOLOGY OF AN ASIAN ELEPHANT (*ELEPHAS MAXIMUS*)

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EFFECTS OF A GONADOTROPIN-RELEASING HORMONE VACCINE ON OVARIAN CYCLICITY AND UTERINE MORPHOLOGY OF AN ASIAN ELEPHANT (*ELEPHAS MAXIMUS*)

Nancy C. Boedeker, D.V.M., Lee-Ann C. Hayek, Ph.D., Suzan Murray, D.V.M, Dipl. A.C.Z.M., David M. de Avila, and Janine L. Brown, Ph.D.

Abstract: This report describes the successful use of a gonadotropin-releasing hormone (GnRH) vaccine to suppress ovarian steroidogenic activity and to treat hemorrhage and anemia associated with reproductive tract pathology in a 59-year-old Asian elephant (*Elephas maximus*). The Repro-BLOC® GnRH vaccine was administered subcutaneously as a series of 4 boosters of increasing dose from 3 to 30 mg of recombinant ovalbumin-GnRH fusion protein given at variable intervals after initial vaccination with 3 mg protein. Efficacy was confirmed over a year after initial vaccination based on complete ovarian cycle suppression determined by serum progestagen analyses. Estrous cycle suppression was associated with a significant increase in GnRH antibody binding and subsequent decrease in serum luteinizing hormone and follicle-stimulating hormone concentrations. Ultrasonographic examinations of the reproductive tract documented a reduction in uterine size and vascularity after immunization. The hematocrit level normalized soon after the initial intrauterine hemorrhage, and no recurrence of anemia has been detected. No substantive adverse effects were associated with GnRH vaccination. The results indicate that GnRH vaccination in elephants shows potential for contraception and management of uterine pathology in older elephants.

Key words: Contraception, elephant, *Elephas maximus,* gonadotropin-releasing, progestagen, vaccine.

INTRODUCTION

Ovarian function in elephants is similar to that in other mammals and is controlled by secretion of the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), presumably under the influence of hypothalamic gonadotropin-releasing hormone (GnRH).[4,5] In general, FSH is responsible for stimulating ovarian follicular growth and development and for the production of estrogens, whereas LH induces ovulation, formation of the corpus luteum, and production of progestagens.[6,25] Elephants are unique in having the longest documented mammalian estrous cycle, of 13–17 wk, and exhibiting two successive waves of follicular development that culminate in two distinct LH surges, with only the second inducing ovulation.[8,9,35]

One documented problem of older female elephants and other long-lived species in captivity is termed asymmetric reproductive aging.[24,28] In the wild, most adult female elephants are either pregnant or lactating; they experience comparatively few reproductive cycles in their lifetime. In captive Asian elephants, primarily nulliparous females over 30 yr of age or those that have not calved within a 10- to 15-yr period, numerous reproductive tract pathologies develop.[24,27–29,44] For example, older Asian elephants often develop endometrial hyperplasia and/or multiple leiomyomata (benign myometrial tumors).[1,27–29,44,45,57] Periodic mucohemorrhagic vaginal discharge can result from these pathologic alterations. These specific conditions have not been reported in wild Asian elephants. Because older cycling cows appear to be particularly susceptible, it has been speculated that continuous steroid hormone (estrogens and/or progestagens) exposure associated with ovarian cyclicity of nonbred females has a negative and cumulative effect on reproductive health and urogenital tract integrity.[1,24,28] To prevent these pathologies from developing, treatments to shut down ovarian activity should be considered if a female is not intended for breeding.

GnRH vaccines have been designed to stimulate the production of anti-GnRH antibodies that

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block binding of endogenous GnRH to gonadotrope receptors in the pituitary gland. This action inhibits the release of FSH and LH from the anterior pituitary, thereby causing the cessation of ovarian steroidogenic activity and reproductive cyclicity. Immunization against GnRH has been used successfully to suppress ovarian function in a number of domestic, laboratory, wildlife, and livestock species.[3,10,18,22,23,30,31,36,42,49,55] The primary structure of the decapeptide GnRH is largely conserved in mammals, but by itself is not immunogenic.[23,46] The vaccine in this study was developed by using a recombinant ovalbumin-GnRH fusion protein, marketed under the trade name Repro-BLOC® (Amplicon Vaccine, LLC, Pullman, Washington 99163, USA).[11,20,33,51,56] The adjuvant is a combination of Freund's incomplete adjuvant with a synthetic cytosine guanine oligodeoxynucleotide that mimics the immunostimulatory effects of bacterial deoxyribonucleic acid because of its relatively high content of unmethylated cytosine guanine dinucleotides.[10,11]

This case report describes the use of Repro-BLOC to suppress ovarian cyclicity to prevent hemorrhage and anemia associated with reproductive tract pathologies in an older female Asian elephant at the National Zoological Park. To date, to the authors' knowledge, there are no published reports on the use of GnRH vaccination to control reproductive activity in female elephants. Possible benefits of using this approach to address problems associated with asymmetric reproductive aging as well as for contraception in elephants are discussed.

CASE REPORT

The subject was a 59-yr-old, approximately 3,300 kg, female Asian elephant who had been at the Smithsonian Institution's National Zoological Park for 46 yr. She was housed with a 33-yr-old female Asian elephant and occasionally that female's 6-yr-old male offspring. This female had a relatively unremarkable medical history, with the exception of chronic dental malposition and a small chronic left elbow abscess. At the time of presentation, she was on long-term, low-dose ibuprofen (3,600 mg p.o. s.i.d.) for management of possible discomfort associated with mastication, and a glucosamine and chondroitin supplement (Cosequin® Equine Concentrated Powder, Nutramax Laboratories, Edgewood, Maryland, 21040 USA) intended for maintenance of joint health.

On 14 January 2007, keepers reported that the elephant had a decreased appetite and stool production, mild lethargy, and reluctance to train, particularly for behaviors that involved lying down. A complete blood cell count (CBC) and chemistry panel were unremarkable other than a hematocrit concentration of 30.8% and hemoglobin level of 10.1 g/dl, both at the low end of her historic range (hematocrit concentration, 30–43.7%, average 36%; hemoglobin level, 10.1–14.9 g/dl, average 12.02 g/dl), and an elevated fibrinogen level of 900 mg/dl (range, 200–700 mg/dl; average, 479 mg/dl). Urinalysis, fecal parasite check, fecal cytology, and fecal occult blood were unremarkable. Mild colic of unknown etiology was initially suspected, and clinical signs were rapidly responsive to a 2-day course of treatment with injectable flunixin meglumine (Banamine® 50 mg/ml, Merck Intervet/Schering Plough Animal Health, Summit, New Jersey 07901, USA; 1,000–3,000 mg i.m. s.i.d.). The hematocrit concentration remained relatively stable, at 30.1% on 16 January, and 31.9% on 22 January 2007.

On 29 January 2007, 15 days after initial presentation, the female's appetite and activity had improved, although it was not completely back to normal levels. However, bloodwork showed that the hematocrit concentration had dropped dramatically, to 19.3%, with a hemoglobin concentration of 6.4 g/dl. Repeated samples confirmed the severity of the anemia. There was no evidence of hemolysis and mean corpuscular volume (MCV) (128/fl), mean corpuscular hemoglobin (MCH) (42 pgm/cell), and mean corpuscular hemoglobin concentration (MCHC) (33 g/dl) remained within her normal ranges, but target cells were subjectively increased. Fibrinogen level had returned to within her normal range, at 500 mg/dl. Fecal parasite check and fecal occult blood tests were negative. Urinalysis, fecal cytology, iron panel, mineral panel, estrogen level, and coagulation panel results were within acceptable limits. Based on the diagnostic test results and clinical signs, a recent episode of acute internal hemorrhage was suspected. Empiric treatment with ceftiofur (Naxcel®, Pfizer Animal Health, New York, New York 10017, USA) was administered at 6–8 g i.m. s.i.d. beginning on 29 January 2007, for a total of 23 days.

By 7 February 2007, 24 days after initial presentation, the elephant's appetite and activity had returned to normal baseline levels and s.i.d. to every other day blood samples showed a gradual increase in the hematocrit concentration, to 28.7%, and in the hemoglobin level, to 9.1 mg/dl. At that time, the elephant was given 30 mg detomidine (Dormosedan® 10 mg/ml; Pfizer Animal Health, New York, New York, 10017,

USA) and 30 mg butorphanol i.m. (Torbugesic® 10 mg/ml; Pfizer) for standing sedation in an elephant restraint device to allow for rectal and transabdominal ultrasound evaluation by an experienced elephant ultrasonographer. An additional 10 mg detomidine and 10 mg butorphanol were administered i.m. as a supplement to deepen sedation at approximately 60 min into the procedure. Transabdominal ultrasound was performed on the left side; no evidence of free fluid was identified, and the spleen, part of the caudal liver lobe, and intestinal loops appeared normal. Transrectal examination of the caudal abdomen was performed by using a 3.5 MHz convex transducer to scan the caudal region of the genital tract and by using a 5.0–7.5 MHz transducer attached to a specially designed probe extension to scan the cranial region. The examination revealed a large mass consistent with a blood clot, estimated at more than 18 L, which expanded the vagina and extended into the uterus (Fig. 1A). The uterus was drawn ventrally into the abdominal cavity and could not be examined in its entirety. Visibly enlarged blood vessels were identified on the dorsal surface of the reproductive tract near the cervix (Fig. 1B). Although these vessels were considered likely to be associated with a vascular tumor, no masses consistent with a tumor could be identified, possibly due to the limited visualization of the ventrally displaced uterus. The caudal lobe of the left kidney appeared normal, and no fluid was noted in the caudal pelvic flexure.

The most likely cause for this elephant's episode of colic followed by severe anemia was considered to be hemorrhage into the vagina and uterus. Underlying reproductive tract pathology was strongly suspected. Differentials included leiomyoma, cystic endometrial hyperplasia, uterine artery rupture, neoplasia, or infection, but the etiology could not be definitively determined due to limited visualization of the reproductive tract, which was expanded and weighed down by the suspected blood clot. The decision was made to resolve the reproductive tract pathologies by using a GnRH vaccine (Repro-BLOC).

MATERIALS AND METHODS

The elephant had been conditioned to venipuncture without sedation, as part of the normal management routine. Blood was collected from an ear vein approximately weekly, generally in the morning. The blood sample was maintained at approximately 4°C, allowed to clot at room temperature, then centrifuged (approximately 1,500 *g*) and frozen. Serum was stored at –20°C or colder until analysis.

The Repro-BLOC vaccine was first administered on 27 March 2007 at a dose and dose interval proven effective to sterilize heifers: 3 mg s.c., with a booster vaccination of 3 mg s.c. given approximately 14 wk later, on 3 July 2007.[11] Based on serial evaluation of serum progestagen concentrations and GnRH antibody titers, 3 additional boosters of increasing dosage (7.5 mg on 29 November 2007, 8 mo after initial injection; 12.5 mg on 27 March 2008, 12 mo after the initial injection; 30 mg on 20 November 2008, 20 mo after the initial injection) were given. The total injection volume increased from 4.5 to 22 ml, with increases in dosage; the volume of each dose was divided in two and administered s.c. by hand injection at two sites on the neck.

Management changes during the study interval included no longer asking the elephant to lie down or perform activities that were thought likely to disrupt the blood clot in her reproductive tract and potentiate further hemorrhage. Ibuprofen and Cosequin treatments were discontinued in case they were contributing to any tendency to bleed; no negative clinical effect of this change was observed. Monitoring by keepers included daily assessment of appetite, attitude, and activity level, and for vaccine site reactions, and for any indication of hemorrhage.

CBC and chemistry panels were obtained at least weekly in 2007, at least twice a month in 2008, and at least monthly with twice monthly hematocrit concentration assessments in 2009. Urine was submitted for urinalysis at least twice monthly throughout the years 2007 to 2009, primarily to monitor for hematuria. Follow-up rectal ultrasound examinations under standing sedation as previously described were performed in April of 2007 and April of 2009.

Anti-GnRH antibody titers were determined in sera at a 1:100 dilution by using a ^{125}I-GnRH radioimmunoassay.[33] Titer results were obtained from samples collected approximately weekly from 19 March 2007 (just before the initial vaccination) through 6 February 2008 and then approximately every 2 wk through 6 January 2010. Serum progestagens were analyzed by using a solid-phase ^{125}I-progesterone radioimmunoassay (Coat-a-Count®, Siemens Diagnostic Products Corporation, Costa Mesa, California 92626, USA) validated for elephants.[7] Serum FSH and LH concentrations were quantified by using double antibody ^{125}I radioimmunoassays validated for elephants.[6] Hormone results were analyzed

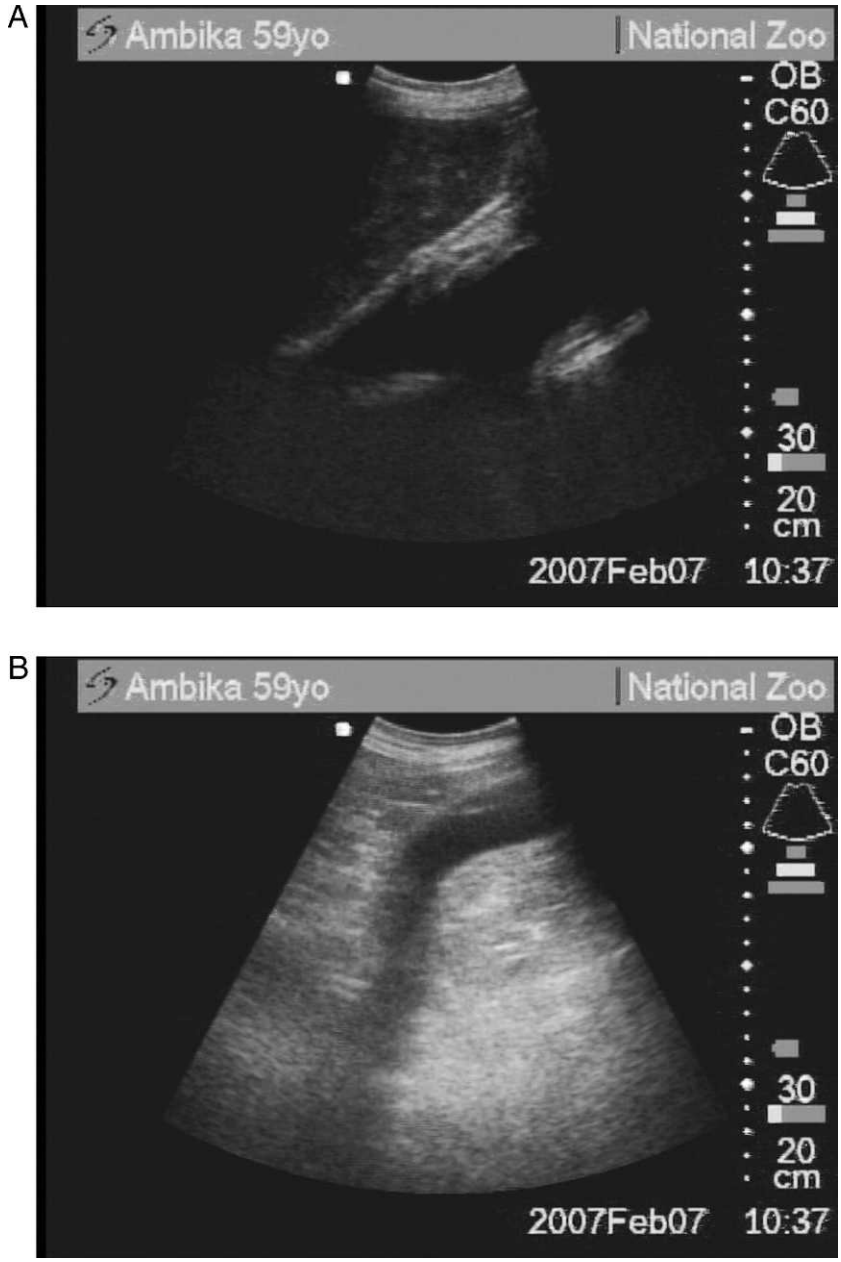


Figure 1. Ultrasound images from the 7 February 2007 examination, showing (**A**) the distended vaginal canal at the top of the image and a suspected blood clot enlarging to the left from the cervix with the urinary bladder below and (**B**) an enlarged blood vessel adjacent to the distended vagina. Images provided by Dr. Dennis Schmitt.

from samples collected approximately weekly from 4 January 2006 to 6 January 2010.

Statistical methods

To determine this elephant's estrous cycle patterns, baseline progestagen concentrations were calculated by using an iterative process previously developed for Asian elephants.[8] All data points with values above the mean plus two times the standard deviation (SD) were removed, and the process was repeated until no values that exceeded the mean plus two times the SD remained. The remaining data points defined the

baseline, and all points above that were considered part of a luteal phase. The highest point in each luteal phase was considered the peak. Estrous cycle duration was calculated as the number of weeks from the first increase in serum progestagens until the next increase. Peaks of LH and FSH levels also were determined by using the same iterative process by excluding values that exceeded the mean plus two times the SD.[8]

Titer and hormone data were subjected to statistical time series analysis and also were modeled with the ASTRA program with SPSS Statistics (ver. 20, 2011, IBM Corp. Armonk, New York 10504, USA) used for simple descriptive statistics, tests of assumptions, and general linear model hypothesis testing and modeling.[50] Autoregressive integrated moving average models were fit to the data for each of the variables. By using these models, the differences in cyclicity over time were examined by trend analysis methods that are related to ordinary least-squares regression.[50] In addition, the relationship over time intervals before and after vaccine introduction was examined. The variables were divided into six intervals based on the timing of vaccine administrations.

RESULTS

Health and ultrasound examinations

The elephant's appetite, attitude, activity, and weight had remained at normal levels with no recurrence of colic throughout the study period. The anemia fully resolved by 14 February 2007, and CBC parameters remained within normal ranges. Chemistry panel values remained unremarkable other than a period of mild intermittent hyperglycemia of undetermined etiology, with a low-level glucosuria in May 2009. Rare trace positive blood results on urine dipstick have been identified without elevations in red blood cells on microscopic urine sediment examination.

Episodes of passage of small volumes of mucohemorrhagic discharge after urination were noted occasionally in 2006 (prevaccination) as well as in 2007 and 2008. Cytologies of this suspected vaginal discharge revealed mild hemorrhage, mild granulocytic, and histiocytic inflammation, and small numbers of gram-positive cocci widely scattered in mucous suggestive of mild vaginitis. Pigment identified within the macrophages was consistent with hemosiderin and suggested breakdown of the ultrasonographically identified blood clot or potentially breakdown of clots from more recent undetected bleeding. This discharge continued intermittently until mid October 2008, approximately 1 mo before administration of the final vaccine booster, and has not been noted in the following 3 yr since this report.

A transient mild localized reaction occurred at both injection sites within 24 hr after the first GnRH vaccination; firm, nonpainful, subcutaneous swellings persisted for approximately 2 mo. A warm, firm, subcutaneous swelling developed as well at both injection sites after the final vaccine administration, which, in contrast to the first vaccination reaction, was sensitive to touch and caused guarded neck movement. The elephant's apparent discomfort resolved with treatment with ibuprofen (6,000 mg p.o. s.i.d.) for 3 days, but the firm subcutaneous swellings have persisted 2 yr later. Other than these local reactions, no negative adverse effects associated with the vaccine had been noted.

A transrectal ultrasound evaluation on 2 April 2007, approximately 11 wk after initial presentation and 6 days after administration of the first GnRH vaccination, revealed an approximately 10-cm-diameter, well-encapsulated, relatively homogenous, soft tissue mass in the cranial vagina. Blood vessels were identified in the capsule but not within the mass itself. Fluid and pseudomembranous tissue surrounded the mass, but there was no evidence of ongoing hemorrhage. Due to the location of the vaginal mass, there was concern that enlargement could compress the urethra and cause obstruction to urine flow and potential renal compromise. The uterus was again difficult to visualize in its entirety but appeared small, irregular, and misshapen, with prominent, visibly enlarged blood vessels, interpreted as being more suggestive of an infectious or neoplastic process than of menopausal changes. Fibrosis and cysts were identified in both kidneys.

A follow-up transrectal ultrasound was performed on 19 April 2009, approximately 5 mo after the last booster was administered and when serum monitoring had indicated cessation of ovarian cyclicity in conjunction with a rise in GnRH antibody titers. A 10-cm diameter vaginal cyst just beyond the opening to the vestibule was observed. Both ovaries appeared small and inactive (Fig. 2A). The uterus was small and indistinct, with a visible decrease in size of the uterine vessels compared with the previous studies (Fig. 2B). The vaginal soft tissue mass identified in April 2007 appeared to have resolved, and no clot or active hemorrhage associated with the reproductive organs was identified. The ultrasound findings supported the hormonal data that indicated cessation of ovarian cyclicity and reproduc-



Figure 2. Ultrasound images from the 19 April 2009 examination, showing (**A**) an inactive ovary and (**B**) the inactive uterus in cross section. Images provided by Dr. Thomas Hildebrandt.

tive quiescence. No progression of the renal changes was identified.

Serum GnRH antibody titer, progestagen, and LH and FSH profiles

GnRH percent binding data in sera diluted at 1:100 is presented in Figure 3A. Time series regression indicated a significant change in binding over time, and a quadratic trend fit to these data showed a monotonically increasing trend ($P = 0.001$). Dividing these time-dependent observations for antibody titers into six time periods starting before the first dosage resulted in significantly different and increasing means ($P < 0.001$), with SDs that increased until time period 4, then decreased (Table 1). The change in GnRH titer remained nonsignificant until after the sec-

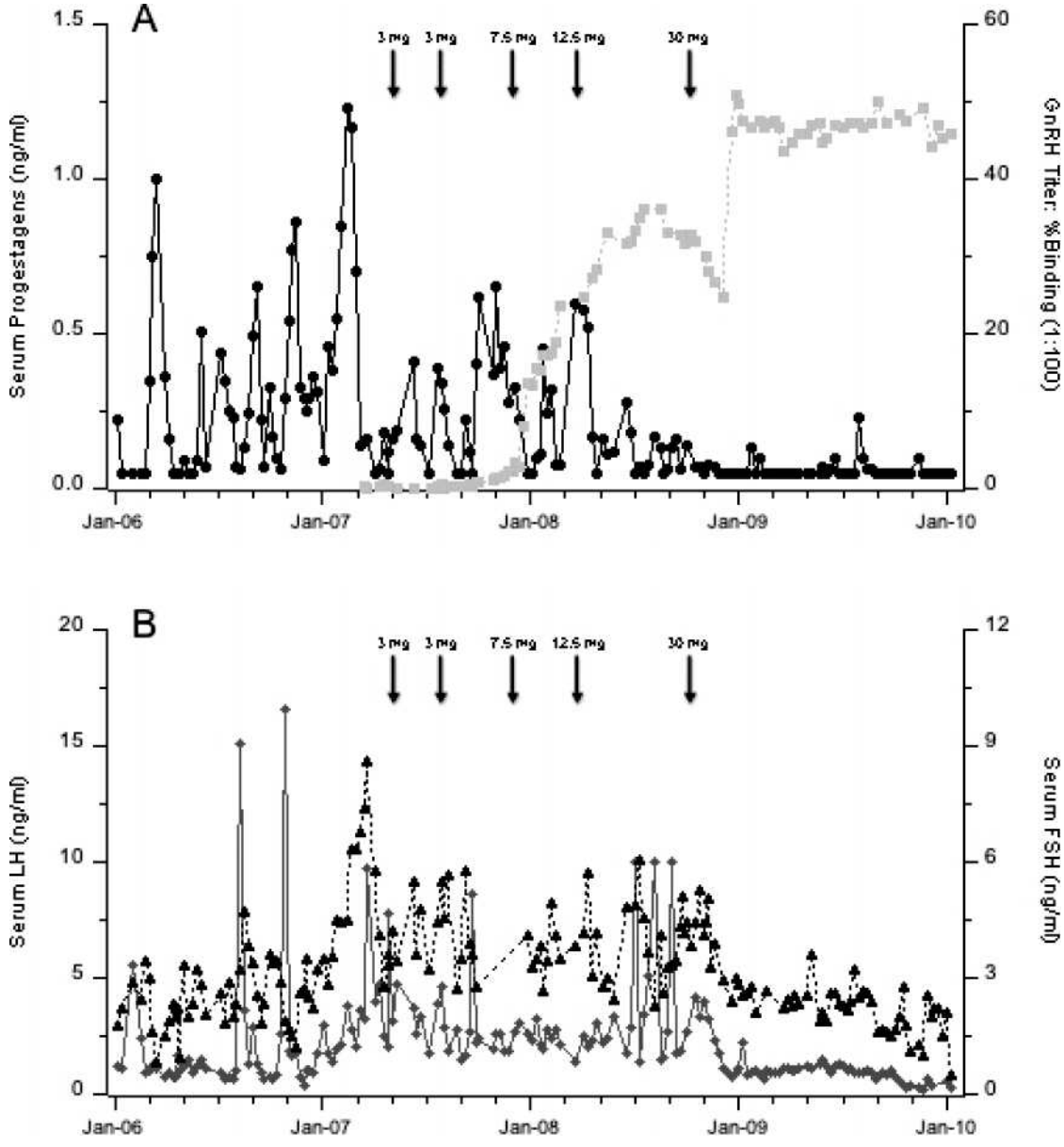


Figure 3. Profiles of (**A**) serum progesterone (●) and gonadotropin-releasing hormone (GnRH) titers (■) indicated by the percentage binding of sera diluted at 1:100; and (**B**) serum luteinizing hormone (LH) (♦) and follicle-stimulating hormone (FSH) (▲) concentrations in an Asian elephant administered a GnRH vaccine. Vaccination dosages were administered at each of five time points indicated by the arrows.

ond 3-mg dose was given on 3 July 2007 in time period 3, after which titers exhibited a continuous and significant increase. The slope of this linear fit was significant as were the two sets of combined observations ($P = 0.001$). The variances, like the means, also were significantly different across these periods ($P = 0.0001$). Although a quadratic trend could be fit, it was apparent that there was a significant change induced after time period 3. In considering all data, there were 2 distinct groups of time periods, which consisted of time periods 1 through 3, and time periods 4 through 6 (Table 1).

The length of the female's estrous cycle before vaccination (time period 1) averaged 14.1 ± 0.5 wk, with a mean peak progestagen concentration of 0.78 ± 0.4 ng/ml (range, 0.45–1.23 ng/ml).

Table 1. GnRH antibody titer, progestagen, LH, and FSH levels, means (SDs) and (ranges), during six time periods, pre- and postvaccination.[a]

Time period	Date range	Vaccine dose (mg)[b]	GnRH antibody Titer (% binding)			Progestagen (ng/ml)			LH (ng/ml)			FSH (ng/ml)		
			n	Mean (SD)	Range	n	Mean (SD)	Range	n	Mean (SD)	Range	n	Mean (SD)	Range
1	1/4/06–3/26/07	0	1	–0.09 ± 0	0.09	53	0.34 ± 0.30	0.05–1.23	53	2.32 ± 3.09	0.34–16.54	53	3.06 ± 1.62	0.83–8.59
2	3/27/07–7/2/07	3	15	–0.01 ± 0.23	–0.32–0.52	10	0.15 ± 0.11	0.05–0.41	10	3.85 ± 1.65	2.05–7.78	12	4.18 ± 0.91	2.77–5.77
3	7/3/07–11/28/07	3	21	0.83 ± 0.91	–0.35–3.28	17	0.28 ± 0.19	0.05–0.65	17	2.82 ± 1.71	1.51–8.63	12	4.25 ± 1.12	2.71–5.77
4	11/29/07–3/26/08	7.5	13	16.55 ± 6.57	2.68–27.13	15	0.20 ± 0.17	0.05–0.60	15	2.81 ± 1.82	1.44–9.14	14	4.27 ± 1.20	2.66–6.85
5	3/27/08–11/19/08	12.5	16	31.31 ± 3.02	24.67–36.11	27	0.14 ± 0.13	0.05–0.58	27	3.45 ± 2.52	1.35–10.00	30	4.03 ± 0.96	2.27–6.07
6	11/20/08–1/6/10	30	29	46.93 ± 1.68	43.66–50.83	56	0.06 ± 0.03	0.05–0.23	53	0.92 ± 0.36	0.16–2.25	48	2.23 ± 0.59	0.49–3.61

[a] GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; SD, standard deviation.

[b] Vaccine doses were administered on the first day of each of the time periods 2 through 5.

Serum progestagen concentrations decreased after GnRH vaccination, with a definitive cessation in ovarian cyclicity and luteal function after the final 30-mg vaccine dose (Fig. 3A; Table 1). The variances, like the means, were significantly different across time ($P = 0.001$). A linear, not a quadratic trend could be fit to the mean values over the time periods ($P = 0.0001$). However, it was apparent that there was a significant change in progestagen concentrations after time period 3 (Table 1). When considering the prevaccine time period 1 as a baseline, Dunnett's test showed that time periods 3 and 4 were not different from baseline, whereas the subsequent time periods 5 and 6 were lower ($P = 0.013$). The average estrous cycle length through time period 4 was 13.9 ± 0.6 wk, with a mean peak progestagen concentration of 0.45 ± 0.06 ng/ml (range, 0.40–0.65 ng/ml). In time periods 5 and 6, after the 12.5-mg dose, only 4 clear cycles were observed; the average length had decreased to 10.1 ± 0.3 wk, and progestagen peak concentrations had decreased from a high in time period 4 of 0.60 ng/ml to successive declining peak concentrations of 0.28, 0.17, 0.16 and 0.14 ng/ml (Fig. 3A). Thereafter, serum progestagen concentrations remained at or near baseline (<0.07 ng/ml).

After an initial increase compared with the prevaccination baseline (time period 1), mean LH and FSH concentrations decreased, with the greatest effect observed after the final 30-mg booster, more than 1.5 yr after the initial vaccination (Fig. 3B; Table 1). When examined as serial data, only time periods 1 and 6 were statistically different from the remainder ($P < 0.001$). Mean LH, which was more variable, declined to 0.92 ng/ml at time period 6, from a baseline of 2.32 ng/ml, whereas mean FSH declined to 2.23 ng/ml at time period 6, from a baseline of 3.06 ng/ml. The decline in gonadotropin secretion occurred coincident with the suppression of serum progestagen concentrations. This decrease in mean concentrations was associated with a dampening of pulsatile LH and FSH secretion in time period 6, as evidenced by a decrease in the SDs (Table 1). Peak concentrations of LH exceeded 6 ng/ml nine times, and peak concentrations of FSH exceeded 4 ng/ml eight times through time period 5. No significant pulses of LH or FSH were observed in time period 6 after the 30-mg dose was given.

DISCUSSION

This is the first published report, to the authors' knowledge, on the effects of a GnRH

vaccine on health and reproductive function of a female Asian elephant. Altogether, GnRH antibody titer, serum hormone, ultrasonographic, and clinical results strongly suggest that the cessation of ovarian cyclicity and the associated decrease in uterine size and vascularity occurred as a result of GnRH vaccination. The significant increase in GnRH antibody binding was inversely related to a sustained suppression in LH and FSH secretion and in serum progestagens after the final booster was administered. Based on time-series analyses, effects of the vaccine on GnRH and progestagens were evident after the 12.5-mg booster (time period 5), approximately a year after the initial injection. Significant effects on LH and FSH secretion were observed after the final 30-mg booster. The vaccinations were well tolerated, with no adverse effects other than relatively minor localized reactions at the injection sites after two of the five vaccinations. Any appreciable discomfort associated with these subcutaneous swellings resolved quickly. Thus, the results of this current study suggest that GnRH vaccination is a safe and effective method for resolving significant health problems associated with uterine pathologies likely caused by continuous ovarian cyclicity.

Caution in not overinterpreting these results is advised, however, because only one female was studied. A definitive diagnosis of the underlying pathology that led to the strongly suspected hemorrhage into this elephant's reproductive tract and associated severe anemia could not be determined. In addition, it cannot be determined with certainty if this elephant would have hemorrhaged again without therapy. Although analysis of limited laboratory data suggests that female Asian elephants can continue cycling normally into their 60s (Brown, unpubl. data), it is possible that this geriatric female would have stopped cycling naturally without vaccine administration. However, the strong correlation in timing of the rise in GnRH antibody titer with the cessation of ovarian cyclicity, appearance of reproductive quiescence on ultrasound and resolution of intermittent mucohemorrhagic vaginal discharge makes this possibility seem unlikely.

GnRH vaccination has potential for treating reproductive tract disorders, such as endometriosis and ovarian tumors, as well as prostate and breast cancer and other diseases driven by gonadal steroids.[21,34,46,53] In elephants, common pathologies of the reproductive tract, particularly in nulliparous females, include leiomyomata and cystic endometrial hyperplasia. Although these processes are thought to be generally benign, they are suspected to contribute to infertility and could lead to erosion of vessels and associated hemorrhage.[24,28] Cessation of cycling through GnRH vaccination would be expected to decrease the likelihood of negative outcomes with these pathologic conditions, and the likelihood of significant negative adverse effects is considered low.[11,16,18,20,26,39]

Many factors can influence the immune response to GnRH vaccination, including individual genetic and immune status variation, patient age, vaccine dose, species variation in regard to optimal adjuvants, and the conjugant and specific design used in the manufacture of the vaccine.[17,19,20,40,41,48,54] Preliminary evaluation of a GnRH vaccine for control of aggression and musth behavior in bull elephants has shown some degree of variation in the response of individuals, and this phenomenon has been noted in a variety of other species.[2,13,23,30,32,52] More work clearly is needed to optimize the dose and frequency of vaccination when using the Repro-BLOC formulation in elephants, because it took more than a year for the effects to be realized. It is not clear why this elephant required multiple boosters before the desired effect of vaccination occurred. The initial doses administered may have been too low, the dose intervals could have been suboptimal, the adjuvant used may not be as immunostimulatory in elephants as in beef cows, or this individual's immune response could have been compromised due to age or disease status. Other GnRH vaccines are available, some of which are being designed for multiyear contraceptive effects after a single injection and act more rapidly in tested species, within weeks (e.g., GonaCon™, National Wildlife Research Center, USDA-APHIS Wildlife Services, Fort Collins, Colorado 80521, USA), but they have not been evaluated in female elephants.[36,37,41,43] The initial doses of Repro-BLOC used in this study were based on data in cattle in which peak antibody titers were reached 2 wk after administration of a 1.5-mg booster (16 wk after initial injection of 1.5 mg).[11] Based on the progestagen and possibly the FSH results, suppression of ovarian activity in this elephant was not evident until after the 12.5-mg dose, whereas a significant effect on LH was evident only after the final 30-mg dose. However, it cannot be concluded that 12.5 mg is the optimal dosage or that the cumulative effects of multiple doses over time were not significant. But once the response occurred, it has been sustained, and the elephant continues to be reproductively suppressed. Ongoing routine progestagen monitoring that continues to date, con-

tinues to show that concentrations average less than 0.07 ng/ml, 3 yr after the final booster. The reversibility of this vaccine approach in elephants now remains to be determined.

Based on studies in other species, GnRH vaccination also offers exciting possibilities as a new option for potentially reversible contraception in female elephants.[3,18,30,36,41,43] In elephant bulls, GnRH vaccination has shown efficacy in suppressing testosterone production and musth behaviors, presumably by similarly inhibiting pituitary control of gonadal steroidogenic activity.[2,13,14] A carefully managed contraception strategy would be of particular value in areas of high elephant density in close proximity to humans to mitigate human-elephant conflicts and in game parks where protected space and resources are limited. It would be beneficial to compare the efficacy, duration of effect, reversibility, long-term health and genetic effects, and social and behavioral effects of GnRH vaccination in elephants with those of porcine zona pellucida vaccination, which prevents fertilization by blocking sperm binding to the egg and is currently used in female elephants for contraception.[2,12,14,15,38,47] Porcine zona pellucida vaccination would not be efficacious for resolving health problems associated with ovarian steroidogenic activity, however, because contracepted females continue to cycle.

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Effect of gonadotropin releasing factor suppression with an immunological on growth performance, estrus activity, carcass characteristics, and meat quality of market gilts

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ABSTRACT: Objectives were to evaluate the administration of an anti-gonadotropin releasing factor (GnRF) analog on suppression of estrus, consistency of feed intake, and growth performance in market gilts and to investigate the impact the physiological changes would have on carcass characteristics and fresh meat quality. Gonadotropin releasing factor stimulates the anterior pituitary to release luteinizing hormone that acts on the ovary to induce follicle development and indirectly initiates ovulation. Improvest (Zoetis, Kalamazoo, MI) contains an incomplete version of naturally occurring GnRF and causes the production of anti-GnRF antibodies that bind to the GnRF receptor and thus render GnRF inactive. This in turn suppresses estrus in female pigs. Gilts were initially separated into 10 blocks based on age and then within each block allotted to a pen (n = 114; 5 pigs/pen) based on BW. Gilts received the first dose at 12 wk of age and the second dose at 16 wk of age, were exposed to a boar daily from 20 to 26 wk of age, and were slaughtered at 26 wk of age (10 wk after second dose). Meat quality was analyzed on the 2 gilts closest to pen average ending live weight in 5 of the 10 blocks. Pen served as the experimental unit for all data analysis. During the 15-wk finishing period, ADG was 0.03 kg greater ($P < 0.01$) and G:F was 0.009 greater ($P = 0.02$) in gilts administered GnRF suppression (treated) compared with untreated gilts (control). The majority of improvements in growth performance were observed from 16 to 20 wk of age (4 wk after second dose), as ADG was 0.07 kg greater ($P < 0.001$) and G:F was 0.021 greater ($P < 0.01$) in treated gilts compared with control gilts. Ovarian weights were reduced ($P < 0.0001$) by 64.15% and gilts exhibiting puberty were reduced by 87.80% ($P < 0.001$) in treated gilts compared with control gilts. Back fat depth was 3.78 mm greater ($P < 0.0001$) and estimated lean was 1.31 percentage units less ($P < 0.0001$) in treated gilts compared with control gilts. With the exception of subjective color, there were no differences ($P \geq 0.12$) in meat quality parameters between treated and control gilts. Subjective color was darker ($P = 0.03$) in treated gilts compared with control gilts. These data suggest market gilts treated with an anti-GnRF analog had suppressed estrus and episodical changes in ADFI, while they had improved feed efficiency, increased ADG, and increased back fat depth when compared with gilts without an anti-GnRF analog treatment.

Key words: estrus suppression, gilt, gonadotropin releasing factor, Improvest

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INTRODUCTION

Historically, 3-way crossbred gilts (0.69 kg/d) grew 5.8% slower than contemporary barrows (0.73 kg/d) from weaning until 91 kg BW (Comstock et al., 1944). Today, gilts consume 11.3% less feed, grow 7.3% slower, and yield a 1.53 percentage unit leaner carcass than counterpart barrows (Boler et al., 2014; Puls et al., 2014). Management strategies to increase growth rate and increase feed intake while maintaining feed efficiency in market gilts would be desirable to pork producers in managing pig flow and marketing of barrows and gilts.

Anti-gonadotropin releasing factor (**GnRF**) analog (Improvest; Zoetis, Kalamazoo, MI) is currently used for temporary immunological castration and reduction of boar taint in intact male pigs intended for slaughter.

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However, GnRF is a compound both male and female pigs produce naturally; therefore, the opportunity to use an anti-GnRF analog in market gilts exists. In females, GnRF stimulates the anterior pituitary to release luteinizing hormone that acts on the ovary to induce follicle development and indirectly initiates ovulation. Improvest contains an incomplete version of naturally occurring GnRF and causes the production of anti-GnRF antibodies to competitively bind to the receptor, thus suppressing estrus in female pigs. The estrous period accompanying puberty lasts between 4 and 5 d (Eliasson, 1989), and feed intake and growth are typically decreased during this time (Hinson et al., 2012). To date, there has been limited research analyzing the effectiveness of managing market gilts with an anti-GnRF analog to suppress estrus. Even so, gilts managed with an anti-GnRF immunological consumed 5.99% more feed and grew 5.55% faster than control gilts when slaughtered at 125 kg (Latorre et al., 2013). Therefore, the objective of this study was to determine the effects of GnRF suppression for the management of market gilts on growth performance, estrus activity, carcass characteristics, and fresh meat quality.

MATERIALS AND METHODS

Experimental procedures involving animals were approved by the North Carolina State University Institutional Animal Care and Use Committee. No approval was obtained from The Ohio State University Institutional Animal Care and Use Committee for this experiment because only fresh meat was used in that portion of the experiment.

Live-Phase Experimental Design

Five-hundred seventy crossbred gilts (62.1 ± 1.3 d of age; Landrace × Large White × Yorkshire sows mated with 3 crossbred boars composed of Duroc, Hampshire, Pietran, and Spot breeds) were allocated into 10 blocks over a 20-wk period. Blocks were based on farrowing group (age) at the date of allocation. Each block consisted of 3 to 8 replications, based on the number of available gilts, with 5 gilts per pen. Pens contained 4.09 m^2 of useable animal space, so each pig was initially provided 0.82 m^2 of floor space. Each pen had 2 nipple drinkers and a 2-hole feeder to provide ad libitum access to feed throughout the experiment. Two finisher barns were used and each finisher barn had a curtain-sided, underslat ventilation system equipped with thermostatically controlled cooling/stirring fans and misters. At 9 wk of age, gilts were individually weighed, examined, and selected for inclusion of the study (Fig. 1). Within each age block, gilts were allocated by BW and provided a 3-wk acclimation period. At 12 wk of age half of the replicates

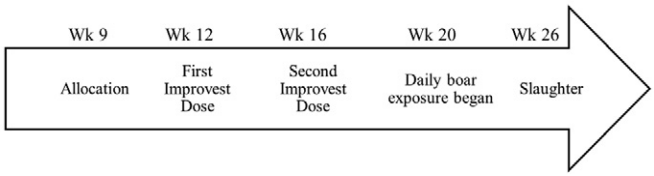


Figure 1. Timeline of study.

in each block were randomly assigned to the GnRF suppression treatment group (treated) and administered 2 mL of an anti-GnRF immunological product (Improvest; each milliliter contains 0.2 mg GnRF analog–diphtheria toxoid conjugate, 150 mg of diethylaminoethyl-dextran hydrochloride, 1 mg chlorocresol, sodium hydroxide as needed to adjust pH, and water for injection; Zoetis, Kalamazoo, MI) while the remaining half were assigned to the untreated group (control). At 16 wk of age, gilts were weighed and treated gilts were administered a second 2-mL dose of the anti-GnRF immunological product (Improvest) and control gilts remained untreated. No placebo injection was administered to control gilts. At 20 wk of age, gilts were weighed and daily boar exposure began. Boar exposure consisted of allowing a mature boar (3 yr old and 340 kg) to move freely in the alley in front of 2 to 3 pens for 10 to 15 min. Daily boar exposure continued until gilts were 26 wk of age. Feed remaining in the feeders was weighed and recorded as feed not consumed on the same day gilts were weighed during wk 9, 12, 16, 20, and 23. Feed remaining in the feeders was weighed on the day after gilts were sent to slaughter. At 26 wk of age, gilts were transported to the processing facility at each of the scheduled slaughter dates and were humanely slaughtered using electrical immobilization and exsanguination. Ovarian weights, ovarian morphology, and HCW (which included weight of the head) were obtained at the slaughter facility.

Carcass Characteristics

Back fat depth and LM area were estimated using real-time ultrasonography at approximately 25 wk of age. Real-time ultrasonography (Aloka 500 V, 3.5 MHz; Corometrics Medical Systems, Inc. Wallingford, CT) was conducted 2 to 5 d before scheduled slaughter date depending on the schedule of the ultrasound technician. These measurements, along with HCW, were used to predict estimated lean weight with the following equation (Burson and Berg, 2001): estimated lean, kg = 5.7769 + (0.401 × HCW, lb) – (18.838 × 10th rib fat depth, in.) + (4.357 × 10th rib loin muscle area, sq. in.) + (1.006 × sex of pig; gilt coefficient = 2). This procedure likely underestimates estimated lean, because HCW of this study included weight of the head. Estimated percent lean was determined by dividing estimated lean by HCW.

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Estrus Activity

Ovarian activity was evaluated using 3 different methods. First, paired ovarian weights were obtained at the slaughter facility. Second, each gilt was given a numerical score based on ovarian morphology according to the following scale: 0 = no visible follicles; 1 = only small follicles present (≤3.0 mm in diam.); 2 = medium follicles present (3.1–5.9 mm in diam.); 3 = large follicles present (≥6.0 mm in diam.); 4 = corpora lutea, corpora hemorrhagica, or corpora albicantia present (1 ovulation cycle); and 5 = corpora lutea or corpora hemorrhagica and corpora albicantia (2 ovulation cycles). Third, the percentage of pens within each treatment that had at least 1 gilt ovulating during the study and the percentage of all gilts within treatments that ovulated were determined.

Meat Quality

Gilts were loaded on trucks on the morning of slaughter and transported approximately 60 km. Gilts were provided a short rest (<2 h) on arriving before slaughter. Meat quality was assessed in 5 of the 10 blocks. The 2 pigs in each pen of a selected block with ending live weights closest to the pen mean weight were selected for meat quality evaluation. A total of 116 loin sections were shipped from the slaughter facility to the meat science laboratory at The Ohio State University. Sections were cut from the posterior section of the 10th rib, vacuum packaged, and shipped under refrigeration. Sections remained vacuum packaged and were stored in a refrigerated cooler (4°C) on arrival. Fresh meat quality was assessed at 7 d postmortem. Initially, sections were faced and allowed at least 15 min to bloom. The piece removed from face was used to determine proximate composition (percentage moisture and fat). The loin sample was trimmed of all subcutaneous fat and homogenized in a food processor. A 10-g sample was oven-dried at 100°C for at least 24 h to determine moisture percentage. The dried sample was washed multiple times in warm petroleum ether to remove fat using the modified procedure from Novakofski et al. (1989). The fresh cut surface was evaluated for subjective color (1 = pale pinkish gray to white, 2 = grayish pink, 3 = reddish pink, 4 = dark reddish pink, 5 = purplish red, and 6 = dark purplish red; NPPC, 1999), marbling (1 through 10 corresponding to intramuscular lipid content; NPPC, 1999), and firmness (1 = soft; cut surface distorts easily; 2 = firm; cut surface tends to hold shape; and 3 = very firm; cut surface were very smooth with no distortion of shape; NPPC, 1999) by trained Ohio State University personnel and recorded in whole numbers. The surface was evaluated for ultimate pH using a handheld Hanna HI 99163 pH meter (Hanna Instruments; Woonsocket, RI.) and was evaluated for objective CIE L*, a*, and b* with a Minolta CR-400 (Konica Minolta; Chiyoda, Tokyo, Japan) using a D65 light source and a 0° observer and an aperture size of 50 mm. The remaining section was then cut into 1 chop cut 1.25 cm thick and 1 chop cut 2.54 cm thick. Water holding capacity was evaluated using the drip-loss method where the 1.25 cm thick chop was suspended from a fish hook in a Whirl-pak bag (Nasco; Fort Atkinson, WI) for approximately 24 h at 4°C. Chops were weighed before and immediately after suspension for 24 h. Results were reported as a percent of weight loss. Chops designated for Warner-Bratzler shear force (2.54 cm thick) testing were vacuum packaged and immediately frozen until further evaluation. Twenty-four hours before analysis, chops were removed from the freezer and placed in a cooler at 4°C to thaw. Chops were trimmed of excess fat and cooked on a clamshell-type grill. Chops were cooked to a final internal temperature of 70°C. Internal temperature was monitored using copper-constantan thermocouples (Type T; Omega Engineering, Stanford, CT) connected to a digital scanning thermometer (model 92000-00; Barnant Co., Barington, IL). Next, chops were allowed to cool to 25°C and four 1.25-cm diam. cores were removed parallel to the orientation of the muscle fibers. Cores were sheared using a Texture Analyzer TA.HD Plus (Texture Technologies Corp., Scarsdale, NY/Stable Microsystems, Godalming, UK) with a blade speed of 3.3 mm/s and a load cell capacity of 50 kg. A single shear force was determined on each of the 4 cores. Shear force was reported as the average of the 4 cores. Cook loss was determined by weighing chops used for shear force immediately before and after cooking. Reported values are percent weight lost during cooking.

Statistical Analysis

Data were analyzed with the MIXED procedure in SAS (SAS Inst. Inc., Cary, NC) as a randomized complete block design with the fixed effect of treatment and the random effect of replication nested within block. Pen served as the experimental unit for all analysis, with the exception of gilts exhibiting puberty, which used pig as the experimental unit. Statistical differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$ using a 2-tailed test.

RESULTS

Growth Performance

Results for growth performance are presented in Table 1. From 12 to 16 wk of age (time between first and second dose), ADG was not different ($P = 0.52$) between treated and control gilts. Average daily feed intake was 0.09 kg (3.80%) less ($P = 0.04$) in treated gilts. Feed efficiency (G:F) was not different ($P = 0.19$). From 16 to 20 wk of age (0–4 wk after second dose, before boar exposure),

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Table 1. Effect of immunological gonadotropin releasing factor suppression on growth performance of market gilts during a 15-wk finishing period

	Management			
Parameter	Control	Treated[1]	SEM	*P*-value
Pens, *n*	57	57		
12–16 wk[2]				
ADG, kg/d	0.82	0.82	0.02	0.52
ADFI, kg/d	2.37	2.28	0.03	0.04
G:F	0.350	0.361	0.008	0.19
16–20 wk[3]				
ADG, kg/d	0.82	0.89	0.02	<0.001
ADFI, kg/d	2.82	2.87	0.06	0.28
G:F	0.295	0.316	0.010	<0.01
20–23 wk[4]				
ADG, kg/d	0.83	0.88	0.03	0.01
ADFI, kg/d	3.10	3.22	0.06	0.01
G:F	0.271	0.279	0.012	0.16
23–26 wk[5]				
ADG, kg/d	0.83	0.82	0.03	0.73
ADFI, kg/d	3.24	3.25	0.08	0.89
G:F	0.258	0.255	0.006	0.66
12–26 wk				
ADG, kg/d	0.82	0.85	0.01	<0.01
ADFI, kg/d	2.82	2.83	0.03	0.67
G:F	0.291	0.300	0.003	0.02
16–26 wk[6]				
ADG, kg/d	0.82	0.86	0.02	<0.01
ADFI, kg/d	3.02	3.08	0.04	0.11
G:F	0.272	0.281	0.004	0.03

[1]Gilts received a 2-mL subcutaneous injection of Improvest (Zoetis, Kalamazoo, MI) at 12 and 16 wk of age (initial BW at 12 wk of age = 38.23 kg). Gilts were slaughtered at 10 wk after second dose.

[2]Twelve- to sixteen-week period was between first and second Improvest dose (16-wk BW = 61.76 kg).

[3]Sixteen- to twenty-week period was after second injection but before boar exposure (20-wk BW = 85.63 kg).

[4]Twenty- to twenty-three-week period was after initiation of boar exposure but likely before estrous period (23-wk BW = 102.20 kg).

[5]Twenty-three- to twenty-six-week period was after initiation to boar exposure and likely during estrous activity (26-wk BW = 118.51 kg).

[6]Sixteen- to twenty-six-week period was after second injection until slaughter.

ADG was 0.07 kg (8.54%) greater ($P < 0.001$) in treated gilts. Average daily feed intake was not different ($P = 0.28$). Feed efficiency (G:F) was 0.021 (7.12%) greater ($P < 0.01$) in treated gilts. From 20 to 23 wk of age (4–7 wk after second dose and during boar exposure), ADG was 0.05 kg (6.02%) greater ($P = 0.01$) in treated gilts. Average daily feed intake was 0.12 kg (3.87%) greater ($P = 0.01$) in treated gilts. Feed efficiency (G:F) was not different ($P = 0.16$). From 23 to 26 wk of age (7–10 wk after second dose and during boar exposure), ADG was not different ($P = 0.73$). Average daily feed intake was not different ($P = 0.89$). Feed efficiency (G:F) was not different ($P = 0.66$). During the entire finishing period (12 to 26 wk of age), ADG was 0.03 kg (3.66%) greater ($P < 0.01$) in treated gilts. Average daily feed intake was not different ($P = 0.67$). Feed efficiency (G:F) was 0.009 (3.09%) greater ($P = 0.02$) in treated gilts. Overall, treated gilts grew faster and were more feed efficient throughout the finishing period than control gilts (Table 1).

Table 2. Effect of immunological gonadotropin releasing factor suppression on carcass characteristics of market gilts

	Management			
Parameter	Control	Treated[1]	SEM	*P*-value
Pens, *n*	57	57		
Initial live weight,[2] kg	38.43	38.02	0.68	0.18
Final live weight,[3] kg	117.39	119.63	1.18	0.05
HCW (including head weight), kg	96.19	97.62	1.00	0.16
Back fat depth,[4] mm	21.75	25.53	0.05	<0.0001
LM area,[4] cm^2	45.13	45.20	0.64	0.91
Estimated lean,[5] kg	48.46	48.11	0.44	0.46
Estimated lean,[5] %	50.53	49.22	0.21	<0.0001

[1]Gilts received a 2-mL subcutaneous injection of Improvest (Zoetis, Kalamazoo, MI) at 12 and 16 wk of age. Gilts were slaughtered at 10 wk after second dose.

[2]Initial weight was taken at 12 wk of age.

[3]Final weight was taken at 26 wk of age.

[4]Real-time ultrasonography images were used to estimate LM area and back fat depth 2 to 5 d before slaughter.

[5]Estimated lean was calculated using a modified version of procedure 6 of the procedures for estimating pork carcass composition (Burson and Berg, 2001).

Carcass Characteristics

Results for carcass characteristics are presented in Table 2. Tenth rib LM area was not different ($P = 0.91$) between treated and control gilts. Tenth rib back fat depth was 3.78 mm (17.38%) greater ($P < 0.0001$) in treated gilts. Hot carcass weight was not different ($P = 0.16$). Estimated weight of the lean (an equation including LM area, back fat depth, and HCW; Burson and Berg, 2001) was not different ($P = 0.46$). Yet estimated lean percentage was 1.31% units less ($P < 0.0001$) in treated gilts. Overall, these data suggest that when slaughtered at 10 wk after second dose, treated gilts had more fat and a reduction in estimated carcass lean percentage compared with control gilts; however, muscling and estimated lean weight were not different between treated and control gilts (Table 2).

Estrus Activity

Results for estrus activity are presented in Table 3. Ovarian weights were 6.80 g (64.15%) lighter ($P < 0.0001$) in treated gilts compared with control gilts. Ovarian activity scores were 1.80 (75.00%) less ($P < 0.0001$) in treated gilts. Pens with at least 1 pubertal gilt were 28.10% units less ($P < 0.001$) in treated gilts. Gilts exhibiting puberty were reduced ($P < 0.001$) by 18.00% units in treated gilts. Overall, administering the

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Table 3. Effect of immunological gonadotropin releasing factor suppression on estrus activity

Parameter	Management		SEM	*P*-value
	Control	Treated[1]		
Pens, *n*	57	57		
Ovarian weight, g	10.60	3.80	0.40	<0.0001
Ovarian activity score[2]	2.40	0.60	0.10	<0.0001
Pens with a pubertal gilt, %	40.40	12.30	6.60	<0.001
Gilts exhibiting puberty, %	20.50	2.50	0.03	<0.001

[1]Gilts received a 2-mL subcutaneous injection of Improvest (Zoetis, Kalamazoo, MI) at 12 and 16 wk of age. Gilts were slaughtered at 10 wk after second dose.

[2]Numerical scoring system of 0 = no visible follicles; 1 = only small follicles present (≤3.0 mm in diam.); 2 = medium follicles present (3.1–5.9 mm in diam.); 3 = large follicles present (≥6.0 mm in diam.); 4 = corpora lutea, corpora hemorrhagica, or corpora albicantia present (1 ovulation cycle); and 5 = corpora lutea or corpora hemorrhagica and corpora albicantia present (2 ovulation cycles).

Table 4. Effect of immunological gonadotropin releasing factor suppression on fresh meat quality of market gilts

Parameter	Management		SEM	*P*-value
	Control	Treated[1]		
Pens, *n*	26	32		
Subjective evaluations[2]				
Color	2.24	2.53	0.10	0.03
Marbling	2.06	2.19	0.13	0.41
Firmness	2.19	2.19	0.08	0.99
Objective color scores				
L*	55.40	55.75	0.38	0.50
a*	18.94	19.01	0.17	0.78
b*	7.53	7.59	0.15	0.76
pH	5.52	5.52	0.03	0.72
Drip loss, %	1.26	1.17	0.09	0.30
Cook loss, %	18.51	18.49	0.58	0.98
Shear force, kg	2.59	2.54	0.06	0.51
Proximate composition				
Moisture, %	72.68	72.39	0.13	0.12
Fat, %	2.45	2.69	0.13	0.17

[1]Gilts received a 2-mL subcutaneous injection of Improvest (Zoetis, Kalamazoo, MI) at 12 and 16 wk of age. Gilts were slaughtered at 10 wk after second dose.

[2]Values assessed using National Pork Producers Council (NPCC, 1999) standards and recorded in whole numbers.

anti-GnRF analog (Improvest) was an effective method for suppressing estrus in market gilts; however, it did not completely eliminate signs of puberty in market gilts. Possible explanations could be that when slaughtered at 10 wk after second dose (the maximum marketing window for male pigs), the GnRF analog immunity was beginning to diminish and dropped below suppressive antibody levels potentially allowing puberty to develop after antibody titers have degraded to a level lower than the level necessary to elicit an immune response.

Meat Quality

Results for meat quality are presented in Table 4. Subjective color evaluation scores were darker ($P = 0.03$) in treated gilts compared with control gilts. At the same time, objective color scores (L*, a*, and b*) were not different ($P \geq 0.50$) between treated and control gilts. All other meat quality traits evaluated (including marbling, firmness, pH, drip loss, cook loss, shear force, percentage moisture, or percentage fat) were not different ($P \geq 0.12$). Overall, meat quality parameters were not different between gilts administered the anti-GnRF analog (Improvest) and control gilts (Table 4).

DISCUSSION

The improved growth performance (Dunshea et al., 2001; Fàbrega et al., 2010) and carcass cutability (Boler et al., 2012) associated with male pigs immunologically castrated with an anti-GnRF analog in comparison to physically castrated barrows has been well documented. Dunshea et al. (2013) reported, via meta-analysis, that ADG was greater (0.16 kg) and back fat depth was less (2.64 mm) in immunologically castrated males when compared with physically castrated barrows. Immunologically castrated barrows had greater ADFI (0.43 kg), which resulted in greater ADG (0.12 kg), and an improved feed conversion ratio when compared with entire males, although back fat depth was also increased (1.53 mm; Dunshea et al., 2013). Therefore, immunologically castrated barrows have advantages in growth rate when compared to physically castrated barrows and entire males and have less back fat depth than physically castrated barrows but have more back fat depth than entire males.

The management of gilts with an anti-GnRF analog has been considerably less well documented. Puberty of gilts occurs before marketing (less than 180 d) but is very dependent on genetics and management factors (including nutrition, exposure to boars, and growing season; Evans and O'Doherty, 2001). These data suggest gilts managed with an anti-GnRF analog had increased growth rates and improved feed efficiency compared with control gilts. Albeit, when slaughtered at 10 wk after second dose, gilts managed with an anti-GnRF analog had greater back fat thickness and reduced estimated lean percentage compared with control gilts. These findings were comparable with other available data. Among the limited available information, Latorre et al. (2013) reported gilts slaughtered at approximately 125 kg BW (67 kg of BW gain after second dose) managed with an anti-GnRF analog had greater ADG (0.99 vs. 0.94 kg) than control gilts but also had greater ADFI (2.83 vs. 2.67 kg) than control gilts; therefore, feed conversion was not different between treated and control gilts. Differences in response between the 2 studies was likely

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due to the present study ending at a fixed time and Latorre et al. (2013) ending at a fixed weight (125 kg). Even so, treated gilts in the current study grew 5% faster and were over 3% more feed efficient than control gilts without affecting feed intake during the finishing phase from the second injection through slaughter. Gómez-Fernández et al. (2013) reported treated gilts (slaughtered 14 wk after second dose, at approximately 170 kg) had greater ADG (0.84 vs. 0.80 kg), ADFI (3.48 vs. 3.21 kg), and reduced feed conversion (Feed:Gain; 4.16 vs 4.04) compared with control gilts. Furthermore, Gómez-Fernández et al. (2013) reported ending live weight and HCW tended to be greater in treated gilts compared with control gilts. Although Gómez-Fernández et al. (2013) did not report 10th rib LM area and back fat thickness, they did report that trimmed shoulder, loin, and trimmed ham were lower yielding in treated gilts compared with control gilts.

Finally, in a study where gilts were slaughtered at 4 wk after the second dose, treated gilts grew 18% faster than control gilts but also consumed 18% more feed (Oliver et al., 2003). Therefore, feed efficiency was not different between treated and control gilts. The management of market gilts with an anti-GnRF analog is a novel concept to increase ADG and ADFI and in turn increase carcass weight with more desirable levels of carcass fat and marbling without exaggerated detrimental impacts on feed efficiency. In this study, treated gilts had slightly greater back fat and reduced estimated lean percentage and numerically increased fat content (0.24%, or approximately a 10% increase) in the LM when gilts were slaughtered 10 wk after the second dose.

Market gilts tend to have leaner carcasses in comparison to physically castrated barrow counterparts (Ekstrom, 1991). From a U.S. pork packer perspective, gilts are consistently leaner and have more carcass muscle resulting in greater carcass cutability (Boler et al., 2014). However, a greater proportion of gilts can have carcasses that are considered excessively lean for the U.S. packing industry standards and have loin muscles with less marbling or fat content than counterpart barrows (Boler et al., 2014). Additionally, lean gilt carcasses can yield bellies that are excessively lean and thinner than bellies considered ideal to process under commercial conditions to optimize belly value (Kyle et al., 2014). Further research is warranted to better understand appropriate age and timing of slaughter after second dose to optimize lean growth, carcass fat deposition, carcass characteristics, and determine carcass cutability.

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The use of a GnRH vaccine to suppress mare ovarian activity in a large group of mares under field conditions

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Abstract. The aim of this study was to evaluate the effect of active immunisation against GnRH on ovarian activity and serum progesterone concentrations in a large group of mares (10 control and 55 experimental) under field conditions as a model for wildlife species such as zebra and African elephants. Within the experimental group, mares were subdivided into three age categories: Category 1 (4 years and younger, $n = 26$), Category 2 (4–10 years old, $n = 18$), and Category 3 ($\geq$11 years old, $n = 11$). Experimental mares were vaccinated intramuscularly with 2 mL (400 μg) of the GnRH vaccine Improvac (Pfizer Animal Health, Sandton, South Africa). Control mares received the same amount of saline solution. The vaccinations were repeated 35 days later. The ovaries and reproductive tracts of each mare were examined by means of rectal palpation and ultrasonography on Days 0, 35 and 70. Blood was collected weekly for determination of serum progesterone concentration until Day 175. On Day 35 after primary vaccination all of the control mares and 14.5% of the experimental mares showed evidence of ovarian activity on the basis of clinical examination and serum progesterone concentration. On Day 70, all control mares and none of the experimental mares showed evidence of cyclic activity. No age-related effect within treatment groups was found. The serum progesterone concentration indicated that all experimental mares remained in anoestrus until Day 175. Five of the control mares fell pregnant between Days 35 and 70. The five non-pregnant control mares continued to cycle until the end of the observation period. Having achieved such promising results in this trial we now plan to test the GnRH vaccine in Burchell's zebra mares and African elephant cows.

Introduction

Contraception as a means of population management of captive wildlife species is well established. Methods that have been used include separation of sexes, surgical techniques and steroid hormone implants. The safety, practicality and reversibility of methods have enjoyed more attention in recent years, particularly where endangered and rare species are involved. Because of developments during the last 15 or so years it has become possible to successfully apply some methods for the management of free-ranging wildlife populations. Typical examples of these are the use of the porcine zona pellucida (pZP) vaccine to control reproduction in wild horses (Kirkpatrick *et al*. 1982, 1990; Kirkpatrick and Turner 2002) and white-tailed deer (Naugle *et al*. 2002; Rutberg *et al*. 2004).

In mares, the control of reproductive activity is a vital management aspect from both a fertility and behavioural perspective. Anecdotally, oestrous behaviour may negatively influence the performance ability of mares engaged in work and competition events (Elhay *et al*. 2007).

In the past, fertility control and the suppression of aggressive and sexual behaviour in horses was successfully managed by administration of progesterone or progestagens, in oral or injectable formulation, to simulate dioestrus. The high costs and the frequent administration associated with the administration of progesterone or progestagens and the short duration of action made their use impractical. A potential negative effect on future reproduction after repeated administration of progesterone or progestagens in prepubertal fillies has also been described (Skelton *et al*. 1991).

The major alternative to pharmacological manipulation for long-term control of reproductive cyclicity and behaviour involves the surgical removal of both ovaries (ovariectomy). Ovariectomy is invasive and also associated with various complications. After this procedure, some mares may still show signs associated with oestrous behaviour (Ginther 1979; Asa *et al*. 1980; Hooper *et al*. 1993). The factors of cost, associated trauma, potential postoperative complications, production setbacks and the irreversible loss of breeding potential make this method difficult to implement (D'Occhio 1993). Ovariectomy is therefore considered undesirable unless circumstances are exceptional.

Recent studies on the use of gonadotropin-releasing hormone (GnRH) vaccination for suppression of fertility, aggressive and sexual behaviour have shown promising results. Vaccination entails the administration of a modified form of the GnRH hormone in order to stimulate the production of anti-GnRH antibodies. These antibodies then bind to the endogenous GnRH and inhibit the natural binding of this molecule to its

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receptors on the pituitary gonadotropes. This results in suppression of secretion of both follicle-stimulating hormone and luteinising hormone at the level of the pituitary.

Immunocastration by means of GnRH vaccination as an alternative to surgical castration to control unwanted male characteristics and behaviour has been studied in male animals of different species, including bulls (Jago *et al*. 1997), stallions (Malmgren *et al*. 2001; Stout 2001; Turkstra *et al*. 2005; Burger *et al*. 2006), Chinese pigs (Zeng *et al*. 2001), lambs (Janett *et al*. 2003), rats, dogs and rams (Ferro *et al*. 2004), African elephants (Bertschinger *et al*. 2004; Delsink *et al*. 2004), cats (Levy *et al*. 2004; Robbins *et al*. 2004), puppies (Jung *et al*. 2005) and feral swine (Killian *et al*. 2006).

The use of the commercially available GnRH vaccine (Improvac, Pfizer Laboratories, Sandton, South Africa) in boars eliminates boar taint and enhances growth performance in uncastrated male pigs (Dunshea *et al*. 2001; Chumkam and Ravungsook 2003).

In stallions and colts the semen quality of animals vaccinated with a GnRH vaccine deteriorated but the production of semen was never completely suppressed (Dowsett *et al*. 1996; Malmgren *et al*. 2001; Turkstra *et al*. 2005; Burger *et al*. 2006). The shedding status of stallions infected with equine viral arteritis was also converted to non-shedder status in vaccinated stallions (Burger *et al*. 2006).

The effect of GnRH vaccination in female animals of various species (including deer, wild rats and bison) has also been reported. With the use of an early form of a GnRH vaccine it appeared that GnRH vaccination is not an effective means of contraception in deer (Becker *et al*. 1999). However, a larger and more comprehensive study on immunocontraception of white-tailed deer (Miller *et al*. 2000) showed immunocontraception of deer to be effective. Female white-tailed deer treated with GnRH immunocontraceptive vaccine showed fewer oestrus cycles per female than either females treated with a porcine *zona pellucida* (PZP) vaccine or an untreated control group (Curtis *et al*. 2001). In wild rats, GnRH vaccine was found to be more effective than mouse *zona pellucida* peptide vaccine (Miller *et al*. 1997). Finally, a single dose of GnRH vaccine prevented pregnancy in female bison for ≥1 year (Miller *et al*. 2004).

In mares, the few studies to date report a successful but variable effect on suppression of ovarian activity and corresponding prevention of oestrous behaviour in adult mares. These studies have, in most instances, been limited by relatively small numbers of mares: in 10 mares (≥2.5 years of age) (Garza *et al*. 1986), in six mares (2 years of age) (Tshewang *et al*. 1997), in four mares (age range: 6–14 years) (Dalin *et al*. 2002), in seven mares (Stout *et al*. 2003), in 18 mares (age range: 3–17 years) (Imboden *et al*. 2006). In only one study was a relatively large population of 48 mares (age range: 3–12 years) studied (Elhay *et al*. 2007). In addition, an effect of mare age on treatment response has been reported in some studies (Dalin *et al*. 2002; Stout *et al*. 2003), with significant injection-site reactions observed in older mares (Stout *et al*. 2003).

The aim of the present study was to investigate the effects of active immunisation against GnRH on reproductive cyclicity in a large group of mares of different age categories and to monitor the effect of vaccination and mare age on injection-site reactions. A secondary aim of the study was to use the domestic mare study as a model for contraception of zebra mares and African elephant cows.

Materials and methods

Experimental design

A total of 65 mares, of either barren or maiden reproductive status, of various horse breeds and between 3 and 17 years of age from the Mounted Unit of the South African Police Services were used for this trial. The trial was approved by the Animal Use and Care Committee of the Faculty of Veterinary Science of the University of Pretoria (V068/05). The mares were housed in outdoor paddocks or extensive pastures for the duration of the trial. They were assigned to either a control ($n = 10$) or an experimental ($n = 55$) group. For management purposes, all experimental mares were excluded from breeding attempts whereas certain control mares were later assigned for breeding. Within the experimental group, mares were subdivided into one of three age categories: Category 1 (4 years and younger, $n = 26$), Category 2 (5–10 years old, $n = 18$), and Category 3 (≥11 years old, $n = 11$).

GnRH immunisation

On Day 0, mares from all three age categories of the experimental group were injected with 2 mL Improvac (400 µg RnRF-protein conjugate) (Pfizer Animal Health, Sandton, South Africa) intramuscularly into the left gluteus muscle, whereas all mares from the control group received 2 mL of a sterile saline solution using a 20-gauge, $1\frac{1}{2}''$ needle (Terumo Corporation, Tokyo, Japan). The subsequent booster vaccination (2 mL) was administered into the right gluteus muscle 35 days after the primary vaccination (J. F. Kirkpatrick, pers. comm.).

Transrectal monitoring of the reproductive tract

On Day 0, all mares were examined by means of transrectal palpation and ultrasound of the internal genitalia using a 5-MHz linear array probe (Aloka Echocamera SSD-500, Aloka Mitakashi, Tokyo, Japan) to establish their reproductive status. All clinical findings were recorded on a standardised data-capture form.

Ovarian activity was described using mean ovarian volume. The length, height and width of the left and right ovaries were estimated on transrectal palpation for each mare. These were used to calculate the ovarian volume using the prolate ellipsoid formula (length × height × width × 0.523) (Pavlik *et al*. 2000). The mean ovarian volume for each mare was calculated as the average of the left and right ovarian volume. The diameters of all ultrasonographically visible follicles ≥2 cm and the presence and number of *corporae luteae* (CLs) were also recorded.

The dimensions of the uterine horns and body and cervix were also estimated on transrectal palpation and their palpable tone was scored. The observed uterine oedema patterns were scored and the presence of intraluminal uterine fluid and other ultrasonographically visible abnormalities were recorded. A subjective scoring system was used to grade the degree of observed endometrial oedema to reflect changes in circulating oestrogen and progesterone concentrations that define oestrus status (Hayes *et al*. 1985): 0 (no oedema with a homogeneous echo texture), 1 (the least amount of detectable uterine oedema),

2 (moderate amount of oedema throughout), and 3 (most obvious oedema throughout the whole uterus, typical 'wagon-wheel' appearance). The gynaecological data collection was repeated on Days 35 and 70.

Blood sampling

Blood samples were collected by means of jugular venipuncture commencing on Day 0 (6 December), approximately the midway point of the Southern Hemisphere physiological breeding season, and repeated at weekly intervals until Day 175 (29 May), which is approximately the onset of the non-ovulatory season. The blood samples were collected in plain Vacutainer tubes (BD Vacutainer Systems, Plymouth, UK) and left to allow clotting. Serum was separated by centrifugation (4000*g*, 10 min) on the same day as sample collection. The labelled samples were stored at −20°C until assayed for serum progesterone concentration.

Serum progesterone assay

Assay for serum progesterone concentration were conducted by means of Radioimmunoassay (Coat-a-Count, Diagnostic Products Corp, Los Angeles, CA, USA).

Injection site reactions

Mares were observed daily in their enclosures by the same operator for one week after the vaccinations for injection-site reactions. Reactions at the injection site were scored as follows: 0, no visible reaction; 1, visible swelling; and 2, visible swelling accompanied by lameness.

Statistical analysis

All data were analysed using Systat 12 (Systat Software Inc., San Jose, CA, USA), using an analysis of variance (ANOVA) of treatment and age on clinical variables for general linear models to assess the level of significance. Significance was set at $P < 0.05$

Results

Transrectal monitoring of the reproductive tract

On Day 0, all control and experimental mares showed clinical evidence of cyclic reproductive activity on clinical examination. On Day 35 after the primary vaccination, all control mares and only 8 of 55 (14.5%) experimental mares showed evidence of ovarian activity on clinical examination. On Day 70 after the primary vaccination, all control mares and none (0%) of the 55 experimental mares showed evidence of ovarian activity on clinical examination. There was no difference between the volumes of the left and right ovaries within individual mares and therefore the mean ovarian volume of each animal at each examination was subjected to further statistical analysis. The mean ovarian volumes of both groups of mares on the days of primary (Day 0) and booster (Days 35) vaccinations and on Day 70 of the trial are shown in Fig. 1.

There was a significant treatment effect on ovarian volume between the control and experimental mares; no age effect was seen within the experimental groups. All clinical variables of the reproductive tract observations recorded are summarised in Table 1.

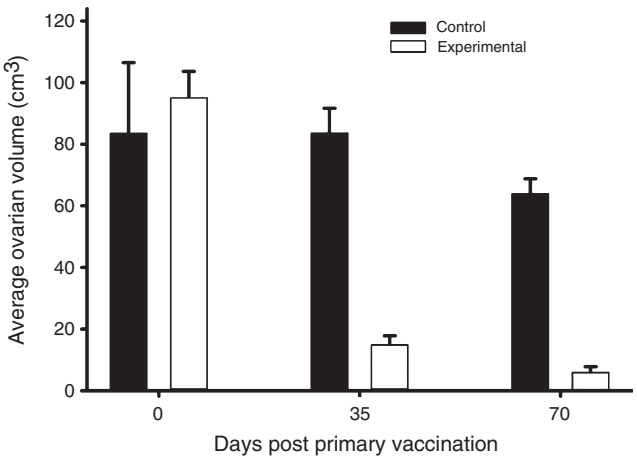


Fig. 1. Mean volume (±s.e.) of the ovaries in control and experimental groups on Day 0 (day of first vaccination), Day 35 (day of second vaccination) and Day 70.

Serum progesterone concentration (SPC)

The serum progesterone concentrations of the control and experimental groups over the 175-day observation period are shown in Fig. 2. Within the experimental group there was no effect of age on SPC ($P = 0.452$) whereas between the two treatment groups there was a significant difference ($P < 0.001$). Five of the control mares were mated and fell pregnant between Days 35 and 70. The remaining five control mares continued to cycle until the end of the observation period (Day 175).

Injection-site reactions

The control mares showed no injection-site reactions to the intramuscular saline injections. Table 2 shows the side effects due to GnRH vaccination in experimental mares. The side effects were transient by nature and by Day 6 were no longer visible. No age-related effect was observed.

Discussion

The effects of GnRH vaccination on a large group of experimental mares on both the reproductive tract (Fig. 1, Table 1) and SPC (Fig. 2) were observed. These effects were shown to be rapid in onset and statistically significant.

Thirty-five days after the primary vaccination, only eight (14.5%) of the experimental mares showed evidence of cyclic ovarian activity. By Day 70 (35 days after the booster vaccination) all experimental mares showed suppressed ovarian activity. For this trial cyclic inactivity was characterised by the absence of any follicle >2 cm in diameter, no visible CL and SPC <1 nmol/L. This effect was significant (and bilateral, as described by Imboden *et al.* 2006), with a marked decrease in the mean ovarian volumes as well as in concurrently observed changes to the dependant variables characterising ovarian structures and the tubular genitalia (cervix and uterus). The ovaries were uniformly small and apparently inactive with follicular atresia, similar to seasonally anoestrus mares (Dalin *et al.* 2002; Elhay *et al.* 2007). The tubular genitalia also showed atrophy typical of seasonal anoestrus. Two GnRH vaccinations (Improvac)

Table 1. The number of control and vaccinated mares allocated by clinical findings at the time of examinations
C, control mares; A1, Age Category 1 (≤4 years); A2, Age Category 2 (5–10 years); A3, Age Category 3 (≥11 years)

Clinical variable category	Day 0				Day 35				Day 70			
	C ($n=10$)	A1 ($n=26$)	A2 ($n=18$)	A3 ($n=11$)	C ($n=10$)	A1 ($n=26$)	A2 ($n=18$)	A3 ($n=11$)	C ($n=10$)	A1 ($n=26$)	A2 ($n=18$)	A3 ($n=11$)
Uterine tone												
0	4	9	9	4	3	21	13	8	1	26	16	8
1	6	11	9	5	4	4	2	2	4	0	1	3
2	0	6	0	2	3	1	3	1	4	0	1	0
3	0	0	0	0	0	0	0	0	1	0	0	0
Cervix tone												
0	2	8	2	2	1	21	12	8	1	26	16	8
1	8	12	8	7	4	4	5	2	4	0	2	3
2	0	6	8	2	4	1	1	1	4	0	0	0
3	0	0	0	0	1	0	0	0	1	0	0	0
Uterine oedema[A,B]												
0	4	12	11	5	6	26	17	10	1	26	18	11
1	3	8	5	4	3	0	1	1	2	0	0	0
2	2	2	2	2	1	0	1	0	1	0	0	0
3	1	2	0	0	0	0	0	0	1	0	0	0
Follicle >2 cm												
Yes	9	20	8	5	9	2	0	1	8	0	0	0
No	1	6	10	6	1	24	18	10	2	26	18	11
CL present												
Yes	8	21	13	11	5	2	1	2	9	0	0	0
No	2	5	5	0	5	24	17	9	1	26	18	11

[A]Two datasets were missing for uterine oedema with examination at Day 0, Age Category A1.
[B]Five control mares were diagnosed pregnant and uterine oedema score for Day 70 of the five control mares not scored.

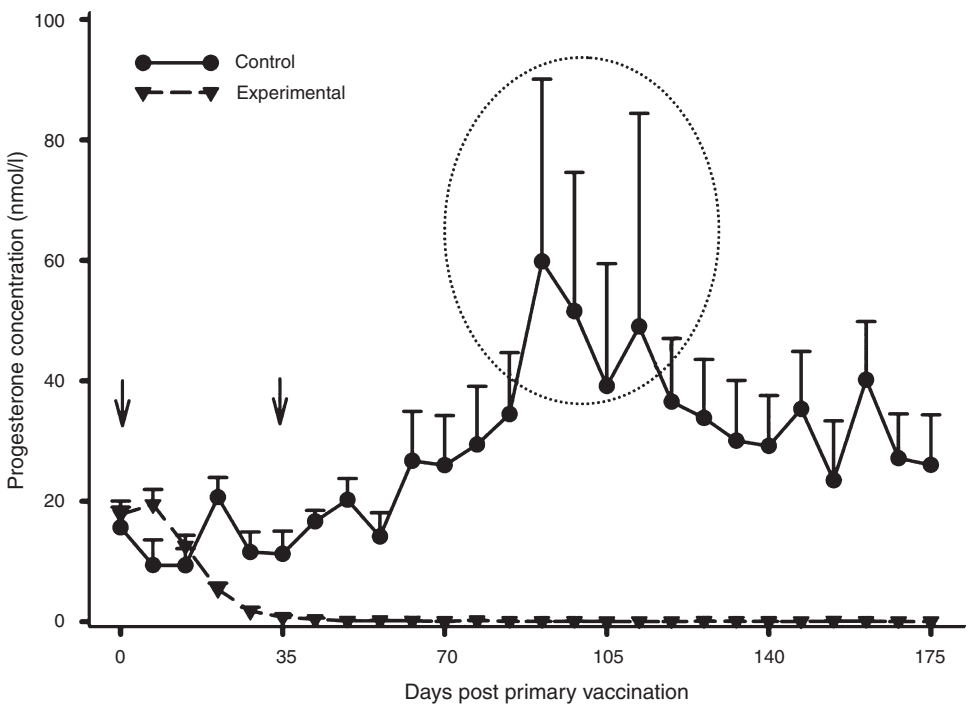


Fig. 2. Progesterone concentrations (±s.e.) of control and experimental mares. Arrows indicate times of vaccinations. The encircled data points reflect raised mean SPC obtained from the control mares because five mares were in the early stages of pregnancy.

Table 2. Injection-site reaction scores (number and percentage) after GnRH vaccination in experimental mares ($n = 55$)

	Score = 0			Score = 1			Score = 2		
Age category	1	2	3	1	2	3	1	2	3
Primary vaccination (*n*)	26	18	11	0	0	0	0	0	0
(%)	(100)	(100)	(100)	(0)	(0)	(0)	(0)	(0)	(0)
Booster vaccination (*n*)	20	17	10	4	1	1	2	0	0
(%)	(76.9)	(94.4)	(90.9)	(15.4)	(5.6)	(9.1)	(7.7)	(0)	(0)

35 days apart apparently resulted in the effective downregulation of ovarian activity in all 55 experimental mares.

The small percentage of mares that still showed evidence of ovarian activity and progesterone secretion after the initial vaccination is probably due to individual variation in cyclic status at the time of initial vaccination. The eight mares (14.5%) that were still cyclic 35 days after the initial vaccination were all in the luteal phase at the time of the vaccination (on the basis of both clinical findings and SPC values of >1 nmol/L). This supports the findings that the stage of oestrous cycle and nature of ovarian structures at first administration of vaccine is of importance in terms of the observed response (Tshewang *et al*. 1997; Dalin *et al*. 2002; Imboden *et al*. 2006).

The SPC was shown to provide a reliable indication of ovarian activity for both group and individual monitoring of the response to GnRH vaccination of a relatively large number of mares. The SPC clearly characterises the effect of GnRH vaccination on the ovarian activity of the mares. Following the primary vaccination, eight mares still showed signs of ovarian activity, whereas after the second vaccination no luteal activity was evident. Baseline SPC persisted until the end of the observation period on Day 175. Once again, there was no age effect within the experimental group, contrary to the findings of Tshewang *et al*. (1997) and Dalin *et al*. (2002). On Days 42, 49 and 56 there were 3, 1 and 1 mares, respectively, with SPC >1 nmol/L. Thereafter, and until the end of the observation period, SPC of all experimental mares remained <1 nmol/L. This is well below the threshold value of 6 nmol/L quoted by Elhay *et al*. (2007). In comparison, all five non-pregnant control mares showed SPC indicative of ovarian cyclicity.

Five of the control mares became pregnant during the course of the observation period. This would explain the large increase in standard error obtained between Days 80 and 110. While the non-pregnant control mares continued to cycle until the end of the observation period (Day 175), none of the experimental mares resumed cyclicity. This probably reflects antibody titres that were still sufficiently high to neutralise endogenous GnRH. As a result, insufficient gonadotropins were available to resume normal ovarian activity. The relatively late stage of the physiological equine breeding season may also have contributed to this observation.

The efficacy achieved with the Improvac GnRH vaccine in our trial was greater than that stated in earlier reports (Garza *et al*. 1986; Tshewang *et al*. 1997; Stout *et al*. 2003). Likely explanations for this are improvements in the antigen and adjuvant used in the final vaccine formulation that evoke a better, more consistent and longer-lasting immune response. The details of the Improvac vaccine are unknown to the authors as they constitute proprietary information with a single exception that the antigen is a RnRF-protein conjugate. Equity (Pfizer Animal Health, West Ryde, NSW, Australia) is manufactured by the same company and consists of a GnRH peptide conjugated to a protein carrier combined with an immunostimulating complex consisting of Saponin Quil A, cholesterol and dipalmitoylphosphatidyl choline as adjuvant (Elhay *et al*. 2007). The results of our trial to date, however, indicate better and more consistent results than those reported with Equity.

No adverse effects were observed in the experimental mares after the initial vaccination, which is consistent with the findings described by Dalin *et al*. (2002), but contrary to the findings of Imboden *et al*. (2006), where injection-site reactions occurred after the initial vaccination. The few animals displaying injection-site reactions during this study were observed only after the booster vaccination. All adverse reactions were found to be transient and mild, and by Day 6 were no longer visible. This is in contrast to the findings of Imboden *et al*. (2006), where the adverse effects to the vaccination with Improvac were found to be quite severe. This can possibly be due to the fact that in the present study all injections were administered intramuscularly into the gluteal muscles (J. F. Kirkpatrick, pers. comm.) and not into the neck, as described by Imboden *et al*. (2006).

This study showed a close relationship between the observed clinical response and SPC within a large group of mares. This finding supports the conclusion that SPC provides an effective method for monitoring the response to GnRH vaccination in horse mares of all ages.

On the basis of the results of our trail in mares, Improvac is a promising prospect for the contraception of zebra, African elephants and perhaps other wildlife species. The vaccine is freely available, cheap, stored at 4°C and no further preparation (e.g. mixing of antigen and adjuvant) is required before injection. Side effects at the chosen site of injection were minimal. Although of a slightly viscous nature, the vaccine has been successfully administered remotely via darting in African elephant bulls with both Dan Inject (Børkop, Denmark) and Pneu-Dart (Williamsport, PA, USA) darts (H. Bertschinger, pers. comm.). Reliable reversibility of anoestrus induced by Improvac has yet to be investigated. Imboden *et al*. (2006) were unable to establish this because the mares were no longer accessible after the initial phase of the trial. Elhay *et al*. (2007) established a 100% return to folliculogenesis 8–28 weeks after the second vaccine with Equity. The mares in the present study will continue to be monitored during the 2007–08 breeding season and the data regarding reversibility of Improvac contraception will

become available in the near future. A likely advantage of a GnRH vaccine used for contraception of wildlife species such as African elephant and zebra is induced anoestrus. The females of species like African elephants and zebra, once they have reached reproduction age, spend most of their lives in a state of anoestrus. African elephants have an average intercalving interval of four years. Of this, 22 months is accounted for by pregnancy and approximately two years by lactation anoestrus (Brown *et al*. 2004). This means that cows usually conceive during the first oestrus once cycling resumes. Thus, an elephant cow probably comes into oestrus approximately only once every four years. The use of pZP immunocontraception in feral horses (Kirkpatrick *et al*. 1990) and African elephants (Delsink *et al*. 2006) causes infertility by interfering with fertilisation and the immunised females continue to exhibit oestrous cycles. The authors of the present study hypothesise that pZP is more likely to result in behavioural and social disturbance because individuals in herds are continually attracting males as a result of coming into heat. Comparative studies are needed to test this hypothesis.

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Immunization of sheep against GnRH early in life: effects on reproductive function and hormones in rams

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Groups of Merino ram lambs were immunized against GnRH either soon after birth (prepubertal) or around puberty (peripubertal) with a prototype commercial preparation and studied over the following 2 years to determine the long-term effects of such immunization on reproductive development, function and hormone concentrations. The treatment was equally effective in suppressing reproductive function and hormones when given at either time. Growth rates of immunized and controls rams were similar throughout the study. Prepubertal immunization delayed testicular growth until the rams were at least 27 weeks of age and some rams had subnormally sized testes even at 115 weeks of age. After peripubertal immunization, the mass of the testes declined and remained regressed until the rams were from 51 to 90 weeks old. There was a significant correlation between total testicular mass at 115 weeks of age and duration (weeks) of suppression of testicular growth (prepubertal, $r = -0.7375$; peripubertal, $r = -0.7132$, $P < 0.001$). Prepubertal immunization markedly delayed separation of the penis from the prepuce. The immunized rams did not display sexual activity until their total testicular mass reached at least 70 g. In control and immunized rams with testicular mass > 100 g, the semen did not differ in the percentage of spermatozoa that were unstained (live), of normal morphology, or showed progressive movement. Compared with the increase in plasma LH and FSH concentrations with age in control rams, the concentrations of these hormones in the immunized animals were lower and remained relatively constant from 46 to 90 weeks of age. Plasma FSH concentration was particularly suppressed in immunized rams and was significantly ($P < 0.05$) lower than the control values at 61 and 90 weeks of age. LH release after injection of 1 μg GnRH at 90 weeks of age was either absent or markedly suppressed in immunized rams compared with that for controls.

Introduction

It is well known that repeated immunization of adult male and female domestic animals against the hypothalamic decapeptide gonadotrophin releasing hormone (GnRH) can prevent the production of gonadotrophins and result in the cessation of reproductive function (Jeffcoate *et al.*, 1978, 1982; Robertson *et al.*, 1979, 1982; Schanbacher, 1982). As a result, considerable attention has been paid to the production of practical methods to induce infertility or control of sexual activity in domestic animals through long-term immunity to GnRH.

Although it has been shown that the suppressive effects of anti-GnRH immunization of adult sheep and cattle (Keeling and Crighton, 1984) on reproductive function is reversible with time, the long-term effects of such immunization when applied to animals early in life has not been thoroughly investigated. Gonadotrophins play a vital role in normal development of the gonads and their cellular components (Courot, 1967), and endogenous GnRH is essential for the maintenance of normal concentrations of pituitary GnRH receptors (Fraser *et al.*, 1982; Popkin and Fraser, 1985). A prolonged reduction in the number of pituitary GnRH receptors following immunization against GnRH (Popkin and Fraser, 1985), together with a concomitant decrease in endogenous LH and FSH concentration during early life, might have some permanent effect on the maturation of the CNS centres controlling reproductive function.

Accordingly, the present study was initiated to examine the duration and severity of effect of an anti-GnRH immunization treatment on subsequent growth, sexual behaviour and reproductive physiology in rams immunized early in life. In addition, the plasma concentrations of the reproductive hormones and the release of LH and testosterone following injection of 1 μg GnRH were compared with those in control rams.

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Materials and Methods

Animals and immunization schedule

Fifty Merino ram lambs born in August were used in this study at Armidale, NSW, until they were approximately 115 weeks of age. They were weaned at 12 weeks of age and maintained at pasture thereafter. Twenty of the lambs each received 1 mg of antigen (see below) in a 2 ml dose (primary dose) at 3–4 weeks of age (prepubertal immunized group) and a boost with the same type and amount of dose at 13–14 weeks of age. Twenty other rams received their primary and booster dose when they were 20–21 weeks and 30–31 weeks of age, respectively (peripubertal immunized group). The remaining ten non-immunized rams served as controls. The primary and booster doses were each injected s.c. at four sites in the neck region. Thirty of these ram lambs (ten from each of the three groups) were used to examine the effects of such immunization on reproductive endocrinology.

Immunogen

Details of the prototype commercial immunization preparation, which was developed in collaboration with Arthur Webster Pty Ltd, Castle Hill, NSW and Peptide Technology Ltd., Dee Why, NSW, are given by Hoskinson *et al.* (1990). Briefly, the immunogen was prepared by conjugation of a carboxyl-containing GnRH analogue to ovalbumin using the diimide reaction. This conjugate was emulsified using a mixture of a solution of 5% DEAE–dextran (pH 7.5) in a 50% water-in-mineral oil emulsion.

Anti-GnRH antibody titre

At frequent intervals (initially 2 weeks, then 4–6 weeks) after booster immunization, 5 ml of blood was taken from the jugular vein in all GnRH-immunized rams to monitor anti-GnRH antibody titre. Antibody titre was determined for all plasma samples from any one sheep in the same radioligand binding assay, which was similar to that reported for steroids (Abraham, 1974) but using GnRH (specific activity: 100 Ci mmol^{-1}). Titre is defined as a dilution of antiserum that bound 50% of the available radioligand.

Body and testicular growth

From about 14 weeks of age, the animals were weighed at frequent intervals (every 4–6 weeks) to assess rate of body growth. Coinciding with these times, estimates of total testicular mass (of the two testes) were also obtained using a palpation method described by Oldham *et al.* (1978), which has been calibrated against actual weights of testes. The period of delayed testicular growth was defined as the time during which no sustainable increase in testicular mass occurred above that at the time of the booster immunization.

Assessment of sexual activity and semen characteristics

Sexual activity of each control and GnRH-immunized ram was evaluated from the total number of services they performed when individually joined with oestrous ewes in pens for 20 min on each of two consecutive days (Mattner *et al.*, 1971). At the end of the study, a semen sample was collected from each ram (where possible) using the electroejaculation procedure (Blackshaw, 1954) to evaluate seminal characteristics. The percentages of unstained (live) and morphologically normal spermatozoa were determined from nigrosin–eosin stained smears of semen (Hancock, 1952). The percentage of spermatozoa exhibiting progressive movement was assessed as described by Mattner and Voglmayr (1962) using prewarmed slides and direct microscopy.

Blood sampling and GnRH challenge

Jugular blood samples (5 ml) were obtained by venepuncture from ten control, ten prepubertal immunized and ten peripubertal immunized rams at intervals of 30 min for 6 h when they were 46, 61 and 90 weeks of age, to measure mean concentrations of the reproductive hormones. The samples were held on ice until centrifugation (at 5°C and 3000 ***g*** for 20 min) within 30 min of collection, and the plasma stored at − 10°C until assay. At 90 weeks of age, 1 μg GnRH (ICI, Melbourne) in 2 ml of 0.9% saline was injected into a jugular vein in each of three control rams and seven GnRH-immunized rams (which were selected from either immunized group to provide a range in testicular mass), to assess the LH and testosterone response to a GnRH challenge. Blood samples (5 ml) were obtained by jugular venepuncture before the GnRH injection and afterwards, at 10 min intervals for 1 h and then at 20 min intervals for a further 2 h.

Hormone assays

All hormones were measured by radioimmunoassay. The sensitivity of the assays for LH (Radford *et al.*, 1987a) and FSH (Radford *et al.*, 1987b) were 0.19 and 0.06 ng ml^{-1}, respectively. The intra-assay and interassay coefficients of variation (at 50% displacement of tracer) were all < 10%.

Testosterone concentrations were measured in duplicate. The samples and standards (20 μl) were extracted for 5 min with 2 ml of toluene:hexane (2:1, v:v), the solvent phase was evaporated to dryness under N_2 and the single antibody technique of Garnier *et al.* (1978) was then used. An antiserum raised in a sheep against testosterone-3-carboxymethyloxime–BSA (M. S. F. Wong and R. I. Cox, CSIRO, Division of Animal Production, Blacktown, NSW) was used at a dilution of 1:60 000. Crossreactivity with dihydrotestosterone, 4-androstene-3β,17β-diol and androstenedione was 31%, 30% and 1.3%, respectively. All oestrogen and progesterone compounds had < 1% crossreactivity. Mean nonspecific binding was 2.8%; the sensitivity of the assay was 0.1 ng ml^{-1}; and for pooled ovine plasma samples with mean concentrations of 0.6, 3.5 and 7.3 ng ml^{-1} the intra-assay and interassay coefficients of variation were < 8% and < 10%, respectively.

Statistical analyses

Data were analysed using standard procedures for repeated measures analysis of variance, one-way analysis of variance,

Table 1. Mean (± SEM) body mass (kg) and mean total testicular mass (g) for control and GnRH-immunized rams at different stages in life

Animals	Age (weeks) 46	61	90	115
Control rams				
Body mass	26.2 ± 1.1	39.8 ± 1.1	51.8 ± 1.3	50.4 ± 1.9
Testis mass	101 ± 12[a]	242 ± 24[a]	214 ± 10[a*]	338 ± 12[a]
Prepubertal immunized rams				
Body mass	25.4 ± 0.8	43.6 ± 1.5	48.7 ± 1.8	50.6 ± 1.8
Testis mass	43 ± 10[b]	112 ± 16[b]	147 ± 20[b]	202 ± 22[b]
Peripubertal immunized rams				
Body mass	28.3 ± 0.8	44.1 ± 1.5	48.3 ± 1.5	49.5 ± 1.4
Testis mass	23 ± 3[b]	79 ± 12[b]	116 ± 20[b]	170 ± 19[b]

Values in any column with different superscripts differ significantly from each other ($P < 0.05$).
*Value probably suppressed owing to effect of season (June) in adult rams (see text).

Student's *t* test and regression analysis (Minitab, Release 7.2, Minitab Inc., Pennsylvania). After GnRH injection, a positive response (rise in LH and testosterone) was said to occur when (i) the concentration of the hormone increased by at least 50% above the preceding baseline value, (ii) there were at least two points between the peak value and the succeeding baseline, and (iii) the rate of decline in concentration after the peak was no greater than that allowed by the half-life of the hormone (Khalid *et al.*, 1987).

Results

During the course of the study, one prepubertal and two peripubertal immunized rams died from causes unrelated to the immunization treatment.

Anti-GnRH antibody titre

In all treated animals, the peak anti-GnRH antibody titre response occurred 2 weeks after the booster dose, after which the titre declined at various rates. There were very great differences in antibody titre response between animals (range of 1:2000 to 1:50 000) and the magnitude of the peak titre values after the boost could not be related to severity or duration of the effect on testicular development. Furthermore, the magnitude of the peak titre value after the boost was not related to the subsequent rate of decline in antibody titre.

The rate of decline in antibody titre tended to be slower in the rams that had testicular masses < 100 g at the end of the study than in those that ultimately developed testes > 100 g. Thus, at 20, 30 and 60 weeks after booster immunization, the prepubertal immunized rams with small testes had mean circulating antibody titres that were 27, 15 and 8%, respectively, of the peak value after the boost, while in all other prepubertal immunized rams, the corresponding values were 9, 4 and 2%, respectively. Similarly, with the peripubertal immunized rams, there was a tendency for those animals with small testes to have a slightly higher mean antibody titre (as a percentage of the peak value after the boost) from 30 weeks after the booster dose, than for other animals in this immune group. By the end of the study, antibody titres were detectable in only three of the 37 GnRH-immunized rams, with the values ranging from 3 to 16% of the peak titre after the boost (1:500 to 1:1000).

Body and testicular growth

Throughout the study, the rate of body growth for the two groups of GnRH-immunized rams did not differ significantly from each other or from that for the control rams (Table 1).

For all treatment groups, mean testicular mass increased significantly with increasing age, but the rate of testicular growth differed between the groups. While the group mean values for testicular mass for the prepubertal and the peripubertal immunized rams did not differ materially from each other from 46 to 115 weeks of age, they were significantly ($P < 0.05$) lower than the corresponding values for the control rams (Table 1).

To understand the trends in testicular development with respect to severity and duration of effect of the treatment, the immunized animals were grouped according to their total testicular mass at 115 weeks of age as follows: those with normally sized testes and in the testicular mass range of the control rams, > 200 g (range for control animals was 220–360 g); those with small testes (juvenile-like), ⩽ 100 g; and those with medium-sized testes that were substantially smaller than those of control animals, > 100 ⩽ 200 g. In the prepubertal ($n = 11$) and in the peripubertal ($n = 6$) immunized rams that ultimately developed testes in the normal range, the treatment delayed testicular growth from 27 to 46 weeks and from 21 to 44 weeks, respectively. For the immunized animals with small testes at 115 weeks, testicular development was delayed from 51 to 99 weeks for the prepubertal immunized group ($n = 4$) and from 64 to 90 weeks in the peripubertal immunized animals ($n = 5$), but some of the rams in both of these groups exhibited little testicular growth even at 115 weeks of age. At 115 weeks of age, there was a significant correlation between the mean testicular mass and the duration

Table 2. Mean (± SEM) service score* for control and GnRH-immunized rams and the percentage of rams serving at various stages in life

Animals	Age (weeks) 61	90	115
Control rams			
Service score	3.0 ± 0.7[a]	3.4 ± 0.5[a†]	6.4 ± 0.6[a]
Rams serving (%)	90	100	100
Prepubertal immunized rams			
Service score	1.3 ± 0.4[b]	3.0 ± 0.6	4.0 ± 0.6[b]
Rams serving (%)	50	72	83
Peripubertal immunized rams			
Service score	0.9 ± 0.4[b]	1.7 ± 0.5[b]	4.1 ± 0.6[b]
Rams serving (%)	25	50	89

*Service score for individual rams is the total number of services performed in two pen tests of 20 min each conducted 24 h apart. Values in any column with different superscripts differ significantly from each other ($P < 0.05$).
†Value probably suppressed owing to effect of season (June) in adult rams (see text).

(weeks) of suppression of testicular growth (prepubertal, $r = -0.7375$; peripubertal, $r = -0.7132$, $P < 0.001$).

Assessment of sexual activity and semen characteristics

The GnRH-immunized rams showed no interest in mounting oestrous ewes until they had attained a testicular mass of at least 70 g. Rams with testes of 70–100 g only occasionally served oestrous ewes. For all treatment groups, the mean service score increased significantly ($P < 0.05$) with increasing age (Table 2). The group mean service score for the prepubertal or the peripubertal immunized rams did not differ significantly from each other at any of the age periods studied, but the values for the two immunized groups at 61 and at 115 weeks of age and for the peripubertal immunized rams at 90 weeks of age were significantly lower ($P < 0.05$) than the corresponding values for the control rams (Table 2). The lack of a difference between control and prepubertal immunized animals at 90 weeks of age was possibly due in part to the suppression of libido that occurs in normal adult rams at this time of year (June) in the nonbreeding season (Mattner, 1977). When only the rams that actually served in pen tests were considered, the mean service scores for the immunized and control rams did not differ significantly.

Semen characteristics

At 46 weeks of age, separation of the penis from the prepuce had occurred in 90% and 37% of animals in the control and prepubertal immunized groups, respectively.

In three GnRH-immunized rams with testes < 100 g at 115 weeks of age, separation of the penis from the prepuce had still not occurred and no semen sample could be obtained. In three other rams in this testis size range in which the penis was free, the ejaculates consisted of seminal fluid devoid of or almost devoid of spermatozoa.

Ejaculates were obtained from all control rams and from all GnRH-immunized rams with testes > 100 g. There was little difference between the control or the immunized animals with respect to the percentage of spermatozoa that were unstained (live), morphologically normal, or showed progressive movement (Table 3).

Although the mean value for the percentage of motile spermatozoa in the ejaculates of the immunized rams with medium-sized testes was low, it was not statistically different from that in ejaculates from control or from the immunized rams with normally sized testes.

Plasma concentrations of testosterone, LH and FSH

Analysis of the data revealed a significant ($P < 0.001$) effect of age on testosterone concentrations, with the mean values increasing markedly in all groups from 46 weeks to 61 weeks of age (Table 4). The decline in mean concentration of this hormone for the control and the prepubertal immunized rams at 90 weeks of age is probably due to a suppressive effect of the nonbreeding season (Mattner, 1977). The mean circulating concentration of testosterone for the peripubertal immunized group of rams at both 46 and 61 weeks of age was significantly lower ($P < 0.02$) than that for the control rams (Table 4). However, in immunized rams (of either group) and control rams with testes of comparable size, mean plasma testosterone concentrations were of a similar order.

The mean plasma concentrations for LH and FSH increased with age in control animals, but the values were relatively constant from 46 to 90 weeks of age in both groups of immunized rams. As a result, the group mean LH values for the two immunized groups at 61 weeks of age and their group mean FSH values at both 61 and 90 weeks of age were significantly lower ($P < 0.002$) than the corresponding values for the control rams (Table 4).

Responsiveness to GnRH injection

The endocrine responses obtained in control and immunized rams to an i.v. injection of 1 μg GnRH are shown (Table 5). In

Table 3. Semen characteristics of ejaculates obtained from control ($n = 10$) and GnRH-immunized rams with medium-sized ($n = 17$) or normally sized ($n = 11$) testes at 115 weeks of age (values are means ± SEM)

Animals	Semen characteristics		
	Live (%)	Normal (%)	Motile (%)
Control rams	73.9 ± 2.5	85.1 ± 3.2	79.5 ± 2.6
GnRH-immunized rams (normal-size testes)	79.6 ± 2.5	74.7 ± 4.2	80.6 ± 5.2
GnRH-immunized rams (medium-size testes)	79.0 ± 4.5	73.8 ± 5.3	68.5 ± 11.5

Data for prepubertal and peripubertal immunized rams are pooled since there were no significant differences between the two immunized groups.

Table 4. Mean (± SEM) concentrations (ng ml^{-1}) of testosterone, LH and FSH for control ($n = 10$) and GnRH-immunized ($n = 20$) rams at different stages in life, and in relation to mean anti-GnRH antibody titre (the percentage of rams with measurable antibody titre is given in parentheses)

Hormone	Age (weeks)	Controls	Prepubertal	Peripubertal
Testosterone	46	0.9 ± 0.2	0.7 ± 0.1	0.3 ± 0.1*
	61	3.2 ± 0.6	2.0 ± 0.5	1.3 ± 0.3*
	90	2.1 ± 0.7†	0.9 ± 0.2	1.5 ± 0.5
LH	46	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
	61	0.6 ± 0.1	0.4 ± 0.1*	0.2 ± 0*
	90	0.8 ± 0.3	0.2 ± 0	0.3 ± 0.1
FSH	46	0.3 ± 0.1	0.3 ± 0	0.2 ± 0.1
	61	0.4 ± 0.1	0.2 ± 0*	0.1 ± 0*
	90	0.5 ± 0.1	0.3 ± 0*	0.1 ± 0*
Mean titre	46		1:500 (70)	1:900 (90)
	61		1:450 (60)	1:600 (80)
	90		1:100 (20)	1:400 (60)

GnRH-immunized rams were treated either soon after birth (prepubertal) or around puberty (peripubertal).
Within rows asterisk denotes a significant difference ($P < 0.05$) from control value.
†Mean possibly lower because of seasonal effects observed in adult rams.

each of the control rams, there was a marked increase in plasma LH concentration within 10 min of the injection of GnRH, followed approximately 20 min later by a rise in plasma testosterone concentration. A similar response did not occur in any of the GnRH-immunized rams with testicular mass < 100 g. GnRH injection into the immunized rams with testicular mass ≥ 100 g resulted in peak plasma LH concentrations that were considerably less than those in the controls, and peak plasma testosterone concentrations that were of a similar order to those in control animals (Table 5). Two of the rams with testes < 100 g had measurable antibody titres that were very low (6 and 8% of the peak antibody titre value after the boost). All other rams injected with GnRH had no measurable antibody titres.

Discussion

Immunization of adult animals against GnRH appears to produce only temporary suppression of gonadal function and sexual behaviour, as reproductive function usually resumes fairly rapidly after decline of antibody titre without any apparent detrimental effects occurring (Robertson *et al.*, 1982; Keeling and Crighton, 1984). In contrast, the present study shows that similar immunization of rams very early in life can produce profound and extremely long-lasting effects on their reproductive capacity, with some of the animals having juvenile-like reproductive organs and a complete lack of libido two years later.

Since many of the immunized rams lacked normal gonadal development long after anti-GnRH antibodies had dissipated, it appears that such immunization early in life may have induced some permanent damage to mechanisms that control reproductive function. Indeed, the results of the present investigation on a selection of these GnRH-immunized animals revealed that their mean plasma gonadotrophin concentration was significantly lower than that of the non-immunized controls. The lower hormone concentrations in the immunized rams at 61 weeks of age is not surprising, since at that time, 14

Table 5. Peak plasma LH and testosterone concentrations (ng ml^{-1}) in control and GnRH-immunized rams injected with 1 μg GnRH at 90 weeks of age

Sheep	Response to injection	Peak LH[a]	Peak testosterone[a]	Testis mass (g)
Control rams	Positive[b]	15.4	7.8	250
	Positive	36.9	9.2	220
	Positive	23.3	7.8	250
GnRH-immunized rams	No response	0.3	0.5	40
	No response	0.4	0.8	50
	No response	0.3	2.1	70
	Positive	2.2	10.7	100
	Positive	8.4	3.0	130
	Positive	1.0	4.8	160
	Positive	1.4	6.8	160

[a]Peak values refer to the maximum concentration of the hormone subsequent to GnRH injection.
[b]Positive response: a rise in concentration of the hormones following injection of GnRH, in accordance with the defined criteria.

of the 20 immunized animals still had some anti-GnRH antibodies present that could be expected to bind at least some of the circulating GnRH (titre range: 2–33% of the peak titre value after the boost). However, at 90 weeks of age, the lower plasma FSH concentrations persisted in both immunized groups of rams, despite the lack of measurable anti-GnRH antibody titres in 12 of the 20 immunized animals and very low titre values for the remainder. Accordingly, these findings indicate that the immunization may have induced some degree of impairment to hypothalamic function, resulting in long-term suppression of GnRH and of gonadotrophin release in the majority of the immunized rams.

The absence of an LH response in the immunized rams with small testes ($\leqslant 100$ g), or the markedly reduced LH release (relative to that for controls) in those with testes $\geqslant 100$ g following injection of GnRH at 90 weeks of age, suggests that the immunization also induced some degree of impairment to pituitary function, resulting in a reduced capacity of the gland either to respond to GnRH stimulation or to produce and release LH and FSH. However, as the concentration of pituitary GnRH receptors appears to be under positive autoregulatory control of GnRH (Clayton and Catt, 1981; Popkin and Fraser, 1985), it is possible that the diminished LH response in the immunized animals to GnRH injection may merely reflect a state of downregulation of pituitary GnRH receptors due to little or no unbound GnRH in the hypophyseal portal circulation.

The significantly lower mean circulating concentrations of testosterone in the peripubertal immunized rams at 46 and at 61 weeks of age would appear to result from many rams in this immunized group having testes of extremely small size rather than to an inability of the testes to produce this hormone (at 46 weeks of age all peripubertal immunized rams and three control rams had testes $\leqslant 70$ g; at 61 weeks of age six peripubertal immunized rams but no control rams had testes $\leqslant 70$ g). Unlike the LH responses in the immunized animals following GnRH injection, the peak plasma testosterone concentrations in those immunized rams with testes $\geqslant 100$ g were of a similar order as those in the controls. Consequently, it appears that the testes of the immunized rams may have become more sensitive to LH stimulation; this feature could account for the ultimate increase in testis mass in the majority of the immunized rams, despite the significantly reduced circulating concentrations of the gonadotrophins. These findings suggest that while the immunization suppressed testicular development, it had no long-term detrimental effect on testicular or Leydig cell function *per se* and that the normal development of the testes was merely delayed owing to insufficient gonadotrophic stimulation. Once testicular development commenced, immunized and control rams with testes of similar size (irrespective of age) had similar mean plasma testosterone concentrations. It seems likely, therefore, that the size of the testes was the main limiting factor to the production of this hormone in the immunized animals.

In both groups of GnRH-immunized rams, the lower mean libido score occurred as a result of the presence of sexually immature animals and other rams that did not serve, despite them having well-developed testes. Some of the immunized rams with estimated testicular mass between 70 and 100 g at 115 weeks of age served oestrous ewes, suggesting that while mechanisms facilitating testicular development may be permanently impaired, maturation of the neural centres that regulate sexual behaviour may not be seriously affected by the absence of gonadotrophin or androgens over a period during early life. Once these centres had been activated, the GnRH-immunized rams soon developed service capacity similar to that of untreated controls.

It is well known that expression of sexual behaviour normally precedes the full development and maturation of the testes. Even though, overall, sperm production was considerably reduced in GnRH-immunized rams owing to reduced testicular size, the quality of semen in those rams that developed a testicular mass > 100 g appeared to be normal. Thus, it is likely that the rams of this class that became sexually active could impregnate at least a proportion of the ewes served by them.

As the anti-GnRH vaccine, whether administered soon after birth or at about the time of onset of puberty, is effective in suppressing gonadal development and sexual activity for considerable periods, commercial producers have wide scope

for the time of immunization. However, the present data show that the suppressive effects on reproductive function may not be reversible in all treated animals. With appropriate booster immunization schedules, the procedure appears to have potential for use in other animal industries, such as companion animals, as an alternative method to conventional spaying when permanent infertility is required.

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Efficacy of dart-delivered PZP-22 immunocontraceptive vaccine in wild horses (*Equus caballus*) in baited traps in New Mexico, USA

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Abstract

Context. Federally protected wild horses on public lands are undergoing population growth that overwhelms the historical management strategy of removal and adoption. Porcine zona pellucida (PZP) has been used as an injectable immunocontraceptive vaccine to induce reversible infertility in free-roaming horses. PZP vaccination during February and March, which is the optimal time for administering current vaccines, is not possible for the herd on Jarita Mesa Wild Horse Territory (JM WHT), New Mexico, due to severe weather, terrain and subject wariness.

Aims. The first goal was to assess bait trapping and remote darting as a minimally disruptive alternative to helicopter gathers for treatment. The second goal was to quantify the efficacy over 2 years following spring treatment with a single injection of PZP-22 (a combination of PZP-adjuvant emulsion and controlled-release pellets) by remote dart delivery.

Methods. Bait trapping and dart delivery of PZP-22 was carried out on JM WHT from 4 April to 16 June 2012. The herd was observed in the summers of 2011, 2013 and 2014 to determine the foaling status of the study mares. Outcome (foal or no foal) as a function of treatment was analysed using Fisher's exact test.

Key results. There were 157 individuals, including 66 females >1 year old, documented in 2011. In 2012, 26 females (including three yearlings) identified by colour and markings were bait trapped and darted with PZP-22. The proportion of treated females foaling was lower than that of untreated females in 2013 and 2014, but the difference was only significant in 2013. Of the treated mares observed in 2013, the two that foaled were the last two treated in 2012. Untreated mares >4 years old were significantly more likely to foal than younger mares.

Conclusions. Bait trapping at JM WHT permitted successful delivery of PZP-22 in a previously inaccessible herd. Dart administration of PZP-22 in April–June induced at least 1 year of measurable infertility.

Implications. This is the first demonstration of the efficacy of an initial treatment of PZP-22 delivered by dart instead of hand injection. Considerations for PZP-22 treatment include seasonal timing of treatments and age of treated mares. Treatments need to take place early enough to allow antibody titers to build to contraceptive levels before the breeding season.

Additional keywords: contraception, fertility control, management strategies, natural resource management, population control, population management, reproductive biology, wildlife management.

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Introduction

Management of wild horses on public lands in the US, as required under the 1971 *Wild Free Roaming Horse and Burro Act* (P.L. 92–195, as amended) has proven challenging. Wild horse herds grow at a rate of 15–20% annually through natural reproduction (Garrott 2018). Growing wild horse populations increase pressure on forage that under the Act they share with wildlife and, where permitted, livestock. Since 1975, the US Bureau of Land Management (BLM) and US Forest Service (USFS) have attempted to control wild horse populations by gathering and removing horses from the range and placing them with adopters, privately funded sanctuaries, contract ranches or

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government-managed long-term holding facilities (Norris 2018). This strategy has not proven sustainable, and the policy of removals may be exacerbating population growth by artificially stimulating reproduction (Kirkpatrick and Turner 1991*a*; Garrott and Oli 2013; National Research Council 2013).

Immunocontraceptives are one possible solution to the control of wild horse populations on public lands. An ideal immunocontraceptive for free-roaming horses is specific, safe for the animal and environment, effective and both cost-effective and practical to administer on a large scale (Kirkpatrick *et al.* 2011; Hobbs and Hinds 2018). First documented to induce infertility in wild and domestic mares by Liu *et al.* (1989), the porcine zona pellucida (PZP) vaccine, tested in a variety of formulations, has been shown to meet many of those requirements (Kirkpatrick *et al.* 1990; Kirkpatrick and Turner 1991*b*; Turner and Kirkpatrick 2002).

Efficacy and cost of administration are key to effective limitation of population size and growth by means of a contraceptive agent. The practicality of delivering PZP to free-roaming mares by hand or dart has been proven in a select group of small, well studied herds (Kirkpatrick *et al.* 1990; Kirkpatrick and Turner 1991*b*; Ransom *et al.* 2011). However, wild horse herds on public lands in the western United States are often challenging to access for vaccine administration because of the rough terrain they inhabit and some horses' avoidance of humans (Turner and Rutberg 2013). In its initial form, consisting of an emulsion of PZP antigen and Freund's Complete Adjuvant, the vaccine required a booster 1 month after the initial injection to reliably decrease fertility for 1 year (Kirkpatrick *et al.* 1990; Turner *et al.* 1997). Extending the period of infertility induced by treatment would eliminate the need for labour-intensive annual boosters and decrease the risk of wary free-roaming horses learning to evade capture and treatment (Naugle and Grams 2013). The goal of extending the longevity of PZP effectiveness has followed two routes. First, PZP has been incorporated into liposomes and delivered with various adjuvants under the trade name SpayVac (IVT, Halifax, NS, Canada). Although SpayVac has shown promise in trials on captive horses (Killian *et al.* 2008; Bechert *et al.* 2013; Roelle *et al.* 2017), it has not yet been field tested on free-roaming wild horses. The second approach has been to add controlled-release components to the PZP emulsion to mimic the effects of boosters. Initial field trials of PZP emulsions combined with PZP embedded in lactide–glycolide polymer microspheres produced 1 year of effectiveness with a single shot, but the microspheres were difficult to handle under field conditions (Turner *et al.* 2001, 2002). Subsequently, a vaccine formulation consisting of the PZP-adjuvant emulsion combined with PZP and QA-21 adjuvant embedded in controlled-release lactide–glycolide polymer pellets ('PZP-22'), delivered by jabstick, induced 2–3 years of substantially reduced fertility with a single injection in January at Clan Alpine HMA, Nevada (Turner *et al.* 2002, 2007). This reduction was followed by a return to a control level of fertility in 4 years (Turner *et al.* 2007). However, attempts to replicate this multi-year effect in mares at three western sites did not meet with the same success (Ransom *et al.* 2011; Rutberg *et al.* 2017). Hypotheses for the observed reduction in effectiveness of PZP-22 primers are currently being tested (Rutberg *et al.* 2017).

Rutberg *et al.* (2017) showed that PZP-22 boosters could be delivered effectively by dart, but remote delivery of PZP-22 priming doses has not been studied. The ability to deliver initial treatments of PZP-22 by dart rifle would permit treatment with less equipment, labour and handling of wild horses.

The aim of the present study was to assess the reduction in fertility of treated mares over 2 years following administration of PZP-22 via dart on unrestrained horses in a corral. Treatments were carried out at Jarita Mesa Wild Horse Territory (JM WHT), Carson National Forest, New Mexico. Access to wild horses for management purposes on JM WHT is challenging. Gathering horses by helicopter is ineffective due to heavily forested terrain and large variations in elevation. JM WHT is generally inaccessible by vehicle in the winter months due to snow cover. Additionally, its resident wild horses avoid direct approaches by people. Thus, access to wild horses on JM WHT requires the development of capture methods other than helicopter gather.

Materials and methods

Study area

The Jarita Mesa Wild Horse Territory (JM WHT) in Carson National Forest, New Mexico, is a 22 200-ha range managed by the U.S. Forest Service (2006; USFS Environmental Assessment). Elevations range from 2300 to 3200 m. Pinon pines (*Pinus* spp.), juniper (*Juniperus* spp.) and sagebrush (*Artemesia tridentata*) dominate the vegetation at lower elevations, and Ponderosa pine (*P. ponderosa*) forest dominates at higher elevations. The JM WHT is home to native mule deer (*Odocoileus hemionus*) and elk (*Cervus elaphus*). As in most western landscapes, JM WHT has a long history of human use, including grazing by domestic livestock; in 2011, the US Forest Service permitted private livestock owners to graze 502 cow–calf pairs and 16 bulls. Appropriate Management Level (AML) for wild horses on JM WHT is 20–70 horses.

Field observations and individual identification

Wild horses were surveyed by vehicle and on foot from 26 May to 17 December 2011, 14 June to 7 August 2013 and 6 June to 7 August 2014. High horse-traffic areas were identified by the presence of tracks and faeces. Two Bushnell (Overland Park, KS, USA) wildlife cameras with 24-h motion activated sensors were placed at heavily used water holes and high traffic areas to document movement of the wild horse groups. When observed directly, wild horses were photographed with a Rebel EOS T3i camera (Canon, Tokyo, Japan) and telephoto (120–400 mm) lens (Sigma, St Louis, MO, USA) and further observed with a Prostaff (20–60 × 82 mm) spotting scope (Nikon, Tokyo, Japan). Horses were photographed for positive identification based on colour, unique face and leg markings, scars and sex. Band composition was recorded, including associations among mares, immatures and stallions. Foals were associated with the dam using affiliative behaviours such as nursing or, occasionally, repeated proximity while grazing or traveling. A few mares were associated with foals by ruling out all other mares present in the group as the possible dam, because they closely associated with other foals present. Foals were aged at first observation to approximately the nearest 2 weeks based on size, tail length and

behaviour. Disturbance of the horses' natural activities by the observer was avoided whenever possible.

The Wild Horse Identification Management System (WHIMS; Wildwise Ecological Solutions, http://wildwisesolutions.com/Whims.aspx) is application-based software used to visually catalogue all the horses identified in a study herd. Each horse is assigned a unique ID number. Information associated with each entry includes sex, age, colour, markings, current and past band associations, dam and photos from each year observed (U.S. Geological Survey 2013). New entries were generated when sufficient photographic evidence was collected during the 2013 and 2014 surveys to prove the animal was not previously identified. New entries were made for all foals of the year.

Capture methods

Groups observed during 2011 field observations were chosen for the PZP-22 treatment regime based on ease of identification, access and regular sightings. As part of a management action that also included capture and removal from the range, eight sites were identified for bait placement based on locations where horses had been observed in the past, road access, horse trails and water sources. Only two sites were set up at a time. Wild horse bands were attracted to gather sites using a proprietary bait formula provided by Mt Taylor Mustangs (Santa Fe, NM), which was hired by USFS under independent contract to treat the JM WHT horses. Timing of bait set-up depended on seasonal horse movements, road condition, snow cover and presence or absence of livestock, which may also be drawn to bait. When a band had acclimated to the bait at a gather site, a 10–13-m diameter catch corral made of portable metal pipe panels was set up at the bait site and the gate left open for several days (typically 3–7), so that horses could come and go freely from the catch corral. Cameras were used to monitor band activity with observers out of the horses' sight. After acclimatisation, when most horses in a group were in the corral, the gate was shut and secured by remote control, with the operator in a nearby blind. All mares trapped in April through mid-June were vaccinated; all mares trapped after mid-June were removed.

Porcine zona pellucida treatment

Females were injected with PZP-22 between 4 April and 16 June 2012 in catch corrals during daylight hours; horses corralled after sunset were held until daylight for darting. All females ≥1 year old that were not removed were treated. The PZP-22 consisted of 100 μg PZP (Science and Conservation Center, Billings, MT, USA) dissolved in 0.5 mL phosphate-buffered saline and emulsified in 0.5 mL modified Freund's Complete Adjuvant (mFCA, Calbiochem, LaJolla, CA, USA) plus three heat-extruded lactide–glycolide polymer pellets containing a total of 450 μg PZP and 625 μg QA-21 (Antigenics, Lexington, MA, USA) (Turner *et al.* 2007). The vaccine was loaded into a 1.0-mL Pneu-Dart (Williamsburg, PA, USA) dart designed with a rod within the 13-gauge, 1.5-inch needle to simultaneously inject the emulsion and the pellets. Darts were delivered into the gluteal musculature via Pneu-Dart X-Caliber rifle at a range of 10–15 m. The corral was videotaped during darting to confirm identification of treated horses and successful dart discharge. Once all mares in a captured group had been treated, the gate was opened and the group was released.

Statistical analysis

For purposes of statistical testing, foaling by PZP-treated and untreated mares was determined using only positively identified females 2 years and older (determined by size and, where known, individual histories) with a definitive foaling status. Mares that were positively identified but had an unclear foaling status were excluded from calculations relating to the outcome variable of foal or no foal.

Fisher's Exact tests (FET) were used to compare outcome (foal or no foal) among treatment groups and years. Because previous studies have not shown a difference in fertility between placebo-treated females and untreated females (Turner *et al.* 1997), the fertility of PZP-22-treated females was compared within a given year with that of individually identified females not captured for treatment. The proportion of females foaling in 2011 (before PZP treatment) also served as a comparison to the post-treatment proportions in 2013 and 2014. Alpha was set at $P = 0.05$ for all tests.

Results

Between 4 April and 16 June 2012, 26 females (three yearlings and 23 mares ≥2 years of age) were darted with PZP-22 in catch corrals and released back to the range. In addition, 78 wild horses were captured and removed; 60 of these (including 15 adult females, and three 2-year old females) had been previously identified in 2011. Horses did not avoid bait areas after corral panels were put in place. Reactions to injection were moderate and non-violent. We saw no abnormalities at or near the injection site on any of the treated mares at any time during the study, and no mares sustained injuries during capture or darting.

Among known mares observed from June to August 2013 (Year 1), the proportion foaling was 79% lower in PZP-treated mares (2/17; 12%) than in untreated mares (15/27; 56%) (FET, $P = 0.0046$). Among known mares observed from June to August 2014 (Year 2), the proportion foaling was 36% lower in PZP-treated mares (6/17; 35%) than untreated mares (14/26; 54%); this difference was not statistically significant (FET, $P = 0.349$). The pooled proportion of all treated and control mares in the sample foaling did not differ among the 3 years of the study (2×3 FET, $P = 0.736$).

Except for the decline associated with removals during trapping activity in 2012, the sample of individually recognised animals continued to grow throughout the study (Fig. 1). Part of that growth was due to identification of new adult animals not observed on the study site in previous years, and part due to birth and recruitment of new foals. The number of females ≥2 years old included in the individually recognised sample was 69 in 2011, 60 (post-removal) in 2012, 78 in 2013 and 86 in 2014. Thus the number of females treated with PZP-22 in 2012 represented only a small proportion of the sample of individually recognised mares on JM WHT (43.3% in 2012, 33.3% in 2013 and 30.2% in 2014).

With respect to the sample of all identified mares, 27 (40.3%) of 66 mares observed had a foal present in 2011 (pretreatment), 16 (37.2%) of 43 mares observed had a foal present in 2013, and

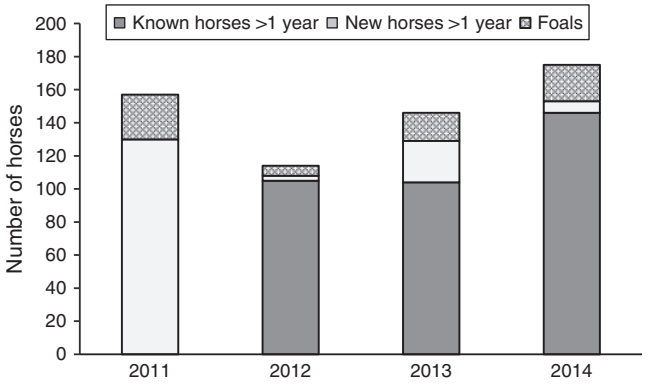


Fig. 1. Total sample of wild horses individually identified on the Jarita Mesa Wild Horse Territory, NM, USA in 2011–14. Numbers of known horses declined in 2011–12 because of removals conducted in 2012, then increased with the addition of newly identified individuals and new foals of the year. Clear, new horses >1 year old; solid grey, horses >1 year old documented in previous years; stippled, foals of the year.

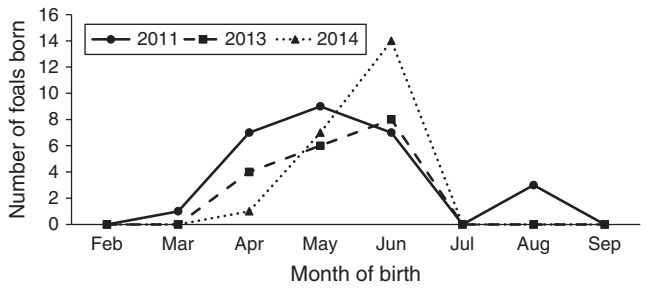


Fig. 2. Dates of foaling for individually identified mares on Jarita Mesa Wild Horse Territory, NM, USA in 2011–14. Dates estimated to within 2 weeks based on foal size, tail length and behaviour at first observation.

20 (46.5%) of 43 mares observed had a foal present in 2014. Untreated mares 4 years old or more had significantly higher foaling rates than mares less than 4 years of age in both 2013 (15/26, 58% for mares ≥4 years of age *v.* 0/5 for females <4 years of age, FET, $P = 0.0098$) and 2014 (13/20; 65% for mares ≥4 years of age *v.* 1/6, 17%, for mares <4 years of age, FET, $P = 0.0192$).

Of 79 foaling dates estimated from 2011 to 2014, 94.5% occurred in April–June (Fig. 2). Estimates of birth dates for the eight known births to treated mares in 2013 and 2014 fell within that window.

Discussion

Single injections of PZP-22 by dart delivery to mares accessed using bait trapping in late spring 2012 reduced foaling rates in the treated mares in the JM WHT for at least 1 year. Although fertility rates for treated mares were lower than those of untreated mares in 2014 (the second year after treatment), this reduction in fertility was not statistically significant. The efficacy data reported here is more similar to those shown in recent work (Ransom *et al.* 2011; Rutberg *et al.* 2017) than those shown in initial tests in the Clan Alpine, Nevada, study (Turner *et al.* 2007). This diminished initial efficacy of PZP-22 could be due to a variety of factors, including variation in the composition and manufacturing of vaccine or the other components of PZP-22, including the adjuvant and polymer used to make the controlled-release pellets (Ransom *et al.* 2011; Rutberg *et al.* 2017).

Timing of delivery also may play some role. Previous field testing of hand-injected PZP-22 at Cedar Mountains Herd Management Area (HMA), UT, USA, and Sand Wash Basin HMA, CO, USA, suggests a relationship between timing of delivery and effectiveness. Hand-injected PZP-22 primers in these studies showed highest effectiveness when delivered in February, moderate effectiveness when delivered in December and lowest effectiveness when delivered in October–November (Rutberg *et al.* 2017). Depending on the timing of the breeding season at a given site, however, there is likely to be a drop-off for the efficacy of late-spring delivery. At JM WHT, where most foaling occurs between April and June, one would expect diminishing first-year efficacy in late May through June, because more treated mares will already be pregnant (given a roughly 11-month gestation period for horses and a 2–3-week time lag in elevation of antibody titres to contraceptive levels; Liu *et al.* 1989, 2005). Anecdotally, the two PZP-22-treated mares observed to have produced offspring in 2013 were treated on 1 and 2 June 2012, which were the latest treatment dates for females observed in 2013 (one gave birth in April and one in June; unfortunately, three mares treated as late as or later than those two in 2012 were not located in 2013). It is also not known whether delivering a PZP-emulsion primer (ZonaStat-H) alone this late in the season would produce a comparable reduction in fertility, i.e. whether the pellet components of PZP-22 enhance the effectiveness of the priming vaccine.

Because this was a pilot study of the efficacy of PZP-22 remote delivery, we did not examine nor expect population-level effects. Fewer than 50% of individually recognised sample mares were treated in 2012, and in a challenging site like JM WHT we are confident that the sample significantly undercounted the population. Either way, treating a larger proportion of the fertile mares in a herd will be necessary to create a substantial and biologically important reduction in overall population fertility.

Another factor affecting the proportion of females foaling in the total sample might have been the preferential removal of younger horses in 2011 and 2012. Mares ≥4 years old were observed to be more fertile; therefore, preferential removal of younger mares could also have driven the sample foaling rates higher post-treatment and post-removal. Similar effects of age on foaling rate have been documented in other wild horse herds on public lands (Ransom *et al.* 2011; Rutberg *et al.* 2017) and, depending on vaccine costs and ease of access to the horses, female age might be a consideration in deciding which mares to treat.

Conclusions and implications

It was shown here for the first time that initial treatments of PZP-22 are effective for at least 1 year when delivered by dart, as long as vaccination occurs early enough for antibodies to reach contraceptive levels before the onset of the local breeding season. We also showed that corral trapping with bait can be

used to access very wary wild horses in remote and challenging terrain, allowing administration of contraception or removal of surplus wild horses, or both.

It has already been shown that PZP boosters (either PZP-22 or simple PZP emulsion formulations) administered by dart to mares primed with PZP-22 provide at least three additional years of contraception (Rutberg *et al.* 2017). The present study implies that two dart-delivered treatments of PZP – an initial PZP-22 primer followed by a PZP booster delivered 2–3 years later – should produce 4–5 years of contraception. This protocol significantly reduces the level of effort relative to the annual darting required by simple PZP emulsions and should extend the range of management applications of dart-delivered PZP. Additional field-testing of PZP-based SpayVac and the gonadotropin-releasing-hormone-based vaccine GonaCon (National Wildlife Research Center, Fort Collins, CO, USA) could also provide foundations for long-term wild horse contraception and management applications (Baker *et al.* 2018; Bechert and Fraker 2018). Ultimately, the use of fertility control for populations to treat and prevent overabundance could decrease the financial burden of humanely protecting wild horses and ease tension among diverse stakeholders seeking to use resources on multiple-use public lands (Bartholow 2007; NRC 2013; Garrott 2018).

Conflicts of interest

The authors declare no conflicts of interest.

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Neutering affects mRNA expression levels for the LH- and GnRH-receptors in the canine urinary bladder

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Abstract

Neutering a bitch increases the incidence of acquired urinary incontinence (AUI) 20-fold. Mechanistically this effect is thought to be related to altered steroid/reproductive hormone concentrations and a recent study showed that gonadotrophin releasing hormone (GnRH) analogue treatment improved continence in bitches with AUI. The aim of this study was to examine mRNA expression levels for luteinizing hormone (LH)- and GnRH-receptors in the canine bladder and the correlation between these and *in vitro* contractility of the bladder using age matched entire and neutered, male and female canines and canines with AUI.

Biopsies from the dome of the bladder were dissected post mortem with informed owner consent. mRNA expression for LH- and GnRH-receptor was quantified by rtPCR (relative to β-actin). Contractility was assessed (cumulative concentration response curve for carbachol) in strips of bladder muscle using standard protocols.

Analysis of variance (Tukey post-test) demonstrated thet neutering was associated with significantly increased levels of expression of LH- and GnRH-receptor mRNA in both sexes ($P < 0.01$). mRNA expression for both receptors was significantly higher in female versus male canines. Neither effect was affected by animals' age and/or weight. A significant inverse correlation (Spearman's test) was found between bladder contractility and mRNA expression for both receptors. This effect was most pronounced in canines with AUI which demonstrated the highest mRNA expression levels yet had the lowest contractility of all animals studied. This suggests that increased LH- and GnRH-receptor mRNA expression is associated with changes in bladder function that increase an animal's predisposition to develop AUI.
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Keywords: Urinary incontinence; Bladder; Bitch; LH; GnRH; mRNA

1. Introduction

Acquired urinary incontinence (AUI) is a debilitating and so far incurable condition that causes significant welfare problems. Two diverse populations have been identified that are particularly susceptible to AUI, post-menopausal women and spayed female canines. Information relating to the exact pathophysiology of the condition in these two groups is not understood well enough to allow determination of commonality of cause; however, both groups are subject to a reduction in gonadal steroids and associated endocrine changes.

Urinary incontinence (UI) is defined as the involuntary leakage of urine [1] and, in the bitch, is an increasingly recognised clinical problem with severe welfare implications that can lead to the ultimate

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euthanasia of the animal. Interestingly, AUI is reported to affect up to 20% of neutered bitches [2] but is seen in less than 1% of intact bitches [3] and is rarely reported in male canines regardless of gonadal status. A direct relationship between neutering and AUI has been reported [4] which is proposed to occur as a consequence of hormonal, vascular or neurological changes [5], rather than mechanical damage of the lower urinary tract, sustained during surgery [6]. Incontinence in the neutered bitch is frequently associated with a decrease in maximal urethral closure pressure [7,8], however, as this is not a defining characteristic of AUI, multiple causative factors are likely to be involved, not all of which will be a consequence of gonadectomy. It has thus been hypothesized that factors in addition to those that decrease urethral closure pressure, lead to a bitch's developing urinary incontinence after neutering. Previous *in vitro* studies have shown that neutering a canine of either gender leads to a decrease in maximal contractile response of the detrusor muscle to both muscarinic and electrical field stimulation as well as a decreased sensitivity to muscarinic stimulation [9]. These decreases in contractile function *in vitro* are similar to those reported in post-menopausal women who suffer from UI due to impaired contractility of the bladder [10].

Given the commonality of endocrine change in the two populations identified as being susceptible to AUI, it is now a widely held hypothesis that the condition may be due to, or be exacerbated by, hormone-mediated effects. This view is supported by the fact that a pharmacological treatment for AUI in the bitch, is steroid replacement [11]. In both spayed bitches and post-menopausal women there is a deficiency of endogenous gonadal steroid hormones which decreases or removes the normal negative feedback to the hypothalamo-pituitary axis, and results in greatly increased production and secretion of gonadotrophin releasing hormone (GnRH) from the hypothalamus, and increased secretion of the pituitary gonadotrophins (luteinizing hormone (LH) and follicle stimulating hormone (FSH)) [12–18]. This increase in plasma concentrations of LH and FSH post-neutering has been demonstrated to have a direct relationship with the development of urinary incontinence in the bitch [19]. A recent paper has described the clinical use of GnRH analogues to decrease LH and FSH concentrations in neutered female canines suffering from AUI. The results of this study also indicated that GnRH analogue treatment resulted in clinical continence, or a decrease in incontinence, during the treatment period [12,13]. This result would suggest that the increase in the concentrations of either GnRH and/or the pituitary gonadotrophins could be a causative factor for AUI in the bitch. Whilst GnRH and the gonadotrophins are classically thought of as hormones of the hypothalamo-pituitary-gonadal axis, the presence of their receptors in tissues such as skin [20], reproductive tract, prostate and mammary gland [21,22] as well as the urinary bladder [23–25] would suggest more widespread actions. In particular the presence of receptors within the urinary tract would indicate that altered concentrations of gonadotrophins could induce local effects within the bladder which could affect function. As specific tissue action and function rely upon the presence of specific receptors in the tissue it is possible that changes in either absolute receptor numbers or receptor–ligand concentration could influence the action of a tissue. In this regard a study in women has reported a decrease in LH-receptor numbers in the bladder of post- compared to pre-menopausal women [25], and a number of recent studies in the canine have reported either a decrease [24,26] or no effect on LH-receptor mRNA concentrations after neutering [17]. However, there are no published reports of mRNA levels for LH-, FSH- or GnRH-receptors in the bladder of bitches known to be suffering from AUI or the correlation between detrusor contractility, a factor known to be involved in UI [10,27], and receptor mRNA expression levels within the urinary bladder.

The aim of this study was to determine the effects of gonadal status and gender on the expression levels of GnRH-, LH- and FSH-receptor mRNA in the canine urinary bladder and to investigate whether these levels are altered in individuals suffering from AUI. A further aim was to see if there is a correlation between the expression of the mRNAs for these receptors and maximal contractility of the bladder, using age matched groups of entire and neutered, male and female canines.

2. Materials and methods

2.1. Animals and tissues

The study was approved by The University of Glasgow Veterinary School's ethical review committee. A total of 78 canines were included in the study, with a mean age of 6.0 years (range 1–16 years) and a mean weight of 22.7 kg (range 8–50 kg). The majority of canines were cross bred, with no pedigree breeds appearing more than once.

In all cases tissue was collected from canines euthanized (intravenous overdose of pentobarbatone),

with full informed owner consent, for reasons other than scientific investigation. The majority of animals were destroyed for severe behavioural problems, the remainder for a number of different complaints, none of which involved the urinary system. In all cases a detailed history of each animal was taken and, within 2 h of death, a gross post mortem study was performed. None of the animals used in the study were receiving medical treatment in the 7 days prior to euthanasia and there was no evidence of previous medical therapy in at least the month prior to this. Biopsies were collected in the area of the bladder dome and samples either stored at 4 °C in Krebs solution for tissue bath studies or immediately frozen and stored at −80 °C until required for mRNA studies. Any animal with a history or signs of gross pathological urinary, neurological or reproductive tract disease (e.g. tumours, cystitis, pyometra) were excluded from the study (except for those suffering from AUI—see below). The remaining animals were split into five groups depending on sex and gonadal status: entire males (ME), entire females (FE), neutered males (MN), neutered females (FN) and neutered females known to be suffering from AUI (FN AUI). All of the entire bitches were considered to be in anoestrous, as the ovaries showed no signs of follicular activity or of an active corpus luteum and all of the neutered animals were at least 6 months post-gonadectomy (determined by history and clinical examination). A diagnosis of AUI was made on the basis of history and clinical signs at time of euthanasia, coupled with full gross post mortem results that ruled out other causes of UI in the bitch such as ectopic ureters, kidney disease, uroliths, cystitis and gross abnormalities of the urinary system.

Ovarian tissue as well as skin biopsies, from the shoulder and groin regions were collected from a subset of animals included in the study, as a control for FSH-receptor mRNA expression [28]. Ovarian and skin samples were handled and stored as described above.

2.2. mRNA extraction and quantitative reverse transcription PCR

Biopsies where placed in 1 ml ice cold TRIzol reagent (Invitrogen, Paisley, UK), homogenised and RNA extracted according to the manufacturers protocol. After extraction, RNA was suspended in 40 μl RNase-free water and the concentration of RNA determined using a NanoDrop ND-1000 spectrophotometer. The purity of RNA (A_{260}/A_{280}) was greater than 1.9 in all samples. 1 μg of each RNA sample was reverse transcribed into cDNA using random hexamers (Promega, Southampton UK), Moloney-Murine Leukaemia virus (M-MLV) reverse transcriptase (Invitrogen, UK) and Rnasin (Promega, UK), as described previously [28]; final reaction volume of 24 μl. Quantitative real time taqman PCR (qPCR) was performed on cDNA for GnRH-, LH- and FSH-receptor with β-actin as the housekeeping gene using the Amplitaq Gold kit (Applied Biosystems, Warrington, UK) and a Strategene MX 3000P thermal cycler. The primers and probes for the three receptor mRNAs were as described by Welle et al. [20], sourced from Eurogentech (Southampton, UK). Prior to use in the current experiment the concentration of primers and florescent FAM-labelled probes against LH-, GnRH- and FSH-receptor mRNAs [20] were optimised relative to the housekeeping gene β-actin, to ensure that amplification of each of the genes of interest matched that of the housekeeping gene. All probes used were at a final concentration of 530 nM, with forward (f) and reverse (r) primers at: LH- and FSH-receptor mRNAs 1000 nM (f), 800 nM (r), GnRH-receptor mRNA 660 nM (f), 800 nM (r), β-actin mRNA 800 nM (f and r). All samples were run in duplicate, PCR conditions used were an initial step of 95 °C for 10 min followed by 45 cycles of 95 °C for 30 s and 60 °C for 1 min. Non-template controls were included for each primer pair to check for significant levels of any contaminants and in all cases were negative.

2.3. Tissue preparation for tissue bath studies

Tissue was used within 48 h of collection. Following removal of the lining urothelium strips of smooth muscle (2 mm × 10 mm) were dissected from the dome of the bladder. The strips were mounted under 4 g resting tension (the optimum tension demonstrated in preliminary previous length-tension studies) on a fixed hook in a 15 ml organ bath bathed at 37 °C in Krebs solution (NaCl 118 mM, KCl 4.8 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 24 mM and glucose 11 mM) gassed with 95% O_2/5% CO_2. Tension was measured with a BIOPAC TSD125C isometric force transducer and displayed using the AcqKnowledge 3.8.1. Biopac data acquisition system (Linton Instruments). Tissues were allowed to equilibrate for 60 min before experiments were begun, during which time the resting tension was re-adjusted to 4 g if required [9]. For all studies, a minimum of 2 muscle strips per animal were included.

2.4. Assessment of carbachol-induced contraction

To determine the response to the non-specific muscarinic agonist carbachol, cumulative concentration

Table 1
Age and weight of animals, by group.

	ME ($n = 27$)	MN ($n = 17$)	FE ($n = 18$)	FN ($n = 13$)	FN AUI ($n = 3$)
Age (years)	5.6 ± 0.7	5.8 ± 0.9	6.1 ± 1.0	6.5 ± 1.0	4.3 ± 2.0
Weight (kg)	23.7 ± 1.8	27.4 ± 3.2	21.9 ± 2.2	21.1 ± 3.1	17. ± 5.0

Mean ± S.E.M. values for age and weight in groups of entire and neutered, male and female canines, and neutered bitches identified as suffering from AUI (ME, MN, FE, FN and FN AUI, respectively). n = number of animals.

response curves to carbachol (1 nm–30 μM) were generated in strips of bladder smooth muscle. Maximal tension was recorded and data calculated as grams tension produced per milligram of wet tissue.

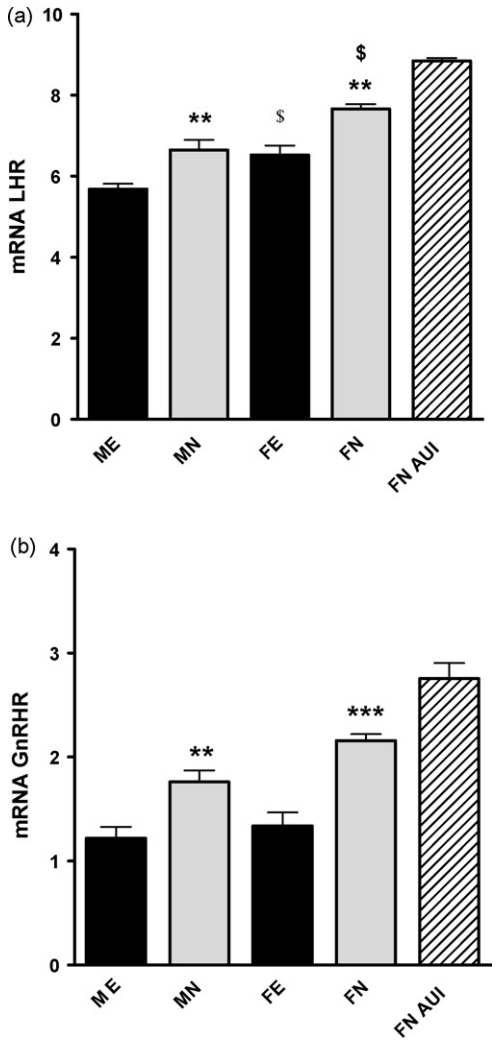


Fig. 1. Mean (±S.E.M.) mRNA expression for: (a) LH-receptor and (b) GnRH-receptor, in entire and neutered male and female canines and neutered female canines identified as suffering from AUI. mRNA values are logarithmically transformed. $^{***}P < 0.001$, $^{**}P < 0.01$ compared entire of same gender. $^{\$}P < 0.05$ compared to male animals of same gonadal status.

2.5. Statistical analysis

Results for both mRNA expression levels and contraction to carbachol were analysed against both age and weight using multivariant analysis with $P < 0.05$ considered significant.

Results are given as mRNA expression levels relative to β-actin using the comparative C_T method (User Bulletin no. 2, PE Biosystems, UK). Data was ln-transformed prior to statistical analysis to equalise the variance between groups and data compared by two-way ANOVA for effects of gender and gonadal status. Post hoc comparisons were conducted using Tukey's test with a significance threshold of $P < 0.05$.

To examine relationships between the levels of mRNA expression of the hormone receptors studied and the ability of the detrusor to contract when stimulated with carbachol, mRNA expression levels of each hormone receptor were plotted against maximal contraction (as grams of tension produced per milligram of wet tissue) and tested for a correlation using Spearman's test with a significance threshold for P (two tailed) <0.05.

3. Results

The mean age and weight of the 78 canines included in this study, 27 entire males (ME), 17 neutered males (MN), 18 entire females (FE), 13 neutered females (FN), and 3 neutered females known to suffering from AUI (FN AUI) are presented in Table 1. There was no statistical difference between the mean ages and weights for the five groups.

The mean levels of mRNA expression for LH- and GnRH-receptor are shown in Fig. 1a and 1b, respectively. Two-way ANOVA indicated significant effects of gonadal status on the expression of the mRNAs for both LH- and GnRH-receptor, whilst there was an additional effect of gender on the expression of LH-receptor mRNA. There was no interaction between the effects of gonadal status and gender. Neutering was associated with significantly ($P < 0.01$) higher levels of mRNA expression for both LH- and GnRH-receptor in

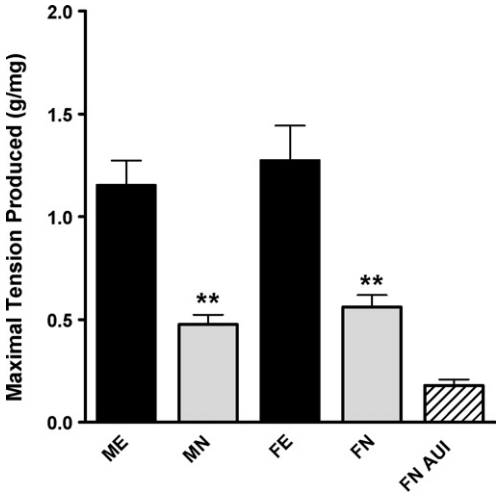


Fig. 2. Mean (±S.E.M.) maximal tension values (g/mg wet tissue) in response to carbachol in entire and neutered male and female canines and neutered female canines identified as suffering from AUI. $^{**}P < 0.01$ compared entire of same gender.

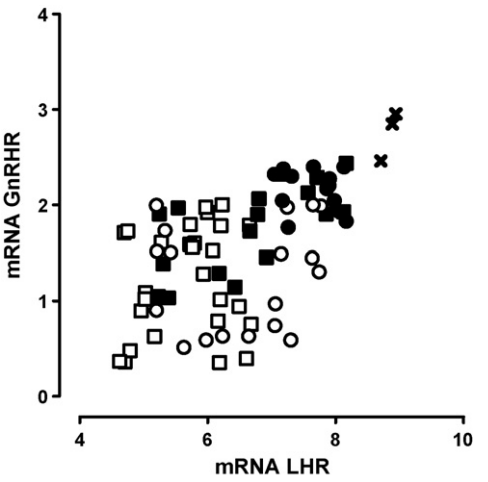


Fig. 3. Correlation between mRNA expression levels of LH- and GnRH-receptor ($P < 0.001$, $r = 607$). (□) Male entire, (■) male neuter, (○) female entire, (●) female neuter, and (×) AUI female neuter.

both genders. Furthermore, female animals showed significantly ($P < 0.05$) greater levels of mRNA expression of LH-receptor, regardless of gonadal status, than their male counterparts (Fig. 1a).

The mRNA expression levels for both LH- and GnRH-receptor for the females identified as AUI were the highest of all the animals in the study and fell above the 95% CI for the continent FN group, although this increase was not considered statistically significant using ANOVA analysis.

Real time rt-PCR was performed for the quantification of FSH-receptor mRNA expression within samples of urinary bladder. In all cases, expression was found to be below the limits of detection of the assay system. To ensure validity of the primers and probes used, they were tested against samples of canine ovary and skin as described in Welle et al. [20]. mRNA for the FSH-receptor was found at very low levels ($3.22E^{-15}$, $n = 2$) in samples of canine skin but was not found in canine ovary.

Carbachol-induced dose dependant contraction of the isolated strips of detrusor muscle from all animals. As in our previous study [9], maximal response was significantly decreased ($P < 0.01$) in neutered compared to entire canines of either gender (Fig. 2). Sensitivity of strips to carbachol, as measured by log EC_{50} values, was also decreased ($P < 0.01$) in neutered compared to entire animals (Table 2). Two-way ANOVA revealed that there was no effect of gender and no interaction between gender and gonadal status. The group of animals identified as suffering from AUI had the lowest maximal contraction of all animals studied, although this was not considered significant using ANOVA.

Statistical analysis revealed a significant positive correlation ($P < 0.001$, $r = 0.607$) between LH- and GnRH-receptor mRNA expression (Fig. 3) and significant negative correlations between the maximal tension produced by detrusor muscle strips and both LH-receptor ($P < 0.001$, $r = -0.671$) and GnRH-

Table 2
Maximum and log EC_{50} values for Carbachol.

	ME ($n = 11$)	MN ($n = 10$)	FE ($n = 10$)	FN ($n = 8$)	FN AUI ($n = 3$)
E_{max}	1.15 ± 0.12	0.49 ± 0.05*	1.27 ± 0.17	0.56 ± 0.06*	0.18 ± 0.03
log EC_{50}	−6.98 ± 0.05	−6.39 ± 0.02*	−7.09 ± 0.06	−6.51 ± 0.07*	−6.26 ± 0.10

Maximum (E_{max}) and log EC_{50} values for carbachol in isolated strips of detrusor muscle from entire and neutered, male and female dogs and neutered females identified as suffering from AUI (ME, MN, FE, FN and FN AUI, respectively). Data are expressed as g/mg of wet tissue with results given as mean ± S.E.M. where n: number of animals.

* $P < 0.01$, indicates different from entire of same gender.

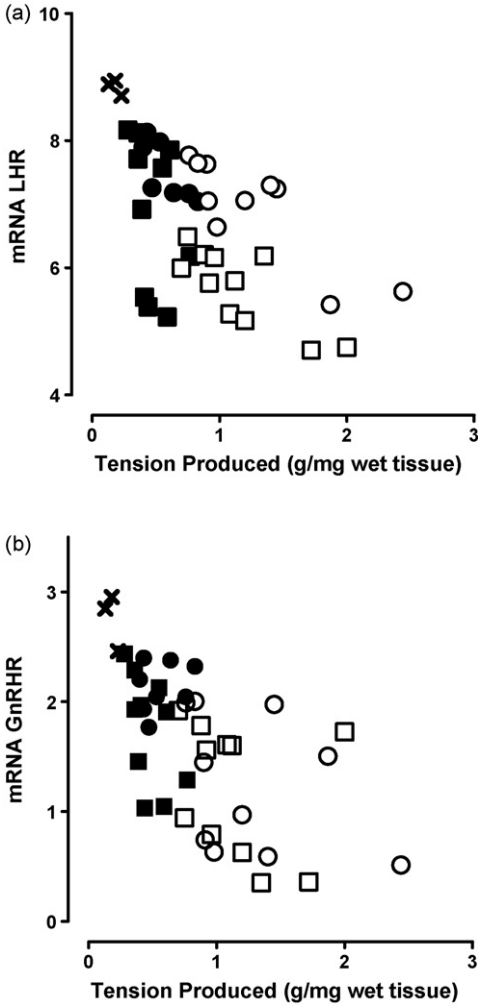


Fig. 4. Correlation between maximal tensions produced by detrusor muscle strips *in vitro* and mRNA expression levels for: (a) LH-receptor ($P < 0.001$, $r = -0.671$) and (b) GnRH-receptor ($P < 0.001$, $r = -0.612$). (□) Male entire, (■) male neuter, (○) female entire, (●) female neuter, and (×) AUI female neuter.

receptor ($P < 0.001$, $r = -0.612$) mRNA expression (Fig. 4a and b, respectively).

Multivariant analysis of the levels of expression of mRNA for both GnRH- or LH-receptor, and the maximum tension produced by detrusor muscle strips indicated no significant effect of age or weight of the animal on the results.

4. Discussion

This is the largest study of the expression levels of the mRNAs for LH- and GnRH-receptors in the canine urinary bladder, and the only study to correlate levels of receptor mRNA expression to bladder contractility (*in vitro*). In addition, although numbers are low, this is the only study to report bladder LH/GnRH-receptor mRNA expression in neutered bitches known to be suffering from AUI.

Bladder contractility is thought to play a significant role in the development of clinical signs of UI [29]. We have previously shown that the maximal contractile response of isolated strips of detrusor muscle to muscarinic and neurogenic stimulation was significantly decreased in neutered compared to entire canines of either gender [9]. We have also demonstrated that sensitivity of the detrusor to muscarinic stimulation is decreased in neutered compared to entire animals of either gender. These results are similar to those reported for studies conducted with tissues collected from women who suffer from UI due to impaired contractility of the bladder [10], a condition known to cause decreased speed and magnitude of detrusor contractions during the emptying phase of micturition. This leads to residual urine retention, a shortened storage phase and predisposes the patient to cystitis [30]. That decreased contractility and sensitivity of the detrusor to muscarinic stimulation in neutered canines may be involved in AUI is supported by the results of this study, looking at animals known to be suffering from AUI, as these had the lowest contractility of all the animals studied. Although impaired bladder contractility has been implicated in UI, the exact mechanisms responsible for its development have not been reported and we hypothesise that the muscarinic receptor effector pathway is involved, its function being altered due to effects in reproductive hormones and the expression of their receptors in the bladder post-neutering/menopause.

It was first suggested that gonadotrophins could influence bladder function in a study which showed that post-menopausal women, who are most likely to suffer from UI, had lower numbers of LH mRNA transcripts and receptors in their bladders than their premenopausal counterparts [25]. As post-menopausal women have a similar hormonal profile to neutered canines, and as both post-menopausal and post-neutering UI are thought to be hormonally related, it follows that a similar aetiology and pathophysiology may link both conditions. Our results clearly show that neutering a canine, regardless of gender, significantly increases levels of mRNA expression for both LH- and GnRH-receptors, and that in general, female animals, regardless of gonadal status, have higher levels of mRNA expression for these receptors than their male counterparts. Although our results, for female canines, are in contrast with those of Tao et al. [25] it should be noted

that in addition to the species difference our study include age matched groups of healthy entire and neutered animals as well as those neutered female canine suffering from AUI, whereas the study by Tao et al. compared women of two distinct age groups, with a number of patients in both groups suffering from UI and genital prolapse, therefore an additional explanation for the difference in the two studies could be an effect of age and mechanical stress on the lower urinary tract. Interestingly, the results of the FN animals in this study known to be suffering from AUI suggest that incontinent neutered bitches may have higher levels of LH-receptor mRNA than their continent counterparts. These findings support the hypothesis that altered gonadotrophins have a role in the pathophysiological mechanism of post-neutering UI as suggested by the efficacy of GnRH analogues in the treatment of canine AUI [13].

In addition to being different from the study reported in humans our results also contradicts with two recent canine studies that reported either no change [24] or a decrease [26] in mRNA expression for these receptors, respectively, in neutered canines. The differences between the three canine studies may again reflect differences in the study populations and conditions. In both of the studies by Reichler et al. [24] and Ponglowhapan et al. [26] a substantially smaller number of animals were used. In addition, the study by Reichler et al. included entire bitches in varying stages of oestrus, thus making comparisons between the groups and studies more complex. Whereas the study by Ponglowhapen et al. looked at relatively acute effects of neutering (24–40 weeks), and thus it is possible as plasma concentrations of FSH and LH continue to increase until week 42 post-neutering [16] and some structural changes may take even longer [31], that there will be significant effects of time post-neutering on the observed mRNA changes.

A further difference between this study and those of Tao et al. and Ponglowhapan et al. is methodological, in that the current study used real time PCR with fresh frozen tissue to quantify mRNA expression levels, whilst the former two studies used in situ hybridisation with formalin fixed, paraffin-embedded tissue. It is known that degradation of target nucleic acids [32,33] and artificial mutations [34] can occur during formalin fixation and paraffin-embedding procedures, which again could have influenced the results, relative to those seen from fresh frozen tissue [35].

The changes in expression of mRNA for LH- and GnRH-receptors observed in our study could support a role for altered expression of GnRH/LH-receptors in the development of AUI in the neutered bitch. Although it is worthy of note that increases in LH- and GnRH-receptor mRNA expression also occurred in the bladders of neutered male canines, which do not typically become incontinent, the magnitude of increase was smaller and the final levels of expression were only marginally above that of entire female canines which also rarely become incontinent. The sex difference in the propensity to develop AUI may also reflect an interaction between post-neutering effects on smooth muscle function and anatomical differences in the urethra of a male canine as has been previously hypothesized [36]. It may be the case however that there is a threshold level of LH- and GnRH-receptor expression, above which UI is more likely to develop. This possibility is supported by the fact that the three animals known to be suffering from AUI had the highest LH- and GnRH-receptor mRNA expression levels observed in this study.

The exact role of the LH-receptor in the urinary bladder has not yet been described. It is known that the plasma concentrations of LH increase in both post-menopausal women and in neutered dogs [16,37], although it is still released in a pulsatile manner. It is also known that this subset of the population is most prone to developing UI. In this study we have shown that neutering causes an increase in mRNA expression levels for the LH-receptor in the canine urinary bladder, and that these expression levels are highest in bitches known to be suffering from AUI. We have also shown that maximal bladder contractility is decreased in neutered animals and is lowest in bitches suffering from AUI and that there is a direct inverse correlation between bladder contractility and mRNA expression levels for LH. It therefore follows that the increase in mRNA for the LH-receptor may be implicated in the decrease in contractility of the detrusor muscle in the canine, either directly or via interactions with another mechanism, possibly involving the muscarinic receptor effector pathway, the primary pathway involved in bladder contraction [38].

The exact role of the GnRH-receptor within the urinary bladder has not been characterised in any species. However, based on a recent study that reported increases in bladder threshold volume following administration of GnRH analogues to neutered continent bitches it has been hypothesized that it may have a role in determining bladder function [12]. This could occur if GnRH-receptor expression modulated the bladders sensitivity to muscarinic stimulation to affect the bladder threshold volume. This hypothesis is supported by the data contained within our study,

where a decrease in *in vitro* detrusor muscle sensitivity to muscarinic stimulation was seen in neutered canines when GnRH-receptor mRNA expression levels were found to be high. Thus, the increase in GnRH-receptor expression could be associated with a decreased sensitivity of the detrusor which would allow further relaxation of the bladder and increased capacity.

Although our study did not identify mRNA for FSH-receptor in the canine bladder two other studies [20,23] have reported its expression within this tissue. The reason for these reported differences studying FSH-receptor mRNA expression, could be methodological, as Ponglowhapan et al. [23] used in situ hybridization whilst our study used real time PCR techniques. Alternatively it could be related to differences in the FSH mRNA sequences used, as our results were similar to those described by Welle et al. [20], who used the same primers and probes and reported expression in canine skin, and inconsistent expression within the canine bladder [20] whilst Ponglowhapan et al. used a different molecular sequence as the basis for their probe. As FSH is known to express multiple isoforms [39] it is a possibility that the primers and probes used in this study and that of Welle et al. [20] were designed against part of the mRNA sequence of an FSH-receptor isoform that is expressed in skin but not in the bladder. As part of the validation of the probe in the current study, in addition to testing the primer and probes against bladder and skin, ovarian tissue was also included as the ovary is a key target organ for the actions of FSH [40]. The ovary, however, was also found to be devoid of significant quantities of the FSH-receptor mRNA expression that could be identified using these primers and probes. Thus, at this point, we are unable to make any definitive conclusions with regard to the effects of neutering on FSH-receptor mRNA expression within the bladder wall.

In conclusion we have shown that neutering a canine, regardless of gender, is associated with an increase in the levels of mRNA for LH- and GnRH-receptors in the canine urinary bladder, and that female animals, regardless of gonadal status have higher LH-/GnRH-receptor mRNA expression levels than males. A further novel observation shown by this study is the positive correlation between increased mRNA expression for LH- and GnRH-receptors and the negative correlation between expression of these receptors and contractility of the detrusor to muscarinic stimulation *in vitro*. It is hypothesized that the changes in mRNA expression for both GnRH- and LH-receptor may have a role to play in the development of post-neutering AUI and this hypothesis is supported by data from a limited number of neutered female canines identified as suffering from AUI which had the highest levels of LH- and GnRH-receptor mRNA expression and the lowest contractility to muscarinic stimulation of all the animals studied.

Acknowledgements

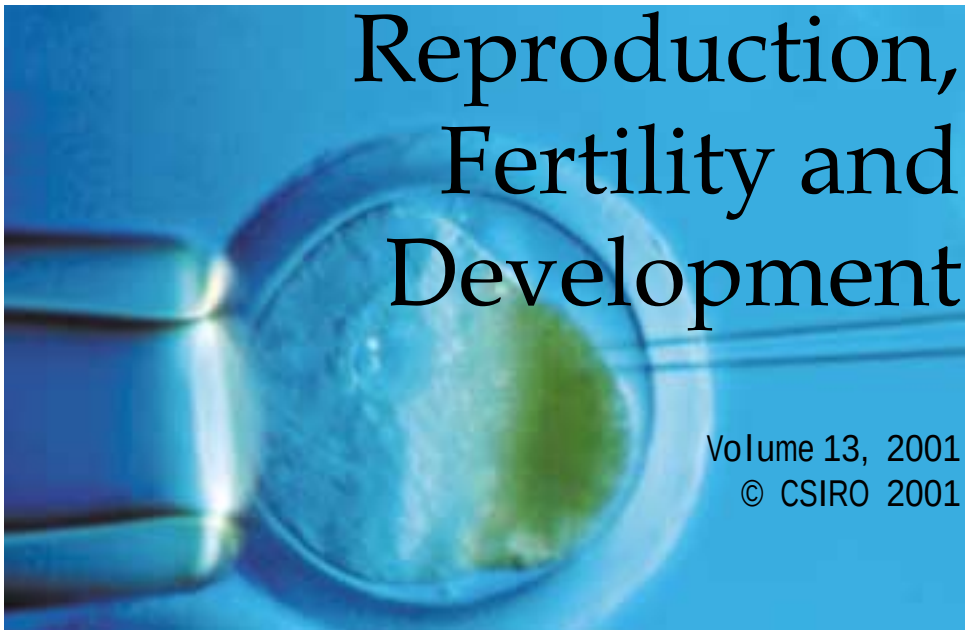
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Genetics, biotechnology and population management of over-abundant mammalian wildlife in Australasia

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Abstract. Wildlife management involves regulation of population numbers of wild vertebrate species. In some cases there are too many animals and in others there are too few. Genetic issues arise in both instances. The historical and genetic evidence for the number of mammals that were in the founder populations of successful colonizing species in Australia and New Zealand is reviewed here. Small numbers have often given rise to large populations, despite the concomitant loss of genetic variability. Restriction of the number of over-abundant and pest species by either physical or chemical methods frequently constitutes very strong artificial selection, which leads to rapid genetic change; an example of major importance in the two countries is sodium monofluoroacetate (compound 1080). Pathogenic agents, surgical sterilization, hormonal contraceptives and translocation have all been used with varying degrees of success. The strengths and weaknesses of these techniques are assessed. A method that has received much attention is immunocontraception. We argue that this attempt to use the animals' own immune system to modulate reproduction is incompatible with the basic biological function of protection against infectious disease. Immune function genes are highly variable in vertebrates, and so often genetic change in the population subjected to immunocontraception is likely to be even more rapid than is the case with lethal agents. Selection for failure to respond to the immunocontraceptive will occur, and will change immune function in general. Poor scientific description of ecosystem complexity makes it difficult to predict the consequences of immunocontraception on wildlife populations.

Extra keywords: deslorelin, fertility control, immunocontraception, myxomatosis, sodium monofluoroacetate.

Introduction

The term Australasia was originally used to describe Australia and New Zealand. In recent years it has also been used to include neighbouring areas of Asia. For our purposes this is serendipitous because Wallace's line in the Indonesian archipelago defines the border of the biologically unique region of Australia, New Guinea, New Zealand and the islands of the south-west Pacific. This is sometimes called Wallacia (van Oosterzee 1997). Wallacia's long isolation after the fragmentation of Gondwana has produced endemic flora and fauna on which Australians and New Zealanders place high conservation value. These endemic species are now strongly challenged by the arrival of Northern Hemisphere species, especially in the last two centuries. In scientific terms, Australia and New Zealand constitute a kind of inadvertent laboratory for the study of factors influencing the success of colonizing species. One such factor is the size of the founder population. We review some of the historical evidence on this question from the two countries, which indicates that the most over-abundant introduced pest species can result from very small founder populations. A major aspect of wildlife management in Australia and New Zealand is aimed at reducing the number of introduced species that are most damaging to indigenous species. Management techniques include shooting, poisoning, the introduction of pathogens, translocation, and, most recently, attempts at using biotechnological products. In this review, we will explore the underlying genetic issues and comment on the extent to which successful management is limited by them. Our focus is a mixture of scientific and wider social issues, an approach that is so richly exemplified in the scientific career of Roger Short.

Genetics and small populations

Small population size results in a loss of genetic variability, equivalent to $(2Ne)^{-1}$ per generation, where Ne is the effective population size. Ne is a measure of the number of independent genes in a population and is usually smaller, often much smaller, than the number of actual individuals. It is determined by the number of individuals that reproduce (Crow 1986; Li and Graur 1991). There are a number of species that have colonized New Zealand and Australia from small foundation numbers (Table 1). Australian brushtail possums in New Zealand and English rabbits and foxes in Australia have become major pests. Rapid population expansion after introduction would have prevented excessive loss of heterozygosity. In principle, the accuracy of the

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historical records can be checked with genetic techniques, but this has rarely been done. Le Page *et al.* (2000) carried out a microsatellite genetic marker study of the New Zealand Bennett's wallaby population, which was recorded as being founded on three animals. The study showed that the founder number was unlikely to have been as small as three. Nonetheless, the bottleneck was severe, with probably three to five pairs of wallabies for one to three generations.

Small population size leads to a degree of linkage disequilibrium, which is proportional to Ne. Linkage disequilibrium is the non-random association of genes at different loci, usually closely linked. It can now be detected and measured using microsatellite markers. A methodology for calculating Ne in the founder population using linkage disequilibrium has been described by Hill (1981) and applied to a fish population (Bartley *et al.* 1992) and cetaceans (Waples 1991). The Hill approach could be extended to colonizing species for which there is no historical record of founder population. Although a large undertaking, this would allow a comprehensive assessment of the extent to which colonizing species are founded on small numbers. It would also allow, as in the case of the New Zealand Bennett's wallaby, the historical record to be checked. The results might have relevance to the question of the influence of population size on the fate of species endangered by small population size.

New Zealand possums are of both Tasmanian and mainland origin, which are usually thought of as subspecies. Thus, part of their success could be the result of heterosis though hybridization between subspecies. Subspecies crosses can negate loss of variation owing to small population size. The red deer and wapiti (North American Elk) hybridize in nature in New Zealand and so similar remarks apply to this species. Red deer were, however, well established before this occurred.

Table 1. Populations founded on small numbers in Australia and New Zealand

Country	Species	Number
New Zealand	Bennett's wallaby	3
	Brushtail possum	200–300
	Hares	6
	Hedgehog	Some tens
	Chamois	8
	Tahr	30
	Red deer	250
	Wapiti	18
	Sika deer	6
	Sambar deer	2
	Rusa deer	8
	Fallow deer	50–60
	White-tailed deer	19
Australia	Kangaroo Island koalas	18
	Rabbit	13
	Water buffalo	80
	Banteng cattle	20

All New Zealand information is from a volume edited by King (1990). The other references are: rabbits (Palmer-Austin 1993); koalas (Martin and Handasyde 1999); water buffalo and banteng cattle from a volume edited by Strahan (1975).

Genetics, biotechnology and over-abundant populations

For the species of most concern in Australia and New Zealand, a number of management techniques are currently used (Table 2). All of these techniques involve large sections of the population, at least locally. There is therefore the potential for genetic selection to take place. The kind of selection and physiological systems that are likely to be affected are very heterogenous (Table 3).

Sodium monofluoroacetate (*1080*)

Various poisons are being used to control wild animals around the world. By far the most widely used and effective poison in Australia and New Zealand is sodium monofluoroacetate (1080) (Eason *et al.* 1994; Seawright and Eason 1994). This has the effect of inhibiting the enzyme aconitase in the mitochondrion, which governs the step, citrate to isocitrate, in the tricarboxylic acid cycle for the aerobic production of energy. It is one of the most poisonous substances known on a weight-for-weight basis: 5 mg will kill a fox, for example, but death is by no means painless. 1080 is cheap, can be used in small quantities and degrades rapidly in the environment.

In various parts of western, central and northern Australia, native plants of the genera *Gastrolobium* and *Acacia* accumulate sodium monofluoroacetate in their flowers and seeds. This is presumably a defence against over-browsing in what are frequently very arid environments (Twigg and King 1991). Many Western Australian vertebrates have been shown to have high 1080 tolerance, and Western Australian animals often have a much higher tolerance than their eastern conspecifics (Table 4). This unusual situation now forms the basis of a highly successful fox control programme in Western Australia, where 1080-containing baits have been used to reduce fox densities to the point where they no longer threaten small terrestrial marsupials (Bailey 1996; Morris *et al.* 1998). It is also used greatly in eastern Australia for foxes and in New Zealand for possum control even though native species are not immune.

There are two serious reservations concerning the use of 1080. First, it is widely viewed as inhumane. Second, as the Western Australian resistant strains of widely occurring species demonstrate, selection for resistance is inevitable. This may have occurred slowly in Western Australia, and may even have been the result of the inhibition of reproduction rather than lethality, if only small amounts were consumed at any one time (Twigg and King 1991). Both reservations might be ameliorated by adding an anaesthetic

to the baits being used (Marks *et al.* 2000). This would cause death to occur much more humanely. If an anaesthetic that also up-regulates energy production could be found, death may occur more quickly. Less 1080 may be necessary and since resistance must develop to two agents, the anaesthetic and 1080, the rate of selection for resistance may slow.

Resistance to 1080 can evolve within the lifetime of a species (Table 4). May and Dobson (1986) draw attention to the finding that under very strong selection pressure, of the kind imposed by biocontrol agents such as insecticides, rodenticides and herbicides, noticeable resistance appears in 5–100 generations. They explain this by deriving a formula for the rate of appearance of resistance based on a two allele monogenic model. This formula shows that the rate is logarithmically dependent on its main determinants. These are the initial frequency of the allele, the threshold at which resistance appears and the selection strength. The last is determined by dosage levels and the degree of dominance of the resistance gene. The conclusion is that despite there being likely orders of magnitude in the range of these values, the time scale of appearance of resistance is compressed. A general account of the ecological and evolutionary aspects of insecticide resistance is given by McKenzie (1996); the data described therein demonstrate the rapidity with which resistant phenotypes can appear when selection is intense.

Myxomatosis and calicivirus

These viral pathogens have been particularly effective in reducing the large rabbit populations of Australia. Myxomatosis was deliberately introduced into Australia in 1950 (Fenner 1983), and it has been possible to study the co-evolution of host resistance and pathogen virulence (Fenner 1972; Kerr and Best 1998). By 1983, natural selection had resulted in a balance between the two (Fenner 1983). Resistance in rabbits followed with the emergence of less virulent strains, which allowed survival of moderately resistant rabbits (Kerr and Best 1998). Resistance is likely to be owing to an enhanced innate immune response, probably the cellular immune response (Kerr *et al.* 1999). Calicivirus was accidentally introduced into the wild in South Australia in 1995. However, there has been too little time to study the

Table 2. Mammalian wildlife species currently subject to management practices in Australia and New Zealand

Common name	Country	Current control
European rabbit	New Zealand, Australia	Myxomatosis Calicivirus Shooting 1080
Brushtail possum	New Zealand, urban Australia	1080 (NZ) Other poisons (NZ) Shooting (NZ) Trapping (NZ) Translocation (Aus)[a]
Feral cat	New Zealand, Australia	1080 Shooting Fencing
Wild deer (various species)	New Zealand, Australia	Shooting Translocation to farms
Mustelids (stoats, ferrets, weasels)	New Zealand	Trapping 1080 Warfarin and derivatives
Wild ungulates (e.g. pigs, goats, horses)	New Zealand, Australia	Shooting 1080
Kangaroos and wallabies (various species)	New Zealand, Australia	Shooting Surgical sterilization (Aus) 1080
Rodents (rats, mice)	New Zealand, Australia	Warfarin and derivatives 1080
Red fox	Australia	1080 Shooting Fencing
Koala	Australia	Translocation Surgical sterilization

[a]Illegal, but probably widespread. 1080, sodium fluoroacetate; calicivirus, rabbit haemorrhagic disease (RHD) virus.

Table 3. Possible evolutionary consequences of management techniques

Technique	Consequence
Shooting	Altered behaviour
1080	Bait shyness (neophobia)
	Alteration to fatty acid metabolism, entry to mitochondrion, detoxification mechanisms, substrate specificity
Steroid or peptide hormones	Alteration to receptor structure
Warfarin and derivatives	Altered clotting mechanism
	Bait shyness
Pathogens (e.g. myxomatosis, calcivirus)	Many possibilities
	Increased immunocompetence for specific antigens (MHC linked)
	Increased immunocompetence in general (not MHC linked)
Immunocontraception	Reduced immunocompetence
Surgical sterilization	Loss of variability if bottlenecks created

host–pathogen interaction in this case. Young rabbits are protected by maternally derived immunity (Cooke *et al.* 2000). The epidemiology of the disease appears to be influenced by the presence of a so far unidentified pre-existing non-virulent rabbit calicivirus closely related to the virulent form (Nagesha *et al.* 1995).

Surgical sterilization

Surgical sterilization has become a popular form of fertility control in situations where total removal of the species is not the goal. This is particularly the case in areas of Australia where marsupial populations have grown too large and require active management, for example recent wide-scale control operations on Kangaroo Island involving surgical sterilization (via tubal ligation) of female koalas (Ellis 1998).

This method has the advantage that it is permanent and the exact specifications of the treated portion of the population can be planned and put into practice (Menkhorst *et al.* 1998). Surgical sterilization may be more publicly acceptable in particular situations than other options such as culling, and is generally more readily available than other fertility control methods currently undergoing the research and development process. The permanence of this form of treatment may also be a disadvantage in some circumstances, for example when the genetic diversity of a population is low. Other disadvantages are the invasive nature and high cost of these procedures.

Table 4. Resistance to 1080 in eastern versus Western Australian vertebrates (Twigg and King 1991)

Species	LD50 (mg kg^{-1})	
	Western race	Eastern race
Tiliqua rugosa	500, >800	214
Varanus rosenbergi	200–300	40
Antechinus flavipes	12.5	3.5
Isoodon obesulus	20	7
Trichosurus vulpecula	125	0.75
Macropus eugenii	5, 20	3
Rattus fuscipes	20, 80	1.1

Hormonal contraceptives

Hormonal contraceptives can be divided into steroidal and non-steroidal contraceptives. Synthetic progestins are the most widely used form of steroidal contraceptives, particularly in zoo environments, because of their efficiency and relatively low cost (Dutton and Allchurch 1998). Synthetic progestins have several modes of action. They reduce the amount of the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), thereby inhibiting follicular maturation, development and ovulation. They also prevent the migration of spermatozoa through the cervical canal by reducing the volume of cervical mucus and increasing its viscosity, and they inhibit implantation by interfering with the development of the endometrium (Segal *et al.* 1991; Nave *et al.* 2000).

Synthetic progestins are usually given in the form of silastic implants (Dutton and Allchurch 1998). Minor surgery under local anaesthetic is often required to administer the contraceptive, for example in tammar wallabies (Nave *et al.* 2000), although there have been published reports of remote delivery using 'biobullet' technology for example in white-tailed deer (DeNicola *et al.* 1997). Contraceptive success can be close to 100% in some species, such as tammar wallabies (Nave *et al.* 2000), koalas (Menkhorst *et al.* 1998) and white-tailed deer (DeNicola *et al.* 1997), once the appropriate dose has been determined (DeNicola *et al.* 1997). The duration of effect for tammar wallabies was found to be at least 36 months (Nave *et al.* 2000).

Commentaries on the use of synthetic progestins as a contraceptive for wildlife often urge caution based on the reports of adverse effects in some species, including

pathology of the reproductive tract (Dutton and Allchurch 1998; Tuyttens and Macdonald 1998). Most of these reports are limited to case reports of animals displaying clinical signs in zoo environments (reviewed by Dutton and Allchurch 1998; Tuyttens and Macdonald 1998). The rate of occurrence of these adverse effects in different species is not clear from the literature and should be reviewed. Recommendations on the use of this form of contraception should be based on sound scientific data from individual species, as it is not clear whether all species are prone to the same negative effects.

Gao and Short (1993*a*, 1993*b*, 1994*a*, 1994*b*) have examined the possibility that steroids (oestrogen, androgen, gestagen, antigestagen) in bait form could be used to control rodent populations. This approach deserves further analysis and trialing, particularly with regard to cost and feasibility for large-scale use in agricultural environments.

Non-steroidal contraceptive agents include analogues of gonadotrophin-releasing hormone (GnRH). Gonadotrophin-releasing hormone is a peptide that is produced and secreted by the hypothalamus. When GnRH agonists are administered continuously, they result in a down-regulation of pituitary GnRH receptors and a subsequent decline in the production of LH and FSH. The end-point of these changes is a decline in the concentration of gonadal steroids, failure of ovulation in females and inhibition of spermatogenesis in males (Fraser 1993).

Gonadotrophin-releasing hormone agonists have been shown to inhibit reproduction in both sexes of many species. They are most effective in the form of a controlled-release implant, administered via subcutaneous injection. Recent advances in slow-release technology (Peptech Pty Ltd, North Ryde, Australia; details in US patent no. 6,211,152) mean that contraception may be effective for one to two years in many species. Gonadotrophin-releasing hormone agonists can be administered by a simple subcutaneous injection once an animal has been captured, and so far there have been no significant negative side-effects documented. As GnRH agonists work by reducing the production of steroids, there are potential changes in behaviour, in particular sexual behaviour, which may affect social dominance hierarchies. This may not be desirable in wild populations of some species in which dominance may confer better access to resources within the environment, such as food, nesting sites and home range size (Tuyttens and Macdonald 1998). The manner and degree to which any behavioural changes will affect the welfare of individuals within a population will be dependent on the social structure of the species in question.

Slow-release implants containing the GnRH agonist, Deslorelin (Peptech Pty Ltd, North Ryde, Australia), are currently being trialed in a range of marsupial species, including tammar wallabies, grey kangaroos and koalas. Preliminary results to date suggest that this is a promising contraceptive for female kangaroos and wallabies (C. A. Herbert and D. W. Cooper, unpublished results).

Hormonal contraceptives, both steroidal and non-steroidal, have the potential to be a relatively inexpensive fertility control option as the costs of research and development are often borne by human or domestic species research. This will make the development phase much less expensive than for other wildlife-specific agents. However, these savings may be offset by the cost of physically catching and treating individual animals if remote delivery is not possible. The reversible nature of this form of fertility control may be viewed as either an advantage or a disadvantage depending on the level of population control that is required. Peptides may be more expensive than steroid derivatives and may have a shorter duration of action. However, they do not have the same potential for negative side-effects as steroidal agents. The incidence of negative side-effects may affect the public perception of steroidal contraceptives and their usefulness for wildlife, particularly with respect to highly valued species such as the koala.

Variation in responses to hormones of all kinds is potentially under genetic control (Spearow *et al.* 1999; Westberg *et al.* 2001). Failure of contraception could result in selection for such genes in the population. All trials of hormonal contraceptives have so far been on a small scale (DeNicola *et al.* 1997; Dutton and Allchurch 1998; Nave *et al.* 2000). These need to be expanded, with particular attention being paid to the heritability of any non-response.

Translocation

Translocation has been used as a management tool for a number of species in Australia and New Zealand. One of the most notable cases was the translocation of koalas from offshore islands in south-eastern Australia to areas of suitable habitat that remained unoccupied following the major population decline in the early 20th century. This programme was extremely successful at restoring koalas to areas of their former range in Victoria, so successful, in fact, that there is now a shortage of suitable release sites (Menkhorst *et al.* 1998; Martin and Handasyde 1999). Translocation of wild deer to deer farms has been used in both Australia and New Zealand, but this strategy is very dependent on the economic viability of the deer industry.

There are a number of animal welfare issues related to translocation of wildlife. There is stress related to the capture and transport of animals, the potential post-translocation mortality, the probable need to sterilize animals so they will not affect the gene pool at their new location, and the environmental impacts of introducing new animals (Edwards and Oogjes 1998). The possible spread of disease is another issue. Furthermore, this is an expensive solution to the problem, which is short-sighted and does not deal with long-term management.

Immunocontraception

This method involves immunizing an animal, usually the female, against some components of the reproductive system (Bradley 1994; Tyndale-Biscoe 1995; Grant and Malin 1997). These can be of sperm, egg or hormonal origin and, as a result, fertilization or mating does not take place. To have long-term usefulness, this method, as for any other control method, should have near 100% effectiveness in the first few generations. Evidence is accumulating that this is not the case (Table 5). In each case, females were immunized and non-responders, in the sense that reproduction has occurred, were present at high levels, probably sufficiently high to prevent effective population decrease (Barlow 1997). Even with a moderate level of heritability, rapid selection for these non-responders would occur if immunocontraception were continued in succeeding generations. One of the largest studies carried out is with white-tailed deer (Miller *et al.* 2000*a*, 2000*b*). Porcine zona pellucida induced a high level of infertility, but recombinant antigens did not produce any. All of the antigens used have been injected in adjuvant, which greatly enhances the level of the immune response. None of the studies where variation in response to immunocontraceptives has occurred have attempted to examine the heritability of response/non- response. This should be done in any future research programme.

Apart from non-responders and the necessity for adjuvant to obtain an effective response, there are other barriers to effective immunocontraception. Multiple immunizations are needed. The cost of the antigen is likely to be higher than for poisons. The mode of delivery is also of concern. If an organism is used as a vector, species specificity becomes difficult to assure. Baits are expensive to deliver, especially if multiple immunizations are needed.

The most difficult problem to assess is the effect of the likely selection for an altered immune response. In some cases there may be increased immunocompetence, but a reduction is more likely. Will such a reduction lead to an increased susceptibility to pathogens that affect the target species itself, as suggested by Nettles (1997)? Or will it lead to the species being a carrier for pathogens of other species in the same ecosystem? One of the two main reasons for wanting to reduce possum density in New Zealand is because of the presence of bovine tuberculosis among them. At least eight genes affect responses to tuberculosis in humans (McNicholl *et al.* 2000). Are responses to tuberculosis and immunocontraceptives related? Any attempt to implement immunocontraception in New Zealand must address this question. Similar remarks apply to koalas in Australia. These are over-populated in some areas, where they are already badly affected by *Chlamydia* (Martin and Handasyde 1999). The general question of the relationship between disease susceptibility and vaccine responses is illustrated by HIV-1. The course of the disease is affected by HLA genotype. Heterozygotes are at an advantage and the haplotype B*35-Cw*04 is at a disadvantage (Carrington *et al.* 1999). The HLA system also affects responses to vaccination against hepatitis B (Kruskall *et al.* 1992; McDermott *et al.* 1999). Attempts to enhance the capacity of a mouse virus to act as a successful vector for an immunocontraceptive by incorporating a cytokine gene (IL-4) have lead unexpectedly to the creation of a lethal virus (Jackson *et al.* 2001). This has caused widespread concern (Finkel 2001), and it raises the question as to whether we have enough fundamental understanding to manipulate the immune system to allow immunocontraception to be used without 'side-effects'. Other reservations have been expressed by Tuyttens and Macdonald (1998). In both

Table 5. Relative fertility of immunocontracepted females in seven mammalian species

Species	Immunogen	Number (Control, Exptl)	Relative fertility[a] (%)	Reference
Baboon	$LDHC_4$ + promiscuous epitope	14, 13	38	O'Hern *et al.* (1997)
Brushtail possum	Whole sperm	16, 16	17	Duckworth *et al.* (1998)
African elephant (1st schedule)	Porcine ZP	19, 18	47	Fayrer-Hosken *et al.* (2000)
African elephant (2nd schedule)	Porcine ZP	10, 18	23	Fayrer-Hosken *et al.* (2000)
White-tailed deer	Porcine ZP	25, 109	25	Miller *et al.* (2000*a*)
White-tailed deer	Porcine ZP	8, 8[c]	13	Miller *et al.* (2000*b*)
	RC55[b]	7, 8[c]	72[d]	
	RC75a[b]	8, 8[c]	73[d]	
	Combined antigens[b]	8, 8[c]	106[d]	
Rabbit	ZPB	(24)[e]	(54)[e]	Kerr *et al.* (1999)
Burro	Porcine ZP	11, 16	11	Turner *et al.* (1996)
Horse	Porcine ZP	83,73	23	Turner *et al.* (1997)

[a]Relative fertility = (No. offspring per immunized female) / (same parameter for control females); [b]recombinant zona pellucida antigens; [c]same animals in controls; [d]not significantly different from 100%; [e]contracepted animals only; no explicit controls. ZP, zona pellucida; LDH, lactate dehydrogenase; ZPB, rabbit zona pellucida B.

Australia and New Zealand, legislation on biosafety and the use of biotechnology is being put into effect. Whether immunocontraception of wildlife would be allowed under this legislation requires detailed examination. The fact that wildlife disease can often be spread as a result of human activity will influence this examination (Daszak *et al.* 2000).

Management of over-abundant wildlife presents a series of difficult problems. There is no one solution. In both Australia and New Zealand, the established techniques of poisoning by 1080 and, in Australia, the use of pathogens are likely to continue to be used for a long period wherever large-scale control is needed. Newer, more 'high-tech' approaches, such as the use of hormone analogues, may have use for high value animals that can be caught relatively easily. We doubt whether immunocontraception will be efficacious; if it is used on a large scale, we are concerned that this could have undesirable ecological consequences.

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Roger Short's influence on this work is one of scientific method and philosophy. In the last quarter century or so, scientific influence has increasingly been wielded by specialists. His career has gone against this long-term trend. Many of his greatest contributions have been holistic and integrative. This is true of his published work and of the groups he has influenced. We argue that his kind of approach will be the basis of a mature wildlife management science.

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REPRODUCTION
REVIEW

Immunocontraception of mammalian wildlife: ecological and immunogenetic issues

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Abstract

Immunocontraception involves stimulating immune responses against gametes or reproductive hormones thus preventing conception. The method is being developed for the humane control of pest and overabundant populations of mammalian wildlife. This paper examines three fundamental issues associated with its use: (1) the difficulties of obtaining responses to self-antigens, (2) the likely evolution of genetically based non-response to immunocontraceptive agents, and (3) the possible changes in the array of pathogens possessed by the target species after generations of immunocontraception. Our review of the literature demonstrates that the barriers to an effective immunocontraceptive are at present very basic. Should they be overcome, the effects of immunocontraception on the immunogenetic constitution of wildlife populations through the selection for non-responders must be examined. We suggest that the attempt to use the animal's own immune system to modulate reproduction may be incompatible with the basic biological function of protection against infectious disease. Research programs on mammalian immunocontraception should involve measurement of the heritability of non-response and an assessment of the likely change in the response of the contracepted population to possible pathogens.

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Introduction

The regulation of human and animal population numbers constitutes a difficult and largely unsolved contemporary problem. In the developed world, steroid contraceptives for humans are both widely used and efficacious. Elsewhere they are too costly. The development of less expensive methods is considered necessary (Aitken *et al.* 1993). One such method is immunocontraception, i.e. the vaccination against sperm, eggs, or reproductive hormones to prevent either fertilization or the production of gametes. Attempts to design human immunocontraception have a long history (Joshi 1973, Stevens 1975, Basten 1988, Gupta & Talwar 1994). The targets include sperm antigens, oocyte antigens, especially zona pellucida proteins (PZP), gonadotropin riboflavin carrier protein, gonadotropins and gonadotropin releasing hormones (Delves *et al.* 2002). The most advanced method involves immunization against human chorionic gonadotropin, in reality a method of very early pregnancy termination (Baird 2000). It now seems likely that problems associated with autoimmune disease and variability of response will prevent any widespread use of immunocontraception in humans in the foreseeable future (Rao 2001, Aitken 2002). Women's health advocates have objected to all forms of immunocontraception because of perceived health risks and the potential for political abuse of the vaccine (Richter 1994). Human male immunocontraceptives have received much less attention and do not appear to be feasible in the near future (Delves *et al.* 2002).

Immunocontraceptives for wild animals have a different objective than those for humans. Their main aim is to check population growth rather than to contracept particular individuals. If some animals are irreversibly sterilized so much the better whereas such an effect in human medicine would be ethically most undesirable. Immunocontraceptives for animals are ostensibly humane and could potentially be used on the large scale required for wildlife population regulation. Research progress to date has been reviewed in Tyndale-Biscoe (1991, 1994), Barber & Fayrer-Hosken (2000), Barlow (2000), and Cooper & Herbert (2001). Three fundamental questions remain to be addressed: (1) Can sufficiently strong immune responses be provoked against the antigens (immunogens) of gametes or reproductive hormones to cause contraception in a proportion of animals large enough for effective

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population management? (2) How rapidly will variation in these responses lead to the evolution of failure to respond to the immunocontraceptive agent? (3) What will be the ecological consequences of the likely changes to the immunogenetic constitution of the population as a result of selection for non-responders? In particular, will the endemic pathogens of the species change? There is considerable information which allows us to answer at least in part the first two questions. The third is of fundamental importance but even a preliminary answer is not possible at present.

Target species

Population control of native and exotic mammals is generally justified by environmental degradation, competition with and predation on native wildlife, conflicts with humans over food production, potential spread of pathogenic infectious diseases and the possibility of population crashes of over-abundant fauna or of wildlife populations near urban areas. Although still in its infancy, immunocontraception is regarded as being more humane than the traditional methods of wildlife population control, such as shooting, trapping, poisoning, or pathogenic agents and its use has strong support from influential animal welfare agencies worldwide (Oogjes 1997, Grandy & Rutberg 2002). Table 1 lists mammalian species for which immunocontraception is being investigated and for which the method could be applied. In all these species, there are at present no completely efficacious, cost-effective, or socially acceptable methods for population regulation available.

Immune responses to self-antigens

Responses to self-antigens are unusual and mainly weak. This constitutes a major barrier to the development of an immunocontraceptive. Table 2 summarizes data on attempts to induce immunocontraception using a variety of antigens in 14 mammalian species. The data in Table 2 show that in most cases a significant proportion of the population is not contracepted by administration of the immunocontraceptive antigen. The reason for this could be either genetic or environmental. In either case it indicates that a fraction of the population will continue to breed despite the administration of the contraceptive. In most cases, there is likely to be at least in part genetic causes underlying lack of response. If so, the genes for lack of response will be selected for and in a comparatively small number of generations most of the population will be non-responsive. This implies that the immunocontraceptive can be useful for only a short period of time.

All studies summarized in Table 2 involve the use of some kind of adjuvant, i.e. a substance or array of substances designed to enhance the immune response. There are no reports of successful immunocontraception without some form of adjuvant. Moreover, most immunizations were boosted at least once (see Table 2). The need for multiple injections and the dependence upon adjuvant to achieve the necessary level of response renders the whole approach impractical at present. The most commonly used adjuvant, Freund's Adjuvant, also induces a range of undesirable side effects and its use is being challenged on animal welfare grounds (Leenaars *et al.* 1994, 1998). There is at present no feasible or acceptable method of promoting responses to self-antigens sufficient to cause immunocontraception.

Jackson *et al.* (2001) attempted to overcome the problem of lack of immune response to self-antigens in the absence of adjuvant by inserting the cytokine interleukin-4 into mousepox virus with the intention of increasing the humoral response. The virus was then inserted into the mice with the unwelcome outcome that the mice all died very quickly. This work caused alarm because of the possibility that this technology could lead to a method for simple conversion of relatively innocuous viruses into lethal ones, which could be used in biological warfare (Finkel 2001).

Another possible problem with virus-vectored immunocontraception is the potential for the horizontal transfer of the immunocontraceptive gene into viruses affecting other species (Becker 2000). While it may be possible to create genetically modified organisms without adverse effects on the target animals, the effects they might have on related species they come in contact with make any use of this approach questionable.

Variation in response and genetic change

Variation in response to biocontrol agents is a widespread phenomenon. This variation has frequently led to evolution of a degree of resistance so that the agent is no longer useful. The evolution of resistance to insecticides has been reviewed by McKenzie (1996). He draws a distinction between biocontrol agents with responses within the phenotypic range and those with responses outside the phenotypic range of the target species. An agent which initially kills all members of the target species is acting outside the normal phenotypical range, while one which kills only a fraction of the population is acting within the normal phenotypical range. He points out that when resistance appears in the former case it is frequently monogenic, while in the latter case a number of different genetic regions are involved, i.e. it is probably multifactorial. The basic genetic parameter to be estimated in either case is heritability, i.e. the extent to which genetic variation is controlled by genetic as opposed to environmental factors. The relative fertility of the immunocontracepted animals in Table 2 is >10% in 27 out of the 32 data sets. A proportion of non-responders is characteristic of most species. Only three species (Tammar wallaby, Fallow deer and Norway rat) out of 14 had no non-responders (Table 2). Following McKenzie's (1996)

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Table 1 Immunocontraception: target species and justification for fertility control.

Species	Location	Management issues	Population control needs	References
K-selected species				
Koala	Australia; local overpopulations	Habitat degradation; likely destruction of own habitat; highly regarded species	Control methods with public acceptance	Martin & Handasyde (1999)
African elephant	Southern Africa	Habitat degradation; public safety and health concerns; highly regarded species	Control methods with public acceptance	Hanks (2001)
Wild horse	USA, Australia	Habitat degradation; conflicts with livestock, timber and mining industry interests (USA); highly regarded species	Control methods with public acceptance	Berger (1986), Dobbie *et al.* (1993), Furbish & Albano (1994)
White-tailed deer	USA	Habitat degradation; public safety and health concerns; high frequencies of deer-vehicle collisions; crop and garden damage	Control methods with public acceptance	McShea *et al.* (1997), Warren (1997)
Feral donkey/Burro	USA, Australia, Africa	Habitat degradation; public safety and health concerns; highly regarded species	Broad-scale control over large, remote and inaccessible areas (Australia)	McCool (1981), Berger (1986), Freeland & Choquenot (1990)
Brushtail possum	New Zealand; major introduced pest species	Habitat degradation (New Zealand); public, livestock and wildlife health concerns; reservoir for bovine tuberculosis	Broad-scale control over large, often inaccessible, areas (New Zealand). Alternative to poison baits (1080)	Montague (2000)
Macropods	Australia	Public, livestock and wildlife health concerns; high frequencies of kangaroo-vehicle collisions; highly regarded species	Control methods with public acceptance	Dawson (1995), Pople & Grigg (1999)
European red fox	Australia; major introduced pest species	Predation on native wildlife (Australia); public, livestock and wildlife health concerns	Control over continental area (Australia). Alternative to poison baits (1080)	Saunders *et al.* (1995)
Pinnipeds	Worldwide	Possible contribution to the depletion of fish stocks	Contraception suggested as humane alternative to culling	Butterworth *et al.* (1988), Brown *et al.* (1996), Mohn & Bowen (1996)
Feral cat	Worldwide; major introduced pest species in Australia	Predation on native wildlife (Australia); public and wildlife health concerns	Control over continental area (Australia) Alternative to poison baits (1080)	Newsome (1991), Bomford *et al.* (1996), Mahlow & Slater (1996)
Feral dog	Worldwide	Public and livestock safety and health concerns; predation on native wildlife (Australia)	Control methods with public acceptance	Fleming *et al.* (2001), Sabeta *et al.* (2003)
Feral pig	Worldwide; major introduced pest species in Australia	Habitat degradation; damage to economic resources; public, livestock and wildlife health concerns	Control methods with public acceptance	Choquenot *et al.* (1996)
Badger	UK	Public, livestock and wildlife health concerns; reservoir for bovine tuberculosis	Control methods with public acceptance	Krebs *et al.* (1998), Donnelly *et al.* (2003)
Grey squirrel	UK; introduced species	Habitat degradation; threat to the native Red squirrel	Control methods with public acceptance	Moore *et al.* (1997)
r-selected species				
European rabbit	Worldwide; major vertebrate pest species in Australia	Habitat degradation; major cost to agriculture; public, livestock and wildlife health concerns	Broad-scale control over large areas	Lawson (1995), Williams *et al.* (1995)
Rodents	Worldwide	Major damage to economic resources, incl. crops, pastures, stored grain, livestock, buildings and infrastructure; public, livestock and wildlife health concerns	Broad-scale control over large areas. Species-specific alternatives to rodenticides	Caughley *et al.* (1998), Chambers *et al.* (1999), Seamark (2001)

argument, this implies that non-response is likely to be multi-factorial in genetic terms. There are no data which will allow estimates of the heritability of non-response to immunocontraception in any of the species in Table 2. We are unable to predict the rate at which this characteristic will increase in any one of these populations under the selective influence of immunocontraception. However, some idea of the likely change per generation given the initial frequency of non-responders can be found using Falconer's (1965) model for threshold characters (Table 3).

Table 2 Relative fertility of immunocontracepted females in 14 mammalian species.

Species	Immunogen	Adjuvant	No. of immunizations	Reproductive performance (control, treated)	Statistical significance[a]	Reduction in relative fertility (%)[b]	Reference
Baboon	LDH-C_4+ promiscuous epitope	CGP11637 emulsified w. Squalene:Arlacel -A (4:1)	3	Offspring/females 10/13, 4/14	$P<0.02$	62	O'Hern *et al.* (1997)
Brushtail possum	Whole sperm	Freund's complete (FCA), Freund's incomplete (FIA) in boosters	3	Offspring/females 12/16, 2/16	$P<0.001$	83	Duckworth *et al.* (1998)
Tammar wallaby	Porcine ZP	FCA, FIA in boosters	5	Offspring/females 4/6, 0/6	$P<0.05$ ($n=6$)	100	Kitchener *et al.* (2002)
African elephant							
First schedule	Porcine ZP	Adjuvant used, type not given	3	Offspring/females 16/18, 8/18	$P=0.005$	50	Fayrer-Hosken *et al.* (2000)
Second schedule	Porcine ZP	Adjuvant not mentioned	2	Offspring/females 2/10 (no true control)	$P=0.001$	77	Fayrer-Hosken *et al.* (2000)
Wild horse	Porcine ZP	FCA, FIA in boosters	3–4	Pregnancy rate 3/6, 1/14	Not stated	85	Kirkpatrick *et al.* (1991)
White-tailed deer							
First schedule	Porcine ZP	FCA, FIA in booster	2	Fawns/doe years 30/16, 4/16	$P<0.0001$	87	Miller *et al.* (2000*a*)
Second schedule	RC55	FCA, FIA in booster	2	Fawns/doe years 30/16, 19/14	$P<0.05$	28	Miller *et al.* (2000*a*)
Third schedule	RC75a	FCA, FIA in booster	2	Fawns/doe years 30/16, 11/8	$P<0.01$	27	Miller *et al.* (2000*a*)
Fourth schedule	Combined antigens	FCA, FIA in booster	2	Fawns/doe years 30/16, 16/8	$P>0.05$	0	Miller *et al.* (2000*a*)
White-tailed deer	KLH-GnRH	FCA, FIA in boosters	2–4	Fawns/doe years 35/19, 5/24	$P<0.01$	89	Miller *et al.* (2000*b*)
White-tailed deer	Porcine ZP	FCA, FIA in boosters	2–3	Fawns/doe years 35/19, 25/57	$P<0.01$	76	Miller *et al.* (2000*c*)
White-tailed deer							
First schedule	GnRH	FCA, FIA in boosters	3	Fawns/doe years 110/90, 36/118	$P<0.0005$	75	Curtis *et al.* (2002)
Second schedule	Porcine ZP	FCA, FIA in boosters	3	Fawns/doe years 72/56, 10/60	$P<0.0005$	87	Curtis *et al.* (2002)
Fallow deer	SpayVac	FCA	1	Pregnancy rate 322/334, 0/22	$P<0.0001$	100	Fraker *et al.* (2002)
Burro	Porcine ZP	FCA, FIA in boosters	2–3	Offspring/females 6/11, 1/16	$P<0.05$	88	Turner *et al.* (1996)
Grey seal	SIZP (SpayVac)	FCA	1	Pups/female 2.76, 0.22	$P<0.001$	92	Brown *et al.* (1996, 1997)
Tule elk	Porcine ZP	FCA, FIA in boosters	3–4	Calves/cow years 53/91, 5/104	Not stated	91	Shideler *et al.* (2002)
Cat	Porcine ZP	FCA, FIA in boosters	5	Pregnancy rate 2/2, 1/5	Not stated	50	Ivanova *et al.* (1995)
Cat							
First schedule	SpayVac	FCA	1	Mean litter size 5.2, 4.5	$P=0.8859$	13	Gorman *et al.* (2002)
Second schedule	SpayVac	Alum	1	Mean litter size 5.2, 4.4	$P=0.8859$	15	Gorman *et al.* (2002)
European rabbit	Myxoma vectored ZPB	FCA, FIA in boosters	3	Mean litter size 7.4, 7.0	Not stated	5	Kerr *et al.* (1999)
Norway rat							
First schedule	MZPP/KLH	FCA, FIA in boosters	3	Pregnancy rate 7/8, 4/8	$P>0.05$	40	Miller *et al.* (1997)
Second schedule	GnRH/KLH	FCA, FIA in boosters	3	Pregnancy rate 7/8, 0/8	$P<0.004$	100	Miller *et al.* (1997)
Wild mouse	KLH-mZP3	FCA, FIA in boosters	5	Pregnancy rate 8/15, 7/30	$P=0.046$	56	Hardy *et al.* (2002*b*)
BALB/c mouse							
First schedule (71–81 days)	Murine rFA-1	FCA, FIA in boosters	4	Mean litter size 8.9, 3.2	$P<0.0001$	64	Naz & Zhu (1998)
Second schedule (283 days)	Murine rFA-1	FCA, FIA in boosters	4	Mean litter size 8.6, 9	$P>0.05$	0	Naz & Zhu (1998)
BALB/c mouse							
First schedule	sp56FLAG	FCA, FIA in boosters	6	Offspring/females 55/14, 12/5	$P=0017$	39	Hardy & Mobbs (1999)

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Table 2 *Continued.*

Species	Immunogen	Adjuvant	No. of immunizations	Reproductive performance (control, treated)	Statistical significance[a]	Reduction in relative fertility (%)[b]	Reference
Second schedule	sp56FLAG	FCA, FIA in boosters	4	Offspring/females 55/14, 19/5	Not stated	3	Hardy & Mobbs (1999)
BALB/c mouse							
First schedule	MBP-polyepitope A	FCA, FIA in boosters	4	Mean litter size 5.2, 3.3	Not stated	37	Hardy *et al.* (2002*a*)
Second schedule	MBP-polyepitope B	FCA, FIA in boosters	4	Mean litter size 5.2, 2.1	$P<0.05$	60	Hardy *et al.* (2002*a*)
Third schedule	6XHis-polyepitope A	FCA, FIA in boosters	4	Mean litter size 6.5, 6.3	Not stated	3	Hardy *et al.* (2002*a*)

Investigations have been conducted on approximately 70 species. This table includes only true experiments, i.e. studies with an immunized group compared with a control group.
[a]The *P* values are those given in the references cited. [b]Relative fertility is defined as the mean no. of offspring for females in the vaccinated group divided by the same figure for the control group (i.e. unimmunized females).

Reproduction is a good example of a threshold character; it is an all-or-none attribute which can be affected by a variety of underlying genetic and environmental factors. If heritabilities are high, rapid selection occurs. This is shown by the high percentage of non-responders that occur within one generation (Table 3).

A limited place for immunocontraception in wildlife management could be in species with long generation times. Genetic changes in them, if they occur, will take decades. Claims have been made for the potential efficacy of immunocontraception for African elephants (Fayrer-Hosken *et al.* 2000, Delsink *et al.* 2002), although this view has been challenged on demographic grounds (Pimm & van Aarde 2001). Long-term studies on immunocontraception in wild horses report 78–94.2% contraceptive efficacy (Kirkpatrick *et al.* 1995, Turner *et al.* 1997, 2002, Turner & Kirkpatrick 2002).

Zoo animals are convenient for immunocontraception studies of wild species, because of their long-term accessibility, although the small numbers usually available make controls hard to find. This is illustrated by an investigation involving 27 females from 10 felid species. Immunization with PZP and Freund's Complete Adjuvant gave several kinds of adverse reaction but no convincing evidence of an effect upon fertility (Harrenstien *et al.* 2004).

Table 3 Predicted proportion of non-responder daughters after one generation of selection by immunocontraception of mothers given various heritabilities (after Falconer 1965).

	Heritability (%)			
Non-responder mothers (%)	100	80	60	50
5	15	11	10	8
10	23	19	16	15

The prediction has been arrived at by entering the table in Falconer (1965) which relates heritability for a threshold trait to incidences in parent and offspring. The response to selection (predicted percentage of non-responders) obtained has been halved because selection is being carried out on one sex.

Delves & Roitt (2005) review attempts to immunocontracept mammals and conclude that GnRH is the most promising target, because of its evolutionary conserved sequence.

Immunogenetic issues

Infectious diseases are assumed to be one of the main classes of selective forces which act upon genes controlling immune responses (Klein *et al.* 1993). Immunocontraceptive agents also have the potential to influence the genetic constitution of a population with respect to the ability to mount immune responses. The two are similar in that pathogens which cause plagues and immunocontraceptive agents are both capable of exerting very strong selective pressure with the potential for rapid genetic change. However, they differ in two important respects. First, most pathogens are cellular and antigenically more complex than most immunocontraceptive agents, which consist of one or a few proteins and in some cases associated carbohydrate. Second, they have opposite directions of selection; resistance to a pathogen involves a positive response, whereas resistance to an immunocontraceptive involves non-response. The consequences of this kind of selection imposed by an immunocontraceptive agent require study. It seems likely that it will alter the immunogenetic constitution of the target species. The existence of genes governing response to pathogens is well documented in humans for malaria (Hill 2001), tuberculosis (Blackwell 1998, Bellamy 2003), and HIV (McNicholl & Cuenco 1999, Carrington & O'Brien 2003). Relevant examples are found in New Zealand Red Deer in which susceptibility to tuberculosis has high heritability (Mackintosh *et al.* 2000) and in the NRAMP1 association with the human response to leishmaniasis (Bucheton *et al.* 2003). The complexities of the co-evolution of pathogens and hosts and its biomedical significance are beginning to be unraveled (Woolhouse *et al.* 2002). Both experimental (Lively & Dybdahl 2000) and theoretical analyses (e.g. Nowak

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& May 1994) point to the inherent difficulties of prediction of the course of these interactions. Prediction and detection of the ecopathological consequences of the use of immunocontraception of wild animals will also be made difficult by the spread of emerging infectious diseases as a result of human activity (Daszak *et al.* 2000).

The effect of immunocontraception upon genetic diversity could be significant. There is the possibility that restriction of breeding to a small group of animals which are closely related will result in localized inbreeding. This will be especially likely if their capacity to resist the immunocontraceptive is the result of shared uncommon genotype. Acevedo-Whitehouse *et al.* (2003) have shown that in California sea lions, inbreeding is associated with a wide range of diseases. They suggest that inbred individuals could act disproportionally as reservoirs of infectious agents.

Selection based upon immune responses could be on one of two parts of the genome: the MHC (major histocompatability complex) region which governs responses to specific immunogens, or other genes, e.g. *NRAMP* which govern the functioning of the immune system in general (Bellamy 1999). The tightly linked *MHC* genes and the resultant linkage disequilibrium mean that selection on one gene will result in changes in gene frequencies at other loci. This could either raise or lower susceptibility to other pathogens. Understanding of the non-MHC genetic component of variability of the immune response is much less advanced than for the MHC component. This understanding is needed to attempt any predictions concerning immunocontraception-based selection.

Experimental approaches to this question have until now been very difficult. The existence of genetic maps of some wild animals (e.g. Tammar wallaby (Zenger *et al.* 2002)) may now allow a genomic approach, in which whole genome DNA typing may allow the identification of changes in gene frequency which accompany the application of immunocontraception or a pathogen, through comparing treated and control populations. A concomitant survey of pathogens in the two groups may identify susceptibility regions, whose existence could be further tested in lab-based investigation. The genome would thus be assayed for these genes, and test the extent to which the same genes are involved in responses to different pathogens and to immunocontraception.

A good model system to address these questions is wildlife tuberculosis which is of economic significance in several countries, e.g. Britain (Delahay *et al.* 2002), New Zealand (Buddle *et al.* 2002), and the United States (Palmer *et al.* 2002). Considerable information on the genetic control of response to mycobacterial antigens is available (North & Medina 1998, Kramnik *et al.* 2000, Bellamy 2003). The possibility of obtaining results relevant to human mycobacterial susceptibility may also encourage use of this system.

We conclude that attempting to use the immunological system to modulate reproduction could be incompatible with the basic biological function of resisting pathogens. We have not discussed some of the practical issues. For example, all fertility control methods have the problem of delivery of the control agent. Highly valued animals must be treated without harming them. When this is the case, fertility control methods with fewer concomitant problems, such as surgical sterilization or the use of steroids or gonadotropin-based hormones, would be competitive with immunocontraception (Cooper & Herbert 2001).

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THE ECOLOGY OF STRESS

The ecology of stress: effects of the social environment

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Summary

1. Many aspects of the social environment affect hypothalamic-pituitary-adrenal (HPA) axis function and increase circulating glucocorticoid concentrations. In this review, we examine the relationships between the social environment and the function of the HPA axis in vertebrates.

2. First, we explore the effects of the social environment on glucocorticoid secretion in territorial (primarily non-social) species, with an emphasis on the effects of variation in population density, as modified by environmental factors such as predation risk and food availability. In general, high population density or frequent territorial intrusions are associated with increased glucocorticoid secretion in a wide range of taxa, including mammals, birds, fish and reptiles, although there is considerable variability across species.

3. Second, we consider the effects of social interactions and dominance rank on glucocorticoid secretion in social species, mostly in birds and mammals. We review studies that have detected an association between social status and glucocorticoid levels – sometimes with higher glucocorticoid levels in low-ranking individuals, and sometimes with higher glucocorticoid levels in dominant individuals. The relationship between dominance and glucocorticoid levels varies among species, populations and years, in a manner that depends on the stability of the social hierarchy, environmental conditions, the type of breeding system, and the manner in which high rank is obtained and maintained.

4. Finally, we discuss the concept of allostasis and consider interactions between social effects and other environmental factors, noting that there is relatively little research on these interactions to date. For both non-social and social species, we identify priorities of future research. These priorities include more complete descriptions of HPA function that move beyond measurements of basal glucocorticoid concentrations (which will generally require field experiments), to studies that examine organizational effects of social stressors, that directly test the relationship between HPA function and fitness, and that examine how glucocorticoid responses affect population dynamics.

5. Although several lines of evidence suggest that glucocorticoid responses can affect the fitness of individuals and therefore can alter the dynamics of populations, the effect of glucocorticoid responses on population dynamics remains essentially unstudied.

Key-words: allostasis, dominance, glucocorticoid, hypothalamic-pituitary-adrenal axis, sociality, social behaviour, stress, territoriality

Introduction

Animals are faced with a constant flood of information from their external environment. Although much of this information can be ignored, some of it can be used in an adaptive fashion. In vertebrates, one of the primary mechanisms by which external environmental information elicits such an adaptive response is through initiating a physiological cascade of internal changes mediated in part through the hypothalamic-pituitary-adrenal (HPA) axis.

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The HPA axis is the neuroendocrine pathway by which stress hormones (glucocorticoids) are released from the adrenal cortex. The structure and function of the HPA axis are reviewed elsewhere (e.g. Sapolsky, Romero & Munck 2000; Sapolsky 2002). The HPA axis integrates environmental information and the downstream release of glucocorticoids can potentiate an adaptive behavioural response (Monclús *et al.* 2005; Schulkin, Morgan & Rosen 2005) or alteration in life-history strategy (Wingfield *et al.* 1998; Boonstra 2005).

The social environment is one of the primary sources of information that can induce a physiological stress response. In both social and non-social animals, the frequency and type of interaction with conspecifics as well as status in social species can affect HPA axis activity. In this review, we examine the relationships between the social environment and the function of the HPA axis in vertebrates. First, we explore the effects of the social environment on glucocorticoid secretion in territorial species, with an emphasis on the effects of variation in population density, as modified by environmental factors such as predation risk and food availability. Second, we consider the effects of social interactions and dominance rank on glucocorticoid secretion in social species. Many studies have detected an association between social status and glucocorticoid levels – sometimes with higher glucocorticoids in low-ranking individuals, and sometimes with higher glucocorticoids in dominant individuals. Many studies also consider the particular traits associated with dominance or subordination that might be causally related to variation in glucocorticoid secretion. Other studies suggest that the general social environment (e.g. the stability or instability of social relationships and the type of dominance hierarchy) can affect the association between glucocorticoids and rank. Finally, we discuss the concept of allostasis and consider interactions between social effects and other environmental factors, noting that there is relatively little research on these interactions to date. Thus, this review reveals relationships between glucocorticoid secretion, environmental variables such as predation risk, population variables such as density or the rate of territorial intrusion and social variables such as dominance rank, and behavioural variables such as the rate of aggression. Clearly the HPA response is a physiological mechanism that might mediate a wide range of fitness effects. The review also reveals considerable variability among species in the response of glucocorticoid levels to these factors and suggests new hypotheses that might explain this variation.

Effect of the social environment on the stress axis in territorial vertebrates

NON-SOCIAL SPECIES: TERRITORIALITY

The patchy distribution of limited but defendable resources such as food, mates or preferable habitats can favour the evolution of territoriality. Territoriality is the exclusive use of an area (i.e. 'ecological' territoriality) or the active defence of an area from conspecifics using various visual, acoustic, or chemical signals that indicate ownership (i.e. 'behavioural' territoriality; Brown & Orians 1970). Signals advertising ownership may deter intrusions by conspecifics of the same gender or both genders and can also attract opposite-sex conspecifics, which can allow for the monopolization of mates and other limited resources.

Many vertebrate species exhibit either temporary or extended periods of territoriality. Although territoriality implies exclusiveness from conspecifics of the same or opposite gender, the true degree of non-sociality (i.e. avoidance of conspecifics) exhibited by territorial vertebrate species is variable. For example, males of many avian species breeding in temperate climates establish breeding territories but aggregate in large flocks at times outside of the breeding season (Nice 1941). The breeding territories of male songbirds are often defended from male but not female conspecifics, which results in male birds spending a significant portion of each year exclusive of other males. Similarly, females of some small mammal species (e.g. *Microtus*, *Clethrionomys,* and *Peromyscus* spp.) defend nesting territories during the breeding season, but will live in groups of related or unrelated individuals during the non-breeding season (Ostfeld 1990; Wolff 1993). At the other end of the continuum of non-sociality are species that defend year-round territories from both intra- and inter-sexual intruders. For example, both female and male North American red squirrels (*Tamiasciurus hudsonicus*) defend exclusive food-based territories year-round (Smith 1968). Female red squirrels are spontaneous ovulators, and they only appear to allow male intruders on their territories when they are in behavioural oestrous, which is approximately 1 day per year (Smith 1968; Lane *et al.* 2008). Thus, the degree of non-sociality in territorial vertebrate species can range from highly non-social (red squirrels) to tolerance of both sexes for most of the year except during the breeding season (temperate breeding avian species and some small mammal species). There is similar variation in the degree of non-sociality in species that actively defend mates (mate-defence polygyny) rather than actively defending a territory (resource-defence polgyny or monogamy). In mate-defence polygyny (e.g. impala, *Aepyceros melampus*), males typically attack other males during the mating season, but aggregate in large herds with little aggression outside of the mating season (Jarman 1974). Because social interactions can strongly affect the activity of the HPA axis, variation in the degree of non-sociality may interact with stress physiology. Here, we focus on the effects of the social environment on the activity of the HPA axis in vertebrate species that exhibit some degree of non-sociality due to territoriality (Fig. 1).

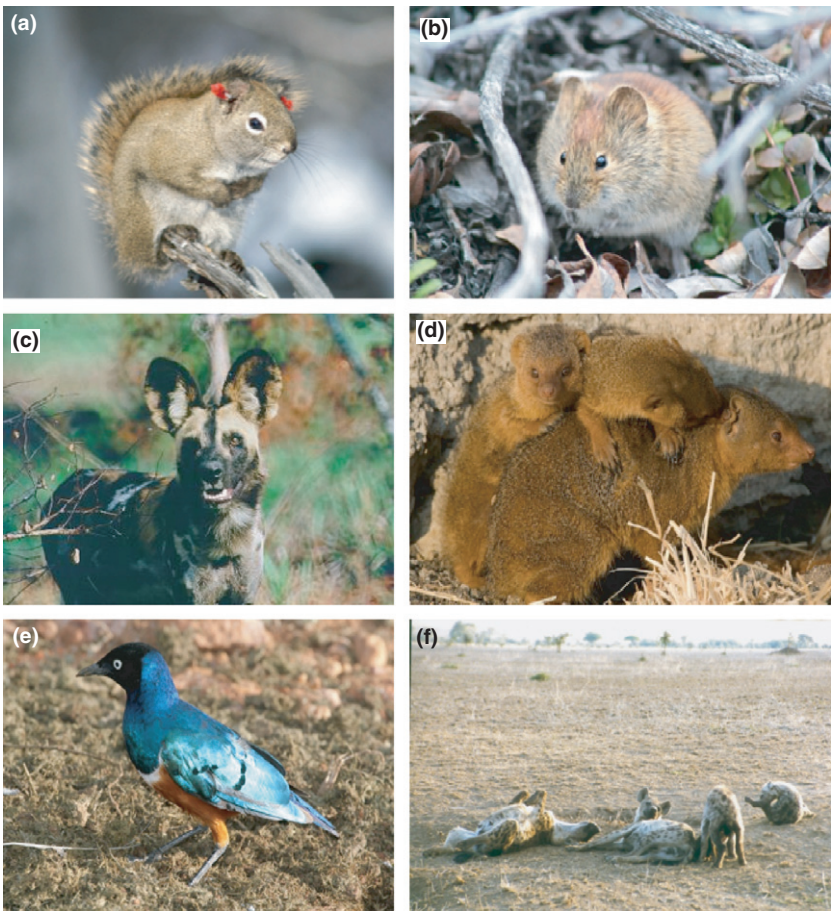


Fig. 1. (a, b) Population density and glucocorticoid levels are usually positively associated in non-social species, although the degree to which territorial vertebrate species are non-social is highly variable. For example, both male and female North American red squirrels (a) defend year-round food-based territories. In other small mammal species such as the northern red-backed voles (b), females only defend nesting territories during the breeding season (photos by R.W. Taylor). In many social species, behavioural dominance is strongly correlated with reproductive success but dominance is also associated with heightened glucocorticoid levels. For example, within groups of African wild dogs (c) and dwarf mongooses (d), only the behaviourally dominant individual of each gender is assured of breeding (photos by S. Creel). However, dominant wild dogs and dwarf mongoose also engage in more aggressive interactions and their glucocorticoid levels are higher than those of subordinates, which may impose fitness costs. In many social species, the relationship between social status and glucocorticoid levels can be complicated by both environmental variation and the method by which social status is acquired and maintained. (e) For example, in cooperatively breeding superb starlings, annual differences in rainfall and resource availability not only influence glucocorticoid levels, but they may do so differently in dominants and subordinates (photo by D. R. Rubenstein). (f) Unlike most other carnivores, social rank in spotted hyenas is inherited from the mother, which complicates the positive relationship between social status and age/size that is seen in many other species (photo by W. Goymann).

FACTORS AFFECTING ACTIVITY OF THE HPA AXIS IN TERRITORIAL SPECIES

In territorial vertebrates, population or breeding density is one of the best documented factors that influence the activity of the HPA axis. Christian (1950) first used Selye's description of stress (or general adaptation syndrome; Selye 1936) to describe how the adrenocortical response to population density might cause the density-dependent population cycles exhibited by some small mammal species. Christian (1950) hypothesized that increases in population density in small mammal species such as voles led to increased antagonistic social interactions, which caused an increase in adrenocortical activity. Christian further predicted that the consequences of this heightened adrenocortical activity were a decrease or suppression of reproduction and an increase in mortality due to decreased resistance to disease or other limiting factors (Christian 1950; Christian & Davis 1964). As a result, population growth ceased or declined during peak population densities due to heightened mortality and decreased fecundity. Christian and colleagues (Christian 1950, 1956, 1961, 1964, 1971; Christian & Davis 1964, 1966) provided extensive empirical documentation in territorial mammalian species of the relationship between adrenal gland mass (a proxy of adrenocortical activity: Bronson & Eleftheriou 1964) and population density and/or the frequency of antagonistic interactions.

Christian's pioneering studies were instrumental in connecting how environmental factors influence the HPA axis

activity of individuals, which in turn affected population dynamics. Groundbreaking studies in a variety of mammalian taxa (primarily rodents) found significant positive relationships between population density and adrenal mass (reviewed by Christian 1971; To & Tamarin 1977) or *in vitro* adrenal secretion of glucocorticoids (Andrews 1968). However, Christian's research has been criticized because it was largely performed on captive animals often in a laboratory setting (Krebs & Myers 1974; Lee & McDonald 1985). Nonetheless, more recent studies in other mammalian species have documented a similar positive relationship between population density and plasma glucocorticoids (Boonstra & Boag 1992; m 1998) or faecal glucocorticoid metabolite concentrations (Novikov & Moshkin 1998; Harper & Austad 2004; Bian *et al*. 2011) in free-living individuals. For example, Boonstra & Boag (1992) found that populations of meadow voles (*Microtus pennsylvanicus*) at the highest densities had the highest free plasma corticosterone concentrations and lowest corticosterone-binding globulin concentrations. Support for this positive relationship between population density and stress axis activity in territorial mammalian species is not universal (e.g. To & Tamarin 1977; Harper & Austad 2004; Kuznetsov *et al*. 2004; Charbonnel *et al*. 2008), and we have little information about the relationship between population density and stress axis activity in some mammalian orders that include many solitary species (e.g. carnivores, of which more than 80% of species are solitary: Gittleman 1984).

The relationship between population density and the activity of the stress axis is best known from studies of mammals. This is not surprising given the initial focus of Christian and colleagues (see above). Although less well-studied than mammals, birds often show similar relationships between density and HPA function. With regard to stress physiology, most of the well-studied avian species breed in temperate regions exhibit territoriality only during the breeding season, and only defend territories from same-gender conspecifics. Although there are fewer studies of birds than mammals, a positive relationship between population density and plasma (Silverin 1998a) or yolk glucocorticoids (Love *et al*. 2008) has been reported for several species, while others have detected no relationship (Beletsky, Orians & Wingfield 1990, 1992). However, further support for the predicted positive relationship between density and stress axis activity in avian species may come from simulated territorial intrusion (STI) experiments. In these experiments, a territorial male is presented with an unfamiliar caged male (or a stuffed male) within his territory, paired with an audio playback of the species' song. Several studies in temperate-breeding birds have found that STIs cause a pronounced increase in plasma glucocorticoids (e.g. Silverin1993, 1998b; Van Duyse *et al*. 2004; Canoine & Gwinner 2005; Landys *et al*. 2007), whereas others have not found such a relationship (e.g. Harding & Follett 1979; Wingfield 1985; Pärn *et al*. 2008; Scriba & Goymann 2010). Because antagonistic interactions and other territorial intrusions are predicted to increase as population density rises (e.g. Bretagnolle, Mougeot & Thibault 2008), we therefore would predict that population density is also positively correlated with stress axis activity in species that responded to STIs with elevated glucocorticoid production. To date, very few studies in avian species that exhibit year-round territoriality have directly tested the relationship between glucocorticoids and population density. However, some studies have found that STIs cause a significant increase in plasma glucocorticoids in bird species exhibiting year-round territoriality (e.g. Gill, Costa & Hau 2008; Landys *et al*. 2010), providing indirect evidence for a positive relationship between population density and stress axis activity.

Other taxa, including amphibians, reptiles and fish, also offer insights into the relationship between population density and HPA function. Amphibians and reptiles often defend resource-based territories and are non-social as adults. Many species only interact with conspecifics during the larval stage or during breeding as adults. Some studies in amphibians and reptiles also support the positive relationship between population or breeding density and stress axis activity. For example, increased population density during mating in anurans (Leary, Garcia & Knapp 2008) or nesting density in turtles (Jessop, Limpus & Whittier 1999) is associated with increased plasma glucocorticoids. High population density either were associated with increased or decreased plasma glucocorticoids in female side-blotched lizards (*Uta stansburiana*) depending upon the female reproductive tactic (Comendant *et al*. 2003). In contrast, experimentally increased densities in breeding salamanders did not elicit elevated plasma glucocorticoids (Cooperman, Reed & Romero 2004). It is important to note, however, that increased glucocorticoid concentrations during breeding aggregations might be driven by changes associated with reproduction that are not related to density (e.g. glucocorticoid concentrations rise during gestation in many mammals; Creel, Winnie & Christianson 2009; Dantzer *et al*. 2010; or glucocorticoid concentrations rise during intense mate competition in marsupials; Bradley, McDonald & Lee 1980; Bradley 1987). Evidence for positive effects of population density on stress axis activity in amphibians and reptiles also comes from laboratory studies. For example, heightened competition over burrows in captive spotted salamanders (*Ambystoma maculatum*; Cooperman, Reed & Romero 2004) or increased housing density in American alligators (*Alligator mississippiensis*; Elsey *et al*. 1990) was associated with increased plasma glucocorticoids (see also Hayes 1997; Glennemeier & Denver 2002). In some lizard species, forced antagonistic interactions with conspecifics in laboratory experiments can elevate plasma glucocorticoids (Klukowski & Nelson 1998; Schuett & Grober 2000; Yang & Wilczynski 2003), which suggests that stress axis activity would be elevated under high density conditions due to a heightened

frequency of antagonistic interactions. In many ways, such studies of forced interaction are similar to early studies of 'social stress' in captive animals that we discuss in the second part of this review.

Finally, it is well established that crowding increases stress axis activity in captive or hatchery reared fish (Barton & Iwama 1991), but we are aware of no studies examining the relationship between population or breeding densities and stress axis activity in free-ranging territorial fish. This is not surprising given the logistical difficulties of such a study. However, future studies in fish species in which males provide paternal care and actively defend brooding sites from conspecifics such as bluegill (*Leopomis macrochirus*; Neff & Knapp 2009) and smallmouth bass (*Micropterus dolomieu*; Hanson *et al*. 2009; Dey *et al*. 2010) should investigate how breeding or nesting density affects stress axis activity.

REMAINING QUESTIONS IN TERRITORIAL SPECIES

In the above section, we have detailed how population or breeding density increases HPA activity in vertebrate taxa and in some cases how forced antagonistic interactions between conspecifics can elevate stress axis activity. However, the positive relationship between population density or the frequency of antagonistic interactions and HPA axis activity is not universal across different taxa. Departures from the general pattern of increased density leading to increased HPA activity could be due to the confounding effects of reproductive condition, seasonality or recent extreme weather events on HPA axis activity or responsiveness (Kenagy & Place 2000; Romero 2002; Romero *et al*. 2008) or to the effects of trapping/handling stress on glucocorticoid levels (Kenagy & Place 2000; Sheriff *et al*. 2011a). As such, future studies examining relationships between population density and HPA axis activity should sample their study animals carefully to avoid these potentially confounding effects.

A central remaining question is to identify if the relationship between population density and HPA axis activity that has been found in many species is due to an increase in antagonistic interactions between conspecifics at high density, decreased per capita food abundance, or even increased attraction of predators. For example, food-abundance often co-varies with population density such that increased overall food abundance is associated with increased reproductive output and/or population density, but perhaps constant or decreased per capita food availability. As a result, determining the actual mechanism that induces heightened HPA axis activity under high population density conditions will require experimental manipulations of the potential causal variables, or careful sampling designs with multiple populations.

Few studies have experimentally manipulated population density, food-abundance or predator densities in free-living non-social territorial animals. In a recent study using a non-social species, the North American red squirrel, population density was positively correlated with faecal cortisol metabolite concentrations (B. Dantzer, unpublished data). Experimental increases in both actual (using long-term food supplementation) and perceived population density (using audio playbacks of territorial vocalizations to simulate high density conditions) also caused a significant increase in faecal corticosteroid metabolites (Dantzer *et al*. unpublished data). This suggests that, at least in this species, the actual variable causing changes in glucocorticoids (and also density-dependent behavioural responses; Dantzer *et al*. in press) is the perception of social competition and not food abundance or predator densities.

In addition to population density, predation risk may affect HPA axis activity in territorial species. For example, predator density affects plasma free glucocorticoid concentrations in snowshoe hares (*Lepus americanus*) (Boonstra *et al*. 1998; Sheriff, Krebs & Boonstra 2011b) and total plasma glucocorticoid concentrations in African stonechats (Scheuerlein, Van't Hof & Gwinner 2001). In elk, (*Cervus elaphus;* Creel, Winnie & Christianson 2009), predator density did not detectably affect faecal corticosteroid metabolites concentrations, although progesterone concentrations and calf production did decline in response to predation risk (Creel *et al*. 2007). To date, too few studies have examined the relationship between HPA activity and predation risk to infer general relationships with confidence. However, recently Zanette *et al*. (2011) simulated a strong increase in the perceived risk of predation using predator vocalization playbacks and observed behavioural changes in territorial female song sparrows, associated with a nearly 40% reduction in clutch size at fledgling. The impact of predation pressure in natural situations may be more subtle than in this experiment, because it simulated a situation with severe predation pressure by broadcasting predator vocalizations steadily for 130 days on a 4 day on/off basis (Zanette *et al*. 2011). This reduction in clutch size may be through a stress-mediated pathway because previous studies have found that glucocorticoids were elevated in response to increased predator abundance (Clinchy *et al*. 2004, 2011). This study experimentally confirms observational studies that have shown strong effects of predation risk on individual reproduction in wild populations, although these are not necessarily stress-mediated (Creel *et al*. 2007). We suggest that future studies examining relationships between population density and the activity of the stress axis should also consider how food abundance and predation risk co-vary with population density and HPA activity (see also McColl 1998).

As with studies of social species (see below), broader assessments of HPA function and tests for fitness effects in non-social species remain important. Although the initial focus of Christian and colleagues was to examine how population density or environmental variation influences the population dynamics of non-social animals through HPA axis activity, no studies that we are aware of have actually explored how HPA axis activity of individuals affects population dynamics as the focus of most field

studies to date has been on *individuals*. While it is reasonable to assume that effects on individual fitness often have consequences for population dynamics, it will be productive to return to the ideas of Christian and explore how the glucocorticoid responses of individuals to environmental variation can influence population or even community dynamics (Hawlena & Schmitz 2010). This will require careful experimentation of population densities and even HPA axis activity with tractable study species to examine if the glucocorticoid responses of individuals to environmental variation affect population density.

Effect of the social environment on the stress axis in group-living vertebrates

SOCIAL SPECIES: SOCIAL STATUS AND DOMINANCE RANK

Animals living in social groups often form dominance hierarchies. An individual's position within the dominance hierarchy can have strong effects on fitness, because social dominance often confers priority of access to mates, food and other resources (Fig. 1). In many cooperatively breeding birds and mammals, the probability of breeding is almost one for the socially dominant individual of each gender and almost zero for social subordinates. Given these clear benefits of social dominance, many early studies of aggression, rank and physiological stress responses were based on the hypothesis that low social status is stressful. Early research on captive rodents and primates supported this hypothesis (Bronson & Eleftheriou 1964; Louch & Higginbotham 1967; Manogue, Leshner & Candland 1975). In paired contests between laboratory rats, mice and monkeys, aggression increased glucocorticoid secretion in both winners and losers, but the effect was significantly stronger among losers. Thus, as field studies began to examine stress responses in the wild in the 1980s, a plausible hypothesis was that the 'stress of subordination' might underlie the reproductive suppression of social subordinates in many cooperatively breeding species.

However, social hierarchies and agonistic behaviour evolve to avoid the costs of overt aggression. This general explanation for the evolution of agonistic behaviour is predicated on the assumption that contests among group members can carry costs (Rubenstein & Shen 2009). The costs of engaging in agonistic or aggressive interactions (and more generally the costs of a given social position) are often not equal for both of the individuals involved, and this observation raises an interesting range of possibilities. If winning a direct contest provokes a weaker stress response than losing, but dominant individuals engage in more contests, then the total effect may be that dominants have higher mean circulating glucocorticoid levels than subordinates. In addition to direct contests, it is plausible that the maintenance of high rank requires more monitoring or 'policing' of the behaviour of others than is required for animals with low rank, simply due to differences in the number of group mates who are subordinate to individuals of different rank. Observations of wild groups commonly show that dominant animals do engage in higher rates of aggressive and agonistic behaviour in many species (e.g. most social carnivores: Creel *et al*. 1997; Sands & Creel 2004), so it is plausible to hypothesize that social stress is greater for dominants than for subordinates, but the fitness costs are offset by the other benefits of high rank.

Given that one might plausibly hypothesize either positive or negative correlations between social status and glucocorticoid secretion, empirical data from wild populations are necessary to advance the science. Robert Sapolsky, one of the pioneers of social stress research in free-living animals, found that subordinate male olive baboons (*Papio anubis*) are more likely to be physiologically stressed than dominants and show chronically elevated levels of glucocorticoids (Sapolsky 1982, 1983). As a consequence of chronically elevated glucocorticoids, subordinate baboons are more likely to develop stress-related pathologies than dominants (Sapolsky, Alberts & Altmann 1997; Sapolsky & Spencer 1997). In follow-up studies, Sapolsky and coworkers discovered that this rank-related pattern in glucocorticoid physiology may be modified by styles of dominance (Ray & Sapolsky 1992), or may be suspended by environmental impacts, such as during a severe drought during which all members of the social group devoted most of their time to foraging, leaving little opportunity for social harassment (Sapolsky 1986). Most notably, Sapolsky (1992a) suggested that the relationship of glucocorticoid concentrations to rank depended on the stability or instability of baboon hierarchies. In stable hierarchies, the glucocorticoid levels of subordinates were higher than the glucocorticoid levels of high-ranking baboons, but this pattern was reversed during periods of social instability. Sapolsky's early results from free-ranging baboons supported the prevailing view from laboratory studies (e.g. Manogue, Leshner & Candland 1975) that subordinates are generally more likely to suffer from social stress than dominants. More importantly, these studies highlighted the role that social stability has on glucocorticoid levels in some species.

With the availability of non-invasive methods to measure hormone metabolites from urine or faeces, studies on the relationship between social rank and glucocorticoid levels of free-living animals have become more common over the past two decades. Some of these studies challenged the traditional view of the relationship between glucocorticoids and social rank in free-living animals that subordinates tend to have higher levels than dominants, arguing instead that dominants experience more psychosocial stress and often express higher levels of glucocorticoids than subordinates (Creel 2001). For example, glucocorticoid levels were higher in dominants than in subordinates in free-living African wild dogs (*Lycaon pictus*) and dwarf mongoose (*Helogale parvula*) (Creel, Creel & Monfort 1996; Creel *et al*. 1997). Both species are cooperative breeders and live in groups with '*despotic hierarchies*'

(Sapolsky 2005). Similarly, in cooperatively breeding African cichlid fish (*Neolamprologus pulcher*) dominants had higher glucocorticoid levels than subordinates (Mileva, Gilmour & Balshine 2010). The same pattern has been reported for several wild populations of cooperatively breeding primates (reviewed in Creel 2001) and gray wolves (*Canis lupus*; Sands & Creel 2004). In cooperatively breeding birds, dominants and subordinates often show similar glucocorticoid levels (e.g. Schoech, Mumme & Moore 1991; Wingfield, Hegner & Lewis 1991; Mays, Vleck & Dawson 1991; Malueg, Walters & Moore 2009). Together, these studies demonstrate that social rank has important effects on glucocorticoids in social species, but that the patterns differ among taxa and may vary with the stability and type of dominance hierarchy. Even in closely related pairs of species, pronounced differences have been observed. For example, dominant dwarf mongooses largely monopolize reproduction (Creel & Waser 1991; Creel *et al.* 1992), although dominant individuals have higher mean glucocorticoid levels than subordinates. When subordinate females do breed, their offspring are often killed, but social interactions with the breeding subordinate do not detectably change. In contrast, glucocorticoid levels are higher in subordinate females of alpine marmots (*Marmota marmota*), which are frequently attacked by dominant females (Hackländer, Möstl & Arnold 2003) and slender meerkats (*Suricata surricata*), who are subject to violent eviction from their group if they become pregnant (Young *et al.* 2006). Sapolsky (2005) reviewed the conditions under which social stress is stronger for dominants or stronger for subordinates and suggested several social conditions or individual traits that promote differences in social status related glucocorticoid patterns (resource inequity, maintenance of dominance, breeding style, stability of social ranks, subordinate coping strategies, subordinate avoiding mechanisms of dominants, subordinates' use of alternative strategies, stress of dominating mating, atmosphere of culture and personality). While much has been learned, further work is needed to fully disentangle the relationships between glucocorticoid secretion, social status and the behavioural causes and consequences of rank in social vertebrates (Creel 2001; Goymann & Wingfield 2004; Sapolsky 2005; Rubenstein & Shen 2009).

Social status and social interactions can be described in many ways. Some studies treat social status as a dichotomy, pooling subordinates into one category and comparing them to the top-ranked (alpha) individual in each group. Other methods produce a rank-order, treating social status as an ordinal variable. Still other methods consider quantitative variation, treating rank as a continuous variable. Most studies to date have tested for a categorical difference in the glucocorticoid levels of alphas and subordinates, or for a linear relationship between glucocorticoid levels and rank. Empirical research is just beginning to evaluate the possibility that glucocorticoid secretion may relate to social rank in a more complex manner. A recent, long-term study of savannah baboons (*Papio cynocephalus*) illustrates this point well. Although the highest-ranking (alpha) males had elevated glucocorticoid levels, high-ranking males other than alphas tended to have lower glucocorticoid levels than low-ranking males (Gesquiere *et al.* 2011). Studies of other species have also reported that the glucocorticoid levels of alphas are elevated, with no detectable relationship between rank and glucocorticoids among non-alphas (e.g. in Yellowstone wolves; Sands & Creel 2004). Still other studies have found that the endocrine profiles of alphas and betas (second-ranked) animals are similar, with little relationship among lower ranks (for example in African wild dogs; Creel *et al.* 1997). Interestingly, the patterns that Gesquiere *et al.* (2011) observed for baboons were not affected by the stability or instability of the hierarchy, in contrast to the results of Sapolsky (1992a) for the same species.

LIVING IN SOCIAL GROUPS MAY REDUCE STRESS

An important, but often neglected, aspect in the discussion of the effect of the social environment on stress physiology is that the social environment may not only produce stress, but in fact it may reduce overall stress levels. For example, social support may buffer glucocorticoid levels in baboons (Alberts, Sapolsky & Altmann 1992; Ray & Sapolsky 1992; Virgin & Sapolsky 1997) and in greylag geese (*Anser anser*), the size of the family unit led to a decrease in the stress response during competitive feeding situations with other flock members (Scheiber *et al.* 2005; Scheiber, Kotrschal & Weiss 2009; see also a review on social buffering of the HPA-axis by Hennessy, Kaiser & Sachser 2009).

HOW SOCIAL STATUS IS ACQUIRED AND MAINTAINED MAY AFFECT THE RELATIONSHIP BETWEEN SOCIAL STATUS AND GLUCOCORTICOIDS

It is often assumed that success in aggressive interactions and the achievement of high dominance status are always a function of morphological and physiological characteristics. However, it has now become clear that social rank means different things in different species or even populations of the same species. Rank as a descriptor of social status does not specify the process by which social roles are obtained or maintained. Thus, behavioural strategies related to the acquisition and maintenance of rank, rather than the rank *per se*, are likely to determine the degree of social stress (and glucocorticoid levels) associated with a particular social status (von Holst 1998; Creel 2001; Goymann *et al.* 2001, 2003; Abbott *et al.* 2003; Goymann & Wingfield 2004; Sapolsky 2005).

In groups where dominance rank is inherited or dominance is achieved via queuing conventions social status is unlikely to have a major effect on glucocorticoid levels,

as long as there are no acute challenges (Goymann & Wingfield 2004; Sapolsky 2005). A similar scenario may occur in many 'high skew' cooperatively breeding species where a single dominant pair breeds and the other group members assist rearing the offspring (Vehrencamp 1984; Keller & Reeve 2004). In some species (e.g. many cooperatively breeding birds), adult subordinates are mainly offspring from prior years (e.g. Brown 1978). In other species, group structures are more complex and subordinates encompass a wide range of ages and degrees of genetic relatedness, including immigrants from other groups (e.g. in African wild dogs; Creel & Creel 2002). In cooperatively breeding species with high skew in reproductive success, reproductive suppression is experienced by most group members at some stage of their lives, and many individuals remain socially subordinate for their entire lives. Neuroendocrine mechanisms that operate mainly within the hypothalamic-pituitary-gondal axis are the prime mediators of reproductive suppression and glucocorticoid levels play only a minor role (e.g. Faulkes & Abbott 1997; Saltzman, Digby & Abbott 2009). Hence, similar to species with rank-inheritance or queuing conventions, rank-related patterns of glucocorticoid levels are unlikely to be the cause of reproductive suppression, and as long as there are no acute challenges, the correlation between rank and glucocorticoids could be positive, negative or neutral (Creel, Creel & Monfort 1996; Goymann & Wingfield 2004; Sapolsky 2005).

In contrast to stable social societies where rank is inherited or reproductive suppression is common, when there is conflict about reproduction in cooperative species, rank-related patterns of glucocorticoids may arise. For example, dominant pregnant female meerkats (*Suricata suricata*) aggressively target older and more distantly related subordinate females that represent a threat to their own reproduction. These subordinates show an impaired function of the hypothalamic pituitary gonadal axis, which may be caused by chronic elevation of glucocorticoid levels (Young *et al.* 2006). A similar situation occurs in many plural breeding species (where there are normally multiple breeding individuals within the group). Here, reproductive options exist for more than one breeding pair in a group and social dominance is acquired and maintained with low level or overt aggression, and subordinates are frequently threatened by dominants. Here, a social status related pattern of acute and chronic elevation of glucocorticoid levels is commonly observed in subordinates (Abbott *et al.* 2003; Goymann & Wingfield 2004; Sapolsky 2005). Such patterns are more likely to occur in societies with strong group cohesion as opposed to fission-fusion groups (Goymann & Wingfield 2004; Sapolsky 2005). In summary, current research suggests that it may not be the dominant or subordinate position itself, but rather the process by which social status is acquired and maintained that determines the physiological and psychosocial effects associated with a given social status (Sapolsky 1992b; Abbott *et al.* 2003; Goymann & Wingfield 2004).

INTERACTIONS BETWEEN SOCIAL AND ENVIRONMENTAL FACTORS

Social living is strongly influenced by ecological and environmental factors. In particular, resource distribution in time and/or space drives dispersal decisions, movement patterns and patterns of association in many taxa (Jarman 1974; Emlen & Oring 1977; Rubenstein & Lovette 2007; Jetz & Rubenstein 2010). These same ecological and environments factors also affect glucocorticoid levels in social species. In cooperatively breeding superb starlings (*Lamprotornis superbus*) (Fig. 1), annual differences in rainfall and resource availability not only influence glucocorticoid levels, but they may do so differently in dominant and subordinate individuals (Rubenstein 2007). However, these rank-related differences in glucocorticoid patterns may be more related to environmentally driven differences in levels of social conflict than to environmental factors directly (Rubenstein & Shen 2009). Similarly, in ring-tailed lemurs (*Lemur catta*), where social stressors and rank influenced glucocorticoid levels in captivity (Starling *et al.* 2010), socially-mediated glucocorticoid levels were also differentially impacted by food availability and environmental stress in the wild (Pride 2005). Although glucocorticoid levels are strongly influenced by social rank in savannah baboons (Gesquiere *et al.* 2011), environmental stressors also had strong effects on the patterns (Gesquiere *et al.* 2008). Thus, while social factors like rank and dominance interactions shape glucocorticoid levels in many species of birds and mammals, environmental factors like resource availability often interact with social factors to shape hormone profiles. One of the future goals in comparative studies of stress physiology in social species should be to disentangle the effects of social and environmental factors on hormone levels and social roles. So far, few studies have experimentally altered social (population density) or environmental factors (predator densities or food-abundance) in social species and investigated the consequences on demography, reproductive success and glucocorticoid secretion For example, food supplementation increased reproductive success in Florida scrub jays (*Aphelocoma coerulescens*). Breeding females supplemented with food started to reproduce earlier, laid larger clutches, and the survival of their offspring was increased (Schoech *et al.* 2008). Glucocorticoids possibly play a role in the mediation of this enhanced reproductive success, as jays supplemented with a high fat and high protein diet had lower glucocorticoid levels than controls and jays supplemented with a diet high in fats but low in proteins (Schoech, Bowman & Reynolds 2004; Schoech 2009). As a consequence, food supplementation may increase local population densities of this threatened species perhaps by lowering glucocorticoid levels and enhancing reproduction.

ALLOSTASIS, SOCIAL STATUS AND GLUCOCORTICOIDS

Separating the effects of social and ecological factors on glucocorticoid levels in free-living social animals may be difficult. The theoretical framework of allostasis has been proposed to help simultaneously study the effect of social and ecological factors on glucocorticoid levels in group-living species (Goymann & Wingfield 2004; Rubenstein & Shen 2009). Allostasis is a controversial theory that emerged from the biomedical literature (McEwen & Wingfield 2003) and has been applied more broadly to other biological disciplines, including studies of vertebrate social behaviour (Goymann & Wingfield 2004; Rubenstein 2007; Rubenstein & Shen 2009; Saltzman, Digby & Abbott 2009). Allostasis, which means achieving internal stability through bodily change of state, supports homeostasis by adjusting set-points to unpredictable stressors away from homeostatic baselines (Sterling & Eyer 1988). McEwen & Wingfield (2003) have used this allostasis framework to describe the physiological costs of coping with social and environmental challenges, and how physiological mediators such as glucocorticoids regulate responses (McEwen & Wingfield 2003). The allostasis framework has been extended by Romero, Dickens & Cyr (2009) in the reactive scope model to build from the allostasis model and address some of its criticisms. Together these models provide a starting place for exploring how predictable and unpredictable social and environmental stressors influence physiology and behaviour in social species.

Models of allostasis treat social interactions as a type of unpredictable social stressor (McEwen & Wingfield 2003; Goymann & Wingfield 2004; Romero, Dickens & Cyr 2009; Rubenstein & Shen 2009). Laboratory experiments have shown that unpredictable or uncontrollable stressors (such as electric shocks) provoke stronger glucocorticoid responses than the same stimuli when they are predictable or controllable (e.g. Weiss 1970). Although it is obvious that intense, short-term stressors like storms are unpredictable challenges that organisms cannot anticipate, many types of social interactions may also be relatively unpredictable. Animals that live in groups will experience affiliative social interactions daily (e.g. foraging, grooming, sleeping), and although these interactions may increase seasonally, they are still generally predictable events because animals can largely anticipate their occurrence. However, for social animals living within a dominance hierarchy that defines reproductive roles, changes in social status are often triggered by violent, escalated aggression. These escalated fights might be less predictable (although they are more likely under certain circumstances, such as the annual breeding period: Creel & Creel 2002; Young *et al.* 2006). In response to such interactions, an individual will rapidly shift its level of glucocorticoids to a new optimum in order to cope with the stressor. Thus, an acute social conflict might be analogous to a somewhat unpredictable environmental challenge (McEwen & Wingfield 2003; Goymann & Wingfield 2004; Romero, Dickens & Cyr 2009).

Allostasis models also specifically address the idea we described above that the physiological and psychosocial effects associated with a given social status are the result of the process by which social status is acquired and maintained rather than the resulting dominance rank *per se*. The process of rank acquisition is reflected in the level of allostatic load, or the cumulative physiological burden exacted on the body, as individuals adjust behaviour to aggressive social interactions (Goymann & Wingfield 2004). Allostatic load can be reflected in glucocorticoid levels (McEwen & Wingfield 2003; Goymann & Wingfield 2004; Rubenstein & Shen 2009). A comparative study by Goymann & Wingfield (2004) demonstrated that across a large sample of social bird and mammal species, the relative levels of glucocorticoids in dominants and subordinates are related to allostatic load and the way that rank is achieved and maintained in different societies. This work was extended theoretically and empirically by Rubenstein & Shen (2009) to predict the environmental conditions under which dominants or subordinates are expected to have higher allostatic load. This work also demonstrated that group structure (i.e. the ratio of breeders to non-breeders in a group) could also influence allostatic load and relative glucocorticoid levels. Together, these studies suggest that the allostatic load framework may help explain not only the different rank-related patterns seen in glucocorticoid patterns in vertebrates, but also the environmentally driven intra-specific variation in levels seen across years in long-term studies of individual species. The allostasis framework may also prove fruitful in studying the effects of factors on stress physiology in social species, such as the role of ontogeny and maternal effects during development.

SOCIAL ENVIRONMENT DURING ONTOGENY: PRENATAL AND POSTNATAL DEVELOPMENT

The effect that the social environment may have during the ontogeny of animals has received little attention in studies of free-living social species. The hormonal stress-axis of the mother and the offspring during early life may play a role in shaping the expression of phenotypic traits. Phenotypic plasticity, or the ability of an individual organism to alter its phenotype in response to changes in environmental conditions (Stearns 1989; Garland & Kelly 2006), is relatively understudied at both the functional and mechanistic levels in social species. In social species, individuals may adapt their phenotype to the social conditions encountered during ontogeny. A variety of laboratory studies have demonstrated that stressors acting on the mother during pregnancy or lactation (or during oogenesis in birds) may result in long-lasting effects on the offspring (reviewed in de Kloet *et al.* 2005; Catalani *et al.* 2011; Henriksen, Rettenbacher & Groothuis 2011). Whereas the mammalian literature generally takes a biomedical

perspective with the prevailing view that environmental stressors lead to pathologies, or deviations from the norm, the avian literature typically takes a more evolutionary perspective (Groothuis *et al.* 2005), with the prevailing view that environmental influences during early ontogeny (e.g. maternal effects) may help offspring to adapt to the current environment (Henriksen, Rettenbacher & Groothuis 2011). In captive animals, such as guinea pigs (Kemme, Kaiser & Sachser 2007, 2008), manipulations of the stability of social groups had no effect on HPA activity. Interestingly, however, male guinea pigs whose mothers lived in an unstable social environment during pregnancy and lactation had higher levels of testosterone later in life compared to males whose mothers lived in a stable social environment during this period. A study in spotted hyenas (Fig. 1) found that androgen metabolite levels are higher in dominant female spotted hyenas during pregnancy, which may affect offspring aggression (Dloniak, French & Holekamp 2006). However, the observed effect on aggressive behaviour was small, and potential effects on the HPA axis of the offspring were not investigated. A study of adoption in free-living spotted hyenas found that the influence of the social status of the surrogate mother on offspring behaviour was much stronger than the effects of the prenatal hormonal environment (East *et al.* 2009). In birds, development occurs in both the egg and during the nest stage (when direct hormonal effects from the mother are unlikely to occur). In cooperatively breeding Florida scrub-jays, parental provisioning rates directly influence baseline glucocorticoid levels in nestling (Rensel, Boughten & Schoech 2010b). The stress response in this species varies in different developmental stages (Rensel, Wilcoxen & Schoech 2010a) and is quite repeatable across different developmental periods (Rensel & Schoech 2011). Therefore, early life and developmental experiences in the nest may influence stress-physiology throughout adulthood.

Developmental effects on stress physiology in social species may not just be limited to circulating hormone levels. In studies of laboratory rats, variation in maternal care altered the expression of genes that regulate behavioural and hormonal stress responses, forming the basis of consistent individual differences in stress reactivity (Meaney *et al.* 1996; Meaney 2001). Similar programming may occur during adolescence. In guinea pigs, housing conditions from early adolescence through adulthood had profound effects on behaviour and hormone physiology of males: males that spent the adolescence period in mixed gender colonies showed a reduction in stress responsiveness compared to males that were housed with a single female during adolescence (Kaiser *et al.* 2007; Lürzel, Kaiser & Sachser 2010). It is unclear if these laboratory studies can be generalized to free-living animals, but it is possible that this kind of programming of the offspring stress responsiveness may be adaptive. For example, the social status of the mother, the stability of the social hierarchy she lives in, or the amount of alloparental care provided by helpers could influence offspring provisioning and/or care. This variation in offspring care may then transduce a social status related signal to the offspring, which could in turn, programme the offspring's reactivity to environmental stressors. Rank-related differences in maternal care that may potentially lead to such programming are known from wild populations (Pusey, Williams & Goodall 1987), and in wild baboons, persistent effects of female rank on offspring HPA activity have been demonstrated (Onyango *et al.* 2008). In summary, laboratory studies suggest that the social environment during embryonic development, in the nursing or nestling phase, and during adolescence may programme the hormonal stress response. Because it is currently unclear whether such programming may affect fitness or what consequences this programming may have on population demography, studies of free-living animals are needed to answer these questions.

Future directions

LINKING HPA FUNCTION AND FITNESS IN THE WILD

For both social and non-social species, much of our interest in relationships between social or environmental conditions and glucocorticoid concentrations is motivated by a desire to understand the physiological mechanisms by which the environment affects fitness. However, it is difficult to establish causal relationships between glucocorticoid levels and social behaviour or environmental conditions. Likewise, understanding the relationship between glucocorticoids and fitness is not straight-forward. Prolonged elevated baseline levels of glucocorticoids are commonly viewed to be detrimental, costly and decrease fitness (Sapolsky, Romero & Munck 2000). However, there is little consistent evidence for a negative association between elevated baseline glucocorticoids and fitness (Bonier *et al.* 2009a). Part of the variation among species in the glucocorticoid/fitness relationship could be attributed to a variety of demographic, social (e.g. social rank, age, group size) or environmental factors (e.g. resource availability). Additionally, seasonal patterns of glucocorticoid release could also obscure the relationship. For example, many of the studies of HPA-fitness relationships reviewed by Bonier *et al.* (2009a,b) were conducted in the breeding season. Although many species show elevated baseline levels of glucocorticoids during breeding (Romero 2002), this does not necessarily indicate that these breeders are chronically stressed. Rather, elevated levels of glucocorticoids during breeding may play a role in the regulation of enhanced metabolic needs during reproduction. During breeding, elevated baseline levels of glucocorticoids may actually be predictive for high reproductive success in some species (Bonier *et al.* 2009b; Ouyang *et al.* 2011). Just as the accumulation of studies across a range of species and conditions altered our understanding of relationship between HPA function and rank, we suggest that studying the relationship between HPA function and fitness across a range

of species and conditions will yield new insights. Studies along ecological or social gradients (e.g. variation in population density or variation in group size) will be of particular value.

HYPOTHALAMIC-PITUITARY-ADRENAL FUNCTION AT MULTIPLE LEVELS

In addition to looking at glucocorticoids in different life history stages, it may also be important to look at other components of the HPA-axis. Studies that incorporate a range of methods [baseline glucocorticoid measurements, glucocorticoid responses to acute stressors, adrenocorticotropic hormone (ACTH) challenge and dexamethasone suppression] will be of particular value. For example, baseline and maximal stress-induced glucocorticoid levels bind to different types of receptors (Romero 2004) and may have fundamentally different effects on behaviour. In cooperatively breeding, superb starlings, baseline, maximal levels and stress responses tended to show similar patterns in individuals of different social rank (Rubenstein 2007), but the same may not be true in all species. What may be most relevant to understanding the relationship between glucocorticoids and fitness in social species is the response to stress and the feedback regulation of glucocorticoid release (reviewed in Romero 2004), which has been demonstrated for baboons (Sapolsky & Altmann 1991; reviewed in Sapolsky, Romero & Munck 2000; Sapolsky 2002). Although dominant baboons typically expressed lower baseline glucocorticoid levels than subordinates, when faced with a stressor, glucocorticoid levels not only increase much faster in dominants than in subordinates, but dominants also terminated the release of glucocorticoids more quickly after cessation of the stressful event (Sapolsky & Altmann 1991; Sapolsky 1993). These differences between dominants and subordinates were neurally driven and could not have been elucidated simply with studies of circulating hormones levels. When injected with corticotropin-releasing factor (CRF), the hypothalamic peptide that induces release of ACTH from the anterior pituitary, dominants responded with a larger increase in ACTH than subordinates, which then led to a much faster increase in glucocorticoid production. Shutting down the adrenocortical stress response via negative feedback was more efficient in dominants. Sapolsky demonstrated this by administering dexamethasone, a synthetic glucocorticoid that artificially stimulates negative feedback of the HPA axis (Sapolsky & Altmann 1991; Sapolsky 1993). To our knowledge, these data are the only ones relating negative feedback regulation of the HPA-axis with fitness and social status in a free-living vertebrate. Data from marine iguanas (*Amblyrhynchus cristatus*) suggest that individuals with an effective negative feedback regulation were more likely to survive a severe environmental stressor than individuals with a less effective negative feedback regulation (Romero & Wikelski 2010). Thus, for baboons and marine iguanas, the effectiveness of negative feedback regulation of the HPA-axis can be a predictor of fitness. These studies demonstrate that field studies can examine not only circulating glucocorticoid levels, but also feedback mechanisms. Broad inferences will not be possible until more studies have been performed, because HPA sensitivity can vary with age, and despite their generally inhibitory effects on CRF, glucocorticoids can have locally positive feedbacks on CRF release (e.g. in the central amygdala, which is involved in social behaviour: Schulkin, Morgan & Rosen 2005).

Conclusion

In summary, the increase in the number of studies of glucocorticoids in free-living vertebrates in the past two decades has greatly influenced our understanding of stress physiology. However, many exciting avenues for future research exist. More complete descriptions of HPA function and a better understanding of the complex relationships between glucocorticoids and fitness will be fundamental. Determining these relationships will require long-term studies of organisms with known ages in different life history stages and demographic, social and environmental contexts, as well as comparisons among populations or experimental manipulations of the putative causal variables that affect HPA axis activity. Maternal and organizational effects on neuroendocrine and epigenetic components of stress physiology in free-living animals are also areas ripe for exploration. To achieve these and other goals, we must move beyond simply assaying plasma glucocorticoid or faecal glucocorticoid metabolite levels. Studies should simultaneously examine natural and experimentally induced stress responses and the associated negative feedbacks. Moreover, studies of glucocorticoid receptors and binding globulins are mostly lacking in field studies and would help to fully understand the complexities of stress physiology. Much of what we know about stress comes originally from the biomedical literature. For several decades, extending concepts from biomedical research to studies in the wild has been a productive way to learn how individuals respond to unpredictable social and environmental challenges, and how these responses influence fitness or population demography. While much has been learned, well-identified avenues for future research remain.

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Comparative effects of GnRH and porcine zona pellucida (PZP) immunocontraceptive vaccines for controlling reproduction in white-tailed deer (*Odocoileus virginianus*)

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Fawning rates and mating behaviour were compared between white-tailed deer (*Odocoileus virginianus*) treated with GnRH and porcine zona pellucida (PZP) immunocontraceptive vaccines from 1997 to 2000. Female deer from a herd of 102 deer at Seneca Army Depot, near Romulus, New York, were treated with prime and booster injections of PZP ($n = 22$) or GnRH vaccine ($n = 32$), or remained untreated as controls ($n = 34$). During the summers after booster treatment, observed fawning rates for adult female deer were similar for both PZP-treated (0.10–0.11 fawns per female) and GnRH-treated (0.13–0.22 fawns per female) female deer, and were significantly lower ($t = -8.93$ and $t = -9.73$; $P \leqslant 0.0005$, respectively) than those observed for control female deer (1.22–1.38 fawns per female). During the second (0.36 fawns per female) and third summers (0.61 fawns per female) after the last booster injection, GnRH-treated female deer still produced significantly fewer fawns than did the controls (1.38 and 1.31 fawns per female, respectively). In one breeding season after treatment, five of 18 (28%) females vaccinated with PZP produced fawns, similar to the rate for GnRH-treated females (29%). In addition, females treated with GnRH had fewer oestrous cycles per female (0.06, $P \leqslant 0.05$) than did either control (0.22 cycles per female) or PZP-treated deer (0.36 cycles per female). Initial PZP treatment followed by a booster dose 5–7 months later reduced fawn production and prolonged the breeding season as females repeatedly returned to oestrus, similar to results reported in other studies.

Introduction

White-tailed deer (*Odocoileus virginianus*) densities have reached unprecedented levels in some suburban communities (Curtis and Richmond, 1992) and deer populations in North

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America are still increasing. Safety concerns and ethical issues have rendered deer population regulation via managed recreational hunting impractical in many residential areas, and have increased interest in the use of non-lethal methods, such as immunocontraceptive vaccines for controlling birth rates in deer (Warren *et al.*, 1995; Miller, 1996; Curtis *et al.*, 1997; Cowan *et al.*, in press).

Fertility control as a tool in wildlife management has been the subject of research for many years (Harder and Peterle, 1974; Kennelly and Converse, 1997) with variable results (Bomford, 1990; Turner *et al.*, 1997; Warren *et al.*, 1997). Porcine zona pellucida (PZP) has been the most commonly used immunocontraceptive antigen for fertility control in female mammals (Sacco, 1987) and has been administered experimentally to > 70 species of wild mammals, including several captive ungulates (Kirkpatrick *et al.*, 1997; Miller *et al.*, 2000a). The continuing expansion of humans into deer habitat and vice versa has resulted in much recent research with PZP vaccines being focused on widespread and growing concerns with deer in suburban settings (Turner *et al.*, 1992, 1996; McShea *et al.*, 1997; Underwood and Verret, 1998).

A second immunocontraceptive antigen, GnRH, has been shown to have potential for fertility control in domestic animals (Jeffcoate *et al.*, 1978, 1982; Adams and Adams, 1992; Brown *et al.*, 1994; Meloen *et al.*, 1994), companion animals (Ladd *et al.*, 1994), and a variety of wildlife species including Norway rats (*Rattus norvegicus*, Miller *et al.*, 1997), African elephants (*Loxodonta africana*, Brown *et al.*, 1993), rainbow trout (*Oncorhynchus mykiss*, Riley and Secombes, 1993) and the marmoset monkey (*Callithrix jacchus*, Hodges and Hearn, 1977). GnRH has been evaluated for efficacy in captive white-tailed deer (Killian, 1998; Miller *et al.*, 2000b); however, little research has been conducted with GnRH vaccines in free-ranging white-tailed deer.

The issue of remotely delivering an immunocontraceptive antigen and following that event with timely delivery of booster doses to the same free-ranging animal is an obvious challenge (Rudolph *et al.*, 2000). PZP booster injections were delivered by dart rifle during early autumn 1997 before the breeding season, 5–7 months after the initial injection. We hypothesized that a similar reduction in fawning rates would be observed for PZP-treated deer and GnRH-treated females that received both prime and booster doses a few weeks apart in autumn 1996.

The aims of the present study were to compare the efficacy of PZP and GnRH vaccines for reducing fawn production in the same herd of white-tailed deer. Fawning rates and breeding behaviour of PZP- and GnRH-treated female deer were compared between treatments and also with untreated control deer. A direct comparison of these vaccines under similar herd and field conditions provides an optimal setting for determining whether either or both of these immunocontraceptive antigens have potential for further development as a tool for wildlife fertility control.

Materials and Methods

The study was conducted in the quarantine area (QA) of the 4000 ha Seneca Army Depot (SAD) located near Romulus, New York. The 263 ha QA is enclosed by three 2.4 m parallel, security fences and consists of 5% paved roads, railroads and buildings, 17% woodland and 78% open grasslands. Natural forage was abundant, and was further supplemented with apples, apple mash, corn, alfalfa hay and salt to ensure that deer were in good condition during the breeding season and winter months. These food items were strategically placed to serve also as bait sites that were used for darting of deer and behavioural observations.

Experimental animals included 61 adult (≥ 2.5 years) and 27 yearling (1.5 years) female deer, and eight adult and five yearling males. Deer were captured with rocket nets (Hawkins *et al.*, 1968), dart guns (Pneu Dart, Inc., Williamsport, PA) and Clover traps (Clover, 1954) from

Table 1. Immunocontraceptive treatment schedule for female white-tailed deer vaccinated with porcine zona pellucida (PZP) or GnRH antigens at Seneca Army Depot, Romulus, New York from 1996 to 1998

Experimental group	Sample size	Prime dose	First booster	Second booster
PZP	22	6 March – 1 May, 1997[a]	26 August – 28 October, 1997	3 September – 30 October, 1998
GnRH	32	31 August – 9 October, 1996	20 September – 9 November, 1996	10 September – 17 October, 1997

[a]The prime dose of PZP vaccine was injected by hand into female deer at the time of capture and marking. All other vaccinations were delivered remotely by dart-syringe and rifle.

surrounding SAD property during winter and early spring in 1996 and 1997. Deer captured in rocket nets or Clover traps were restrained under the net and immobilized with 2.2 mg xylazine hydrochloride kg^{-1} (Rompun; Miles Laboratories, Shawnee Mission, KS) administered i.m. The antagonist yohimbine hydrochloride (Yobine; Lloyd Laboratories, Shenandoah, IA) was administered i.v. at 0.11 mg kg^{-1} upon release of the deer (Mech *et al.*, 1985). For immobilization with a dart rifle, disposable 2 cc darts with 1.9 cm needles and gelatin collars were used. These syringe darts injected either pure xylazine hydrochloride, or a mixture of xylazine hydrochloride tiletamine hydrochloride and zolazepam hydrochloride (Telazol; Fort Dodge Labs, Fort Dodge, IA) at 2.2 mg kg^{-1}. No antagonist was administered at release when the Telazol mixture was used for deer immobilization. Each deer was fitted with a numbered collar for individual identification before release into the research enclosure.

For the field study, female deer were assigned to GnRH (1996–2000), PZP (1997–2000) or control (1996–2000) groups (Table 1). All vaccine treatments were administered in the hip region either by injection by hand at capture (PZP) or by remote delivery later with self-injecting 1 cc darts (Pneu Dart Inc., Williamsport, PA). In this investigation, a prime dose of PZP vaccine was administered during winter 1997 when deer were food-stressed and more easily lured to bait sites, thereby increasing darting efficiency. Deer were darted from canvas shelters at baited sites (< 35 m) near dawn and dusk, or from a vehicle at night with the aid of a spotlight at distances of 14–45 m along roadsides. All treatments followed positive identification of individual deer.

GnRH experiments

The immunocontraceptive vaccines used in the present study were prepared by L. A. Miller (US Department of Agriculture-National Wildlife Research Center, Fort Collins, CO). The 10 amino acid GnRH was made immunogenic by coupling it to the carrier keyhole limpet haemocyanin (KLH, Miller *et al.*, 2000b). A glycine was added at the C-terminus of GnRH as a spacer, and a cysteine was added to provide a coupling agent to maleimide on KLH. Maleimide-activated KLH was purchased from Pierce Chemical Co. (Rockford, IL) and the C-terminal Cys-GnRH was coupled to the activated KLH following the manufacturer's instructions. Both KLH-maleimide and the peptide were lyophilized and rehydrated in a 1:1 (w:w) ratio for coupling. The 1 cc prime dose of GnRH vaccine contained 65 µg KLH–GnRH in saline mixed with Freund's complete adjuvant (FCA) at a 1:1 ratio (Miller, 1997). The 1 cc booster dose contained 65 µg KLH–GnRH mixed with Freund's incomplete adjuvant (FIA) in a 1:1 ratio. The prime dose (GnRH + FCA) was remotely injected into 32 female deer and the first booster shot (GnRH + FIA) was administered 3–4 weeks later, before the breeding season

during September and October 1996. A second booster dose (GnRH + FIA) was delivered by remote injection during September 1997. No GnRH booster treatment was provided in September 1998 or 1999; however, fawning rates were again monitored in the summer of 1999 and 2000 to determine any residual effects of the GnRH vaccine in female deer at 1 and 2 years after treatment.

PZP experiments

Female deer ($n = 22$) were immunized with purified PZP, which was prepared by and purchased from B. S. Dunbar (Baylor College of Medicine, Houston, TX; Skinner *et al.*, 1994). The 1 cc prime dose of PZP vaccine consisted of 0.5 cc saline containing 65 μg PZP mixed with 0.5 cc FCA. Similarly, a 1 cc booster dose contained 65 μg PZP in 0.5 cc saline mixed with 0.5 cc FIA at a 1:1 ratio. The prime dose (PZP + FCA) injection was administered by hand at the time of capture during late winter 1997 (February–April), as deer were readily caught at bait sites during the winter. The effectiveness of a prolonged period between administration of the prime and the initial booster dose was determined by administering the initial booster (PZP + FIA) by remote injection 5–7 months later in September 1997. A second booster treatment (PZP + FIA) was delivered by dart-rifle in September and October 1998. These deer were monitored for mating and subsequent fawning activities from autumn 1997 to summer 2000.

Fawn observations

Fawn searches were conducted in the 263 ha enclosure each day from May to September 1997–2000. Maternity was determined on the basis of repeated observations of fawns following females and by females displaying maternal behaviour toward fawns. A minimum of three doe–fawn interactions was used to determine parentage. Parturition dates were initially based on fawn motor skills and size at first observation relative to fawns of known age (McShea *et al.*, 1997). In September, October and December of each year, fawns were captured and removed from the research enclosure. These fawns were classified by age on the basis of incisor development (Severinghaus, 1949). Adjustments to initial estimated parturition dates were made on the basis of incisor development.

From 1996 to 1998, observations of deer breeding activity were conducted each day at dawn and dusk from mid-October until all males had shed their antlers (early March 1997 and late March 1998). A female was considered to be in oestrus when she was observed being tended (Hirth, 1977) or mounted by a male. The breeding season was divided into five, 1 month periods beginning with the date on which a male was first observed tending a female. If the oestrous cycle overlapped two periods, the doe was placed only in the earlier period.

For analysis of parturition and dates of observed oestrus, the Wilcoxon rank sum test (Z) or Kruskal–Wallis test (chi-squared) for multiple groups were used. All percentages were subjected to angular (arcsine) transformation (Steele and Torrie, 1960) before ANOVA.

Results

Fawn production

Only four of 32 (13%) and four of 30 (13%) GnRH-treated female deer produced fawns in 1997 and 1998, respectively (Table 2). None of nine GnRH-treated yearlings, and only four of 23 (17%) GnRH-treated adults, produced fawns in 1997. The apparent value of providing a booster treatment is shown by comparing fawn production in boosted, sexually mature adults with those deer that did not receive the booster. During the fawning seasons of 1997 and 1998

Table 2. Fawn production for female white-tailed deer treated with porcine zona pellucida (PZP) or GnRH immunocontraceptive vaccines, and for control females at Seneca Army Depot, Romulus, New York from 1997 to 2000

Experimental group	Year	Sample size (yearlings:adults)		Percentage producing fawns (yearlings:adults)		Total fawns produced (yearlings:adults)		Avearge number of fawns per female (yearlings:adults)	
PZP	1998	3	19	33	11	1	2	0.33	0.11
	1999	–	20	–	10	–	2	–	0.10
	2000[a]	–	18	–	28	–	5	–	0.28
GnRH	1997	9	23	0	13	0	5	0.00	0.22
	1998	–	30	–	13	–	4	–	0.13
	1999[b]	–	28	–	29	–	10	–	0.36
	2000[b]	–	28	–	57	–	17	–	0.61
Control	1997	15	19	53	79	12	26	0.80	1.37
	1998	–	27	–	81	–	33	–	1.22
	1999	–	16	–	88	–	22	–	1.38
	2000	–	13	–	85	–	17	–	1.31

[a]PZP-treated female deer that received their last booster injection in autumn 1998, so that fawning rates for summer 2000 occur > 20 months after treatment.

[b]GnRH-treated female deer that received their last booster injection in autumn 1997, so that fawning rates in summer 1999 occur > 20 months after treatment, and those in summer 2000 occur > 32 months after the last booster injection.

after booster treatments during the previous autumn, only eight of 62 females (13%) treated with GnRH produced fawns and only one of the eight females produced twins. During summer 1999, after one breeding season without a booster, eight of 28 (29%) adult females previously treated with GnRH produced fawns and of these, two does produced twins, which is the common litter size for adult females in good health (Verme, 1969). In the second breeding season after treatment, 16 of 28 females (57%) bore fawns. During these same four reproductive seasons, 110 fawns were produced by control does (n = 38 in 1997; 33 in 1998; 22 in 1999; and 17 in 2000; Table 2).

For females treated with PZP, three of 22 (14%) and two of 20 (10%) produced offspring during 1998 and 1999, respectively. One of three (33%) PZP yearlings and two of 19 (11%) PZP adults produced fawns in the summer of 1998. During the reproductive seasons immediately after booster treatments the previous autumn, none of the PZP-treated females that produced fawns had twins. One breeding season after treatment, five of 18 females (28%) bore young, which was similar to the rate for GnRH-treated females (29%).

During summers immediately after autumn booster treatments, fawn production for both GnRH- and PZP-treated females was significantly lower than that of control females ($t = -8.93$; $P < 0.0005$ and $t = -9.73$; $P < 0.0005$). In addition, in the second reproductive season after booster injections, GnRH- and PZP-treated females still produced significantly fewer ($P < 0.0005$) fawns than did the controls.

There was no difference in parturition dates between 1997, 1998 and 1999 (chi-squared = 1.58; 2 df; $P > 0.1$; Table 3) among control females, as most fawns were born before 1 July. Fawning dates for the GnRH- and PZP-treated females were later in 1998 than for the 1997 GnRH group (chi-squared = 8.34; 2 df; $P \leq 0.03$) and later than the control groups in 1997 and 1998 (chi-squared = 3.87; 1 df; $P \leq 0.05$). There was no difference in parturition dates between any of the three groups in 1999 (chi-squared = 0.00; 2 df; $P > 0.995$).

Table 3. Parturition dates for female white-tailed deer treated with porcine zona pellucida (PZP) and GnRH immunocontraceptive vaccines, and for control females, at Seneca Army Depot, Romulus, New York from summer 1997 to 1999

	Parturition dates				
Experimental group	Before 15 June	15 June – 1 July	1 – 15 July	15 July – 1 Aug	1 – 15 Aug
PZP					
1998	1	1	1	0	0
1999	1	0	1	0	0
GnRH					
1997	4	0	0	0	0
1998	0	1	1	1	1
1999[a]	5	2	1	0	0
Control					
1997	10	10	3	0	0
1998	13	7	3	0	0
1999	9	5	0	0	0

[a]GnRH-treated female deer that received their last booster injection during autumn 1998.

Table 4. Observed dates for the resumption of oestrous cyclicity in female white-tailed deer treated with porcine zona pellucida (PZP) and GnRH immunocontraceptive vaccines, and for control females, at Seneca Army Depot, Romulus, New York during 1997 and 1998

		Occurrence of oestrus				
Experimental group	Year	November	December	January	February	March
PZP	1997	2	2	3	1	0
	1998	4	10	3	2	1
GnRH	1997	0	1	1	0	0
	1998[a]	4	1	0	0	0
Control	1997	2	3	1	0	0
	1998	4	0	0	0	0

[a]GnRH-treated female deer that received their last booster treatments during autumn 1997.

Mating activity

There was no difference in observed timing of oestrus for the control females between 1997 and 1998, or for the PZP-treated females between 1997 and 1998 ($Z = 1.49$, $P = 0.136$ and $Z = 0.406$, $P = 0.682$, respectively; Table 4). However, females treated with GnRH for a second year cycled later than females in their first year of treatment with GnRH ($Z = 1.93$; $P = 0.05$). Observed oestrous cyclicity for PZP-treated females was significantly later than that for control females ($Z = 2.62$; $P = 0.009$) and marginally later than that for GnRH-treated females ($Z = 1.78$; $P = 0.075$). In 1997, females treated with PZP showed significantly more mating activity (0.36 oestrous cycles per doe) than those treated with GnRH (0.06 oestrous cycles per doe) and control females (0.22 oestrous cycles per doe).

Discussion

The results from this study demonstrate successful inhibition of fertility in semi-free ranging white-tailed deer with a remotely delivered GnRH vaccine. The observed fawning rates (0.13–0.22 fawns per female) in the present study were similar to those reported by Miller *et al.* (2000b) for hand-injected, captive deer in Pennsylvania (0.21 fawns per female) with an 88% reduction in fawn births as compared with control females.

PZP treatments with 5–7 months between prime and booster injections markedly reduced fawn production, and reduced the incidence of twin births for those females that produced fawns. PZP treatment also prolonged the breeding season due to an increased number of oestrous cycles in each doe; this finding is similar to results reported in studies with 2–4 weeks between prime and booster injections (Turner *et al.*, 1992, 1996; McShea *et al.*, 1997; Killian, 1998). Miller *et al.* (2000a) also noted that the best immune response to PZP vaccination often occurs in the second year after original antigen exposure and that infertility may be retained for 1–4 years in some females without additional booster treatments.

GnRH and PZP vaccines reduced fawn production in white-tailed deer to a comparable degree. However, the GnRH treatment was apparently more effective for yearling females, and more adult females responded to PZP treatment. Three of the four GnRH-treated adult females that did not respond to vaccine injection and produced fawns in 1997 also produced fawns in 1998, and all four females again produced fawns in 1999. Apparently, either a small proportion of the adult female population did not respond to the GnRH vaccine (Bomford, 1990; Becker and Katz, 1997) or the first, second or both remotely delivered injection failed to deliver a complete dose.

Suppression of reproductive hormones with GnRH may be more effective in yearlings (deer classified as 1.5 years old), which generally ovulate less frequently than do adults (Hesselton and Jackson, 1974), and do not reach full reproductive potential until adulthood (deer classified as 2.5 years or older; Verme and Ullrey, 1984). None of the nine females immunized with GnRH as yearlings produced fawns during their first or second year as adults. Adams and Adams (1992) and Brown *et al.* (1994) reported that immunization of domestic stock against GnRH very early in life (prepubertal) may be more efficacious and longer lasting than immunization later in life. Warren and White (1995) suggested that treating prepubertal fawns may increase the efficiency of a contraceptive management programme.

Contraceptives delivered orally will probably be necessary for practical wildlife management in the future (Warren *et al.*, 1995; Miller, 1996; Curtis *et al.*, 1997; Linhart *et al.*, 1997; Miller *et al.*, 2000a), and, thus, may be administered to deer of all ages via bait consumption. Therefore, it is important for future investigators to examine the potential impacts of immunization on all age classes (fawns, yearlings, adults) of white-tailed deer. However, the risk of unintentional treatment of non-target wildlife species is much greater with oral delivery than for injection or implants (Guynn, 1997). Species specificity could be achieved either by genetically engineering the antigen, or by designing a bait station to exclude non-target animals. Much work is still required to develop safe and effective delivery systems for contraceptive drugs or vaccines (Cowan *et al.*, in press).

Parturition dates for PZP-treated females were not substantially delayed, as all three births occurred before early July. The few deer that did not develop a sufficient antibody response to inhibit reproduction must have conceived on their first or second oestrous cycle. Jackson and Hesselton (1973) reported that 95% of females (including fertile fawns) in New York gave birth before 19 July, which is similar to the results obtained for the control females.

Variation in the response to GnRH among adult females or incomplete injections may also explain the delayed breeding in 1997 and resulting delayed parturition in 1998 shown by

GnRH-treated females. Only two oestrous cycles were observed for GnRH-treated females in 1997; however, the first was not observed until December. Cyclicity in deer in all other experimental groups was first observed in November. If GnRH injections administered in autumn 1996 were incomplete in the four non-responding females and insufficient to block reproduction (all four females fawned in early June), then administration of the third injection to these females in autumn 1997 may have acted as a single-injection treatment. If this was the case, only a short-lived antibody response would be produced and, thus, there would be only a delay in the onset of oestrus and subsequent fawning. Miller *et al.* (2000b) noted variations in antibody titre and immune response for captive female deer treated with GnRH vaccines. These observations were consistent with past immunocontraceptive studies and are thought to be due to genetic differences among individual animals. Feral horses treated with GnRH showed delayed foaling (Goodloe, 1991), and captive red deer stags showed a delayed rut (Atja *et al.*, 1992; Freudenberger *et al.*, 1993). McShea *et al.* (1997) also noted delayed fawning for PZP-treated females that received only a single injection.

Females treated with GnRH for a second year and PZP-treated females breeding late in the season showed reduced rates of twin births. This finding may have resulted from decreased sperm production often experienced by males during late winter (Mirarchi *et al.*, 1977; Knox *et al.*, 1988), which may be associated in part with loss of body weight (Gittleman and Thompson, 1988). GnRH can also function as a contragestation agent depending on timing of the vaccine delivery and immune response of the individual deer (Miller *et al.*, 2000b). Therefore, GnRH could have inhibited implantation or even the maintenance of pregnancy in some female deer resulting in fetus resorption and, thus, smaller litter sizes.

GnRH-treated females that did not receive a booster treatment in autumn 1998 showed an earlier onset of oestrus than did GnRH-treated females that received a booster injection immediately before the breeding season. Similarly, 14 of 20 (70%) of the oestrous cycles observed for second year PZP-treated females occurred before the end of December compared with only four of eight (50%) for the first year PZP-treated does. This finding may be attributable to non-parous and non-lactating does entering the breeding season in better body condition and, therefore, showing an earlier onset of breeding activity (Verme and Ullrey, 1984; McShea *et al.*, 1997).

Only eight of 28 (29%) GnRH-treated female deer bore fawns in 1999, indicating that GnRH continued to inhibit reproduction in 71% of females 1 year after treatment. If the four non-responsive GnRH-treated females that consistently produced fawns during the study are excluded, then only four of 24 (17%) of females produced young in the second fawning season after booster treatment. Miller *et al.* (2000b) noted that infertility was directly related to anti-GnRH serum antibody titres above 64 000 with infertility lasting 2 or more years without booster treatment. However, studies in domestic stock revealed that short-term anti-GnRH immunization was reversible (Keeling and Crighton, 1984), and thus unlikely to be accompanied by a prolonged period of reduced fertility after treatment.

Turner *et al.* (1996) reported that PZP-mediated infertility for white-tailed deer was reversible within 2 years for 75% of treated females. In contrast, Miller *et al.* (2000a) observed that PZP produced variable infertility for up to 4 years. Overall, birth rates were reduced by 76% during the 6 year study, and there was an 89% reduction in fawn production during the first 2 years of active immunization. For this captive herd in Pennsylvania, immune responses among deer were variable, especially in the first year of treatment (Miller *et al.*, 2000a).

The obvious impact of a residual effect and the ultimate reversibility of contraception are of considerable importance to population modelling efforts (Barlow, 2000; Hobbs *et al.*, 2000). A predictable natality rate is a cornerstone for accuracy in building and understanding wildlife population models (Courchamp and Cornell, 2000; Hobbs *et al.*, 2000; Cowan *et al.*, in

press). Although Nettles (1997), Kirkpatrick *et al.* (1997) and other workers have alluded to this issue, additional studies of the long-term effects of both PZP and GnRH vaccines, as well as other candidate agents, are needed. In addition, recent modelling of virus vectors and bait delivery for immunocontraceptive agents highlighted the importance of delivery systems to the overall success for field application of fertility control programmes (Courchamp and Cornell, 2000; Cowan *et al.*, in press).

In summary, both PZP and GnRH vaccines were effective for inhibiting reproduction in white-tailed deer when administered as described here. In addition, the GnRH vaccine was responsible for a reduced fawning rate for female deer 1 and 2 years after treatment. Hobbs *et al.* (2000) showed that fertility control with long-lived or irreversible agents may be more efficient than culling for regulation of ungulate populations. However, longer term studies and pathological examination of deer treated with immunocontraceptive vaccines may be required to reveal potential detrimental health effects resulting from interference with the reproductive system of white-tailed deer (Seal, 1991; Muller *et al.*, 1997).

Our experience and data, plus recent reports, indicate that implementation of an immunocontraceptive programme using current protocols, even in a semi-free ranging but enclosed deer herd, would be expensive and perhaps impractical due to high equipment and labour costs (Rudolph *et al.*, 2000; Pooler *et al.*, in press). The need for a single dose, oral contraceptive agent for free-ranging animals has been highlighted in a number of studies (Warren *et al.*, 1995; Miller, 1996; Bradley *et al.*, 1997; Curtis *et al.*, 1997; Linhart *et al.*, 1997). Further research to develop such a single-dose, fertility control agent that can be delivered orally and at lower cost is warranted.

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Physiological effects of gonadotropin-releasing hormone immunocontraception on white-tailed deer

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Abstract: Before immunocontraceptives can be considered safe to use on wildlife species, potential health risks should be assessed. Gonadotropin-releasing hormone (GnRH) immunocontraceptive has successfully reduced fertility of white-tailed deer (*Odocoileus virginianus*); however, associated deer physiology has rarely been examined. We conducted gross necropsy examinations, histology, and blood chemistry comparisons on euthanized deer previously vaccinated with immunogenic GnRH (n = 18 females and n = 4 males), or left as untreated controls (n = 7 females and n = 6 males). Granulomas were found at injection sites of most deer, even 3 years post-treatment. There were no significant differences in ovary ($F_{2,22}$ = 0.31, P = 0.73), or pituitary weights ($F_{2,22}$ = 0.30, P = 0.75) between treatment groups. Ovaries from control females had significantly more secondary follicles ($F_{2,21}$ = 20.56, $P \leq$ 0.001), but not Graafian follicles ($F_{2,22}$ = 2.22, P = 0.13). Immunized males had significantly lower mean testes weights, a number of morphologic abnormalities, and varying degrees of aspermatogenesis with fewer mature spermatozoa. We do not recommend treating male deer with anti-GnRH immunocontraceptive vaccines.

Key words: deer–vehicle collision, GnRH, gonadotropin-releasing hormone, human–wildlife conflict, immuno-contraception, *Odocoileus virginianus*, reproduction, white-tailed deer

POPULATION DENSITIES of white-tailed deer (*Odocoileus virginianus*) in many suburban communities are reaching unprecedented levels (Curtis and Richmond 1992, DeNicola et al. 2008) and continue to escalate throughout North America. In suburban settings, where deer densities are often highest, safety concerns and ethical issues have rendered population regulation via recreational hunting impractical (DeNicola and Williams 2008). Higher deer densities have caused increases in deer–vehicle collisions (DVCs; Hussain et al. 2007, Mastro et al. 2008), and there is no easy way to reduce the frequency of these collisions (Mastro et al. 2008, Ng et al. 2008). In response, efforts to control growth of suburban deer herds with nonlethal methods, such as immunocontraception, have intensified (Warren et al. 1995, Curtis et al. 1997, Cowan et al. 2003, Miller et al. 2008, Rutberg and Naugle 2008). However, recent modeling efforts have shown that it will be very difficult to attain sufficient numbers of treated deer to have measurable reductions in open populations experiencing emigration and immigration (Merrill et al. 2006). Furthermore, community residents' attitudes towards fertility control as a deer management option are not fixed, and after stakeholders received additional information concerning the effectiveness or humaneness of contraception, their perceived acceptance of this technique often declined (Lauber and Knuth 2004). Consequently, it is imperative that communities and wildlife management agencies study all aspects of immunocontraception so that informed decisions can be made about applying these technologies for suburban wildlife management.

Fertility control as a tool in wildlife management has been investigated for many years (Harder and Peterle 1974, Kennelly and Converse 1997) with variable results (Bomford 1990, Turner et al. 1997, Warren et al. 1997).

However, success with effective fertility control of individual deer has been achieved by vaccinating them with the immunocontraceptive antigens gonadotropin-releasing hormone (GnRH; Killian 1998, Miller et al. 2000*a*, Curtis et al. 2002, Killian et al. 2006) or porcine zona pellucida (PZP; Kirkpatrick et al. 1997, McShea et al. 1997, Miller et al. 2000*b*, Rutberg et al. 2004). While many researchers have documented successful fertility suppression, few investigators have considered the effects of immunocontraceptive vaccine treatment on deer physiology or histopathology (McShea et al. 1997, Killian et al. 2006). Kirkpatrick and Rutberg (2001) even stated that, "the absence of significant health side effects" is an important characteristic of any contraceptive vaccine. Hence, in this study, we conducted detailed necropsies on anti-GnRH-immunized deer to document potential pathological impacts.

Materials and methods

In accordance with an amendment approved by the Cornell University Institutional Animal Care and Use Committee (Protocol Number 96-10-99), postmortem examinations were conducted on a sample of euthanized, free-ranging, white-tailed deer (n = 18 females and 4 males) that had been injected with an anti-GnRH vaccine or left as untreated controls (n = 7 females and 6 males) during 1996–2000. The deer were included in a fertility control study contained within a 263-ha fenced, natural area at Seneca Army Depot near Romulus, New York (Curtis et al. 2002).

The immunocontraceptive vaccines used for the study were prepared by L. A. Miller (USDA/APHIS/Wildlife Services' National Wild-life Research Center). A 10-amino acid GnRH complex was made immunogenic by coupling it to the carrier keyhole limpet hemocyanin (KLH; Miller et al. 2000*a*). A glycine residue was added at the C-terminus of GnRH as a spacer, and cysteine residue was added to provide a coupling agent to maleimide on KLH. Maleimide-activated KLH was purchased from Pierce Chemical Company (Rockford, Ill.) and the C-terminal Cys-GnRH was coupled to the activated KLH following the manufacturer's instructions. Both KLH-maleimide and the peptide were lyophilized and rehydrated in a 1:1 (weight:weight) ratio for coupling. Each 1-ml dose of anti-GnRH vaccine contained 0.5 mg of KLH-GnRH in 0.5 ml of phosphate-buffered saline (PBS) solution emulsified with 0.5 ml of Complete Freund's Adjuvant (CFA) or Incomplete Freund's Adjuvant (IFA).

Treated deer had received a primary vaccination (KLH-GnRH + CFA) in the hip region with remotely delivered, self-injecting, 1-ml darts (Pneu Dart Inc., Williamsport, Pa.). We administered the first booster shot (KLH-GnRH + IFA) 3 to 4 weeks later during September and October 1996 prior to the breeding season. A second booster dose (KLH-GnRH + IFA) was delivered by remote injection during September 1997. No other booster treatments were given until 7 female deer were revaccinated in 2000 (KLH-GnRH + IFA) to compare the effects of recent vaccination to deer previously treated 3 years earlier. No males were revaccinated during 2000.

With a permit from the New York State Department of Environmental Conservation (NYSDEC), we administered euthanasia of deer for necropsy in October 2000 using a single, lethal shot from a high-powered rifle fired either out of a blind or from a vehicle (Beaver et al. 2001). We collected blood samples via heart puncture immediately at the time of death, and we stored them in vials on ice for transport. Deer (n = 35) were quickly transported (usually within 1–2 hours after collection) to the Cornell University College of Veterinary Medicine (CUCVM), Necropsy Lab, and placed in a cooler at 4.4°C. Most necropsies were conducted the day the deer were collected.

Blood chemistry analysis was performed by the CUCVM, Animal Health Diagnostic Center, on all of the collected deer. Anti-GnRH titer (1/titer x 1000) levels were determined via an enzyme-linked immunosorbent assay (ELISA), while progesterone and testosterone were determined by radioimmunoassay (RIA; Miller et al. 2000*a*). These values were then compared to the deer's histology and to its ovarian, testes, and pituitary weights.

Gross examinations included an accurate assessment of deer's age by tooth wear (Severinghaus 1949), evaluation of bone marrow fat (Cheatum 1949), examination of injection sites, and documentation of any abnormalities.



Figure 1. Buck treated with GnRH immunocontraceptive vaccine, Romulus, New York, USA. Note malformed antlers covered in velvet during the rut.

Ovaries, testes, pituitary gland, internal iliac lymph node, popliteal lymph node, and thyroid glands were stripped of fat and connective tissue then weighed immediately (to the nearest mg). Morphologic analysis was performed on tissues fixed in formalin, embedded in paraffin, sectioned to 7 microns, and stained with hemotoxylin and eosin, Masson's Trichrome, or acid-fast stains. Each ovary, testis, and pituitary gland was halved before fixation in formalin, and each half was embedded in paraffin. Multiple sections (but not step sections) were made of each surface to be examined with the previously mentioned stains, and if a suspected lesion was observed grossly, a section was also made through the lesion.

The total number of secondary and Graafian follicles were enumerated from 2 cross-sectional slices from each ovary from each deer. Any normal-appearing follicle with a corona radiata (a single layer of columnar cells anchoring the oocyte) or total follicular size >0.5 cm was classified as a Graafian follicle. Follicles with greater than 10% of their granulose cells (small cells that form the wall of an ovarian follicle) exhibiting cell death (apoptosis) were classified as atretic ovarian follicles (degenerated prior to maturity). Apoptosis was characterized as granulose cells with dark, small consolidated (hyperchromatic) nuclei.

A single layer of cuboidal epithelium (surface) lined several large fluid filled follicles. Because none of these follicles exceeded 1 cm in diameter, they were classified as atretic Graafian follicles; however, they could have been small follicular cysts. Also, some larger Graafian follicles with diminished numbers of granulosa cells may have also been early follicular cysts. However, we found no advanced cyst to confirm this.

Results

Gross necropsy observations

Blood chemistry. There was no significant difference in any of 28 standard blood chemistry measures between control and anti-GnRH-treated female (Table 1) or male deer (Table 2). Although there was some variation for selected blood parameters, these values fell within normal ranges, and patterns were unremarkable.

Body condition. On the basis of body weight, external visibility of individual rib bones, and subcutaneous fat observed during gross necropsy, most animals were judged in good to excellent condition and had a bone marrow fat score of >85%. Two exceptions were: (1) female no. 66, an anti-GnRH revaccinate in year 2000, had poor body condition and was grossly underweight. She also had very poor teeth and had raised twins that summer. (2) Control female no. 323 had good body condition; however, her bone marrow fat score was <50%.

Injection sites. Injection sites for the year 2000 revaccinates were compared to those for deer vaccinated in 1997 to evaluate current and former dart site lesions induced by Freund's adjuvant. While abscesses were more evident and larger in recent revaccinates, granulomas could be found at the injection site of nearly all vaccinated deer.

Lung abscess. A 4x4-cm-circumscribed lung abscess, greenish in color, was noted during the necropsy of deer no. 25 (anti-GnRH-vaccinated female, year 2000). Tissue sections demonstrated this to be a giant cell granuloma containing acid fast bacilli similar to those found at the injection site.

Parasites. Three deer (nos. 13, 19, and 378) had liver cysts, most likely associated with the tapeworm *Echinococcus granulosus*, and 1 deer had an encysted parasite in skeletal muscle consistent with sarcosporidiosis (*Sarcocystis* spp.; no. 20). Each of these animals was in good to excellent body condition.

Table 1. Means, standard errors, and *t*-test statistics for blood parameters of female white-tailed deer in control and anti-GnRH-treated groups at Seneca Army Depot, Romulus, New York, 2000.

Blood parameter	Control group ($\bar{x}$ ± SE)	GnRH group ($\bar{x}$ ± SE)	*t*-test results	
Sodium (mEq/L)	141.00 ± 1.48	142.56 ± 1.04	$t = 0.81$	$P = 0.43$
Potassium (mEq/L)	11.77 ± 0.97	10.40 ± 0.84	$t = 0.93$	$P = 0.36$
Chloride (mEq/L)	103.50 ± 2.11	101.94 ± 0.92	$t = 0.80$	$P = 0.43$
Bicarbonate (mEq/L)	23.00 ± 2.21	24.06 ± 0.71	$t = 0.61$	$P = 0.55$
Cation-anion difference (mEq/L)	26.33 ± 4.18	27.53 ± 0.94	$t = 0.41$	$P = 0.69$
Urea nitrogen (mg/dL)	8.00 ± 1.51	13.82 ± 2.15	$t = 1.54$	$P = 0.14$
Creatinine (mg/dL)	1.27 ± 0.12	1.42 ± 0.06	$t = 1.24$	$P = 0.23$
Calcium (mg/dL)	9.75 ± 0.21	9.68 ± 0.17	$t = 0.21$	$P = 0.83$
Phosphate (mg/dL)	8.75 ± 1.11	8.16 ± 0.37	$t = 0.66$	$P = 0.52$
Magnesium (mg/dL)	2.55 ± 0.09	2.45 ± 0.07	$t = 0.75$	$P = 0.46$
Total protein (g/dL)	6.32 ± 0.36	6.75 ± 0.16	$t = 1.29$	$P = 0.21$
Albumin (g/dL)	3.13 ± 0.14	3.09 ±0.06	$t = 0.34$	$P = 0.74$
Globulin (g/dL)	3.18 ± 0.22	3.66 ± 0.14	$t = 1.73$	$P = 0.10$
Albumin:globulin ratio (A:G)	1.00 ± 0.04	0.87 ± 0.04	$t = 1.90$	$P = 0.07$
Glucose (mg/dL)	186.00 ± 82.08	155.06 ± 22.14	$t = 0.52$	$P = 0.61$
AST/PST[a] (U/cL)	11.47 ± 5.29	10.85 ± 4.18	$t = 0.08$	$P = 0.94$
SDH[b] (U/L)	67.18 ± 13.56	73.42 ± 20.31	$t = 0.18$	$P = 0.86$
Alkaline phosphatase (U/L)	197.87 ± 132.52	81.71 ± 11.99	$t = 1.49$	$P = 0.15$
GGT[c] (U/L)	45.33 ± 2.80	51.88 ± 6.42	$t = 0.59$	$P = 0.56$
Total bilirubin (mg/dL)	0.17 ± 0.02	0.19 ± 0.04	$t = 0.40$	$P = 0.69$
Direct bilirubin (mg/dL)	0.05 ± 0.03	0.05 ± 0.02	$t = 0.00$	$P = 0.99$
Indirect bilirubin (mg/dL)	0.12 ± 0.03	0.14 ± 0.04	$t = 0.44$	$P = 0.66$
Creatine kinase (U/cL)	130.10 ± 66.44	159.41 ± 74.64	$t = 0.22$	$P = 0.83$
Iron (µg/dL)	155.67 ± 18.30	168.71 ± 10.98	$t = 0.61$	$P = 0.55$
TIBC[d] (µg/dL)	318.67 ± 28.24	353.94 ± 13.71	$t = 1.24$	$P = 0.23$
% saturation	48.50 ± 3.18	48.24 ± 3.01	$t = 0.05$	$P = 0.96$
Lipemia	32.50 ± 8.48	13.18 ± 2.67	$t = 2.91$	$P = 0.01$
Hemolysis	103.83 ± 34.68	115.59 ± 32.12	$t = 0.20$	$P = 0.84$

Note: A Bonferroni-corrected ($n = 28$ tests) alpha level of 0.05 is 0.002.
[a]aspartate aminotransferase; [b]sorbitol dehydrogenase; [c]gamma-glutamyltransferase; [d]total iron-binding capacity

Multifocal lymphocytic infiltrates. Focal infiltrates and nongiant cell granulomas were seen in kidney, skeletal muscle, and liver. These lesions were consistent with immunological action against migrating parasites in tissues.

Female deer

Neither combined ovarian weights ($F_{2,22} = 0.31$, $P = 0.73$) nor pituitary weight ($F_{2,22} = 0.30$, $P = 0.75$) differed significantly between treatment groups and showed no correlation with antibody titer (Table 3). Mean anti-GnRH antibody titers were greater in the year 2000 vaccinates than for control females (Table 3; $t_1 = 2.31$, $P = 0.03$). The average titer for female deer treated in 1997, including 2 of 8 deer that had no detectable titer, was not significantly different from control females ($t_1 = 0.13$, $P = 0.90$).

The average total number of secondary follicles was less in both 1997 and year 2000 vaccinates than for control females ($F_{2,22} = 17.8$, $P \leq 0.001$). The mean number of Graafian follicles ($F_{2,22} = 2.22$, $P = 0.13$), and average total follicle count did not differ between treatment groups ($F_{2,22} = 1.60$, $P = 0.23$).

Male deer

When collected in October 2000, most of the male deer were in breeding condition with hardened antlers and swollen necks. Blood analysis confirmed appreciable levels of testosterone in the bucks sampled. Average testosterone levels of the control males (312 ng [nanograms]/100 ml) were considerably higher

Table 2. Means, standard errors, and *t*-test statistics for blood parameters of male white-tailed deer in control and anti-GnRH-treated groups, Seneca Army Depot, Romulus, New York, USA, 2000.

Blood parameter	Control group ($\bar{x}$ ± SE)	GnRH group ($\bar{x}$ ± SE)	*t*-test results
Sodium (mEq/L)	140.67 ± 1.98	143.50 ± 1.44	$t = 1.04$ $P = 0.33$
Potassium (mEq/L)	9.47 ± 0.84	10.22 ± 1.26	$t = 0.52$ $P = 0.61$
Chloride (mEq/L)	101.17 ± 1.17	102.00 ± 0.91	$t = 0.51$ $P = 0.62$
Bicarbonate (mEq/L)	25.00 ± 1.46	27.00 ± 0.41	$t = 1.08$ $P = 0.31$
Cation-anion difference (mEq/L)	23.83 ± 1.74	25.00 ±2.52	$t = 0.40$ $P = 0.70$
Urea nitrogen (mg/dL)	9.67 ± 2.59	6.25 ± 0.85	$t = 1.03$ $P = 0.33$
Creatinine (mg/dL)	1.57 ± 0.13	1.52 ± 0.20	$t = 0.18$ $P = 0.86$
Calcium (mg/dL)	9.98 ± 0.20	10.18 ± 0.44	$t = 0.44$ $P = 0.67$
Phosphate (mg/dL)	7.48 ± 0.52	9.10 ± 1.14	$t = 1.46$ $P = 0.18$
Magnesium (mg/dL)	2.30 ± 0.08	2.30 ± 0.16	$t = 0.00$ $P = 0.99$
Total protein (g/dL)	7.87 ± 0.29	7.82±0.57	$t = 0.07$ $P = 0.94$
Albumin (g/dL)	3.43 ± 0.10	3.18 ± 0.10	$t = 1.74$ $P = 0.12$
Globulin (g/dL)	4.43 ± 0.32	4.65 ± 0.65	$t = 0.33$ $P = 0.75$
Albumin:Globulin ratio (A:G)	0.80 ± 0.07	0.72 ± 0.10	$t = 0.64$ $P = 0.54$
Glucose (mg/dL)	184.83 ± 46.46	79.50 ±123.89	$t = 1.72$ $P = 0.12$
AST/PST[a] (U/cL)	5.17 ± 2.16	4.96 ± 2.35	$t = 0.06$ $P = 0.95$
SDH[b] (U/L)	39.30± 7.48	40.58 ± 12.41	$t = 0.09$ $P = 0.93$
Alkaline phosphatase (U/L)	61.17 ± 16.86	67.25 ± 8.78	$t = 0.27$ $P = 0.79$
GGT[c] (U/L)	57.00 ± 6.35	51.50 ± 4.56	$t = 0.63$ $P = 0.55$
Total bilirubin (mg/dL)	0.25 ± 0.05	0.20 ± 0.04	$t = 0.65$ $P = 0.54$
Direct bilirubin (mg/dL)	0.10 ± 0.03	0.10 ± 0.00	$t = 0.00$ $P = 0.99$
Indirect bilirubin (mg/dL)	0.15 ± 0.03	0.10 ± 0.04	$t = 0.93$ $P = 0.38$
Creatine kinase (U/cL)	53.24 ± 25.89	49.26 ± 32.48	$t = 0.10$ $P = 0.93$
Iron (μg/dL)	115.67 ± 36.96	159.75 ± 29.50	$t = 0.85$ $P = 0.42$
TIBC[d] (μg/dL)	336.00 ± 16.59	417.75 ± 56.64	$t = 1.66$ $P = 0.14$
% saturation	39.50 ± 8.42	39.00 ± 7.45	$t = 0.04$ $P = 0.97$
Lipemia	14.50 ± 4.28	16.00 ± 8.09	$t = 0.18$ $P = 0.86$
Hemolysis	41.50 ± 16.71	46.75 ± 19.17	$t = 0.20$ $P = 0.84$

Note: A Bonferroni-corrected ($n = 28$ tests) alpha level of 0.05 is 0.002.
[a]aspartate aminotransferase; [b]sorbitol dehydrogenase; [c]gamma-glutamyltransferase; [d]total iron binding capacity

than the vaccinated males (100 ng/100 ml) even 3 years post-treatment.

The mean combined testicular weight for anti-GnRH-treated male deer ($\bar{x}$ = 70.3) was significantly lower ($t_1 = 3.56$, $P = 0.007$) than for the control group ($\bar{x}$ = 130.5). Similarly, the average testes:pituitary index for anti-GnRH-treated ($\bar{x}$ = 69.2) male deer was less ($t_1 = 2.52$, $P = 0.04$) than for the control group ($\bar{x}$ = 130.0). However, there was no difference in pituitary weights ($t_1 = 0.30$, $P = 0.77$) for anti-GnRH-treated males ($\bar{x}$ = 1.0) and control ($\bar{x}$ = 1.0) bucks.

Morphologic alterations were observed in the testes of anti-GnRH-treated males (Table 4). The pathologies included diffuse lymphocytic infiltrate with associated seminiferous tubule degeneration, segmental tubular aspermatogenesis (where large contiguous areas of the testes were seen with only sertoli-

Table 3. Mean left and right ovary weights (milligrams), pituitary gland weights, anti-GnRH antibody titers, and follicle counts for control does vaccinated during 1977 or 2000 with anti-GnRH at Seneca Army Depot, Romulus, New York, 2000.

	Control does	Anti-GnRH does				
		1997	2000	*F*	*df*	*P*
	n = 7	*n* = 11	*n* = 7			
Left ovary	502.4	421.2	505.4	0.71	2,22	0.50
Right ovary	497.1	446.4	475.2	0.15	2,21	0.86
Pituitary gland	788.1	729.0	733.9	0.30	2,22	0.75
Titer (1/titer x 1,000)	0.0	1,700	31,100	3.37	2,18	0.06
Secondary follicles						
Normal	8.1	3.4	5.9	3.38	2,22	0.05
Atretic	10.3	3.1	3.3	11.2	2,22	0.0004
% Atretic	55.1	55.3	38.5			
Graafian follicles						
Normal	0.6	3.2	5.7	2.65	2,22	0.09
Atretic	1.6	4.4	2.1	2.34	2,22	0.12
% Atretic	84.2	42.3	32.2			

cell-lined tubules); subepithelial concretions in the collecting tubules, decreased cross-sectional diameter of seminiferous tubules, decreased cytoplasmic volume of Leydig cells, interstitial fibrosis, and total aspermatogenesis. Histology of testes from control males revealed none of these abnormalities. Among the treated males, one had only segmental aspermatogenesis with none of the associated pathologies. The remaining 3 males all had ≥2 of the abnormalities noted above.

Male no. 31, had malformed (atypical) antlers still covered in velvet (Figure 1), exhibited total aspermatogenesis with small fibrotic testes, and vacant epididymi. This same male had no detectable anti-GnRH antibody titer, but also lacked any measurable blood testosterone level. The 3 other anti-GnRH vaccinated males did have some epididymal spermatozoa.

Discussion

Observations for both male and female deer

Lesions. A variety of infectious, parasitic, and noninflammatory lesions were observed in our study animals. Most noteworthy was the formation of granulomas at the injection site with all the characteristics of tuberculosis. These granulomas were characterized by the presence of a necrotic core surrounded by mixed inflammatory cells and fibrous connective tissue. All granulomas had Langerhans giant cells typical of a tuberculosis condition. Likewise, giant cell inflammation was seen in the regional (popliteal) and deep (intral iliac) lymph nodes draining these sites. Acid fast bacilli were documented in these giant cells.

These injection site lesions and reactions, including the production of multi-nucleated giant cells associated with the presence of *Mycobacteria* in the adjuvant in other species vaccinated with CFA have been previously described (Stills and Bailey 1991, Kleinman et al. 1993, Munson et al. 2005, Stills 2005). In other studies, remote lesions comparable in composition to the Freund's injection sites had also been documented in lungs and other organs (Rigdon and Schadewald 1972, Broderson 1989, Kleinman et al. 1993, Malaga et al. 2004).

When evaluating the safety of immunocontraceptive vaccines, potential complications associated with treatment should be considered. Killian et al. (2006) reported that pulmonary disease was the most common cause of death in their captive deer herd and that males treated with anti-GnRH vaccine had significantly higher mortality than did control deer. Microbes associated with pneumonia were endemic in their captive herd and anti-GnRH-treated bucks appeared less resistant to infection.

Table 4. Presence (+) or absence (-) of different features in the testes of control (n = 6) and anti-GnRH-treated bucks at Seneca Army Depot, Romulus, New York, 2000.

	Deer identification number	Estimated age at start of trial	Diffuse lymphocytic infiltrate genesis (sertoli cells only)	Segmental tubular aspermatogenesis	Subepithelial concretions	Total aspermatogenesis seminiferous tubules	Decreased cross-sectional diameter	Decreased cytoplasm in Leydig cells	Interstitial fibrosis
Controls		3 ½	-	-	-	-	-	-	-
GnRH	22	2 ½	-	+	-	-	-	-	-
	31	3 ½	+	+	+	+	+	+	+
	37	3 ½	-	-	+	-	+	-	-
	4	3 ½	+	-	+	-	+	-	+

Likewise, Miller et al. (2000*b*) reported that 3 female deer died of complications resulting from pneumonia during a study of porcine zona pellucida (PZP) immunocontraceptive with Freund's adjuvant. It was unclear if there was an association between the pneumonia cases and treatment with Freund's adjuvant. Bearing in mind the warning by Billiau and Matthys (2001) that too few studies consider the biological effects of using CFA when evaluating experimental vaccines, we feel that future researchers should examine the possibility that injections with CFA could predispose deer to pneumonia.

Hypothalamic-pituitary response. Normally GnRH released from the hypothalamus circulates into the anterior pituitary gland where it activates production of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by gonadotrophs. Antibodies to GnRH interacted with neurons secreting GnRH from the hypothalamus at terminals within the median eminence. These GnRH release points are accessible because they are not within the blood-brain barrier that otherwise protects the hypothalamus from circulating antibodies. One study demonstrated degeneration of the neuron cell bodies (perikarya) and a sharp decrease of the GnRH immunochemical reactivity in the terminals within the median eminence of hyperimmunized hogs (*sus scrofa*; Molenaar et al. 1993). With hymotoxylin and eosin stain on fixed pituitary gland tissue, gonadotrophs appear as large cells with secretory granules of moderate and variable size. Cells with little or no cytoplasmic staining (chromophobes) are likely degranulated chromophilic cells. Thus, we classified large granule-bearing cells as gonadotrophs in this study, and found large variation in their numbers among females in each treatment group. A more precise staining method would involve immunoperoxidase staining with anti-LH/FSH. This would allow simultaneous quantification of cell numbers and the relative intensity of intracytoplasmic LH/FSH stores.

Female deer

Titers in 5 of the 7 deer revaccinated in 2000, and 6 of the 8 deer last vaccinated in 1997 were well below the level of 64,000 to 128,000 identified in deer with successful contraception, or the 16,000 to 32,000 level for contragestation (Miller et al. 2000*a*). Because the revaccinates in 2000 were retreated <4 weeks prior to collection, it is possible that the immune response was incomplete and that a peak anti-GnRH titer would be greater. Lower anti-GnRH titer in females vaccinated in 1997 than those vaccinated in the 2000 treatment group validates the assumption that, if not boosted, the anti-GnRH vaccine treatment is reversible for most deer. There were no identifiable health concerns associated with anti-GnRH treatment for female deer.

Male deer

Little has been reported about the effects of anti-GnRH vaccination on the reproductive physiology of male deer. Miller et al. (2000*a*) injected 4 male deer with the anti-GnRH vaccine, and testes size observed for treated bucks was <50% testes size in control bucks. Killian et al. (2006) also observed similar physical differences in testicular size and qualified those observations reporting histological changes in the testes "which resembled the testes of males during the non-breeding season." They further noted that the Leydig cells, which produce testosterone, appeared inactive in anti-GnRH treated males. Similar testicular pathologies have also been documented in research conducted on hogs (Molenaar et al. 1993, Meloen et al. 1994).

It is clear from testes weights and histology that sperm production was reduced, even 3 years after the last anti-GnRH vaccination. The significantly lower average weight of testes in this study correlated with a number of persistent morphologic abnormalities and decreased (qualitative assessment) spermatozoa in epididymi. Observing these effects 3 years after vaccination suggested that anti-GnRH vaccination early in the adult lives of deer may be sustained over several years. Similarly, in a study on 3- to 4-year-old sheep vaccinated with anti-GnRH antigen as neonates, Clarke et al. (1998) reported that in these study animals males had small gonads and females had no large follicles or corpora lutea, and titers had dropped to undetectable levels.

Immunized bucks had reduced cross-sectional diameter of seminiferious tubules and varying degrees of aspermatogenesis, including

buck no. 31 with total aspermatogenesis that was vaccinated at 3.5 years of age. This latter animal had no detectable anti-GnRH titer, suggesting a possible permanent alteration in the hypothalamic-pituitary-testes axis. The other 3 anti-GnRH treated bucks had normal outward physical traits and detectible mature spermatozoa, although the latter were fewer than normal. While it was not possible to assess their actual fertility from this study, this evidence suggests that anti-GnRH contraception is reversible; however, some portion of treated males may become permanently sterilized.

Variations in the residual immune response and the extent of physiological, morphological, or pathological differences among the treated deer are thought to be due to genetic differences among individual animals (Miller et al. 2000*a*). In fact, substantial research has been conducted using anti-GnRH vaccines to chemically castrate hogs as an alternative to surgical castration typically used to control boar taint, with considerable variations in efficacy documented (Molenaar et al. 1993, 1994; Oonk et al. 1995, 1998).

Management implications

Many factors affect the success and potential pathology associated with an immunocontraceptive vaccine, including formulation of the immunogen, concentration of the immunogen, adjuvant used, and species treated. Therefore, before judgments can be made concerning the effectiveness and health risks associated with a given vaccine for a particular species, adequate evaluation must be conducted.

Curtis et al. (2002) demonstrated that vaccinating free-ranging female white-tailed deer with anti-GnRH immunocontraceptive vaccines can be effective at reducing fawn production. We further evaluated anti-GnRH vaccinated deer and identified no detrimental, morphologic effects that could jeopardize the normal health of vaccinated females. Vaccinating female deer with anti-GnRH vaccines eliminates the potential problems associated with repeated estrous cycl-ing commonly observed in deer treated with PZP immunocontraceptive vaccines (McShea et al. 1997, Miller et al. 2000*b*, Kirkpatrick and Rutberg 2001). Baker et al. (2004) studying leuprolide, a GnRH agonist, which would cause similar effects as the anti-GnRH immunocontraceptive vaccine used in this study, also reported no significant adverse health impacts to female deer resulting from the reduced systemic GnRH. Furthermore, if GnRH could be administered to newborn female fawns, as Clarke et al. (1998) were able to do with neonatal lambs, considerable long-term efficacy could be achieved.

Morphologically, anti-GnRH-treated males essentially became neutered and exhibited behavioral and physiological traits consistent with castrated males (Pooler 2001, Killian et al. 2006). Antlers on treated males did not harden or shed velvet and became atrophied (Killian 1998, Miller et al. 2000*a*, Curtis et al. 2002, Killian et al. 2006). Such antlers are spindly, atypical, and possibly pose a health risk to deer from frost bite when they freeze and break off during winter. Treated male deer also could develop adverse health effects including increased risk for pneumonia. Killian et al. (2006) recommended that anti-GnRH vaccines should not be used on male deer due to these concerns. Our results were consistent with these previous studies, and we also do not recommend treating male deer with anti-GnRH vaccines.

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Immunization against GnRH in Mature Mares: Antibody Titres, Ovarian Function, Hormonal Levels and Oestrous Behaviour

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Abstract

The aim of the present study was to investigate the effect of active immunization against GnRH in mature Standardbred mares (three experimental and one control mare) on antibody titres, ovarian function, hormonal levels and oestrous behaviour. The mares were individually teased with a stallion once each day. During the first part of the experiment (period I: late April until November), blood was sampled every third day during the first 3 months, thereafter once per week. In the second part of the experiment (period II: December until August), sampling was carried out every second week. Progesterone, oestradiol-17β and LH were analysed. Rectal gynaecological examination was made with the same intervals as the blood samplings and included palpation of the genital organs and ultrasonography. The experimental mares were immunized against GnRH with a GnRH–BSA conjugate. Equimune® (Vetrepharm, Bracetown, Business Park, Clonee, Co. Meath, Republic of Ireland) was used as adjuvant. The mares were immunized on four occasions (20–30 day intervals) and GnRH antibody titre was determined. All immunized mares produced antibodies against GnRH but the maximum titres as well as the duration of a greater than 10% binding capacity varied between the mares (1 : 1600 to 1 : 50 000; 5 to 12 months, respectively). After the first injection, all mares showed one oestrus and ovulated at the regular time. In two of the mares, the immunization resulted in ovarian atrophy. Their hormone levels of progesterone, oestradiol-17β and progesterone decreased to basal levels and the cyclical hormone pattern was interrupted from approximately 30 days onwards. They continued to show oestrous signs but with irregular durations and intervals. The third mare showed ovarian suppression only for short periods and not in both ovaries at the same

time; the hormone levels were basal for only about 20 days (days 50–70) and the mare ovulated on day 75 after start of immunization. The other mares ovulated after 13.5 and 15 months, respectively. It is concluded that the effect of immunization against GnRH in mature mares was individual concerning antibody titre response and the suppression of ovarian activity and hormone levels. Mares with totally inactive ovaries continued to show oestrous signs but with irregular intervals and durations.

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Use of an anti-GnRF vaccine to suppress estrus in crossbred Iberian female pigs

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Sow

ABSTRACT

Antibodies against GnRF elicited by vaccination with Improvac are expected to have the same effects on the gonads of female pigs as those observed in males and thus suppress estrus. The objective of this study was to evaluate whether a 3- or a 4-dose regimen of Improvac was effective in inducing and maintaining suppression of estrus in intact Iberian female pigs through 14 months of age. Sixty 18-week-old gilts were randomly assigned to one of the following three treatment groups: control (PBS injected), V3 (3-dose Improvac regimen), and V4 (4-dose Improvac regimen). Animals were tested for the presence of standing estrus in the presence of a boar three times per week until study completion at 60 weeks of age. Blood samples were regularly collected to determine the serum levels of anti-GnRF antibodies and progesterone. The weight and size of the uterus and the weight and activity of the ovaries were assessed at the time of euthanasia. For both treatment groups V3 and V4, a large reduction in the incidence of standing estrus was observed when compared to the control group ($P < 0.0001$). No significant differences between the V3 and V4 groups were observed. Both V3 and V4 groups had higher serum anti-GnRF antibody levels than the control group from 28 days after the first vaccination until the end of the study. In addition, both V3 and V4 groups had lower levels of progesterone than the control group from Day 112 until the end of the study ($P < 0.0001$ in all cases). Mean ovary weights at study end in treatment groups V3 and V4 were significantly lower ($P < 0.0001$) than those for the control group and not significantly different between the V3 and V4 groups. An onset of immunity of 2 weeks after the second vaccination and a long-lasting immunity of at least 20 weeks after the third vaccination were observed. In summary, both the 3-and 4-dose Improvac regimens were efficacious in reducing the incidence of standing estrus, serum progesterone levels, and the development of the uterus and ovaries.

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1. Introduction

The Iberian pig is a native breed of the Iberian Peninsula, which originates from *Sus scrofa mediterraneous* and is reared in La Dehesa, an extensive system. This is a Mediterranean ecosystem based on an oak forest (*Quercus*

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rotundifolia and *Quercus suber*) with herbaceous species, mainly grasses and legumes where animals can graze [1]. The Iberian pig is characterized by its fat-production ability [2] and high rusticity. This enables the animals to cope better with climatic hazards. The Iberian pig has been raised for centuries to produce dry-cured meat. The whole reproductive cycle of the animals is planned to provide an adequate physiological status capable of taking advantage of La Dehesa during the late fattening phase which starts when pigs are around 10 months of age and 90- to 120-kg live weight. However, the age range during this stage varies widely, from 10 months to 2 years [2]. In fact, the requirements to be classified as an Iberian pig product prescribes a minimum slaughter age of 14 months and a minimum carcass weight of 115 kg per animal (180 kg per animal in purebred Iberian pigs) [3].

There is limited information available on the precise time of onset of estrus in Iberian gilts. It has been stated that the Iberian female pig begins puberty at an average age of 207 days (7 months) [1] or 180 days [4]. It is clear that males and females are reared for several months after becoming fertile. As a result, for different reasons, males are traditionally castrated and females are spayed. The negative effect on growth due to estrous behavior in Iberian females [5,6] or in other breeds slaughtered late [7] has been used to justify the need for spaying, but this has not scientifically examined. However, the main reason to justify the spaying of Iberian pigs is the presence of wild boars in the La Dehesa. Wild boars cohabitating with intact females can produce a high percentage of pregnancies which is undesirable from health, welfare, and economic perspectives. As a result, spaying was traditionally seen as the best solution. However, Council Directive 2008/120/EC restricts this practice resulting in the need for other alternatives as it is not always possible to avoid cohabitation with the wild boars.

Improvac, a vaccine inducing the production of antibodies against GnRF, was developed for the control of boar taint in intact male pigs and subsequently licensed in Europe and elsewhere. In males, vaccination reduces production of LH and FSH by the pituitary gland, reducing the production of sex hormones by the testes [8,9]. The immunization regimen comprises two subcutaneous injections in the neck, at least 4 weeks apart. The first vaccination only primes the pig's immune system; the second one (usually administered 4–5 weeks before slaughter) stimulates high levels of anti-GnRF antibodies that neutralize the pig's endogenous GnRF which inhibits testicular function [10]. This regimen ensures freedom from boar taint at slaughter and allows pigs to spend most of their fattening period as functional males before puberty. In commercial systems where the vaccine is currently used, pigs are usually given their second dose before 24 weeks of age which has been found to be highly effective [11]. However, in older animals such as those from Iberian ham production systems, delaying the second dose until 4 to 5 weeks before slaughter is likely to result in a considerable period during which animals express marked sexual behavior. This is because the animals would be immunized well after reaching sexual maturity. Claus et al. [12] found that the vaccine was efficacious for at least 10 weeks after the second vaccination, and Zamaratskaia et al. [13] confirmed an even longer time. Both cases, however, showed that the 2-dose vaccination regimen is effective for only a limited time and that if administered early would be unlikely to last until slaughter age in a significant proportion of Iberian Pigs. In consequence, Allison et al. [11] propose using a three-dose vaccination regimen for male pigs slaughtered at 14 months of age.

In female pigs, antibodies against GnRF are expected to reduce LH and FSH production and thus to have similar effects in the gonads of females as those in males, resulting in suppression of estrus. First evidence for this was provided by the administration of a GnRF-tandem dimer peptide [14] to males at 10 to 13 weeks and 8 weeks later which resulted in a decrease of serum LH levels and in a suppression of reproductive functions. However, not all the animals responded to immunization with this experimental formulation. Furthermore, immunization against GnRF has been investigated under experimental conditions in Chinese female pigs as a practical alternative to surgical castration [7].

On the basis of these data, the present study was conducted with the objective of testing whether either a 3- or a 4-dose regimen of Improvac is effective in maintaining suppression of estrus and estrus-related behavior in Iberian female pigs until 60 weeks of age in comparison to non-treated animals. In addition, concentrations of progesterone and antibodies against GnRF in blood samples were measured throughout the study, and reproductive organs were assessed at the end of the study.

2. Materials and methods

2.1. Animals

Sixty intact Iberian × Duroc crossed female pigs were transported at the age of 16 weeks from Extremadura in the south of Spain to the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) facilities in Monells, Catalonia, Spain. On arrival, pigs were individually weighed, identified with a double ear tag (one tag in each ear), and randomly allotted into four groups of 15 animals each. Five animals from each pen were randomly assigned to the control, the 3-dose (V3), or the 4-dose (V4) vaccination regimen with Improvac. Floor space was 1.7 m^2 per pig with *ad libitum* access to food and water during the whole study.

At the end of the study, 290 days after arrival, pigs were euthanized in the experimental abattoir of IRTA in four batches of 15 animals each (one per pen) with a difference of 7 days from the first to the fourth slaughter day. Animals were individually weighed before being stunned with a carbon dioxide (90%) stunning system.

This study was approved by both the Institutional Animal Care and Use Committee of IRTA and Zoetis Ethical Review Committee according to Directive 2010/63/EU on the protection of animals used for scientific purposes. Animals were kept in line with regulations applicable in Spain regarding housing and animal welfare.

2.2. Application of treatment

The control group received 2 mL of a PBS solution subcutaneously. The V3 and V4 groups received 2-mL Improvac

subcutaneously. The treatment schedule is listed in Table 1. The first injection was at 18 weeks of age, Day 0 of the study. The second injection was performed 28 days later, at 22 weeks of age. Subsequent vaccinations occurred at 12-week intervals as applicable. Pigs in group V3 received a total of three vaccinations, and those in group V4 received a total of four vaccinations. In all cases, injections were performed in the neck using disposable needles of 19-mm length and a 16-ga diameter.

2.3. Estrus detection

Gilts in estrus were assessed for presence of estrus using the back-pressure test. The test was performed three times per week in the presence of a teaser boar from the start of the study until Day 290. The boar was led past the gilts' pens and allowed to have head-to-head contact from the corridor during the test. Estrus detection was assessed by rubbing the flank and pressing the back of the gilt in the presence of the boar. Gilts were deemed to be in estrus when they remained in standing response, immobile with arched back, and cocked ears. The following scoring system was used: 0 = no estrus; 1 = doubtful estrus; 2 = clear (standing) estrus. Staff performing estrus detection were blinded to treatment groups. Two different boars were alternately used. They were housed in separate facilities without visual or olfactory contact with the females except during estrus testing.

2.4. Serum analysis

A plain blood sample was taken before the first injection on Day 0 from all pigs. Blood samples were collected before vaccine administration on the day of the second, third, and fourth vaccination. In addition, blood samples were collected from all pigs 2 and 5 weeks after the second, third, and fourth vaccinations, 10 weeks after the fourth injection, and just before study end. Blood samples were allowed to clot for 24 hours at 4 °C and then centrifuged at 3500 rpm for 10 minutes to obtain the serum. The serum samples were divided into two aliquots: one was used for progesterone determination and the other for GnRF antibody testing. All aliquots were coded to ensure appropriate blinding during analysis. Aliquots were stored at −20 °C until they were analyzed.

The amount of progesterone in porcine serum was quantified by the progesterone Enzyme Immunoassay (EIA) Kit from Enzo Life Sciences (Farmingdale, NY, USA; catalog number, ADI-900-011 Progesterone EIA). This test is a competitive immunoassay which uses an antibody to bind in a competitive manner with progesterone. After a simultaneous incubation, the excess reagents were washed away and the substrate was added. After a short incubation time, the enzyme reaction was stopped and the yellow color generated was read on a microplate reader. The intensity of the bound yellow color was inversely proportional to the concentration of progesterone in either standards or samples. The measured optical density for the samples was used to calculate the concentration of progesterone in the samples. The detailed method, validation data from the supplier of the kit, and publications can be found in http://www.enzolifesciences.com/ADI-900-011/progesterone-elisa-kit. The laboratory in charge of testing Zoetis samples took care to check the intra-assay coefficient of variation (CV; duplicates on the same plate) and to check the interassay CV between titers of samples tested "fresh" and titers of the same samples stored frozen. The CVs were respectively below 10% and 12%.

The amount of antibody against GnRF in porcine serum was measured by a noncompetitive ELISA, which uses GnRF peptide as the capture phase. Dilutions of sera from pigs were added to plates coated with GnRF peptide. After several washes, the plates were incubated with horseradish peroxidase–labeled antiswine immunoglobulin. Finally, 3,3′,5,5′-tetramethylbenzidine substrate was added. After a short incubation time, the enzyme reaction was stopped and the color generated was measured spectrophotometrically. The amount of color produced was directly proportional to the amount of antibody present in the sample. The limit of quantification is 18.56 U/mL. The laboratory took care to check the interassay CVs which were below 10% (8.4%). They also checked the intra-assay CVs which were below 10% (5.9%) in the linear phase of the standard curve.

Table 1
Treatments applied in the study.

Treatment	Dosage	Study day	Age of pigs (wk)	Route of administration	Pigs per treatment group
Control	2 mL	0	18	Subcutaneous	20
	2 mL	28	22	Subcutaneous	20
	2 mL	112	34	Subcutaneous	20
	2 mL	196	46	Subcutaneous	19
V3	2 mL	0	18	Subcutaneous	20
	2 mL	28	22	Subcutaneous	20
	2 mL	112	34	Subcutaneous	19
V4	2 mL	0	18	Subcutaneous	20
	2 mL	28	22	Subcutaneous	20
	2 mL	112	34	Subcutaneous	20
	2 mL	196	46	Subcutaneous	20

2.5. Reproductive organs

At study end, the uterus and ovaries were removed and weighed, and the length of the uterine horns was recorded after straightening them. Each ovary was scored in relationship to the presence and size of the developing follicles and CL: 0 = no follicles; 1 = immature follicles: 3 to 4 mm of diameter; 2 = mature follicles: 8 to 11 mm of diameter; and 3 = mature follicles and luteal tissue [15]. The person responsible for assessing these variables was blinded to treatment groups.

2.6. Statistical analysis

Statistical analysis was carried out with Statistical Analysis System (SAS Release 9.2, SAS Institute, Cary, NC, USA). All treatment differences were assessed at the two-sided 5% significance level. The experimental unit for analysis was the individual animal.

Standing response (estrus score = 2) in each time period between the vaccinations and throughout the study was analyzed using the Cochran–Armitage test adjusted for block. All possible pairwise comparisons were made if the overall effect of treatment was significant. At each time point, the proportions of pigs with titers of antibodies against GnRF and progesterone levels below the level of quantification in each treatment group were calculated. The log-transformed GnRF antibody titers and progesterone levels were analyzed using a linear mixed model for repeated measures, with treatment, time, and their interaction as fixed effects and block, block-by-treatment interaction (pen term), animal within block and treatment, and residual as random effects. All possible pairwise treatment comparisons were made at each time point if the treatment and/or treatment by time point effect(s) was significant. For animals with anti-GnRF antibody titers below the level of quantification, these were defined as half the level of quantification and included in this analysis. The weight of uteri and ovaries and uterine horn length were analyzed using a general linear mixed model with treatment as a fixed effect and random effects of block, block-by-treatment interaction, and residual. If the treatment effect was significant, then all possible pairwise treatment comparisons were made.

3. Results

3.1. Estrus detection

During the study, 17 of 20 animals in the control group showed standing estrus (score 2) on at least one occasion. In treatment group V3, three of 20 animals showed standing estrus (score 2) on a single occasion. One animal did so on Day 29 (1 day after the second vaccination), another one on Day 36 (8 days after the second vaccination), and the third one on Day 244 (132 days after the third vaccination). In treatment group V4, not a single animal had an estrus score greater than one at any time point. Both groups V3 and V4 showed a significantly lower incidence of standing estrus compared to cover the entire study period ($P < 0.0001$). The difference between the V3 and V4 groups was not significant ($P = 0.3236$).

When analyzed by time period (with periods related to the injection day), there were no differences between the V3 and V4 groups in the percentages of sows showing no estrus for any period. Both Improvac groups showed significantly less estrus ($P < 0.0001$) than the control group (Fig. 1).

3.2. Serum analysis

The level of anti-GnRF antibodies in serum was not significantly different between the treatment groups on Day 0. Thereafter, both treatments V3 and V4 had higher anti-GnRF antibody levels at all time points ($P < 0.0001$), including Day 28 before the second vaccination (Fig. 2), when concentrations were low but detectable. Differences between the V3 and V4 groups were not significant until Day 210 of the study; beyond that point, antibody levels in treatment V4 were significantly higher ($P < 0.0001$) than those in treatment V3.

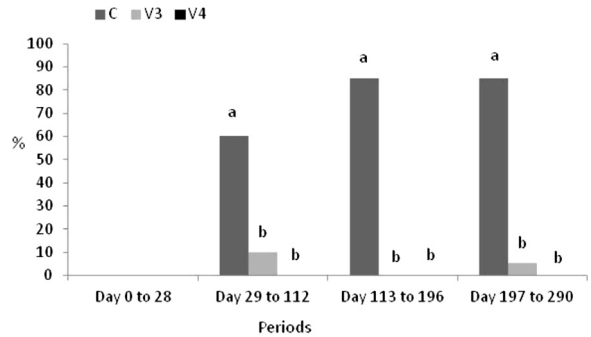


Fig. 1. Percentage of sows that showed standing estrus (score = 2) when tested three times weekly during the different periods of the study in the control group (C) and groups vaccinated with a 3-dose regimen (V3) or a 4-dose regimen (V4). Different letters annotate significant differences between the treatments at $P < 0.001$ in each study period.

The level of progesterone in serum remained low in treatments V3 and V4 throughout the study. In the control group, it started to rise from Day 42 (approximately 24 weeks of age) onward as gilts approached sexual maturity. There were no significant differences in progesterone levels between any of the three treatment groups until Day 63. From Day 112 onward, the progesterone levels were significantly ($P < 0.0001$) lower in both treatments V3 and V4 compared to the control group (Fig. 3). There were no significant differences in progesterone levels in the serum between V3 and V4 at any time point.

3.3. Reproductive organs

On average, ovary weights in treatment groups V3 and V4 were significantly lower ($P < 0.0001$) than those in the control (Table 2). No significant difference was observed when comparing weights of treatment groups V3 and V4 ($P = 0.3250$). In the control group, follicles were present in all ovaries. In group V3, one animal had mature follicles on both ovaries (Table 3). In group V4, no follicles were observed on any of the scored ovaries.

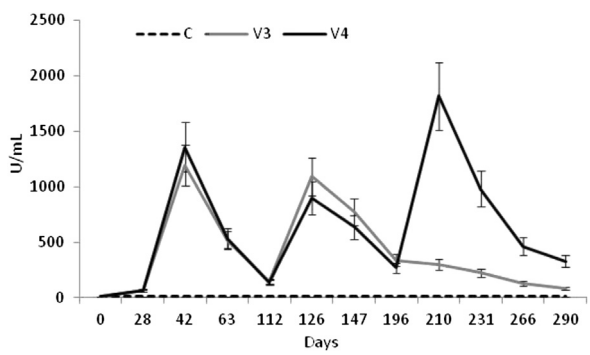


Fig. 2. Anti-GnRF antibodies (U/mL) detected in serum samples taken from control gilts (C) and gilts vaccinated with a 3-dose regimen (V3) or a 4-dose regimen (V4).

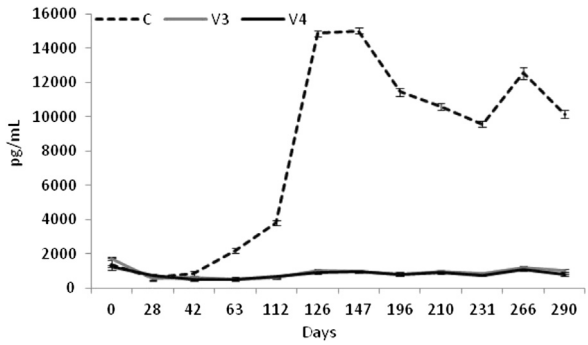


Fig. 3. Progesterone (pg/mL) detected in serum samples taken from control sows (C) and sows vaccinated with a 3-dose regimen (V3) or a 4-dose regimen (V4).

Comparisons between the treatment groups showed a significant reduction in uterus weight and horn length (Table 2) for treatment groups V3 and V4 versus control ($P < 0.0001$ for both measures and for both pairwise comparisons). Weight and length of the uterus were not significantly different between treatment groups V3 and V4 ($P = 0.3498$ and 0.9737, respectively).

4. Discussion

Although progesterone levels were not significantly different between vaccinated and control animals until Day 112, estrus was effectively inhibited starting around 10 days after the second injection. Dunshea et al. [16] also described, in male pigs, clear effects of Improvac within 2 weeks after administration of the second dose. It therefore appears that the time needed for the efficacy of the vaccine to appear is similar in both sexes.

There were no significant differences in standing estrus behavior between the treatment groups V3 and V4 in the last phase of the study, from Day 197 to Day 290 (178 days after the third injection). This suggests that a 3-dose regimen is sufficient for animals up to 14 months of age to suppress estrus and its related behavior. The continuous reduction seen in anti-GnRF antibodies of group V3 from Day 126 does suggest that efficacy will eventually wane and that a 4-dose regimen might prove more efficacious in ensuring consistent estrus suppression if the growing period is further prolonged. The duration of immunity against GnRF has been studied in males by Claus et al. [12] and Zamaratskaia et al. [13]. Their research suggested a duration of immunity for Improvac of at least 10 weeks after a second injection and a duration of 16 and 22 weeks, respectively. In the study presented in this paper, the duration of immunity shown in the females was up to 20 weeks after the third injection in 93% of V3-treated animals and at least 13 weeks after a fourth injection in 100% of V4-treated animals. This duration of immunity is as least as long as that observed in male pigs.

Table 2
Ovary weight, uterus weight, and horns length after study end for control sows (C) and sows vaccinated with a 3-dose regimen (V3) or a 4-dose regimen (V4).

Measure	C	V3	V4
Ovaries weight, g	20.8 ± 2.13^{a}	1.5 ± 0.36^{b}	1.2 ± 0.12^{b}
Uterus weight, g	821.0 ± 93.86^{a}	46.9 ± 12.83^{b}	34.48 ± 3.73^{b}
Horn length, mm	2844 ± 273.3^{a}	797 ± 216.5^{b}	787 ± 211.2^{b}

[a,b]Different letters annotate significant differences between the treatments at $P < 0.001$ in each study period.

Table 3
Presence and size of follicles in the ovaries of sows of control group (C) and groups vaccinated with a 3-dose regimen (V3) or a 4-dose regimen (V4) after study end.

Treatment	Follicle size score				Total (n)
	0	1	2	3	
C, n (%)	0 (0)	15 (39)	12 (32)	11 (29)	38
V3, n (%)	34 (94)	0 (0)	2 (6)	0 (0)	36
V4, n (%)	40 (100)	0 (0)	0 (0)	0 (0)	40

Benito et al. [1] stated that puberty in Iberian female pigs begins at an average age of 207 days. On the other hand, Gonzalez-Añover et al. [4] concluded that Iberian females can reach puberty before 180 days of age. In our study, the Iberian females showed first estrus behavior at around 160 days of age. This is in accordance with studies in other commercial breeds, such as those of Patterson et al. [17], in which first estrus was detected from Day 154 to Day 167 in a high percentage of the animals if boar contact commenced on Day 140. According to Kummer [18], with good boar contact, 73% of gilts can be cyclic within 40 days of daily boar exposure. These numbers are consistent with our results in which contact with a boar three times per week started at an age of 126 days and around 30 days later, the first gilts began to show estrus behavior. This could be one of the main reasons why the time of sexual maturation observed in the present study was earlier compared to previously reported times [1]. Obviously, in commercial conditions, contact with the boar normally starts later. van Wettere et al. [19] suggested that the optimal timing and synchrony of puberty attainment occur when boar contact commences at 160 days of age. So under commercial conditions, the first signs of estrus would be expected a little later. Another possible explanation for the early maturation observed in this study is the fact that animals were a cross between Iberian and Duroc. However, there is no clear information about the real impact of this specific cross on the sexual maturation of these animals. For instance, Gonzalez-Añover et al. [4] described that the onset of puberty was earlier in Iberian gilts compared to a Large White × Landrace commercial cross which differed from the traditional assumptions.

In the control group, three animals did not show a standing estrus at any time during the study. Inspection of the serum progesterone levels, weight of ovaries and uteri, and follicle size score after the end of the study (Day 290) showed that these animals developed a reproductive cycle, despite the fact that no standing estrus was observed during any of the testing days. Hemsworth et al. [20] described an ability to detect estrus by the back-pressure test in the presence of a boar in 81% of gilts when the boar was continuously housed in a pen adjacent to the females and only separated by a wire-mesh wall. A similar

result was obtained in the present study. However, in our case, the boar was housed in a separate building without continuous visual or olfactory contact with the females, so results closer to 100% would have been expected in these conditions [20]. The effect of hierarchy or fear should be considered as possible reasons to explain the lack of clear standing estrus detection in these animals. In fact, Pedersen et al. [21] concluded that both heat detection and mating may be impaired in subordinate sows when they were pair housed. This effect could even be higher in animals housed in groups of 15 individuals as in this study.

4.1. Conclusions

The use of Improvac in Iberian female pigs will reduce the risk of unwanted mating by wild or domestic boars, being a suitable alternative to spaying in sows.

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AN ECONOMIC MODEL DEMONSTRATING THE LONG-TERM COST BENEFITS OF INCORPORATING FERTILITY CONTROL INTO WILD HORSE (*EQUUS CABALLUS*) MANAGEMENT PROGRAMS ON PUBLIC LANDS IN THE UNITED STATES

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AN ECONOMIC MODEL DEMONSTRATING THE LONG-TERM COST BENEFITS OF INCORPORATING FERTILITY CONTROL INTO WILD HORSE (*EQUUS CABALLUS*) MANAGEMENT PROGRAMS ON PUBLIC LANDS IN THE UNITED STATES

Charles W. de Seve, Ph.D., and Stephanie L. Boyles Griffin, M.S.

Abstract: In recent years, the Bureau of Land Management's (BLM) Wild Horse and Burro Management program costs have increased dramatically due to a rise in the number of animals removed from public lands coupled with significantly decreased adoption rates. To assist with development and implementation of effective, cost-containing management programs, a robust economic model to project the costs and optimize outcomes of various management scenarios was created. For example, preliminary demonstration model runs show that by gradually replacing "removal-only" programs with contraception-and-removal programs on one hypothetical Herd Management Area (HMA), the BLM could save about US$8 million over 12 years while maintaining an area target population of 874 horses. Because the BLM estimates that more than 38,000 wild horses roam on 179 HMAs in the United States, the use of this economic model could result in a cost-savings of tens of millions of dollars if applied broadly across all HMAs.

Key words: Bureau of Land Management, economic model, *Equus caballus*, fertility control, porcine zona pellucida, wild horses.

BRIEF COMMUNICATION

The Wild Free-Roaming Horses and Burros Act of 1971 (Public Law 92–195)[13], as amended, directs the Bureau of Land Management (BLM) to manage wild horse herds by setting appropriate management levels (AMLs). AMLs are estimates of the optimum number of wild horses which results in a "thriving natural ecologic balance and avoids deterioration of the range" for each Herd Management Area (HMA). As of February 2012, the BLM estimates that approximately 37,300 wild horses and burros (31,500 horses and 5,800 burros) exist on 179 HMAs that comprise 31.6 million acres of public lands, of which 26.9 million are under BLM management.[10]

Herds can grow at an average rate of 20% annually.[2,3,4] When herd populations exceed established AMLs, the BLM conducts roundups to remove excess animals from HMAs, and roundups occur every 3–5 yr to ensure BLM complies with the requirements of the amended act. Removed animals are transported to long-term government holding pastures in the Midwest where they are cared for in perpetuity or, to a lesser extent each year, adopted by private individuals.

From 1971 to 2012, the BLM has removed over 300,000 wild horses and burros from the range and adopted out more than 230,000 animals.[10,12] However, in recent years the number of animals removed from the range and placed in long-term holding has far exceeded the number adopted or sold; adoption demand has fallen in response to the U.S. economic recession and other factors. According to the BLM's data reports for 2000 through 2012, over 121,000 animals were removed from the range while only 62,000 were adopted or sold. In Fiscal Year (FY) 2012, 66% fewer animals (i.e., 2,598) were adopted compared to FY 2002 when the BLM adopted out 7,746 animals.[10]

The remaining 46,500 animals have been placed in government holding facilities and, as a result, off-the-range wild horse and burro management costs have increased dramatically.[10] In FY 2012 alone, the cost of holding and caring for these animals exceeded $43 million—59% of the $72.4 million in total annual expenditures for the entire wild horse and burro management program.[10]

BLM is committed to caring for these animals, but the costs associated with their care will continue to increase because mortality rates in captivity are low, the average life span of wild horse and burros in captivity is approximately 30 yr, and every year the agency continues to remove more animals from the range and place them in additional holding facilities. This situation will

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result in exponentially increasing holding costs that will continue to rise as holding facilities become scarce and operators increase their prices, a trend now evident. Unless the agency develops and implements long-term management strategies to reduce the number of removals by reducing population growth rates on-the-range, the current removal-only approach seems doomed by inevitable budget constraints off-the-range.

Fertility control is a viable alternative to the current wild horse management regime. In early 2012, the Environmental Protection Agency (EPA) registered the first contraceptive vaccine for wild horses commonly known as porcine zona pellucida (PZP) and registered under the brand name ZonaStat-H.[11] Fertility control techniques and immunocontraception vaccines have been shown to be safe, humane, reversible,[8] and capable of stabilizing and reducing wild horse populations over time, either when used alone or when combined with other wild horse population growth suppression methods.[5,6,9] A United States Geological Survey (USGS) economic study[1] also demonstrated that reducing wild horse population growth rates through the combined use of fertility control drugs and sex ratio adjustments may reduce the need and frequency of removals which, in turn, could lead to long-term reductions in off-the-range management costs. This study estimated that fertility control on-the-range could reduce total wild horse and burro management costs by 14%, saving $6.1 million per year. The question is how to implement a humane and efficient cost-saving management plan.

Currently the BLM uses WinEquus, a stochastic wild horse population model, to evaluate possible management actions and select preferred alternatives, but the model does not calculate any costs associated with various management options.[7] The model cannot optimize management options, compare the combined removal and holding costs of alternative management plans, loop through multiple ranges to help build an efficient overall strategy, or simulate multi-year planning.

To assist the BLM in developing and implementing a long-term plan to maintain healthy wild horse and burro herd populations, a robust economic model to project the costs and outcomes of various management regimes was created which can be used by the agency to plan effective, cost-beneficial programs. The management system is designed to simulate range populations and alternative BLM management interventions and costs in either a single HMA or over multiple HMAs. It employs not only simulation methods but also optimization. Thus, it can help make sound management decisions under uncertainty, including allocating resources and reducing costs. The modeling incorporates stochastic simulations, conventional optimization, and stochastic optimization. There are four sub-models in the overall model framework: The Dynamic Population Simulation Model, The Economic Costing Model, The Management Intervention and Optimization Model, and The Population and Range Database System.

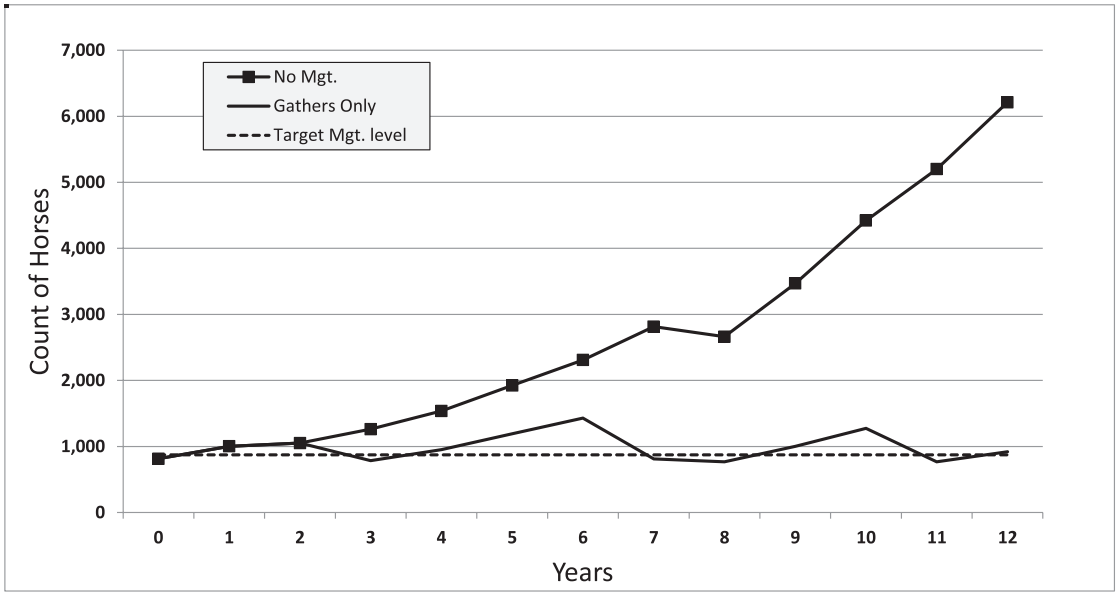
Recognizing the sparse data currently available for wild horse populations, the database system incorporates techniques for simulating missing data. It also guides the user to track range management results and enter additional data as the years pass, helping to initiate sound data collection procedures on the range and from contractors hired to conduct gathers. As with range data, missing cost items can be approximated or simulated using appropriate BLM assumptions.

BLM could use the model to decide on the optimum number and locations of round-ups, age–sex gather targets, fertility control elements, and remove:release ratios in order to allocate a fixed total budget, thus selecting the "best" limited set of gathers, or allocate to minimize cost in a single year or over multiple years to reach annual or overall population targets. The model might also optimize to control costs by suggesting adjustments to consider to HMA target populations or AMLs.

To demonstrate, using a hypothetical HMA with a target population of 874 animals, initial model runs over a 12-yr period demonstrate that the "no management" alternative, meaning no round-ups, removals, or contraception (or any combination thereof), is incapable of maintaining the target population and would lead to an exponential population growth rate that is not desirable or sustainable, as the population would rise from under 874 to over 6,000 in 12 yr (Fig. 1a, b). A model run of a typical "removal-only" management regime, where animals are removed from the HMA at years 3, 7, and 11, shows that such a scenario is capable of maintaining a population at or near the target population level (Fig. 1a.) but at a cost of over $13 million over 12 yr (Table 1).

A lower cost approach uses a contraception-and-removal management program where during a gather some animals are removed, selected by age and sex, but a high proportion of the mare population is treated with fertility control drugs and released. Population projections for years 3,

a.



b.

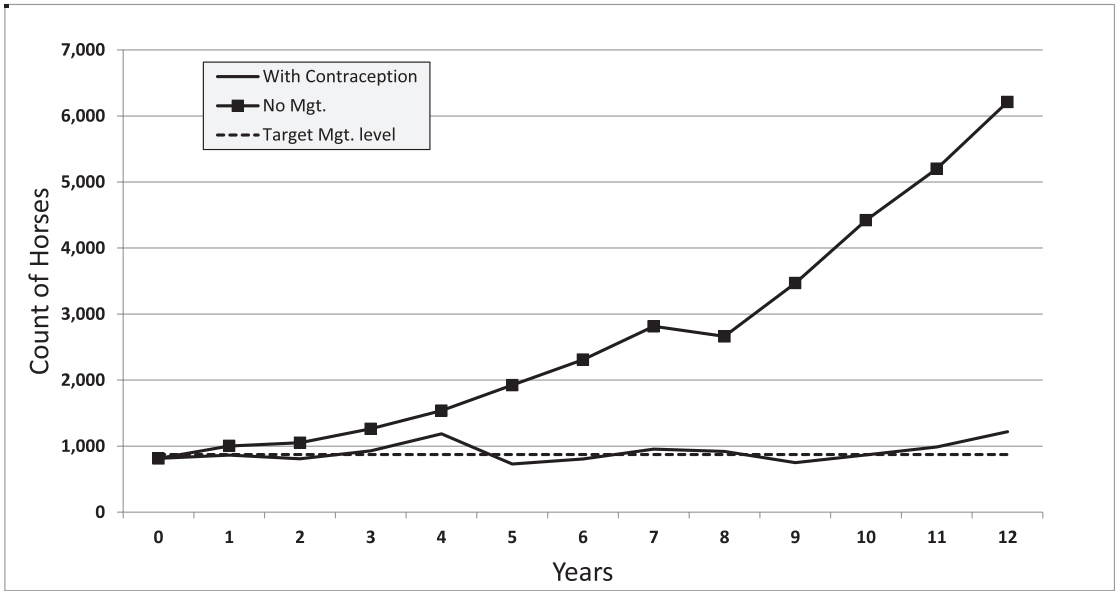


Figure 1. Example economic model output for a hypothetical Herd Management Area (HMA) with a target management population of 874 animals. The model simulates the difference in population growth rates over a 12-yr period when no management is conducted on the HMA versus (**a**) removal-only and (**b**) contraception-and-removals at years 3, 7, and 11.

7, and 11 show that such a management scheme will both maintain the target population level (Fig. 1b.) and reduce management costs by approximately $8 million over 12 yr—or $5.9 million present value compared to the "removal only" scenario (Table 1). Note that even a suboptimal effectiveness of PZP can achieve substantial population control and cost savings.

The model is flexible and, while these demonstration runs are based on realistic working assumptions provided by experts in the field, virtually all inputs can be adjusted to see the

Table I. Example of economic model output results of 12-yr contraception-and-removals versus removals-only scenarios.

	Contraception and removals	Removals only	Change (%)
Total horses to holding over 12 yr	798	1,790	−55.4
Average range pop. over 12 yr	910	982	−7.3
AML[a]	874	874	
Average difference from AML (%)	4.1	12.4	
Total gather and treatment costs	$2,435,523	$2,634,579	−7.6
Total holding costs	$5,560,426	$10,694,712	−48.0
Total cost (sum of years)	$7,995,949	$13,329,290	−40.0
Total cost (present value)	$5,852,135	$9,314,090	−37.2

[a] AML = appropriate management levels.

impact of other conditions, to test the limits of the model, the risks of other outcomes, or to use other assumptions. The examples here describe the population in an individual HMA while the gold-standard model would be a simultaneous collection of model runs for multiple, or all, HMAs in order to calculate the full population impact and the total cost to the agency's budget.

BLM estimates that there are approximately 38,000 wild horses and burros roaming on 179 HMAs located on public rangelands in 10 Western states. If about $8 million could be saved by implementing the proposed management scheme at one HMA over 12 yr, it stands to reason that such an approach could result in a cost-savings of tens of millions of dollars, if applied across all HMAs, recognizing the size differences among ranges.

Given current budget constraints imposed on federal and state wildlife and land management agencies, the hybrid population and economic model describe here could be used to develop and support the implementation of cost-effective and long-term wildlife and land management programs. It is clear to us that BLM and other agencies should invest more resources into both modeling and population growth suppression methods.

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ORIGINAL ARTICLE

The Heart: A Novel Gonadotrophin-Releasing Hormone Target

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Gonadotrophin-releasing hormone (GnRH) is a hypothalamic hormone transported by the hypophyseal portal bloodstream to the pituitary gland, where it binds to GnRH receptors. However, GnRH receptors are expressed in multiple extrapituitary tissues, although their physiological relevance is not fully understood. GnRH agonists are employed extensively in steroid deprivation therapy, especially to suppress testosterone in prostate cancer. Because GnRH agonist treatment is associated with increased coronary heart disease and myocardial infarction, we investigated the impact of GnRH on cardiomyocyte contractile function. Cardiomyocytes were isolated from mouse hearts and mechanical and intracellular Ca^{2+} properties were evaluated, including peak shortening amplitude (PS), time-to-PS (TPS), time-to-90% relengthening (TR_{90}), maximal velocity of shortening/relengthening (± dLdt), electrically-stimulated rise in Fura-2 fluorescence intensity (ΔFFI) and Ca^{2+} decay. GnRH (1 ng/ml) increased PS, ± dL/dt, resting FFI and ΔFFI, and prolonged TPS, TR_{90} and Ca^{2+} decay time, whereas 1 pg/ml GnRH affected all these cardiomyocyte variables, except TPS, resting FFI and ΔFFI. A concentration of 1 fg/ml GnRH and the GnRH cleavage product, GnRH-[1–5] (300 pg/ml), had no effect on any cardiomyocyte parameter. The 1 pg/ml GnRH-elicited responses were attenuated by the GnRH receptor antagonist cetrorelix (10 μM), the protein kinase A (PKA) inhibitor H89 (1 μM) but not the protein kinase C inhibitor chelerythrine chloride (1 μM). These data revealed that GnRH is capable of regulating cardiac contractile function via a GnRH receptor/PKA-dependent mechanism. If present in the human heart, dysfunction of such a system may play an important role in cardiac pathology observed in men treated with GnRH agonists for prostate cancer.

Key words: GnRH, cardiomyocyte shortening, intracellular Ca^{2+}, PKA, PKC.

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Prostate cancer is the most common noncutaneous cancer in the USA and is second only to lung cancer in carcinoma-associated mortality. Because most prostate cancers are androgen-dependent, it is essential to suppress circulating testosterone. Continuous exposure to GnRH causes the pituitary gland to desensitise to the stimulatory signal, resulting in reduced gonadotrophin release and, consequently, testosterone secretion. Gonadotrophin-releasing hormone (GnRH) agonists are thus used therapeutically to chemically castrate men. This treatment is accompanied by various side effects (hot flashes, weight gain and decreased libido), and has recently been associated with an overtly increased cardiovascular risk, although this effect was ascribed to the loss of circulating testosterone (1–3). However, an ensemble of epidemiological studies has provided convincing evidence of a significant increase in the prevalence of coronary heart disease, myocardial infarction and sudden cardiac death in patients receiving GnRH agonist treatment compared to untreated and castrated men (4–8), resulting in an Food and Drug Administration warning (20 October 2010). In addition, oral anti-androgen monotherapy is not associated with negative cardiac outcomes (9), favouring the hypothesis that the GnRH agonists *per se*, rather than the loss of androgenic effects, are directly responsible for the negative health outcome.

Similar to many other hypothalamic peptides, the attributed function of GnRH focuses on gonadotrophin release from the pituitary (10). However, the peptide hormone and its receptor are synthesised in several sites outside of the hypothalamus and pituitary gland, including other regions of the brain (11–13), the reproductive

system (12,14,15), bladder (16–20), spleen (21), pancreas (22) and heart (23,24). Nonetheless, the physiological significance of GnRH within these extrapituitary sites remains unclear. Accordingly, the present study was designed to examine the impact of GnRH exposure on murine cardiomyocyte contractile function and intracellular Ca^{2+} homeostasis. This model is particularly suitable for this purpose because neither GnRH II nor the GnRH type II receptor is synthesised in mice (25).

Materials and methods

Isolation of adult murine cardiomyocytes

The experimental procedure used in the present study was approved by the University of Wyoming Animal Use and Care Committee (Laramie, WY, USA). Adult male FVB mice (approximately 22 g) were anaesthetised with ketamine/xylazine (5 : 3, 1.32 mg/kg i.p.). Hearts were rapidly removed and perfused (at 37 °C) through the aorta with Krebs–Henseleit bicarbonate (KHB) buffer (in mM: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 HEPES, 11.1 glucose, pH 7.4). The hearts were then perfused via the aorta for 15 min with KHB containing Liberase Blendzyme 4 (Hoffmann-La Roche, Indianapolis, IN, USA). After perfusion, left ventricles were removed and minced. Dispersed cardiomyocytes were filtered through a nylon mesh (300 μm). Extracellular Ca^{2+} was added slowly and incrementally to 1.25 mM over 30 min. A total of 12 mice were used for this study.

Cell shortening/relengthening

Mechanical properties of cardiomyocytes were assessed using an IonOptix™ soft-edge system (IonOptix, Milton, MA, USA). Myocytes were placed in a chamber mounted on the stage of an Olympus IX-70 microscope (Olympus, Tokyo, Japan) and superfused (approximately 2 ml/min at 25 °C) with a KHB buffer containing 1 mM $CaCl_2$. Mammalian GnRH (GnRH 1; American Peptide Co, Sunnyvale, CA, USA; 1 ng/ml, 1 pg/ml, 1 fg/ml or no GnRH in KHB buffer) was applied for 10 min before the myocytes were field stimulated at 0.5 Hz (80 V) for the duration of 3 ms. Cell shortening and relengthening were assessed, including peak shortening (PS), time-to-PS (TPS), time-to-90% relengthening (TR_{90}) and maximal velocities of shortening/relengthening (± dL/dt).

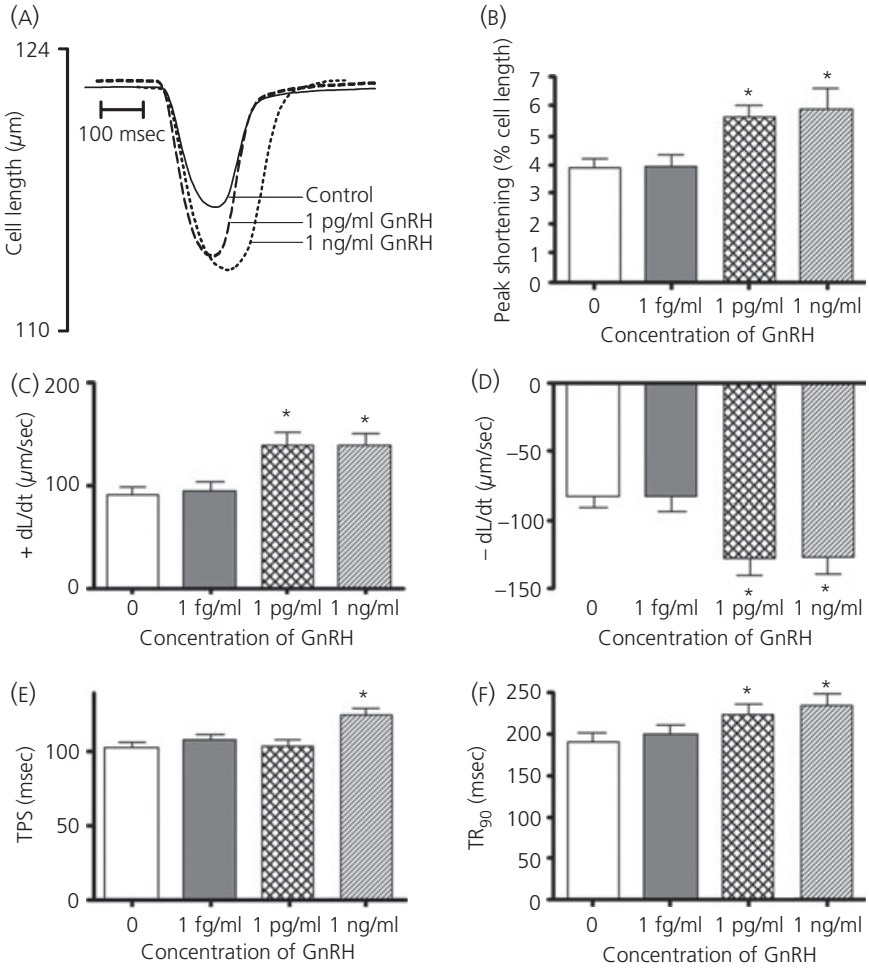


Fig. 1. Effect of acute exposure to gonadotrophin-releasing hormone (GnRH) (1 fg/ml–1 ng/ml) on cardiomyocyte contractile function. (A) Representative traces of cell shortening in the absence or presence of GnRH (1 pg/ml and 1 ng/ml in Krebs–Henseleit bicarbonate); (B) peak shortening normalised to resting cell length; (C) maximal velocity of shortening (+ dL/dt); (D) maximal velocity of relengthening (–dL/dt); (E) time-to-PS (TPS); and (F) time-to-90% relengthening (TR_{90}). Mean ± SEM, n = 74–76 cells per group, *P < 0.05 versus control (0 GnRH).

Effect of cetrorelix, protein kinase inhibitors and GnRH-[1–5]

Both protein kinase A (PKA) and protein kinase C (PKC) have been shown to participate in GnRH-regulated reproductive function (26–29). To determine whether these two kinases and the GnRH receptor *per se* are involved in the GnRH-induced cardiac contractile response, the effect of acute GnRH exposure on cardiomyocyte shortening was re-examined in the presence of the GnRH receptor antagonist cetrorelix (10 μM; Anaspec, San Jose, CA, USA), the PKA inhibitor H89 (1 μM; Calbiochem, San Diego, CA, USA) or the PKC inhibitor chelerythrine chloride (1 μM; Calbiochem). These pharmacological agents were all applied for 20 min alone and then in the presence or absence of GnRH (1 pg/ml) for 10 min before the 0.5-Hz stimulation.

GnRH can be enzymatically cleaved into GnRH-[1–5], which is a pentapeptide with biological activity independent of the GnRH receptor (30). To investigate whether the observed effects could be mediated by this pentapeptide, GnRH-[1–5] was synthesised (Michael Flora, Biomedical Instrumentation Center, Uniformed Services University of the Health Sciences, Bethesda, MD, USA) and applied (300 pg/ml) to cardiomyocytes for 10 min before the 0.5-Hz stimulation.

Intracellular Ca^{2+} transients

A cohort of myocytes was loaded with Fura-2/AM (0.5 μM; 10 min) and fluorescence intensity was recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). Myocytes were placed onto an Olympus IX-70 inverted microscope and imaged through a Fluor × 40 oil objective. GnRH (1 ng/ml, 1 pg/ml, 1 fg/ml or no GnRH in KHB buffer) was applied for 10 min before electrical stimulation. Cells were exposed to light emitted by a 75-W lamp and passed through either a 360 or 380 nm filter, at the same time as being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected in the range 480–520 nm and qualitative change in Fura-2 fluorescence intensity (FFI) was inferred from the FFI ratio at the two wavelengths (360/380 nm). Fluorescence decay time was calculated as an indicator of $[Ca^{2+}]_i$ clearing (11).

Statistical analysis

Data are presented as the mean ± SEM. Statistical significance ($P < 0.05$) for each variable was independently estimated by analysis of variance (ANOVA) followed by a Tukey's post-hoc analysis to correct for the probability of making a type I error.

Results

Effect of GnRH exposure on cardiomyocyte contractile and intracellular Ca^{2+} response

Acute exposure (10 min) of cardiomyocytes to GnRH had no effect on resting cell length (data not shown). Although GnRH at 1 fg/ml had no effect on cardiomyocyte contractile function, GnRH at the levels of 1 pg/ml and 1 ng/ml significantly increased PS and ± dL/dt, as well as prolonged TPS and TR_{90} in murine cardiomyocytes (Fig. 1). The GnRH-induced cellular response was maximal within 10 min of exposure and was maintained for up to 60 min.

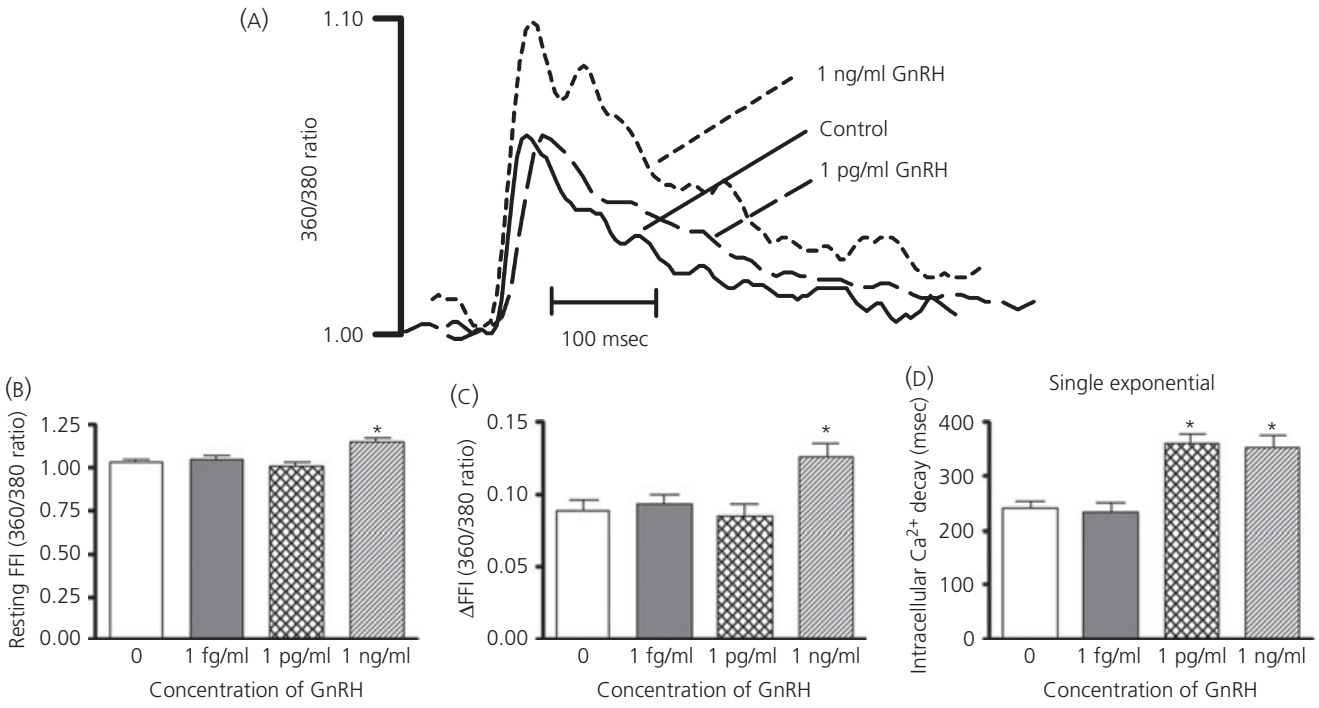


Fig. 2. Effect of acute exposure to gonadotrophin-releasing hormone (GnRH) (1 fg/ml–1 ng/ml) on intracellular Ca^{2+} homeostasis. (A) Representative traces depicting the changes in intracellular Ca^{2+} in response to a 0.5 Hz stimulus in the presence of different concentrations of GnRH; (B) resting Fura-2 fluorescence intensity (FFI); (C) electrically-stimulated rise in FFI (ΔFFI) and (D) single exponential decay of intracellular Ca^{2+}. Mean ± SEM, n = 60 cells per group, *$P < 0.05$ versus control (0 GnRH).

These responses were reversible upon a 20 min washout (data not shown). To explore the possible mechanism of action behind GnRH-induced mechanical response, intracellular Ca^{2+} homeostasis was monitored using the fluorescent dye Fura-2/AM. The results shown in Fig. 2 revealed that higher doses of GnRH elevated resting intracellular Ca^{2+} and electrically-stimulated rise in intracellular Ca^{2+} as well as prolonged intracellular Ca^{2+} decay.

Effect of cetrorelix, protein kinase inhibitors and GnRH-[1–5]

In the absence of GnRH, cetrorelix, H89 and chelerythrine chloride had no overt effect on cell shortening over 30 min (Fig. 3). The GnRH-induced cardiomyocyte mechanical responses were abolished or significantly attenuated by cetrorelix and H89 but not chelerythrine,

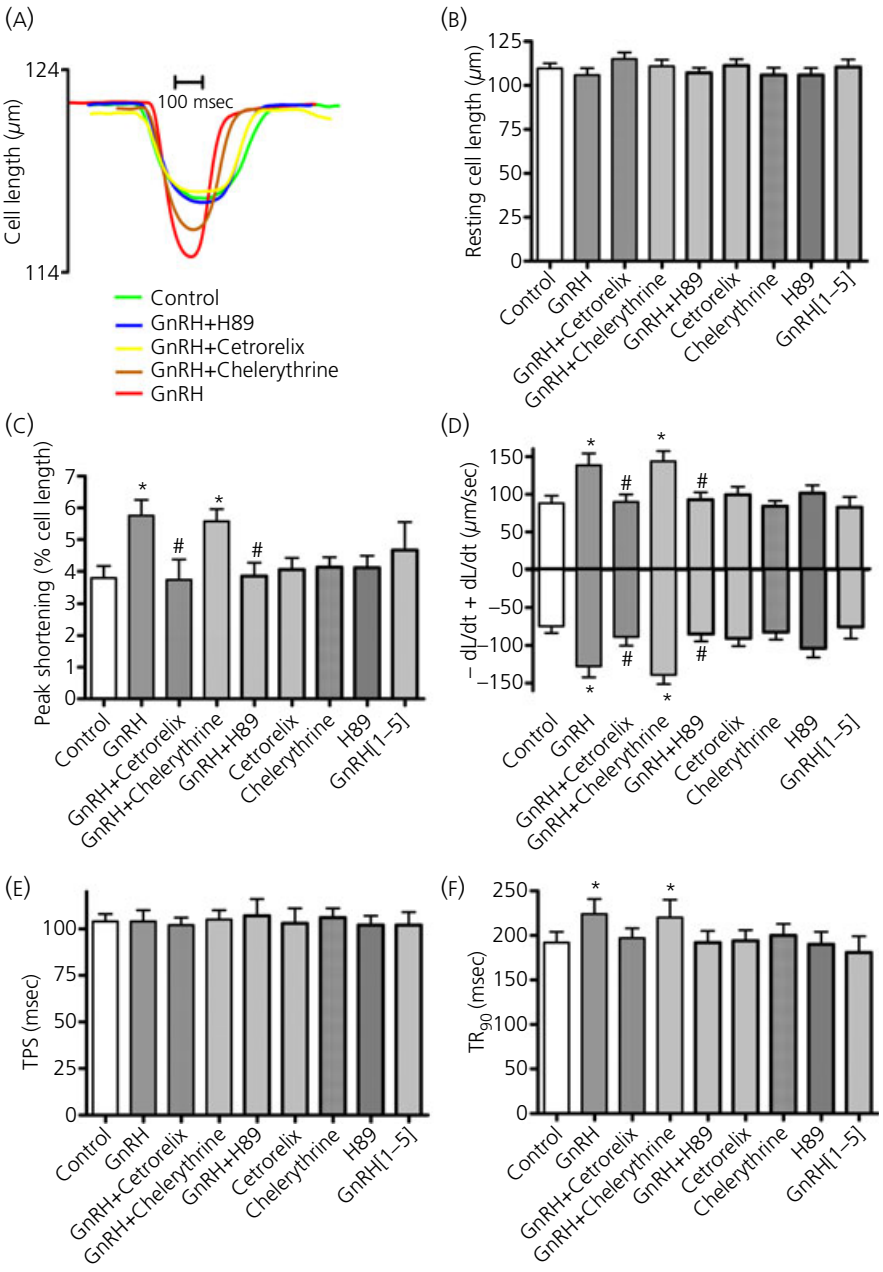


Fig. 3. Effect of the gonadotrophin-releasing hormone (GnRH) receptor antagonist cetrorelix (10 μM), the protein kinase C inhibitor chelerythrine chloride (1 μM) and the protein kinase A inhibitor H89 (1 μM) on acute GnRH (1 pg/ml) exposure-induced cardiomyocyte contractile response. Effect of the truncated form of GnRH (300 pg/ml GnRH-[1–5]) was also exhibited. All pharmacological inhibitors were applied 10 min before GnRH addition. (A) Representative traces depicting GnRH-elicited cell shortening response in the absence or presence of pharmacological inhibitors; (B) resting cell length; (C) peak shortening normalised to resting cell length; (D) maximal velocity of shortening/relengthening (± dL/dt); (E) time-to-PS (TPS); and (F) time-to-90% relengthening (TR_{90}). Mean ± SEM, n = 37–47 cells per group, *P < 0.05 versus control; #P < 0.05 versus GnRH group.

suggesting an involvement of GnRH receptor and PKA (but unlikely PKC) in the GnRH-elicited cardiomyocyte contractile responses. GnRH-[1–5] had no effect on cardiomyocytes (Fig. 3). Similar to the cardiomyocyte mechanical responses, the GnRH-elicited increase in electrically-stimulated intracellular Ca^{2+} (ΔFFI) and prolongation in intracellular Ca^{2+} decay were abolished by H89, but not chelerythrine, thus providing further support for the involvement of PKA (but unlikely PKC) in the GnRH-elicited intracellular Ca^{2+} response in cardiomyocytes (Fig. 4).

Discussion

The present study suggests that that GnRH has positive cardiomyocyte contractile effects in the mouse. This effect appears specific to the intact GnRH molecule because the enzymatically cleaved GnRH product, GnRH-[1–5], a pentapeptide with biological activity independent of the GnRH receptor (30), failed to affect cardiomyocyte mechanical responses, whereas the GnRH type I receptor antagonist, cetrorelix, blocked the GnRH-mediated mechanical response. These observations favour a GnRH receptor-mediated mechanism for the GnRH-modulated cardiac contractile response. The GnRH treatments used in the present study correspond well with the circulating concentrations of a GnRH agonist (leuprorelin) produced by GnRH agonist therapies. Specifically, leuprorelin concentrations of 430–190 pg/ml were evident after a 3-month depot Lupron implant and 930 pg/ml after a 12-month implant (31). Similarly, a different 4-month leuprorelin-releasing implant produced mean serum levels between 1 ng/ml and 100 pg/ml, from day 3 to day 112, with individuals ranging from 50 pg/ml to 5 ng/ml (32). However, it remains to be determined whether GnRH has a physiologically important cardiac role in mammals or whether such effects have become vestigial and only become evident in the face of excess exposure to GnRH or its agonists.

The GnRH-elicited cardiomyocyte contractile response in mice is reminiscent of that seen in isolated bullfrog aortas (33) and guinea pig papillary muscles (34). In rats, GnRH was reported to increase heart rate and blood pressure (35), whereas GnRH antagonists trigger hypotension and bradycardia (36). However, these haemodynamic responses were attributed to indirect effects of GnRH in the cardiovascular system (35,36). In male rats treated for 2 months with the GnRH agonist, goserelin, the mean and maximal aortic velocities (37), as well as the end systolic pressure (38), were significantly decreased, with the effects being attributed to the loss of steroids. Most recently, Wojniusz *et al.* (39) reported that chronic

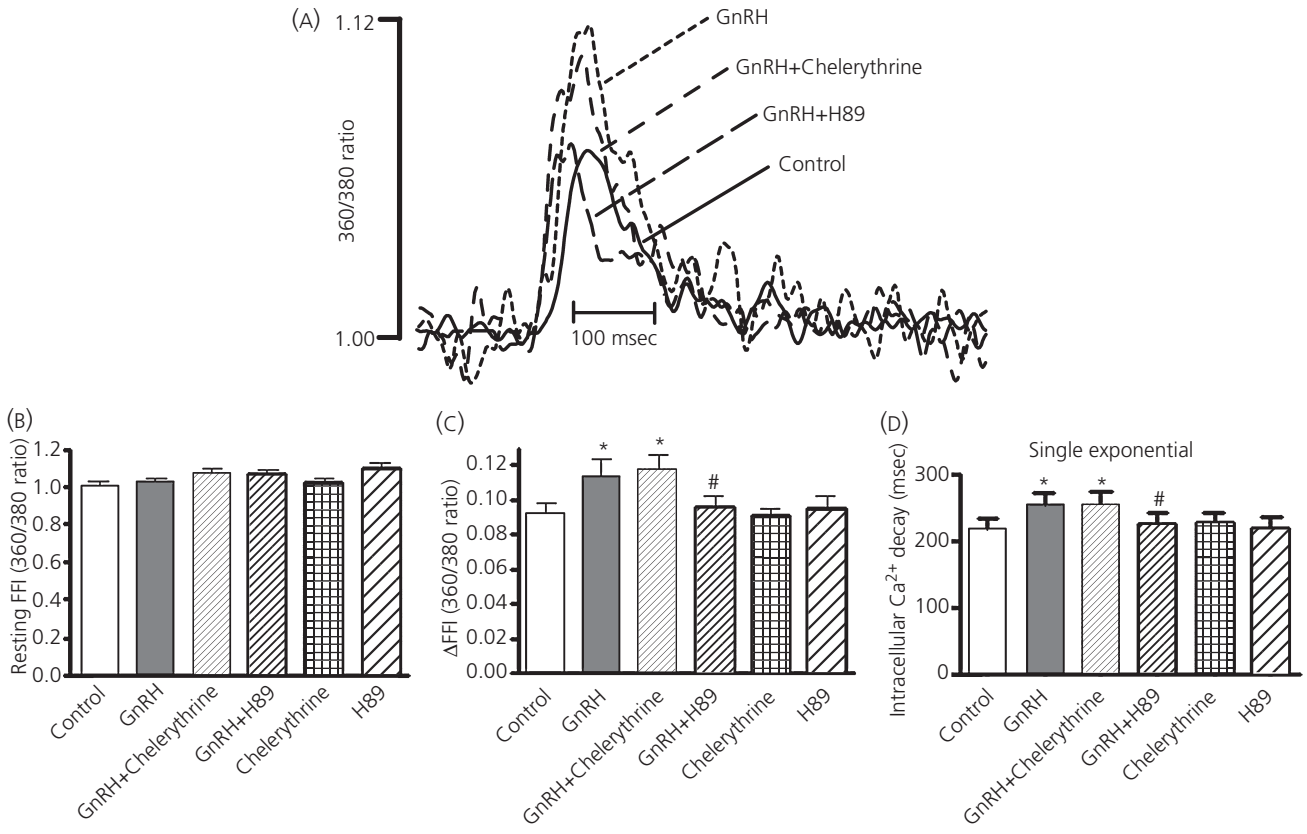


Fig. 4. Effect of the protein kinase C inhibitor chelerythrine chloride (1 μM) and the protein kinase A inhibitor H89 (1 μM) on acute gonadotrophin-releasing hormone (GnRH) (1 pg/ml) exposure-induced cardiomyocyte intracellular Ca^{2+} homeostasis. All pharmacological inhibitors were applied 10 min before GnRH addition, which was added 10 min before the 0.5-Hz stimulation. (A) Representative traces depicting GnRH-elicited intracellular Ca^{2+} transient response in the absence or presence of pharmacological inhibitors; (B) resting Fura-2 fluorescence intensity (FFI); (C) electrically-stimulated rise in FFI (ΔFFI); and (D) single exponential decay of intracellular Ca^{2+}. Mean $\pm$ SEM, n = 57–58 cells per group, *P < 0.05 versus control; #P < 0.05 versus GnRH group.

(10 months) exposure to goserelin increased heart rate variability in male sheep, although it was not determined whether this effect was direct or dependent on testosterone.

Evidence concerning the hemodynamic and cardiac effects of GnRH in humans is sparse. Reports vary from little effect of acutely administered GnRH on heart rate and blood pressure (40), to a notable increase in heart rate (41). In the setting of chronically administered GnRH agonists, it was suggested that the electrocardiographic QT interval may be prolonged in men (42) and, in women, peak flow velocity and cardiac index (stroke volume × heart rate) were significantly reduced after 1 month of treatment (43). Both systolic and diastolic blood pressure were decreased following 3 months of Lupron in women and, notably, this was not prevented with oestrogen replacement (44). As indicated earlier, a plethora of evidence suggests that GnRH treatment may be associated with an increased risk of cardiac dysfunction (1–3,45–47). Such phenomenon of compromised heart function accompanying GnRH therapy is usually attributed to the accompanying androgen deprivation (1–3). However, Keating *et al.* (4,9) provide compelling support for the hypothesis that GnRH *per se* may be a major contributor to the cardiac pathology.

The present study suggests that the PKA pathway (but not the PKC pathway) is involved in the GnRH-mediated increased contractility. In the pituitary gland, the inositol trisphosphate-PKC pathway is known to be the major intracellular pathway activated by GnRH to induce secretion from gonadotrophs (26), although there is accumulating evidence that GnRH also activates PKA within gonadotrophs to affect nonsecretory responses (27–29). It is noteworthy that a previous study suggested the involvement of PKA in a GnRH-associated cardiac response (48). PKA has multiple targets for phosphorylation in cardiomyocytes, including phospholamban (49), the L-type Ca^{2+} channel on the sarcolemma (50) and components of the contractile apparatus (51). PKA also phosphorylates the ryanodine receptor/Ca^{2+} release channel in cardiomyocytes, which in turn regulates the channel opening probability and leads to increased sensitivity to Ca^{2+}-induced activation (52). In the present study, higher doses of GnRH elevated resting intracellular Ca^{2+} and, together with the electrically (0.5 Hz)-stimulated rise in intracellular Ca^{2+}, as well as the prolonged intracellular Ca^{2+} decay, this may be a reflection of increased sarcoplasmic reticulum Ca^{2+} release and, consequently, cardiac contractility.

GnRH receptors have been reported in the heart of the sea hare, *Aplysia californica* (53) (P.S. Tsai, personal communication), and octopus, *Octopus vulgaris* (54,55), where GnRH is shown to exert powerful cardiac effects (56,57), and is well documented in piscine cardiac tissue (58–62). GnRH analogue binding has been reported in rodent hearts (13,63–68) and GnRH receptor mRNA has been noted in the rat (69) and dog (Navratil AM, Roberson MS, personal communication). GnRH receptor mRNA (23,70) and immunoreactive receptor (24,71) have also been demonstrated in human hearts. Taken together, there is compelling evidence for an evolutionarily conserved role for GnRH in the heart.

In summary, our data favour that GnRH promotes cardiomyocyte contractile function through a GnRH receptor/PKA-mediated pathway. The accumulating evidence of multiple extrapituitary GnRH targets allows the consideration of a broader evolutionary hypothesis suggesting that GnRH may not have evolved solely with a reproductive function (53). Moreover, as proposed three decades ago (72), the hypophyseal portal system may not only have evolved solely as a means to deliver hypothalamic peptides to the pituitary gland, but also as a way to restrict their delivery precisely to that target.

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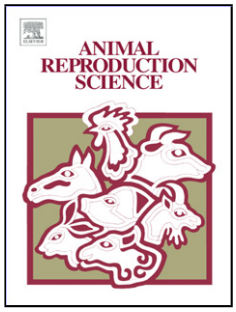
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Effects of a commercial canine gonadotropin releasing hormone vaccine on estrus suppression and estrous behavior in mares

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15 **Contents**

16 We investigated the effect of immunization against gonadotropin releasing hormone
17 (GnRH) using a commercial canine GnRH vaccine on estrus suppression and unwanted estrous
18 behavior in mares. In experiment 1, mares were immunized (n=6) twice with vaccine (5mL)
19 given intramuscularly 4 weeks apart or received a control diluent (n=5). Transrectal
20 ultrasonographic examination of the reproductive tracts were performed three days a week for 40
21 weeks after initial vaccination. Blood samples were collected weekly for GnRH antibody titer
22 and progesterone concentration determination. In experiment 2, privately-owned mares (n=12)
23 were immunized twice with vaccine (1mL) given intramuscularly 4 weeks apart. Blood samples
24 were collected prior to each vaccination as well as 12 and 20 weeks after initial treatment, and
25 transrectal ultrasonographic examinations of the reproductive tracts were performed 12 weeks
26 after the first vaccination. Vaccinated mares in experiment 1 responded with a GnRH antibody
27 titer, progesterone concentrations significantly lower than controls, and cessation of ovarian
28 activity. Vaccinated mares in experiment 2 also responded with a GnRH antibody titer,
29 progesterone concentrations that remained basal for the duration of the study, and cessation of
30 ovarian activity. Owners of vaccinated mares in experiment 2 reported that the number of
31 unwanted estrous behaviors present before vaccination significantly decreased following
32 vaccination. In conclusion, GnRH immunization using a canine GnRH vaccine is an effective
33 method for suppressing estrus and unwanted estrous behavior.

34

35 Keywords: antibody; estrous cycle; GnRH immunization; horse; immunocontraception;
36 progesterone

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Introduction

Unwanted behavioral changes in mares during estrus affect handling and performance. Reproductive estrous behaviors exhibited by the mare in the presence of a stallion include raising the tail, clitoral eversion, and urinating (Ginther, 1992). However, mares also exhibit non-reproductive estrous behaviors that limit their performance potential, which include hyperexcitability, oversensitivity, abdominal discomfort, and aggression (McDonnell, 1992). Non-surgical estrous behavior suppression is most commonly achieved via daily treatment with an oral progestin, altrenogest (Regu-Mate®, Intervet Inc., Millsboro, DE) (Pryor and Tibary, 2005). However, daily oral administration is costly and can be impractical for horse owners (Burger et al., 2008). In addition, altrenogest is readily absorbed through human skin, which is a potential human safety concern (Hazan, 2011). Also, since altrenogest does not inhibit follicular activity, some mares continue to display unwanted estrous behavior (Pryor and Tibary, 2005).

Gonadotropin releasing hormone (GnRH) controls ovarian activity by regulating the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Therefore, preventing GnRH from stimulating the release of LH and FSH may be a viable approach for the suppression of estrus. Immunization against GnRH to subsequently cease ovarian activity has been investigated in the mare. Two commercial GnRH vaccines, Improvac® (Pfizer Animal Health Australia) and Equity® (Pfizer Animal Health Australia) have demonstrated suppression of reproductive cyclicity (Botha et al., 2008; Elhay et al., 2007; Imboden et al., 2006). Improvac®, labeled for the prevention of boar taint in swine, was shown to suppress ovarian activity in the mare for at least 23 weeks (Botha et al., 2008; Imboden et al., 2006). However, one study reported a high percentage of adverse vaccine reactions (Imboden et al., 2006), while

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the other study reported a small number of transient side effects (Botha et al., 2008). Equity®, labeled for the control of estrus in mares, showed no adverse reactions, a significant decrease in estrous behavior, and ovarian activity suppression for at least three months (Elhay et al., 2007). However, neither of these products are commercially available in the United States, so there remains a need for a safe and cost-effective method for long-term estrus suppression in mares.

In 2004, a commercial GnRH vaccine was launched in the United States (Canine Gonadotropin Releasing Factor Immunotherapeutic®; Pfizer Animal Health USA). This vaccine was labeled for the treatment of benign prostatic hyperplasia in intact male dogs (Pfizer Animal Health, 2004). The vaccine decreases serum testosterone concentrations and testicular volume in intact male dogs (Donovan et al., 2012) and has been used for pregnancy termination in bitches as well (Chew and Purswell, 2010). Recently, this vaccine has also been shown to be effective in male llamas and alpacas, decreasing serum testosterone concentrations, testicular volume and intermale aggressive behavior (Donovan et al., 2013).

The objectives of the current study were to determine (a) the effect of this canine GnRH vaccine on equine ovarian cyclicity and (b) the effect of the canine GnRH vaccine on equine non-reproductive estrous behavior. It was hypothesized that the canine GnRH vaccine would safely and effectively suppress estrus (both ovarian cyclicity and subsequent estrous behavior) for a prolonged duration.

Materials and Methods

Animals

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Eleven mares (mean age 13.36 years) were obtained for experiment 1 and twelve privately-owned mares (mean age 9.91 years) that had histories of displaying unwanted behaviors during the estrous period were recruited for experiment 2. All mares had histories of normal reproductive cyclicity.

Experimental Design

In experiment 1, which began in May (spring in the Northern hemisphere), reproductive tracts were monitored by transrectal palpation and ultrasonography three days a week for four weeks. After initial monitoring, mares were either given 5mL of the canine GnRH vaccine (5 times the labeled canine dose; n=6) or a placebo (n=5) twice four weeks apart. Reproductive tracts continued to be monitored three days a week for 40 weeks after initial vaccination, until spring of the following season, and venous blood samples were collected at each session.

Experiment 2 was a clinical study and began in January-April (winter-early spring in the Northern hemisphere). All mares received 1mL of the canine GnRH vaccine (a similar antigenic mass as the Equity® vaccine; n=12) twice four weeks apart. Venous blood samples were collected prior to vaccination (week 0 and 4) and at weeks 12 and 20. Transrectal ultrasonographic examinations of the reproductive tracts were also performed at week 12.

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Oregon State University (ACUP #3699). For the clinical study (experiment 2), the vaccine was used in an extra-label manner by the attending veterinarian and each owner signed a consent form to participate in the study.

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Vaccine

The canine GnRH vaccine (Canine Gonadotropin Releasing Factor Immunotherapeutic®, Pfizer Animal Health, Exton, PA) consists of the GnRH peptide conjugated to diptheria toxoid as the protein carrier and combined with a proprietary adjuvant of plant-based origin (Hashimi et al., 2008; Russo, 2008). Each 1 mL dose contains 200μg peptide conjugate (Hashimi et al., 2008). The placebo was a sterile diluent provided by the vaccine manufacturer for use in this experiment. Vaccines were administered into the semimembranosus muscle with not more than 2.5 mL of vaccine or diluent injected at one site (experiment 1) or the middle of the neck (experiment 2).

Vaccine Reactions

In experiment 1, mares were monitored twice daily for two days then once daily for one week for adverse reactions after each injection. The injection site was observed visually and digitally for warmth and swelling, and gait was monitored for lameness. In addition, the general appearance, behavior, and appetite of each mare was observed. If any (even subtle) adverse reactions were present, they were noted by investigators. If no adverse reactions were present at the time of each examination, this was also noted by investigators. In experiment 2, mares were monitored for adverse reactions by their owners using the same criteria as experiment 1.

Hormone Analysis

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Blood samples from both experiments were collected into Vacutainer® clot tubes (02-685-A, Fisher Scientific Co.) and centrifuged upon clotting. Sera were separated, aliquoted, and frozen at -20°C until analysis.

In experiment 1, GnRH antibody titers were measured monthly from the time of initial vaccination in June until all mares were seronegative. In experiment 2, titers were measured at the four time points blood samples were collected. Titers were determined by ELISA using a technique modified from Elhay et al. (2007). Pooled serum from unvaccinated horses served as the negative control and pooled serum from vaccinated mares with a known high antibody titer served as the positive control. Briefly, 96-well microtiter plates were coated with 100 µL of 5 µg/mL of LH-RH (71447-49-9, Sigma, St. Louis, MO, USA) in sodium bicarbonate buffer (pH 8.0) at 4ºC overnight. After incubation, plates were washed with phosphate-buffered saline containing 0.05% Tween-20 (TPBS) (pH 8.0). Plates were then incubated for 1 hour at 20ºC with serum samples diluted in a buffer containing 0.5% bovine serum albumin (9048-46-8, Sigma, St. Louis, MO, USA) to yield final serum dilutions ranging from 1:8 to 1:1024. After tapping dry, antibodies were detected using horseradish peroxidase protein G conjugate (HRP) (10-1223, Invitrogen, Camarillo) diluted at 1:2000 in serum dilution buffer for 1 hour at 37ºC. After a final wash with TPBS, HRP was visualized with 100 µL of ABTS peroxidase substrate (50-66-01, KPL, Gaithersburg, MD, USA). Absorbances were read at 405 nm using a spectrophotometer (FLUOstar Omega, BMG Labtech Inc., San Francisco, CA, USA) and each serum sample was measured in duplicate. The cutoff for seropositivity, defined in this study as the upper limit of a 99% confidence interval above the mean negative control level, was calculated using the methods of Frey et al. (1998). Serological results were expressed as the reciprocal of the highest

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144 twofold serial dilution above the calculated cutoff and linearized using a base-2 logarithmic
145 scale.

146 In experiment 1, progesterone concentrations were measured weekly from the time of
147 initial vaccination in June until the end of the breeding season in October for a total of 17 weeks.
148 In experiment 2, progesterone concentrations were measured at the four time points blood
149 samples were collected. Serum samples were analyzed for progesterone using a commercially
150 available kit (Immulite® Progesterone, Siemens) designed for an enzyme-amplified
151 chemiluminescence assay system (Immulite® 1000, Diagnostic Products Corporation) and
152 performed according to the manufacturer's protocol. The interassay coefficient of variation
153 ranged from 5.8% at 7.2 ng/mL to 16% at 0.81 ng/mL; the intraassay coefficient of variation
154 ranged from 6.3% at 7.9 ng/mL to 16% at 0.81 ng/mL, respectively. The detection limit was 0.2
155 ng/mL.

156

157 *Ovarian Activity*

158 Ovarian activity was monitored in experiment 1 mares three days a week for 44 weeks by
159 ultrasonography and transrectal palpation. In experiment 2, ovarian activity was observed by
160 ultrasonography at week 12, during the spring. At each observation, follicle diameter on both
161 ovaries were measured and the presence of a corpus luteum was noted. In experiment 1, each
162 mare was given a weekly score to reflect cyclicity:

163 Score 0: Anestrus-like. Follicles remain < 20 mm in diameter with no corpus luteum
164 present.

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Score 1: Diestrus or estrus-like. Follicles reach > 20mm in diameter, corpus luteum or dominant follicle may be present.

Behavioral Analysis

Mares in experiment 2 were evaluated for the presence of estrous behavior. Owners scored their mare's estrous behavior (pre-vaccination score) reflecting the specific number of behaviors each mare exhibited during estrus. At the end of the study, owners again scored their mare's estrous behavior (post-vaccination score) reflecting the specific number of behaviors that were still present through the duration of the study.

Statistical Analysis

In experiment 1, the presence of a GnRH antibody titer was compared between the vaccination and control group using Fisher's exact test (GraphPad QuickCalcs Software, La Jolla, CA, USA). Ovarian activity scores were analyzed using the non-parametric Wilcoxon rank sum test in SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA). Progesterone concentrations were analyzed as a repeated measure in time design using PROC MIXED in SAS. Fixed effects in the model were whether the animal was vaccinated, time after first vaccination, and the interactions between vaccination and time. A first order heterogeneous autoregressive variance-covariance structure was fitted for repeated measurements within animals.

In experiment 2, estrous behavior was analyzed using the non-parametric sign test in SAS. The presence of a GnRH antibody titer and progesterone concentrations were analyzed as a

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repeated measure in time design using PROC MIXED in SAS. The fixed effect in the model was time after first vaccination. A first order heterogeneous autoregressive variance-covariance structure was fitted for repeated measurements within animals.

Results

Experiment 1

There were no local or systemic vaccine reactions experienced in either the vaccinated or control mares following the first or second injection. All eleven mares demonstrated normal ovarian cyclicity during the four weeks of monitoring pre-vaccination, and control mares continued to cycle normally for the duration of the breeding season (17 weeks). Circulating progesterone fluctuated throughout the season in control mares as expected. Within days after receiving the booster injection, largest mean follicle size began to decrease in the treated mares (Figure 1). By three weeks post-booster, the largest mean follicle size was consistently < 20mm. In addition, no corpora lutea were present based upon ultrasonographic evaluation and serum progesterone concentrations (Figure 2) for all six vaccinated mares throughout the breeding season. Ovarian activity scores for vaccinated mares were significantly less than controls ($p < 0.0001$).

Of the vaccinated mares, 50% (3/6) resumed normal cyclicity in the following spring, 40 weeks after the initial vaccination. The remaining mares displayed continued cessation of ovarian cyclicity. Control mares resumed cyclicity in the following spring.

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All mares were seronegative for antibodies against GnRH prior to the first vaccination and all control mares remained seronegative for the duration of the study. Compared to control mares, antibody titers of vaccinated mares were significantly greater post-vaccination (Figure 3). One mare had an antibody titer until week 20, three mares had an antibody titer until week 32, one mare had an antibody titer until week 36, and one mare maintained an antibody titer for the duration of the study (40 weeks). The three mares that did not resume normal cyclicity the following spring had antibody titers that lasted 32, 36, and 40 weeks, respectively.

Experiment 2

There were no local or systemic vaccine reactions experienced following the first or second injection. Of the 12 mares vaccinated, all but one was examined by transrectal ultrasonography 12 weeks after the initial vaccination, during the spring. All of these mares had follicles < 20mm and no corpora lutea were present based upon ultrasonographic evaluation.

As expected, progesterone concentrations were basal (<1.0 ng/mL) in all mares when they were vaccinated initially during the winter-early spring. However, progesterone concentrations remained basal through the late spring and early summer (weeks 12 and 20). All mares were also seronegative for antibodies against GnRH prior to the first vaccination and all developed a GnRH antibody titer post-vaccination that peaked at week 12 (Figure 4).

Owner pre-vaccination and post-vaccination estrous behavior scoring was available from nine mares (Table 1). Common behaviors reported included poor performance under saddle, distractability, irritability, aggression towards other horses or handler, and frequent attempts to evade work. Estrous behaviors diminished completely in all mares, a significant decrease

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227 compared to pre-vaccination (p=0.004) (Table 1). Furthermore, all owners reported that they
228 would be interested in yearly revaccination.

229

230 **Discussion**

231 In the United States, there is a need for a safe, effective, and long-lasting method to
232 suppress estrous ovarian cyclicity and subsequent unwanted estrous behavior in mares. This
233 study demonstrated that immunizing mares against GnRH using a vaccine labeled for dogs safely
234 elicited GnRH antibody formation that suppressed estrus. The clinical study component also
235 demonstrated owner satisfaction with the product.

236 Forty weeks after initial vaccination, 50% of the vaccinated mares in experiment 1
237 returned to normal cyclicity whereas the remaining 50% of mares had sustained estrus
238 suppression. Differences in individual responses with regard to estrus suppression in mares was
239 also observed when using Equity® (Elhay et al., 2007) and Improvac® (Imboden et al., 2006) as
240 well as a non-commercial GnRH vaccine (Dalin et al., 2002). Variation in response to GnRH
241 immunization has also been reported in other female species, including the queen (Levy et al.,
242 2011), heifer (Prendiville et al., 1995), and deer (Miller et al., 2000). It has been speculated that
243 genetic differences among individual animals is responsible for variations in immune response
244 (Miller et al., 2000); however, what these differences are has not yet been elucidated.
245 Regardless, differing individual responses should be expected when immunizing against GnRH.

246 The reversibility and the effect of GnRH immunization on fertility is unknown. Equity®
247 does not recommend vaccinating mares later intended for breeding (Pfizer Animal Health
248 Australia, 2008) considering response to vaccine is variable. One study investigating the

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reversibility of Improvac® in mares found that 47/51 mares returned to normal cyclicity by 103 weeks after initial vaccination with a mean of 60 weeks; of the 4 mares that were still not cycling, all were ≤ 4 years of age (Schulman et al., 2013). Another study observed the effect of Equity® on fertility and achieved high rates of pregnancy for the two seasons following the season the mares were vaccinated (Card et al., 2007). While the current study was unable to continue monitoring the vaccinated mares that did not resume cyclicity, the three vaccinated mares that did resume cycling the following spring were artificially inseminated to determine whether their fertility had been compromised. Pregnancy was achieved in all three mares on the first cycle, which was in agreement with the findings regarding fertility following use of Equity® (Card et al., 2007).

With regards to behavior, mares display estrous behaviors even when they are not in the vicinity of a stallion, and these behaviors adversely affect performance (Jorgensen et al., 1996; Spiker, 2009). While these non-reproductive estrous behaviors are variable between mares, individuals have a relatively consistent style of estrus from one cycle to the other (Pryor and Tibary, 2005), allowing for owners to make definitive differentiations between non-reproductive estrous behaviors and other unrelated behaviors. The most frequently reported unwanted estrous behaviors in this study are in concordance with other reported behaviors as a result of estrus in performance mares (Jorgensen et al., 1996). By demonstrating diminished non-reproductive estrous behaviors in multiple mares as recognized by the owners, we were able to establish high owner satisfaction with the product and a desire to continue yearly vaccination regimens.

In conclusion, immunization against GnRH using Canine Gonadotropin Releasing Factor Immunotherapeutic® is a safe, effective, and long-lasting method for suppressing estrus in mares. Variability in duration of suppressed estrus is to be expected.

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Conflict of interest statement

The authors have declared no conflicts of interest.

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344 **Tables**

Mare	Number of Estrous Behaviors Present Pre-Vaccination	Number of Estrous Behaviors Present Post-Vaccination
1	3	0
2	2	0
3	1	0
4	3	0
5	2	0
6	4	0
7	2	0
8	2	0
9	3	0

345 Table 1. Number of estrous behaviors present pre- and post-vaccination. There was a significant
346 overall decrease in behavior score (p=0.004).

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Figure Legends

Figure 1. Mean largest follicle diameter (±SD) in control (□) and vaccinated (●) mares, measured three days a week until 3 out of 6 vaccinated mares and all control mares resumed cyclicity (44 weeks total). Dotted lines indicate times of vaccination.

Figure 2. Progesterone concentrations (Mean±SD) in control (□) and vaccinated (●) mares, measured every week from time of initial vaccination (week 0) to the end of the breeding season (week 17). There was an overall significant difference in progesterone concentration between vaccinated and control mares ($p<0.0001$).

Figure 3. GnRH antibody titer (Mean±SD) in control (□) and vaccinated (●) mares prior to each injection (0 and 4 weeks) and at weeks 8-36 following initial treatment. $^{*}p<0.05$ compared to controls.

Figure 4. GnRH antibody titer (Mean±SEM) in experiment 2 mares, measured prior to each injection (0 and 4 weeks) and at weeks 12 and 20. $^{*}p<0.05$ compared to week 0.

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Figure 1

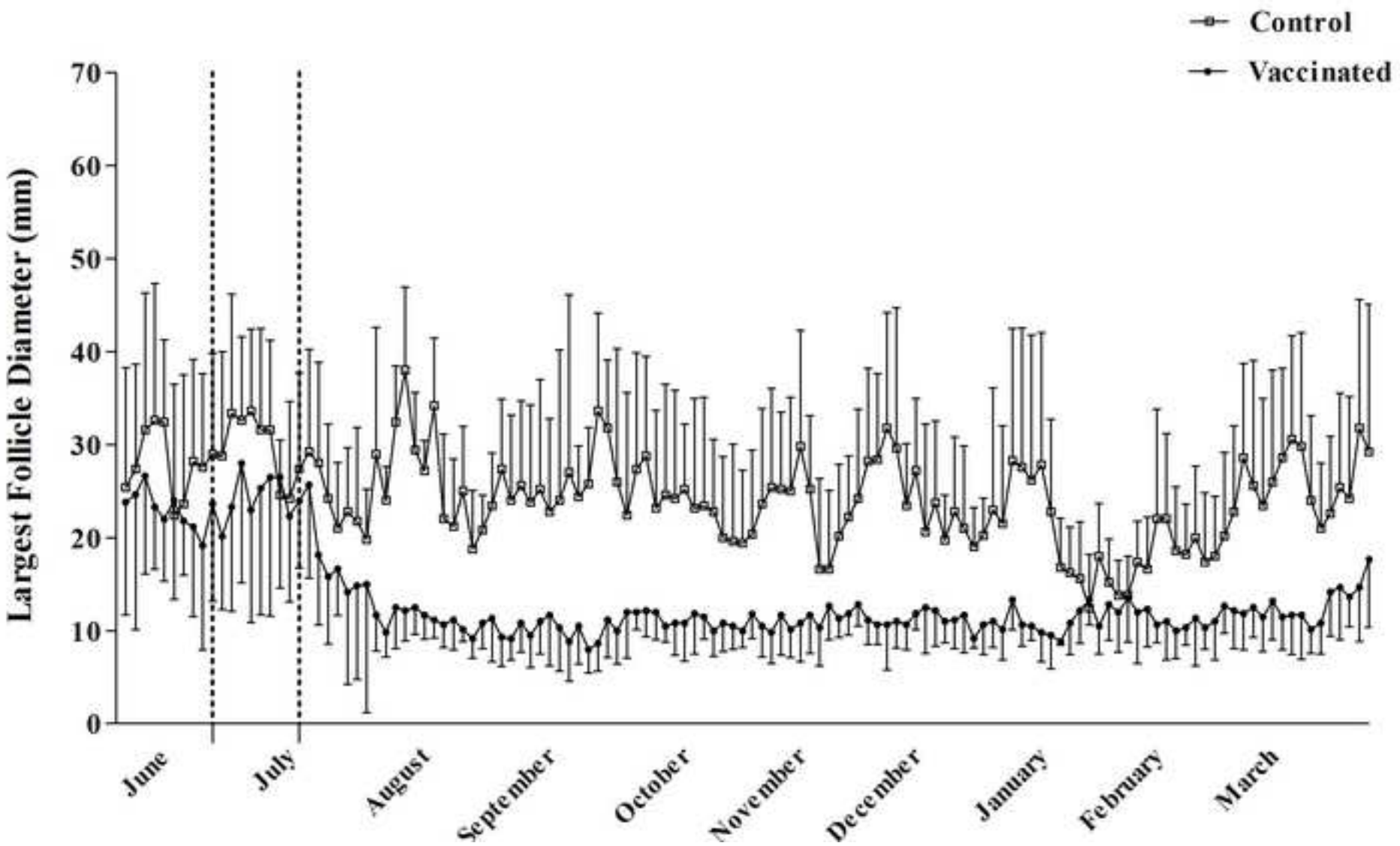

Control
Vaccinated
Largest Follicle Diameter (mm)
70
60
50
40
30
20
10
0
June
July
August
September
October
November
December
January
February
March

Figure 2

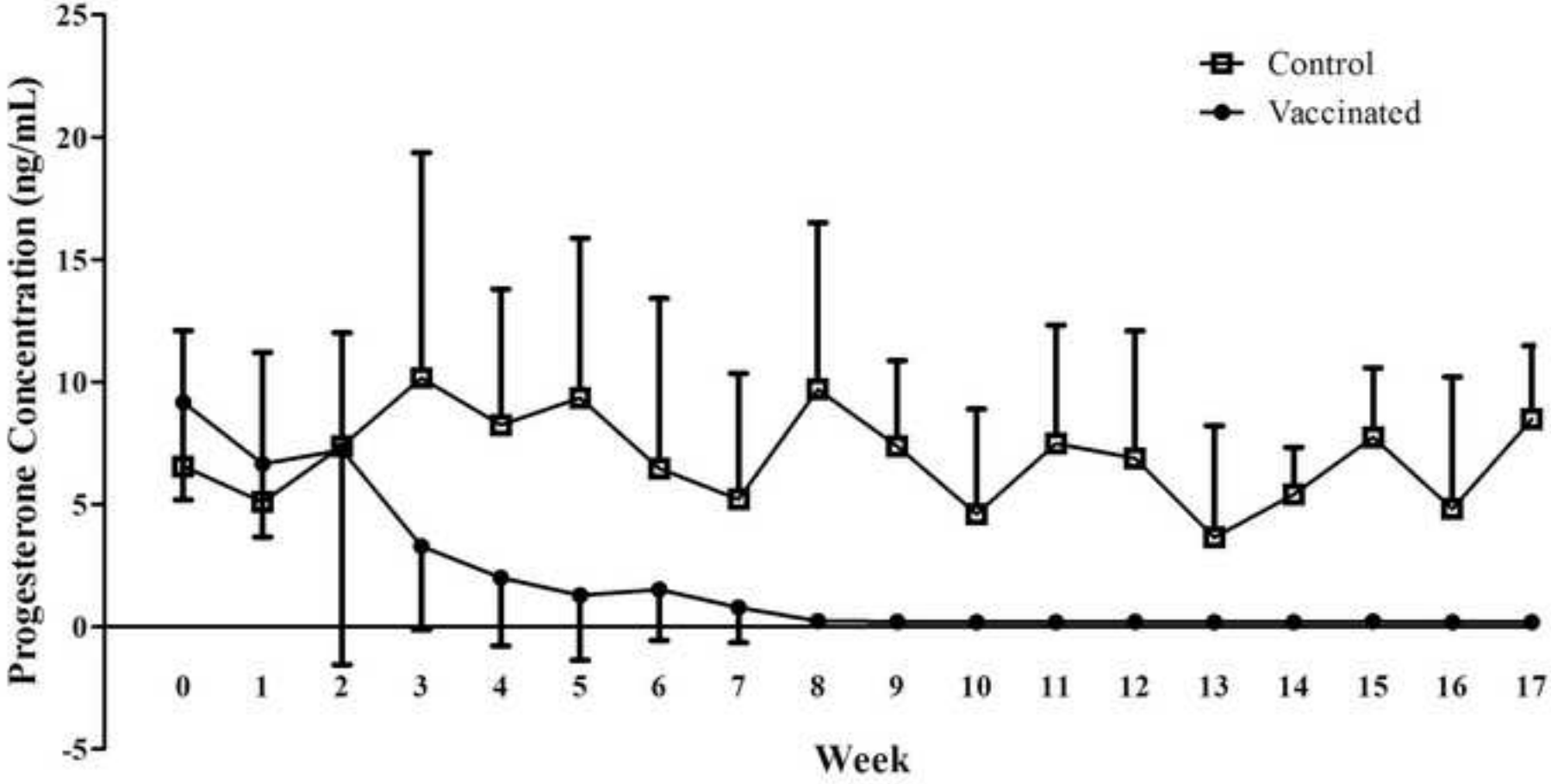

Control
Vaccinated
Progesterone Concentration (ng/mL)
25
20
15
10
5
0
-5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Week

Figure 3

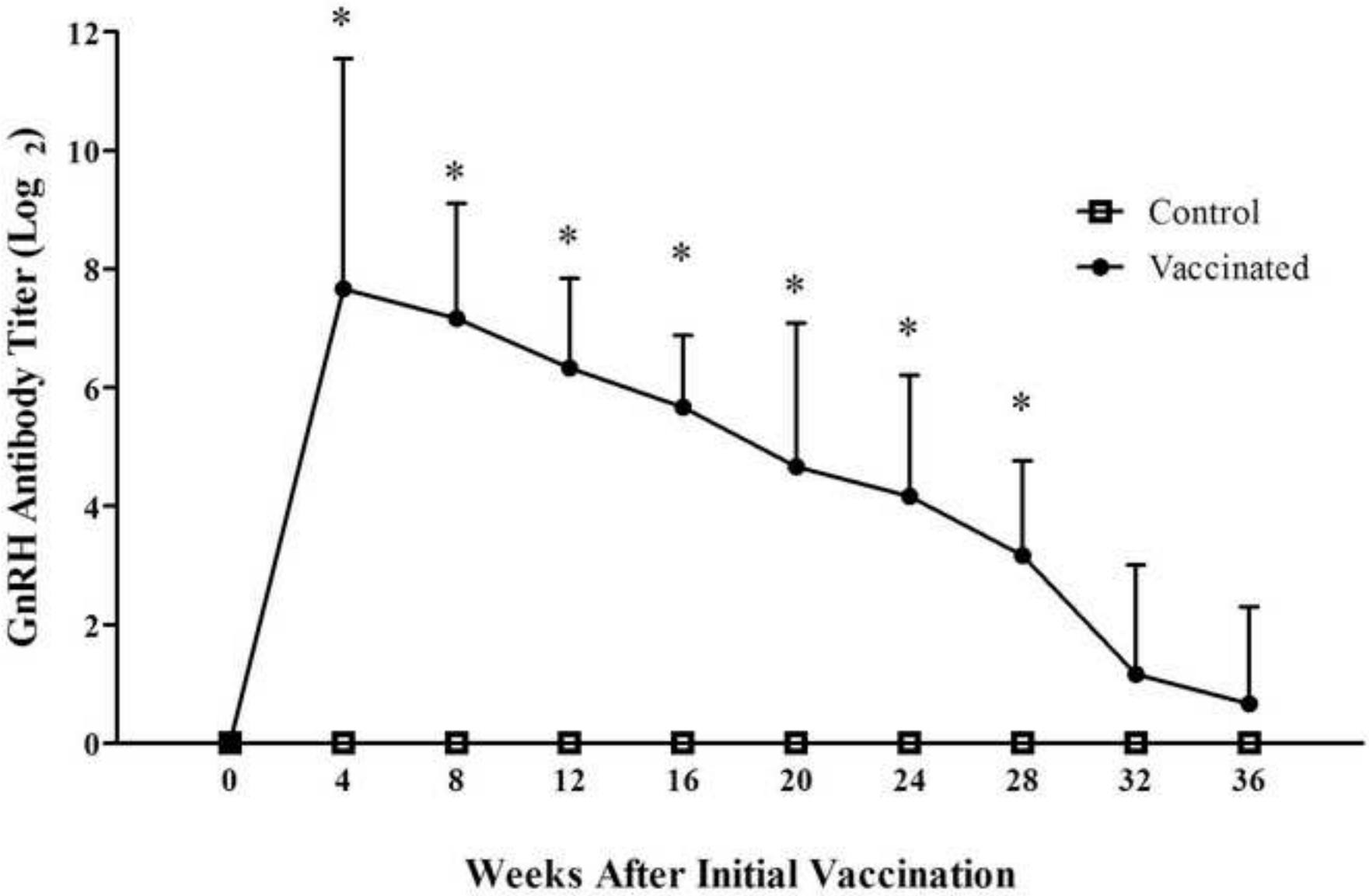
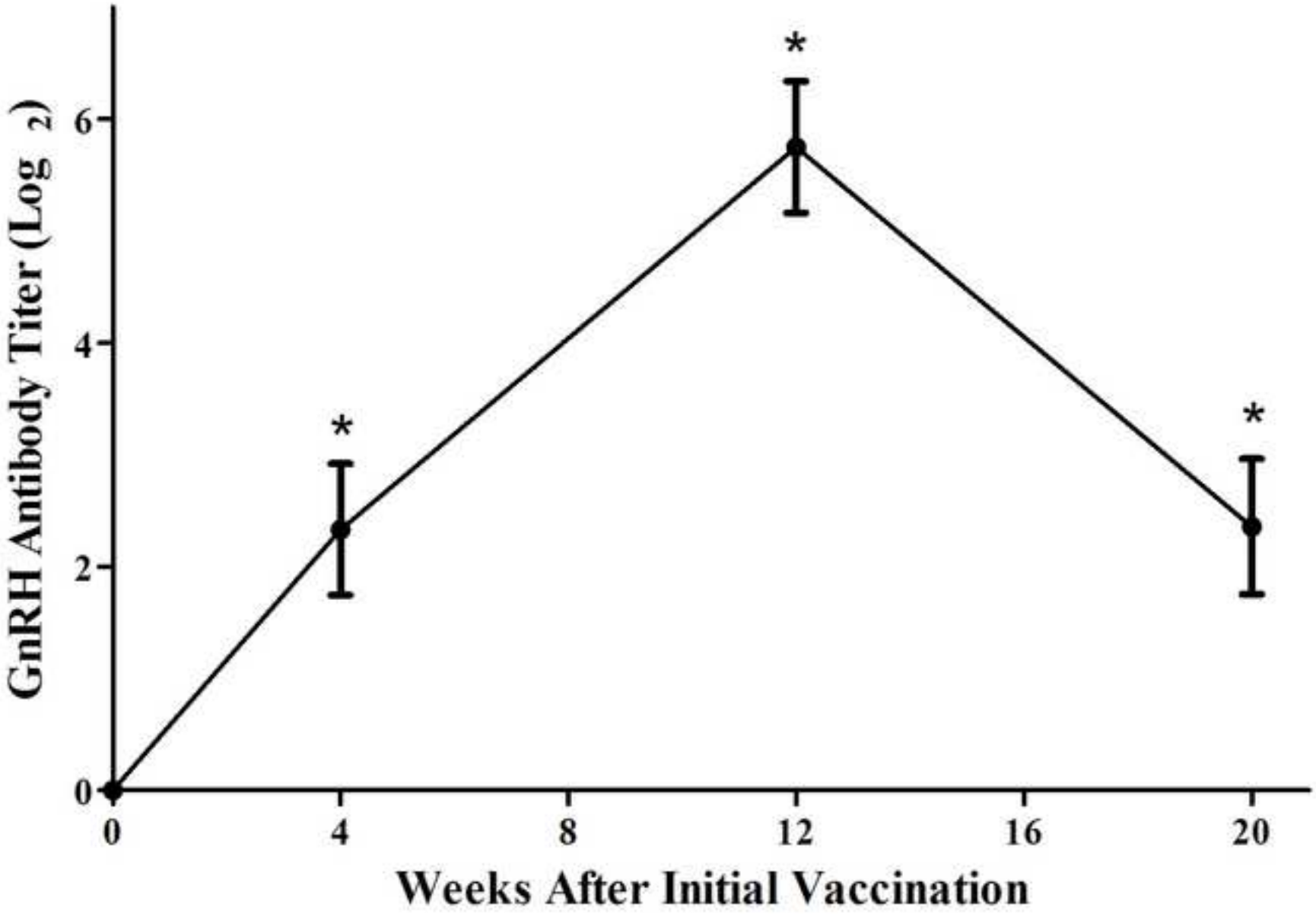

GnRH Antibody Titer (Log 2)
12
10
8
6
4
2
0
*
Control
Vaccinated
0
4
8
12
16
20
24
28
32
36
Weeks After Initial Vaccination

Figure 4


GnRH Antibody Titer (Log 2)
0
2
4
6
*
*
*
0
4
8
12
16
20
Weeks After Initial Vaccination

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Effects of prolonged immunocontraception on the breeding behavior of American bison

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In late 2009, the Catalina Island Conservancy began using fertility control to replace periodic removals to manage an introduced population of American bison (*Bison bison*) on the island. Through the application of the immunocontraceptive vaccine porcine zona pellucida (PZP), population growth was slowed within 1 year, and halted over time. In response to lingering questions about the use of PZP to manage large, free-ranging wildlife populations, we sought to determine the reversibility of PZP by ceasing the annual application to a subset of 15 bison cows and monitoring for subsequent calf arrival, and to document changes in the timing and length of the breeding season in response to PZP by monitoring breeding behavior and assessing fecal progesterone (FP) levels for all 60 resident cows over a 13-month period. As of June 2017, no new calves had been observed on the island, suggesting that, following repeated annual treatment with PZP (3 or 4 years), bison do not resume normal reproduction for at least 4 or 5 years, and that fewer treatments would be advisable if a faster return to fertility is desired. Based on observations of bull and cow behavior, and FP levels, cows displayed estrous cycles consistently throughout the study period, indicating that bison may ovulate year-round when conception and its consequences, e.g., lactation and presence of calves, are blocked. Because there is little evidence that an extended breeding season would negatively impact the health of bulls or result in large numbers of out-of-season births on Catalina, PZP appears to be a highly effective tool for managing the population of introduced bison on the island. However, the extended period of contraception and breeding activity of both cows and bulls may make PZP less suitable in high-latitude, predator-rich environments where bison conservation remains a top priority.

Key words: *Bison bison*, Catalina Island, immunocontraception, non-lethal population control, PZP, reproductive behavior

Historically, humans have altered wildlife abundance in many ways, including eliminating predators to protect themselves and their livestock, over-harvesting game species, and introducing both non-native and domestic species to new areas where they outcompete or displace native wildlife. The resulting population imbalances require informed management to prevent habitat destruction caused by overgrazing, and the extinction of native species from competition or predation (Asa and Porton 2005). Lethal control, used historically for wildlife species considered pests, is no longer socially acceptable in many parts of the world, and translocation may result in the death of the translocated animals or of other animals in their new range (Conover 2002; Asa and Porton 2005). Translocation efforts can also transport diseases or parasites between populations (Woodford and Rossiter 1993).

Contraception, and especially immunocontraction, is increasingly used for non-lethal control of wildlife populations, especially for species that are non-native or risk overabundance. Using the same principles as disease prevention through vaccination, immunocontraceptive agents are administered in small doses that stimulate the immune system of the target animal to produce antibodies that target and disrupt an essential aspect of reproduction (Kirkpatrick and Turner 1991). Porcine zona pellucida (PZP) vaccine, one of several immunocontraceptive vaccines registered or in development, has been widely used in zoos (Frank et al. 2005) and for free-ranging wildlife (Kirkpatrick and Turner 1991). PZP works by disrupting the zona pellucida, the non-cellular capsule that surrounds all mammalian eggs. This membrane is composed of glycoproteins that act as sperm receptors to facilitate sperm attachment and subsequent fertilization of the egg. When injected into the muscle of the target female, the PZP vaccine (derived from eggs of domestic swine *Sus scrofa domesticus*) stimulates

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her immune system to produce antibodies against the proteins in the vaccine. These antibodies target the similar protein in the zona pellucida of her eggs, attach to sperm receptors, and thereby block fertilization (Liu et al. 1989; Barber and Fayrer-Hosken 2000). The widespread use of PZP can be attributed to its success in controlling reproduction of wild horses (*Equus caballus*—Kirkpatrick et al. 1990; Turner et al. 1997; Ransom et al. 2011) and white-tailed deer (*Odocoileus virginianus*—Turner et al. 1996; McShea et al. 1997; Miller et al. 1999).

The American bison (*Bison bison*) was nearly driven to extinction by overexploitation (Hornaday 1889). Conservation efforts to recover bison populations continue throughout much of North America, but the growth of localized wild herds in some areas, including locations outside of their native range, has created conflict with human interests and other management objectives (Garrott et al. 1993—Plumb et al. 2009). One such area is Santa Catalina Island (hereafter, Catalina), 1 of the 8 California Channel Islands, United States. In 1924, approximately 14 bison were introduced to the island, and were immediately embraced by the island's residents as a possible draw for tourists. Genetic variation within the herd was supplemented through the addition of a total of 45 bison between 1934 and 1996, and, at its largest known size in 1987, the herd exceeded 520 individuals (Sweitzer et al. 2005; Duncan et al. 2013). However, in response to the cumulative ecological impacts of introduced mammalian herbivores on the island, beginning in 1969 bison were periodically captured and shipped to the mainland, with the aim of keeping the herd relatively small and minimizing their effects on native plants and animals (Sweitzer et al. 2005).

Since 1972, the Santa Catalina Island Conservancy (hereafter, Conservancy) has been responsible for managing ecological resources on the island, including the bison herd (O'Malley 1994). To better understand their ecological effects, in 2002, the Conservancy commissioned a multiyear population study (Sweitzer et al. 2003) to examine multiple management approaches, all of which included the maintenance of a bison population on Catalina at some level. Sweitzer et al. (2003) suggested that approximately 142 bison could be maintained within their current range of 16,200 ha on the island's East End, while "providing an acceptable level of protection for the unique natural resources on the island, including endemic plants and animals" (Sweitzer et al. 2003). In an attempt to balance potential ecological impacts with the demand for public viewing opportunities, the Conservancy set a goal of maintaining the herd at approximately 150–200 individuals on the East End (Duncan et al. 2013). However, maintaining this target herd size through occasional removals became unsustainable due to the high costs, concerns about the potential stress on animals during transport, and the expansion of the herd between shipments (Duncan et al. 2013). Additionally, genetic testing suggested a relatively high prevalence of cattle gene introgression in the Catalina herd, which raised concerns about shipping bison to mainland herds with no historical or genetic evidence of hybridization with cattle (Vogel et al. 2007). Given these circumstances, the Conservancy investigated potential methods of fertility control, including immunocontraception, as an alternative to translocation and culling (Duncan et al. 2013). PZP was selected because decades of research on wild horses had demonstrated its safety in pregnant mares, and because it had been shown to increase body condition and longevity in mares treated chronically (Kirkpatrick and Turner 2002, 2003; Turner and Kirkpatrick 2002). The reversibility of PZP, i.e., the ability of an individual to reproduce after cessation of treatment, had also been documented in multiple species, including horses (Liu et al. 1989; Kirkpatrick et al. 1991; Kirkpatrick and Turner 2002), white-tailed deer (Turner et al. 1996), and African elephants (*Loxodonta africana*—Fayrer-Hosken et al. 2000).

Based on its well-documented safety, efficacy and reversibility characteristics, its low cost, and the ability to deliver the vaccine remotely, PZP was first administered to cows in the Catalina herd during 2 roundups in 2009. The first (September 2009) facilitated a final shipment of 149 bison off the island and reduced the island herd to approximately 120 individuals (Table 1). The second (November 2009) supported sample collection, ear tagging, and the application of PZP to approximately 76% of the remaining bison cows (Duncan et al. 2013). The protocol developed by the Science and Conservation Center (Billings, Montana) for species such as bison, with a fairly well-defined breeding season, required a minimum of 2

Table 1.—Annual calving and adult mortality rates calculated for a free-ranging bison (*Bison bison*) herd on Catalina Island, California, prior to porcine zona pellucida (PZP) vaccination (2010) and post-PZP vaccination (2011–2016). Data from 2010 to 2012 from Duncan et al. (2013).

Year	Bison population[a]	Calving-age cows (≥ 2 years)	Calves produced	Calving rate (%)	Known mortalities	Mortality rate (%)
2010	120	43	29 (15F:14M)	67.4	5 (1F:4M)	4.2
2011	144	48[b]	5 (5F:0M)	10.4	7 (2F:5M)	4.9
2012	142	61[c]	2 (0F:2M)	3.3	6 (5F:1M)	4.2
2013	138	61[d]	1 (0F:1M)	1.6	3 (1F:2M)	2.2
2014	136	60	0 (0F:0M)	0.0	4 (1F:3M)	2.2
2015	132	59[e]	0 (0F:0M)	0.0	7 (1F:6M)	5.3
2016	125	58	0 (0F:0M)	0.0	5 (1F:4M)	4.0

[a]Island bison population estimate at the beginning of each calendar year.
[b]Four juvenile cows from 2010 reached calving age, 1 unmarked adult cow was discovered.
[c]Two geriatric cows died and 15 female calves from 2010 reached calving age.
[d]Five juvenile cows from 2011 reached calving age and 5 geriatric cows died.
[e]One of the 15 Reversal cows died in December 2014 from a leg injury.

inoculations during the 1st year of treatment. The 1st inoculation (primer) was administered 1–2 months prior to breeding activity or rut, with the 2nd inoculation 2–6 weeks later, but no later than 1–2 weeks prior to the onset of breeding activity. Infertility could then be maintained by administering a single booster annually, or could be reversed by ceasing booster inoculations (Kirkpatrick and Turner 2002).

By early 2010, all calving-age cows (≥ 2 years old; $n = 43$) in the population had received the vaccine during the roundups or remotely via darting. Pregnancy testing, completed during the November 2009 roundup, confirmed that 67% (29) of these cows were already pregnant when they received their 1st dose and all successfully produced calves in the spring of 2010 (Duncan et al. 2013). The resulting calving rate of 67.4% (29/43) was identified as the pre-PZP calving potential for the herd. The 2010 calving rate was somewhat higher than that estimated by Sweitzer et al. (2003) in 2001 (49%) and 2002 (35%), presumably due to the reduced herd size (120 versus 303–379 animals) and improved resource availability in 2010. By 2011, the calving rate was reduced to 10.4%, and to 3.3% by 2012 (Table 1). The arrival of calves in 2011 and 2012 was attributed to difficulty in identifying unmarked individuals during early vaccine administration efforts, and a single cow that apparently did not respond to the vaccine during her 1st year of treatment. These problems were resolved through follow-up vaccinations, and the efficacy of PZP in bison on Catalina has been consistently 100% since (Duncan et al. 2013). PZP had been shown to be effective in captive bison (Frank et al. 2005), but our study was the 1st time that PZP had been administered to a large, free-ranging bison herd.

Although the bison contraception program met its initial goal of halting population growth by reducing the calving rate, several key problems remained to be addressed to demonstrate that PZP could be an effective method for long-term, population-wide control of fertility in bison. First, the use of contraceptives to manage high-profile species has raised public concerns about the long-term health of treated populations, and their ability to resume reproduction successfully when the vaccine is no longer administered (Kirkpatrick and Turner 2002). Reversibility has been documented in many but not all species treated with PZP, and the length of time required for the resumption of fertility has varied considerably. On Assateague Island National Seashore, Maryland, Kirkpatrick and Turner (2002) assessed reversibility in wild horse mares after 1–7 consecutive years of treatment with PZP. Of the 53 mares treated with PZP for 1, 2, or 3 consecutive years, 100%, 100%, and 69%, respectively, became fertile within 4 years of the cessation of treatment, and all of the mares that received PZP for 4 or 5 consecutive years returned to fertility within 8 years (Kirkpatrick and Turner 2002). Lyda et al. (2013) showed that female Dall sheep (*Ovis dalli dalli*) and domestic goats (*Capra hircus*) maintained high antibody titers to PZP antigens for 3 years after a single year's treatment (primer and booster), but that antibody titers in lowland wisent (*Bison bonasus*) and Javan banteng (*Bos javanicus*), species that are more closely related to bison, returned to relatively low levels within only 9 months. Lyda et al. (2013) emphasized the variability among ungulate species and recommended a conservative approach to the application of PZP if a quick return to fertility is desired. In light of this interspecific variability, it was imperative to determine the reversibility of PZP in bison.

A 2nd concern was that PZP would alter the reproductive behavior of bison, specifically, the timing and duration of the breeding season. Adverse changes associated with social behaviors, aggression, and movement and activity patterns have been documented in animals treated with hormone-based fertility control agents (Powell 1999). However, PZP is not hormone-based and the antigen is extremely specific and does not cross-react with other tissues or hormone proteins (Palm et al. 1979; Barber and Fayrer-Hosken 2000). Further, PZP acts upon physiological processes that occur near the end of the reproductive cycle, and therefore does not interfere with other reproductive events (Kirkpatrick et al. 2011). PZP therefore was not expected to elicit behavioral changes as a direct result of vaccine administration. Nonetheless, it is conceivable that the successful act of blocking conception and the absence of calves in the population might alter the behavior of both males and females. These changes may be subtle or inconsequential in captivity, but may be significant for free-ranging populations. Potential concerns include the belief that prolonged or out-of-season breeding behavior as a result of blocked conception and repeated estrous in female ungulates could reduce energy reserves of competing males, especially in populations subject to periods of resource scarcity (McShea et al. 1997; Nuñez et al. 2009; Delsink et al. 2013). Others have speculated that once PZP antibodies decline, offspring could potentially arrive out of season or asynchronously, leaving them more susceptible to adverse weather or predators (Nuñez et al. 2010; Ransom et al. 2013).

Out-of-season breeding activity has been documented in studies of PZP application to free-ranging elk (*Cervus elaphus*—Heilmann et al. 1998) and white-tailed deer (McShea et al. 1997). However, inferences from the elk study are limited because few individuals were treated with PZP, and, in cervids, the breeding season tends to be more regulated by the testicular-antler rhythms of males and is therefore more finite (McShea et al. 1997). Long-term treatment of wild horse mares on Assateague Island did not significantly alter their social organization or behavior (Powell 1999; Kirkpatrick et al. 2011). In other locations, however, PZP-treated mares changed social bands more frequently outside of the breeding season than untreated mares (Nuñez et al. 2009), had different time-activity budgets than untreated ones (Ransom et al. 2010), and cycled and produced foals outside of the normal season (Nuñez et al. 2010; Ransom et al. 2013). Although these studies have been criticized for their small sample sizes and inadequate controls (Kirkpatrick et al. 2011), at minimum they suggest that PZP might alter reproductive behavior of some wildlife species.

In the continental climatic conditions that characterize their native range in the Great Plains of North America, bison generally breed from late June or early July through September, with the majority of breeding activity or copulations occurring

from mid-July through mid-August (Meagher 1986). Out-of-season births have been recorded in many bison herds, but the calving season generally extends from early April through early June, with births concentrated from late April to mid-May (Meagher 1986; Pac and Frey 1991; Berger and Cunningham 1994). The timing of the breeding season of bison on Catalina has been poorly documented. Lott and Minta (1983) reported that bison on Catalina initiated breeding much earlier in 1975 (30 May–24 July [55 days]) than in most mainland herds, presumably because of California's milder Mediterranean climate and seasonality of available resources. More recently, Sweitzer et al. (2003) identified the 90% calving period (the shortest interval in which 90% of the calves were born) in 2002 as occurring over a 3- to 4-week period from mid-April to early May (approximately 24 days), suggesting that, with a gestation period of about 275 days, conception occurred from about 14 July to 7 August 2001. Using a similar approach in 2010, prior to the administration of PZP, we identified the 90% calving period as occurring from 5 March to 17 May 2009 (73 days), suggesting that most conception occurred from early June to mid-August the previous year. These data suggest a somewhat earlier onset and expanded breeding season compared to bison in other locations and from previous research on Catalina; however, a distinct, synchronous calving season has always been apparent. Given the variability between studies, additional data were needed to determine how cessation of conception using PZP would affect breeding behavior and the seasonality of reproduction of bison on Catalina.

Our goals were to investigate the reversibility of PZP in bison after several years of treatment by ceasing administration of the vaccine to a subset of cows and monitoring for new calves, and to determine if the successful blocking of conception by PZP altered the length of the bison breeding season. We hypothesized that bison cows kept infertile for 3 or more consecutive years through PZP treatment would produce calves within 3 years of cessation of booster inoculations. Moreover, at the population level, we predicted that the administration of PZP would not significantly alter the length or seasonality of the breeding season. We interpreted our results in terms of their implications for the continued maintenance of bison on Catalina, and assessed the relevance of our results for efforts to manage bison populations elsewhere.

Materials and Methods

All animal handling and PZP application procedures were performed under an approved protocol from the Institutional Animal Care and Use Committee at California State University Fullerton, and were consistent with ASM guidelines for research on mammals (Sikes et al. 2016).

Study area.—Santa Catalina Island (194 km^2), located approximately 40 km west of Los Angeles, California, United States, has a rugged mountainous terrain, with a central ridge running its length (Fig. 1). The elevation ranges from sea level to 640 m, and a narrow isthmus (< 800 m wide) separates the island into 2 geographically distinct sides: the larger East End, comprising 84% of the island, and the smaller West End, comprising the remaining 16% (Schoenherr et al. 1999). The island has a semiarid Mediterranean climate, with average annual precipitation from 2013 to 2015 of 146.6 mm (WRCC 2016), most of which falls between November and April. Common plant communities include coastal sage scrub, coastal bluff scrub,

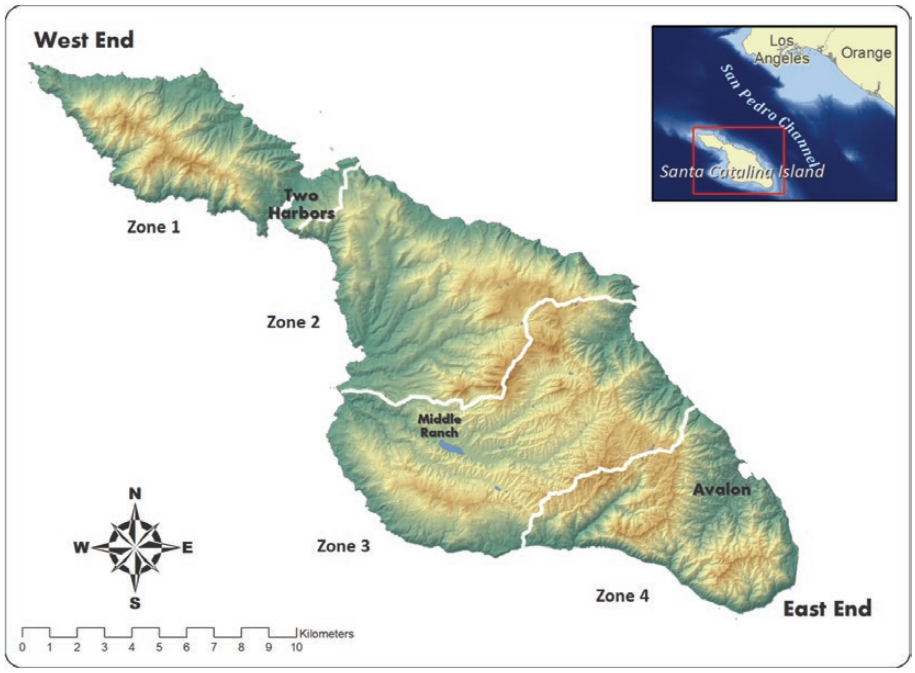


Fig. 1.—Map of Santa Catalina Island, California, illustrating locations of 3 cross-island fences that partition the island into 4 zones. Zone 1 is free of bison (*Bison bison*), whereas Zones 2, 3, and portions of 4 are occupied by bison. Avalon and Two Harbors are the largest human communities on the island. Inset shows the location of Catalina in relation to mainland southern California.

island chaparral, island woodland, riparian woodland, and coastal grassland (Schoenherr et al. 1999). Catalina is home to more than 60 endemic species of plants and animals. It has a resident human population of approximately 4,050, > 90% of which live in the town of Avalon (King et al. 2014), and receives an average of 800,000 visitors annually, making it the most populous of the California Channel Islands.

Although no large mammalian herbivores are native to the island, non-native grazers were introduced over the past 2 centuries. Feral goats were established on the island in the early 1800s, and domestic cattle, horses, and sheep (*Ovis aries*) were present by the 1890s. Feral pigs (*Sus scrofa*) were initially established in the 1930s (Schuyler et al. 1998). Domestic cattle (*Bos taurus*) operations ceased by the 1950s (Schoenherr et al. 1999), and pigs and goats were eradicated between 1990 and 2004 (Schuyler et al. 1998; Sweitzer et al. 2005). Mule deer (*Odocoileus hemionus*) were introduced to the island in 1928 and have been managed since 1998 through a private land management hunting program (Stapp and Guttilla 2006).

Breeding behavior and reproductive physiology of bison.—Bison are sexually dimorphic, with males (544–907 kg) weighing 60–70% more than females (Meagher 1986). The mating system has been characterized as male dominance polygyny (Lott 1981). Sexual maturity in both female and male bison generally occurs at 2–4 years of age; however, bulls usually do not have the opportunity to breed until age 6 (Meagher 1986). Mixed-sex herds comprised of females and young or subdominant bulls (usually the offspring of cows within the group) may be observed throughout the year. Mature bulls often seek solitude or are found in small bachelor groups during the non-breeding season, and then join the larger cow groups during the rut, when herd sizes are at their peak (Lott 1981). Bulls can identify cows in or near estrus by sniffing or licking their urine or anogenital region and performing *flehmen*, a lip curl and inhalation response that exposes the vomeronasal organ to a scent or pheromone (Mahan et al. 1978; Lott 1981; Mooring et al. 2006).

Once a bull becomes interested in a specific cow, he will tend or guard her, remaining in close proximity, often impeding her movement and warding off other bulls until he is either permitted to copulate with her or is displaced by a rival (Lott 1981; Wolff 1998). Bulls communicate their dominance and desire to mate through posturing, bellowing, pawing, rubbing, scent-urinating, and wallowing (Mooring et al. 2006). A tended cow often attracts rival bulls and head-to-head battles may ensue (Wolff 1998). The breeding season or rut can be particularly taxing for bulls as they may forgo feeding, deplete fat reserves through increased activity, and be injured during battles (Lott 1979; Roden et al. 2011).

Immediately following copulation, which lasts only a few seconds, the cow arches her back, voids a small volume of milky secretions from the vulva (presumably semen), and erects her tail (Lott 1981; Berger and Cunningham 1994; Wolff 1998). Cows elevate their tails for other reasons (urination, defecation, or agitation), but this post-copulation 'tail-up' response is distinctive in that the tail is raised at an angle of approximately 135° or higher and is maintained for 1–2 days (Komers et al. 1992; Wolff 1998; Mooring et al. 2006). This behavioral indicator of copulation is very reliable and can be used to infer copulation (Berger and Cunningham 1991; Wolff 1998; Vervaecke and Schwarzenberger 2006). Though uncommon, superficial oval-shaped wounds caused by a mounting bull's hooves scraping the cow's back have been observed in recently bred cows and may also be an indicator of breeding (Lott 1981). Cows remain sexually receptive for 9–28 h (Haugen 1974).

Unlike domestic cattle, bison were initially thought to be monestrous (Rutberg 1986) or to only rarely undergo a 2nd estrus in a given year (Haugen 1974). Kirkpatrick et al. (1991a) used urinary steroid metabolites to document definitive estrous cycles of captive bison and reported that 7 of 8 cows became pregnant during their 2nd cycle and subsequently produced calves. The arrival dates for those calves also supported the occurrence of a 2nd seasonal estrus. Vervaecke and Schwarzenberger (2006) used fecal progesterone (FP) metabolites to identify 3 ovulatory periods, and consequently, bison are now considered seasonally polyestrous.

The reproductive physiology of bison is similar to that of domestic cattle. The estrous cycle, as described by Turner (2014), averages 21 days in length (range 17–24). A period of standing heat or estrus, marked by a rise and peak in estrogen secretion, initiates the cycle. Estrus lasts 12–24 h, and ovulation occurs approximately 12 h after the end of estrus. After ovulation, cells that make up the ovulatory follicle differentiate to form the corpus luteum (CL), which, in turn, produces progesterone. Progesterone levels rise substantially and peak after approximately 15–16 days when the CL is fully mature. After approximately 16 days of elevated progesterone, prostaglandin is released by the uterus, resulting in the lysis of luteal tissue and the regression of the CL. Circulating progesterone begins to decline and the luteal phase of the estrous cycle ends. The decline in progesterone triggers increased pulses of gonadotropin-releasing hormone, which, in turn, stimulates the production of follicle-stimulating hormone and luteinizing hormone (LH). Increases in these hormones support the development of another ovulatory follicle, which results in an increase in estrogen. Once estrogen peaks, a surge of LH is released and ovulation occurs again.

Photoperiod has been identified as a cue for reproduction for many wild ungulates, and plant phenology and predation have been implicated as factors contributing to birth synchrony (Rutberg 1984; Green and Rothstein 1993; Zerbe et al. 2012). The breeding season, or rut, of bison has been estimated several ways, such as recording the timing of specific breeding behaviors (tending, copulations, and post-copulation 'tail-up' responses of cows), by subtracting gestation length (approximately 265–285 days—Haugen 1974; Rutberg 1986) from parturition, or by monitoring changes in reproductive hormones. Each approach has its own challenges and assumptions. For instance, subtracting gestation length from parturition can provide an estimate of the date of successful conception, but does not yield information on unsuccessful copulation attempts. Brief actions or signs associated with breeding behavior may

go undetected when attempting to monitor a large free-ranging population. Assessment of changes in hormones may expand the period of detection, but the collection of adequate blood, urine, or fecal samples systematically from large populations of wild bison can be difficult, dangerous, and expensive. For these reasons, we used a combination of observational and endocrine-based methods to assess changes in the seasonality of breeding behavior in response to PZP application.

PZP vaccine preparation and administration.—The native PZP antigen was prepared at the Science and Conservation Center from porcine oocytes, using the modified method of Dunbar et al. (1980). Antigen samples were shipped to Catalina on dry ice and stored frozen (−10°C) until time of use, under the authority of the Food and Drug Administration Investigational New Animal Drug Exemption (No. 8840-G003-004). The vaccine consisted of 0.5 ml standard phosphate buffer solution containing 100 µg of PZP emulsified with 0.5 ml Freund's Modified Adjuvant (FMA; Calbiochem, La Jolla, California) for all initial (primer) inoculations, and 0.5 ml Freund's Incomplete Adjuvant (FIA; Sigma, St. Louis, Missouri) for all subsequent booster inoculations. The emulsion was prepared within minutes prior to injection using 2 10-ml glass syringes joined with a Luer-Loc connector. After 100 plunger strokes, the emulsion was loaded into 1.0-ml Pneu-Dart type "P" darts with 3.8-cm barbless needles (Pneu-Dart, Williamsport, Pennsylvania) to facilitate remote delivery using an X-Caliber gauged CO_2 rifle (Pneu-Dart). At the beginning of each darting season, the tail fin of each dart was painted red or orange using Rust-Oleum specialty fluorescent spray paint and numbered to facilitate dart recovery and tracking of vaccinations. Immediately after loading the dart a small dab of petroleum jelly was applied to the tip to prevent PZP leakage. Prepared vaccine was stored in a refrigerator and transported to the field in an ice chest. Treated cows were darted in the caudal thigh or gluteus muscle.

Following the roundup and shipment of bison off-island in late 2009, all calving-age (≥ 2 years old) cows were treated with PZP each year from 2010 to 2012 (Table 2). In 2013, the herd consisted of approximately 138 individuals, 61 of which were calving-age cows. Primer inoculations of PZP + FMA were administered in March 2013 to 5 bison cows that were born in 2011. Booster inoculations of PZP + FIA were administered from February to April 2013, to 51 of 61 cows; from March to April of 2014, to 45 of 60 cows; and from March to April 2015, to 45 of 59 cows (Table 2). These represented all breeding-age cows in the Catalina herd each year. Bison cows were identified through a combination of numbered ear tags (Richey Livestock ID, Brighton, Colorado; applied during previous roundups) and photographs highlighting key features such as horn characteristics, pelage color, and natural markings.

Reversibility testing.—To assess the reversibility of PZP in bison, the vaccine was withheld from 10 cows (ages 5–11 years) beginning in 2013, and 5 additional cows (ages 8–14 years) in 2014. All but 1 of the Reversal cows were within the range of ages in which the highest fecundity in bison cows has been documented (3–13 years old—Berger and Cunningham 1994). One of the 10 initial Reversal cows died from a leg injury, naturally caused, in late 2014. The vaccine was withheld from the 14 surviving individuals again in 2015, and no cows received PZP in 2016 (Table 2). Reversal cows had received PZP inoculations for 3 (initial 10 cows) or 4 (additional 5 cows) consecutive years; because of the staggered entry (young cows) and exits (deaths) from the program, PZP-treated cows were

Table 2.—Annual treatment schedule and delivery method of porcine zona pellucida (PZP) to free-ranging, breeding-aged bison (*Bison bison*) cows on Catalina Island, California, from 2009 to 2012 (Duncan et al. 2013) and 2013 to 2015. The number of treated cows was not necessarily the number of cows in the population that year because cows might have died between January and the darting period. FMA = Freund's Modified Adjuvant; FIA = Freund's Incomplete Adjuvant.

Year	Month(s)	Activity	Delivery method	Primer/booster	Adjuvant	Cows
2009	Sep.–Nov.	Roundup	Syringe	Primer	FMA	35
2010	Feb.–May	Field darting	Dart	Primer	FMA	12 of 13[a]
2010	Apr.–Jul.	Field darting	Dart	Booster	FIA	45 of 47[b]
2011	Apr.–Jun.	Field darting	Dart	Booster	FIA	48[c]
2011	Dec.	Roundup	Syringe	Primer/booster	FMA/FIA	13[d]/4
2011	Dec.	Field darting	Dart	Primer	FMA	1[d]
2012	Jan.	Field darting	Dart	Primer	FMA	1[d]
2012	Apr.–May	Field darting	Dart	Booster	FIA	60[e]
2013	Mar.	Field darting	Dart	Primer	FMA	5[f]
2013	Feb.–Apr.	Field darting	Dart	Booster	FIA	51[g]
2014	Mar.–Apr.	Field darting	Dart	Booster	FIA	45[h]
2015	Mar–Apr.	Field darting	Dart	Booster	FIA	45[i]

[a]Additional unmarked adult cow was present but not detected in 2010.
[b]One geriatric cow died early in the year.
[c]One additional adult bison cow was discovered in the population.
[d]Fifteen juvenile cows born in 2010 reached maturity and were added to the program.
[e]Three geriatric cows died between October 2011 and February 2012, and 5 additional geriatric cows died between June and December 2012.
[f]Five juvenile cows born in 2011 reached maturity and were added to the program.
[g]PZP vaccine withheld from 10 adult bison cows (reversibility testing).
[h]One geriatric cow died in late 2013 and PZP vaccine was withheld from 5 additional adult bison cows (reversibility testing).
[i]PZP vaccine withheld from same 14 remaining Reversal cows.

vaccinated annually a total of 6 times (22 cows), 5 times (2 cows), 4 times (16 cows), and 3 times (5 cows) between 2010 and 2015. All cows in the population were monitored regularly from 2013 through November 2016 to document calf arrival. Using Kirkpatrick and Turner's (2002) 8-year study of reversibility in wild mares as a guide, we expected approximately 6 (67%) of the initial 9 cows, last treated in 2012, and 1 (20%) of the additional 5 cows, last treated in 2013, to produce calves by the end of 2016.

Monitoring of breeding behavior.—We monitored behavior and fecal hormone levels of all 60 surviving breeding-aged cows in the Catalina herd as of 1 June 2014. Observations and fecal-sample collections were carried out from a vehicle and on foot over a 13-month period from 1 June 2014 to 30 June 2015. Our aim was to record breeding behavior and signs from all 60 cows every 3–4 days, and to collect a fecal sample from each cow biweekly to determine FP levels. Global positioning system (GPS) collars (G2110E Iridium; Advanced Telemetry Systems, Isanti, Minnesota) were fitted on 5 mature cows on 10–11 May 2014 to assist with locating smaller groups of bison and to assess movement patterns and resource use. Monitoring of the movements of these 5 collared cows allowed us to effectively locate and sample approximately 85–90% of the Catalina herd at all times (Duncan 2017). However, adverse weather conditions, access limitations, safety concerns, and the approachability of the bison all constrained our ability to collect behavioral observations and samples systematically.

For each cow, we recorded the following potential signs of breeding activity (adapted from Vervaecke and Schwarzenberger 2006):

- Tended lightly by bull: A bull, dominant or subdominant but not one of her offspring, remained in close proximity to and followed a cow for longer than 30 min.
- Tended heavily by bull: A dominant bull remained in close proximity to a cow, and often impeded her movements. This activity was accompanied by one or more attempts to mount, cow placing her chin on the bull's back, and frequent nudging, bellowing, pawing, scent urination, as well as defensive behavior against rival bulls. This level of tending behavior occurred for a prolonged period (> 30 min) and continued until copulation or the end of an observation period.
- Tail-up partial: The cow was observed with a slightly elevated, less than horizontal, tail at the beginning of an observation period and maintained it for 1 h or more.
- Tail-up high: The cow held her tail at a horizontal angle or higher, for an extended period of several hours to several days.
- Copulation: The bull mounted the cow with intromission.
- Fresh mounting sores (FMS): The presence of fresh (new, moist, bloody) wounds on the lower back, point of hip region on either side of a cow.
- Healing mounting sores (HMS): The presence of healing (dry, scabbing) wounds, presumably > 2 days old, on the lower back, in the point of hip region on either side of a cow.

Heavy tending, tail-up high, and copulation behaviors have all been confirmed as reliable indicators of ovulation (Kirkpatrick et al. 1991a; Vervaecke and Schwarzenberger 2006), and were used independently to assess the timing of ovulation in monitored cows. Tail-up partial and light tending were not included as subcategories in previous studies, meaning that these less intensive behaviors were either included within the more general categories of "tending" and "tail-up" or were dismissed altogether. These subcategory behaviors were recorded in the field so that they could be examined separately during analysis, and to help identify individuals that were observed close to the date of ovulation.

Collection and analysis of fecal samples.—During behavioral observations, cows frequently voided feces. We marked the location of newly voided feces by fixing the location of the feces in the crosshairs of a rifle scope attached to a tripod. We used a range finder to record the distance between the tripod and the target cow. When it was considered safe to approach (within 15 min, on average, and always within 50 min of deposition), a fecal sample was collected to fill about three-quarters of a 30-ml plastic container (Sarstedt Inc., Newton, North Carolina), and placed in a cooler with ice packs. Each collection cup and lid was labeled with the cow's identification code, date, and time of fecal deposition just prior to sample collection. To ensure a homogeneous sample, fecal material was mixed thoroughly on the ground prior to collection using a single-use wooden tongue depressor. Samples were transferred to a freezer at the end of an observation period and stored frozen (−10°C) until time of shipment. Fecal samples were shipped to the San Diego Zoo Institute for Conservation Research (Escondido, California) within 6 weeks of collection for progesterone radioimmunoassay analysis.

Fecal samples were lyophilized for 72 h in a Flexi-Dry microprocessor manifold lyophiliter (FTS Systems, Inc., Stone Ridge, New York) to reduce variability in water content. Extraneous material was removed from the lyophilized feces by sifting each sample through a copper mesh screen (2 × 2 mm). A 0.1-g sample was weighed and suspended in 5 ml of 90% aqueous ethanol and boiled immediately for 20 min at 80°C. The sample was then centrifuged at 1,000 × *g* for 10 min in a Legend XTR centrifuge (Thermo Fisher Scientific, Waltham, Massachusetts). The supernatant was recovered and the pellet resuspended in 5 ml of 90% aqueous ethanol, vortexed briefly and then centrifuged. The combined supernatants were dried under a stream of filtered air and dissolved in 1 ml of 100% ethanol.

Samples were analyzed using a monoclonal progesterone antibody produced against 4-pregnen-11-ol-3,20-dione hemisuccinate:BSA (Grieger et al. 1990), which cross-reacts 100% with progesterone, 96% with allopregnanolone, and, to a lesser degree, with a number of other progesterone metabolites (Grieger et al. 1990; Wasser et al. 1994). Tritiated progesterone (10,000 cpm/0.1 ml; PerkinElmer, Waltham, Massachusetts) was used to compete against standard progesterone (7–100 pg/0.05 ml; Sigma, St. Louis, Missouri).

One microliter of the fecal extract was assayed in duplicate and incubated overnight at 4°C. After incubation, the

competitive reaction was stopped and the bound and unbound hormone were separated by adding 0.25 ml of char-dex solution to the assay, which was then incubated for 30 min at 4°C. The assay was then centrifuged at 1,000 × *g* for 15 min and the supernatant poured into scintillation vials. Scintillation fluid (3.5 ml; MP Biomedicals, Solon, Ohio) was added to each tube and the assay counted on a Beckman LS 6500 liquid scintillation counter (Beckman Coulter, Brea, California) to determine the FP level.

Data analysis.—We plotted FP levels and breeding behaviors for all 60 cows individually to detect errors and identify possible cycles. For behavioral purposes, a cow was considered to be in estrus if any 1 of 3 characteristic behaviors (heavy tending by bulls, copulation, tail held high) was observed on a given day. To produce a conservative estimate, we considered observations of these behaviors to represent different estrous cycles if they were separated by at least 20 days (Isobe et al. 2005; Turner 2014).

Using the approach of Brown et al. (2001), we estimated a baseline FP value for each cow using an iterative process in which we first calculated the mean and *SD* of all values and then excluded ones that exceeded the mean plus 1.5 *SD*. A new mean was then calculated and these steps were repeated until none of the remaining values exceeded the mean plus 1.5 *SD*. Brown et al. (2001) identified the onset of the luteal phase by the occurrence of FP values that exceeded the baseline by 50% and remained elevated for at least 2 weeks. However, they studied captive animals that were monitored frequently so that increases in FP could be easily identified. To conservatively identify estrous cycles in wild bison cows, we classified FP values as occurring in the luteal phase if they were at least 2 times higher than baseline FP. Given that, in cattle, FP is significantly higher than baseline for approximately 19 days during the estrous cycle (Isobe et al. 2005), we considered elevated FP values to be from different cycles if they were at least 20 days apart.

We were unable to calculate a baseline FP value using the Brown et al. (2001) approach for 8 cows (4 PZP-treated, 4 Reversal). Six of the 8 cows had consistently elevated FP values throughout the study period (71–100% of values > 1,000 ng/g; see "Results"). One cow (Red38) was frequently isolated from the rest of the herd and another (9046) died from a leg injury in December 2014, and so were monitored less frequently. To document the presence of estrous cycles in these 8 cows, we used the mean baseline value (338 ng/g, *SD* = 100) calculated from the other 52 cows. Data from these 8 cows were included in summaries of behavior and FP values, but were excluded from the analysis of temporal patterns in cycles across the herd.

Results

As of 1 June 2017, no calves had been detected in the herd. This included the 9 surviving Reversal cows that had not been vaccinated since 2012 (60–62 months since last booster; treated for 3 years and off for ~5 years) and the 5 Reversal cows that had not been vaccinated since 2013 (50–51 months since last booster; treated for 4 years and off for ~4 years).

For both behavioral observations and FP sampling, the number of days sampled and the number of total observations and samples collected tended to increase over the course of the 13-month study period (see Supplementary Data SD1). The fewest samples and observations were in summer 2014, whereas, except for a peak in October 2014, the most observations and samples were in spring 2015.

Breeding behavior and FP levels were plotted together for all 60 cows. Representative profiles of PZP-treated and Reversal cows (see Duncan (2017) for profiles of all cows) are shown in Fig. 2, and a summary of behavioral observations and FP sampling for all 60 cows is given in Appendix I. On average, PZP-treated and Reversal cows were sampled over similar numbers of days (67 versus 61 days, respectively). Reversal cows tended to be heavily tended and copulated more than PZP-treated ones, but the differences were relatively small. For both groups, tail-up high behavior was observed much more frequently than heavy tending or copulations (Appendix I). Fewer than one-half (43%) of all cows were observed copulating, compared to more than 70% that were tended heavily by bulls or observed with their tails held high. Tending behavior by bulls (including light tending) occurred throughout the study period, whereas most copulations were observed between September 2014 and March 2015 (Fig. 3A). Considering all types of breeding behaviors displayed by bulls and adjusting for sampling effort (number of days of behavioral observations per month), bulls apparently were most responsive to females from June 2014 to January 2015, followed by a decline through May 2015, and then increasing again in June 2015 (Fig. 3B).

The presence or absence of mounting sores was determined to not be a good indicator of breeding or ovulation. Although ovulation and breeding behavior were documented in all 60 cows, 20% (12) never developed sores, and sores were rare on many others, despite abundant evidence of breeding activity. Moreover, sores were often persistent and could not be used to indicate the specific timing of copulation. In many instances, sores were re-aggravated or maintained by scratching on trees or bushes, wallowing, or licking, and by mounting activities that were not associated with breeding.

FP values varied greatly within and among individuals (see Supplementary Data SD2). On average, 18.4 samples (range 7–29, $n = 60$) were collected from each cow (Appendix I). Reversal cows tended to have a lower minimum and baseline, and a higher maximum and range of FP values than PZP-treated cows, although these differences were not statistically significant (*t*-tests, $P > 0.135$).

We tentatively identified estrous cycles first based on behavioral observations and FP values separately. Based on behavior alone, on average, Reversal cows displayed more cycles than PZP-treated ones, but the difference was small (2.9 versus 2.5; Appendix I). Using FP levels, Reversal cows had, on average, 1 more cycle than PZP-treated cows (5.9 versus 4.9; Appendix I), but this difference was not significant ($t_{50} = 2.01$, $P = 0.15$). Fewer cycles were identified using behavior than FP values and the 2 measures only occasionally overlapped (Appendix I).

To estimate the total number of estrous cycles observed for each cow, we summed the number of behavioral and FP cycles

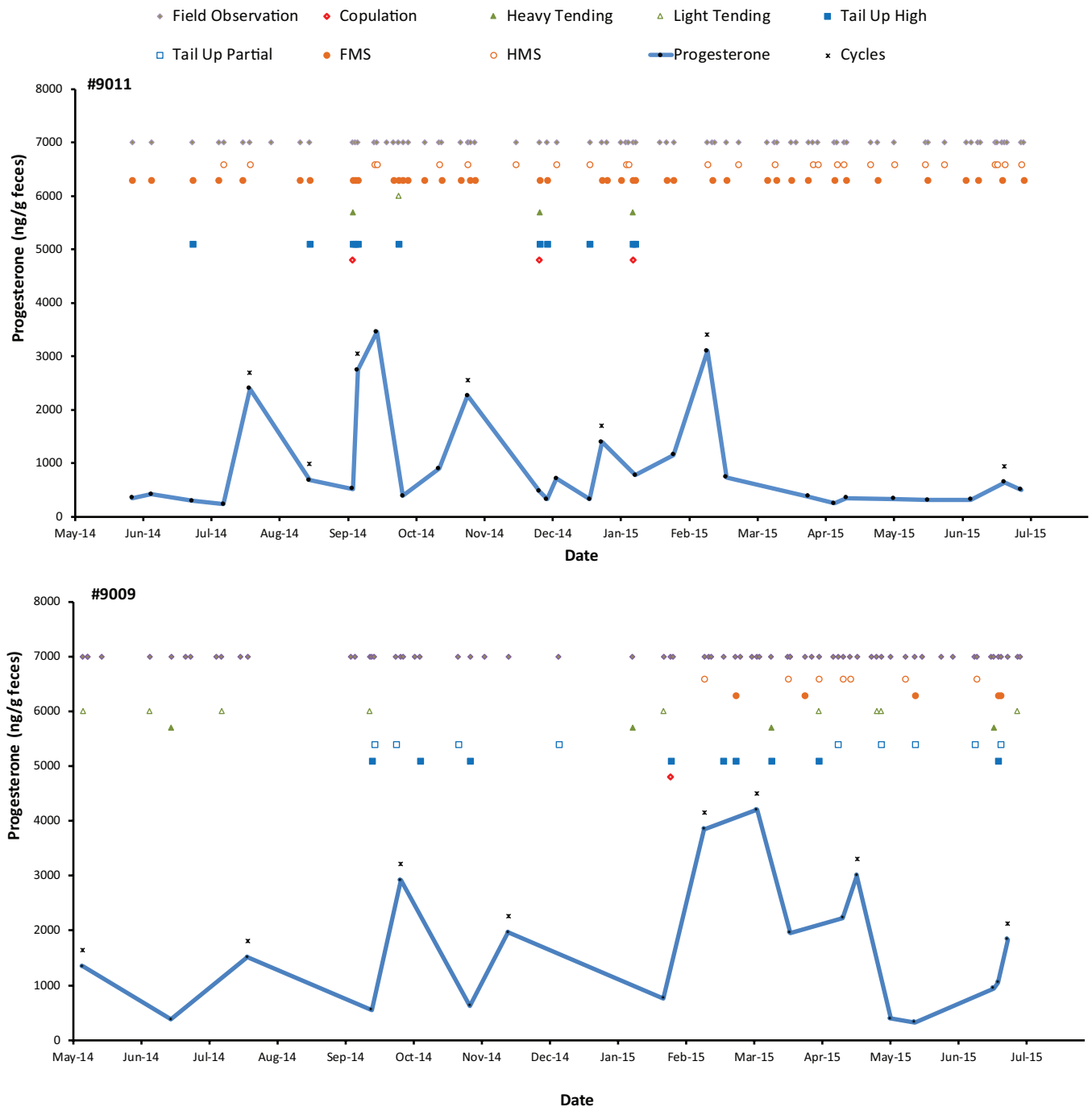


Fig. 2.—Behavioral and FP profiles of representative PZP-treated and Reversal bison (*Bison bison*) cows on Catalina Island. (top) PZP-treated cow 9011, for which 4 cycles were detected based on behavior, 7 cycles were detected based on FP levels (black stars), with 2 occasions in which behavior and FP overlapped. (bottom) Reversal cow 9009, for which 7 cycles were detected based on behavior, 8 cycles were detected based on FP levels (black stars), with 6 occasions in which behavior and FP overlapped. FP = fecal progesterone; PZP = porcine zona pellucida.

and subtracted the number that overlapped. Considering only the 52 cows with reliable FP data, individual PZP-treated cows displayed 2–12 cycles ($\bar{X}$ = 6.2, *SD* = 2.0, *N* = 41), whereas Reversal cows displayed 4–11 cycles ($\bar{X}$ = 7.3, *SD* = 2.2, *N* = 11; Appendix I), a difference of approximately 1 cycle that was not statistically significant (t_{50} = 1.45, *P* = 0.15). Estrous cycles were recorded throughout the 13-month study period (Fig. 4A). Adjusting for the number of cows studied each month (behavior: 18–44; FP: 24–49), a substantial proportion (30–70%) of the resident bison cows were in estrus each month except for April and May 2015 (Fig. 4B).

Discussion

Administration of PZP to bison cows quickly reduced and, within 3 years, completely halted calf production on Catalina Island, demonstrating for the 1st time the efficacy of PZP as a method of controlling population growth of a large, free-ranging

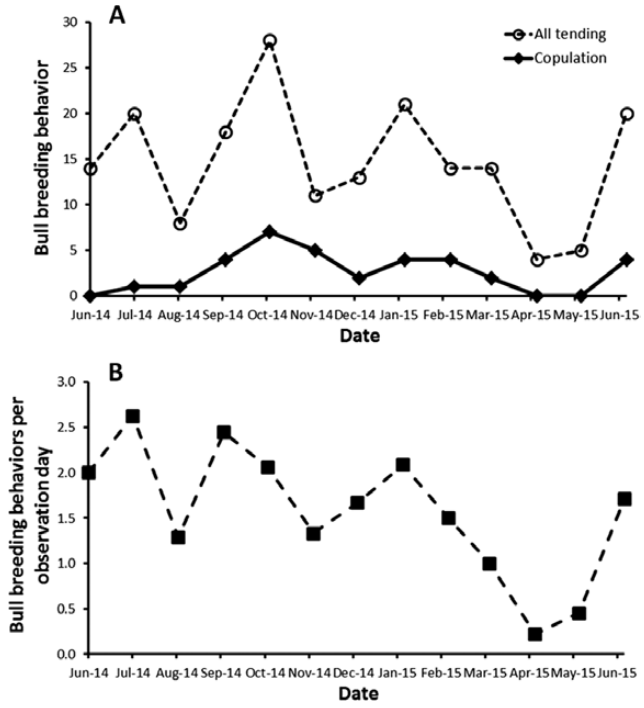


Fig. 3.—(A) Observations of light and heavy tending and copulations by bison (*Bison bison*) bulls, and (B) all bull breeding behaviors (all tending and copulations) per day of observation, tallied by month, in response to PZP treatment in cows on Catalina Island from 1 June 2014 to 30 June 2015. PZP = porcine zona pellucida.

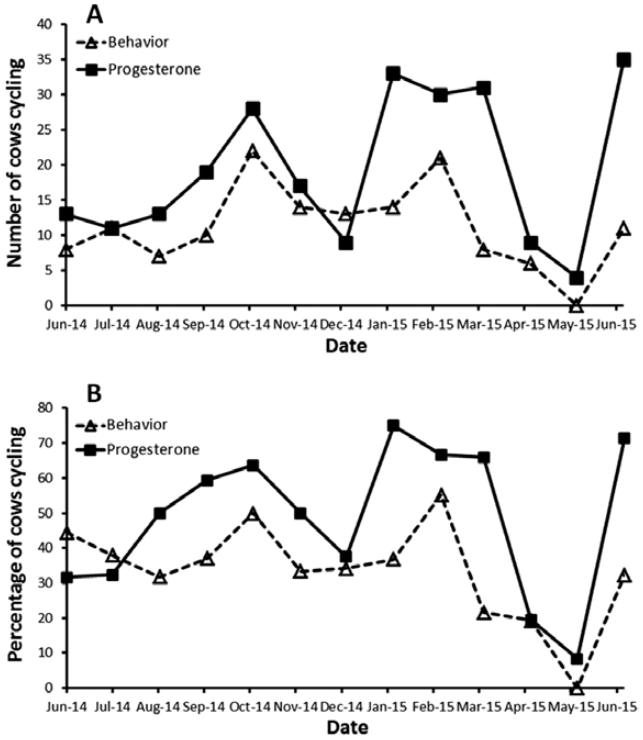


Fig. 4.—(A) Number and (B) percentage of bison (*Bison bison*) cows displaying estrous cycles, tallied by month, on Catalina Island from 1 June 2014 to 30 June 2015 in response to PZP treatment. Percentage was calculated as a fraction of the number of cows sampled in a given month. Prior to PZP treatment, the typical calving season on Catalina was from March to mid-May, indicating breeding from June to mid-August. PZP = porcine zona pellucida.

bison herd. Our success can be attributed in part to the fact that the population was closed demographically, that we were able to consistently identify and treat the entire female population, and that the agent's contraceptive effects were persistent. However, some 4–5 years have passed since PZP was administered to one-fourth (15) of the resident cows and, as of June 2017, no calves have been born on the island. Based on the documented reversibility of PZP in wild horses (Kirkpatrick and Turner 2002), we anticipated that roughly one-half of the Reversal cows would have produced calves by 2016. The shorter (9 months) return to pretreatment PZP antibody titer levels in 2 bovid species closely related to bison (Lyda et al. 2013) also encouraged us that bison might return to fertility relatively quickly. However, Ransom et al. (2013) recently found that the duration of contraception for wild horses increased with the number of years of PZP treatment, such that mares treated with PZP for 3–4 years, like the bison in our study, would not be expected to have their 1st foal until about 5–6 years later. If this pattern applies to bison, calves may not be expected in significant numbers on Catalina for several more years, especially because many cows (24; 40%) were treated for longer (5–6 years) and until as late as 2015. Based largely on our results, the Conservancy elected not to administer PZP boosters to any bison cows in 2016 and will re-assess the administration of PZP when calves are again born on the island.

It is also possible that resumption of fertility in Reversal cows has been delayed because severe drought conditions that persisted on Catalina from 2012 to 2016 reduced the amount and quality of forage. Some of the lowest pregnancy and calving rates recorded in free-ranging bison herds were documented on Catalina Island (35% in 1975 and in 2002) and on Antelope Island, Utah (46% from 1987 to 1997), and in these instances, nutritional deficiencies were suggested as the primary cause (Lott and Galland 1987; Wolfe et al. 1999; Sweitzer et al. 2003). However, the Conservancy provided bison with access to water and modest amounts of supplementary food annually throughout the drought, which should have kept bison in reasonable condition.

Because bison were reported to have at most 2 or 3 estrous cycles in a given year, we anticipated that blocking conception with PZP would, at most, modestly extend the breeding season. Aside from a brief period of low reproductive behavior and progesterone levels in mid-April through May 2015, our results instead suggest that, like domestic cattle (Garverick and Smith 1993), bison are capable of cycling nearly year-round when conception and its consequences, e.g., lactation and presence of calves, are blocked by PZP. This was evident in the behavior of the bulls as well, which continued to copulate with and tend females intensively throughout the year.

Two concerns that have been raised about an extension of the breeding season in PZP-treated ungulates are the potential impacts on the condition of males as a result of an extended rut (Nuñez et al. 2009, 2010; Delsink et al. 2013), and risks

associated with out-of-season calving (McShea et al. 1997; Nuñez et al. 2010; Ransom et al. 2013). A closer examination of the behavior of bison, specifically the frequency of shifts in dominance among bulls, and the survival of offspring born out-of-season in species subject to harsh winters, may help address these concerns, especially in comparison to the consequences of other management approaches such as removals or culling that might be applied to the Catalina herd.

Bison bulls that are 7–13 years old and weigh > 750 kg are the dominant age group, and are responsible for approximately 95% of copulations (Wolff 1998). Both tending and challenging bulls feed less during peak rut than during early rut, lose considerable amounts of weight during rut, and may sustain debilitating or life-threatening injuries during battles (Wolff 1998; Roden et al. 2011). In most herds, dominance rankings among bulls change from year to year as younger bulls mature and grow in size, and begin to challenge older or weaker bulls. Dominance may only be maintained for 2–3 weeks during the rut before an individual is displaced (Wolff 1998). Once a dominant bull is displaced, time off from tending or fighting often allows him to regain enough weight and strength to again challenge for breeding rights (Wolff 1998). Prolonged or continuous ovulation in females therefore may speed up this cycle or the rotation of bulls through periods of dominance, but it may not necessarily affect health or survival of bulls significantly.

Although we did occasionally observe head-to-head battles, in the absence of a long non-breeding period, we speculate that dominance among Catalina bulls was likely maintained through non-contact behaviors such as posturing, bellowing, head swaying, and scent urination, until a challenger reached a size and condition to confidently compete with a tending bull. Abscesses, presumably associated with battles, were occasionally observed, but dominant bulls with more serious injuries were not seen. Due to the high profile of the bison herd on Catalina, and the frequency in which they are observed by Conservancy staff, island tours, and residents, conspicuous injuries, such as a limp or open wound, are unlikely to go undetected. Based on these reports and our own informal observations, we found no evidence that the frequency of injuries or mortalities has increased as a result of the PZP program. Adult bison on Catalina have no predators, and remain brucellosis and tuberculosis free, but they still are subject to marked seasonal variations in the availability of forage and water, which were exacerbated by the drought. Given our close monitoring of the herd since the inception of the PZP program in 2009, any negative effects of an extended rut on Catalina should have been apparent by now.

At the individual level, photoperiod, temperature, vegetation green-up, and female body condition influence the timing of parturition in many ungulate species, whereas resource availability is believed to be the primary driver of birth season length in populations in highly seasonal habitats (Rutberg 1987; Green and Rothstein 1993; English et al. 2012; Zerbe et al. 2012). Because of Catalina's latitude and coastal position, these factors are mitigated to some extent compared to the Great Plains, but strong seasonal variability still occurs, in terms of pronounced wet and dry seasons. During non-drought periods vegetation green-up on Catalina generally occurs in late winter (January–February), with nutritious vegetation persisting until summer (July–August). As a result, the calving season on the island begins sooner than in most mainland herds (March versus April), but lasts about the same amount of time (Meagher 1986; Pac and Frey 1991). Out-of-season births occur in many herds (Meagher 1986; Berger and Cunningham 1994), and birth synchrony and birth season length have been found to vary due to forage availability (Green and Rothstein 1993) and population size (Meagher 1973). On Catalina, calves have been observed occasionally in September and December (Sweitzer et al. 2003) and in 2010, 2 calves were recorded in mid-February. However, the milder winter and lack of predators poses less of a mortality risk to calves born out of season on Catalina than in much of their native range. In some species, PZP application led to more out-of-season births and changes in birth synchrony (McShea et al. 1997; Nuñez et al. 2010; Ransom et al. 2013), whereas, in others, no changes were seen (Kirkpatrick and Turner 2003; Gray et al. 2010). Because we observed breeding-related behaviors and estrous cycles throughout the year, it is conceivable that declining PZP antibody titers would eventually result in more out-of-season calves on Catalina. However, until bison cows on the island again produce calves, we have no evidence that this occurs.

We underscore that bison are not native to Catalina and that some aspects of their biology on the island are quite different than in their natural range. Because of the special cultural significance of bison to island residents, and their possible role in reducing fuel loads and minimizing damage from wild fires (Sweitzer et al. 2005), it is unlikely that bison will be removed from the island in the foreseeable future. However, the bison contraception program represented an opportunity to reduce the ecological damage caused by a large, historically overabundant herbivore on the island, and our research has shown that PZP is much more cost-effective than shipments of bison off-island (Duncan 2017) and less controversial than culling. In similar circumstances elsewhere, the prolonged period of contraception apparently afforded by PZP may represent an advantage over other methods of population control. Nonetheless, we caution that the differences in climate and predators between coastal southern California and most of the native range of bison may have important implications for the application of PZP to herds elsewhere. For example, immunocontraception has been proposed as a method for reducing transmission of brucellosis in the Greater Yellowstone Area because it could prevent bison cows infected with *Brucella abortus* from becoming pregnant and thus spreading the pathogen to other bison, elk, and livestock by the shedding of infected birthing tissues and fluids and aborted fetuses (Rhyan et al. 2013). APHIS (2012) rejected PZP as a potential contraceptive agent for Yellowstone because of concerns that it would prolong the breeding season, resulting in increased activity by dominant bulls and PZP-treated cows during fall and early winter that increased mortality (Miller et al. 2004). Although the high costs associated with out-of-season breeding activity remain to be elucidated fully, our work confirms that PZP treatment does result in year-round breeding

activity of both cows and bulls, making this a legitimate concern in high-latitude, continental climates where large predators are still present.

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Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—Sampling effort (number of days, numbers of observations, and FP samples collected) on Catalina Island from 1 June 2014 to 30 June 2015.

Supplementary Data SD2.—Range of fecal progesterone values for 60 bison (*Bison bison*) cows on Catalina Island between 1 June 2014 and 30 June 2015.

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Appendix I

Summary of behavioral and fecal progesterone (FP; ng/g) measurements of 60 bison (*Bison bison*) cows on Santa Catalina Island, between 1 June 2014 and 30 June 2015. Duration is the number of days between the 1st and last behavioral observations. Overlap indicates the number of estrous cycles which were identified by both behavior (Behavior) and FP using the Brown et al. (2001) approach. The total number of cycles detected was estimated by summing the number of behavioral and FP cycles and subtracting the instances of overlap. No baseline FP value could be calculated for the last 8 cows (below the *Baseline*), so the number of FP and overlapping cycles were estimated using the mean baseline FP (338 ng/g) for the other 52 cows.

						BEHAVIOR			FP (ng/g)					CYCLES[a]			
Bison ID#	Status	Age in 2014	No. years of PZP treatment	No. days observed	Duration (days)	Heavy tending	Copulation	Tail high	No. samples	Minimum	Baseline[a]	Maximum	Range	Behavior	FP	Overlap	(Behavior + FP) − Overlap
7913	PZP	3	3	67	391	2	0	2	27	246	356	3,138	2,891	3	6	2	7
9003	PZP	6	5	65	396	0	0	1	25	183	304	977	795	1	3	0	4
9004	PZP	10	6	66	397	0	0	0	22	214	299	2,022	1,808	0	5	0	5
9005	PZP	3	3	69	392	1	0	1	15	357	448	3,186	2,829	2	4	0	6
9006	PZP	3	3	69	392	1	1	1	19	225	373	2,557	2,331	2	2	1	3
9007	PZP	5	6	74	391	3	1	2	21	294	355	3,296	3,002	3	8	2	9
9008	PZP	7	6	56	379	5	1	13	14	367	403	3,841	3,474	7	4	3	8
9011[b]	PZP	4	4	77	372	3	3	11	29	227	318	3,460	3,233	4	7	2	9
9012	PZP	3	3	74	392	0	0	3	17	222	370	2,711	2,489	3	3	0	6
9013	PZP	4	4	53	389	0	0	0	13	154	308	5,640	5,487	0	2	0	2
9014	PZP	10	6	92	340	0	0	1	20	173	308	3,348	3,175	1	3	0	4
9016	PZP	4	4	82	388	2	1	3	24	288	417	1,793	1,505	2	5	0	7
9017[b]	PZP	4	4	82	374	3	0	3	22	168	238	2,919	2,751	3	3	1	5
9018	PZP	4	4	74	387	5	0	4	12	203	466	2,114	1,912	5	2	1	6
9019[b]	PZP	4	4	72	392	3	0	0	17	208	473	6,271	6,062	3	5	2	6
9021	PZP	8	6	38	395	1	0	2	12	201	305	1,313	1,111	2	2	0	4
9023	PZP	4	4	81	394	1	0	0	19	64	272	2,395	2,331	1	5	1	5
9025	PZP	4	4	57	391	6	0	0	20	76	264	2,593	2,517	3	5	3	5
9027[b]	PZP	13	6	70	390	1	0	3	18	64	236	2,548	2,484	2	6	2	6
9029[b]	PZP	4	4	73	389	2	1	1	19	67	164	5,689	5,622	2	7	1	8
9030[b]	PZP	4	4	59	349	1	1	4	18	92	349	4,469	4,377	4	6	3	7
9032	PZP	4	6	85	392	3	1	1	23	210	470	6,002	5,792	3	7	1	9
9033	PZP	4	4	55	392	5	0	2	19	63	372	1,302	1,239	4	5	1	8
9036	PZP	4	4	79	392	0	0	0	22	128	316	3,928	3,800	0	7	0	7
9038	PZP	12	6	48	384	0	0	1	18	77	269	3,275	3,198	1	5	0	6
9040	PZP	12	6	69	394	1	1	6	19	58	283	5,419	5,361	4	7	4	7
9041[b]	PZP	5	6	60	387	0	0	1	12	44	285	873	829	1	1	0	2
9042[b]	PZP	5	6	64	389	1	1	1	7	156	497	4,321	4,165	1	3	0	4
9044	PZP	12	6	74	405	0	0	0	24	136	237	2,186	2,050	0	6	0	6
9045	PZP	5	6	62	392	3	0	2	20	57	336	5,042	4,985	4	6	2	8
9047	PZP	10	6	67	405	0	0	7	25	345	592	5,305	4,960	4	7	2	9
9048	PZP	6	6	66	375	6	1	5	10	275	602	3,065	2,790	5	2	2	5
9053[b]	PZP	19	6	75	405	1	0	1	25	223	454	2,787	2,564	1	6	1	6
9054	PZP	9	6	74	390	0	0	1	22	50	205	1,294	1,244	1	6	0	7
9056	PZP	4	4	62	397	2	2	3	16	42	223	2,581	2,539	2	5	2	5
9057	PZP	4	4	65	394	4	1	10	22	153	332	3,371	3,218	6	7	5	8
9058[b]	PZP	4	4	61	382	2	1	3	18	128	362	5,454	5,326	3	5	2	6

Appendix I. *Continued*

Bison ID#	Status	Age in 2014	No. years of PZP treatment	No. days observed	Duration (days)	BEHAVIOR			FP (ng/g)					CYCLES[a]			
						Heavy tending	Copulation	Tail high	No. samples	Minimum	Baseline[a]	Maximum	Range	Behavior	FP	Overlap	(Behavior + FP) − Overlap
9059[b]	PZP	4	4	78	387	1	0	4	23	92	343	4,244	4,152	2	6	1	7
9060	PZP	7	5	77	392	1	1	8	17	60	414	6,994	6,934	3	4	0	7
9061	PZP	9	6	97	397	6	3	13	27	63	203	3,532	3,469	8	9	5	12
UM13	PZP	3	3	80	391	0	0	1	22	159	481	5,197	5,038	1	4	0	5
9001	Reversal	8	3	76	390	1	1	4	17	140	214	5,489	5,348	4	5	3	6
9009	Reversal	8	3	73	405	4	1	9	18	323	507	4,206	3,881	7	8	6	9
9015[b]	Reversal[c]	10	4	53	292	0	0	2	14	212	267	3,014	2,802	2	7	2	7
9020[b]	Reversal	9	3	78	387	2	0	3	28	260	376	4,320	4,060	2	11	2	11
9022[b]	Reversal[c]	10	4	66	394	1	0	3	16	156	217	1,648	1,492	3	4	3	4
9026	Reversal	12	3	60	389	3	1	4	20	119	304	3,758	3,639	4	6	3	7
9037[b]	Reversal	8	3	71	389	5	1	4	17	115	283	3,146	3,031	4	7	2	9
9039[b]	Reversal[c]	8	4	45	377	1	1	2	17	125	264	4,768	4,643	2	2	0	4
9049	Reversal	10	3	64	392	3	1	2	19	83	223	1,859	1,776	4	7	3	8
9051[b]	Reversal	8	4	61	387	3	2	4	20	218	251	4,724	4,506	5	5	1	9
9052[b]	Reversal	6	3	67	392	4	2	3	13	59	370	8,134	8,075	3	3	0	6
9002	PZP	19	6	34	381	0	0	0	11	2,206	*338*	8,635	6,429	0	7	0	7
9043	PZP	14	6	54	384	0	0	3	21	18	*338*	3,379	3,361	3	4	1	6
9055[b]	PZP	10	6	70	389	2	0	0	16	268	*338*	4,422	4,154	2	10	1	11
Red38[b]	PZP	19	6	23	392	0	0	1	8	540	*338*	3,808	3,268	1	4	0	5
9046[b,d]	Reversal	7	3	23	188	0	0	0	7	251	*338*	3,227	2,976	0	5	0	5
9010[b]	Reversal[c]	14	4	50	387	4	2	2	12	332	*338*	4,996	4,664	3	7	1	9
9028[b]	Reversal	10	3	61	384	1	1	1	21	172	*338*	4,923	4,751	1	10	0	11
9050[b]	Reversal	8	3	70	395	0	0	0	17	364	*338*	4,461	4,097	0	11	0	11
PZP-treated ($N = 45$)																	
$\bar{X}$		7.5	4.9	67.3	388.2	1.7	0.5	2.9	18.9	214.3	348.8	3,615.5	3,401.1	2.5	4.9	1.3	6.2
SD		4.5	1.1	14.1	11.9	1.9	0.8	3.4	5.1	322.3	101.3	1,678.2	1,565.2	1.8	1.9	1.4	2.0
Reversal ($N = 15$)																	
$\bar{X}$		9.1	3.3	61.2	369.9	2.1	0.9	2.9	17.1	195.3	297.8	4,178.2	3,982.7	2.9	5.9	2.3	7.3
SD		2.0	0.5	14.1	56.6	1.7	0.7	2.2	4.7	94.5	88.8	1,572.5	1,579.1	1.9	2.5	1.7	2.2

[a]Mean and *SD* values do not include 8 cows (bottom rows; below double line) for which individual baseline values could not be calculated. Therefore, $N = 41$ and 11, respectively, for porcine zona pellucida (PZP)-treated and Reversal cows for these variables.

[b]Cows with cattle mtDNA, based on Derr et al. (2012).

[c]No longer received PZP boosters after 2013. All other Reversal cows were last administered PZP boosters in 2012.

[d]#9046 died in December 2014 from a leg injury.

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Suppression of behavioural and physiological oestrus in the mare by vaccination against GnRH

M ELHAY,[a] A NEWBOLD,[a] A BRITTON,[a] P TURLEY,[b] K DOWSETT,[c] and J WALKER[a]

Objective To examine the immunogenicity of an equine immunocontraceptive vaccine and its efficacy in controlling hormone-related behaviour.

Design A total of 24 mares at two sites in Australia were vaccinated with an immunocontraceptive vaccine comprising gonadotrophin releasing hormone (GnRH) conjugated to a carrier protein in immunostimulating complex as an adjuvant. Twelve animals at each site received a placebo of adjuvant alone and served as controls for seasonal oestrus, hormonal and behaviour patterns. Animals were observed for injection site reactions, ovarian and follicular activity, and serum levels of antibody, 17β-oestradiol and progesterone in the weeks following vaccination. Mares were also examined for oestrous behaviour by teasing with a stallion.

Results All mares responded to vaccination. Two weeks following the second vaccination there was a peak in antibody response to GnRH that declined gradually over the following weeks. Commensurate with the elevated anti-GnRH antibody there was a marked effect on ovarian activity with a reduction in 17β-oestradiol and progesterone levels in the 24 vaccinated mares. There was also a reduction of oestrus-related behaviour as determined by a teaser stallion. This effect lasted a minimum of 3 months and correlated with the initial level of antibody response.

Conclusion Following a conventional two-dose immunisation regime this commercially available equine immunocontraceptive vaccine was effective at inhibiting oestrous behaviour for at least 3 months. This vaccine has a high level of safety since there were no significant local reactions nor were there any adverse systemic responses to vaccination.

Key words: vaccination, gonadotrophin releasing hormone, mares, equine reproduction

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EIA	Enzyme immunoassay
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
LH	Luteinising hormone
TU	Titre unit(s)

The onset of oestrus in mares and fillies is often associated with behavioural changes such as leaning, urinating, aggressiveness and responsiveness to nearby stallions that can cause considerable concern to the animal's owner, trainer or handlers. Gonadotrophin releasing hormone (GnRH) has a central role in the control of fertility and sexual behaviour in mammals and thus oestrous behaviour in mares. GnRH exerts this effect indirectly by controlling the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. Disruption of the effects of GnRH by manipulation of GnRH receptor signals either by agonists or antagonists, has raised concerns about their expense and/or longevity of effect.[1] Neutralisation of GnRH by specific antibody elicited by vaccination represents an alternative approach and should lead to a dramatic reduction in levels of FSH and LH and subsequent effects on gonadal function.

Previously, studies with experimental formulations have demonstrated that vaccination against GnRH can have significant effects on reproductive function of colts and stallions[1–6] and prevention of the development of oestrus in the mare.[1,7–11] These studies demonstrated the feasibility of immuno-contraception in male and female horses, confirming significant physical and endocrine effects. The immunocontraceptive effects of these vaccines were often observed only following repeated, prolonged immunisation regimes that were often associated with unacceptable site reactions. There is a need, therefore, for an immunocontraceptive vaccine that would be effective and have an acceptable safety profile for general use in horses.

The feasibility of developing GnRH vaccines for use in domestic species has now been established in the swine[12] where such a vaccine is used to control boar taint in uncastrated male pigs. This vaccine has the additional benefit that it increases production gains and improves carcass characteristics, primarily through its action in suppressing sexual behaviour including fighting. A natural extension of this observation in pigs would be to apply this technology to other species where control of sexual behaviour is desired.

Materials and methods

Animals

Mares aged between 3 and 12 years were obtained at sales in South Eastern Queensland or leased from a horse buyer in Victoria (24 mares per site). The reproductive status of each mare was assessed by rectal examination and ultrasonography, before mares were admitted into the trial, to ensure they were cycling. For teasing, a miniature stallion (Queensland) or a Shetland pony stallion (Victoria) was used.

Experimental location and timing

Two trials were run in the late spring to early summer in two locations. Study 1 was conducted at Woodhill, Queensland (latitude 27°53′S) starting on 5 October 1999. Study 2 at

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Woodend, Victoria (latitude 37°21′S) began two weeks later on 19 October 1999.

Experimental design

For both studies the experimental design was identical. In each study 24 mares were identified by neck tag and other markings such as brand or coat colour. Each mare was given one of two treatments in coded syringes, either immunocontraceptive vaccine or adjuvant placebo, randomly, so that there were two groups of 12 at each site. Clinical observations were related to animal number so that those making the observations were unaware of which treatment animals had received. The code was revealed at the end of the study. During the study mares were run together in the same paddock. Each 1 to 2 weeks they were examined by ultrasonography for ovarian structure and teased to elicit oestrus cycle-related behaviour. At key time points (0, 28 and 42 days into the studies) serum samples were taken for antibody and hormone analysis. Mares were assessed for 16 weeks after first vaccination.

Vaccine

The GnRH vaccine examined (Equity™, Pfizer Animal Health P/L, West Ryde, NSW, Australia), comprises GnRH peptide conjugated to a protein carrier and admixed with immunostimulating complex as adjuvant. Immunostimulating complexes are formed from Saponin Quil A (Brenntag Biosector, Frederikssund, Denmark), cholesterol (Avanti Polar Lipids, Inc, Alabaster, Alabama) and dipalmitoylphosphatidycholine (Avanti Polar Lipids, Inc). Each 1 mL dose contained 200 µg peptide conjugate, 300 µg immunostimulating complexes, and 0.01% thiomersal, as preservative, and isotonic buffered solution to volume. The placebo vaccine was similarly formulated but with the omission of GnRH peptide conjugated to a protein carrier.

Vaccines were administered intramuscularly in the middle of the neck on day 0 and day 28.

Injection site reactions

Mares were examined daily for the first 4 days then weekly thereafter for injection site reactions. The area of vaccination was observed by eye and palpated for any swellings undetectable to the eye. Injection site reactions were scored as follows: 0, no observable or palpable reaction; 1, reaction palpable but not visible; 2, visible swelling.

Serum assays

Blood was collected into serum separating tube gel vacutainers (BD, Australia) and serum prepared by centrifugation. Serum anti-GnRH antibody titres were determined by enzyme immunoassay (EIA). Briefly, 96-well microtitre plates (Maxisorb, Nunc, Denmark) were coated overnight at 4°C with 10 µg/mL GnRH peptide in bicarbonate coating buffer pH 5.0. Plates were washed in wash buffer (0.1 M Tris buffered saline containing 0.05% Tween 20). Plates were then blocked for 1 h at room temperature with 1% sodium casein in wash buffer. Plates were washed again then incubated with serial dilutions of test mare serum for 1 h at room temperature. Plates were washed and bound antibody detected by incubating plates for further 1 h at room temperature with goat anti-horse IgG conjugated to horseradish peroxidase (HRP) (Kirkegaard-Perry Laboratories, USA) then washed. Bound goat anti-horse-HRP was visualised with tetramethylbenzidine (TMB) Microwell Peroxidase Substrate System (Kirkegaard-Perry Laboratories). A standard with a nominal titre of 1/45,000 served as a reference for unknown samples. Non-vaccinated mare serum served as a negative control.

Assays of serum progesterone were conducted using the Bioclone Elegance Progesterone EIA kit according to the manufacturer's instruction (Bioclone Australia Limited, Australia). During winter anoestrus, serum progesterone levels are typically between 3 to 6 nmol/L (Elhay and Walker, unpublished observations). Therefore, 6 nmol/L was selected as the cut-off value, below which it was regarded that an individual mare was in anoestrous, with respect to level of serum progesterone.

Serum 17β-oestradiol was measured by Victorian Veterinary Pathology Services (Clayton, Victoria, Australia), using a microparticle EIA technique.

Ovarian and follicular activity

Ovarian and follicular activity were determined by transrectal ultrasonography and palpation. Ovarian activity was measured systematically as follows: follicles and other structures were first recorded for the left ovary, and then for the right ovary. Follicles were recorded as diameter in cm. Other structures were recorded as follows: msf = many small follicles < 1 cm in diameter, mf = many follicles ≥ 1 cm in diameter, CL = corpus luteum present, na = no activity detected in/on the ovary, nd = not determined.

Each mare's cycle status was then summarised as follows:

Score 0. Anoestrus-like: small follicles, < 1 cm, or no palpable or detectable follicles; ovaries generally showing no echogenic structure by ultrasonography.

Score 1. Cycling but not in oestrus: a number of follicles, ≥ 1 cm to 2.5 cm, with no dominant follicle on ultrasonographic assessment; follicles could vary in size, but needed to be distinct and clearly delineated.

Score 2. Oestrus-like: at least one large follicle greater than 2.5 cm in diameter beginning to predominate or a follicle judged to be close to ovulation.

Behavioural activity

Oestrous behaviour was elicited with a miniature stallion or Shetland pony stallion. This induced behaviour was classified as follows:

Score 0. Anoestrous-like: if there was no interest from the mare. Some mares showed a few low-level, positive signs, such as the ears inclined forward, but which were generally passive. These mares were judged as being in an anoestrous-like state.

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Score 1. Dioestrus-like: the following behaviours, typical of dioestrus, were recorded: ears held back, aggressive squealing, aggressive kicking, aggressive biting, jamming tail down.

Score 2. Oestrus-like: the following behaviours, typical of oestrus, were recorded: ears to the side, non-aggressive/passive squealing, lifting tail, leaning toward teaser, squatting and urinating, clitoral 'winking'.

Using all of the above parameters, an estimate was then made of an individual mare's oestrous state; whether they were in oestrus (score 2), dioestrus (score 1), or in an anoestrous-like state (score 0). An intermediate score of 1.5 was recorded when a mare's behaviour indicated she was just entering oestrus or had just ovulated. A score of 0.5 was recorded for a mare which had little interest in the teaser or which kicked out at the teaser when forced to stand for teasing.

Return to oestrus

Following completion of the first part of the study, eight mares from the vaccinated group and 4 mares from the placebo vaccinated control group from each site were then kept to observe for return to oestrus activity before the onset of seasonal anoestrus. Duration of this phase of the study was 18 weeks in Queensland and 14 weeks in Victoria. These mares were examined for ovarian activity and oestrous behaviour as described above.

Statistical analyses

Statistical analysis of data was carried out by Beresford Computing and Statistical Services Pty Ltd (Melbourne, Australia). Data for serum progesterone and 17β-oestradiol were analysed by random effects regression analysis and ovarian and behavioural differences by the non-parametric Wilcoxon rank sum test (both using Stata 8.0 statistical package). It was expected that serum hormone levels would rise and fall in unvaccinated animals according to their oestrus cycle therefore random effects regression was used to determine if consistent low hormone levels were associated with vaccination. Also the oestrous cycle length of each mare was not determined and mares were examined on the same day so cycle length variation between mares could not be catered for. The two studies were analysed independently, as there were a number of variables that were different in each study including different lots of vaccine.

Adjustments were made for repeated measures in the same mare; the random effect considered was the mare. The threshold for statistical significance was at the 5% level.

Results

Injection site reactions

In the Woodend (Victoria) trial, injection site reactions were observed in 2 mares 1 week following the first vaccination and consisted of a raised area (score 2) measuring 1 or 2.5 cm in diameter and raised 0.5 cm (mares 11C and 13C respectively). One animal (19C) had a palpable (score 1) reaction, 2 cm in diameter and 0.5 cm high, 1 week following the second vaccination. All three mares were from the treatment group.

In the Queensland study conducted at Woodhill, six mares from the treatment group (mares 1, 7, 8, 9, 11, and 14) had reactions with a score of 2 (slightly raised and flat swelling of the skin approximately 5 cm in diameter) following the first vaccination. These reactions had resolved within a week. Another mare (19), vaccinated with immunocontraceptive vaccine had a reaction that was not visible to the eye though palpable (score 1) at 1 and 2 weeks post first vaccination. Finally, one of the placebo-vaccinated mares had palpable (score 1) reaction that resolved within the week following first detection. There were no injection site reactions in any of the mares at either site following the second vaccination.

Anti-GnRH antibody titres

Serum antibody was measured from samples taken at approximately 2 weekly intervals starting on the day of first vaccination. Antibody was measured by EIA and referenced to standard serum assigned a titre of 45,000 titre units (TU). Mares that received placebo vaccine had no detectable antibody response. All of the mares vaccinated with anti-GnRH vaccine developed an antibody response with maximum titres achieved by 2 weeks following the second immunisation (Figure 1). Maximum antibody levels at 2 weeks after the second vaccination were quite heterogeneous amongst animals, ranging from a low titre of 1064 through to a maximum of 1.8×10^6 TU. More often, the maximum antibody level was between 10^4 to 10^5 TU. Nine of the 24 vaccinated mares developed an antibody response following one injection only (data not shown). This antibody waned quickly and was then masked by the antibody that developed rapidly following the second immunisation.

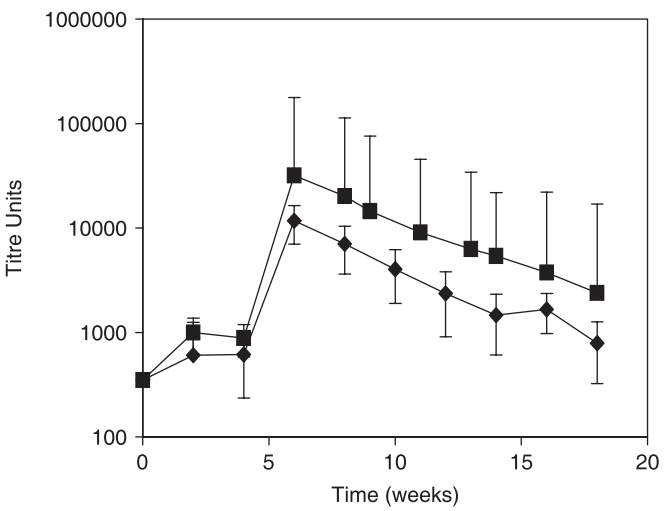


Figure 1. Antibody responses to anti-GnRF vaccination in two sites, Woodhill in SE Queensland (-◆-QLD) and Woodend in Central Victoria (-■- VIC). Twelve mares at each site were vaccinated at week 0 and week 4 with equine immunocontraceptive vaccine. Antibody is presented as the group geometric mean in Titre Units ± SEM.

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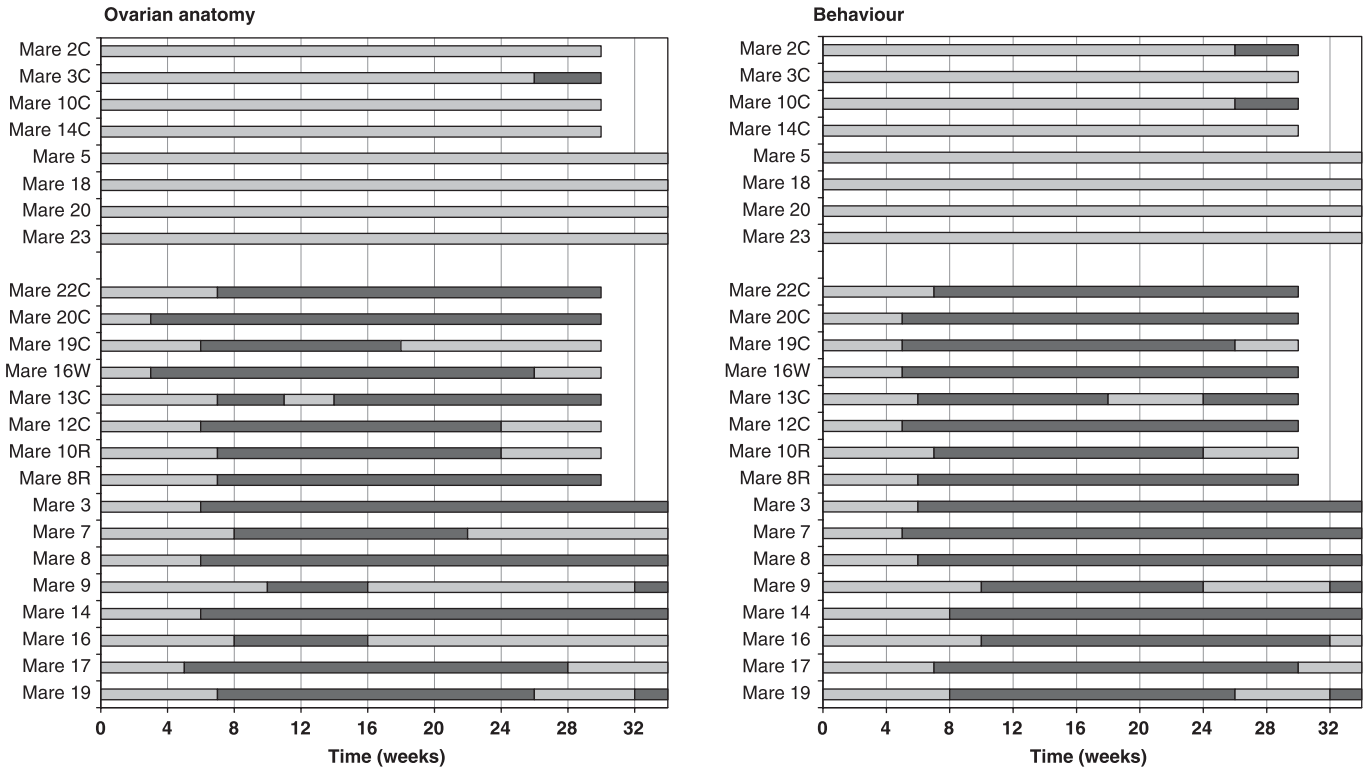


Figure 2. Time in weeks of reproductive activity of individual mares kept for 29 weeks (in Victoria) and 34 weeks (Queensland), after vaccination at Weeks 0 and 4. Each horizontal line represents one mare. Mares shown as having oestrus or dioestrus like activity (light grey) or anoestrus like activity (dark grey). The upper block represents placebo mares (mare numbers 2C to 23) and the lower block represents treated mares (mares 22C to 19). Mares with alphanumeric identification are from the trial run in Victoria.

Ovarian activity

All mares were observed to cycle in both groups during the 2 weeks post first vaccination (Figure 2). Placebo vaccinated controls continued to cycle normally throughout the trial, as expected for the time of year (Figure 3).

The effect of vaccination on ovarian activity was apparent in all treated mares within 2 weeks of second vaccination. In general, there was a significant reduction in the size of the ovaries and any follicles that could be palpated or distinguished by ultrasonographic imaging (Woodhill, Queensland $P < 0.001$, Woodend Victoria, $P < 0.001$). This process of ovarian change continued for several weeks until no folliculogenesis could be detected. For most mares, by 4 weeks post second vaccination, the ovaries resembled those of seasonally anovulatory mares, and no copora lutea were identified.

Oestrous behaviour

A significant (Woodhill, Queensland $P < 0.001$, Woodend Victoria, $P = 0.012$) effect of vaccination on oestrous/dioestrus behaviour was observed following second vaccination (Figure 4). A number of vaccinated mares in the Victorian study were recorded as showing signs of cycling mares. In all cases, these mares showed dioestrus-like signs (kicking out at the teaser) that may have been indicative of forced teasing in the crush.[13] This behaviour stopped once mares were allowed greater freedom in a pen rather than a crush with the teaser separated from them by a fence. For the Queensland study all teasing was conducted within a pen with the mare and miniature stallion on a lead. In this study the onset of anoestrus-like behaviour more closely followed the decline in ovarian activity (Figure 3).

Serum progesterone levels

Results of the serum progesterone assay are shown in Figure 5. Mares vaccinated with GnRH vaccine showed serum progesterone levels below 6 nmol/L from 2 weeks following the second dose of vaccine, and by 4 weeks post boost, none of the 24 GnRH vaccine immunised mares had significant levels of serum progesterone. At no stage thereafter did serum progesterone levels exceed the threshold value of 6 nmol/L.

Placebo vaccinated mares exhibited varying levels of progesterone. Statistical analysis by random effects regression analysis indicated a highly significant difference between the means of the placebo and treated groups at both sites (Woodhill, $P < 0.001$ and Woodend, $P = 0.003$).

Serum 17β-oestradiol levels

Serum 17β-oestradiol levels were reduced from those observed before vaccination, but there was always a detectable level of 17β-

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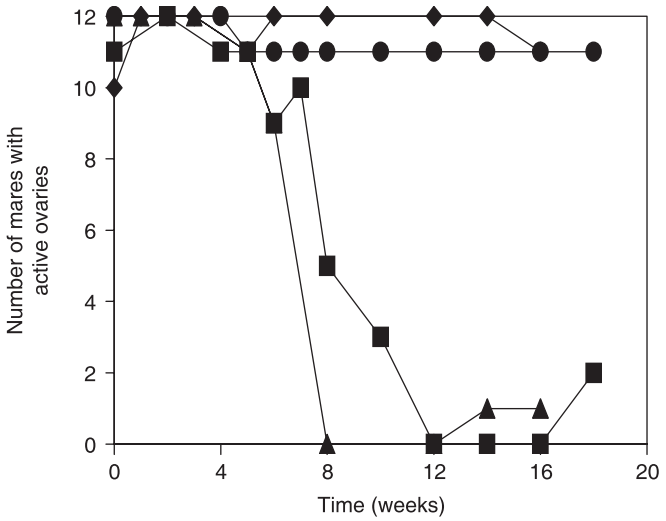


Figure 3. Number of mares with ovarian activity (Score 1 or 2) as measured by trans-rectal palpation and ultrasonography following vaccination with equine immunocontraceptive vaccine. Mares were vaccinated on week 0 and 4 with either vaccine (-■- QLD, -▲- VIC) or placebo (-●- QLD, -◆- VIC).

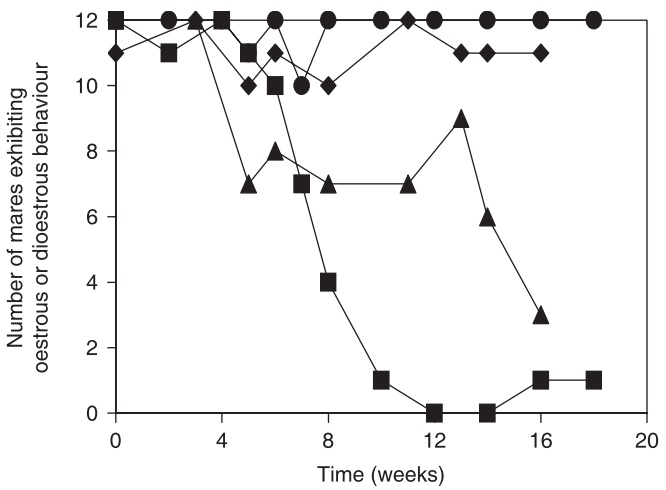


Figure 4. Number of mares exhibiting oestrous or dioestrous behaviour (Score 1 or 2), measured by teasing with a Shetland pony stallion (VIC) or miniature stallion (QLD) following vaccination with GnRH vaccine (-■- QLD, -▲- VIC) or placebo (-●- QLD, -◆- VIC).

oestradiol in some of the vaccinated mares during the period of immune anoestrus. Mean 17β-oestradiol levels were reduced by 16.5 pmol/L for the Queensland trial and 10.9 pmol/L for the Victorian trial. Random effects regression analysis of the differences between the means of the 2 groups indicated a significant effect of vaccination ($P = 0.005$ and $P = 0.02$ for the Queensland and Victorian trials respectively).

Return to oestrus

In the mares that were kept to observe for return to oestrus activity before the onset of seasonal anoestrus, the duration of

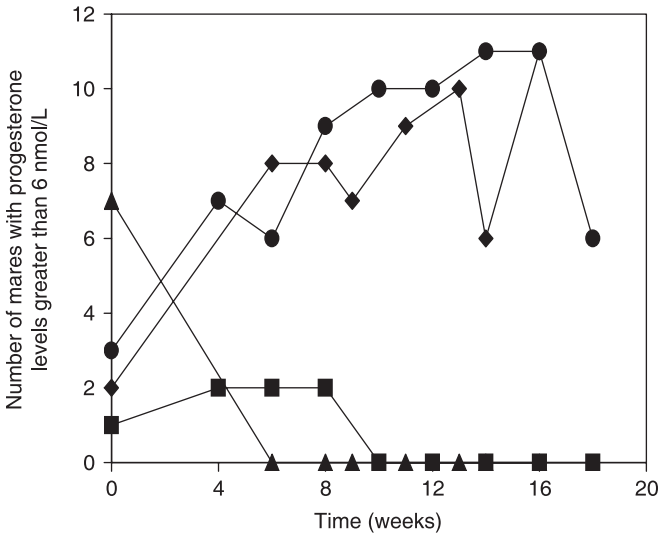


Figure 5. Number of mares with progesterone levels greater than 6 nmol/L. Mares were regarded as being equivalent to anoestrus if progesterone was less than 6 nmol/L. Vaccine treated mares: -■- QLD, -▲- VIC; placebo treated mares: -●- QLD, -◆- VIC).

ovarian shutdown varied from 4 to 23 weeks in 10 of the 16 vaccinated mares (Figure 2). The remaining 6 (3, 8 14, 8R, 20C, 22C) did not return to a cycling status for the rest of the trial.

Three mares (9, 16 and 13C) appeared to have a relatively short period of GnRH vaccine-induced ovarian dormancy lasting approximately 6, 8 and 4 weeks respectively. Thereafter, their ovaries indicated that folliculogenesis and cycling may have begun. These mares also had some of the lowest initial anti-GnRH antibody titres. However, despite this correlation between circulating anti-GnRH antibody and ovarian anatomy, there was no immediate return of sexual behaviour. Sexual behaviour was not observed in these mares for another 8, 14 and 8 weeks following first signs of ovarian activity for mares 9, 16 and 13 C respectively (Figure 2).

The duration of immune anoestrus as measured at the level of behaviour was always longer in duration than the effect on ovarian activity.

The apparent onset of seasonal anoestrus at the level of ovarian anatomy and/or teasing behaviour was observed in three of the mares (mares 13C, 9 and 19). Two of the control mares (2C and 10C) appeared to enter behavioural seasonal anoestrus within the study period, and one (3C) ceased ovarian activity.

Discussion

Immunisation of all 24 mares with two doses of GnRH vaccine elicited antibody to GnRH and significant changes to the levels of ovarian hormones and oestrous behaviour.

The effect of vaccination with the GnRH vaccine was evident in most mares within 2 weeks following second vaccination. Immunological vaccine efficacy was 100%, as reflected in antibody titre, which rose following vaccination to a peak at 2 weeks

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post-boost in all animals. Neutralisation of GnRH in all likelihood resulted in the diminished production of FSH and LH,[7] and as a consequence, due to the absence of these hormonal stimulators, there was distinct regression of both ovaries in all vaccinated mares. In fact, their ovaries began to resemble those observed in seasonally anovulatory mares.

Commensurate with the antibody response and ovarian changes, there was a reduction in the levels of sex steroids in serum from vaccinated mares. Progesterone showed the clearest effect indicating lack of FSH dependent folliculogenesis and LH dependent luteal maintenance. At no stage thereafter did serum progesterone levels exceed the threshold value of 6 nmol/L, indicating that the cycle status of the mares had been suspended.

All vaccinated mares showed a significant reduction in their ovarian activity due to vaccination. This was evident as a dramatic reduction in the size of the ovary and a reduction in follicular activity. These results indicate that the effect of vaccination against GnRH was to prevent folliculogenesis and the development of the corpus luteum and thereby prevent progesterone production.

17β-oestradiol levels were not reduced to the same degree as was observed for progesterone. This may indicate that there was an extra-ovarian source for this hormone. The adrenal cortex is a known source of oestrogens and other steroids and may be an alternate source of oestrogen in the mare.[13–16] It has been suggested that adrenal 17β-oestradiol may be responsible for the sexual behaviour displayed by seasonally anovulatory mares as this behaviour can be ablated by dexamethasone treatment of ovariectomised mares.[15]

Another extra-ovarian source of 17β-oestradiol may have been from steroid precursors in the adrenal. The precursor androstenedione is produced by the ovarian cells from 17α-hydroxyprogesterone. Androstenedione is then converted to oestrone or testosterone, either of which are precursors of oestradiol. Pre-antral follicles, arrested in their development by anti-GnRH vaccination,[10] may have served as a source of serum androstenedione which in turn may have acted as a precursor for 17β-oestradiol. Tshewang and coworkers[10] found no effect of vaccination on serum androstenedione levels in mares and fillies. Colts treated with another GnRH vaccine on the other hand had negligible levels of serum androstenedione suggesting a different mechanism of hormonal control of these steroids in mares from colts.[5]

Without further specific investigation, it is not possible to say if one or a combination of these mechanisms may have contributed to the results observed in these studies. Importantly, there was a reduction in the total amount of 17β-oestradiol in vaccinated mares. Oestrous related behaviour, which is believed to be driven by 17β-oestradiol in the mare, was markedly reduced, indicating that this behaviour was inhibited by vaccination.

Sixteen mares (eight from each trial) were retained beyond 4 months post primary course of vaccination. The duration of the effect of treatment, as measured by a return of folliculogenesis detected by ultrasonography, ranged from 6 to 28 weeks with the most frequent duration of immune anoestrus being 23 weeks for 5 of 16 mares (Figure 3).

This reduction in ovarian activity was reflected in the results for oestrous behaviour. Mares vaccinated with GnRH vaccine showed suppressed oestrous behaviour such that their behaviour resembled that of seasonally anovulatory mares. This effect lasted from 12 to at least 29 weeks with 11 of 16 mares clustered around 22 to 29 weeks duration of immune anoestrus.

The observation that the duration of immune anoestrus as measured by behaviour was always longer in duration than the effect on ovarian anatomy, suggests that reactivation of the ovary and folliculogenesis is a necessary precursor to the onset of oestrus-related behaviour.

For those mares that did return to cycling behaviour, as the immune response waned, there was an increase in ovarian activity including folliculogenesis and ultimately, ovulation. For some mares (three in Victoria and four in Queensland), there was a clear return of seasonal oestrous behaviour in late summer or early autumn. One of these mares in Victoria and two in Queensland then proceeded to enter apparent seasonal anoestrus in autumn after having earlier returned to oestrus cycling activity. It is expected that duration of efficacy of the vaccine may have overlapped with the onset of seasonal anoestrus in these mares.

The study described demonstrates that an anti-GnRH vaccine has been developed that is safe and effective. It is unique in that it uses a proprietary adjuvant system, immunostimulating complex, and to our knowledge is the first vaccine of its type for use in horses.

Acknowledgments

We thank Dr Peter Little and Mr Andrew Hodge, Veterinary Medicines Research and Development, Pfizer Animal Health for their helpful comments and discussion on the studies described in this manuscript.

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United States
Environmental Protection Agency
Office of Prevention, Pesticides and Toxic Substances
(7505P)

Pesticide Fact Sheet

Name of Chemical: **Mammalian Gonadotropin Releasing Hormone (GnRH)**

Reason for Issuance: **New Chemical**
Nonfood Use

Date Issued: **September 2009**

1. Description of Chemical

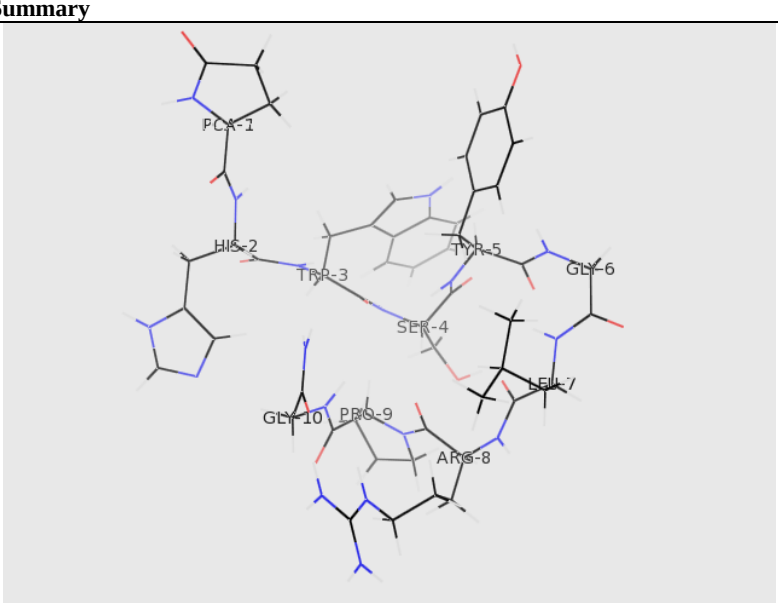
Peptide Chain:	pyroGlu1 -His2-Trp3- Ser4 -Tyr5- Gly6 -Leu7-Arg8-Pro9- G ly10NH2 [GnRH]
Common Name:	Mammalian Gonadotropin Releasing Hormone (GnRH)
EPA PC Code:	116800
Chemical Abstracts Service (CAS) Number:	9034-40-6
Chemical Class:	Sterilant/Hormone
Registration Status:	New Chemical, nonfood use
Pesticide Type:	Mammalian Contraceptive
U.S. Producer:	U.S. Department of Agriculture, APHIS, Pocatello Supply Depot 238 East Dillon Street Pocatello, ID 83201

2. Use Pattern and Formulations

Mode of Action	The active ingredient, Mammalian Gonadotropin Releasing Hormone (GnRH) is conjugated into a large protein that initiates an immune response in the animal with its own GnRH resulting in contraceptive effects for a minimum of one year.
Application Sites	GonaCon will be used to control wild white-tailed deer *Odocoileus virginianus*) populations in areas where they have become a nuisance (e.g, urban and suburban settings).
Methods of Application	The vaccine will be administered to restrained female deer using preloaded syringes with an 18 or 19 gauge stainless steel hypodermic needle intramuscularly into a large muscle mass by hand injection only. GonaCon is classified as a Restricted Use Pesticide. Use is restricted to USDA APHIS Wildlife Services or state wildlife management personnel or persons working under their authority.
Application Rate:	Female deer are injected with a single injection containing 1.0 ml of GonaCon at least two to three months prior to the onset of rut for full contraceptive effect. If multi year contraceptive effects are desired, a second vaccination may be given 30 to 60 days after the first injection or during the following year. Two formulations (basic and alternate) are proposed for registration.

3. Science Findings

Available data supporting the use and registration of Mammalian Gonadotropin Releasing Hormone including product chemistry, toxicology, efficacy, and ecological effects and environmental fate are summarized below.

Table 1. Product Chemistry Summary	
Peptide structure	
Common name	Mammalian Gonadotropin Releasing Hormone (GnRH)
CAS Reg. No.	9034-40-6
Color	white
Physical State	Active: solid: powder EU: liquid: somewhat creamy in appearance
Melting Point	N/A Waiver request
Boiling Point	Waiver request: Solid at room temperature.
Odor	Active: odorless EU: odorless
Stability to Normal and Elevated Temperatures, Metal, and Metal Ions	N/A Waiver request
Oxidation/Reduction Action	N/A Waiver request
pH	Active: NA EU: pH = 6.49
Flammability	N/A Waiver request
Explodability	N/A Waiver request
Vapor pressure	N/A Waiver request
Water Solubility	N/A Waiver request
Storage stability	N/A product's shelf life will be $\leq$ 6 months so not necessary
Corrosion Characteristics	N/A product's shelf life will be $\leq$ 6 months so not necessary

TOXICOLOGY SUMMARY

The Registrant submitted the studies listed in Tables 2, which include a number of toxicity studies. The Registrant submitted waiver requests which were granted for acute inhalation and dermal sensitization.

Acute Toxicity Data GnRH

Table 2. Acute Toxicity			
Guideline No.	**Study Type**	**Results**	**Toxicity Category***
OPPTS 870.1100	Acute Oral Rat	All test animals survived 1 mL exposure.	IV
OPPTS 870.1200	Acute Dermal Rat	All test animals survived 1 mL exposure.	IV
OPPTS 870.1300	Acute Inhalation Rat	WAIVED	IV
OPPTS 870.2400	Primary Eye Irritation Rabbit	No corneal opacity or iritis was observed. 1-hour post-instillation: 3/3 treated eyes exhibited conjunctival redness (score 1-2) and discharge (score 1). No "positive" grade irritation was noted at the 24-hour observation. Treated eyes were free of all eye irritation by 72-hours.	IV
OPPTS 870.2500	Primary Skin Irritation Rabbit	No edema was observed at any treated site. Very slight erythema (score 1) was noted at all 3/3 test sites within 1-hour post-pad removal. Irritation severity decreased thereafter. No irritation was noted at the 72-hour observation. The PDII was 0.5.	IV
OPPTS 870.2600	Dermal sensitization	WAIVED	--

- Toxicity Category IV = No precautions required

Chronic toxicity data requirements were waived. There is no human exposure from use of GonaCon, therefore no toxicity endpoints were selected because of the very limited potential worker and dietary exposure.

ECOLOGICAL EFFECTS

Waivers were submitted to fulfill required ecological effects and environmental fate guideline studies for the registration of GonaCon because of the limited potential for environmental releases. Since the product is labeled only for injection to deer by hand and the substance is expected to be rapidly metabolized in treated animals, the limited

potential risks to non-target organisms resulting from the proposed registration of GonaCon are not expected to exceed the Agency's concern levels.

The proposed registration of GonaCon is expected to have no effect on endangered or threatened species

EFFICACY

GonaCon is intended to render a vaccinated female white-tailed deer infertile for a minimum of one year following vaccination. The label claims that GonaCon will not affect existing pregnancies but should cause infertility of the vaccinated animal in subsequent years. If multi-year contraceptive effects are desired, a second vaccination may be given 30-60 days after the first injection or during the following year. The label claims that there is a chance that some vaccinated females will become permanently sterile.

Product performance studies were conducted by APHIS's National Wildlife Research Center (NWRC) both in the field over a two year period and in a laboratory over multiple years to compare two different formulations.

The field test, initiated in 2004 used two sites consisting of fenced federal land near Silver Spring, MD. Female deer in the vaccinated group were dosed with the labeled rate of the vaccine during July and August 2004. All deer were marked with ear tags and radio telemetry collars equipped with mortality sensors. Reproductive status was assessed in the summers of 2005 and 2006 with visual inspection of the udders for signs of lactation.

Results showed 88% efficacy in the summer after vaccination with lactation evident in 3 of the 26 does vaccinated (12%). Of the control group, reproductive status was able to be determined in 13 of the 15 deer and lactation was evident in 11 of those 13 doe (85%).

In the second summer after vaccination lactation was evident in 10 of the remaining 19 vaccinated deer (53%) equating to an efficacy rate of 47%. In the control group, 10 of the 10 remaining deer (100%) had reproductive success.

The lab study results showed the alternate formulation to be more effective than the basic formulation. The alternate formulation had a 100% success rate (5/5 does remained contracepted for two years) compared to the basic formulation with a 60% success rate after two years (3/5 does contracepted). Due to economic and supply reasons however, it is necessary for the product to carry both formulations.

4. Summary of Regulatory Position and Rationale

Available data provide adequate information to support the conditional registration of GnRH as an effective population control method for nuisance white-tailed deer.

White-tailed deer have been classified by EPA as a public health pest because they are a host for deer ticks that may cause Lyme disease and they are a cause of vehicle accidents.

In many urban and suburban areas white-tailed deer populations have become over abundant and are considered a year-round nuisance causing many human-wildlife conflicts. Since these areas are typically non hunting areas, where building and population densities do not allow for safe shooting zones, local laws prohibit guns or shooting or public opinion does not support hunting, there has not been an economically feasible solution to control overabundant deer populations.

In many of these urban and suburban areas, deer have been known to inflict significant economic damage to gardens and landscapes of homeowners associations, golf courses, private residential yards, etc. When deer are in numbers exceeding 20 per square mile, as they are becoming in many areas, they can inflict severe damage to resident flora and fauna. This can adversely affect the ecosystem and the wildlife that depend on that ecosystem.

Since deer in these areas are so well fed, female deer, which normally give birth to twins may be becoming more fertile which is evident by an increase in triplet births. This, and the fact that deer in developed areas appear to be more nocturnal to avoid human contact, is contributing to rapidly increasing populations and problems.

According to a 2006 study, the National Highway Traffic Safety Administration reported that there are about 1.5 million car accidents with deer resulting in over $1 billion of damage and 150 human fatalities annually. GnRH will not only help prevent increases in deer numbers through contraception, but should decrease the number of car accidents during the fall mating season - which is associated with a surge in car collisions with deer as bucks chase fertile does, sometimes for miles.

Deer can also play a role in spreading disease as they are a main host for blacklegged ticks (*Ixodes scapularis*), more commonly known as deer ticks, which are a carrier of Lyme disease. There has been a sharp increase in cases of Lyme disease in suburban/urban areas with significant deer populations.

Contraception of nuisance deer using GnRH is not intended to replace hunting (where it is an option) as a population control method. GnRH is intended to be used in combination with other population management techniques since it cannot alone reduce already over abundant populations. Since GnRH is a nonlethal alternative to population control, many animal rights groups enthusiastically support this registration.

The registration of GonaCon has received letters of support from the Department of Natural Resources at Cornell University and White Buffalo, Inc. a non-profit wildlife organization based out of Connecticut. At Cornell University, deer have caused damages to plants in research and teaching plots as well as dozens of car accidents. Currently, the university attempts population management of deer by capturing and surgically sterilizing deer via tubal ligation. This method however is both time consuming and costly, with expenses of approximately $1,000 per deer along with the impracticality of finding surgery staff and anesthesiologists during the early and late hours that deer are typically captured. The use of GnRH is also supported by the Humane Society of the United States and the Wildlife Society as a humane and “necessary” method of deer population control.

GonaCon was mentioned in an April 2009 article in the Washington Post Magazine as a possible population control method for deer in Rock Creek Park in Washington, DC and suburban Maryland.

5. Labeling Restrictions

To mitigate any risks, the following requirements have been imposed:

- Restricted -Use Pesticide classification due to non-target injection hazard.
- Application is restricted to USDA APHIS Wildlife Services or state wildlife management agency personnel or persons working under their authority only.
- Administration of vaccine is only by hand injection to mitigate any non-target or environmental risks that occur with administration with darts.
- Use restricted to only one species: white-tailed deer (*Odocoileus virginianus*).
- PPE requirements include: long sleeved shirt and long pants, gloves and shoes plus socks to mitigate occupational exposure.
- Children are not allowed in areas where product is used
- A warning that pregnant women should not be involved in handling or injecting GonaCon and that all women should be aware that accidental self-injection may cause infertility.

6. Conditional Data Requirements

Because of the unique chemical nature of GnRH additional preliminary analysis and certified limits data are necessary. The registrant must submit this data to the Agency upon completion.

Conditional data required for GonaCon consists of:

- Guideline 830.1700 Validating the method of analysis of the formulation and additional preliminary analysis
- Guideline 830.1750 Certified Limits

Contact Person at USEPA

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Office of Pesticide Programs
Registration Division (7505P)
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2777 S. Crystal Drive
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703-305-5314

DISCLAIMER: The information in this Pesticide Fact Sheet is for information only and is not to be used to satisfy data requirements for pesticide registration. The information is believed to be accurate as of the date on the document.

APPENDIX I

GLOSSARY OF TERMS AND ABBREVIATIONS

ADNT	Acute delayed neurotoxicity
a.i.	Active Ingredient
aPAD	Acute Population Adjusted Dose
ARI	Aggregate Risk Index
BCF	Bioconcentration Factor
CAS	Chemical Abstracts Service
ChE	Cholinesterase
ChEI	Cholinesterase inhibition
cPAD	Chronic Population Adjusted Dose
%CT	Percent crop treated
DAT	Days after treatment
DEEM-FCID	Dietary Exposure Evaluation Model - Food Consumption Intake Database
DNA	Deoxyribonucleic acid
DNT	Developmental neurotoxicity
DIT	Developmental immunotoxicity
DWLOC	Drinking Water Level of Comparison.
EC	Emulsifiable Concentrate Formulation
EEC	Estimated Environmental Concentration. The estimated pesticide concentration in an environment, such as a terrestrial ecosystem.
EPA	U.S. Environmental Protection Agency
FQPA	Food Quality Protection Act
GLC	Gas Liquid Chromatography
GLN	Guideline Number
LC_{50}	Median Lethal Concentration. A statistically derived concentration of a substance that can be expected to cause death in 50% of test animals. It is usually expressed as the weight of substance per weight or volume of water, air or feed, e.g., mg/l, mg/kg or ppm.
LD_{50}	Median Lethal Dose. A statistically derived single dose that can be expected to cause death in 50% of the test animals when administered by the route indicated (oral, dermal, inhalation). It is expressed as a weight of substance per unit weight of animal, e.g., mg/kg.
LOAEL	Lowest Observed Adverse Effect Level
LOAEC	Lowest Observed Adverse Effect Concentration
LOC	Level of Concern
LOD	Limit of Detection
LOQ	Limit of Quantitation
mg/kg/day	Milligram Per Kilogram Per Day
mg/L	Milligrams Per Liter
MOE	Margin of Exposure

MRID	Master Record Identification (number), EPA's system of recording and tracking studies submitted
MTD	Maximum tolerated dose
NA	Not Applicable
NOEC	No Observable Effect Concentration
NOEL	No Observed Effect Level
NOAEL	No Observed Adverse Effect Level
NOAEC	No Observed Adverse Effect Concentration
NPDES	National Pollutant Discharge Elimination System
OP	Organophosphate
OPP	EPA Office of Pesticide Programs
OPPTS	EPA Office of Prevention, Pesticides and Toxic Substances
PAD	Population Adjusted Dose
PAG	Pesticide Assessment Guideline
PAM	Pesticide Analytical Method
PHED	Pesticide Handler's Exposure Data
PHI	Preharvest Interval
ppb	Parts Per Billion
PPE	Personal Protective Equipment
ppm	Parts Per Million
PRZM/EXAMS	Tier II Surface Water Computer Model
RAC	Raw Agriculture Commodity
RBC	Red Blood Cell
RED	Reregistration Eligibility Decision
REI	Restricted Entry Interval
RfD	Reference Dose
SCI-GROW	Tier I Ground Water Computer Model
SF	Safety Factor
TGAI	Technical Grade Active Ingredient
UF	Uncertainty Factor
µg	micrograms
µg/L	Micrograms Per Liter
µL/g	Microliter per gram
USDA	United States Department of Agriculture
WPS	Worker Protection Standard

APPENDIX II

Citations Considered Part of the Data Base Supporting the Registration of GonaCon.

MRID	Citation	Receipt Date
47649600	United States Department of Agriculture's Animal and Plant Health Inspection Service (USDA APHIS) (2009) Submission of Product Chemistry, Toxicity, Efficacy and Residue Data in Support of the Application for Registration of GonaCon Immunocontraceptive Vaccine. Transmittal of 36 Studies.	22-Jan-2009
47649601	O'Hare, J.; Eisemann, J. (2008) Description of Materials Used to Produce: "GoneCon Immunocontraceptive Vaccine". Project Number: P4417. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 168 p.	22-Jan-2009
47649602	O'Hare, J.; Eisemann, J. (2008) Description of Formulation Process Used to Manufacture: "GonaCon Immunocontraceptive Vaccine". Project Number: M8546, BT016/02. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 99 p.	22-Jan-2009
47649603	O'Hare, J.; Pilon, J.; Eisemann, J. (2008) Discussion of the Formation of Impurities "GonaCon Immunocontraceptive Vaccine". Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlive Research Center. 56 p.	22-Jan-2009
47649604	O'Hare, J.; Eisemann, J.; Pilon, J. (2008) Preliminary Analysis and Certified Limits "GonaCon Immunocontraceptive Vaccine": Final Report. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 9 p.	22-Jan-2009
47649605	O'Hare, J.; Eisemann, J. (2008) Enforcement Analytical Method: "GonaCon Immunocontraceptive Vaccine". Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 40 p.	22-Jan-2009
47649606	O'Hare, J.; Eisemann, J. (2007) Product Chemistry: Color, Physical State, Odor, and pH - USDA APHIS GonaCon Immunocontraceptive Vaccine (EPA Reg. No. 56228-xx): Final Report. Project Number: QA/1421. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 31 p.	22-Jan-2009
47649607	Warren, J.; Stephens, S. (2008) Stability to Normal and Elevated Temperatures, Metal, and Metal Ions: (Gonadotropin Releasing Hormone). Unpublished study prepared by US Dept. of Agriculture, APHIS, WS. 8 p.	22-Jan-2009
47649608	Warren, J.; Stephens, S. (2008) Oxidation/Reduction Chemical Incompatibility: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by US Dept. of Agriculture, APHIS, WS. 8 p.	22-Jan-2009
47649609	Warren, J.; Stephens, S. (2008) Flammability: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009

47649610	Warren, J.; Stephens, S. (2008) Explodability: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649611	Warren, J.; Stephens, S. (2008) Miscibility: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649612	Warren, J.; Stephens, S. (2008) Corrosion Characteristics: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649613	Warren, J.; Stephens, S. (2008) Dielectric Breakdown Voltage: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649614	Warren, J.; Stephens, S. (2008) UV/Visible Absorption: (GonaCon Immunocontraceptive). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649615	Warren, J.; Stephens, S. (2008) Viscosity: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649616	Warren, J.; Stephens, S. (2008) Melting Point: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649617	Warren, J.; Stephens, S. (2008) Boiling Point: (GonoCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649618	Warren, J.; Stephens, S. (2008) Density/Relative Density/Bulk Density: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649619	Warren, J.; Stephens, S. (2008) Dissociation Constant in Water: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649620	Warren, J.; Stephens, S. (2008) Octanol/Water Partition Coefficient: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649621	Warren, J.; Stephens, S. (2008) Water Solubility: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649622	Warren, J.; Stephens, S. (2008) Vapour Pressure: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649623	Eisemann, J.; O'Hare, J. (2008) Chemical Identity: "GonaCon Immunocontraceptive Vaccine". Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: Natiional Wildlife Research Center. 27 p.	22-Jan-2009
47649624	Eisemann, J.; O'Hare, J. (2008) Direction for Use: GonaCon Immunocontraceptive Vaccine. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Research Center. 12 p.	22-Jan-2009
47649625	Lowe, C. (2008) GonaCon Immunocontraceptive Vaccine: Acute Oral	22-Jan-

	Toxicity - Limit Dose Procedure in Rats. Project Number: P320/USDA, 23211. Unpublished study prepared by Product Safety Laboratories. 28 p.	2009
47649626	Lowe, C. (2008) GonaCon Immunocontraceptive Vaccine: Acute Dermal Toxicity - Limit Dose Procedure in Rats. Project Number: 23212, P322/USDA. Unpublished study prepared by Product Safety Laboratories. 28 p.	22-Jan-2009
47649627	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Acute Inhalation Toxicity: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649628	Lowe, C. (2007) GonaCon Immunocontraceptive Vaccine: Primary Eye Irritation Study in Rabbits. Project Number: P324, 22806. Unpublished study prepared by Product Safety Laboratories. 29 p.	22-Jan-2009
47649629	Lowe, C. (2008) GonaCon Immunocontraceptive Vaccine: Primary Skin Irritation Study in Rabbits. Project Number: P326, 22807. Unpublished study prepared by Product Safety Laboratories. 27 p.	22-Jan-2009
47649630	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Dermal Sensitization: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649631	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Chronic Dietary Toxicity: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649632	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Gene Mutation: (GonaCon Immunocontraceptive Vaccine): (Human). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649633	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Stuctural Chromosomal Aberration: (GonaCon Immunocontraceptive Vaccine): (Human). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649634	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Other Genotoxic Effects: (GonaCon Immunocontraceptive Vaccine): (Human). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649635	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Acute Delayed Neurotoxicity: (GonaCon Immunocontraceptive Vaccine): (Hen). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649636	Gionfriddo, J.; Eisemann, J.; O'Hare, J. (2008) Product Performance: Field Test of a Single-Injection of GonaCon Immunocontraceptive Vaccine In Female White-Tailed Deer. Project Number: QA/1112. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 31 p.	22-Jan-2009

United States
Environmental Protection Agency
Office of Prevention, Pesticides and Toxic Substances
(7505P)

Pesticide Fact Sheet

Name of Chemical: **Mammalian Gonadotropin Releasing Hormone (GnRH)**

Reason for Issuance: **New Chemical**
Nonfood Use

Date Issued: **September 2009**

1. Description of Chemical

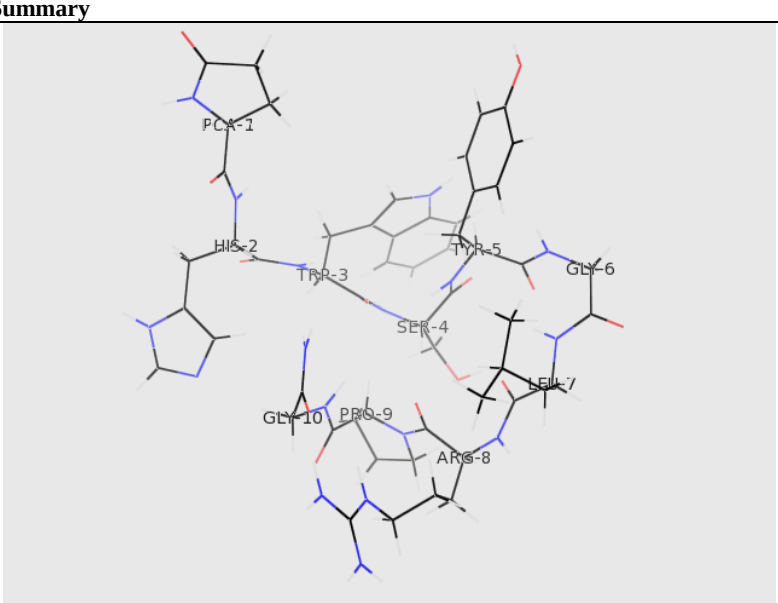
Peptide Chain:	pyroGlu1 -His2-Trp3- Ser4 -Tyr5- Gly6 -Leu7-Arg8-Pro9- G ly10NH2 [GnRH]
Common Name:	Mammalian Gonadotropin Releasing Hormone (GnRH)
EPA PC Code:	116800
Chemical Abstracts Service (CAS) Number:	9034-40-6
Chemical Class:	Sterilant/Hormone
Registration Status:	New Chemical, nonfood use
Pesticide Type:	Mammalian Contraceptive
U.S. Producer:	U.S. Department of Agriculture, APHIS, Pocatello Supply Depot 238 East Dillon Street Pocatello, ID 83201

2. Use Pattern and Formulations

Mode of Action	The active ingredient, Mammalian Gonadotropin Releasing Hormone (GnRH) is conjugated into a large protein that initiates an immune response in the animal with its own GnRH resulting in contraceptive effects for a minimum of one year.
Application Sites	GonaCon will be used to control wild white-tailed deer *Odocoileus virginianus*) populations in areas where they have become a nuisance (e.g, urban and suburban settings).
Methods of Application	The vaccine will be administered to restrained female deer using preloaded syringes with an 18 or 19 gauge stainless steel hypodermic needle intramuscularly into a large muscle mass by hand injection only. GonaCon is classified as a Restricted Use Pesticide. Use is restricted to USDA APHIS Wildlife Services or state wildlife management personnel or persons working under their authority.
Application Rate:	Female deer are injected with a single injection containing 1.0 ml of GonaCon at least two to three months prior to the onset of rut for full contraceptive effect. If multi year contraceptive effects are desired, a second vaccination may be given 30 to 60 days after the first injection or during the following year. Two formulations (basic and alternate) are proposed for registration.

3. Science Findings

Available data supporting the use and registration of Mammalian Gonadotropin Releasing Hormone including product chemistry, toxicology, efficacy, and ecological effects and environmental fate are summarized below.

Table 1. Product Chemistry Summary	
Peptide structure	
Common name	Mammalian Gonadotropin Releasing Hormone (GnRH)
CAS Reg. No.	9034-40-6
Color	white
Physical State	Active: solid: powder EU: liquid: somewhat creamy in appearance
Melting Point	N/A Waiver request
Boiling Point	Waiver request: Solid at room temperature.
Odor	Active: odorless EU: odorless
Stability to Normal and Elevated Temperatures, Metal, and Metal Ions	N/A Waiver request
Oxidation/Reduction Action	N/A Waiver request
pH	Active: NA EU: pH = 6.49
Flammability	N/A Waiver request
Explodability	N/A Waiver request
Vapor pressure	N/A Waiver request
Water Solubility	N/A Waiver request
Storage stability	N/A product's shelf life will be $\leq$ 6 months so not necessary
Corrosion Characteristics	N/A product's shelf life will be $\leq$ 6 months so not necessary

TOXICOLOGY SUMMARY

The Registrant submitted the studies listed in Tables 2, which include a number of toxicity studies. The Registrant submitted waiver requests which were granted for acute inhalation and dermal sensitization.

Acute Toxicity Data GnRH

Table 2. Acute Toxicity			
Guideline No.	**Study Type**	**Results**	**Toxicity Category***
OPPTS 870.1100	Acute Oral Rat	All test animals survived 1 mL exposure.	IV
OPPTS 870.1200	Acute Dermal Rat	All test animals survived 1 mL exposure.	IV
OPPTS 870.1300	Acute Inhalation Rat	WAIVED	IV
OPPTS 870.2400	Primary Eye Irritation Rabbit	No corneal opacity or iritis was observed. 1-hour post-instillation: 3/3 treated eyes exhibited conjunctival redness (score 1-2) and discharge (score 1). No "positive" grade irritation was noted at the 24-hour observation. Treated eyes were free of all eye irritation by 72-hours.	IV
OPPTS 870.2500	Primary Skin Irritation Rabbit	No edema was observed at any treated site. Very slight erythema (score 1) was noted at all 3/3 test sites within 1-hour post-pad removal. Irritation severity decreased thereafter. No irritation was noted at the 72-hour observation. The PDII was 0.5.	IV
OPPTS 870.2600	Dermal sensitization	WAIVED	--

- Toxicity Category IV = No precautions required

Chronic toxicity data requirements were waived. There is no human exposure from use of GonaCon, therefore no toxicity endpoints were selected because of the very limited potential worker and dietary exposure.

ECOLOGICAL EFFECTS

Waivers were submitted to fulfill required ecological effects and environmental fate guideline studies for the registration of GonaCon because of the limited potential for environmental releases. Since the product is labeled only for injection to deer by hand and the substance is expected to be rapidly metabolized in treated animals, the limited

potential risks to non-target organisms resulting from the proposed registration of GonaCon are not expected to exceed the Agency's concern levels.

The proposed registration of GonaCon is expected to have no effect on endangered or threatened species

EFFICACY

GonaCon is intended to render a vaccinated female white-tailed deer infertile for a minimum of one year following vaccination. The label claims that GonaCon will not affect existing pregnancies but should cause infertility of the vaccinated animal in subsequent years. If multi-year contraceptive effects are desired, a second vaccination may be given 30-60 days after the first injection or during the following year. The label claims that there is a chance that some vaccinated females will become permanently sterile.

Product performance studies were conducted by APHIS's National Wildlife Research Center (NWRC) both in the field over a two year period and in a laboratory over multiple years to compare two different formulations.

The field test, initiated in 2004 used two sites consisting of fenced federal land near Silver Spring, MD. Female deer in the vaccinated group were dosed with the labeled rate of the vaccine during July and August 2004. All deer were marked with ear tags and radio telemetry collars equipped with mortality sensors. Reproductive status was assessed in the summers of 2005 and 2006 with visual inspection of the udders for signs of lactation.

Results showed 88% efficacy in the summer after vaccination with lactation evident in 3 of the 26 does vaccinated (12%). Of the control group, reproductive status was able to be determined in 13 of the 15 deer and lactation was evident in 11 of those 13 doe (85%).

In the second summer after vaccination lactation was evident in 10 of the remaining 19 vaccinated deer (53%) equating to an efficacy rate of 47%. In the control group, 10 of the 10 remaining deer (100%) had reproductive success.

The lab study results showed the alternate formulation to be more effective than the basic formulation. The alternate formulation had a 100% success rate (5/5 does remained contracepted for two years) compared to the basic formulation with a 60% success rate after two years (3/5 does contracepted). Due to economic and supply reasons however, it is necessary for the product to carry both formulations.

4. Summary of Regulatory Position and Rationale

Available data provide adequate information to support the conditional registration of GnRH as an effective population control method for nuisance white-tailed deer.

White-tailed deer have been classified by EPA as a public health pest because they are a host for deer ticks that may cause Lyme disease and they are a cause of vehicle accidents.

In many urban and suburban areas white-tailed deer populations have become over abundant and are considered a year-round nuisance causing many human-wildlife conflicts. Since these areas are typically non hunting areas, where building and population densities do not allow for safe shooting zones, local laws prohibit guns or shooting or public opinion does not support hunting, there has not been an economically feasible solution to control overabundant deer populations.

In many of these urban and suburban areas, deer have been known to inflict significant economic damage to gardens and landscapes of homeowners associations, golf courses, private residential yards, etc. When deer are in numbers exceeding 20 per square mile, as they are becoming in many areas, they can inflict severe damage to resident flora and fauna. This can adversely affect the ecosystem and the wildlife that depend on that ecosystem.

Since deer in these areas are so well fed, female deer, which normally give birth to twins may be becoming more fertile which is evident by an increase in triplet births. This, and the fact that deer in developed areas appear to be more nocturnal to avoid human contact, is contributing to rapidly increasing populations and problems.

According to a 2006 study, the National Highway Traffic Safety Administration reported that there are about 1.5 million car accidents with deer resulting in over $1 billion of damage and 150 human fatalities annually. GnRH will not only help prevent increases in deer numbers through contraception, but should decrease the number of car accidents during the fall mating season - which is associated with a surge in car collisions with deer as bucks chase fertile does, sometimes for miles.

Deer can also play a role in spreading disease as they are a main host for blacklegged ticks (*Ixodes scapularis*), more commonly known as deer ticks, which are a carrier of Lyme disease. There has been a sharp increase in cases of Lyme disease in suburban/urban areas with significant deer populations.

Contraception of nuisance deer using GnRH is not intended to replace hunting (where it is an option) as a population control method. GnRH is intended to be used in combination with other population management techniques since it cannot alone reduce already over abundant populations. Since GnRH is a nonlethal alternative to population control, many animal rights groups enthusiastically support this registration.

The registration of GonaCon has received letters of support from the Department of Natural Resources at Cornell University and White Buffalo, Inc. a non-profit wildlife organization based out of Connecticut. At Cornell University, deer have caused damages to plants in research and teaching plots as well as dozens of car accidents. Currently, the university attempts population management of deer by capturing and surgically sterilizing deer via tubal ligation. This method however is both time consuming and costly, with expenses of approximately $1,000 per deer along with the impracticality of finding surgery staff and anesthesiologists during the early and late hours that deer are typically captured. The use of GnRH is also supported by the Humane Society of the United States and the Wildlife Society as a humane and “necessary” method of deer population control.

GonaCon was mentioned in an April 2009 article in the Washington Post Magazine as a possible population control method for deer in Rock Creek Park in Washington, DC and suburban Maryland.

5. Labeling Restrictions

To mitigate any risks, the following requirements have been imposed:

- Restricted -Use Pesticide classification due to non-target injection hazard.
- Application is restricted to USDA APHIS Wildlife Services or state wildlife management agency personnel or persons working under their authority only.
- Administration of vaccine is only by hand injection to mitigate any non-target or environmental risks that occur with administration with darts.
- Use restricted to only one species: white-tailed deer (*Odocoileus virginianus*).
- PPE requirements include: long sleeved shirt and long pants, gloves and shoes plus socks to mitigate occupational exposure.
- Children are not allowed in areas where product is used
- A warning that pregnant women should not be involved in handling or injecting GonaCon and that all women should be aware that accidental self-injection may cause infertility.

6. Conditional Data Requirements

Because of the unique chemical nature of GnRH additional preliminary analysis and certified limits data are necessary. The registrant must submit this data to the Agency upon completion.

Conditional data required for GonaCon consists of:

- Guideline 830.1700 Validating the method of analysis of the formulation and additional preliminary analysis
- Guideline 830.1750 Certified Limits

Contact Person at USEPA

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2777 S. Crystal Drive
Arlington, VA 22202
703-305-5314

DISCLAIMER: The information in this Pesticide Fact Sheet is for information only and is not to be used to satisfy data requirements for pesticide registration. The information is believed to be accurate as of the date on the document.

APPENDIX I

GLOSSARY OF TERMS AND ABBREVIATIONS

ADNT	Acute delayed neurotoxicity
a.i.	Active Ingredient
aPAD	Acute Population Adjusted Dose
ARI	Aggregate Risk Index
BCF	Bioconcentration Factor
CAS	Chemical Abstracts Service
ChE	Cholinesterase
ChEI	Cholinesterase inhibition
cPAD	Chronic Population Adjusted Dose
%CT	Percent crop treated
DAT	Days after treatment
DEEM-FCID	Dietary Exposure Evaluation Model - Food Consumption Intake Database
DNA	Deoxyribonucleic acid
DNT	Developmental neurotoxicity
DIT	Developmental immunotoxicity
DWLOC	Drinking Water Level of Comparison.
EC	Emulsifiable Concentrate Formulation
EEC	Estimated Environmental Concentration. The estimated pesticide concentration in an environment, such as a terrestrial ecosystem.
EPA	U.S. Environmental Protection Agency
FQPA	Food Quality Protection Act
GLC	Gas Liquid Chromatography
GLN	Guideline Number
LC_{50}	Median Lethal Concentration. A statistically derived concentration of a substance that can be expected to cause death in 50% of test animals. It is usually expressed as the weight of substance per weight or volume of water, air or feed, e.g., mg/l, mg/kg or ppm.
LD_{50}	Median Lethal Dose. A statistically derived single dose that can be expected to cause death in 50% of the test animals when administered by the route indicated (oral, dermal, inhalation). It is expressed as a weight of substance per unit weight of animal, e.g., mg/kg.
LOAEL	Lowest Observed Adverse Effect Level
LOAEC	Lowest Observed Adverse Effect Concentration
LOC	Level of Concern
LOD	Limit of Detection
LOQ	Limit of Quantitation
mg/kg/day	Milligram Per Kilogram Per Day
mg/L	Milligrams Per Liter
MOE	Margin of Exposure

MRID	Master Record Identification (number), EPA's system of recording and tracking studies submitted
MTD	Maximum tolerated dose
NA	Not Applicable
NOEC	No Observable Effect Concentration
NOEL	No Observed Effect Level
NOAEL	No Observed Adverse Effect Level
NOAEC	No Observed Adverse Effect Concentration
NPDES	National Pollutant Discharge Elimination System
OP	Organophosphate
OPP	EPA Office of Pesticide Programs
OPPTS	EPA Office of Prevention, Pesticides and Toxic Substances
PAD	Population Adjusted Dose
PAG	Pesticide Assessment Guideline
PAM	Pesticide Analytical Method
PHED	Pesticide Handler's Exposure Data
PHI	Preharvest Interval
ppb	Parts Per Billion
PPE	Personal Protective Equipment
ppm	Parts Per Million
PRZM/EXAMS	Tier II Surface Water Computer Model
RAC	Raw Agriculture Commodity
RBC	Red Blood Cell
RED	Reregistration Eligibility Decision
REI	Restricted Entry Interval
RfD	Reference Dose
SCI-GROW	Tier I Ground Water Computer Model
SF	Safety Factor
TGAI	Technical Grade Active Ingredient
UF	Uncertainty Factor
µg	micrograms
µg/L	Micrograms Per Liter
µL/g	Microliter per gram
USDA	United States Department of Agriculture
WPS	Worker Protection Standard

APPENDIX II

Citations Considered Part of the Data Base Supporting the Registration of GonaCon.

MRID	Citation	Receipt Date
47649600	United States Department of Agriculture's Animal and Plant Health Inspection Service (USDA APHIS) (2009) Submission of Product Chemistry, Toxicity, Efficacy and Residue Data in Support of the Application for Registration of GonaCon Immunocontraceptive Vaccine. Transmittal of 36 Studies.	22-Jan-2009
47649601	O'Hare, J.; Eisemann, J. (2008) Description of Materials Used to Produce: "GoneCon Immunocontraceptive Vaccine". Project Number: P4417. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 168 p.	22-Jan-2009
47649602	O'Hare, J.; Eisemann, J. (2008) Description of Formulation Process Used to Manufacture: "GonaCon Immunocontraceptive Vaccine". Project Number: M8546, BT016/02. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 99 p.	22-Jan-2009
47649603	O'Hare, J.; Pilon, J.; Eisemann, J. (2008) Discussion of the Formation of Impurities "GonaCon Immunocontraceptive Vaccine". Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlive Research Center. 56 p.	22-Jan-2009
47649604	O'Hare, J.; Eisemann, J.; Pilon, J. (2008) Preliminary Analysis and Certified Limits "GonaCon Immunocontraceptive Vaccine": Final Report. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 9 p.	22-Jan-2009
47649605	O'Hare, J.; Eisemann, J. (2008) Enforcement Analytical Method: "GonaCon Immunocontraceptive Vaccine". Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 40 p.	22-Jan-2009
47649606	O'Hare, J.; Eisemann, J. (2007) Product Chemistry: Color, Physical State, Odor, and pH - USDA APHIS GonaCon Immunocontraceptive Vaccine (EPA Reg. No. 56228-xx): Final Report. Project Number: QA/1421. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 31 p.	22-Jan-2009
47649607	Warren, J.; Stephens, S. (2008) Stability to Normal and Elevated Temperatures, Metal, and Metal Ions: (Gonadotropin Releasing Hormone). Unpublished study prepared by US Dept. of Agriculture, APHIS, WS. 8 p.	22-Jan-2009
47649608	Warren, J.; Stephens, S. (2008) Oxidation/Reduction Chemical Incompatibility: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by US Dept. of Agriculture, APHIS, WS. 8 p.	22-Jan-2009
47649609	Warren, J.; Stephens, S. (2008) Flammability: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009

47649610	Warren, J.; Stephens, S. (2008) Explodability: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649611	Warren, J.; Stephens, S. (2008) Miscibility: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649612	Warren, J.; Stephens, S. (2008) Corrosion Characteristics: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649613	Warren, J.; Stephens, S. (2008) Dielectric Breakdown Voltage: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649614	Warren, J.; Stephens, S. (2008) UV/Visible Absorption: (GonaCon Immunocontraceptive). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649615	Warren, J.; Stephens, S. (2008) Viscosity: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649616	Warren, J.; Stephens, S. (2008) Melting Point: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649617	Warren, J.; Stephens, S. (2008) Boiling Point: (GonoCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649618	Warren, J.; Stephens, S. (2008) Density/Relative Density/Bulk Density: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649619	Warren, J.; Stephens, S. (2008) Dissociation Constant in Water: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649620	Warren, J.; Stephens, S. (2008) Octanol/Water Partition Coefficient: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649621	Warren, J.; Stephens, S. (2008) Water Solubility: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649622	Warren, J.; Stephens, S. (2008) Vapour Pressure: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 8 p.	22-Jan-2009
47649623	Eisemann, J.; O'Hare, J. (2008) Chemical Identity: "GonaCon Immunocontraceptive Vaccine". Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: Natiional Wildlife Research Center. 27 p.	22-Jan-2009
47649624	Eisemann, J.; O'Hare, J. (2008) Direction for Use: GonaCon Immunocontraceptive Vaccine. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Research Center. 12 p.	22-Jan-2009
47649625	Lowe, C. (2008) GonaCon Immunocontraceptive Vaccine: Acute Oral	22-Jan-

	Toxicity - Limit Dose Procedure in Rats. Project Number: P320/USDA, 23211. Unpublished study prepared by Product Safety Laboratories. 28 p.	2009
47649626	Lowe, C. (2008) GonaCon Immunocontraceptive Vaccine: Acute Dermal Toxicity - Limit Dose Procedure in Rats. Project Number: 23212, P322/USDA. Unpublished study prepared by Product Safety Laboratories. 28 p.	22-Jan-2009
47649627	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Acute Inhalation Toxicity: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649628	Lowe, C. (2007) GonaCon Immunocontraceptive Vaccine: Primary Eye Irritation Study in Rabbits. Project Number: P324, 22806. Unpublished study prepared by Product Safety Laboratories. 29 p.	22-Jan-2009
47649629	Lowe, C. (2008) GonaCon Immunocontraceptive Vaccine: Primary Skin Irritation Study in Rabbits. Project Number: P326, 22807. Unpublished study prepared by Product Safety Laboratories. 27 p.	22-Jan-2009
47649630	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Dermal Sensitization: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649631	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Chronic Dietary Toxicity: (GonaCon Immunocontraceptive Vaccine). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649632	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Gene Mutation: (GonaCon Immunocontraceptive Vaccine): (Human). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649633	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Stuctural Chromosomal Aberration: (GonaCon Immunocontraceptive Vaccine): (Human). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649634	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Other Genotoxic Effects: (GonaCon Immunocontraceptive Vaccine): (Human). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649635	O'Hare, J.; Eisemann, J.; Stephens, S. (2008) Acute Delayed Neurotoxicity: (GonaCon Immunocontraceptive Vaccine): (Hen). Unpublished study prepared by USDA APHIS. 4 p.	22-Jan-2009
47649636	Gionfriddo, J.; Eisemann, J.; O'Hare, J. (2008) Product Performance: Field Test of a Single-Injection of GonaCon Immunocontraceptive Vaccine In Female White-Tailed Deer. Project Number: QA/1112. Unpublished study prepared by US Dept. of Agriculture, APHIS, WS: National Wildlife Research Center. 31 p.	22-Jan-2009

United States
Environmental Protection Agency
Office of Chemical Safety and Pollution Prevention
(7505P)

Pesticide Fact Sheet

Name of Chemical:	**Porcine Zona Pellucida (PZP)**
Reason for Issuance:	**New Chemical Nonfood Use**
Date Issued:	**January 2012**

1. Description of Chemical

Glycoprotein Complex:	ZP1 (80,000-90,000 KD), ZP2 (60,000-65,000 KD), ZP3 (55,000 KD), and ZP4 (20,000 – 25,000 KD)
Common Name:	Porcine Zona Pellucida (PZP)
EPA PC Code:	176603
Chemical Class:	Contraceptive
Registration Status:	New Chemical, nonfood use
Pesticide Type:	Mammalian Contraceptive
U.S. Technical Registrant:	Humane Society of the United States 2100 L St. NW Washington, DC 20037

2. Use Patterns and Formulations

Mode of Action:	PZP antigen is the glycoprotein layer that surrounds the oocyte and is weakly antigenic by itself. Therefore, PZP is emulsified with an adjuvant (mFCA for the primary vaccination and mFIA for booster

vaccinations) which stimulates a stronger immune response. This results in the creation of anti-zona pellucida antibodies which bind to the zona pellucida of the oocyte, alter their conformation, and block sperm attachment to the zona pellucida receptors.

Application Sites: ZonaStat-H will be used to control female wild and feral horse and burros privately or publicly owned, in areas where they have become a nuisance and are capable of doing environmental damage.

Methods of Application: The vaccine will be injected intramuscularly in hip or gluteus muscles by hand-held syringe, syringe mounted on a jabstick, or by syringe dart fired from a CO_2 or cartridge-powered projection system.

Application Rate: The application rate is 1.0 cc of PZP + adjuvant (modified Freund's complete adjuvant for the initial application, then modified Freund's incomplete adjuvant for follow-up applications). A second administration is given 2 to 4 weeks after the initial priming dose, then annually thereafter.

3. Science Findings

Available product chemistry data supporting the use of ZonaStat-H including product chemistry, toxicology, efficacy, and ecological effects and environmental fate are summarized below in Tables 1 and 1.1.

Table 1. Product Chemistry Summary

Common name	Porcine Zona Pellucida (PZP)
Color	Clear
Physical State	Active: Aqueous solution or powder EU: Thick, white aqueous emulsion
Odor	Odorless
Oxidation/Reduction Action	Denatured by acid or base, no incompatibility
pH	7.0 – 7.04
Flammability	Nonflammable (protein)
Explodability	Not explosive (protein)
Storage stability	Frozen liquid (or powder in desiccant) is viable for 2 years.
Corrosion Characteristics	No corrosive activity.

TOXICOLOGY SUMMARY

The Registrant submitted waiver requests for the acute oral, acute dermal, acute inhalation, primary eye irritation, primary dermal irritation, and dermal sensitization studies. The waiver requests were reviewed and determined to be acceptable.

Table 2. Acute Toxicity Data

GUIDELINE NO.	STUDY TYPE	MRID NO.	RESULTS	TOXICITY CATEGORY
870.1100	Acute Oral Toxicity	47859803	Waived	IV
870.1200	Acute Dermal Toxicity	47859803	Waived	III
870.1300	Acute Inhalation Toxicity	47859803	Waived	III
870.2400	Acute eye irritation	47859803	Waived	III
870.2500	Primary skin irritation	47859803	Waived	III
870.2600	Dermal sensitization	47859803	Waived	Negative

- Toxicity Category III = Precautionary Statements Required

Chronic toxicity data were not submitted. There is no human exposure from use of ZonaStat-H, therefore no toxicity endpoints were selected because of the very limited potential worker and dietary exposure.

ECOLOGICAL EFFECTS

Waivers were submitted to fulfill required ecological effects and environmental fate guideline studies for the registration of ZonaStat-H because of the limited potential for environmental releases. Since the product is labeled only for injection to target animals by hand or dart, is expected to be deactivated in the digestive tract, and has a short half-life in treated mammals, the limited potential risks to non-target organisms resulting from the proposed registration of ZonaStat-H are not expected to exceed the Agency's concern levels.

Risk to Endangered Species

The following table summarizes the conclusions of potential concerns for direct and indirect effects to federally-listed threatened and endangered species (listed species). Because the proposed uses cannot be geographically limited, all federally listed species are assumed to be potentially indirectly affected. The available data suggest that potential exposures to non-target animals is not expected to result in any significant risk concerns to terrestrial or aquatic organisms from the proposed use. However, indirect effects (potentially beneficial or negative) to Listed species could not be precluded.

Table 3. Potential Effects to Federally Listed Taxa

Listed Taxa	Direct Effects	Indirect Effects
Terrestrial and semi-aquatic plants – monocots and dicots	No	Yes
Terrestrial invertebrates	No	Yes
Birds (surrogate for terrestrial-phase amphibians and reptiles)	No	Yes
Mammals	No	Yes
Aquatic vascular plants	No	Yes
Aquatic non-vascular plants	No	Yes
Freshwater fish (surrogate for aquatic-phase amphibians)	No	Yes
Freshwater invertebrates	No	Yes
Freshwater benthic invertebrates	No	Yes
Estuarine/Marine fish	No	Yes
Estuarine/Marine crustaceans	No	Yes
Estuarine/Marine mollusks	No	Yes

EFFICACY

As ZonaStat-H does not bear claims to control pests that may pose a threat to human health, pursuant to OPPTS 810.1000(b)(2), the requirement for demonstration of efficacy is waived. In lieu of efficacy studies, the registrant provided various peer-reviewed published articles demonstrating ZonaStat's efficacy as a contraceptive for wild horses and burros.

The principle of efficacy of PZP in horses was first demonstrated by Liu et al. (1989) by inhibiting fertility in 12 of 14 captive fertile domestic and wild mares (*Eqqus caballus*), which persisted for 7 months. The researchers inoculated the mares with 4 hand injections of PZP with aluminum hydroxide gel. As the aluminum hydroxide gel was found to be only moderately effective in most of the horses, it was therefore substituted by FCA and FIA at 2-4 week intervals. A fifth booster injection was administered 6-9 months after the fourth injection. This study also demonstrated that anti-PZP antibody titers of 64% or greater were associated with effective contraception, and that a decline in contraceptive effect correlated with a decline in antibody titers.

Kirkpatrick et al. (1990) demonstrated PZP effectiveness in a study conducted at Assateague Island National Seashore (ASIS), MD in which 26 mares were remotely injected with a priming dose of 65-100 µg PZP in FCA and either one or two boosters of PZP in FIA at three-week intervals based on the determination by Liu et al. (1989) that at least two inoculations are required in horses so antibody titers are raised high enough for a minimum of 6 months. Upon the first inoculation, antigen recognition is initiated which increases antibody titers temporarily. Then, the second inoculation causes increased titers that last for several months, with each follow-up inoculation prolonging the duration of high titers (Kirkpatrick, et al. 1990).

During this study, 14 of the 26 treated mares were already pregnant upon inoculation and gave birth to healthy foals approximately 1 – 3 months after the last inoculation. By October 1998, there was only one pregnancy out of the 26 treated mares, as indicated by analysis of urinary steroids, with zero pregnancies among the 18 receiving 3 inoculations, and one pregnancy out of the 8 receiving two inoculations. The following spring, August 1989, only one of the 26 treated mares produced foals (Kirkpatrick, et al. 1990). Of the 26 treated mares, 14 were boosted again a year later with a single remotely delivered dart containing PZP in FIA. Only 1 of the 14 boosted mares was pregnant and produce a foal the following year, compared to 10 of 22 "sham-treated and untreated mares (45.5%) (Kirkpatrick, et al. 1991). Additional studies were carried out during the next 6 years which demonstrated foaling rates of 3.8% (4 foals in 105 mare-years) among PZP-treated mares compared to 46.2% in untreated mares (Kirkpatrick, et al. 1991). Zero population growth was achieved in 2 years, with an initial decline in the population becoming apparent in 8 years of inoculations and by year 11, the population declined from 175 to 135 horses, a decrease of 22.8% (Kirkpatrick and Turner 2008).

Turner et al. (1996) conducted a study at Virgin Islands National Park, St. Johns, VI (VINP) on free-roaming feral burros (*Eqqus asinus*) to assess the effectiveness of PZP as a contraceptive with results comparable to those seen in the Assateague Island studies. In this study, 16 female burros were treated with PZP contraceptive. Burros were given an initial one- or two-injection PZP treatment and, after 10 – 12 months, were given a one-injection PZP booster treatment. Initial treatment consisted of: (1) two separate injections (3 weeks apart) of a 1.0 mL emulsion, containing 65 µg PZP plus FCA (first injection) followed by a booster of FIA (n = 13); or (2) a single injection containing 130 µg PZP emulsified in FCA (n = 3). The single injection was a time-released method with release rates projected to be continuous across 4 weeks, with greatest release in weeks 1 and 4 followed by a booster shot at the end of the 4 weeks (Turner et al., 1996).

Zero of 13 females darted with a priming dose of 65 – 100 µg in FCA and a booster of 65 – 100 µg PZP in FIA produced foals in the period 12 – 24 months after treatment, while 1 of the 3 females receiving the single dose produced foals. Furthermore, 6 of 11 control females gave birth in that time period. Unlike wild and feral horses, feral burros are not seasonal breeders, and some of the burros were pregnant at the time of treatment.

The results of this study indicate the two-injection protocol was more effective than the single-injection in preventing pregnancies.

The effectiveness of the adjuvant used is an important factor in how efficacious the PZP epitope is as an immunocontraceptive (Lyda, et al. 2005). Since 1998, PZP has been used in captive free-ranging wild horses with a high degree of efficacy, utilizing Freund's Complete Adjuvant (FCA) as the adjuvant of choice for the initial inoculation and Freund's Incomplete Adjuvant (FIA) for booster inoculations. The use of FCA has resulted in 90% or greater efficacy, however two side effects can occur from its use: 1) Injection site reactions, including open abscesses and 2) false-positive tuberculosis (TB) tests in treated animals. The primary ingredient in the FCA is *Mycobacterium tuberculosis* which can cause antibodies against the TB organism. As a result of these side effects, the United States Department of Agriculture (USDA) has voiced opposition to the use of FCA.

Therefore, modified Freund's Complete Adjuvant (mFCA) has been substituted for FCA in titer trials of captive mares. These trials demonstrated no significant difference between mares hand-injected with 65-100 µg PZP in mFCA followed by a booster shot of 65-100 µg in FIA and mares treated with 65-100 µg PZP in FCA followed by a booster of 65-100 µg in FIA. Lyda et al. (2005) reported that 7 of 8 (87.5%) of mares treated with PZP and mFCA remained above the contraceptive titer threshold 10 months after treatment. The effectiveness of mFCA as an adjuvant was verified with these studies.

4. Summary of Regulatory Position and Rationale

Available data provide adequate information to support the unconditional registration of ZonaStat-H as a tool for management of nuisance feral and wild horses, and burros.

Like other animals (e.g. deer, Canada geese, etc.), horses may be pests in some situations. As a result of Federal protection, lack of natural predators, and fecundity (herd sizes can double in about four years), wild horse and burro herd populations have significantly increased, exceeding the BLM appropriate population levels of 27,200 in BLM managed lands. To help control these populations, BLM removes wild horses and burros and transfers them to private ownership or maintains them in BLM holding facilities.

With high population levels and the inability to sell or adopt out all captured wild horses and burros, the BLM has expressed that there is an explicit need to manage wild horse and burro populations because uncontrolled populations may lead to adverse environmental effects such as degradation of wildlife and native vegetation habitat. Additionally, these populations may lead to conflicts with other rangeland uses such as cattle grazing and recreation.

With these factors in mind, EPA is proposing to register ZonaStat-H and PZP for use to control wild and feral horse and burro populations. The Agency feels that ZonaStat-H will provide BLM a much needed alternative control method for wild horse and burro populations. The Agency believes that ZonaStat-H and PZP meet the standard for unconditional registration in FIFRA § 3(c)(5) including that it will not cause any unreasonable adverse effects on the environment. Therefore, the Agency proposes to grant this registration with the labeling requirements below.

5. Labeling Restrictions

To mitigate any risks, the following requirements have been imposed:

- Restricted-Use Pesticide classification limiting application to Department of Interior, and all its designated agents (i.e., National Park Service, Bureau of Land Management, U.S. Fish &Wildlife Service); State departments of agriculture/livestock and wildlife, and their designated agents; Federally recognized Indian tribes, and their designated agents; Department of Defense and its designated agents; Public and private wild horse sanctuaries and reserves; Humane Society of the United States designated agents; USDA and all its designated agents (i.e., U.S. Forest Service, Animal and Plant Health Inspection Service).

- Use limited to only two animals: Wild and feral horses (*Eqqus caballus*) and feral burros (*Eqqus asinus*).

- Label statement restricting the application of ZonaStat-H to horses or burros that will not be used as food or feed.

- Personal Protective Equipment requirements include: long sleeved shirt and long pants, gloves and shoes plus socks to mitigate occupational exposure.

- A warning that pregnant women must not be involved in handling or injecting ZonaStat-H and that all women should be aware that accidental self-injection may cause infertility.

6. Data Requirements

The registrant has fulfilled all data requirements, resulting in an unconditional registration of ZonaStat-H.

7. CONTACT PERSON AT EPA

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DISCLAIMER: The information presented in this Pesticide Fact Sheet is for informational purposes only may not be used to fulfill data requirements for pesticide registration and reregistration. The information is believed to be accurate as of the date on the document.

APPENDIX I

GLOSSARY OF TERMS AND ABBREVIATIONS

ADNT	Acute delayed neurotoxicity
a.i.	Active Ingredient
aPAD	Acute Population Adjusted Dose
ARI	Aggregate Risk Index
BCF	Bioconcentration Factor
CAS	Chemical Abstracts Service
ChE	Cholinesterase
ChEI	Cholinesterase inhibition
cPAD	Chronic Population Adjusted Dose
%CT	Percent crop treated
DAT	Days after treatment
DEEM-FCID	Dietary Exposure Evaluation Model - Food Consumption Intake Database
DNA	Deoxyribonucleic acid
DNT	Developmental neurotoxicity
DIT	Developmental immunotoxicity
DWLOC	Drinking Water Level of Comparison.
EC	Emulsifiable Concentrate Formulation
EEC	Estimated Environmental Concentration. The estimated pesticide concentration in an environment, such as a terrestrial ecosystem.
EPA	U.S. Environmental Protection Agency
FQPA	Food Quality Protection Act
GLC	Gas Liquid Chromatography
GLN	Guideline Number
LC_{50}	Median Lethal Concentration. A statistically derived concentration of a substance that can be expected to cause death in 50% of test animals. It is usually expressed as the weight of substance per weight or volume of water, air or feed, e.g., mg/l, mg/kg or ppm.
LD_{50}	Median Lethal Dose. A statistically derived single dose that can be expected to cause death in 50% of the test animals when administered by the route indicated (oral, dermal, inhalation). It is expressed as a weight of substance per unit weight of animal, e.g., mg/kg.
LOAEL	Lowest Observed Adverse Effect Level
LOAEC	Lowest Observed Adverse Effect Concentration
LOC	Level of Concern
LOD	Limit of Detection
LOQ	Limit of Quantitation
mg/kg/day	Milligram Per Kilogram Per Day
mg/L	Milligrams Per Liter
MOE	Margin of Exposure
MRID	Master Record Identification (number), EPA's system of recording and tracking studies submitted
MTD	Maximum tolerated dose
NA	Not Applicable
NOEC	No Observable Effect Concentration
NOEL	No Observed Effect Level

NOAEL	No Observed Adverse Effect Level
NOAEC	No Observed Adverse Effect Concentration
NPDES	National Pollutant Discharge Elimination System
OP	Organophosphate
OPP	EPA Office of Pesticide Programs
OPPTS	EPA Office of Prevention, Pesticides and Toxic Substances
PAD	Population Adjusted Dose
PAG	Pesticide Assessment Guideline
PAM	Pesticide Analytical Method
PHED	Pesticide Handler's Exposure Data
PHI	Preharvest Interval
ppb	Parts Per Billion
PPE	Personal Protective Equipment
ppm	Parts Per Million
PRZM/EXAMS	Tier II Surface Water Computer Model
RAC	Raw Agriculture Commodity
RBC	Red Blood Cell
RED	Reregistration Eligibility Decision
REI	Restricted Entry Interval
RfD	Reference Dose
SCI-GROW	Tier I Ground Water Computer Model
SF	Safety Factor
TGAI	Technical Grade Active Ingredient
UF	Uncertainty Factor
µg	micrograms
µg/L	Micrograms Per Liter
µL/g	Microliter per gram
USDA	United States Department of Agriculture
WPS	Worker Protection Standard

APPENDIX II

Citations Considered to be Part of the Data Base Supporting the Registration of Porcine Zona Pellucida.

MRID	Citation	Receipt Date
47859801	Grandy, J. (2009) ZonaStat-H (Porcine Zona Pellucida): Product Efficacy: (Wild Horses and Burros). Unpublished study prepared by The Humane Society of the United States.	September 17, 2009
	Liu, I.K.M., M. Bernoco, and M. Feldman. 1989. Contraception in mares heteroimmunized with pig zonae pellucidae. Journal of Reproduction and Fertility. 85:19-29.	September 17, 2009
	Kirkpatrick, J.F., I.K.M. Liu, and J.W. Turner, Jr. 1990. Remotely delivered immunocontraception in feral horses. Wildlife Society Bulletin. 18:326-330.	September 17, 2009
	Kirkpatrick, J.F., I.K.M. Liu, T.W. Turner, and M. Bernoco. 1991. Antigen recognition in feral mares previously immunized with porcine zonae pellucidae. Journal of Reproduction and Fertility Supplement. 44:321-325.	September 17, 2009
	Kirkpatrick, J.F. and A. Turner. 2008. Achieving population goals in a long-lived wildlife species (*Eqqus caballus*) with contraception. Wildlife Research. 35:513-519.	September 17, 2009
	Turner, J.W., I.K.M. Liu, and J.F. Kirkpatrick. 1996. Remotely delivered immunocontraception in free roaming feral burros (*Eqqus asinus*). Journal of Reproduction and Fertility. 107:31-35.	September 17, 2009
	Lyda, R.O., J.R. Hall, and J.F. Kirkpatrick. 2005. Comparison of Freund's complete and Freund's modified adjuvants used with a contraceptive vaccine in wild horses (*Eqqus caballus*). Journal of Zoo and Wildlife Medicine. 36:610-616.	September 17, 2009
47859802	Grandy, J. (2009) ZonaStat-H (Porcine Zona Pellucida): Product Identity and Composition. Unpublished study prepared by Humane Society of the United States.	September 17, 2009
47859803	Grandy, J. (2009) ZonaStat-H (Porcine Zona Pellucida): Toxicology – Acute. Unpublished study prepared by Humane Society of the United States.	September 17, 2009
47859804	Grandy, J. (2009) ZonaStat-H (Porcine Zona Pellucida): Human Exposure. Unpublished study prepared by Humane Society of the United States.	September 17, 2009
47859805	Grandy, J. (2009) ZonaStat-H (Porcine Zona Pellucida): Ecological Effects. Unpublished study prepared by Humane Society of the United States.	September 17, 2009
47859806	Grandy, J. (2009) ZonaStat-H (Porcine Zona Pellucida): Human Exposure. Unpublished study prepared by Humane Society of the United States.	September 17, 2009
47859807	Grandy, J. (2009) ZonaStat-H (Porcine Zona Pellucida): Environmental Fate. Unpublished study prepared by Humane Society of the United States.	September 17, 2009

56228-41 1/10/2013 1/3

U.S. ENVIRONMENTAL PROTECTION AGENCY
Office of Chemical Safety and Pollution Prevention
Registration Division (7505C)
1200 Pennsylvania Ave., N.W.
Washington, D.C. 20460

NOTICE OF PESTICIDE:
_X_ Registration
___ Reregistration
(under FIFRA, as amended)

EPA Reg. Number:	Date of Issuance:
56228-41	JAN 10 2013

Term of Issuance:
Unconditional

Name of Pesticide Product:
Gonacon - Equine

Name and Address of Registrant (include ZIP Code):

U.S. Department of Agriculture, Animal and Plant Health Inspection Service
Environmental Services, Unit 149
4700 River Road
Riverdale, MD 20737

Note: Changes in labeling differing in substance from that accepted in connection with this registration must be submitted to and accepted by the Registration Division prior to use of the label in commerce. In any correspondence on this product always refer to the above EPA registration number.

On the basis of information furnished by the registrant, the above named pesticide is hereby registered/reregistered under the Federal Insecticide, Fungicide and Rodenticide Act.

Registration is in no way to be construed as an endorsement or recommendation of this product by the Agency. In order to protect health and the environment, the Administrator, on his motion, may at any time suspend or cancel the registration of a pesticide in accordance with the Act. The acceptance of any name in connection with the registration of a product under this Act is not to be construed as giving the registrant a right to exclusive use of the name or to its use if it has been covered by others.

This product is unconditionally registered in accordance with FIFRA section 3(c)(5) provided that you:

1. Submit and/or cite all data required for registration/reregistration of your product when the Agency requires all registrants of similar products to submit such data.

2. Make the following label change before you release the product for shipment:
- Revise the EPA Registration Number to read, "EPA Reg. No 56228-41."

Signature of Approving Official:

for Meredith Laws, Chief
Insecticide-Rodenticide Branch, Registration Division (7505P)

Date: JAN 10 2013

EPA Form 8570-6

3. Submit one copy of your final printed labeling before you release the product for shipment.

If these conditions are not complied with, the registration will be subject to cancellation in accordance with FIFRA section 6(e). Your release for shipment of the product constitutes acceptance of these conditions. If you have any questions, please contact Autumn Metzger at 703-305-5314 or metzger.autumn@epa.gov.

A stamped copy of the label is enclosed for your records.

Meredith Laws, Chief
Insecticide-Rodenticide Branch
Registration Division (7505P)

Enclosure

PRECAUTIONARY STATEMENTS

HAZARDS TO HUMANS AND DOMESTIC ANIMALS

Keep away from humans, domestic animals and pets. Wear protective gloves when handling. If pregnant, do not handle or administer product. Accidental injection may cause infertility in women. Wash all implements used for handling or applying product with detergent and water. Do not use these implements for mixing, holding, or transferring food or feed.

ENVIRONMENTAL HAZARDS

Do not apply this product directly to water, or to areas where surface water is present or to intertidal areas below the mean high water mark

PERSONAL PROTECTIVE EQUIPMENT (PPE)

Applicators and other handlers must wear:

-long sleeved shirt and long pants
-gloves
-shoes plus socks

DIRECTIONS FOR USE

Restricted Use Pesticide

It is a violation of Federal law to use this product in a manner inconsistent with its labeling. A copy of this label must be in the possession of the user at the time that the product is applied.

READ THIS LABEL: Read this entire label and follow all use directions and precautions.

IMPORTANT: Do not expose children, pets, or other non-target animals to this product. To help prevent accidents:

1) Keep children out of areas where this product is used.
2) Store product not in use in a location out of reach of children and pets.
3) Apply product only according to the directions authorized.
4) Dispose of product container and spoiled or unused product as specified in the "STORAGE AND DISPOSAL" section on this label.

GonaCon™ - Equine is used to manage fertility in reproductively mature female wild or feral horses *(Equus caballus)* and burros *(Equus asinus)*.

Use caution to prevent accidental self-injection when administering GonaCon™ - Equine to wild or feral horses and burros.

Pregnant women should not be involved in the handling or injection of GonaCon™. Do not ingest. Avoid contact with eyes.

Do not apply this product to food or feed.

Applicators: Additional State regulations and/or permitting may apply to the use of this product in wild or feral horses and burros. All applicable State authorities must be contacted prior to use. GonaCon™ renders vaccinated reproductively mature female wild or feral horses and burros infertile for a minimum of one year following vaccination.

(See right panel for application instructions)

RESTRICTED USE PESTICIDE

DUE TO NON-TARGET INJECTION HAZARD

For use by employees of USDA APHIS Wildlife Services and Veterinary Services, U.S. Bureau of Land Management, U.S. Fish and Wildlife Service, U.S. National Park Service, U.S. Department of Defense, Federally recognized Indian Tribes, State agencies responsible for wild or feral horse and burro management, public and private wild horse sanctuaries, or persons working under their authority.

GONACON™ - EQUINE

For managing fertility in female wild or feral horses (Equus caballus) and burros (Equus asinus)

ACTIVE INGREDIENT

Mammalian Gonadotropin Releasing Hormone	0.03%
OTHER INGREDIENTS	99.97%
TOTAL	100%

ACCEPTED
JAN 10 2013
Under the Federal Insecticide, Fungicide, and Rodenticide Act, as amended, for the pesticide registered under:
EPA Reg. No: 56228-41

KEEP OUT OF REACH OF CHILDREN

CAUTION

Have the product container or label with you when calling a poison control center or doctor, or when going for treatment

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage or disposal.

PESTICIDE STORAGE: Keep preloaded GonaCon™ - Equine in a refrigerator (36 °F to 45 °F) until ready for use. In the field, keep preloaded GonaCon™ - Equine in a cooler on ice as long as possible prior to use.

PESTICIDE DISPOSAL: If not used within 6 months of manufacture when held under refrigeration (36°F to 45°F), or if not maintained on ice in the field, disable and dispose of unused GonaCon™ - Equine material and preloaded syringes as medical waste according to applicable Federal, State, and/or Local regulations.

CONTAINER DISPOSAL: Nonrefillable container. Do not reuse or refill container. Disable and dispose of expired material, preloaded syringes or darts, used syringes, darts and needles as medical waste according to applicable Federal, State, and/or Local regulations.

APPLICATION INSTRUCTIONS

GonaCon™ can be administered at any time throughout the year and should not adversely affect an existing pregnancy, but should cause infertility of the vaccinated animal in the subsequent year and possibly longer.

Administer a single vaccination (2 ml) of GonaCon™ at least two to three months prior to the onset of breeding for full contraceptive effect. If longer contraceptive effect is desired, a second vaccination may be given 30 or more days after the first injection or during the following year with no known adverse health effects to the vaccinated animal. The effects of the vaccine may wear off during the second year or sometimes later and vaccinated females may once again become fertile. However, re-immunization with GonaCon™ can extend infertility.

There is a chance some vaccinated females will become permanently sterile. Accidental injection of males will result in infertility.

Two-milliliter (2 ml) doses of GonaCon™ are packaged in pre-loaded, 3-ml plastic syringes.

GonaCon™ can be administered by hand injection, jab stick or remote delivery (darting). Inject 2.0 ml of GonaCon™ by intramuscular injection (IM) into a large muscle mass (e.g., rump, neck).

Hand Injection: Inject IM 2 ml GonaCon™ using the packaged preloaded syringe and a 1.5-inch 18G or 19G stainless steel hypodermic needle.

Jab Stick Delivery: Transfer 2 ml GonaCon™ from the preloaded syringe into the jab stick syringe. Inject IM using a 1.5-inch 14G stainless steel hypodermic needle.

Remote Darting: Recommended dart specifications for this use are a 2 cc dart with a 1.25- or 1.5-inch 14G gelatin barb needle. Transfer 2 ml GonaCon™ from the preloaded syringe into the dart. Deliver IM using an appropriate projection device.

If remote delivery is used, the applicator must make every attempt to recover all darts. If possible, all darts that are discharged and drop from the horses at the shooting site must be recovered before another darting occurs. In exceptional situations, such as an onset of inclement weather, loss of daylight, applicator safety concerns, or other urgent circumstances, the site of a lost dart may be noted and marked, and recovery efforts made at a later time. Examine all fired darts after recovery to determine if the charge fired and the plunger fully expelled its vaccine content.

Syringes must be individually labeled with the following language:

Restricted Use: Injection Hazards
CAUTION
GonaCon™ - Equine
Active Ingredient: Gonadotropin Releasing Hormone (0.03%)
KEEP OUT OF REACH OF CHILDREN
EPA Reg. No. 56228-XX, EPA Est. No. 26228-CO-1
See Full Product Label for Application Instructions.
Vaccine expires 6 months from: _____

UNITED STATES DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
Riverdale, MD 20737-1237
EPA Est. No. 56228-CO-1
EPA Reg. No. 56228-XX
Net Contents: 2 milliliter (0.066 fl. ounce)
Batch Code No.:

3/3

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

November 19, 2015

David S. Reinhold
Chief
Environmental and Risk Analysis Services
USDA-APHIS
4700 River Road
Riverdale, MD 20737

Subject: Label and CSF Amendment – Calculation corrections on CSF and subsequent label changes
Product Name: GonaCon – Equine
EPA Registration Number: 56228-41
Application Date: 7/22/2015, 11/2/2015
Decision Numbers: 508908, 510842

Dear Mr. Reinhold:

The amended label and CSFs referred to above, submitted in connection with registration under the Federal Insecticide, Fungicide and Rodenticide Act, as amended, are acceptable. This approval does not affect any conditions that were previously imposed on this registration. You continue to be subject to existing conditions on your registration and any deadlines connected with them.

A stamped copy of your labeling is enclosed for your records. This labeling supersedes all previously accepted labeling. You must submit one copy of the final printed labeling before you release the product for shipment with the new labeling. In accordance with 40 CFR 152.130(c), you may distribute or sell this product under the previously approved labeling for 18 months from the date of this letter. After 18 months, you may only distribute or sell this product if it bears this new revised labeling or subsequently approved labeling. "To distribute or sell" is defined under FIFRA section 2(gg) and its implementing regulation at 40 CFR 152.3.

Please note that the record for this product currently contains the following CSFs:

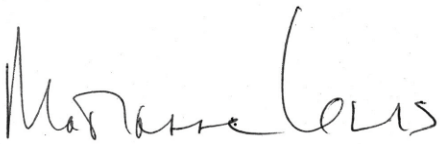
- Basic CSF dated 07/22/2015
- Alternate CSF 1 dated 07/22/2015

Should you wish to add/retain a reference to the company's website on your label, then please be aware that the website becomes labeling under the Federal Insecticide Fungicide and Rodenticide Act and is subject to review by the Agency. If the website is false or misleading, the product would be misbranded and unlawful to sell or distribute under FIFRA section 12(a)(1)(E). 40 CFR 156.10(a)(5) list examples of statements EPA may consider false or misleading. In addition, regardless of whether a website is referenced on your product's label, claims made on the

website may not substantially differ from those claims approved through the registration process. Therefore, should the Agency find or if it is brought to our attention that a website contains false or misleading statements or claims substantially differing from the EPA approved registration, the website will be referred to the EPA's Office of Enforcement and Compliance.

Your release for shipment of the product constitutes acceptance of these conditions. If these conditions are not complied with, the registration will be subject to cancellation in accordance with FIFRA section 6. If you have any questions, please contact Jacquelyn Marchese by phone at 703-347-0559, or via email at marchese.jacquelyn@epa.gov.

Sincerely,



Marianne Lewis,
Acting Product Manager 07
Invertebrate & Vertebrate Branch 3
Registration Division (7505P)
Office of Pesticide Programs

Enclosure

PRECAUTIONARY STATEMENTS

HAZARDS TO HUMANS AND DOMESTIC ANIMALS

Keep away from humans, domestic animals and pets. Wear protective gloves when handling. If pregnant, do not handle or administer product. Accidental injection may cause infertility in women. Wash all implements used for handling or applying product with detergent and water. Do not use these implements for mixing, holding, or transferring food or feed.

ENVIRONMENTAL HAZARDS

Do not apply this product directly to water, or to areas where surface water is present or to intertidal areas below the mean high water mark.

PERSONAL PROTECTIVE EQUIPMENT (PPE)

Applicators and other handlers must wear:

- long-sleeved shirt and long pants
- gloves
- shoes plus socks

USE RESTRICTIONS

Restricted Use Pesticide

It is a violation of Federal law to use this product in a manner inconsistent with its labeling. A copy of this label must be in the possession of the user at the time that the product is applied.

READ THIS LABEL: Read this entire label and follow all use directions and precautions.

IMPORTANT: Do not expose children, pets, or other non-target animals to this product. To help prevent accidents:

1) Keep children out of areas where this product is administered.
2) Store product not in use in a location out of reach of children and pets.
3) Apply product only according to the directions authorized.
4) Dispose of product container and spoiled or unused product as specified in the "STORAGE AND DISPOSAL" section on this label.

GonaCon - Equine (GonaCon) is used to manage fertility in reproductively mature female wild or feral horses *(Equus caballus)* and burros *(Equus asinus)*.

Use caution to prevent accidental self-injection when administering GonaCon to wild or feral horses and burros.

Pregnant women should not be involved in the handling or injection of GonaCon. Do not ingest. Avoid contact with eyes.

Do not apply this product to food or feed.

Applicators: Additional State regulations and/or permitting may apply to the use of this product in wild or feral horses and burros. All applicable State authorities must be contacted prior to use.

GonaCon renders vaccinated reproductively mature female wild or feral horses and burros infertile for a minimum of one year following vaccination.

(See right panel for DIRECTIONS FOR USE)

RESTRICTED USE PESTICIDE

DUE TO NON-TARGET INJECTION HAZARD

For use by employees of USDA APHIS Wildlife Services and Veterinary Services, U.S. Bureau of Land Management, U.S. Fish and Wildlife Service, U.S. National Park Service, U.S. Department of Defense, Federally recognized Indian Tribes, State agencies responsible for wild or feral horse and burro management, public and private wild horse sanctuaries, or persons working under their authority.

GONACON - EQUINE

For managing fertility in female wild or feral horses (Equus caballus) and burros (Equus asinus)

ACTIVE INGREDIENT

Mammalian Gonadotropin Releasing Hormone	0.032%
OTHER INGREDIENTS	99.968%
TOTAL	100.000%

KEEP OUT OF REACH OF CHILDREN

CAUTION

Have the product container or label with you when calling a poison control center or doctor, or when going for treatment

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage or disposal.

PESTICIDE STORAGE: Keep preloaded GonaCon - Equine in a refrigerator (36 °F to 45 °F) until ready for use. In the field, keep preloaded GonaCon - Equine in a cooler on ice as long as possible prior to use.

PESTICIDE DISPOSAL: If not used within 6 months of manufacture when held under refrigeration (36 °F to 45 °F), or if not maintained on ice in the field, disable and dispose of unused GonaCon - Equine material and preloaded syringes as medical waste according to applicable Federal, State, and/or Local regulations.

CONTAINER DISPOSAL: Nonrefillable container. Do not reuse or refill container. Disable and dispose of expired material, preloaded syringes or darts, used syringes, darts and needles as medical waste according to applicable Federal, State, and/or Local regulations.

DIRECTIONS FOR USE

GonaCon can be administered at any time throughout the year and should not adversely affect an existing pregnancy, but should cause infertility of the vaccinated animal in the subsequent year and possibly longer.

Administer a single vaccination (2 ml) of GonaCon at least two to three months prior to the onset of breeding for full contraceptive effect. If longer contraceptive effect is desired, a second vaccination may be given 30 or more days after the first injection or during the following year with no known adverse health effects to the vaccinated animal. The effects of the vaccine may wear off during the second year or sometimes later and vaccinated females may once again become fertile. However, re-immunization with GonaCon can extend infertility.

There is a chance some vaccinated females will become permanently sterile. Accidental injection of males will result in infertility.

Two-milliliter (2 ml) doses of GonaCon are packaged in pre-loaded, 3-ml plastic syringes.

GonaCon can be administered by hand injection, jab stick or remote delivery (darting). Inject 2.0 ml of GonaCon by intramuscular injection (IM) into a large muscle mass (e.g., rump, neck).

Hand Injection: Inject IM 2 ml GonaCon using the packaged preloaded syringe and a 1.5-inch 18G or 19G stainless steel hypodermic needle.

Jab-Stick Delivery: Transfer 2 ml GonaCon from the preloaded syringe into the jab-stick syringe. Inject IM using a 1.5-inch 14G stainless steel hypodermic needle.

Remote Darting: Recommended dart specifications for this use are a 2 cc dart with a 1.25- or 1.5-inch 14G gelatin barb needle. Transfer 2 ml GonaCon from the preloaded syringe into the dart. Deliver IM using an appropriate projection device.

If remote delivery is used, the applicator must make every attempt to recover all darts. If possible, all darts that are discharged and drop from the horses at the shooting site must be recovered before another darting occurs. In exceptional situations, such as an onset of inclement weather, loss of daylight, applicator safety concerns, or other urgent circumstances, the site of a lost dart may be noted and marked, and recovery efforts made at a later time. Examine all fired darts after recovery to determine if the charge fired and the plunger fully expelled its vaccine content.

Syringes must be individually labeled with the following language:

Restricted Use: Injection Hazard
CAUTION
GonaCon - Equine
Active Ingredient: Gonadotropin Releasing Hormone (0.032%)
KEEP OUT OF REACH OF CHILDREN
EPA Reg. No. 56228-41, EPA Est. No. 26228-CO-1
See Full Product Label for Application Instructions.
Vaccine expires 6 months from: _____

ACCEPTED
Nov 19, 2015
Under the Federal Insecticide, Fungicide and Rodenticide Act as amended, for the pesticide registered under EPA Reg. No. 56228-41

UNITED STATES DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
Riverdale, MD 20737-1237
EPA Est. No. 56228-CO-1
EPA Reg. No. 56228-41
Net Contents: 2 milliliter (0.066 fl. ounce)
Batch Code No.: _____

Revised 11-16-2015



International Wild Equid Conference

Vienna 2012

18-22 September 2012
in Vienna, Austria

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International Wild Equid Conference



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Research Institute of Wildlife Ecology, University of Veterinary Medicine

18-22 September 2012, in Vienna, Austria

International Wild Equid Conference

Research Institute of Wildlife Ecology, University of Veterinary Medicine

Contributed Poster

Vienna 2012

Delayed Reversibility of PZP (Porcine Zona Pellucida) in Free-Ranging Przewalski's Horse Mares

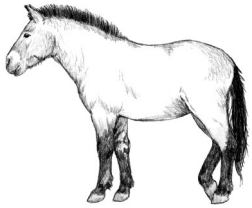
Claudia Feh

Contraception can be important for endangered species management, both in captivity and for reintroductions, reversibility being essential for planned breeding. PZP (porcine zona pellucida) has been successfully employed as a contraceptive in 80 species, in both captive and wild populations. Studies on its efficiency, safety and reversibility show no major detrimental effects. Following 3 years of treatment, feral mares returned to fertility 3.7 years later on average. PZP has been used in Przewalski's horses in zoos, but published data on reversibility is missing. We contracepted 20 Przewalski's mares belonging to two free-ranging subpopulations from the same origin, 8 mares at Le Villaret (France) and 12 re-introduced mares in Khomiin Tal (Mongolia). We administered 65 µg doses of PZP remotely, the primo-injection mixed with Freund's Complete adjuvant, subsequent injections with Freund's Incomplete adjuvant. We treated 9 mares for one year, 7 mares for two years and 4 mares for 3 years. Eight mares were 2 years old at the start of treatment, 1 mare 3, 9 mares between 4 and 8, two mares 11 and 18. All mares were in good to excellent body condition throughout and after treatment. Eleven out of the 20 mares have still not produced a foal after an average time span of 6.7 years following contraception, regardless whether PZP was administered for one, two or three years. The other 9 mares gave birth after an average of 3.2 years. All 12 mares aged 3 years or more at the start of contraception had foaled at least once beforehand. Only 4 of these reversed. The 8 remaining 2 year old mares obviously had no previous foals. All mares were mated regularly throughout the breeding season. All 10 stallions associating with the 20 mares sired foals with other mares. The inbreeding coefficient of mares not producing foals was slightly lower (mean 0.174) compared to mares having foals (mean 0.183). Foaling rate of untreated mares in the same population over 9 years was 0.64 for adults, similar to the 0.72 observed prior to the start of contraception over the same time span. The reaction of Przewalski's mares to PZP seems to differ compared with feral domestic mares, reversibility being delayed and probably compromised in a high percentage of individuals even after a short administration time. Although our study is not experimental and other factors may influence conception, we were able to control for population and previous mare fecundity, mare inbreeding coefficient and stallion fertility. A soft-release approach to reintroductions, including releasing preformed family groups, proved beneficial to survival in many projects. This implies applying contraceptives before shipping to avoid the risk of transporting pregnant females, but PZP seems not ideally suited for Przewalski's mares.

Corresponding author:

Claudia Feh, PhD

Association pour le cheval de Przewalski
Station biologique de la Tour du Valat
Le Sambuc, 13200 Arles
France
Tel: +33.490.972313
E-mail: feh@takh.org



E. ferus przewalskii

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BEHAVIOURAL PROCESSES
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Male infanticide and paternity analyses in a socially natural herd of Przewalski's horses: Sexual selection?

C. Feh [a,*], B. Munkhtuya [b]

[a] *Association pour le cheval de Przewalski: TAKH, Station Biologique de la Tour du Valat, Le Sambuc, 13200 Arles, France*
[b] *Charles University in Prague, Ovocny trh 5, Praha 1, 116 36, Czech Republic*

Received 18 April 2007; received in revised form 7 December 2007; accepted 24 December 2007

Abstract

The sexual selection hypothesis explains infanticide by males in many mammals. In our 11-year study, we investigated this hypothesis in a herd of Przewalski's horses where we had witnessed infanticidal attacks. Infanticide was highly conditional and not simply linked to takeovers. Attacks occurred in only five of 39 cases following a takeover, and DNA paternity revealed that, although infanticidal stallions were not the genetic fathers in four cases out of five, stallions present at birth did not significantly attempt to kill unrelated foals. Infanticide did not reduce birth intervals; only in one case out of five was the infanticidal stallion, the father of the next foal; mothers whose foals were attacked subsequently avoided associating with infanticidal stallions. Therefore, evidence for the sexual selection hypothesis was weak. The "human disturbance" hypothesis received some support, as only zoo bred stallions which grew up in unnatural social groups attacked foals of mares which were pregnant during takeovers.
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Keywords: DNA paternity analysis; Human disturbance; Male infanticide; Przewalski's horse (*Equus ferus przewalskii*); Sexual selection

1. Introduction

Observations on many species show that some males, after taking over a female group, kill vulnerable infants dependent on their mothers. The sexual selection hypothesis received strong support to explain infanticide by mammalian males, mainly in primates, carnivores and rodents (Van Schaik, 2000a). The overall assumption states that infanticidal males produce offspring at a faster rate than non-infanticidal males. Therefore, if male infanticide is adaptive, the following conditions should be fulfilled (Hausfater and Hrdy, 1984).

1. The probability that the infanticidal male had sired the attacked infants was zero or close to zero.
2. The mother can be impregnated earlier than if the infant had lived.
3. The infanticidal male has an increased probability of siring the next infants relative to the current offspring.

The seven extant Equids should be at a low risk regarding infanticide. In contrast to typical species where infanticide is of high prevalence (Van Schaik, 2000a), foals are precocious, mares only give birth to one offspring at a time, show post partum oestrous and, in the mean, gestation time (11 months in horses) generally lasts longer than mean lactation time (8 months in horses).

Despite being classified as low risk species, male infanticides in relation to takeovers have been reported in Plains zebras (Pluhacek and Bartos, 2000, 2005; Pluhacek et al., 2006), Mountain zebras (Penzhorn, 1984), Przewalski's horses (Ryder and Massena, 1988; Zarkikh, 1999; Kolter and Zimmermann, 1988; Boyd, 1991) and Camargue horses (Duncan, 1982), although all actual observations of stallions killing foals were based on studies with human induced experimental male replacement. Some authors tended cautiously towards favouring the classical sexual selection hypothesis (Duncan, 1982; Pluhacek and Bartos, 2000; Ryder and Massena, 1988), while others did not (Kolter and Zimmermann, 1988; Zarkikh, 1999). Infanticide has never been witnessed in studies on natural freeliving populations of equids (Berger, 1983; Duncan, 1982; Feist and McCullough, 1976), despite the considerable length and intensity of the observational studies.

In this study, we first investigated whether infanticide in stallions was linked to takeovers, a first prerogative, and under what conditions. Indeed, as has been shown in experimental studies on rodents, infanticide rates can be highly conditional. Both mating

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E-mail address: feh@tourduvalat.org (C. Feh).

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doi:10.1016/j.beproc.2007.12.009

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UNCORRECTED PROOF

with the future mother and familiarity during gestation, which are thought to enhance paternity probability, reduce infanticide rates regardless of genetic paternity (Huck et al., 1982; Perrigo and Cully Bryant, 1990; Elwood, 1980). Therefore, we studied links between infanticide and the stallions' presence at time of conception, their presence during gestation in general and the gestational stage of the mother. We then tested whether males preferentially attacked unrelated foals, whether the interbirth interval was reduced following infanticide, and whether infanticidal males were the genetic fathers of the foals to be born to the mother of the attacked foals.

Furthermore, we examined the two, somehow contradictory, female defence strategies mentioned in the literature with regard to the influence of male infanticide on mammalian social systems. Do mares whose foals suffered infanticidal attacks subsequently associate with the infanticidal male in an exclusive one-male unit, such as suggested for mountain gorillas and other species (Watts, 1989)? Or do they engage in multi-male matings as a consequence to infanticide, as suggested by many authors (Wolff and MacDonald, 2004)?

Finally, we discuss alternatives to the sexual selection theory for infanticide by males, such as the "social pathology" hypothesis.

Although the sample size of infanticidal behaviour in our study is low, this is the case in most studies on the subject. For example, Van Schaik (2000b) synthesis of this type of behaviour in primates is based on 55 observed cases from 17 species. Moreover, if infanticide brings an evolutionary advantage to males killing unrelated offspring after takeovers of females, situations where infanticide occurs after male takeovers should be compared to situations where this behaviour does not occur, and the reproductive success of males committing infanticide should be compared to that of non-infanticidal males. To our knowledge, this is the first study on a socially natural mammalian population which investigates these aspects of infanticide.

2. Materials and methods

2.1. Study animals

Between 1993 and 1994, we released 11 horses (five subadult stallions, six adult mares) originating from eight different zoos on a 200 ha pasture at Le Villaret, Causse Méjan (Lozère), a high plateau of the Massif Central. The pasture area was increased to 380 ha in 2001. The herd grew to a maximum of 55 animals in 2003. Human intervention was kept to a minimum (e.g. supplementary feeding on two occasions only, no anti-parasite treatment, veterinary care only for severely sick and isolated horses). The sex-ratio was slightly biased towards males in all age-classes. The horses quickly formed one-male family groups (3–5 over the different years), and bachelor male groups (1–2), similar to all feral horses living under natural conditions. Sixty-five foals were born between 1993 and 2003, of which 12 died in the first month. Five of these foals were stillborn, they never breathed, and one other foal was found dead on its day of birth, the cause could not be identified despite an autopsy. These foals were excluded from the infanticide analysis, as they had died before the stallions could potentially attack them.

2.2. Observation methods and definitions

We carried out a systematic individual behavioural follow-up over all 11 years, from 1993 to 2003. Except for weekends, we made observations every other day, 1 h in the morning and 1 h in the evening, for a total number of 2692 h. We recorded group composition at the beginning of every observation period. Groups were defined as spatial units where the diameter of the group was inferior to the distance from the next group. All individual horses then were recorded as belonging to a group. We classified horses that stayed at a distance exceeding the diameter of the group as solitary. During the foaling season, we checked the herd every day, and newborn foals were closely watched for 2 days.

We defined takeovers as observations when we saw a mare or a group of mares together in the same group with a new stallion, who had previously been a member of a different group, or classified as solitary.

Gestation in Przewalski's mares lasts for a mean of 48.6 weeks or 340 days (range 47–50 weeks) (Monfort et al., 1994). We calculated conception time backwards from the day of parturition, and added 15 days in each direction for "presence at conception time" by the stallions, as oestrous can last for 1 week in mares and we wanted to make sure to cover uncertainties with regard to actual fertilisation. We calculated interbirth intervals based on the same data.

Between 290 and 29 days before parturition, oestrogen levels exceed the highest levels found during oestrous (Monfort et al., 1994). This allowed us to separate "high hormone level" gestation periods from "low hormone level" gestation periods. Indeed, it is known from domestic stallions and even geldings that they are able to detect oestrous in mares from olfactory cues (Stahlbaum and Houpt, 1989), so they may well be capable of detecting gestation, at least when hormone levels are high.

2.3. Infanticidal behaviour

Infanticidal attacks on foals cannot be mistaken for any other behaviour. The stallions lunge at the foal and viciously bite it in repetitive actions, often at the head or neck. They then frequently toss the foal in the air and attack it again once on the ground, with teeth and sometimes hooves. These attacks can be repeated many times, sometimes over several days. The inflicted wounds range from open or internal haemorrhages to broken jaws, skulls, vertebrae or limbs. We witnessed five foals being attacked in this way. Two foals survived due to the energetic interventions of their mothers who, at that stage, were older and dominant over the infanticidal stallion. For the three other foals, their mothers tried equally to intervene, in one case helped by the half-sister of the mother. Our observer team tried to keep the stallion at bay during infanticidal attacks on two foals, without much success (the stallion took profit of a short absence). One foal died shortly after repeated attacks, and we euthanised the two others as they suffered from lethal wounds. Similar descriptions of infanticidal

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attacks by equid stallions are found in the literature (Pluhacek and Bartos, 2000; Ryder and Massena, 1988; Zarkikh, 1999; Duncan, 1982; Kolter and Zimmermann, 1988).

For our analysis on infanticide, we only included witnessed attacks. Thanks to the high observation pressure, it is highly unlikely that we missed out others.

2.4. DNA paternity analysis

DNA was extracted from blood samples or skin biopsies taken mostly with the help of a hypodermic gun. ETH (Swiss Federal Institute of Technology, Zürich) and Labogena (Jouy-en Josas, France) performed the genetic analysis based on the 17 standard commercial microsatellites used for pedigree analysis in domestic horses. We were able to establish the complete pedigree of our herd, including the 11 original horses and the 65 foals born at Le Villaret. We therefore have no uncertainty about paternities despite several adult stallions being present. We checked the paternity of each foal against all potential fathers 2 years or older present in the herd, as this is the age of physiological sexual maturity in Przewalski's stallions in zoos (Monfort et al., 1994).

2.5. Statistical analysis

We analysed infanticidal behaviour from the foals' point of view and therefore treated foals as independent samples. Even if several foals had the same mother or the same father, this should not influence its probability of being attacked or not with regard to takeovers by new males.

We used non-parametric tests for all analyses, as we were unable to test for distribution because of the relatively small sample size. We calculated exact probabilities with the help of StatExact software programme (CYTEL) for the same reason. All tests are two-tailed.

3. Results

3.1. Takeovers and infanticidal behaviour

Twenty live births out of 59 (18 different mothers) were not preceded by male takeovers of the mother between the time of conception and 30 days after parturition. None of these 20 foals were attacked by stallions. Out of the remaining 39 foals, whose mothers had changed their reproductive partner, five foals (three males, two females), were attacked by stallions. There was no significant relation between takeovers and infanticide (Fisher's exact, $p = 0.1558$).

3.2. Conditionality of infanticidal behaviour (paternity probability)

Exclusive mating opportunity with the mother at the time of conception, when followed by a separation during gestation, did not prevent the stallions from attacking foals (Fisher's exact, $p = 0.3716$). We found no relation between infanticidal behaviour and the continuous presence of the stallion during gestation, regardless of his presence at conception time. (Fisher's exact, $p = 0.1558$). A continuous association between the stallion and the future mother during the high level hormone gestation period compared to low hormone level gestation period, without being present at conception, had no influence on infanticide (Fisher's exact, $p = 0.2663$). But then, when stallions were present during the time of conception and during high hormone level gestation periods, or formed a permanent association from the time of conception to the day of birth, foals had a significantly lower probability of being attacked, regardless of takeovers during other periods (Fisher's exact, $p = 0.0036$).

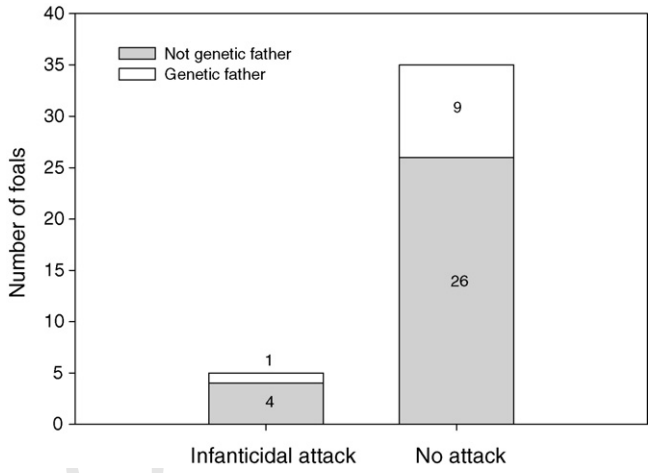


Fig. 1. Number of foals attacked/not attacked by stallions present at birth following takeovers.

3.3. DNA paternity analysis and infanticide

With the exception of two foals out of 59, genetic fathers had associated with the mother during the time of conception, although in 11 cases, two alternate stallions were present. For 26 out of 35 foals, the stallion present from the day of birth to 30 days after parturition was not its genetic father but he did not attack the foal after a takeover (Fig. 1). Although four out of five infanticidal attacks were directed towards unrelated foals (one stallion killed his own foal), unrelated foals were not significantly targeted compared to foals sired by the stallion (Fisher's exact, $p = 0.4165$).

3.4. Interbirth intervals and infanticide

Interbirth intervals following infanticide were not significantly different from interbirth intervals before or after infanticide for the mares whose foals were attacked (Permutation test for paired replicates, $n = 5$, $p = 0.375$).

3.5. DNA paternity analysis of offspring following infanticide

Subsequent to the attack on an infant, only one foal out of five was sired by the stallion which previously had attacked a foal of the same mare.

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3.6. Characteristics of infanticidal stallions

Out of the nine stallions that formed their own one-male reproductive units over the 11 years, only two attacked foals. Both infanticidal stallions originated from zoos, but then, two other stallions of the same origin were never observed to show infanticidal behaviour. We never saw the five reproductive stallions of the second generation, born at Le Villaret, killing foals, despite forming their units, or increasing their groups, after takeovers of pregnant mares (eight cases) or of mares with vulnerable foals (three cases). The latter stallions were neither the genetic fathers of the foals nor had they associated with the mothers during the time of conception or gestation.

One and a half years after attacking foals, one of the infanticidal stallions died accidentally due to a cause not related to the set-up of the project, so his behaviour could not be monitored further. The other one continued to be a reproductive stallion for 3 years without attacking foals before he was definitely replaced by another stallion and subsequently joined a bachelor group where he still lives, 4 years later. Anecdotally, DNA paternity revealed that the infanticidal stallion which we were able to follow during his whole tenure length had a lower life-time reproductive success (five foals sired, three survived) compared to the two non-infanticidal stallions of the same cohort and the same zoo origin (16 and 9 foals sired, respectively, 12 and 7 survived).

3.7. Female defence strategies

Mothers whose foals had been attacked avoided associating with the infanticidal stallion in the years after (sign-test, $n = 17$, $p = 0.049$). In 11 cases, two stallions were present during the time of conception, but we found no tendency for mothers to engage preferentially in multi-male matings during conception time after one of their foals had been attacked (Fisher's exact, $p = 0.563$).

4. Discussion

Infanticide by males in this herd of Przewalski's horses was highly conditional and not simply linked to takeovers. Attacks on foals were significantly reduced only when stallions associated with the future mother at the time of conception, exclusively or not, and during the high hormone gestation period, whether the stallion was the genetic father or not. Although most infanticidal behaviour was directed against unrelated foals, 74 % of unrelated foals (26 cases out of 35) were not attacked following takeovers by the stallions present at birth. Mothers were not fertilized earlier after infanticides, and the infanticidal stallion was the father of the next foal in one case out of five only. Moreover, mothers whose foals had been attacked avoided associating with the infanticidal stallion in the years after. Therefore, the evidence in support of the classical sexual selection hypothesis was weak at most.

Van Schaik (2000a) summarizes the other hypotheses that were suggested as explanations for infanticide by males, four of which can be excluded for evident reasons. We eliminate

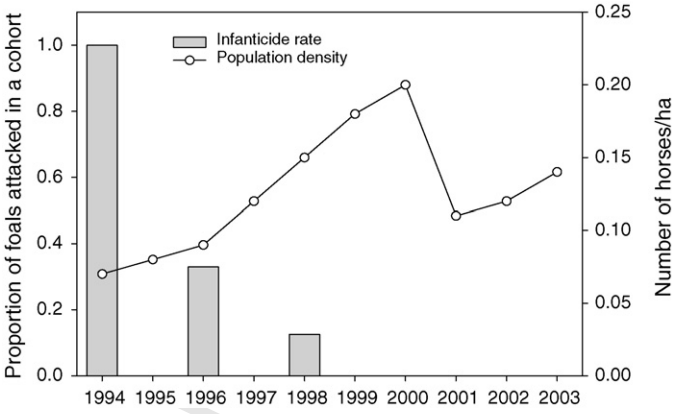


Fig. 2. Infanticide rate and population density.

the "exploitation" hypothesis, as cannibalism obviously never occurred, as well as the "resource competition" hypothesis; in this study, population density and therefore grazing competition increased, while infanticide decreased (Fig. 2). We rule out the "elimination of future rivals", as two out of the five attacked foals were females, an observation confirmed by other studies on equids (Duncan, 1982; Pluhacek and Bartos, 2000; Zarkikh, 1999). Infanticide as a "by-product of male aggression" mainly aimed at females can be rejected. The attacks we observed were without doubt directed towards the foals, not their mothers. The "social pathology" hypothesis states that infanticide is either linked to crowding or to human disturbance. As mentioned above, population density in our herd increased nearly threefold while infanticide disappeared after the first 6 years. However, only zoo bred stallions committed infanticide. Interestingly, all witnessed records of male infanticide in equids concern stallions which have not grown up in socially natural herds, the main difference being that male foals born into a group are separated at a relatively early age from their father and sometimes their mother, before acting as reproductive stallions. Moreover, in all cases of witnessed infanticides, the reproductive partner of the infanticidal stallion, a pregnant mare, was always imposed by humans (Pluhacek and Bartos, 2000; Ryder and Massena, 1988; Kolter and Zimmermann, 1988; Zarkikh, 1999; Duncan, 1982). Infanticide by males in equids therefore seems clearly to contain an element of human "disturbance" or intervention, even if it may have gone unobserved in some populations due to its rarity.

Uncited references

Elwood and Ostermeyer (1984), Feh et al. (2001), Klingel (1972), Sherman (1982), Thomas and Wolff (2004) and Wolff (2004). Q2

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Optimal Control of Wild Horse Populations with Nonlethal Methods

Robert Fonner and Alok K. Bohara

ABSTRACT. *Effective management of wild horse populations has proven difficult for land managers. This paper develops and evaluates dynamic optimization models of wild horse management for three nonlethal population control methods: removals, fertility control, and a hybrid method combining removals and fertility control. Two state variables are included in the models to account for horses on the range and horses in off-range holding facilities. The optimization problems are solved and dynamic simulations are generated for each method. The results indicate that a strategic approach to nonlethal wild horse population control has the potential to significantly improve management efficiency.* (JEL Q28, Q57)

I. INTRODUCTION

Wildlife populations provide a variety of amenities to society, ranging from food and recreation to ecosystem regulation and existence values. Wildlife populations can also impose costs on society through interference with human activities, degradation of habitat, and spreading of disease. Species that both provide a valuable resource and impose societal costs are classified in the literature as multi-use species (e.g., Horan and Bulte 2004; Zivin, Hueth, and Zilberman 2000). Some multi-use species require active management to keep populations from imposing net societal costs. Striking a balance between the costs and benefits of multi-use species can involve active population management. Direct population control is commonly carried out through increased regulated hunting or trapping (Rondeau 2001). However, when multi-use species are endeared to the public, lethal population control methods may not be palatable from a societal perspective. In these cases, wildlife can be captured then relocated, or captured then held in captivity. A third alternative, fertility control, is an emerging nonlethal population control method that can be implemented without displacing animals from their range.

This study evaluates alternative nonlethal strategies for controlling wild horse populations in the American West—a multi-use species for which traditional population control methods are not a politically feasible option. Dynamic economic models of optimal wild horse management are developed for two nonlethal management methods: horse removals and fertility control. A third "hybrid" model is also developed to investigate the effectiveness of using removals and fertility control in tandem. The models account for how management decisions and horse biology influence the dynamics of horse populations on the range, and the population of horses in holding facilities. The objective functions incorporate the costs of on-range management, the costs of off-range holding, and the net benefits associated with wild horse populations. Model parameters are calibrated using published studies on wild horse population biology and Bureau of Land Management (BLM) expenditure data. The value of ongoing status quo management is also estimated, and policy simulations are generated for horse-removal, fertility-control, and hybrid management scenarios. Additional scenarios are developed to assess the sensitivity of model results to shorter time horizons and a cost minimization specification. The simulation results are then examined to glean insights for effectively managing wild horse populations.

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This study fits within a growing body of literature examining the dynamics of multi-use species management. A key characteristic of these analyses is that the shadow price of multi-use species can be either positive or negative, which can lead to multiple potentially optimal equilibria (Rondeau 2001; Zivin, Hueth, and Zilberman 2000). Prior studies have investigated the dynamics of multi-use species management in the context of controlling beaver, deer, and wild pig populations (Huffaker, Bhat, and Lenhart 1992; Rondeau and Conrad 2003; Zivin, Hueth, and Zilberman 2000); wolf reintroductions (Rondeau 2001); and invasive species management with biological controls (Fenichel, Horan, and Bence 2010). This article contributes to the dynamic multispecies management literature an analysis of nonlethal population control using removals with off-site holding, and fertility-control methods.

II. BACKGROUND

Free-roaming horses are an enduring icon of the American West, but an effective and sustainable population management plan has eluded land managers for decades. Western wild horse populations are descendants of the domestic animals that were introduced by Spanish explorers in the early 1500s. Once introduced, the horse populations grew quickly, and by the year 1800 wild horses roamed in large herds from Texas to California (Dobie 1952).

Rapid settlement of the western United States and the subsequent development of western lands led to conflicts with wild horse populations in the 1800s and 1900s. During this time, wild horses were sought out and destroyed or captured for commercial slaughter to prevent their interference with grazing and agricultural activities (Phillips 2012). By 1971, the total U.S. wild horse population was reduced to approximately 9,500 a (Pitt 1984). Some wild horse advocates viewed wild horse management practices as inhumane, and by the mid-twentieth century these advocates had organized to lobby Congress for wild horse protections. The campaign garnered public interest and media coverage; and in 1959 Congress passed legislation to prohibit the use of aircraft or motorized vehicles to hunt horses and burros on public lands (Pitt 1984).

In 1971 Congress passed the Wild and Free-Roaming Horses and Burros Act, legislation that continues to guide the BLM's management of wild horse populations. The act banned private horse gathers and tasked the BLM with "protection, management and control of wild free-roaming horses and burros on public lands" (National Research Council [NRC] 2013). The bill's language guides the BLM to manage horses and burros at "the minimal feasible level" to "achieve and maintain a thriving natural ecological balance on the public lands" (NRC 2013). These directives must be balanced with the BLM's mandate to manage public lands for multiple uses. Without active control, horse populations can become a stress on grazing land (Pimentel, Zuniga, and Morrison 2005). To prevent rangeland degradation, the BLM actively gathers wild horses and removes them from the range with the goal of keeping populations within predetermined appropriate management level (AML) bounds. Adoptive homes are sought for removed horses, and unadopted animals are sent to long-term holding facilities. Current BLM policy does not support selling unwanted horses for slaughter.

In recent years, unwanted horses have been accumulating at holding facilities. During the 2015 fiscal year, 66% of the Wild Horse and Burro Program's (WHBP) $77.2 million budget was dedicated to maintaining captive horses (BLM 2016b). In 2000, by comparison, holding accounted for 46% the $19.8 million total program budget (Government Accountability Office 2008). Furthermore, Garrott and Oli (2013) estimated the total net present value (NPV) cost of caring for the horses currently in holding, if no more were added, at nearly $350 million. Under the existing management program, these authors estimate that the total 2013–2030 costs of maintaining unadopted horses in captivity would total $1.1 billion.

Facing an uncertain future, the BLM requested that the NRC scientifically evaluate the challenges facing the WHBP (NRC 2013). In their report, the council offered guidance for developing dynamic models that could assist the BLM with identifying cost-effective management options. Specifically, a need was

expressed for a model that evaluates the dynamics of on-range and off-range populations, and the costs and consequences of existing and alternative management regimes (NRC 2013).

The findings of the NRC report have not yet led to appreciable shifts in the BLM's wild horse management strategy. Meanwhile, horse populations remain above the AML in most areas, and over 45,000 horses are held in off-range facilities (BLM 2015c). The unsustainable nature of this situation recently garnered coverage by several major media outlets, heightening awareness of wild horse management issues (*The Economist* 2016; Phillips 2016). In September 2016, the citizen-led Wild Horse and Burro Advisory Board recommended euthanizing horses in holding after offering them for adoption. However, following public outcry from animal advocates, the BLM stated that it does not intend to change its policy against wild horse euthanization (Chokshi 2016). Here, we evaluate management alternatives using nonlethal strategies to inform future management actions under current BLM policy.

III. MODELS OF OPTIMAL WILD HORSE POPULATION CONTROL

This section presents dynamic models of optimal wild horse population control for two management methods: removing horses from the range and treating horses with a fertility-control vaccine. A hybrid management regime combining removals with fertility control is then developed. The optimal management policy under a given set of assumptions is to choose a sequence of annual population control efforts that maximizes the net benefits associated with wild horse populations over time.

Both removal and fertility-control methods require that horses be gathered on the range. With removal, gathered horses are transported to short-term facilities where adoptive homes are sought for the animals. Horses that go unadopted are shipped to long-term holding facilities. One of the most promising and practical fertility-control methods for wild horse populations is the long-lasting formulation of porcine zona pellucida vaccine (PZP-22), an imunocontraceptive that is administered through injection after horses are gathered. Treated horses are released back to the range, where the contraceptive remains about 85% effective after 22 months (NRC 2013).

The annual cycle of horse management in the optimization models coincides with the fiscal year. Management actions begin October 1 and continue through the end of February. Wild horse foaling (i.e., birthing) occurs from March 1 to June 30, and the BLM prohibits helicopter gathers during that period (BLM 2010). Net population benefits are assumed to accrue from July 1 through September 30.

Optimal Horse Removal

The NPV of a population of wild horses managed in perpetuity with removal methods is maximized where the chosen sequence of removals $\{Y_0,\ldots,Y_\infty\}$ solves the problem in [1].

$$\max_{\{Y_t\}} \sum_{t=0}^{\infty} \rho^t [B(X_t) - C^{\mathrm{g}}(Y_t) - C^{\mathrm{I}}(I_{t-1},X_{t-1},Y_t) - C^{\mathrm{TR}}(X_{t-1},Y_t) - C^{\mathrm{MISC}}(X_{t-1},Y_t) - CADPT\gamma_t - SC_t], \quad [1]$$

subject to

$$X_t - X_{t-1} = F(X_{t-1}) - Y_t\left[1 + \frac{F(X_{t-1})}{X_{t-1}}\right],$$

$$I_t - I_{t-1} = -I_{t-1}m_{\mathrm{I}} + Y_t\left[1 + \frac{F(X_{t-1})}{X_{t-1}}\right] - \gamma_t,$$

$$Y_t \le \min\{Y^{\max}, \delta X_{t-1}\} \forall t,$$

$$X_{-1} \text{ is given,}$$

$$I_{-1} \text{ is given,}$$

$$X_t^{\min} \ge AML \forall t,$$

$$SC_t = \begin{cases} 0 \text{ if } Y_t = 0 \\ SC \text{ if } Y_t > 0 \end{cases}.$$

Initial population X_{-1} is the population size when the first management cycle begins. The choice variable Y_t is the number of horses gathered during the first five months of period

t. In the absence of management, the horse population grows by $F(X_{t-1})$ during period t foaling. Under optimal removal management, the population transitions to size $(X_{t-1} - Y_t)$ after removals are implemented and then grows to size X_t before benefits are realized in the latter part of period t. Many of the female horses removed from the range are pregnant and give birth off-range rather than on-range. Thus the effective impact of removals on horse population growth is $Y_t[1 + F(X_{t-1})/X_{t-1}]$, where $F(X_{t-1})/X_{t-1}$ approximates the per capita growth increment for horses in a population of size X_{t-1}. This also implies that removing Y_t horses from the range results in $Y_t[1 + F(X_{t-1})/X_{t-1}]$ captive horse births during period t. Furthermore, the total cost of gathering horses, $C^{g}(Y_t)$, depends on the number of horses gathered. Initiating management actions at the beginning of a management period is associated with setup costs $SC_t = SC$.

The inventory of horses in long-term holding at the beginning of period t equals I_{t-1}. These horses are held in long-term facilities until the end of the period when a discrete mortality event occurs at rate m_{I}. The horse inventory in short-term holding during period t equals effective removals in that period, $Y_t[1 + F(X_{t-1})/X_{t-1}]$. Adoptions occur at the end of the period, and the remaining horses are shipped to long-term holding. The costs of off-range holding during time t are given by $C^{\mathrm{I}}(I_{t-1}, X_{t-1}, Y_t)$. The costs of shipping horses from short-term to long-term holding are $C^{\mathrm{TR}}(X_{t-1}, Y_t)$.

The parameter γ_t represents the horses adopted in period t, and γ is the annual state adoption demand. The first γ removed and captive-born horses are adopted and the rest go to long-term holding facilities. Thus, the number of horses adopted in period t equals $Y_t[1 + F(X_{t-1})/X_{t-1}]$ if effective removals (i.e., removed plus captive-born horses) are less than or equal to γ, and equals γ otherwise. The costs associated with administering an adoption are $CADPT$, and total adoption costs in period t equal $\gamma_t CADPT$. When removals plus captive births exceed adoption demand, adoption costs are a fixed cost of management. The miscellaneous costs related to compliance inspections, horse preparation, and additional holding are given by $C^{\mathrm{MISC}}(X_{t-1}, Y_t)$.

Wild horses are associated with economic values beyond the costs of population management. Wild horses provide recreation benefits for wildlife viewers and nonuse benefits for individuals who value knowing that wild horses roam free on the western range. Horse populations can also impose economic costs through displacement of wildlife species and domestic livestock, and through degradation of rangelands. The net economic benefits provided by a wild horse population of size X_t are given by the function $B(X_t)$. It is assumed that $B(X_t)$ is a strictly concave, single-peaked function that reaches its maximum where $X = \bar{X}$. Thus, the marginal benefits function $B_x > 0$ for $X \in [0, \bar{X})$ and $B_x < 0$ for $X \in (\bar{X}, \infty]$.

Several constraints are placed on the optimal horse management solution. During a given period, the horse population is smallest after removals occur but before foaling begins. The models specify that this minimum population level, $X_t^{\min}$, must be above the low-range AML set by the BLM. Wild horse managers may be constrained by the number and proportion of animals that can be gathered in a given year (NRC 2013). Constraint parameter δ is the maximum proportion of the population that can be gathered in a single period. Gathers are also constrained in the model by the maximum total number of horses that can be gathered in a single period, $Y^{\max}$. Finally, the discount factor ρ reflects time preference and is bound between 0 and 1 (inclusive).

Optimal Fertility Control

The optimal fertility-control program is a sequence of fertility treatment choices that maximizes the following problem:

$$\max_{\{Y_t\}} \sum_{t=0,2,4,\ldots}^{\infty} \rho^t \left[B(X_t) + \rho B(X_{t+1}) - C^{\mathrm{g}}(Y_t) - C^{\mathrm{fc}}(Y_t, HMA) - C^{\mathrm{I}}(I_{t-1}) - SC_t \right], \quad [2]$$

subject to

$$X_{t+2} - X_t = F_{t+1}(X_t, Y_t) + F_{t+2}(X_{t+1}, Y_t),$$
$$I_{t+1} - I_{t-1} = I_t(1 - m_{\mathrm{I}}) - I_{t-1},$$
$$Y_t \le \min\{Y^{\max}, \delta \tilde{X}_t\},$$
$$X_{-1} \text{ is given},$$
$$I_{-1} \text{ is given},$$
$$\tilde{X}_t \ge AML \forall t,$$
$$SC_t = \begin{cases} 0 \text{ if } Y_t = 0 \\ SC \text{ if } Y_t > 0 \end{cases}.$$

As in the removals model, the horse population is of size X_{-1} when fertility-control management commences, and Y_t horses are gathered during the first five months of period t. All gathered horses are released back to the range after the mares (i.e., female horses) are treated with contraceptives. Gathers are assumed to occur once every two periods to coincide with the approximate effectiveness duration of PZP-22.

Fertility-control treatments prevent conception but do not interfere with the development of horses in utero (Kirkpatrick and Turner 2003). Thus, fertility treatments applied in period t begin to impact population growth in period $t+1$. Because of this delayed impact, the population grows to size $X_0 = F(X_{-1}) + X_{-1}$ by the beginning of the second period regardless of the initial level of fertility-control effort. The fertility-control formulation considered in this model is effective for two periods, so the treatments applied in period t impact population growth in periods $t+1$ and $t+2$. Fertility-control treatments directly impact the population growth rate. The population growth increments impacted by fertility control undertaken in period t are given by $F_{t+1}(X_t, Y_t)$ and $F_{t+2}(X_{t+1}, Y_t)$. There are no horse removals in the fertility-control optimization model. Thus, the initial horse inventory decays according to mortality rate m_{I} until there are no horses left in holding. The costs of horse holding are determined in the same manner as in the removals model, except that gathers and population size have no impact on holding costs and no are horses held in short-term holding.

Costs specific to fertility-control management (C^{fc}) are assumed to be a function of the number of horses gathered for treatment (Y_t) and the number of BLM herd management areas (*HMA*) where fertility control is undertaken. Gather constraints under fertility-control management mirror the constraints under removals, with one notable exception. The delayed effect of fertility control necessitates a more restrictive gather constraint to facilitate dynamic programming of the solution. The constraint $\tilde{X}_{t-1}$ is the lower bound of the previous period population.

Hybrid Management: Combining Removals and Fertility Control

Horse-removal and fertility-control methods have thus far been considered independently. Each of these approaches has advantages that could be leveraged in a population management regime utilizing both removals and fertility control. The following section discusses the strengths and weaknesses of removals and fertility control and proposes a hybrid management regime that combines both methods.

Compared to removals, fertility control is a more benign method of population management that better aligns with the BLM's mandate to manage horses at the "minimum feasible level." Fertility control may also represent a low-cost population control alternative as it does not require off-range management of horses. However, reducing or stabilizing horse populations with fertility control alone requires that a large share of the population be frequently gathered and treated (NRC 2013).

Removal management directly decreases the base from which a population grows and prevents removed pregnant females from giving birth on the range. Furthermore, removals have an immediate impact on the population compared to the delayed effect of fertility control. These characteristics make removal management a more effective means of reducing horse populations in terms of population reduction per unit of gathering effort. Management with removals also has drawbacks. It requires costly long-term management of off-range horses and is a more intensive management alternative compared to fertility control.

Because removals and fertility each have respective strengths and weaknesses, a management alternative that allows for both removals and fertility control may be preferred to management under a single method. Specifically, a "hybrid" management program could allow for population reductions while reducing the number of horse removals required to maintain populations at a desirable level. Hybrid management may also reduce the intensity of population management subject to maintaining ecological balance on the range.

The NRC's report describes a possible hybrid management scenario (NRC 2013). Following this description, the hybrid scenario is specified to involve several years of intensive removals to reduce the population, followed by a management regime that incorporates fertility control. Specifically, hybrid management involves z consecutive years of removals at the $Y_t = \min\{Y^{\max}, \delta\tilde{X}_t\}$ level until the population is within the AML range. Following the initial reduction phase, horses are managed on a two-year gather cycle with a fixed number of removals and optimally chosen fertility-control effort. The hybrid management model is presented in the Appendix. The number of horses removed at the beginning of each management cycle, q, is the minimum removal effort that maintains a population below carrying capacity in the long term under optimally chosen fertility control.

IV. MODEL CALIBRATION

The objective of this article is to inform wild horse management through development and evaluation of dynamic bioeconomic models. Because the form of the defined optimization problems makes derivation of analytical solutions impractical,[1] we proceed by exploring the problems empirically and develop policy insights through dynamic simulations. This section specifies functional forms, parameters, and assumptions for the optimal horse management models.

[1] These characteristics include having objective functions that are nonmonotone in X, nonlinear transition functions for X and I, and multiple state variables.

Separate models were developed for Nevada and Oregon based on data availability. Thus, all of the herds within in a state were modeled as a single meta-population. This treatment is appropriate given the significant migration that can occur between herds (e.g., see BLM 2015b), but it also imposes no migration between states and equal density dependence (i.e., X/K) across herds.

The biological parameters specified for the models are based primarily on studies of the Beatys Butte herd in eastern Oregon. Due to data limitations, the models do not incorporate burro-specific cost or population biology parameters. Monetary costs and benefits in the models are inflation-adjusted to 2015 dollars. The discount factor (ρ) reflects a 4% discount rate in the base model. The functional forms and associated parameter values specified for the models are summarized in Table 1.

Wild Horse Population Biology

Eberhardt and Breiwick (2012) examined growth data from four wild horse populations, including one in the western United States. They found that the populations grew according to a theta logistic growth function, with the parameter controlling the inflection point equal to two, as shown in equation [3]. The carrying capacity (K) is the maximum population that can be sustained by an environment given available resources, and the intrinsic rate of growth (r) is equal to $b - m$, the birth rate minus the mortality rate.

$$F(X_{t-1}) = X_{t-1} r\left[1 - \left(\frac{X_{t-1}}{K}\right)^2\right]. \quad [3]$$

The theta logistic growth function exhibits properties that add intuition to the equation. The term $X_{t-1}r$ represents the intrinsic increment of growth of the population, and $X_{t-1}r(X_{t-1}/K)^2$ captures the density-dependent population effects. As carrying capacity K goes to infinity, the population is unconstrained by density effects, and population growth is equal to the intrinsic increment of growth. As the population approaches the carrying capacity, growth goes to zero.

TABLE 1
Model Calibration

Description	Functional Form	Parameter Values	References
Discount factor	$\rho = \frac{1}{1+\delta}$	$\delta = 0.04$	
Initial population	X_{-1}	$X_{-1}^{NV} = 41{,}161; X_{-1}^{OR} = 3{,}841$	BLM 2016a
Initial holding inventory	I_{-1}	$I_{-1}^{NV} = 24{,}335; I_{-1}^{OR} = 2{,}707$	BLM 2015c
Appropriate management level ranges	$AML_{low}; AML_{high}$	NV: $AML_{low} = 7{,}597, AML_{high} = 12{,}811$; OR: $AML_{low} = 1{,}374, AML_{high} = 2{,}715$	BLM 2016a
Annual adoption demand	γ	$\gamma^{NV} = 75; \gamma^{OR} = 196$	BLM 2015a
Population growth function	$F(X_{t-1}) = X_{t-1} r\left[1 - \left(\frac{X_{t-1}}{K}\right)^2\right]$	$r = 0.28; K^{NV} = 61{,}595; K^{OR} = 13{,}054$	Eberhardt and Breiwick 2012
Population transition function with removals	$X_t - X_{t-1} = F(X_{t-1}) - Y_t\left(1 + \frac{F(X_{t-1})}{X_{t-1}}\right)$		
Population transition with biannual fertility control	$X_{t+2} - X_t = F_{t+1}(X_t, Y_t) + F_{t+2}(X_{t+1}, Y_t); r_{t+1}(X_t, Y_t) = r_0 - \frac{\pi_1 \theta Y_t}{X_t}; r_{t+2}(X_{t+1}, Y_t) = r_0 - \frac{\pi_2 \theta Y_t}{X_{t+1}}$	$r_0 = 0.28; \theta = .354; \pi_1 = 0.94; \pi_2 = 0.82$	Eberhardt, Majorowicz, and Wilcox 1982; Eberhardt and Breiwick 2012; Bartholow 2007
Holding inventory transition function	$I_t - I_{t-1} = -I_{t-1} m_I + Y_t\left[+\frac{F(X_{t-1})}{X_{t-1}}\right] - \gamma$	$m_I = 0.0952$	Garrot and Oli 2013
Net population benefits	$B(X_t) = aX_t \ln\left(\frac{b}{X_t}\right)$	NV: $a = 25{,}85.5, b = 34{,}823.9$; OR: $a = 25{,}85.5, b = 7{,}380.1$	Bastian et al. 1999; Rondeau and Conrad 2003

(table continued on following page)

TABLE 1
Model Calibration *(continued)*

Description	Functional Form	Parameter Values	References
Gather costs	$C^{g}_{NV}(Y_t) = \beta_1 Y_t + \beta_2 Y_t^2$; $C^{g}_{OR}(Y_t) = \beta_1 Y_t$	NV: $\beta_1 = \$343.5, \beta_2 = \$0.07$; OR: $\beta_1 = \$489.5$	BLM 2015a
Holding costs	$C^{I}(I_t, X_{t-1}, Y_t) = I_{t-1} CLT + Y_t CST_g + Y_t \frac{F(X_{t-1})}{X_{t-1}} CST_{cb}$	$CLT = 1.52 \frac{\$}{day} * 365$ days; $CST_g = 5.37 \frac{\$}{day} * 290$ days; $CST_{cb} = 5.37 \frac{\$}{day} * 150$ days	BLM 2016b
Transport costs	$C^{TR}(X_{t-1}, Y_t) = CTR\left[S_I^{ST} Y_t \left(1 + \frac{F(X_{t-1})}{X_{t-1}}\right) - \gamma\right]$	$CTR = \$123.4; S_I^{ST} = 0.968$	Arneson, Beutler, and Hurst 2002; Garrot and Oli 2013
Miscellaneous costs	$C^{MISC}(X_{t-1}, Y_t) = CMISC\left[Y_t \left(1 + \frac{F(X_{t-1})}{X_{t-1}}\right) - \gamma\right]$	$CMISC = \$210$ if adopted; $CMISC = \$726$ if unadopted	BLM 2014
Gather startup costs	SC	NV: $SC = \$45,447.4$; OR: $SC = \$154,842.3$	BLM 2015a
Adoption costs	$\gamma * CADPT$	$CADPT = \$2,582$	Zeigler 2012; BLM 2014
Biannual fertility-control costs	$C^{fc}(Y_t) = HMA * M + \rho HMA * M + CFC * 0.5 Y_t$	$HMA^{NV} = 84; HMA^{OR} = 18$; $M = \$6,997; CFC = \$299$	Bartholow 2007

For the Beatys Butte herd in eastern Oregon, Eberhardt and Breiwick (2012) estimated an intrinsic growth rate of 0.28 and a carrying capacity of 1,202 horses. The models developed in this study assume that in the absence of management, state horse populations grow according to equation [3] with $r = 0.28$. The states are also assumed to have the same carrying capacity to high-bound AML ratio as the Beatys Butte herd.

The models specify the initial population size based on the BLM's population estimates from March 1, 2016. Because the models require initial population estimates from the beginning of the fiscal year, the BLM population estimates are assumed to grow under model assumptions without management. In the optimization models, $X_{-1} = F(X_{\mathrm{BLM}}) + X_{\mathrm{BLM}}$, where X_{BLM} is the 2016 BLM estimate and X_{-1} is the population at the start of management.

Wild Horse Gather Costs

The annual cost of conducting horse gathers depends on the number of horses gathered. State-level gather expenditure data were obtained from the BLM for fiscal years 2009 through 2015 to parameterize this relationship. The gather expenditure data were escalated to the 2015 fiscal year and then merged with gather statistics from the WHBP website (BLM 2016a). Linear models regressing gather expenditures on the number of horses gathered were estimated for two candidate specifications: a linear specification to represent constant marginal gather costs, and a quadratic specification to represent increasing marginal costs. The quadratic specification provides a superior model fit for the Nevada data, and the linear specification provides a better fit for the Oregon data, as determined by the adjusted R^2 values of the regressions. The final Oregon and Nevada fitted regression lines explain 75% and 87% of the variation in annual gather costs, respectively, according to the final model R^2 values. The model constants are estimates of gather setup costs (SC), and the model coefficients are marginal gather cost estimates. Setting up gather operations is estimated to cost approximately $45,000 Nevada and $155,000 in Oregon. The marginal cost of gathers in Oregon is constant at $490 per horse. In Nevada, marginal gather costs are increasing. The cost of gathering the first horse is $345, and the marginal cost of gathering the 5,000th horse is $1,078. Other potential determinants of state-level gather costs were investigated by sequentially including additional covariates in the regressions described above. However, none of the candidate covariates improved model fit, according to the adjusted R^2, suggesting that gather costs are best predicted by the number of horses gathered without additional covariates.

The gather constraint parameters are specified as follows. The maximum number of horses that can be gathered in a given period ($Y^{\max}$) is set just under the maximum number gathered in a single fiscal year in Oregon ($Y^{\max} = 1{,}250$) from 2009 to 2015. In Nevada, where the current population is a greater share of the AML, the constraint is set to 140% of the maximum number gathered in a single fiscal year in Nevada from 2009 to 2015 ($Y^{\max} = 8{,}000$). Further, no more than half of the pregather population can be removed in a single period in either state ($\delta = 0.5$).

Off-range Horse Inventory

Horses removed from the range are transferred to short-term holding facilities where adoptive homes are sought for them. Horses that go unadopted are transported to long-term holding pastures to live out the rest of their lives. Thus, the inventory of horses in holding over time depends on the number of horses removed from the range, the number of captive-born horses, off-range horse mortality (assuming no reproduction), and the initial inventory of horses.

The models assume zero mortality for removed and captive born horses during the period they were gathered. Mortality for horses in long-term holding occurs as a discrete event at the end of each period. The rate of annual mortality for horses in holding (m_1) was specified as follows. First, inventory accrual was simulated over a 35-year period by looping over 1996–2015 removals and adoptions for Nevada and applying the gender-age-specific survival rates from Garrott and Oli (2013).

The simulations assumed that half of the horses removed were mares and that the age of removed horses is a Poisson process with $\lambda = 2$. The simulations yielded age distributions of horses in holding by gender. The mortality rate for horses in holding, $m_I = 0.095$, was then calculated as the overall annual mortality rate in the 36th year, given the age distributions derived in the simulations and the gender-age-specific survival rates. The initial holding inventories for Nevada and Oregon were approximated as the product of the total number of horses in holding as of September 2015 times the state's share of the total horse population, where $I_{-1}^{NV} = 24{,}335$ and $I_{-1}^{OR} = 2{,}707$.

Off-range Management Costs

Horses removed from the range are transported to short-term holding corrals where they remain until they are adopted or transported to long-term pastures. The models assume that adoptions occur as a discrete event just before the end of each period, and that all unadopted horses are transported to long-term holding facilities before the next period begins. The models also assume that future annual adoption demand remains constant at the 2010–2014 average and that long-term holding capacity is not limiting.

Gathers occur between October and February, and captive-born horses are foaled during the spring of the same period in which their mothers are gathered. To reflect this timing the models assume that I_{t-1} horses are held in long-term holding for the entirety of period t, removed horses are held in short-term holding for 290 days, and captive-born horses are held for 150 days of period t.

Off-range management costs were parameterized with WHBP adoption, horse holding, and expenditure data from the BLM. Per-horse adoption expenditures averaged $2,707 annually for fiscal years 2011–2015, and adopters of wild horses paid a $125 fee (BLM 2016b). Annual adoption demand in Nevada and Oregon averaged 75 and 196 horses, respectively, from fiscal years 2010–2014 (i.e., $\gamma^{NV} = 75$; $\gamma^{OR} = 196$). During fiscal years 2011–2015, long-term holding expenditures averaged $1.52 per day and short-term holding expenditures averaged $5.37 per day.[2] The cost of shipping unadopted horses to long-term facilities is $3.29/mi/40 horses (Arneson, Beutler, and Hurst 2002). The typical distance from short-term facilities to long-term facilities is approximately 1,500 miles, roughly the distance from short-term holding facilities in Reno, Nevada, or Burns, Oregon, to long-term holding pastures the midwestern United States. Horses removed from the range are associated with miscellaneous costs related to compliance inspections, horse preparation, and additional holding. Total miscellaneous costs are $210 for adopted horses and $726 for unadopted horses (BLM 2014).

Fertility-Control Effectiveness and Duration

The relationship between horse population growth and fertility-control effort is specified next. Recall that Eberhardt and Breiwick (2012) estimated the intrinsic growth rate for the Beatys Butte herd at $r = 0.28$ without managment. Another study of the Beatys Butte herd (Eberhardt, Majorowicz, and Wilcox 1982) estimated the average annual survival rate at 0.926. Thus, the intrinsic rate of growth at Beatys butte equals 0.28 with no fertility control, and equals –0.074 when every female in the herd receives effective contraception. In reality, PZP-22 is not 100% effective at preventing horse pregnancy. Bartholow (2007) reports that PZP-22 was 94% effective in the first year after treatment and 82% effective in the second year for Nevada's Clan Alpine herd.

To maintain tractability, the optimal fertility-control model assumes an equal sex ratio in horse populations, homogeneity across gender-specific gather production, and no gender selectivity in gathers. These assumptions yield the simplifying condition that half of the horses gathered are treated with fertil-

[2] For each fiscal year from 2011 to 2015, the number of horses in holding (short and long) is calculated as the average of monthly holding inventories reported by the BLM. The per day holding cost is then calculated as the average number of horses in holding divided by the total (short and long) holding expenditures reported by the BLM for that fiscal year.

ity-control agents and the other half are returned to the range.

Contraceptive applications occur on a biannual cycle in the fertility-control model so that two foaling seasons occur between management actions. In the first mating period after fertility-control application, fertility-control effectiveness is $\pi_1 = 0.94$ and the intrinsic rate of growth is r_{t+1}. In the second mating period after management, fertility-control effectiveness is $\pi_2 = 0.82$ and the intrinsic rate of growth is r_{t+2}. A linear functional form is specified between the growth rate and the proportion of females treated with contraceptive. Combined with the previously defined parameters, a linear functional form yields equations [4] and [5].

$$r_{t+1}(X_t,Y_t) = r_0 - \pi_1 0.354\frac{Y_t}{X_t}, \quad [4]$$

$$r_{t+2}(X_{t+1},Y_t) = r_0 - \pi_2 0.354\frac{Y_t}{X_{t+1}}. \quad [5]$$

The transition of the horse stock over time is then given by the theta logistic population growth function in equation [3], where the intrinsic rate of growth is $r_{t+1}(X_t,Y_t)$ in the second foaling period after fertility-control management and $r_{t+2}(X_{t+1},Y_t)$ in the third foaling period after management.

Fertility-Control Costs

The costs of fertility-control management are parameterized as follows. The combined cost of obtaining contraceptives and administering them on the range is approximately $300 per application (Bartholow 2007). Fertility-control management requires additional population monitoring compared to management with removals. The cost of fertility-control monitoring (M) is approximately $7,000 per HMA per year (Bartholow 2007). There are 18 distinct HMAs on BLM lands in Oregon and 84 in Nevada. Because management is considered at the state level, the models assume all HMAs on BLM lands are monitored for the two years after a fertility control cycle is initiated. The second year of fertility monitoring is discounted, so that if fertility control is initiated in fiscal year t, then monitoring costs per HMA for that management cycle equal $M_t = M + \rho M$. If fertility control is not initiated, then no monitoring costs are incurred during that management cycle.

Horse Population Net Benefits

Wild horse populations are associated with significant societal benefits. On the other hand, large horse populations can impose costs on stakeholders through displacement of alternative land uses and degradation to range lands. Pimentel, Zuniga, and Morrison (2005) estimated that U.S. wild horse and burro populations cause $5 million in forage losses annually. Bastian et al. (1999) found that the marginal opportunity costs of additional horses on the range in excess of target population levels are well over $4,060 in in 2015 dollars. Wild horses also provide use and nonuse benefits to wild horse supporters. However, these benefits are challenging to measure because their value is not revealed through market transactions.

The function describing horse population net benefits, $B(X)$, represents the aggregation of all costs and benefits attributable to that population. The shape of $B(X)$ depends on the shape of the marginal benefits and marginal costs functions. Bastian et al. (1999) found that the marginal costs of adding horses to a population were increasing through the population size associated with the $4,060 figure mentioned above. Intuitively, adding horses to a small population under BLM management (i.e., management subject to an overall grazing limit) would displace less valuable wildlife and livestock resources compared to when the horse population is already large. Conversely, attempts in the economic literature to measure benefit functions for wildlife populations and habitats support decreasing marginal benefits, (e.g., Layton, Brown, and Plummer 1999; Rollins and Lyke 1998), particularly when the wildlife population is above its minimum viable population (e.g., Bandara and Tisdell 2005; Ojea and Loureiro 2009). Decreasing marginal costs and increasing marginal benefits imply that the total net benefits function for horse populations, $B(X)$, is a single-peaked function. This analysis fol-

lows Rondeau and Conrad (2003) in assuming that total net benefits can be represented by a Gompertz function where

$$B(X) = \begin{cases} 0 \text{ if } X = 0 \\ aX\ln\left(\frac{b}{X}\right) \text{ if } X > 0 \end{cases}. \quad [6]$$

The Gompertz function is a single-peaked function that satisfies the characteristics of net horse benefits specified previously (i.e., that $B_X > 0$ for $X \in [0,\bar{X})$ and $B_X < 0$ for $X \in (\bar{X},\infty]$). The high-bound AML is assumed to be the population level $\bar{X}$ that corresponds with the maximum of $B(X)$. The marginal opportunity cost of an additional wild horse at high population levels is approximately \$4,060 (Bastian et al. 1999), and the models assume that this is the marginal net benefit at carrying capacity. The resulting system of equations (i.e., $B_X(\bar{X}) = 0$ and $B_X(K) = -4{,}060$) was then solved for parameters a and b. In the Nevada model, parameters a and b are equal to 2,586 and 34,824, respectively. The resulting function implies that a state population of 10 horses in Nevada produces \$211,000 in annual net benefits, while a population at the high-range AML produces \$33.1 million in annual net benefits. At carrying capacity, the Nevada horse population yields net benefits of −\$90.8 million annually. In the Oregon model, parameter a equals 2,586, and parameter b equals 7,380.

V. RESULTS

This section presents simulations of the specified optimal management models and compares them to current management practices. A 50-year time horizon is used to compare the base scenarios, and sensitivity scenarios with shorter horizons are also explored. A long-term time horizon is appropriate for identifying optimal management strategies because horse removals conducted in the current period are associated with holding costs that extend decades into the future.

Solving the Dynamic Optimization Problem

The solution to the removals problem in [1] is identified by solving the associated Bellman equation in [7], subject to the previously defined constraints and the terminal condition $V(T+1,X_t) = 0$, where T is the finite time horizon.

$$\begin{aligned} V(t,X_{t-1},I_{t-1}) = \max_{\{Y_t\}} \{ & \rho^t [B(X_t) - C^{\mathrm{g}}(Y_t) \\ & - C^{\mathrm{I}}(I_{t-1},X_{t-1},Y_t) - C^{\mathrm{TR}}(X_{t-1},Y_t) \\ & - C^{\mathrm{MISC}}(X_{t-1},Y_t) - CADPT\gamma_t - SC_t] \} \\ & + \rho^{t+1} V(t+1,X_t,I_t). \end{aligned} \quad [7]$$

The function $V(t,X,I)$ is the maximum achievable NPV benefits starting at time t with population level X and given action Y. All subsequent actions are assumed to be taken optimally, given the action taken in the current period. Bellman equations [8] and [9] are the equivalent for optimal fertility-control and hybrid population management, respectively.

$$\begin{aligned} V(t,X_t) = \max_{\{Y_t\}} \{ & B(X_t) + \rho B(X_{t+1}) - \mathrm{C}^{\mathrm{g}}(Y_t) \\ & - C^{\mathrm{fc}}(Y_t,HMA) - C^{\mathrm{I}}(I_{t-1}) - SC_t \} \\ & + \rho^{t+1} V(t+1,X_{t+1},I_t), \end{aligned} \quad [8]$$

$$\begin{aligned} V(t,X_t) = \max_{\{Y_t\}} \{ & \rho^t [B(X_t) + \rho B(X_{t+1}) - C^{\mathrm{g}}(Y_t+q) \\ & - C^{\mathrm{fc}}(Y_t) - C^{\mathrm{I}}(I_{t-1},\tilde{X}_t,q) - C^{\mathrm{TR}}(\tilde{X}_t,q) \\ & - C^{\mathrm{MISC}}(\tilde{X}_t,q) - 2CADPT\gamma - SC] \} \\ & + \rho^{t+1} V(t+1,X_{t+1},I_t). \end{aligned} \quad [9]$$

The Bellman equations were programmed in MATLAB (MathWorks 2015) and solved with a backward induction algorithm. The terminal period (T) was set to 65 years, so that realization of the terminal condition is not taken into account in the optimal management simulations. Robustness checks with longer-horizon models suggest that the solutions are stationary. The problems were discretized to limit their dimensionality and facilitate their solution via dynamic programming.[3]

The Nevada and Oregon scenarios were simulated with a 50-year planning horizon for

[3] Discretization involved grouping the horses into units and then rounding the state variables to the nearest unit before each value calculation. For removal and hybrid management, the Nevada and Oregon models grouped horses into 1,000-horse and 150-horse units, respectively. The fertility-control models grouped horses into 10-horse units.

both the horse-removal and fertility-control models. The steady-state solutions to the optimal removal and optimal fertility-control models are summarized for Nevada and Oregon in Table 2. The population and holding inventory paths for the optimal solutions and the status quo scenario are presented for Nevada and Oregon in Figure 1 and Figure 2, respectively.

Status Quo Management Scenario

Status quo horse management practices were simulated under model assumptions to provide a baseline for comparison. BLM horse population estimates from 2009 to 2014 and associated management actions from that period were evaluated to characterize current state-level management in Nevada and Oregon. Notably, the intensity of removal efforts varied substantially from year to year in both states. In Nevada, annual gathers ranged from 1.5% to 30.5% of the estimated prefoal population.[4] Annual Oregon gathers ranged from 0.0% to 54.0% of the prefoal population. The status quo management scenarios assume that future gather, removal, and fertility-control efforts reflect a state's recent management practices. Specifically, the scenarios follow a six-year management cycle based on the proportion of horses managed in the six-year period during fiscal years 2009–2014, subject to state gathers not exceeding $Y^{\max}$ or δX_{t-1}.

The periodic nature of removals in the status quo scenarios gives rise to oscillations in horse populations and holding inventories, as displayed in Figures 1 and 2. In terms of long-term trends, populations and inventories increase in the earlier periods before leveling off in the later periods. The cumulative NPV of status quo horse management over a 50-year time horizon is –$1.52 billion in Nevada and –$255 million in Oregon. The cumulative NPV program benefits over time for each scenario are presented in Figure 3, and the cumulative NPV management costs over time are presented in Figure 4.

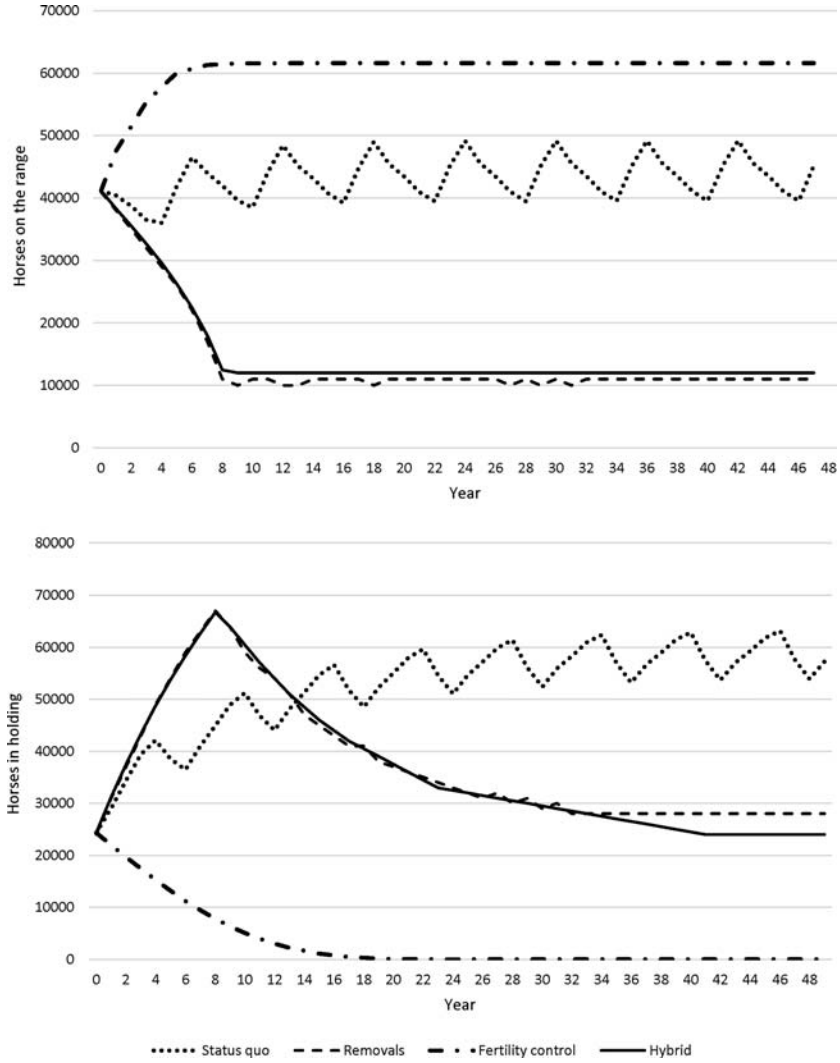
[4] The status quo gather proportions were constructed with the March 1 BLM population estimate in the denominator and the horses gathered over the subsequent 12 months in the numerator.

TABLE 2
Optimal Steady-State Management Programs and Cumulative Optimal Program Benefits

Scenario	Pregather Population	Removals	Fertility Treatments	Total Gathers	Horses in Holding	Percent Gathered	Cumulative Net Benefits[a]	Change from Status Quo[a]
Nevada								
Removals	11,000	2,000	0	2,000	28,000	18	(230.1)	1,290.5
Fertility control	61,600	0	0	0	0	0	(2,005.5)	(484.9)
Hybrid	12,000	3,750	1,000	5,750	24,000	48	(265.1)	1,255.5
Oregon								
Removals	2,250	450	0	450	4,050	20	34.7	290.1
Fertility control	13,050	0	0	0	0	0	(371.6)	(116.2)
Hybrid	2,250	677	150	977	2,100	43	44.3	299.8

[a] Millions of dollars. Parentheses indicate negative values.

FIGURE 1
Population and Holding Inventory Paths for Nevada Simulations

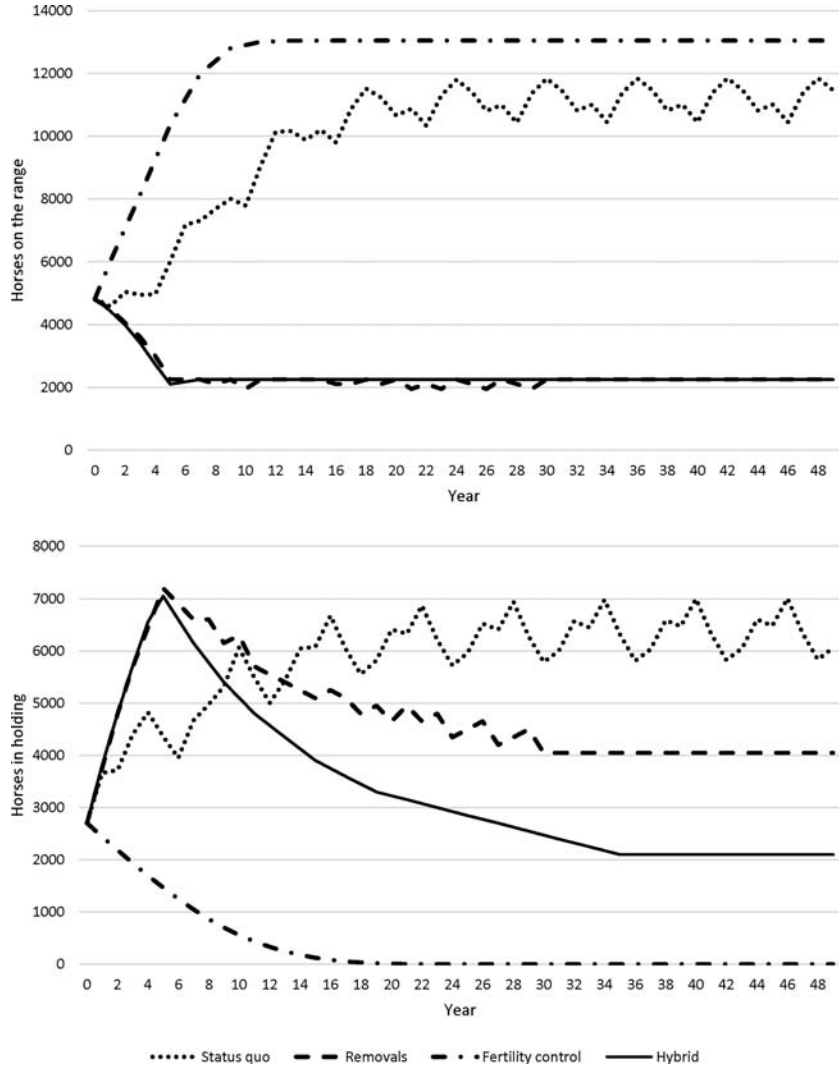


Management Simulations

Horse Removal

The Nevada and Oregon optimal removal scenarios involve an initial reduction of the population to a steady-state equilibrium. In Oregon, the optimal program involves five years of intensive removal effort at or near $Y^{\max}$. At steady-state equilibrium, 450 horses are removed from the range each year to maintain a population of 2,250 horses and a holding inventory of 4,050 horses. The 50-year cumulative NPV of the Oregon optimal removals program is –$34.7 million, a savings of $290.1 million compared to the status quo scenario. Similarly, the optimal removal program in Nevada involves intensive removals over the first eight years of management. Following the initial population reduction, a steady-state management regime commences where 2,000 horses are removed annually to maintain the population at 11,000 and the holding inventory at 28,000. The 50-year cumulative NPV of optimal removals in Nevada

FIGURE 2
Population and Holding Inventory Paths for Oregon Simulations



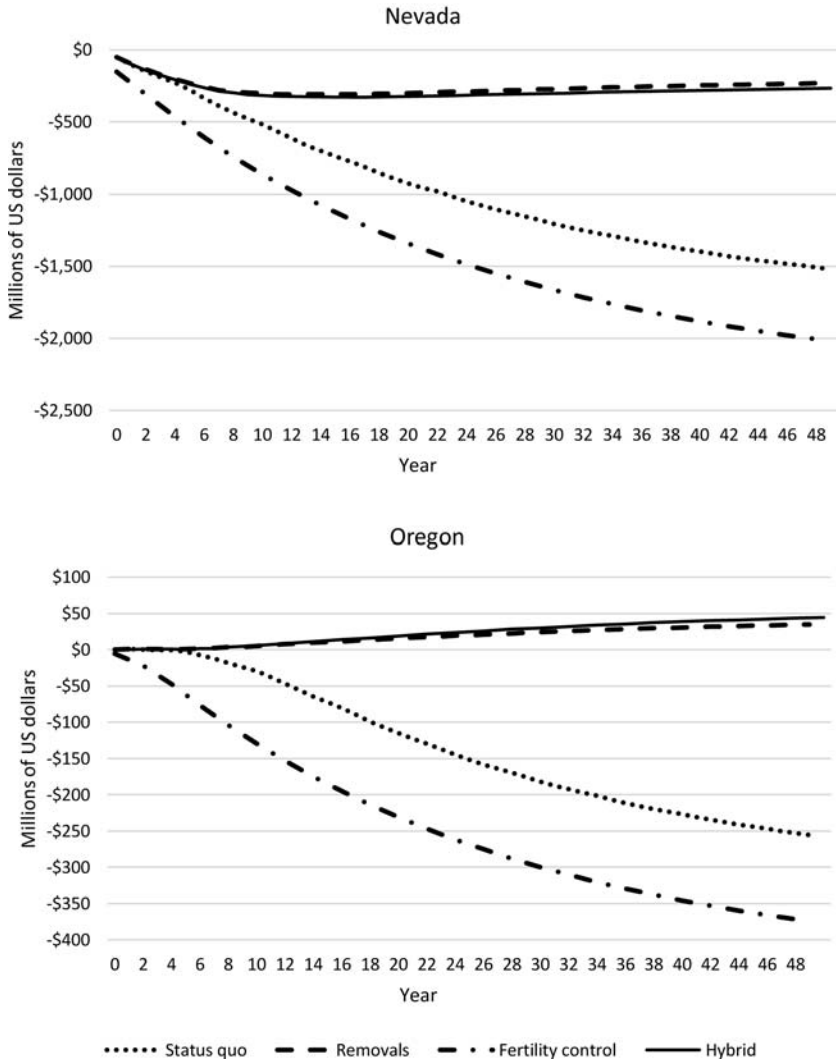
is –$230.1 million, a savings of $1.3 billion over that period compared to the status quo scenario.

As a check to the dynamic programming solutions, optimal steady-state values for removals, population size, and holding inventory were derived numerically using the discrete-time maximum principle (Canon, Cullum, and Polak 1970). While minor differences in the solutions occur due to rounding errors, the numerically derived steady-state values verify the dynamic programming solutions.[5]

The optimal management scenario results yield insights into the trade-offs involved in maximizing population benefits over time. The absolute gather constraint is binding while the populations are reduced in the initial

[5] The steady-state values derived using the maximum principle are as follows: $X_{SS}^{NV} = 10{,}141; I_{SS}^{NV} = 28{,}242; Y_{SS}^{NV} = 2{,}171; X_{SS}^{OR} = 2{,}175; I_{SS}^{OR} = 4{,}163; Y_{SS}^{OR} = 466$.

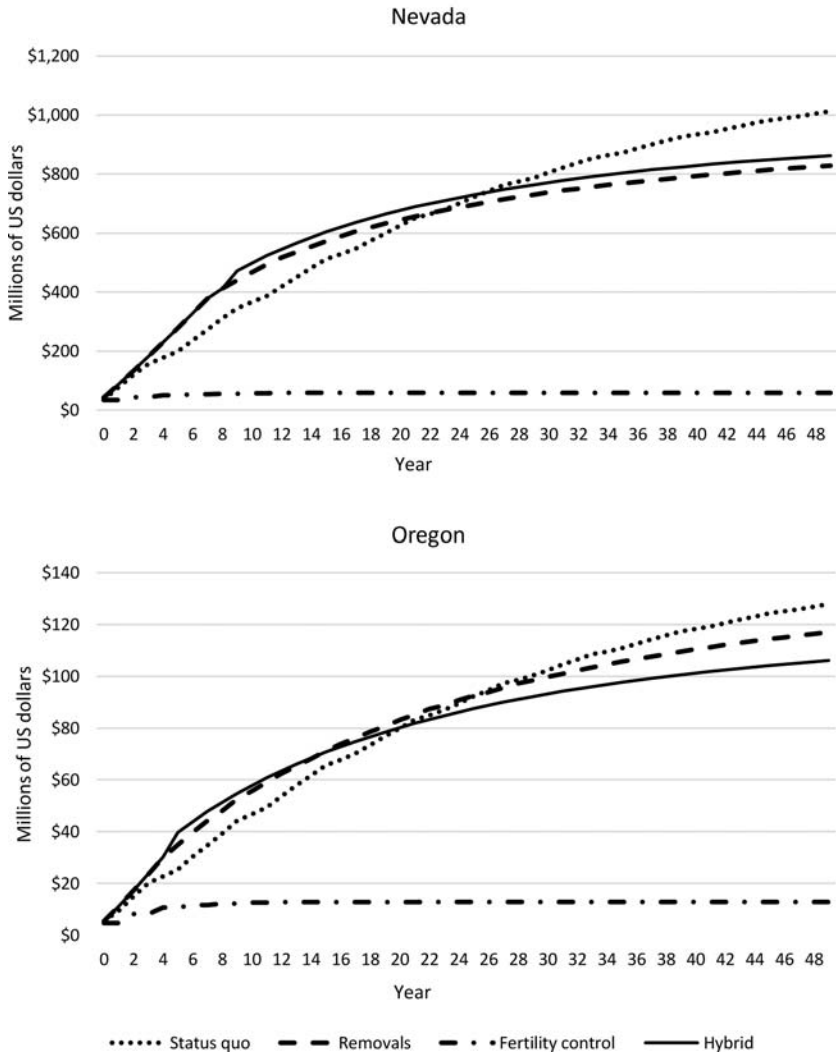
FIGURE 3
Cumulative Net Present Value Net Benefits of Management Programs



periods (i.e., $Y_t = Y^{\max}$). Whereas the removals optimization problem does not meet the sufficient conditions for bang-bang control defined by Spence and Starrett (1975),[6] the removal simulations suggest the optimal management path is indeed to approach a steady state as rapidly as possible. At the optimal steady state, neither the Oregon nor the Nevada optimal removals program is bound by the gather constraints imposed on the model (i.e., $Y^{SS} < \min\{Y^{\max}, \delta X^{SS}\}$). Thus, while the gather constraints regulate the speed with which the population is reduced to steady state, they do not constrain management in optimal steady-state equilibrium.

[6] The Nevada removal model specifies nonlinear gather costs in Y. Both state removal models specify setup costs and stock-dependent variable costs that violate the sufficient conditions of quasi-concavity and separability, respectively.

FIGURE 4
Cumulative Net Present Value of Wild Horse Management Costs



The management costs incurred over a single fiscal year in the optimal removals steady state are higher for the larger Nevada population ($22.4 million) than they are for the smaller Oregon population ($4.3 million). Likewise, in steady state, the larger Nevada population yields higher annual net benefits ($32.8 million) than the Oregon population ($6.9 million). The annual value of net program benefits from optimal removals in steady state is $10.3 million in Nevada and $2.6 million in Oregon.

Horse Fertility Control

The NRC report suggested that fertility control alone cannot address the challenges associated with managing western wild horses. The optimal fertility-control scenarios support this sentiment. The optimal fertility-control management program is to undertake fertility control in early management cycles and then to undertake no management once the population grows toward its carrying capacity. In Oregon, the optimal fertility-control

TABLE 3
Optimal Two-Year Management Cycles at the Steady State (Millions of U.S. Dollars)

Scenario	Net Population Benefits	Management Costs[a]	Net Benefits[a]
Nevada			
Removals	64.3	44.0	20.3
Hybrid	64.1	46.1	18.0
Difference	− 0.4%	4.8%	− 11.3%
Oregon			
Removals	13.6	8.4	5.1
Hybrid	13.2	6.4	6.7
Difference	− 2.7%	− 23.8%	31.4%

[a] Includes costs of off-range holding. For discounting purposes, $t = 0$ in the first year of the optimal management cycle, and $t = 1$ in the second year.

program is to treat around 600 females for the first three cycles and then cease fertility control in all subsequent periods. The optimal fertility-control program in Nevada is to treat 3,940 females in the first period and then undertake no further management actions. Without management, the horse populations grow to their carrying capacity (K) and remain at that level for the duration of the management horizon. The inventory of horses in holding declines to zero deterministically in all of the fertility-control scenarios. Intuitively, it is impossible to reduce the population with fertility control alone given the model gather constraints. Thus, after slowing growth in the initial period(s), it is optimal to just let the populations grow to their carrying capacity instead of applying costly fertility control. Because horse populations are not effectively managed in the fertility-control scenarios, subsequent discussion focuses on the removal and hybrid methods.

Hybrid Management

Fertility-control alone is not a viable population management strategy. The hybrid management scenarios explore the efficacy of fertility control when applied in tandem with horse removals. The hybrid scenarios begin with intensive removals over successive years until the state population is brought within the AML range. Next, a program of fixed removals with optimally chosen fertility-control effort ensues on a two-year cycle. Fixed removals are undertaken at the lowest possible level subject to maintaining a stable population below carrying capacity in the long run. When implemented in concert with removals, fertility control helps stabilize the horse population by decreasing the intrinsic rate of growth. The Nevada hybrid scenario involves nine years of intensive removals followed by a steady-state gather program that stabilizes the population at 12,000. The steady-state program involves gathering 5,750 horses biannually, of which 3,750 are removed and 1,000 are treated with fertility-control agents. Adoptive homes are found for 150 of the removed horses and 3,600 go to long-term holding facilities. A steady-state inventory of 28,000 horses is maintained in holding facilities. The hybrid program in Oregon involves six years of removals followed by a biannual steady-state management program that maintains a pregather population of 2,250 horses. The program involves gathering 977 horses every other year, including 677 removals and 150 horses treated with fertility control. In Oregon, 392 of the removed horses are adopted and 285 are sent to long-term holding facilities. A holding inventory of 2,100 horses is maintained in the steady state.

The NPV costs and benefits of the optimal removal and hybrid management programs over a two-year cycle are presented in Table 3. The steady-state NPV management costs of a two-year hybrid management cycle in Nevada are $46.1 million, an increase of 4.8% compared to the NPV costs removals over the same time period. In Oregon, the NPV of steady-state hybrid management over a cycle is $6.4 million, which represents a 23% re-

duction compared to removals. In terms of program net benefits, a cycle of steady-state hybrid management yields NPV $18.0 million in Nevada and $6.7 million in Oregon. Compared to optimal removals in the steady state, hybrid management decreases two-year net program benefits by 11.3% in Nevada and increases net program benefits by 31.4% in Oregon. The superior performance of the Oregon hybrid scenario is related to Oregon's higher adoption demand, which facilitates a lower steady-state holding inventory.

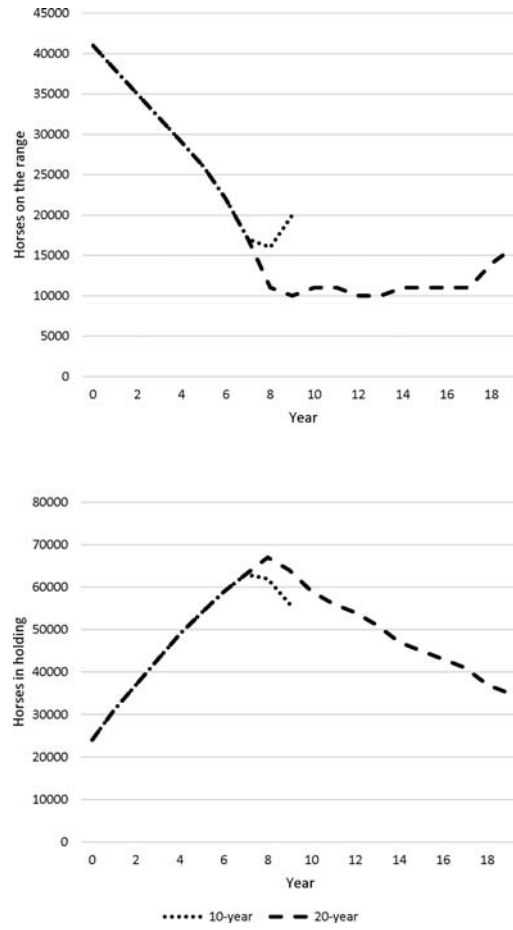
Time-Horizon Sensitivities

A long-term time horizon is appropriate for capturing the economic impact of wild horse management decisions because removals in the current period result in holding costs for the life span of removed horses. In practice, however, land managers may base their management decisions on shorter time horizons, due to budgetary constraints, management uncertainties, or other factors. Additionally, the models assume that specified parameters remain constant over time, and setting a shorter time horizon makes this assumption more likely to hold. The Nevada removals models were solved under 10-year and 20-year time horizons (i.e., $T = 10$ and $T = 20$) to explore how shorter time horizons influence optimal management. The optimal population and holding inventory paths from these simulations are shown in Figure 5. The paths suggest that even when considered under a shorter time horizon, it is optimal to reduce the population rapidly in the early periods to avoid incurring costs (i.e., negative net population benefits) associated with a larger population. The population uptick at the end of the shortened time horizons occurs to satisfy the terminal condition.

Removals under Cost Minimization

Allowing horse populations to become self-limited can reduce population growth rates, and potentially reduce the number of horses removed and sent to holding compared to current management. On the other hand, larger populations grow from a bigger base and thus produce more horses annually for a given growth rate. If managers determine

FIGURE 5
Optimal Paths for Removal Management in Nevada under Alternate Time Horizon



horse populations should not exceed some maximum level, the question arises as to whether it is cost-effective to maintain self-limited populations just below the threshold, or if it is less costly to maintain smaller, but faster growing populations. To explore this question, the next section examines optimal horse removals under a cost minimization objective, where the net population benefits term is dropped from the objective function. Dropping $B(X)$ from [1] and imposing a maximum population constraint frames management decisions in terms of cost-effectiveness, where management costs are minimized subject to maintaining the horse population under some ceiling.

The cost-effectiveness scenarios were run for optimal removals management in Nevada. If the maximum population constraint is set above 50,000, or roughly 80% of carrying capacity, then it is cost-effective to actively manage a large population at the constraint. If the maximum population is set below this level, then it is cost-effective to manage a population of 11,000 horses, the optimal steady-state solution to the model in equation [1].

VI. DISCUSSION

Dynamic, benefit maximizing models of wild horse management were developed for three nonlethal population control methods using published research on wild horse population biology and expenditure data from the BLM. The optimal model solutions were compared to each other and to a scenario that represents status quo management. Finally, optimal removal models were simulated with shorter time horizons, and with a cost-minimization objective to assess the cost-effectiveness of maintaining populations under a specified level.

The model simulation results provide evidence of how to undertake state-level horse management to maximize benefits for stakeholders. Importantly, the results indicate that societally efficient management programs involve an initial reduction of state horse populations from their 2015 levels. This result is robust to the time-horizon specifications, and to the cost minimization specifications where the maximum population is set less than 80% of carrying capacity. The upshot here is that even in the face of costly removals and holding, efficient management involves reducing populations from current levels and maintaining them within their AML ranges. Moreover, the removal and hybrid management simulations suggest that with current technology nonlethal wild horse management requires maintaining off-range populations comparable to, or greater than, the on-range populations.

A second takeaway from the results is that, as defined, fertility control alone is not an effective method for managing horse populations. The reason is that nearly all of the mares must be effectively treated to reduce horse populations using fertility control only. Further, the gather constraints imposed by the model make reducing or stabilizing horse populations with fertility impossible. If all mares in the population could somehow be effectively treated in each period, fertility control has the potential to slowly reduce the population at the mortality rate. However, it is unlikely all mares could be treated, and even if they could, fertility control would not produce a significant population reduction over the short term.

While fertility control alone is not an effective population management strategy, the hybrid management results indicate that fertility control can reduce the total number of horses removed from the range while maintaining comparable management costs and net program benefits. At the optimal steady state, for example, there are 6% fewer horses removed over each two-year hybrid management cycle compared to optimal removals in Nevada, and 25% fewer in Oregon.

When framed in terms of cost minimization, the models yield the cost-effective solution for keeping populations under some specified maximum level. For removals in Nevada, it is cost-effective to reduce populations and maintain them at the base model steady state unless the population constraint is set above 50,000, or roughly 80% of carrying capacity. Thus, maintaining small populations that are not resource limited is less costly than managing large, resource-constrained populations unless the large populations are near the carrying capacity. Further, managing medium-sized populations is costly because many horses need to be removed to stabilize fast-growing populations that are not resource limited.

As shown in Figure 4, cumulative NPV management costs are higher in the optimal scenarios than in status quo management until at least the twentieth management year across scenarios. Thus, realizing the gains associated with optimal management requires enduring higher initial management costs in return for greater population benefits and lower management costs in the future. Furthermore, while the optimal scenarios involve intensive management in the initial periods, the status quo scenario is the most intensive manage-

ment method across the 50-year management horizon. In the Nevada status quo scenario, over 280,000 horses are taken to long-term holding facilities across the management horizon, compared to around 175,000 with optimal removals and 170,000 with hybrid management.

Overall, the simulation results show that significant economic gains can be realized with a more strategic approach to wild horse population management. Relative to the status quo scenario, the results indicate that implementing optimal removals or hybrid management would result in 50-year NPV program savings of $1.3 billion in Nevada. Undertaking optimal hybrid management would save $300 million compared to the status quo in Oregon. Realizing these savings, however, is a matter of incurring short-term costs to realize future benefits.

Separate models were developed for Nevada and Oregon, and comparison of the results across states yields additional management insights. Oregon contains almost 3 million HMA acres compared to 14 million in Nevada. Likewise, the Oregon models specify a smaller carrying capacity, AML, and initial population to carrying capacity ratio compared to the Nevada models. Adoption demand is greater in Oregon than in Nevada despite Oregon having a much smaller horse population. This allows Oregon to send a smaller proportion of removed horses to long-term holding. These state-specific management differences reflect the difficulties inherent in managing the large Nevada population of horses. Though the larger Nevada population yields nearly five times the population benefits at the optimal steady state, Oregon yields greater 50-year program benefits due to the high costs of managing the Nevada population.

Implementing the optimal management programs identified in this study would require allocating additional resources to the WHBP to fund intensive removals in the early periods and associated increases in the horse-holding inventory. The BLM currently has capacity for around 32,000 horses in long-term facilities. Implementing the optimal management programs would require temporarily increasing that capacity to around 70,000 to handle the Nevada population alone (Nevada's horse population represents roughly half of the overall wild horse population in the west.) Securing the resources to implement these programs would surely be challenging. However, the model results suggest that the investment would yield benefits that outweigh the costs.

While the models developed in this article represent a step toward understanding the economic consequences of alternative wild horse management regimes, they also have some notable limitations. First, the models do not capture potentially significant heterogeneity in biological and economic parameters across the state. More granular models may increase overall precision and provide guidance regarding how to best allocate management effort among state HMAs. Second, the models implicitly assume that specified economic and biological relationships remain constant across time. Aside from uncertain shifts in preferences or management costs, horse habitats are likely to change in the future due to climate change. In the Great Basin, where most of Oregon's and Nevada's wild horses reside, climate change is expected to increase overall precipitation, but also increase interannual precipitation variability. In summer, stream flows are expected to decline (Chambers 2008), which could have limiting impacts for wild horse populations. Beschta et al. (2013) suggest that reducing wild horse grazing is a prudent policy for mitigating the expected effects of climate change on western public range lands. Future research efforts could incorporate emerging climate science and related ecosystem impacts into horse management models. Despite its limitations, this article presents significant evidence that current wild horse management is inefficient, and that significant efficiency gains are possible if management actions are undertaken strategically.

APPENDIX: HYBRID POPULATION CONTROL

$$\max_{\{Y_t\}} \sum_{t=z+1,z+3,z+5,\ldots}^{\infty} \rho^t \big[B(X_t)+\rho B(X_{t+1}) - C^{g}(Y_t+q) - C^{fc}(Y_t,HMA) - C^{I}(I_{t-1},X_t,q) - C^{TR}(X_t,q) - C^{MISC}(X_t,q) - 2CADPT\gamma - SC\big], \quad [A1]$$

subject to

$$X_{t+2} - X_t = F_{t+1}(X_t, Y_t) + F_{t+2}(X_{t+1}, Y_t) - q\left[1 + \frac{X_{t+1}}{F_{t+2}(X_{t+1}, Y_t)}\right],$$

$$I_{t+1} - I_{t-1} = (1 - m_I)\left\{I_{t-1}(1 - m_I) + q\left[1 + \frac{\tilde{X}_{t-1}}{F(\tilde{X}_{t-1})}\right] - 2\gamma\right\} - I_{t-1},$$

$$Y_t \leq \min\{Y^{\max}, \delta\tilde{X}_t\},$$

$X_z; I_z$ = states when fertility control begins,

$$\tilde{X}_t^{\min} \geq AML \forall t,$$

where

$$\tilde{X}_{t-1} = \frac{X_t}{(1 + r_0)} + q,$$

$$\tilde{X}_t^{\min} = \frac{X_t}{(1 + r_0)},$$

$$X_{z+1} = X_z + F(X_z) + - q\left[1 + \frac{X_z}{F(X_z)}\right],$$

z = initial reduction periods.

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Porcine and recombinant zona pellucida vaccines as immunocontraceptives for donkeys in the Caribbean

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Immunocontraception has been investigated as an alternative to hormone manipulation and lethal methods of fertility control in wildlife for almost 30 years. Porcine zona pellucida (PZP) vaccine has been shown to utilize an animal's immune system to prevent pregnancies temporarily and reversibly in multiple species. This study investigated PZP vaccination as a method of practical population control in feral donkeys (Equus asinus) in the tropics. In addition, the study investigated a novel recombinant zona pellucida vaccine (recZP) as an alternative for fertility management in donkeys.

Twenty-five feral female donkeys of proven fertility were captured on Nevis and transported to Ross University School of Veterinary Medicine (RUSVM). All animal protocols were approved through RUSVM IACUC review. Jennies allocated to Group 1 (n=9) received the recZP vaccine in complete modified Freund's adjuvant (CMFA) and thereafter two booster vaccines in incomplete Freund's adjuvant (IFA) 5 weeks and in sterile saline 10 weeks later. Jennies allocated to Group 2 (n=8) received the PZP vaccine in CMFA and one booster in IFA 5 weeks later. Those allocated to Group 3 (n=8) received an initial injection of CMFA and second injection of IFA 5 weeks later and acted as controls. All treatments were administered intramuscularly by injection into the left and right gluteal muscles (1st and 2nd injections), and the left pectoral muscles (3rd injection in Group 1). Trans-rectal ultrasonography was performed weekly on each jenny to monitor ovarian and follicular development and cyclical reproductive activity. Five weeks after the final injection, one jack was placed with each group of jennies. Estrus detection, mounting behaviors, and breeding were recorded when observed. Weekly trans-rectal observations were continued to visualize the reproductive tracts and ovaries and to identify pregnancies. Jacks were rotated through the groups every three weeks.

All donkeys were cycling normally prior to injection with obvious estrus signs detected when in proximity to a jack. Sterile abscesses at injection sites were observed in 9/9 (100%) jennies in Group 1, 7/8 (87.5%) jennies in Group 2 and 3/8 (37.5%) jennies in Group 3. Three months after the final injection, 7/9 (78%) jennies in Group 1 and 6/8 (75%) jennies in Group 2 had no detectable follicular development nor estrus signs. No pregnancies were found in either of the treatment groups at twelve weeks post-treatment. In contrast, 6/8 (75%) of the control group were pregnant and all had shown follicular development.

Despite the injection site reactions, both PZP and recZP vaccines were shown to be effective as non-hormone based contraceptives with potential for application in fertility management programs in donkeys. Further investigation of these vaccines, using alternative adjuvants providing similar contraceptive efficacy without the associated adverse reactions, may benefit future population control programs for feral donkeys in the Caribbean.

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Efficacy and safety of native and recombinant zona pellucida immunocontraceptive vaccines in donkeys

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ABSTRACT

Feral and semi-feral donkeys are recognised as a problem in some world regions. The main problem associated with uncontrolled donkey populations is habitat degradation and competition for feed resources, especially in arid climes. Controlling population numbers would reduce the impact of donkeys and other species. While removal by various means is effective, it has been shown to stimulate reproductive rate. Probably the most effective and humane solution is reducing reproduction using minimally invasive methods including immunocontraception. This study tested the immunocontraceptive efficacy and safety of zona pellucida (ZP) vaccines, both recombinant (reZP; three treatments) and native porcine (pZP; two treatments) vaccines formulated with Freund's modified complete (primary) and Freund's incomplete (boosters) adjuvants in donkey jennies. Control jennies received adjuvants only (two treatments). Twenty-five non-pregnant jennies were randomly assigned to reZP (n = 9), pZP (n = 8) or control (n = 8) groups. Weekly monitoring of the reproductive tract and ovaries via transrectal palpation and ultrasound and inspection of injection sites was conducted and anti-pZP antibody titers were measured. Five weeks after last treatment, one donkey jack was introduced to each group and rotated every 21 days. By 232 days after last treatment the number pregnant and median days to pregnancy was 2/9 and 214 (reZP group), 1/8 and 196 (pZP group) and 8/8 and 77 (control group). Median time to ovarian shut-down was 77 (9/9) and 56 (7/8) days for reZP and pZP groups, respectively. This was observed in association with a distinct reduction in mean uterine diameter. The antibody response was equally good for both ZP-treated groups. Incorporation of Freund's adjuvants initially produced a high incidence of side effects from local swelling and intermittent lameness followed weeks later by sterile abscesses (reZP, 9/9; pZP, 7/8; control, 3/8). Both ZP vaccines effectively controlled reproduction in jennies, albeit with a high incidence of adjuvant-associated side effects.

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1. Introduction

Feral and semi-feral donkey populations are described as an important emerging global human-animal conflict scenario [1,2]. These perceived conflicts are reported in various countries (Australia, India, Northern Ireland, South Africa, USA, Zimbabwe) and include deleterious environmental impacts such as erosion, damage to crops, competition for resources with livestock or wildlife [3], road and air traffic hazards and as a perceived nuisance in urban areas. Currently, however, there is a paucity of evidence-based reports supporting these perceptions and the available data are often contradictory [4–7]. Methods reportedly employed in attempting to manage these populations to mitigate their impacts include fencing, fertility control, and lethal approaches such as shooting, immobilization followed by lethal injection, and

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mustering or trapping for transport and slaughter at abattoirs. These methods vary in their humaneness, efficacy, cost-efficacy and impact on non-target species [8].

Fertility control has proved effective in certain herbivore populations, including African elephants (*Loxodonta africana*), that experience density-dependent increases in reproductive rates following short-term removal programs [9]. In these populations, reducing reproductive rates through fertility control may be more sustainable in the long-term. Current methods of fertility control include surgical sterilization, hormonal (chemical) methods and immunocontraception. In the latter, injection of formulations containing antigenic epitopes results in the production of circulating antibodies or immune effector cells that interrupt reproductive processes, causing variable periods of infertility in treated animals [10]. One target is the zona pellucida (ZP) capsule, a complex glycoprotein matrix surrounding the mammalian oocyte [11]. Zona pellucida proteins are weak antigens requiring inclusion of strongly immunogenic adjuvants in vaccine formulations in order to elicit an effective immune response. For immunocontraception, native porcine zona ZP (pZP) proteins are typically emulsified with modified Freund's complete adjuvant (MFCA) for primary and Freund's incomplete adjuvant (FIA) for booster immunizations, respectively [12]. These formulations have been used successfully for contraception in more than 90 species [13], most notably feral equids in the USA [14–18] and African elephants in South Africa [19,20]. In donkeys, to our knowledge, a single study reported the successful use of pZP immunocontraception [21]. Recombinant ZP vaccines based on the expression of porcine ZP3 and ZP4 in *Escherichia coli* have also been developed [22]. Their potential advantages over vaccines using native pZP include production efficiency and avoiding contamination with non-ZP proteins.

The aims of the current study were to evaluate the contraceptive efficacy, ovarian effects and safety of pZP and reZP vaccines, both formulated with MFCA and FIA, in female donkeys (jennies). Results of this study will help to inform choice of method for humane and sustainable reproductive management of feral and semi-feral donkey populations.

2. Materials and methods

The study was reviewed and approved by Ross University School of Veterinary Medicine Institutional Animal Care and Use Committee (protocol no. 15.12.032).

2.1. Animals and management

The study was conducted at Ross University School of Veterinary Medicine (RUSVM) on the island of St Kitts in the federation of St Kitts and Nevis. Twenty-five feral jennies were caught on the island of Nevis. On capture, all appeared to be in good body condition and estimated to be in late gestation which was confirmed by transcutaneous transabdominal ultrasound (US). No additional history was available. Jennies were subsequently transported to St Kitts via water ferry. On arrival at RUSVM, all were microchipped and individually identified with numbered collars. Individual ages in y varied from three to 13, estimated according to the appearance of the incisors on dental inspection [23]. Jennies were housed in outdoor grass paddocks with free access to water and freshly-cut New Guinea grass (*Megathyrsus maximus*). They were monitored twice daily for signs of impending parturition and weekly by transcutaneous transabdominal US until they had foaled. Three clinically healthy jacks between the ages of three to six y (estimated by dental inspection) with two apparently normal intrascrotal testes assessed by inspection and palpation, were chosen for this study. Jacks were housed in a separate outdoor grass paddock with similar access to water and feed.

2.2. Study design

Inclusion criteria for the study were good physical condition, non-pregnant status, a normal reproductive tract and active ovaries on clinical examination. After foaling, all 25 jennies met these criteria and were randomly assigned to one of three treatment groups (Fig. 1) stratified according to age. The groups were housed in separate, adjacent outdoor grass paddocks. The study commenced after all eligible jennies had shown at least one complete estrous cycle. Day 0 of the study was defined as the day of last treatment. On Day 35, one jack was introduced into each group of jennies; jacks were rotated between groups every 21 days. The primary endpoint for the study was a pregnancy diagnosed by transrectal palpation and US of the reproductive tract. The secondary endpoint was 'ovarian shutdown', defined as the presence of bilaterally inactive ovaries with only small follicles (<10 mm) and no corpus luteum (CL) visible on transrectal US [24,25]. Jennies remained in the study until diagnosed pregnant or Day 232, the last day of the study.

2.3. Treatments

2.3.1. Preparation of vaccines

All three vaccine formulations were prepared less than 30 min before administration.

Recombinant ZP_3 and ZP_4 proteins, supplied by the Council for Scientific and Industrial Research, South Africa, were expressed in *E. coli* according to Gupta et al. [22] with several modifications. The reZP vaccine used in the current study comprised recombinant ZP_3 and recombinant ZP_4, containing the promiscuous T-cell tetanus and bovine RNase epitopes at the N-terminus, respectively. The recombinant ZP_3 and ZP_4 proteins were analysed via SDS-PAGE gels (Fig. 2) and confirmed by LC-MS peptide mapping. ZP3 with the TT epitope had 93.3% coverage at 95% confidence, while ZP4 with the bRNase epitope had 92.6% coverage at 95% confidence, analysed with ProteinPilot Software (AB Sciex). The resultant reZP product was lyophilized in vials, each containing 2 mg of each protein. Before administration, individual vials were reconstituted with 5 mL sterile water for injection providing a final concentration of 400 μg/mL of each protein. For the primary dose (V1), 0.5 mL reZP (200 μg per protein) was mixed with 0.5 mL MFCA to provide a stable emulsion. For the booster doses (V2 and V3), 0.5 mL recZP was mixed with 0.5 mL FIA to provide a stable emulsion.

Native pZP was prepared according to a standard method [14] and was supplied lyophilized in 1 mg vials by Trumpeter Farms and Veterinary Service (Winters, CA, USA). Each vial was reconstituted with 5 mL sterile water for injection providing a final protein concentration of 200 μg/mL. For the primary dose (V1), 0.5 mL pZP (100 μg) was mixed with 0.5 mL MFCA to provide a stable emulsion. For the booster dose (V2), 0.5 ml pZP (100 μg) was mixed with 0.5 mL FIA to provide a stable emulsion.

An adjuvant control consisted of 0.5 mL sterile saline emulsified with 0.5 mL MFCA for the primary dose (V1) and 0.5 mL sterile saline emulsified with 0.5 mL FIA for the booster dose (V2).

2.3.2. Vaccinations

reZP group (n = 9; mean age 6.1 y; age range 3–13 y): the primary dose (1 mL; V1) was administered by injection into the gluteal muscle mass on Day −70, the first booster (1 mL; V2) was similarly administered into the contralateral gluteal muscle mass on Day −35 and the final booster (1 mL; V3) was administered into the pectoral muscles on Day 0; pZP group (n = 8; mean age 6.5 y; range 3–13 y): the primary dose (1 mL; V1) was injected into the gluteal

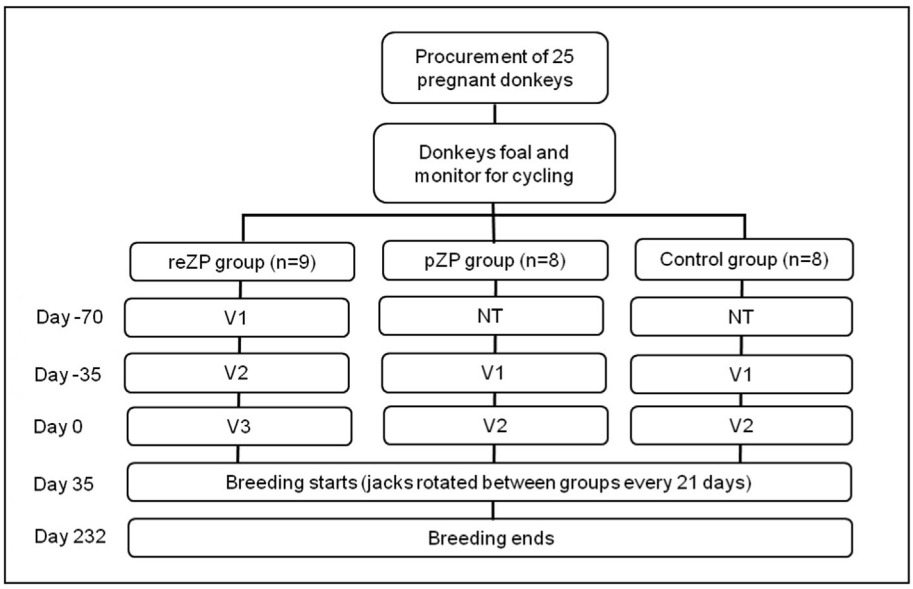


Fig. 1. Overall timeline and treatment protocol for the 25 jennies. NT = no treatment; V1 = primary vaccination; V2 = 1st booster; V3 = 2nd booster.

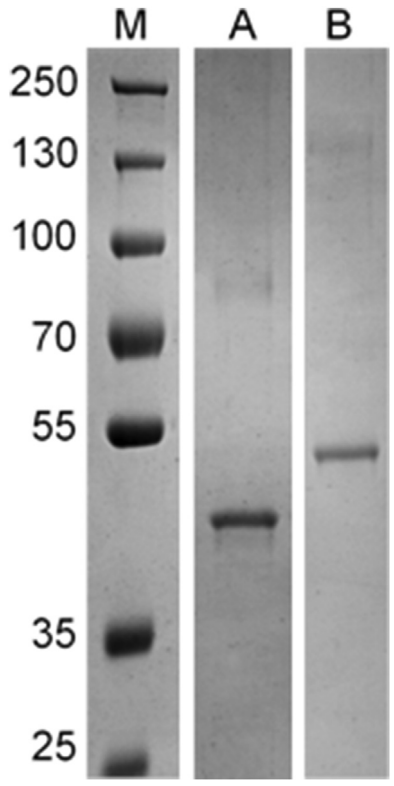


Fig. 2. 10% SDS-PAGE gel of purified (A) ZP3 with the TT epitope and (B) ZP4 with the bRNase epitope. M = PageRuler plus prestained protein ladder (Thermo).

muscle mass on Day −35 and the booster (1 mL; V2), into the contralateral gluteal muscle mass on Day 0; Control group (n = 8; mean age 5.9 y; range 4–10 y): the primary dose (1 mL; V1) was injected into the gluteal muscle mass on Day −35 and the booster (1 mL; V2), into the contralateral gluteal muscle mass on Day 0.

2.4. Observations and sample collection

2.4.1. Transrectal monitoring of the reproductive tract

Clinical examination of the reproductive tract by transrectal palpation and US was performed weekly from Day −105 using a linear 5 mHz probe (Sonosite, Universal). The left and right uterine horn diameters were measured at their base using US. Ovarian findings were recorded as the number of visible follicles (approximate resolution threshold of 5–10 mm) on each ovary and either the presence or absence of a CL. Following introduction of jacks, the jennies were monitored weekly until Day 232 for pregnancy via transrectal US.

2.4.2. Monitoring of injection sites

Jennies were monitored daily for five days after each vaccination for signs of swelling and pain. Thereafter injection sites were monitored weekly until the end of the study.

2.4.3. Serum collection and antibody titer assays

Blood samples from all jennies were collected with plain, evacuated tubes (Terumo™ VENOJECT™ II, Fischer Scientific, Waltham, Massachusetts, USA) by jugular venipuncture at 5-weekly intervals from Day −70 to Day 70 for assessment of anti-pZP antibody titers. Samples were allowed to coagulate at room temperature and serum was separated and stored at −80 °C until assayed. Anti-pZP antibody response was measured by enzyme immunoassay (EIA), using a modification of a method previously described [24]. Positive reference serum consisted of pooled samples collected from the pZP group (n = 8) on Day 35, when maximal antibody responses were anticipated for use as reference standards. Briefly, 96-well plates (MaxiSorp, cat. no. NUN430341) were incubated at 2–8 °C for 16 h with 1 μg purified pZP in 100 μL coating buffer (2.94% NaHCO3, 1.59% Na2CO3, pH 9.6) per well. Plates were washed four times with phosphate buffered saline (PBS) containing 0.05% Tween 20 and then blocked with 0.03% bovine serum albumin in PBS for 16 h at 2–8 °C. Plates were then incubated with serial dilutions of reference standards (1:500–1:64,000) and test samples (1:250–1:32,000) in duplicate at 37 °C for 1 h. Wells containing PBS served as blanks. After washing four times, antibodies were detected by incubating plates with 100μL/well of a

1:10,000 solution (diluted with assay buffer) of a Protein G-horse radish peroxidase conjugate concentrate (1 mg/mL; EMD Millipore Corporation, 28820 Single Oak Drive Temecula, CA 92590 USA) at 37 °C for 1 h. After washing four times, plates were developed with trimethylene blue (SureBlue™, KPL, Gaithersburg, Maryland, USA). The reaction was stopped by adding 50 μL of 2 mol/L H_2SO_4 per well. Absorbance was measured at 450 nm using a microplate photometer (BioTeck ELx800, Winooski, Vermont, USA). Sample antibody response (titer) was expressed as the serum dilution rate which achieved an absorbance 1.

2.5. Data analysis

The proportion of pregnancies and of ovarian shutdowns by Day 232, mean of antibody titers and 95% confidence intervals (95% CI) were computed by treatment group. Comparisons of shutdown and pregnancy proportions were done using a permutation test. Threshold of significance was set at 0.05. Comparisons of antibodies titers were done using Wilcoxon rank-sum test. To take in account multiple comparison, P-value threshold was set up at 0.0056 for these statistical tests (Bonferroni correction). Number of follicles and uterine horn diameter were plotted over time until pregnancy diagnosis or the end of the study (Day 232) using local polynomial regression fitting ('loess') smoothing with 95% CI. Mean of antibodies titers were plotted with an error plot (95% CI). The effect of antibody titers at Day 35 on time to ovarian shutdown was evaluated in the pZP and reZP groups combined using a Cox proportional hazards model. The threshold effect was identified using a penalized spline term. All data analyses were performed in R software [25] using the packages coin, ggplot2 and survival [26–28].

3. Results

3.1. Effects of treatment on pregnancy rate, ovarian function and the uterus

Table 1 summarizes the results for the primary and secondary endpoints for the study. The proportion of pregnant jennies differed across the three treatment groups (P = 0.0004). All in the control group were confirmed pregnant by Day 119. One in the pZP group (Day 196) and two the reZP group (Days 196 and 232) were diagnosed pregnant. The proportion of jennies fulfilling the criteria for ovarian shutdown also differed across the three treatment groups (P < 0.00054). None in the control group, all in the reZP and all but one in the pZP group showed shutdown. The median time (days from Day 0) to shutdown was 56 days for the pZP and 77 days for the reZP group. In the pZP group, the jenny not showing ovarian shutdown was diagnosed pregnant on Day 196. A second jenny in this pZP group had resumed ovarian cyclicity on Day 232. In the reZP group, two jennies resumed ovarian cyclicity after shutdown, by Days 165 and 210, respectively. Both were subsequently diagnosed as pregnant on Days 196 and 232, respectively.

Jennies in both pZP and reZP groups showed a post-treatment reduction in their total ultrasonographically-visible follicle counts compared with those in the control group (Fig. 3). In addition, their mean uterine horn diameter was smaller compared to those in the control group (Fig. 4).

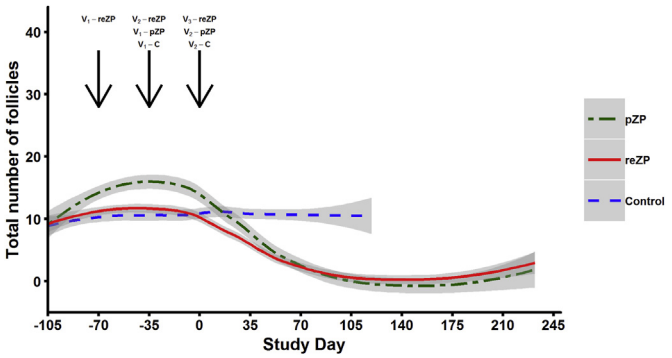


Fig. 3. Total mean follicle counts (≥5–10 mm) of pZP, reZP and control groups from Day −105 (prior to vaccination) to the end of the study (Day 232) after 2 or 3 vaccinations. V_1 = primary vaccination; V_2 = 1st booster vaccination; V_3 = 2nd booster vaccination. Curves were smoothed using loess regression. Grey shading indicates the 95% confidence intervals.

3.2. Injection site reactions

Post-treatment injection site reactions were more frequently observed in ZP-treated jennies than in the controls. Reactions ranged from localized swelling, sometimes accompanied by intermittent lameness during the first five days post vaccination, to abscesses which appeared several weeks later. Subjectively assessed, the swelling and pain exhibited following the primary and first booster treatments was more pronounced in the reZP than the pZP and even more so, the control group. Abscesses were observed in 9/9, 7/8 (87.5%) and 3/8 (37.5%) jennies treated with reZP, pZP and control formulation, respectively. Bacteriological cultures of these abscesses were negative. As a result of the more severe injection site reactions observed after the first booster in the reZP group, a decision was made to substitute FIA with physiological saline for the second booster. As a result, only minor injection site reactions were observed.

3.3. Post treatment antibody titers

The mean antibody titers of each group over time are shown in Fig. 5. Groups pZP and reZP differed significantly from the control group on Days 0, 35 and 70 (P < 0.001 in all cases). There were no significant differences between the mean titers of the pZP and reZP groups on days 0 (P = 0.24), 35 (P = 0.37) and 70 (P = 0.42). Table 2 shows the antibody titers (Day −35 to Day 70) of the jennies that were diagnosed pregnant in the reZP (#10, Day 232; D #23, Day 196) and pZP (#4, Day 196) groups, respectively. It also shows the mean group titers and titers of jennies that were lower (at least on

Table 1
Summary of reproductive outcomes observed in jennies following the final administration (Day 0) of native porcine zona pellucida vaccine (pZP), recombinant zona pellucida vaccine (reZP) or saline control, all formulated with Freund's adjuvants.

Observation	control group (n = 8)	reZP group (n = 9)	pZP group (n = 8)
number (%) pregnant by Day 232	8 (100%)	2 (22.2%)	1 (12.5%)
median time to pregnancy (days)	77 (n = 8)	214 (n = 2)	196 (n = 1)
median time to shutdown (days)	–	77 (n = 9)	56 (n = 7)
Jennies with temporary shutdown - days to resumption of cyclicity (%)	0 (0%)	1 - 165 (11.1%) 1 - 210 (11.1%)	1–232 (12.5%)
Number of jennies with prolonged shutdown	0 (0%)	7 (77.8%)	6 (75%)

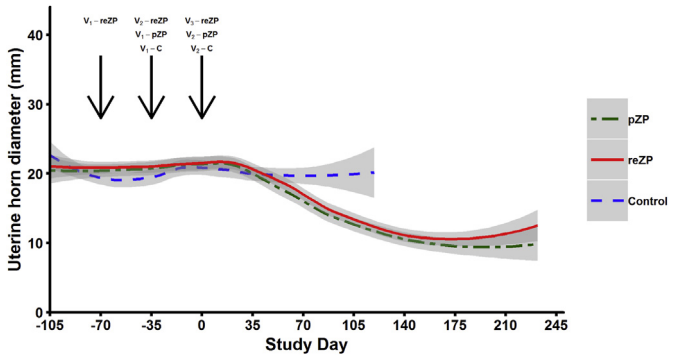


Fig. 4. Mean uterine diameter at the base of the uterine horn in jennies from pZP, reZP and control groups from Day −105 (prior to vaccination) to the end of the study (Day 232) after 2 or 3 vaccinations. V_1 = primary vaccination; V_2 = 1st booster vaccination; V_3 = 2nd booster vaccination. Curves were smoothed using loess regression. Grey shading indicates the 95% confidence intervals.

the last sampling day), than jennies that became pregnant during the observation period. There was a statistically significant negative association between titer at Day 35 and time to shutdown. The "hazard" (instantaneous potential for shutdown) was increased by 13% (95% CI: 2–26%) for every 1000 unit increase in Day 35 titer ($p = 0.02$). This effect varied non-linearly, with a threshold of effect identified at a Day 35 titer of ~17,000 (Fig. 6). This figure excludes the one subject in the pZP group with a very low titer that did not shut down, as the effect of that one subject masked the above threshold effect to a large extent. Donkeys categorized as "high responders" on the basis of this threshold (titers>17,000; n = 10) showed shut down significantly faster than "low responders" (titers<17,000; n = 7), with a median time to shutdown of 59.5 days in high responders and 84 days in low responders (log-rank test p-value = 0.03).

4. Discussion

4.1. Contraceptive efficacy

The most important finding of the study was the contraceptive efficacy of both ZP vaccines. On the last day of the trial (Day 232), 7/9 and 7/8 jennies in the reZP and pZP groups were not pregnant. Compared to the controls, the first conceptions were delayed by more than 100 days. The median time to pregnancy diagnosis from the final treatment was 77 days for the controls, whereas

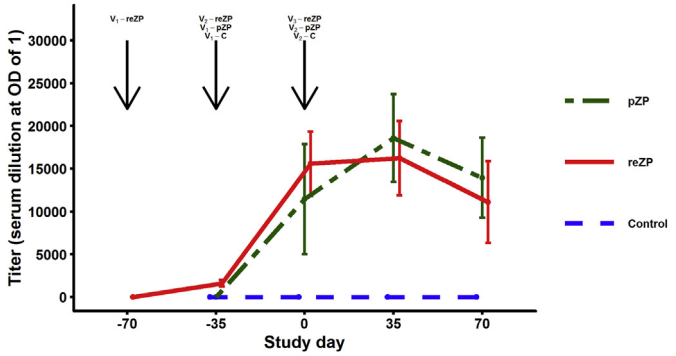


Fig. 5. Mean antibody titers expressed as serum dilution rates of pZP, reZP and control samples which achieved an absorbance of 1 from Day −70 (prior to vaccination) to Day 70 (after 2 or 3 vaccinations). V_1 = primary vaccination; V_2 = 1st booster vaccination; V_3 = 2nd booster vaccination. Vertical bars represent 95% confidence intervals.

Table 2
Antibody titers expressed as serum dilution rates which achieved an absorbance of 1 of pregnant and non-pregnant jennies with low Day 70 titers, and the group means during the study.

Group	ID	Titer			
		Day −35	Day 0	Day 35	Day 70
reZP group	#10 pregnant	2600	8461	19549	9978
	#23 pregnant	1300	13763	12988	6537
	#19 Low not pregnant	1539	8935	7559	4503
	#18 Low not pregnant	1363	18744	8268	4593
	reZP group mean	1604	15577	16229	11103
pZP group	#4 pregnant		7065	7081	9912
	#6 Low not pregnant		1556	13435	7016
	#15 Low not pregnant		18939	19582	7016
	pZP group mean		11437	18574	13941

conceptions in the reZP and pZP groups were recorded on Days 196 and 232, and Day 196, respectively. Given the weekly examination of the jennies, estimated time of conception would have been 2–3 weeks earlier. The contraceptive efficacy was similar to those reported in mares treated using pZP formulated with Freund's adjuvants [14,15,17,29]. In the only other study reporting the immunocontraception of donkeys with pZP in the Virgin Island National Park [21], 16 feral jennies were treated and 11 served as controls. Thirteen jennies were treated by remote delivery of two injections of 65 μg pZP in Freund's complete adjuvant (FCA; primary) and FIA (booster) 3 weeks apart. Another three jennies were treated once with a combination of a 65 μg pZP formulated in a slow-release pellet and 65 μg pZP in FCA. All jennies received another booster in FCA 10–12 months later. The jennies were monitored for pregnancy using a fecal progestogen and total estrogen assays. Of the 16 treated, one was pregnant 12–24 months after the final booster whereas 6/11 control animals were found to be pregnant during the same period. Other than providing definite proof of efficacy, especially during the 12–24 month period, the two studies are difficult to compare. Our jennies were captive whereas theirs were feral. All our females were non-pregnant whereas 4/27 (3 control and 1 treatment) were already pregnant at the commencement of their study. The authors did not report on post-treatment injection site reactions or changes to the reproductive organs.

The current study is novel in that it describes the first successful

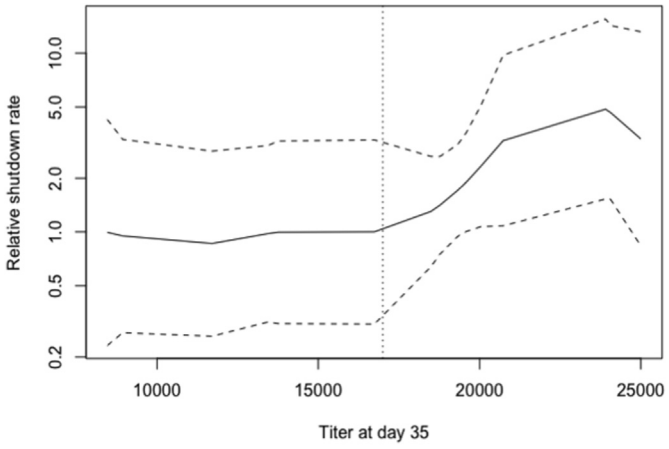


Fig. 6. Effect of antibody titer at Day 35 on time to ovarian shutdown in the pZP and reZP groups combined, excluding one subject with low titers which did not shut down. The solid line shows the shutdown rate relative to the mean antibody titer as the reference. The dashed lines show the 95% confidence intervals. The proposed threshold value of 17,000 is shown by the vertical dotted line.

application of reZP to control fertility in an equid species. This supports reZP as an alternative to native pZP vaccination with its added potential benefits as a non-animal derived product of enhanced production efficacy, antigen protein purity and microbiological safety.

4.2. Ovarian and uterine effects

The previously-presumed mechanism of pZP contraception was largely ascribed to blocking of sperm receptor sites by antibodies in response to ZP_3 protein. Some reports did suggest a limited disturbance in ovarian function, including ovulation failure [15,17] and decreased total urine estrogen concentrations [16]. These consequences were mainly associated with repeated (annual) administration of pZP. It is possible, however, that studies may have overlooked ovarian shut-down because animals were first monitored following a post-treatment delay of ≥3–4 months by which time the presumed ovarian status may have reversed [14,15,17]. Studies in feral mares either did not monitor ovarian function [14,15,29] or did so only indirectly via the measurement of fecal or urinary steroids, and then only for short periods [30].

Recent reports have however associated administration of pZP vaccines in mares with temporary ovarian suppression. Bechert et al. [31] compared two formulations of a single-injection pZP vaccine in MFCA in mares and reported that 93% of mares were non-cyclic 3–4 months after injection. This was associated with basal progesterone concentrations and significantly smaller ovaries with multiple small follicles and no CLs on trans-rectal US. Similarly, Joonè et al. [24] reported that 86% of mares receiving a pZP vaccination protocol similar to this study experienced a period of prolonged anestrus five to 12 weeks post vaccination. Anestrus was characterized by basal serum estradiol and progesterone concentrations and scanty, small follicles on transrectal US. These mares all resumed ovarian activity and reproductive cyclicity by 10 months post-booster injection. On further investigation, it was found that T-helper and cytotoxic cells were associated with the ovarian changes [32]. The current study reports similar findings of ovarian shut-down, thus confirming the findings in the two horse studies. By day 77, all reZP-treated and 88% of the pZP-treated jennies had ceased cyclic ovarian activity. Two reZP and one pZP-treated jenny had resumed ovarian activity by Day 165, 210 and 232, respectively. The pZP jenny diagnosed pregnant on Day 196 had cycled throughout the study.

Although the precise mechanism leading to suppression of ovarian function is currently undefined, these observations suggest that this phenomenon may play an important role in ZP immunocontraception in equids and other species [32]. It would seem that ovarian suppression following ZP immunocontraception is not confined to vaccine formulations using FCMA and FIA, having also been observed in varying levels in mares treated with reZP and pZP formulated with Pet Gel A and Poly(I:C) [33].

The reduction in uterine horn diameter is an interesting hitherto unreported observation in pZP-immunocontracepted mares. These changes were associated with reductions in ovarian size, follicular and luteal activity. We suggest that this change was associated with decreased stimulation of the endometrium by ovarian steroids.

4.3. Anti-pZP antibody titers and infertility

Although good anti-pZP antibody titers are achieved in response to pZP immunocontraception in most species, they vary considerably [35,36]. In general, antibody titers within a species are closely related to infertility [14], but between individuals there is considerable variation. Also, comparison between laboratories is difficult unless universal standards are used. Roelle et al. [37] reported that mean anti-pZP titers of non-pregnant mares treated with a slow release pZP formulation (SpayVac) were 33.7–91.9% higher than those of pregnant mares. However, several mares that developed high maximum titers became pregnant whereas one mare with a maximum titer of only 60.11% failed to conceive. A study by Willis et al. [38] may shed some light on these inconsistencies. Three mares were immunized with pZP (primary and booster after 4.5 weeks). The polyclonal antibodies of each mare were characterized by immunoblots against pZP and equine ZP (eZP). All three mares recognised pZP bands of approximately 90 and 55 kDa. For eZP, one mare reacted to the 75–90 and 40–50 kDa bands while the two others mainly reacted to the 90–106 kDa band, but also the 45–55 kDa band. The differing response may explain why anti-pZP antibody titers are not always accurate in predicting contraceptive efficacy in mares. Speculatively, the same may be true for other species including donkeys. The two pregnant jennies in the reZP group had lower final antibody titers than the group mean. However, there were two other jennies with lower titers on Day 70 that did not fall pregnant within the study period. Similarly, the jenny that was pregnant in the pZP group had a higher titer than two non-pregnant jennies on Day 70. The titers of all three were lower than the group mean on Day 70 (Table 2). From this it would seem that anti-pZP titers can be used as a guideline to predict infertility, however, there is considerable as yet unexplained variation between individuals. On the other hand, antibody titers were found to be significant predictors of ovarian shutdown in mares treated with either pZP or reZP [39]. Similarly, an exploratory analysis in the present study, revealed an apparent distinct threshold of effect at a Day 35 titer of ~17000.

4.4. Injection site reactions

The formulation of reZP and pZP with MFCA for primary and FIA for booster immunizations produced excellent antibody titer responses in this study. The inclusion of Freund's adjuvants in the vaccine formulations was unfortunately also associated with undesirable injection site reactions. The localized swellings and lameness were a common finding in all groups, the tendency increasing after the second treatment. Subjectively assessed, swelling and pain was more pronounced in the reZP group. Subsequently, sterile abscesses developed in many controls, all reZP and most of the pZP jennies. Similar injection site reactions were reported in mares treated with two single-injection pZP formulations [31] and with pZP and reZP formulated with FCMA and FIA [24]. Similarly, these were more common with reZP than pZP treatment [24]. Speculatively, this difference may be partly attributable to the promiscuous T-cell epitopes for tetanus toxoid and bovine RNase in reZP in combination with the Freund's adjuvants. Given the more severe reactions to V1 and V2 in the reZP-group, FIA was substituted with saline for V3. The minor reactions associated with the incorporation of saline may also explain the smaller rise in mean antibody titer following V3.

5. Conclusions

Both immunocontraceptive vaccines successfully reduced pregnancy rates in treated jennies. Significantly, this is the first study reporting the successful application of reZP for fertility control in any equid species. Both vaccines induced ovarian shut-down which may be partly responsible for their observed contraceptive effect. In our opinion the injection site reactions associated with inclusion of Freund's adjuvants with both reZP and pZP are an untenable feature of these formulations. Alternative similarly immunogenic adjuvant formulations without the associated severe local reactions are an essential prerequisite for future application.

The recently-reported use of the commercially-available adjuvants Pet Gel A and Poly (I:C) with pZP and reZP in mares producing antibody titer responses equivalent to those observed with Freund's adjuvants [33,34] may be a suitable alternative for donkey jennies.

Author contribution

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Original Draft and editing, Project administration, Funding Acquisition

Erik Peterson: Methodology, Investigation

Martin Schulman: Conceptualization, Methodology, Writing - Original Draft and Editing,

Supervision

Robyn Roth: Resources

Michael Crampton: Resources

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Silvia Marchi: Investigation

Darryn Knobel: Conceptualization, Methodology, Formal analysis, Editing, Supervision,

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Henk Bertschinger: Conceptualization, Methodology, Resources, Writing – Original Draft

and Editing, Supervision

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CONSERVATION

A Critical Crossroad for BLM's Wild Horse Program

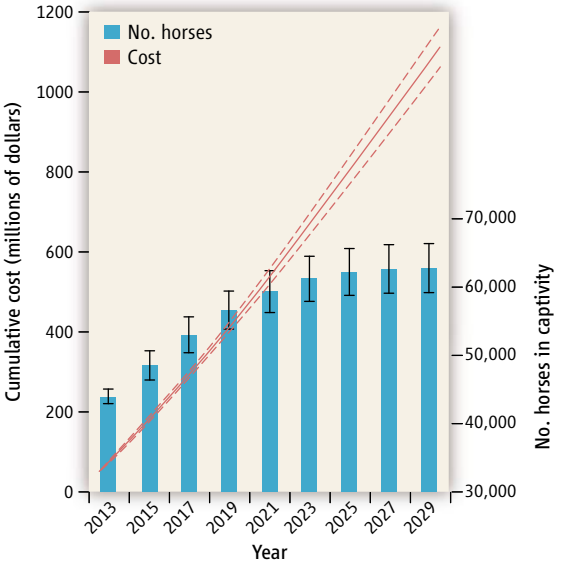
Captive wild horses will cost the United States over $1 billion by 2030 unless management approaches change

Robert A. Garrott,[1]* and Madan K. Oli[2]

Although horses evolved in North America, they went extinct 10 to 12 thousand years before the present. Spanish conquistadors returned domestic horses to the continent in the mid-1500s, and since then, domestic horses have escaped captivity and also were purposefully released onto western rangelands where they thrive today. Most free-roaming horse populations are managed primarily by the Bureau of Land Management (BLM) of the U.S. Department of the Interior (*1*).

In 1971, the Wild Free-Roaming Horses and Burros Act (*2*) was passed, which establishes public ownership of free-roaming horses and instructs federal agencies to "protect and manage" horses as "an integral part of the natural system of the public lands." The act mandates that BLM and other federal agencies monitor horse numbers, determine appropriate population levels, and remove excess horses from public lands "to preserve and maintain a thriving natural ecological balance." However, at present, tens of thousands of horses are not living the life of "wild mustangs" as Congress intended in passing the Wild and Free-Roaming Horses and Burros Act; instead, they have reverted back to the status of captive, domestic livestock, and the cost of the captive horse program is increasingly unsustainable.

The BLM currently reports ~33,000 free-roaming horses in the western United States. The WHB Program has 179 designated Herd Management Areas, with population objectives for each, and an aggregate maximum goal of 23,622 horses (*3*). Since its inception, the WHB Program has been embroiled in controversy over appropriate population goals, management activities, and the accuracy of its estimates of population size and growth rates (*4*–*7*). The BLM monitors numbers using aerial surveys and captures and removes horses from rangelands when horse numbers exceed target population size. As of 2012, BLM has removed >195,000 horses from public lands under this program (*3*). The act requires BLM to place removed horses into "private maintenance and care for which … an adoption demand exists," with unadoptable, old, and excess animals, beyond the adoption demand, either sold or "destroyed in the most humane manner possible" (*2*). The number of horses BLM removes from public lands routinely exceeds adoption demand (*8*). However, because of pressure from horse advocates, administrative directives and congressional appropriation bills prohibit killing healthy horses (*9*); therefore, BLM is left with large numbers of captive horses that must be maintained indefinitely.



Projections of U.S. captive wild horses and costs (with 95% confidence intervals). (see SM for more details).

These policies have led to a complex and costly system for maintaining captive horses. Horses removed from public lands are processed in short-term holding facilities similar to cattle feed lots and held for several months or more. Many mature females are pregnant when captured and foal in the facilities, which adds to the captive population. Most adult males are castrated. Horses can be adopted from the short-term holding facilities, and some are removed through deaths, sales, or release back onto public lands. Excess animals are eventually transferred to private pastures, primarily in the Midwest, that are contracted as long-term holding facilities. Although small numbers are sold, horses typically remain in these facilities until they die of natural causes. Currently, the ~45,000 horses in captivity exceeded the estimated 33,000 free-ranging horses on public rangelands (*3*).

In a recently released National Research Council (NRC) report (*7*), a committee of 14 scientists concluded that, left unmanaged, horse numbers on public lands would triple every 6 to 8 years until food and water became limited. Under these conditions, horses would be in poor health, reproduction would be suppressed, and deaths from starvation and dehydration would become common, with mass mortality events possible during periods of drought. High horse populations and limited resources would also degrade rangelands, which would affect all native species and public uses of these lands. Thus, although the NRC committee concluded that BLM must actively manage wild horse populations, it asserts that continuing "business as usual" will impede effective management of wild horses on public lands and recommends management changes to put the WHB Program on a more sustainable course (*7*).

Although the NRC report focused on scientific issues, a major impetus for changing the WHB Program is the escalating costs of maintaining increasing numbers of horses in captivity. The WHB Program budget grew from $19.8 million in 2000 (*10*) to $74.9 million in 2012 (*3*). More than 60% of the 2012 budget was used to maintain captive horses, 11% expended to capture and remove animals from public lands, and 10% for adoption programs (*3*). To inform the policy debate, we extend the implications of the NRC report to provide a projection of the captive horse population and associated costs if current policies and management approaches continue.

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The Consequences of Business as Usual

We used records of the sex and age of 165,459 horses removed from public lands and placed in captive facilities between 1990 and 2011 to develop demographic projections and associated costs (see the chart) (Fig. 1) [see supplementary materials (SM) for details]. We made two basic assumptions. First, we projected the life span of 33,946 unadoptable horses currently in long-term holding facilities. Under this scenario, BLM will accrue 366,846 horse-years of maintenance costs over a 30-year period before all animals currently in captivity die. Given that $45.9 million was budgeted to maintain 42,835 horses in captivity during 2011 (*10*), annual per-horse maintenance costs would be ~$1074. Thus, the 30-year cost of maintaining horses currently in long-term holding facilities would be ~$394 million ($449 million with 2% annual inflation).

Second, we assumed the current management program would continue, with a mean of 9412 horses in short-term holding facilities and 3803 horses transferred annually to long-term holding facilities. Expenses would be ~$1.1 billion between 2013 and 2030, and annual costs thereafter would be $67 million. Although some citizens may support such expenditures, these projections reinforce assertions (*7*, *8*) that BLM's current approach to managing horse numbers is not sustainable.

A Way Forward

Over 30 years of research on contraceptive agents for feral horses have resulted in the development of effective vaccines that prevent pregnancy in both captive and free-ranging mares for 1 to 3 years (*7*). Experimental application of these vaccines has contributed to the control of a small horse population on an Atlantic Coast barrier island, and limited experiments on western horse herds have also produced encouraging results. The Environmental Protection Agency recently sanctioned two contraceptive vaccines, which enables their routine use for horse population management on western rangelands (*7*). The average life-expectancy of horses entering long-term holding facilities is about 15 years (see SM). Therefore, every horse that does not enter long-term holding facilities would save, because of contraceptive application, $16,110 in maintenance cost (or savings of $1 million for every 62 horses), not considering the modest cost of contraceptive application.

Uncertainties exist when attempting to deliver vaccines to large numbers of horses. Although concerns have been raised regarding behavioral, physiological, and demographic consequences of contraceptive vaccines, none of these concerns preclude their use for managing rapidly increasing populations (*7*). The level of reduction in population growth that can be realistically attained for western horse herds through application of contraceptive vaccines remains unclear. Large-scale management experiments, perhaps within an adaptive management framework, would help address some of this uncertainty (*11*).

We estimate that the typical 15 to 20% annual population increase of western horse herds (*7*) could be halved by means of contraceptive vaccines. If a gather-and-removal effort reduced the western horse population to the BLM's goal of 23,622 horses and aggressive contraception treatment was initiated, then BLM need only remove 2000 to 3000 horses annually to maintain its goal. The number of horses annually removed would then more closely match adoption demand and that would minimize or eliminate the need for long-term maintenance of unadopted animals.

Although broad-scale use of contraceptives can help reduce the number of horses that BLM must remove to control populations, it is not a panacea. Current contraceptive vaccines are most effective when hand-injected, and remote delivery of vaccines via dart is impractical for most free-ranging horse populations (*7*). Accordingly, vaccine delivery will require continuing, and perhaps increasing, the frequency of horse captures. It may also take a decade of experiment and refinement of treatment regimens and contraceptive methods to scale-up management to include the entire population of horses on western rangelands. Furthermore, most populations already exceed management objectives, and population modeling efforts indicate that current contraceptives can only reduce, not stabilize or reverse, population growth. Thus, even with the changes suggested by the NRC report, BLM must continue removing horses from public lands for the foreseeable future.

It is up to society to decide how they want their government to manage wild horses, how many wild horses should be accommodated on public lands, what should be done with excess horses, and how much they are willing to spend. The debate has gone on for many decades; however, we are at a critical juncture. BLM recently announced that because of funding constraints and lack of additional capacity for maintaining captive animals, it will substantially reduce scheduled removals of horses from public lands this year. This decision has been made despite worsening drought conditions that are causing severe shortages of forage and water for horses, wildlife, and livestock (*12*). Terminating or reducing active management will lead to a rapid increase in the number of horses, further exacerbating resource limitations.

The current situation in Australia provides a sobering view of what might be in store for western rangelands in the United States if the current management dilemma is not resolved. The wild horse population in Australia is estimated to exceed 400,000 animals (*13*), and severe drought conditions in central Australia have reportedly forced government agencies to propose shooting 10,000 horses from helicopters to reduce animal suffering and environmental degradation (*14*). One can only hope that there is the political will to change current policy and place BLM's WHB Program on a more sustainable trajectory.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/341/6148/PAGE/DC1

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BIOLOGY OF REPRODUCTION 35, 347–352 (1986)

Active Immunization of Intact Mares against Gonadotropin-Releasing Hormone: Differential Effects on Secretion of Luteinizing Hormone and Follicle-Stimulating Hormone

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ABSTRACT

Five lighthorse mares were actively immunized against gonadotropin releasing hormone (GnRH) to determine the relative importance of this hypothalamic hormone in the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Five mares immunized against the conjugation protein served as controls. Mares were initially immunized in November and received secondary immunizations 4 wk later, and then at 6-wk intervals until ovariectomy in June. All mares immunized against GnRH exhibited an increase ($p<0.01$) in the binding of tritiated GnRH by plasma, an indication that antibodies against this hormone had been elicited. Concentrations of LH, FSH and progesterone in weekly blood samples were lower ($p<0.05$) in GnRH-immunized mares than in controls after approximately 4 mo of immunization. However, the LH concentrations were affected to a greater degree than were FSH concentrations. All five control mares exhibited normal cycles of estrus and diestrus in spring, whereas no GnRH-immunized mare exhibited cyclic displays of estrus up to ovariectomy. All mares were injected intravenously with a GnRH analog (which cross-reacted <0.1% with the anti-GnRH antibodies) in May, after all control mares had displayed normal estrous cycles, to characterize the response of LH and FSH in these mares; two days later, the mares were injected with GnRH. The LH response to the analog, which was assessed by net area under the curve, was lower ($p<0.01$) by approximately 99% in mares immunized against GnRH than in control mares. In contrast, the FSH response to the analog was similar for both groups. The FSH response to GnRH in mares immunized against GnRH was 85% lower than that in control mares ($p<0.05$), an indication that the antibodies against GnRH did prevent much of the injected GnRH from reaching the pituitary. At ovariectomy, combined ovarian weights of GnRH-immunized mares were 57% lower than those of control mares ($p<0.01$); numbers of follicles and corpora lutea were also lower ($p<0.01$) than those of controls. It was concluded that LH and to some degree FSH secretion, and probably LH production, in the mare are dependent upon the bioavailability of GnRH. However, the lack of effect of GnRH-immunization on FSH response to the analog indicates that FSH stores in the pituitary (and perhaps FSH production) are relatively independent of GnRH bioavailability.

INTRODUCTION

In horses, concentrations of luteinizing hormone (LH) in the blood appear to be tightly coupled in some physiologic states to concentrations of follicle-stimulating hormone (FSH). For example, gonadectomy results in increased concentrations of both gonadotropins in blood (Ginther, 1979; Thompson et al., 1979); administration of gonadotropin-releasing hormone (GnRH) results in a rapid increase in concentrations of both hormones (Ginther, 1979; Thompson et al., 1979, 1983d); and spontaneous, simultaneous peaks in concentrations of both hormones have been reported for both mares and stallions (Thompson et al., 1983a, 1986).

Under other conditions, usually involving gonadal steroids, concentrations of LH and FSH in blood appear to be independent. For example, LH concentrations are stimulated by administration of estrogen to gonadectomized horses whereas FSH concentrations are suppressed (Ginther, 1979; Thompson et al., 1979, 1983d; Garza et al., 1985). Moreover,

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FSH production is increased in ovariectomized mares treated with testosterone propionate whereas LH production is not affected (Reville-Moroz et al., 1984).

Another condition under which LH and FSH concentrations in blood differ is seasonal anestrus. Hart et al. (1984) reported that the amount of LH in pituitaries from anestrous mares in winter was only 15% of that in pituitaries of mares in the breeding season. In contrast, pituitary content of FSH did not differ due to season. These results agree well with secretion rates of LH and FSH after administration of GnRH to anestrous mares (approximately 94 times more FSH than LH; Thompson et al., 1986). Hart et al. (1984) suggested that the reduced LH content in the pituitaries of mares in the nonbreeding season was due to the lowered hypothalamic content (and presumably secretion) of GnRH that they found in those same mares.

Given that pituitary content and secretion rates of FSH differ markedly from those of LH during winter, we hypothesized that there is a component of FSH production and/or secretion in the horse that is relatively independent of GnRH's reaching the pituitary. Thus, the present experiment was designed to determine the long-term effects of active immunization of intact mares against GnRH on concentrations of LH and FSH in blood and on their relative responses to exogenous secretagogues.

MATERIALS AND METHODS

Ten lighthorse mares (≥2.5-yr-old) that had exhibited normal estrous cycles during the previous breeding season were used. All mares were kept on pasture and fed grass hay as needed to maintain good body condition throughout the experiment. On November 1, five randomly selected mares were immunized with 4.0 mg of a conjugate prepared from GnRH and bovine serum albumin (BSA) as described by Fraser et al. (1974). The remaining five mares were immunized with 4.0 mg of BSA (controls). The primary immunizations were prepared in Freund's complete adjuvant. Immunizations consisted of multiple intramuscular injections in the neck and/or hip regions of each mare. Secondary immunizations (2.0 mg antigen), similarly in Freund's incomplete adjuvant, were given 4 wk after the primary immunizations, and then every 6 wk thereafter until ovariectomy. Samples (10-ml) of jugular blood were collected from each mare immediately before the primary immunization and then weekly throughout the experiment. The ovaries of all mares were evaluated by rectal palpation once a month for size (length, width and depth) during March, April and May.

Beginning February 1, all mares were checked daily for estrus by exposure to a stallion. After all control mares had exhibited at least two normal estrous cycles in the spring (a normal estrous cycle was defined as at least 3 consecutive days of estrus followed by at least 8 consecutive days of diestrus), each control mare was randomly paired with one GnRH-immunized mare for the timing of all subsequent events. This was because no GnRH-immunized mare exhibited normal estrous cycles during the late winter and spring. Thus, for the following procedures (GnRH-analog administration, GnRH administration, and ovariectomy), one control mare and one GnRH-immunized mare were treated at the same time based on the stage of the control mare's estrous cycle.

On Day 8 or 9 of diestrus in May, one jugular vein of each mare was fitted with a 14-gauge catheter for frequent blood sampling. Three 10-ml samples of blood were collected from each mare at 15-min intervals. Each mare was then administered a GnRH analog (des-Gly^{10}-(im-Bzl-D-His^{6})-LHRH; Sigma Chem. Co., St. Louis, MO) through the jugular catheter at 40 ng/kg of body weight. Blood samples were collected at 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min after the injection of analog. The GnRH analog was chosen because it was found in preliminary trials to cross-react <0.1% with the antibodies in the plasma of these mares when tested with tritiated GnRH. The dosage of GnRH analog was also determined in a preliminary trial (with different mares) and was equivalent to 1.0 μg/kg of body weight of GnRH with regard to LH and FSH response (area under the curve).

Forty-eight h after the injection of GnRH analog, the above procedure was repeated for each mare except that GnRH (Sigma) was injected. The dose of GnRH was 1.0 μg/kg of body weight.

On Day 7 or 8 of the subsequent diestrus (in June), each pair of mares was bilaterally ovariectomized via flank incision under local anesthesia. Each ovary was trimmed of connective tissue, weighed, and serially sectioned (approximately 6-mm sections) for the enumeration of follicles and corpora lutea.

For all blood samples, heparinized plasma was harvested via centriguation and stored at −15°C. Concentrations of LH, FSH and progesterone were

measured by radioimmunoassay as described previously (Thompson et al., 1983a,b,c). The binding of tritiated GnRH to diluted plasma was measured and the cross-reactivities of various GnRH analogs (Sigma) were determined as described previously for estradiol (Thompson and Honey, 1984).

Data for measurements taken over time were analyzed by analysis of variance that accounted for the repetitive nature of the sampling (split-plot design; Gill and Hafs, 1971). Differences between groups for each time period were assessed for significance by the lsd-test. Net areas under the LH and FSH response curves for GnRH and analog injections were calculated as described by Thompson and Nett (1984). Data for nonrepetitive variables were analyzed by one-way analysis of variance (Steel and Torrie, 1960).

RESULTS

The binding of tritiated GnRH by 10 μl of plasma from control and GnRH-immunized mares is presented in Figure 1. After the third injection of antigen in January, GnRH binding increased ($p<0.01$) in plasma of GnRH-immunized mares relative to control mares. There was an increase in binding after each immunization thereafter such that binding was gradually increasing up through June 6 when blood sampling ceased.

The cross-reactivities of several commercially available GnRH analogs were as follows (%): (Gly-OH^{10})-LHRH 12.9 ± 7.8; des-Gly^{10},(D-Leu^{6})-LHRH 0.28 ± 0.24; des-Gly^{10},(D-Trp^{6})-LHRH 0.31 ± 0.23; des-Gly^{10},(im-Bzl-D-His^{6})-LHRH 0.097 ± 0.054; and (Ac-D-pCl-Phe^{1,2}, D-Trp^{3}, D-Arg^{6}, D-Ala^{10}) - LHRH 0.078 ± 0.046. Because up to 5 μg of most of these analogs did not inhibit the binding of tritiated GnRH by 50%, the above cross-reactivities were calculated at the greatest percentage of inhibition obtained with a given analog. The amount of unlabeled GnRH that resulted in 50% inhibition averaged 3.92 ± 0.92 ng.

Concentrations of progesterone, LH and FSH were all suppressed ($p<0.05$) in GnRH-immunized mares relative to control mares during the latter periods of the experiment (Fig. 1). Concentrations of all three hormones in control mares varied due to the stage of the estrous cycle as evidenced by the large fluctuations in values in Figure 1. In contrast, hormonal concentrations in GnRH-immunized mares became relatively constant after approximately 15 wk of treatment.

From February 1 until ovariectomy in June, no GnRH-immunized mare exhibited cyclic displays of estrus (Table 1). In fact, during that time, only one GnRH-immunized mare exhibited estrus, and that was on two separate occasions (March 21 and May 16) for one day only. This mare did not exhibit any rise in LH or progesterone concentrations in association with these two displays of estrus. All control mares exhibited normal estrous cycles (Table 1) with an average duration of estrus of 6.4 ± 0.7 days and an average duration of diestrus of 17.3 ± 0.7 days.

During March, ovarian volume, as assessed by rectal palpation, did not differ ($p>0.05$) between the two groups of mares (Table 1). In April and May, ovarian volumes of GnRH-immunized mares averaged only 18 and 8%, respectively, of control values ($p<0.05$). At ovariectomy, ovarian weights, number of follicles >10 mm in diameter and number of corpora lutea were all lower ($p<0.01$) in GnRH-immunized mares than in control mares (Table 1).

When mares were administered GnRH analog in May, the mean concentrations of FSH before injection were lower ($p<0.05$) in GnRH-immunized mares than in control mares (Fig. 2). However, the FSH response to GnRH analog, as assessed by the change in hormonal concentrations and by the net area under the curve, was similar for both groups. In contrast, when

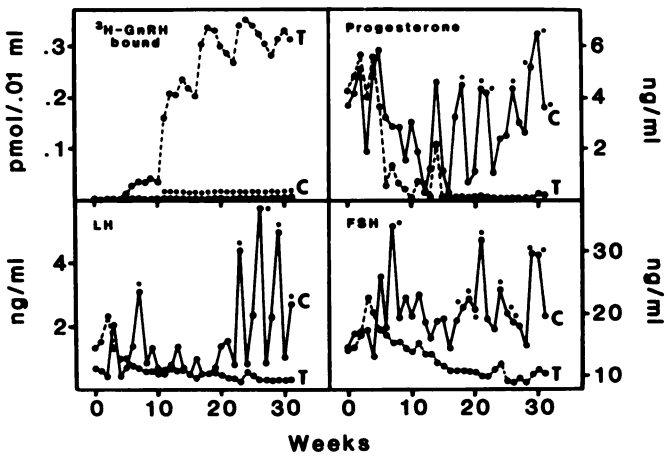


FIG. 1. Binding of tritiated GnRH and concentrations of progesterone, LH, and FSH in plasma of control mares (C; *solid lines*) and GnRH-immunized mares (T; *dasbed lines*). *Asterisks* indicate differences ($p<0.05$) between groups for the indicated period. The primary immunizations (Week 0) were given on November 1, and the final samples (Week 31) were drawn on June 6. Pooled SEM from the analyses of variance were 0.016 pmol and 1.26, 1.16 and 3.55 ng/ml for GnRH-binding and progesterone, LH, and FSH concentrations, respectively.

TABLE 1. Estrous and ovarian characteristics of control and GnRH-immunized mares before and during the breeding season.

Characteristic	Group	
	Control	GnRH-immunized
Estrous cycles/mare[a]	3.6 ± 0.8[b]	0**
Ovarian volume (cm^3)[c]		
March	117 ± 38	64 ± 14
April	189 ± 47	35 ± 5*
May	640 ± 262	54 ± 7*
Ovarian weight (g)[d]	140 ± 23	60 ± 4**
Follicles >10 mm/mare[d]	11.6 ± 3.1	0**
Follicles ≤10 mm/mare[d]	10.6 ± 2.5	9.2 ± 2.3
Corpora lutea/mare[d]	1.4 ± 0.2	0**

[a]From February 1 to ovariectomy in June.

[b]Mean ± SEM.

[c]Sum of both ovaries estimated via rectal palpation (length × width × depth).

[d]At ovariectomy in June.

*$p<0.05$.

**$p<0.01$.

GnRH itself was administered (Fig. 2), the FSH response was reduced ($p<0.05$) in GnRH-immunized mares compared with control mares.

The LH response to GnRH analog (Fig. 3) was essentially nonexistent in GnRH-immunized mares relative to control mares ($p<0.01$). The same lack of response was observed for LH after administration of GnRH. For both LH and FSH, the shape of the response curves differed for the analog and GnRH injections, with the analog resulting in a sustained elevation in concentrations through 6 h after injection.

DISCUSSION

Active immunization of these mares against a GnRH-BSA conjugate resulted in the production of anti-GnRH antibodies, as has been reported for other species (Clarke et al., 1978; Fraser and Baker, 1978; Chappel et al., 1980; Shettigara et al., 1981). Moreover, repetitive injection of antigen at 6-wk intervals resulted in high GnRH binding in plasma of GnRH-immunized mares such that the long-term effects of immunization could be determined. Although the specificity of the anti-GnRH antibodies could not be exhaustively assessed (and was not attempted), it appears from the analog data that minor deviations from the GnRH structure greatly reduced the cross-reactivity of an analog when it was in competition with GnRH. If this apparent specificity was also present in vivo, then we would conclude that only GnRH or closely related compounds in plasma were

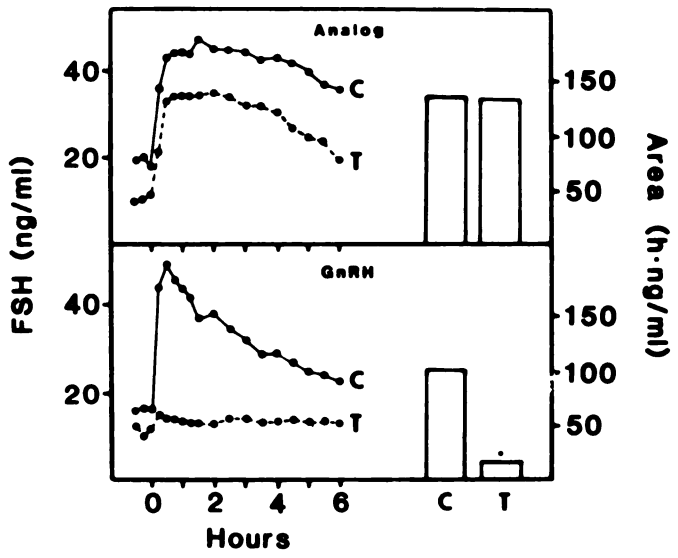


FIG. 2. Concentrations of FSH in plasma of control mares (C; *solid lines*) and GnRH-immunized mares (T; *dashed lines*) before and after an injection of GnRH analog (*top panel*) and GnRH itself (*bottom panel*). *Bar graphs* indicate net areas under the response curves. GnRH injections were given 48 h after analog injections. The *asterisk* indicates a difference ($p<0.05$) between groups. Pooled SEM from the analyses of variance were 3.9 ng/ml for FSH concentrations and 20 units for areas.

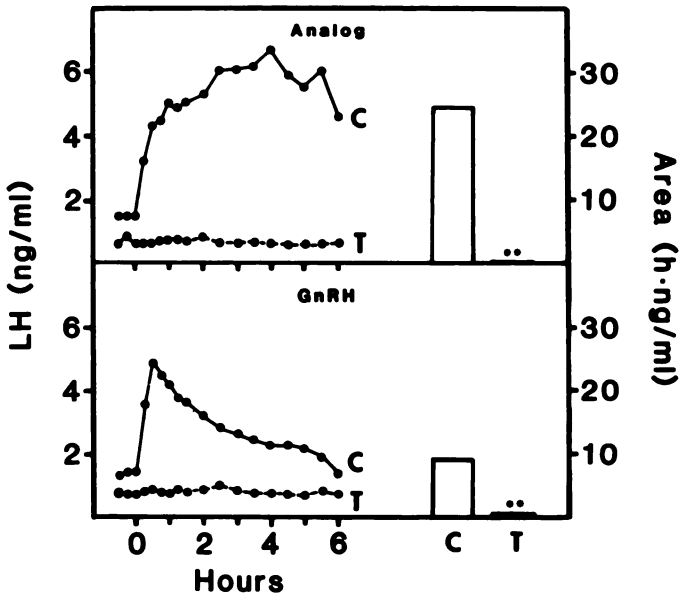


FIG. 3. Concentrations of LH in plasma of control mares (C; *solid lines*) and GnRH-immunized mares (T; *dashed lines*) before and after an injection of GnRH analog (*top panel*) and GnRH itself (*bottom panel*). *Bar graphs* indicate net areas under the response curves. GnRH injections were given 48 h after analog injections. *Asterisks* indicate differences ($p<0.01$) between groups. Pooled SEM from the analyses of variance were 0.64 ng/ml for LH concentrations and 4.5 units for areas.

affected by the antibodies generated against the GnRH moiety of the conjugate.

Because horses are seasonal breeders, the mares in the present experiment were first immunized in the autumn so that they received several secondary immunizations before the onset of the next breeding season. Our goal was to study the long-term effects of immunization on characteristics of LH and FSH secretion, thereby minimizing the importance of pituitary stores and/or turnover rates that could mask any real effects of treatment in the short term. The failure of GnRH-immunized mares to return to normal estrous cycling in the spring is similar to the cessation of normal reproductive activity observed in other species immunized against GnRH (Clarke et al., 1978; Fraser and Baker, 1978; Shettigara et al., 1981). Thus, it appears that the generation of anti-GnRH antibodies in these mares resulted in a biological inactivation of endogenous GnRH. This concept is also supported by the data in Figure 2; i.e, immunization against GnRH reduced the GnRH-induced release of FSH by 85% but did not affect analog-induced FSH release.

Concentrations of both LH and FSH in plasma were suppressed in the long term in GnRH-immunized mares relative to control mares. However, it must be noted that LH concentrations in GnRH-immunized mares were suppressed to undetectable levels (0.3 ng/ml in our assay) whereas FSH concentrations were still easily detectable after 32 wk (approximately 10 ng/ml as compared with the limit of detection of 3.9 ng/ml in the FSH assay). Thus, we conclude that normal secretion of these two gonadotropins is dependent upon GnRH's reaching the pituitary; however, there appears to be a component of FSH secretion that is relatively independent of GnRH. This is in agreement with previous observations in seasonally anestrous mares in which concentrations of plasma LH are generally undetectable whereas concentrations of FSH are not nearly as suppressed (Thompson et al., 1986). A similar dichotomy was reported for LH and FSH secretion in geldings after suppression with testosterone or estradiol (Thompson et al., 1979).

The effect of GnRH-immunization on apparent amount of hormone released in response to injection of GnRH analog differed markedly for LH and FSH. Essentially no LH was secreted by GnRH-immunized mares. In previous studies, the apparent amount of LH secreted in response to exogenous GnRH was closely coupled with the mean amount of LH in the pituitary (Thompson et al., 1979; 1986; Hart et al., 1984). If this relationship had persisted in the present experiment, the amount of LH in the pituitaries of GnRH-immunized mares would have been proportionally lower, which would be indicative of a long-term suppression of LH production. Such a suppression would be similar to that reported for LH in seasonally anestrous mares (Hart et al., 1984).

The FSH response to the injection of GnRH analog was virtually identical in both groups of mares. Again, if this analog-induced change in blood levels of FSH is indicative of pituitary stores, then we conclude that the amount of FSH in the pituitary was relatively independent of GnRH's reaching the pituitary. Because concentrations of FSH in blood in the long term were suppressed by approximately 50%, it might be expected that a concurrent and proportional reduction in FSH production would have to occur to prevent a large accumulation of FSH in the pituitary. Given that FSH secretion after injection of the GnRH analog was not indicative of such an accumulation, we conclude that some reduction in FSH production likely occurred. One possible interpretation of these data is that the production of FSH in the pituitary is coupled to the amount within the gland such that accumulation would not occur. It must also be noted that this FSH was released by the GnRH analog, thus its secretion per se was GnRH-dependent.

The overall characteristics of LH and FSH secretion in the mares immunized against GnRH were analogous to those in seasonally anestrous mares. Thus, our results agree with the speculation by Hart et al. (1984) that the low LH content in the pituitary of mares in the winter is due to the low concentration (and probably secretion) of GnRH in the hypothalamus. Moreover, continued low concentrations of LH resulted in a failure of the mares in the present experiment to return to estrus and to ovulate, which is similar to the seasonally anestrous state.

It is not known whether LH and FSH are produced by one, two or perhaps three cell types within the pituitary of the horse. In rats, at least three cell types appear to be involved with LH and FSH storage (Dada et al., 1983). This important basic information is needed to develop a model of gonadotropin secretion by the equine pituitary. The complexities of the LH vs. FSH data in the present experiment and in previous reports (Thompson et al., 1979, 1986; Hart et al., 1984; Reville-Moroz et al., 1984) tend to refute the

argument that a single cell type is responsible for both LH and FSH secretion in the horse.

In conclusion, long-term immunization against GnRH produces a reproductive state in mares analogous to seasonal anestrus. It appears that LH secretion, and probably production, in mares is highly dependent upon the bioavailability of GnRH from the hypothalamus, because immunization against GnRH markedly reduced LH concentrations in the long term and the LH response to secretagogues. In contrast, FSH concentrations were less affected by immunization, and there appear to be components of FSH secretion and perhaps production that are relatively independent of GnRH bioavailability.

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Health Effects of GnRH Immunocontraception of Wild White-Tailed Deer in New Jersey

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Original Article

Health Effects of GnRH Immunocontraception of Wild White-Tailed Deer in New Jersey

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ABSTRACT We evaluated the health effects of GonaCon™ Immunocontraceptive Vaccine in individual white-tailed deer (*Odocoileus virginianus*) on a fully fenced corporate office campus in suburban New Jersey, USA. We captured and vaccinated adult females, fawns of both sexes, and yearling and adult males, and evaluated their health status through field and necropsy observations, assessment of blood chemistry, and histopathological examination of selected tissues. One 1.0-mL intramuscular injection of vaccine was delivered by hand to the hind limb of each GonaCon-treated deer. Control deer received sham injections (ad F) or no injections (yearling and ad M). Mean body-condition scores of GonaCon-treated adult females and males were greater than those of corresponding control groups. No evidence of limping or impaired mobility was noted in study deer during the 2-year study. No adverse effects of vaccination were detected in major organs, organ systems, body condition, fat deposits, or blood chemistry. Injection-site lesions (granulomatous nodules and sterile abscesses) occurred in the deep hind-limb musculature of >85% of GonaCon-treated and sham-injected deer but were not detectable externally. Reactions at injection sites and in lymph nodes were typical responses to injection of vaccines formulated as water-in-oil emulsions, especially those, like GonaCon, that contain mycobacteria. The formation of injection-site lesions may be a necessary component of the immune response that causes infertility in treated animals. Natural resource managers who use GonaCon to manage deer in settings such as developed areas and public parks will ultimately determine its value and applicability. © 2011 The Wildlife Society.

KEY WORDS contraception, gonadotropin releasing hormone, granuloma, infertility, *Odocoileus virginianus*, overabundance.

Management of overabundant populations of white-tailed deer (*Odocoileus virginianus*) has become a major challenge for natural resource managers in many urban, suburban, and public park settings in the United States. Traditional management methods cannot be used in many such settings because of safety issues, conflicting views regarding the social acceptability of hunting, or laws prohibiting the discharge of firearms (DeNicola et al. 1997). Fertility control may offer a nonlethal management alternative, especially for deer subpopulations that are isolated by fencing or natural barriers. Immunocontraception and other nonlethal methods are increasingly advocated by the public (Stout et al. 1997, Lauber et al. 2007) and hold promise as a means of reducing reproduction in overabundant wildlife populations (Hobbs et al. 2000, Curtis et al. 2002, Fagerstone et al. 2010).

GonaCon™ Immunocontraceptive Vaccine (National Wildlife Research Center, Fort Collins, CO) is a gonadotropin releasing hormone- (GnRH-) based water-in-oil emulsion that has safely induced temporary infertility in many mammalian species, including free-ranging California ground squirrels (*Spermophilus beecheyi*; Nash et al. 2004), captive Norway rats (*Rattus norvegicus*; Miller et al. 1997), domestic cats (*Felis catus*; Levy et al. 2004), domestic and feral swine (*Sus scrofa*; Miller et al. 2003; Killian et al. 2006*c*), wild horses (*Equus caballus*; Killian et al. 2006*a*), bison (*Bison bison*; Miller et al. 2004*a*), elk (*Cervus elaphus*; Killian et al. 2009), and white-tailed deer (Miller et al. 2008*b*; Gionfriddo et al. 2009). It can be administered as one intramuscular injection that suppresses reproduction in treated animals of both sexes for multiple years (Miller et al. 2004*b*). GonaCon was registered by the United States Environmental Protection Agency in September 2009 as a restricted-use product for the contraception of adult, female, white-tailed deer via hand injection (Fagerstone et al. 2010).

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We assessed the health effects of GonaCon treatment in white-tailed deer adults and fawns of both sexes. Adult females were the primary focus of the study because they will be the targets of operational applications of GonaCon vaccine by natural resource managers. Fawns were tested because a GnRH immunocontraceptive agent could potentially be delivered orally in the future, and treated bait might be consumed by deer of all ages. We also tested GonaCon in yearling and adult males. Although GonaCon is not registered or recommended for use in male deer (Killian et al. 2005, Curtis et al. 2008), antlerless males could inadvertently be treated during winter management activities because they can be mistaken for females. Inadvertent treatment of males could also occur if a new GnRH vaccine is registered for remote injection or oral delivery in bait.

This research was part of a larger investigation that also evaluated GonaCon's contraceptive efficacy, which is addressed separately in another manuscript (Gionfriddo et al. 2011). The objective of the research covered in this manuscript was to evaluate the health effects of GonaCon Immunocontraceptive Vaccine in wild white-tailed deer through field observations, necropsy examinations, laboratory assessment of blood chemistry, and histopathological analysis of selected reproductive and other tissues. We tested several hypotheses to determine whether mean body condition scores were higher in some groups of study deer than in others. In addition, we tested a series of hypotheses to determine whether mean values for blood-chemistry parameters differed between treatment groups (GonaCon-injected ad F vs. sham-injected control F) and between years (pretreatment vs. posttreatment). We also tested the hypotheses that the incidence of injection-site lesions was higher 1) in GonaCon-treated than in sham-injected control adult female deer, and 2) in GonaCon-treated adult female deer that became pregnant than in those that did not, for each year. Finally, we tested the hypothesis that the GonaCon-injected rear leg of each deer was greater in circumference near the injection-site location than the other rear leg.

STUDY AREA

The study was conducted on a fully fenced (2.4-m-tall chain-link), 123-ha corporate office campus near Madison in New Jersey, USA. Habitat types included hardwood forest (about 20%), old fields reverted to grasslands and savannah (about 20%), lawns (about 40%), and landscaped areas around buildings. Deer were easily observed from paved and gravel roads located throughout the study area. During the 2-year study, a few deer emigrated or immigrated by running through security gates opened briefly by guards to allow ingress and egress of motor vehicles.

Northern New Jersey experienced a continental type of climate, with cold, damp winters and hot, humid summers. At Morris Plains, about 8.5 km northwest of the study area, mean monthly temperatures (1945–1990) ranged from −2.4° C (January) to 22.4° C (July). Mean annual precipitation (1941–1990) was 128.8 cm, with an annual growing season of about 142 days (Office of the New Jersey State Climatologist 2011; U.S. Department of Agriculture Natural Resources Conservation Service, National Water and Climate Center 2011).

METHODS

Capture and Treatments

All study deer.—We captured deer at night with tranquilizer darts fired from a Pneu-Dart rifle (Model 171; Pneu-Dart, Inc., Williamsport, PA). We used a mixture of Telazol® (4.5 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine hydrochloride (2.5 mg/kg; Bayer, Leverkusen, North Rhine-Westphalia, Germany) to immobilize deer. One of us (AJD) estimated deer body masses in the field by handling deer and by palpating body contours strongly influenced by fat deposits (Riney 1960, Robinson 1960, Audigé et al. 1998). We fitted deer with numbered ear tags and radiotelemetry collars equipped with mortality sensors (Model M2520; Advanced Telemetry Systems, Isanti, MN). We delivered a 1.0-mL injection (GonaCon vaccine or a sham material; see below) by hand to the deep musculature of the upper hind limb of each deer. If a deer had been darted in a hind limb we hand-injected the opposite hind limb. We administered tolazoline hydrochloride (3.0 mg/kg; Lloyd, Inc., Shenandoah, IA) intravenously to reverse anesthesia and hasten recovery of deer before we released them at their capture sites.

Adult females.—During July and early August 2005, we captured all adult females present on the study area ($n = 47$) and collected a blood sample from the jugular or femoral vein of each animal to measure pretreatment blood-chemistry parameters. We injected each female with GonaCon Immunocontraceptive Vaccine ($n = 32$; each 1.0-mL dose of vaccine contained 1,000 μg of conjugated GnRH–mollusk protein) or with a sham material ($n = 15$). The sham material consisted of AdjuVac™ adjuvant (National Wildlife Research Center, Fort Collins, CO) and mollusk stabilizing buffer (i.e., GonaCon vaccine without GnRH and without the mollusk protein that usually serves as the carrier molecule to which GnRH is conjugated).

Fawns and males.—We captured and vaccinated 8-month-old female fawns ($n = 4$) in February 2006 to determine the health effects of vaccination with GonaCon vaccine on young deer treated during their first winter. To evaluate the health effects of treating young fawns with GonaCon, we captured and vaccinated 3- to 4-month-old fawns ($n = 16$) during September–October 2006. We captured and vaccinated yearling and adult males ($n = 10$) during March 2007 to assess the health effects of wintertime treatment of male deer with GonaCon. To provide baseline data on untreated (control) males, we killed (via gunshot to head or neck) and necropsied previously uncaptured males (yearlings and ad; $n = 13$) during October 2007.

Evaluation of Health Effects of Vaccination on Treated Deer

We used field observations, necropsy examinations, laboratory assessment of blood chemistry, and histopathological evaluation of selected reproductive and other tissues to

identify potential health and safety problems associated with GonaCon treatment. We observed all study animals during spring(s) and summer(s) between their capture and October 2007. During field observations we looked for evidence of limping, impaired mobility, reduced range of motion of limbs, other abnormal behavior, and externally visible injection-site reactions. In October 2007, at the conclusion of field activities, we collected 62 deer (including representatives of all of the above treatment and control groups) after shooting them in the head or neck with a high-powered rifle. We shot deer as they foraged or rested, without a chase. We collected blood from each deer via cardiac puncture within 1 min of the animal's death, and we stored blood samples in a cooler or refrigerator before we isolated the serum via centrifugation and shipped it to a laboratory for analysis of blood chemistry. For the original group of adult females (the only experimental deer from which we collected blood at the time of initial capture), we used analysis of variance (ANOVA; mixed model) to compare mean values of blood-chemistry parameters of vaccinated versus control deer. Treatment group and year were treated as fixed effects and individual deer were treated as a random effect in the mixed-model analysis.

An experienced wildlife veterinarian performed standard necropsy examinations (Munson 2010) in the field immediately after deer were killed and blood samples were collected. We fixed and preserved tissue samples in formalin before we shipped them to Colorado, USA, for histopathological evaluation (Colorado Histo-Prep, Fort Collins, CO). Samples collected included ovaries, fallopian tubes, uteri, hypothalami-anterior pituitary glands, mammary glands, and the injection-site tissues and their draining (popliteal and iliac) lymph nodes. We also submitted other apparently abnormal tissues, if present, for histopathological analysis. We used Fisher's exact tests to determine whether the incidence of injection-site lesions was greater 1) in GonaCon-treated than in sham-injected control adult female deer, and 2) in pregnant than in nonpregnant GonaCon-treated adult female deer for each year.

Fat stores and general body condition were evaluated for each deer at necropsy to provide information on individual and general herd health, which may have influenced vaccine efficacy. We measured (to the nearest 0.5 mm) the thickness of the thickest fat layer at the base of the heart and on the ventral surfaces of the kidneys; of the 6 indicator sites used by Kistner et al. (1980), these are the most predictive of body composition (Cook et al. 2001). Based on thickness, we assigned a value of 1–5 to each heart or kidney fat deposit (0.0–2.0 mm = 1, 2.5–4.5 mm = 2, 5.0–7.0 mm = 3, 7.5–9.5 mm = 4, $\geq$10.0 mm = 5). For each deer, we categorized general fat deposits as "little," "little to moderate," "moderate," "moderate to abundant," or "abundant," and we assigned values of 1–5 for these categories ("little" = 1, "abundant" = 5). In addition, the necropsy veterinarian classified each animal's general body condition on a scale of 1 through 4, based upon body size (relative to age), muscularity, and externally perceptible fat stores (1 = poor poor condition, with ribs, spine, and all bony prominences starkly visible, and with no fat cover in the concave rump; 2 = thin condition, with the spine, ribs and bony prominences clearly but not starkly visible, with no muscle development, and with little palpable fat in the flat rump; 3 = fair condition, with the spine visible, the ribs and bony prominences palpable but only slightly visible, and with little muscle development; 4 = good condition, with spine, ribs, and prominences not visible, and with at least moderate muscularity, but not a "fat" animal). None of the deer on this study site was considered "fat" or in "excellent" body condition. For each deer, the 4 values for fat stores and body condition were summed to produce a body condition score that took into account body size, musculature, and both externally apparent and deep internal fat stores. If one of the values was missing, we replaced it with the mean of the other 3 values, rounded to the nearest integer. We used these body condition scores, which ranged from 4 to 16, to assign each deer to a body condition class (4–5 = "poor," 6–8 = "thin," 9–11 = "fair," 12–16 = "good"). Body condition scores usually vary among males, females, and fawns due to differences in their ability to store fat and in their need to metabolize fat stores as the autumn breeding season proceeds (Kistner et al. 1980). Accordingly, we calculated a mean body-condition score for each of the following groups: GonaCon-treated adult females, control adult females, GonaCon-treated adult males, control adult males, male fawns vaccinated at 3–4 months of age, female fawns vaccinated at 3–4 months of age, and female fawns vaccinated at 8 months of age (Table 1). Modified Kistner scores have been used successfully to evaluate body condition in many cervids (e.g., Cook et al. 2001, 2007; Stephenson et al. 2002; Peterson and Messmer 2007). Although our body condition scores may not provide an absolute measure of fat deposits or body condition, they do provide standardized relative measures of these variables that permit valid comparisons of treatment–demographic groups within our study.

To determine whether injections had caused a swelling of hind limbs, or whether limb disuse had caused muscular atrophy, we used a standard vinyl tape measure to determine the circumference of each thigh of each deer at necropsy. We held the end of the tape at the top edge of the patella at the insertion of the quadriceps muscle group and wrapped the tape around the thigh at the narrowest point caudal to the stifle. Although this point was very slightly distal to the injection site, it provided a practical, standardized location for comparative measurements that would detect differences in girth at the injection sites. We used a paired t-test to compare the left and right thigh circumferences of each deer. For all statistical analyses, we used $\alpha = 0.05$. The Institutional Animal Care and Use Committee of the National Wildlife Research Center approved all aspects of this research.

RESULTS

All Study Deer

With one exception, no overt signs of limping, restricted range of motion of limbs, or impaired mobility were observed

Table 1. Body condition scores* of GonaCon-treated and control white-tailed deer in New Jersey, USA, 2005–2007.

Treatment group	Body condition				*N*	Group mean	SEM
	Poor	Thin	Fair	Good			
Ad F: GonaCon	2	7	4	3	16	8.8	0.8
Ad F: control[a]	3	5	2	0	10	6.8	0.6
3- to 4-month-old F fawns: GonaCon	1	1	2	1	5	8.8	1.4
3- to 4-month-old M fawns: GonaCon	1	3	2	0	6	7.7	1.1
8-month-old F fawns: GonaCon	0	1	1	0	2	8.0	1.0
Ad M: GonaCon	0	0	1	3	4	12.0	1.2
Ad M: control[b]	0	3	2	0	5	8.4	0.9
Totals	7	20	14	7	48		

* Body condition scores were based on evaluation of body size (relative to age), musculature, and both externally apparent and deep internal fat stores.
[a] Each control ad F received a sham injection of AdjuVac adjuvant and mollusk stabilizing buffer (i.e., GonaCon vaccine without the GnRH and mollusk hemocyanin).
[b] Control ad M received no injections.

in any vaccinated or control deer in this study. No externally visible evidence of injection-site reactions was observed in live deer in the field or in dead deer prior to dissection of the musculature at injection sites during necropsies. Hind limb circumferences measured at the injection sites did not differ ($P > 0.05$) in any animal in this study.

Major organs and organ systems were normal in most study deer, although several abnormalities (apparently unrelated to capture, handling, and treatment) were noted at necropsy. Livers of 5 (4 GonaCon-treated and 1 control) deer and spleens of 2 (1 GonaCon-treated and 1 control) deer showed evidence of current or previous parasitic infection. Several small nodules were found in the lungs of 2 (1 GonaCon-treated and 1 control) deer and in the parenchyma of the pancreas of 1 (control) deer.

Field estimates of deer body weights at initial capture ranged from 32 kg to 57 kg. Skin was normal on all examined deer, with either no external parasites or a few ticks present. Ovaries and uteri in many GonaCon-treated females (ad and fawns) were small and inactive. Ovarian and uterine quiescence is a normal response to GonaCon because the vaccine disrupts the synthesis of reproductive hormones.

Dissection of the deep musculature (semimembranosus, semitendinosus, and biceps femoris muscles) at injection sites revealed lesions of 2 types within muscles and in fascia between muscles: hard nodules and abscesses. Nodules were partially or fully encapsulated, yellowish-white globular masses that occurred singly or in groups; most were 3–6 mm in diameter. Histopathologic examination confirmed that these nodules were chronic inflammatory granulomas of long duration, characterized by macrophages, multinucleated giant cells, vacuoles, and mineralized and/or necrotic centers. Most abscesses were small (0.5–4 cm^3; $n = 6$) or moderate (30–100 cm^3; $n = 5$) in size, but one was very large (about 1,000 cm^3). During histopathological examinations, abscesses were identified as granulomas that had progressed to sterile abscesses.

Adult Females

Of the 30 adult female deer (18 GonaCon-treated and 12 control) that were collected in October 2007, 26 were assigned body condition scores based on necropsy evaluation (Table 1). None of the 10 control females and only 3 of the 16 GonaCon-treated females were in "good" condition. The mean body condition score for all GonaCon-treated adult females (regardless of pregnancy outcomes) was higher than that of controls ($t_{24} = 1.92$, $P = 0.034$; Table 1). The mean body condition score for GonaCon-treated adult females that became pregnant during the study (1 or both yr) did not differ from that of control females ($t_{16} = 0.47$, $P = 0.323$).

Fat reserves in necropsied study deer generally were meager. Ten of 16 GonaCon-treated and 6 of 8 control females had "little" general body fat and 3 others (1 GonaCon-treated and 2 control) had "little-to-moderate" fat. Only 3 deer (all GonaCon-treated) had "moderate" or "moderate-to-abundant" fat, and none had "abundant" fat. Fat reserves on the heart and kidneys ranged from 0 mm to 10 mm in thickness; most deer had 2–4 mm of fat.

None of the 15 blood-chemistry parameters we measured differed ($P > 0.05$) between GonaCon-treated and control adult female deer at the time of initial capture in July–August 2005 or when deer were collected in October 2007 (Table 2). Although 2005 and 2007 mean values of some blood-chemistry parameters differed, all values were within the normal ranges for adult white-tailed deer (Seal et al. 1981, Kie et al. 1983) except creatinine phosphokinase (CPK), potassium, and phosphorus values for 2007, which were abnormally high.

Injection-site lesions (hard nodules, or hard nodules and abscesses) were found at necropsy in 17 (94%) of 18 adult female deer that had been vaccinated with GonaCon, and in 9 (82%) of 11 adult female deer that had received sham injections (Table 3). The incidence of lesions was not higher in GonaCon-treated females ($P = 0.316$) than in controls. Of the 17 GonaCon-treated females with hard nodules at injection sites, 8 (47%) also had abscesses (2 small, 5 moderate, 1 large). Two (22%) of the 9 sham-injected control females with hard nodules also had abscesses (both small). Among GonaCon-treated adult females, the incidence of lesions was not higher in pregnant ($n_{2006} = 6$; $n_{2007} = 8$) than in nonpregnant ($n_{2006} = 12$; $n_{2007} = 13$) animals for either year ($P_{2006} = 0.333$; $P_{2007} = 0.619$). Injection-site tissue samples from 12 adult females (7 GonaCon-treated

Table 2. Means and standard errors of the means for blood chemical parameters of adult, female, white-tailed deer in GonaCon-treated groups and control groups in New Jersey, USA, 2005–2007. Values marked with different letters (A and B) within a row differed significantly from each other. Note that some values are marked with both A and B. Values for 2005 are pretreatment values. ALK, alkaline phosphatase; CPK, creatinine phosphokinase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.

	2005				2007					
	Control[a]		GonaCon		Control		GonaCon			
Parameter	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Group, P^b	Yr, P^c
Sodium (mEq/L)[d]	142.5 A	3.1	142.0 A	2.2	148.6 A,B	3.5	157.5 B	2.9	0.174	<0.001
Potassium (mEq/L)[e]	4.8 A	0.6	4.9 A	0.4	11.5 B	0.7	10.7 B	0.6	0.562	<0.001
Calcium (mg/dL)	8.7 A	0.3	8.6 A	0.2	9.7 B	0.3	10.3 B	0.3	0.399	<0.001
Phosphorus (mg/dL)	5.7 A	0.6	5.3 A	0.4	11.6 A	0.7	11.6 A	0.6	0.796	<0.001
ALK (U/L)[e]	32.6 A	30.3	26.2 A	20.8	205.5 B	33.9	215.7 B	27.7	0.947	<0.001
Glucose (mg/dL)[d]	142.8 A	25.2	150.0 A	17.3	189.0 A	28.2	183.5 A	23.0	0.972	0.098
Total protein (g/dL)	6.6 A	0.2	6.7 A	0.15	6.7 A,B	0.3	7.3 B	0.2	0.111	0.052
Albumin (g/dL)	2.7 A	0.1	2.62 A	0.1	2.6 A	0.1	2.8 A	0.1	0.576	0.793
Globulin (g/dL)	3.9 A	0.2	4.0 A	0.1	4.1 A,B	0.2	4.6 B	0.2	0.080	0.012
CPK (U/LD)[e]	345.7 A	1,667.4	241.2 A	1,141.6	3,388.3 B	1,864.2	6,332.9 B	1,522.1	0.369	0.005
Total bilirubin (mg/dL)	0.1 A	0.0	0.10 A	0.0	0.3 B	0.0	0.4 B	0.0	0.817	<0.001
ALT (U/L)[d]	26.8 A	5.5	23.5 A	3.8	59.5 B	6.2	45.7 B	5.0	0.104	<0.001
Creatinine (mg/dL)	1.0 A	0.1	1.2 A	0.1	1.4 B	0.1	1.6 B	0.1	0.059	<0.001
BUN (mg/dL)	17.3 A	2.4	18.1 A	1.7	22.0 A	2.7	21.1 A	2.2	0.986	0.094
Amylase (U/L)	106.8 A	10.2	115.9 B	7.0	133.6 A,B	11.3	140.8 B	9.2	0.426	0.006

[a] Means are from the PROC MIXED procedure in SAS.
[b] Between-groups comparison.
[c] Between-yr comparison.
[d] The inverse ($1/x$) was used for ANOVA. The *P*-value reported in the table is the value for the ANOVA of the inverse values.
[e] The natural log (ln) was used for ANOVA. The *P*-value reported in the table is the value for the ANOVA of the natural logs.

and 5 control) were submitted for histopathological examination. Chronic granulomatous inflammation was detected in 11 of these 12 samples, and the remaining sample had a chronic abscess. Necrosis of muscle fibers at injection sites was common.

Injection sites of tranquilizer darts used at initial capture (summer 2005) were found in 7 (24%) of 29 adult female deer (GonaCon-treated and controls) at necropsy in October 2007. A tissue sample from the tranquilizer-dart injection site of an adult female was examined histopathologically and found to have chronic granulomatous inflammation.

At necropsy, the popliteal lymph node in the hind leg that was hand-injected was enlarged or hard in 11 (61%) of 18 GonaCon-treated adult female deer and in 6 (50%) of 12 sham-injected control females. The iliac lymph node associated with the injected hind leg was enlarged or hard in 6 (35%) of 17 GonaCon-treated females and in 6 (50%) of 12 sham-injected females.

Twenty-five popliteal or iliac lymph nodes from 14 (10 GonaCon-treated and 4 control) adult study females were submitted for histopathological examination. Chronic granulomatous inflammation was found in 4 unidentified (popliteal or iliac) lymph nodes from 3 (2 GonaCon-treated and 1 control) deer. Chronic hyperplasia was detected in 2 popliteal lymph nodes (from 2 GonaCon-treated deer) and 1 unidentified lymph node (from a control deer). Acute hemorrhage was noted in 2 unidentified lymph nodes (from 1 GonaCon-treated and 1 control deer). Foreign material was found in one unidentified lymph node (from a GonaCon-treated deer). No significant findings were reported for 5 popliteal, 4 iliac, and 7 unidentified lymph nodes.

In several lymph nodes in our study deer (both sexes), vacuoles were found among the macrophages and multinucleated giant cells or within their cytoplasm. These vacuoles were probably mineral oil droplets from the AdjuVac in GonaCon.

Table 3. Occurrence of injection-site lesions in wild white-tailed deer injected with GonaCon Immunocontraceptive Vaccine or a sham material in New Jersey, USA, 2005–2007.[*,**]

Treatment group[a]	Deer examined	Deer with injection-site lesions	Deer without injection-site lesions
Ad F: GonaCon	18	17 (94%)	1 (6%)
Ad F: sham	11	9 (82%)	2 (18%)
3- to 4-month-old fawns: GonaCon	12	8 (67%)	4 (33%)
8-month-old fawns: GonaCon	2	2 (100%)	0 (0%)
Ad M: GonaCon	6	6 (100%)	0 (0%)
Totals	49	42 (86%)	7 (14%)

[*] Sham material consisted of AdjuVac adjuvant and mollusk stabilizing buffer (i.e., GonaCon vaccine without the GnRH and mollusk hemocyanin).
[**] No injection-site lesions were found in 13 ad M control deer that received no injections.
[a] Ages in this column are ages at time of injection.

Fawns (F, 8 Months Old)

Two of the 4 fawns vaccinated in February 2006 were collected for necropsy in October 2007. Deer number 76 was "thin" based upon body condition score, with "little" general body fat. Deer number 78 was in "fair" body condition, with "little-to-moderate" general body fat (Table 1). Heart and kidney fat deposits were 3–5 mm thick in these 2 deer.

Lesions were found at vaccine injection sites in both deer at necropsy (Table 3). An intramuscular scar caused by a tranquilizer dart also was found in the hind leg of deer number 78. The popliteal and iliac lymph nodes were hard in deer number 78; only the popliteal lymph node was hard in deer number 76.

Fawns (M and F, 3–4 Months Old)

Twelve of the 16 (8 M and 8 F) fawns we captured and vaccinated in September–October 2006 were collected and necropsied in October 2007. Although body condition varied substantially, half of these deer were in "poor" or "thin" condition, and only one was in "good" condition (Table 1). General fat deposits were "little" in half of these deer, and no deer had more than "moderate" fat stores. Heart and kidney fat stores were $\leq$2 mm in thickness in 4 of the 12 necropsied deer, and from 3 mm to 10 mm in the remaining deer ($n = 7$) that we evaluated.

Vaccine injection-site lesions (hard nodules, no abscesses) were found in 8 (67%; 5 [100%] of 5 F and 3 [43%] of 7 M; Table 3), and tranquilizer dart scars were found in 7 (58%) of the 12 GonaCon-treated deer for which these data were recorded. Histopathological examination of tissue samples taken from the vaccine injection sites of 2 deer detected chronic granulomatous inflammation in both cases. One of these 2 deer also had chronic granulomatous inflammation in its other hind limb from the tranquilizer dart injection.

Enlargement of the popliteal lymph node was observed at necropsy in 3 (27%) of 11 examined deer for which data were recorded, and enlargement of the iliac lymph node was detected in 1 of these 3 deer. The popliteal and iliac lymph nodes from 2 (1 M and 1 F) of these 3 deer were examined histopathologically. One (unidentified) lymph node from the male had chronic granulomatous inflammation, and acute hemorrhage was found in one lymph node from the female. The remaining lymph node from each animal showed no signs of pathology.

Adult Males

Of the 10 adult males that were captured and vaccinated with GonaCon during March 2007, 2 died of unknown causes and 2 left the study site before October 2007. Body condition scores were determined for 4 of the 6 remaining males; the mean was higher than that for 5 noninjected control males ($t_5 = 2.31$, $P = 0.032$; Table 1). Heart and kidney fat deposits were 4–8 mm thick in 3 males that had "moderate" general fat deposits, and they were 2–4 mm thick in a male that had "little-to-moderate" general fat deposits.

Vaccine injection-site lesions were observed in all 6 males at necropsy (Table 3), but no tranquilizer dart wounds or scars were found. Two (33%) of these 6 males had small abscesses in addition to hard nodules. Popliteal lymph node reactions were evident at necropsy in 5 (83%) of these males. Enlargement of the iliac lymph node was seen in 2 (33%) of the males. Relatively small testes and delayed or abnormal antler development were observed in 2 of the adult males and in males that had been vaccinated as 3- to 4-month-old fawns (J. P. Gionfriddo, unpublished work).

Control Males

Thirteen previously untreated males (12 of which had never been captured) were collected for necropsy during October 2007 for comparison with GonaCon-treated males. Heart and kidney fat deposits were $\leq$5 mm, and general fat deposits were "little" to "moderate" in 5 control males we evaluated at necropsy. Necropsy observations and photographs of the other 8 control males indicated that they also were generally in "thin" to "fair" body condition (J. P. Gionfriddo, unpublished work).

DISCUSSION

Treatment with GonaCon Immunocontraceptive Vaccine caused no serious adverse effects on deer mobility, major organs and organ systems, or general health and welfare. Intramuscular injection-site lesions were the only adverse effect of vaccination that we detected via field observations, necropsy examinations, laboratory assessment of blood chemistry, and histopathological evaluation of selected reproductive and other tissues collected from study deer. These results are consistent with those of earlier studies of GonaCon vaccination of white-tailed deer (Killian et al. 2006*b*, Gionfriddo et al. 2009). In New York, USA, Curtis et al. (2008) detected no adverse health effects (except injection-site lesions) associated with hand- and remote-vaccination of female white-tailed deer with 2 early formulations of GonaCon. Instead of AdjuVac, Freund's complete adjuvant (FCA) was used in the primary dose and Freund's incomplete adjuvant (FIA) (Freund 1956) was used in the booster injection.

Although transitory limping can be common among animals that receive intramuscular vaccinations that produce injection-site inflammation and granulomas (Macy 1997), we saw no limping or decreased mobility in injected deer in this study. Although histopathological evaluation found chronic granulomas in both rear legs of one of the adult males (from the tranquilizer dart and a GonaCon hand injection), the animal was never seen limping during many field observations in 2007.

Body Condition and Fat Reserves

Prior to the start of our field research, deer had not been actively managed on the study site for many years, and the herd had increased to about 95 animals by early spring 2005 (A. J. DeNicola, White Buffalo, Inc., unpublished data). At capture in July 2005, most deer were noticeably thin, as reflected in their low body masses. Sustained and severe overbrowsing by deer was evident in the condition of native and ornamental vegetation throughout the study site. Thus it was not surprising that fat reserves of study deer at necropsy generally were meager.

GonaCon-treated adult females, especially those that had not been pregnant during the field study, were in better body condition at necropsy than control females. By relieving females of the annual energetic burdens of gestation and lactation, immunocontraception may have enabled treated deer to maintain better body condition than control females that produced and raised fawns (McShea et al. 1997). Body condition was not evaluated in previous GonaCon studies. The effects of porcine zona pellucida (PZP) immunocontraception on body condition have been equivocal. Adult female white-tailed deer contracepted with a PZP vaccine in Connecticut, USA were not in better condition than untreated controls after a 3-year field study (Walter et al. 2003). Among captive white-tailed deer in Pennsylvania, USA, no differences in annual autumn body weights were detected between PZP-treated and control females during a 4-year vaccine trial (Miller et al. 2001). On the other hand, female deer successfully contracepted with PZP in Virginia, USA were heavier 1 year posttreatment than untreated control females that had become pregnant (Kirkpatrick et al. 1997). Many deer treated with PZP undergo repeated estrous cycling that is energetically costly and may adversely affect body condition (Miller et al. 2009). In addition, fertilization during later estrous cycles can produce fawns whose late birthdates could reduce their survivorship during their first winter (McShea et al. 1997). GonaCon does not cause repeated estrous cycling.

The better condition of GonaCon-treated males when compared to control males may be related to the rut and to the relatively short interval between vaccination and necropsy. Control males may have lost body condition through seasonal rut-associated behavior such as fasting, sparring, and chasing. Although GonaCon-treated males generally do not grow as large as untreated males (Killian et al. 2005), males in our study were injected with GonaCon in March 2007 and killed in October 2007, perhaps before some effects of the vaccine (such as reduced muscle development) were manifested.

Blood Chemistry

Blood-chemistry parameters may be influenced by many factors, including sex, age (Johnson et al. 1968), season, reproductive status (Klinger et al. 1986), nutrition (Sams et al. 1998), and capture-related factors such as capture and handling methods (Mautz et al. 1980, Kock et al. 1987*b*, DelGiudice et al. 1990), including the use of immobilizing agents (Marco and Lavín 1999, Poljičak-Milas et al. 2006, Vengušt et al. 2006). Several aspects of our blood-sampling procedures may have affected blood parameters and could have contributed to differences between treatment groups or years. At initial capture in 2005, blood was drawn from the jugular veins of live deer, whereas cardiac puncture of lethally gunshot deer was used at the end of the field study in 2007. The latter collection method may have affected the poststudy (2007) values of some blood parameters (White and Cook 1974). For example, cardiac puncture of a dead animal tends to yield higher values for CPK, potassium, and phosphorus than venipuncture of a live animal, and trauma associated with lethal shooting also can elevate these parameters (Savignano et al. 1969, Seal et al. 1981, Latimer et al. 2003). Creatinine phosphokinase, potassium, and phosphorus were the only blood-chemistry parameters in our study with values outside the normal ranges for adult white-tailed deer. A second potential capture-related source of variation in blood-chemistry results was our use of immobilizing agents in 2005 but not in 2007. These compounds may affect blood parameters and could have influenced our results (Seal and Bush 1987). Finally, observed differences in the values of some blood parameters could be due to seasonal (July vs. October) variation in nutrition and physiology (Seal et al. 1981, Waid and Warren 1984).

Despite the above limitations, our data show that GonaCon caused no serious adverse health effects that were reflected in blood parameters. Other researchers have reported similar results. Curtis et al. (2008) measured 28 standard blood parameters in white-tailed deer in New York and found no differences between GnRH-treated and control animals. Killian et al. (2006*b*) used standardized methodology under carefully controlled conditions to handle captive, GonaCon-treated, white-tailed deer and to sample their blood. Their comparison of the blood chemistry and hematology of 19 captive adult females detected no statistically significant treatment effects for any of the 33 blood parameters tested. As expected, however, seasonal changes in some variables were detected via repeated sampling over a 20-week period (Killian et al. 2006*b*).

Posttreatment values for CPK, potassium, and phosphorus were abnormally high in our study deer (Table 2). Elevated CPK values usually reflect stress or trauma to heart, brain, or skeletal muscle. Lethal gunshot wounds to the head or neck, and cardiac puncture for blood collection immediately after death could have caused CPK to increase dramatically. Stress and tissue damage associated with chronic inflammation at injection sites and in lymph nodes (reactions to injection) also could have contributed to the elevation of CPK. Evaluation of CPK values may not be useful due to the extreme fluctuations that commonly occur. For example, 200-fold increases in serum CPK were observed in penned white-tailed deer 24 hr after deer were physically restrained (Seal et al. 1972).

Elevated concentrations of potassium and phosphorus, like CPK, often are associated with excess destruction or necrosis of cells, and may reflect the extent of tissue (especially muscle) damage (Kock et al. 1987*a*, Latimer et al. 2003). Wilber and Robinson (1958) collected Maryland, USA white-tailed deer via lethal gunshots to neck vertebrae and then immediately sampled blood via heart puncture. They found consistently high concentrations of plasma potassium (similar to our values) and phosphorus, which they attributed to "shock." Using similar methods in Oklahoma, USA, Sams et al. (1998) found very high serum phosphorus concentrations in white-tailed deer. Elevated potassium and phosphorus concentrations in our study deer were likely due to muscle necrosis associated with collection of deer and blood samples.

Mean posttreatment alkaline phosphatase (ALK) values showed a 6-fold increase from pretreatment values in our

study, but they remained within the ranges of values reported for white-tailed deer (e.g., Sams et al. 1998, Curtis et al. 2008) and for our sample of noninjected control male deer ($\bar{x} = 221.3$, SE = 48.5, $n = 13$). A published range of normal values for white-tailed deer could not be found for this variable, but the range is very broad for large mammals and, therefore, ALK is not a sensitive indicator (Latimer et al. 2003).

Injection-Site Lesions

The occurrence of intramuscular injection-site lesions in 5 (29%) of 17 GonaCon-treated deer in a previous field study (Gionfriddo et al. 2009) alerted us to the possibility that deer in the current study might develop such reactions. Necropsy observations and histopathological evaluation revealed that >85% of the GonaCon-treated and sham-injected deer in New Jersey had injection-site granulomas or sterile abscesses in the deep musculature of the hind limbs in October 2007. The similarity in the incidence of injection-site lesions in GonaCon-treated and sham-injected adult female deer strongly suggests that the causative agent(s) was present in both inocula.

Adjuvants and vaccines often cause localized reactions at injection sites, including pain, local inflammation, swelling, necrosis, granulomas, ulcers, and sterile abscesses (Aguilar and Rodríguez 2007). For example, adjuvants that include alum (Gupta et al. 1995, Valtulini et al. 2005, Reed et al. 2008) or *Mycobacterium* spp. (Broderson 1989) often cause reactions at injection sites. Larger amounts of mycobacteria cause proportionately more inflammation and necrosis (Broderson 1989). Water-in-oil emulsions, especially those containing mineral oil, also induce local inflammation and granuloma formation at injection sites (Humphrey 1982, Straw et al. 1985, Yamanaka et al. 1992, Macy 1997, Petrovsky and Aguilar 2004). Injection-site granulomas and other lesions are routinely encountered in livestock, from which they are excised at slaughter (George et al. 1995, Van Donkersgoed et al. 1997, Valtulini et al. 2005). They also commonly occur in humans (Hay 1995, Bordet et al. 2001, Marsee et al. 2008).

GonaCon Immunocontraceptive Vaccine contains AdjuVac, a mineral-oil-based adjuvant developed from a USDA-approved Johnes disease vaccine called Mycopar™ (Fort Dodge Animal Health). AdjuVac contains killed *Mycobacterium avium*, which is needed for GonaCon vaccine to induce a rapid, strong (Miller et al. 2008*a*), and sustained (Perry et al. 2008) contraceptive effect. *Mycobacterium* spp. are commonly used in adjuvants to strengthen the immune response (Gupta et al. 1993, Aguilar and Rodríguez 2007). Like Freund's complete adjuvant (which contains *Mycobacterium paratuberculosis* and has been used extensively in laboratory animals), AdjuVac's combination of water-in-mineral-oil emulsion and killed mycobacteria results in a highly potent adjuvant that stimulates both humoral and cellular immunity (Warren et al. 1986). Freund's complete adjuvant, however, cannot be used in human or veterinary vaccines because it may cause severe pain, fever, abscess formation, and the possibility of permanent organ damage (Edelman 1980). Leenaars et al. (1998), for example, found extensive intramuscular lesions in rabbits (*Oryctolagus cuniculus*) and mice (*Mus musculus*) after injection with FCA and FIA (as prime and boost, respectively), although palpation had detected no swelling or nodules. Wild horses injected with an early formulation of a PZP immunocontraceptive vaccine that contained FCA developed externally visible nodules, swelling, and abscesses at injection sites (Roelle and Ransom 2009). Porcine zona pellucida and GnRH vaccines (early formulations of GonaCon that contained FCA or FIA instead of AdjuVac) also caused granulomas at hand-injection (PZP) and dart-injection (PZP and GnRH) sites in nearly all treated white-tailed deer in New York (Curtis et al. 2007, 2008). The intramuscular injection of large doses of FCA or FIA can cause diffuse necrosis and extensive atrophy of functional muscle groups, impairing function (Broderson 1989). Such severe reactions have not been observed in animals injected with GonaCon (containing AdjuVac), possibly because of the relatively small amount of mycobacteria it contains (Miller et al. 2008*a*), or because GonaCon uses a different mycobacterial species.

Necrosis of muscle fibers at injection sites was observed in many of our study deer in New Jersey and in Maryland (Gionfriddo et al. 2009), and in captive white-tailed deer (Killian et al. 2006*b*) and rabbits (Powers et al. 2007) treated with GonaCon. Exposure to necrotic cells may be necessary for the maturation of dendritic cells and the initiation of an immune response (Gallucci et al. 1999, Sauter et al. 2000). Moreover, the strength of the immune response may be proportional to the extent of local tissue damage caused by the inoculum at the injection site (Schijns 2000). Thus the necrosis of muscle fibers at the injection site by adjuvants containing alum (Walls 1977), mineral oil (Straw et al. 1985, Yamanaka et al. 1992), or mycobacteria (Broderson 1989) may yield important immunological benefits.

Vaccines that contain alum or mycobacteria, or that are formulated as water-in-oil emulsions, also may induce strong immune responses because they form a repository ("depot") at the injection site (Glenny et al. 1931, White 1967, Fukanoki et al. 2000). The vaccine depot slowly releases antigen, which is carried by maturing, migratory dendritic cells via afferent lymph vessels to the paracortical regions of the local draining lymph node, where it is presented to resting, antigen-specific T cells that are activated by the now-mature dendritic cells (HogenEsch 2002). Antigen has been detected in lymph nodes as early as 3 hr after peripheral injection (Dupuis et al. 1998). Antigen may be protected and retained for months or even years on follicular dendritic cells in draining lymph nodes where antibody formation continues (Greene et al. 1975, Tew and Mandel 1979, Burton et al. 1994). Subsequent release of antigen from the depot acts as a secondary stimulus (similar to a booster injection) to the sensitizing action of the antigen released previously.

In response to the presence of the vaccine depot, a granuloma develops as the immune system attempts to wall off the irritating foreign material by enclosing it in a fibrous capsule of thick connective tissue. Granulomas that develop

at injection sites contribute substantially to antibody synthesis because they harbor large numbers of antibody-producing plasma cells (White 1967, World Health Organization 1976). The release rate of antigen from an injection-site granuloma decreases as the granuloma develops and becomes encapsulated, and eventually it becomes insufficient to maintain an optimal antibody response (Herbert 1968, Lascelles et al. 1989). After the granuloma is completely walled off and antigen release stops, excision of the granuloma does not affect antibody formation in the draining lymph node because antigen is already present there (Lascelles et al. 1989, Schijns 2000). Active but unused antigen remains trapped inside the encapsulated granuloma, as demonstrated by researchers who excised material from injection-site granulomas and injected it into the same or different animals, thereby inducing immune responses in the recipients (Harrison 1935, Holt 1950, Herbert 1968).

Lymph Nodes

Popliteal and iliac lymph nodes are the draining and regional lymph nodes for the hind limb area where hand injections were delivered to deer in our study. Enlargement of local lymph nodes is a normal response to infection or other types of antigenic stimulation, and also can be caused by injection of oil emulsions (Yamanaka et al. 1992). Stronger antigens and adjuvants induce greater inflammation of draining lymph nodes (Taub et al. 1970).

In our study, popliteal and iliac lymph nodes of noninjected control male deer were normal, as expected. Many of the lymph nodes of GonaCon-treated and sham-injected deer, however, were enlarged, hard, or otherwise reactive, indicating the involvement of these nodes in the drainage of the intramuscular injection site. These results demonstrate that the material that caused reactions in lymph nodes was present in both GonaCon vaccine and in the sham material (which contained AdjuVac adjuvant and mollusk stabilizing buffer). The mineral oil (Aucouturier et al. 2001) or the *M. avium* (Herbert 1968, Lind 1968) in AdjuVac could have caused the draining lymph nodes to react. Enlargement of draining lymph nodes also was reported in captive rabbits (Powers et al. 2007) and in captive (Killian et al. 2006*b*) and wild (Gionfriddo et al. 2009) white-tailed deer treated with GonaCon vaccine.

In $\geq$6 of our study deer, secondary granulomatous inflammation and hyperplasia occurred in draining lymph nodes to which the irritant at the injection site had been transported by lymph. Similarly, one of 13 captive adult female white-tailed deer vaccinated with GonaCon in Pennsylvania developed granulomatous lymphadenitis in a draining lymph node (Killian et al. 2006*b*). Development of granulomas in draining lymph nodes is common when alum-absorbed adjuvants (Humphrey 1982), FCA (Yamanaka et al. 1992), or FIA (Gaafar and Turk 1970) are used. In extreme cases, granulomatous lesions may replace the majority of the tissue in lymph nodes (Yamanaka et al. 1992). Unanue and Benacerraf (1973) demonstrated that granulomatous reactions may be passively transferred between animals by intravenously injecting lymph node cells from a granulomatous lymph node in one animal into a second animal.

The oil droplets detected in the lymph nodes of several of our study deer probably originated in the inoculum and were transported via lymphatic vessels to the draining lymph nodes. Previous studies have documented the presence of paraffin or mineral oil (used as an adjuvant) in lymph nodes after subcutaneous or intramuscular injection (White et al. 1955, Freund 1956, Broderson 1989). Long-term (probably lifelong) retention of oil in body tissues may result from the use of mineral oil adjuvants (World Health Organization 1976).

MANAGEMENT IMPLICATIONS

GonaCon Immunocontraceptive Vaccine provides a safe and humane way to reduce reproduction in individual adult female white-tailed deer, especially in demographically closed (e.g., fenced or otherwise spatially isolated) populations. The only adverse reactions to vaccination in our field study were normal inflammatory responses at injection sites and in associated draining lymph nodes. These responses were natural and essential expressions of the strong immune response that is required to induce temporary infertility. Safety issues should not impede acceptance of GonaCon as a deer management tool. Operational use of GonaCon Immunocontraceptive Vaccine and other wildlife contraceptives will likely be limited by efficacy, practicality, and politics to settings where traditional management methods cannot be applied. The widespread occurrence of such settings suggests that the potential contribution of contraceptive agents to deer management in developed landscapes is substantial. In addition to its contraceptive effects, GonaCon may improve deer body condition without the PZP-associated disadvantages of repeated estrous cycling and delayed birth of fawns to vaccine nonresponders. Although public demand for safe, humane contraceptive agents like GonaCon will continue to drive their research and development, wildlife management agencies and personnel will ultimately determine the value and applicability of these products.

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Original Article

Efficacy of GnRH Immunocontraception of Wild White-Tailed Deer in New Jersey

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ABSTRACT Safe and effective contraceptive agents are needed to manage overabundant populations of cervids in settings where traditional management methods such as hunting are prohibited or impractical. We used GonaCon™ Immunocontraceptive Vaccine to reduce reproduction in individual white-tailed deer (*Odocoileus virginianus*) on a fully fenced corporate-office campus in suburban New Jersey, USA. In July–August 2005, we captured, marked, injected, and released 47 adult females and then monitored their reproductive performance for 2 years. Thirty-two of these females each received a 1.0-mL injection of GonaCon vaccine, and 15 control females were given sham injections. Field observations of udder condition during summers of 2006 and 2007 were used to determine which adult female deer were lactating; lactation was used as an indicator of imminent or recent parturition. During summer 2006, 8 of 24 GonaCon-treated deer were pregnant, in contrast to 12 of 13 control deer. During summer 2007, 2 years after injections were given, 13 of 23 GonaCon-treated and 10 of 12 control animals were pregnant. We also captured, vaccinated, and released fawns (both sexes) and yearling and adult males and then monitored their reproductive status. Immunocontraception of fawns was unsuccessful. In some GonaCon-treated males (all age classes), serum testosterone concentrations and development of testes and antlers were reduced. Higher anti-gonadotropin-releasing-hormone antibody titers were associated with greater infertility in females and with lower values for reproductive parameters in males. GonaCon reduced reproduction in wild adult female white-tailed deer, but greater contraceptive efficacy may be required for it to gain widespread acceptance and use by natural resource managers. © 2011 The Wildlife Society.

KEY WORDS contraception, gonadotropin-releasing hormone, infertility, *Odocoileus virginianus*, overabundance.

Local populations of white-tailed deer (*Odocoileus virginianus*) have become overabundant in many regions of North America, where they are causing problems for residents and natural resource managers (Crête and Daigle 1999). Deer–motor-vehicle collisions (Etter et al. 2000), severe overbrowsing of native and ornamental vegetation (Rooney and Waller 2003), and adverse effects on local avian communities (McShea and Rappole 2000) are among the problems caused by overabundant deer. Hunting is prohibited in many urban, suburban, and public park settings, and other deer management tools must be used. Contraception and other nonlethal deer management approaches are increasingly preferred by many urban and suburban residents (Stout et al. 1997, Lauber and Knuth 2000). Immunocontraception offers natural resource managers another tool to reduce reproduction in overabundant wildlife populations (Fagerstone et al. 2002).

Although efforts to develop safe and effective wildlife contraceptive agents have been underway for about 50 years (Davis 1961, Vandenbergh and Davis 1962, Balser 1964), only 2 contraceptive products have been registered in the United States for wildlife: GonaCon™ Immunocontraceptive Vaccine (National Wildlife Research Center, Fort Collins, CO) for adult female white-tailed deer, and a nicarbazin-based contraceptive bait ("OvoControl") for Canada Geese (*Branta canadensis*), certain domestic ducks, and pigeons (*Columba livia*; Innolytics, Rancho Santa Fe, CA). Among the obstacles to the development and registration of wildlife contraceptives is the achievement of objectives related to safety, efficacy, practicality, and humaneness of treatment. To be registered by the United States Environmental Protection Agency (EPA) and to gain widespread acceptance among wildlife management professionals, a contraceptive agent must: 1) not adversely affect the health, social behavior, or population structure of the target

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species; 2) not adversely affect nontarget species; 3) provide a complete, long-lasting (but ideally, reversible) contraceptive effect; 4) be cost-effective; and 5) not cause treated food animals to become unfit for human consumption (Fagerstone et al. 2010).

GonaCon Immunocontraceptive Vaccine is a gonadotropin-releasing hormone (GnRH)-based wildlife contraceptive agent that has safely induced temporary infertility in many mammalian species (see refs in Gionfriddo et al. 2011). One intramuscular injection of GonaCon suppresses mammalian reproduction (M or F) for multiple years (Miller et al. 2004). GonaCon was registered with the United States EPA in September 2009 as a restricted-use product for the contraception of adult, female, white-tailed deer via hand injection (Fagerstone et al. 2010). Regarding potential risk associated with consuming deer treated with GonaCon vaccine, the EPA (Farwell 2009:2) has stated "There is little likelihood of exposure to hormonally active compound from deer meat because GnRH is a protein that is digested and not absorbed intact. Like other proteins, GnRH is too large and polar to pass through the membranes of the gastrointestinal tract. Proteins are digested into their component amino acids in the stomach and small intestines."

We tested GonaCon's contraceptive efficacy in white-tailed deer adults and fawns of both sexes. Adult females were the primary focus of the study because they will be the targets of real-world applications of GonaCon vaccine by natural resource managers. Fawns were tested to determine whether their immune systems were sufficiently mature to mount and maintain the strong immune responses to vaccination that are required to achieve and sustain a contraceptive effect. We also tested GonaCon in yearling and adult males. Although GonaCon is not registered or recommended for use in male deer (Killian et al. 2005, Curtis et al. 2008), deer-management field activities in North America often occur during winter when ambient temperatures are lower, deer are more visible (due to reduced vegetative cover), and deer occur in larger groups than at other times of year. Male deer lack antlers during late winter and can be mistaken for females. We vaccinated males to determine the effects of GonaCon treatment on male deer that might inadvertently be treated during winter management programs (if a GnRH vaccine is registered for remote injection or oral delivery in bait).

The objective of this 2-year field study was to evaluate the efficacy of GonaCon Immunocontraceptive Vaccine in individual wild white-tailed deer by determining the proportion of treated individuals that became infertile and the duration of induced infertility. For each year, we tested the hypothesis that pregnancy rates of adult females did not differ between GonaCon-treated and control deer. For male deer, we tested hypotheses to determine whether serum testosterone concentrations or testicular circumferences differed among treatment groups or age classes.

This research differs from our previous studies of GonaCon immunocontraception of white-tailed deer in several ways. Most studies involved captive deer at Pennsylvania State University where optimal diets and veterinary care (including de-worming) were routinely provided. In contrast, our field studies in Maryland, USA (Gionfriddo et al. 2009) and New Jersey, USA (Gionfriddo et al. 2011) involved wild deer subpopulations that lived independently of humans and ranged freely on very large, fenced, partially forested sites where forage was extremely overbrowsed, deer were exposed to natural environmental conditions, and deer received no veterinary care. Important differences between the Maryland and New Jersey field studies included the occupation of a single site by all study deer in New Jersey (GonaCon-treated and control deer occupied adjacent but different fenced sites in Maryland, for political and logistical reasons) and the injection of New Jersey control females with a sham material (controls in Maryland were not injected, again for political and logistical reasons). In addition, only adult female deer were tested in the Maryland study.

STUDY AREA

The study was conducted on a 123-ha corporate office campus near Madison, New Jersey, USA. Vegetation on the site consisted of hardwood forest (about 20%), old fields in various stages of secondary succession (about 20%), lawns (about 40%), and landscaping near buildings. A 2.4-m-tall chain-link fence surrounded the property. Although the site was bounded by public roads on all 4 sides, public access to the campus was prohibited, and security gates and guards controlled access of authorized personnel. Despite the presence of the fence, a few deer entered or left the study area by running through gates opened briefly by guards for vehicular traffic. Most of the study area was accessible via a network of paved and gravel roads that was used during deer capture and observation activities. Limited food availability in forested areas caused deer to forage heavily on lawn areas where they were easily observed with binoculars and spotting telescopes. Long-term patterns of temperature and precipitation for the study area are summarized elsewhere (Gionfriddo et al. 2011).

METHODS

Capture and Treatments

General.—We captured deer at night with tranquilizer darts and fitted each animal with numbered ear tags and a radiotelemetry collar before reversing anesthesia and releasing deer at their capture sites, as described by Gionfriddo et al. (2011). We gave each deer a 1.0-mL injection by hand to the deep musculature of the upper hind limb (see below); if a deer had been darted in a hind limb, we delivered the hand injection to the opposite hind limb. We observed all study deer during the summer(s) between their capture and October 2007, when deer were collected and necropsied.

Adult females.—During July and early August 2005, we captured all adult females ($n = 47$) present on the study area. We injected each female with GonaCon Immunocontraceptive Vaccine ($n = 32$; each 1.0-mL dose of GonaCon contained 1,000 μg of conjugated GnRH-hemocyanin in a water-in-oil emulsion) or with a sham material ($n = 15$) that consisted of AdjuVac™ adjuvant (National Wildlife Research

Center) and mollusk stabilizing buffer (i.e., GonaCon vaccine without GnRH and without mollusk hemocyanin, which usually serves as a carrier molecule for the GnRH). We collected a blood sample from the jugular or femoral vein of each female for measurement of pretreatment serum anti-GnRH antibody titers. Four GonaCon-treated adult females that became pregnant during autumn 2005 were recaptured in October 2006 and given a second vaccine injection to determine the effects of a booster dose on contraceptive efficacy. We assumed that these 4 females would (without boosting) have become pregnant during autumn 2006, and we classified them as pregnant for the 2007 efficacy analysis. Four other GonaCon-treated females were recaptured and reinjected with GonaCon in June 2007, after their reproductive status for spring 2007 had already been determined through observations of udder condition (1 of 4 had lactated in 2007; status was confirmed at necropsy).

Fawns and males.—To investigate the effects of GonaCon vaccination on young deer treated during their first winter, we captured, vaccinated, and released 8-month-old female fawns ($n = 4$) in February 2006. To determine whether GonaCon would elicit strong enough immune responses to induce infertility in 3–4-month-old fawns, and to establish the duration of such responses via evaluation of antibody titers, we captured fawns (8 F and 8 M) during September–October 2006, vaccinated them with GonaCon, and released them. We captured, vaccinated, and released yearling ($n = 3$) and adult ($n = 7$) males during March 2007 to investigate the effects of wintertime vaccination with GonaCon on antibody and testosterone levels, testis size, and antler development in male white-tailed deer. Finally, to provide baseline data on untreated males, we collected previously uncaptured males (4 yearlings and 9 ad) during October 2007 for necropsy evaluation.

Evaluation of Contraceptive Efficacy

We evaluated the contraceptive efficacy of GonaCon vaccine in experimental female deer during 2006 and 2007 by using spring and summer field observations of udder condition in vaccinated and control deer to identify animals that were lactating. Lactation was used as an indicator of imminent parturition (i.e., current pregnancy) or recent pregnancy (Whitten 1995, Pojar and Bowden 2004, Gionfriddo et al. 2009). By repeatedly observing the udders of deer throughout the fawn-bearing and fawn-rearing seasons in 2006 and 2007, we were able to determine which individuals became pregnant, although we were unable to tell how many fawns were born to each female, or whether the fawn(s) survived. We used 1-tailed Fisher's exact tests ($\alpha = 0.05$, 1 df) to compare, for each year, the proportion of GonaCon-treated females that lactated with the proportion of control females that lactated. The experimental unit was the individual deer.

Serum concentrations of anti-GnRH antibodies also can be used to assess immunocontraceptive efficacy (Miller et al. 2000). We used enzyme-linked immunosorbent assays (ELISAs) to measure serum antibody titers (Muller et al. 1997) in blood collected from vaccinated and control deer of both sexes at the conclusion of field activities in October 2007. We collected blood samples by cardiac puncture within 1 min of death after deer were shot in the head or neck with a high-powered rifle as they rested or foraged (no chase). We stored blood samples in a cooler or refrigerator until we isolated serum by centrifugation and shipped it to the National Wildlife Research Center in Fort Collins, Colorado, USA for analysis. An experienced wildlife veterinarian conducted field necropsies by following standard procedures described by Munson (2010). Examination of reproductive tracts during necropsy provided additional information on the reproductive status of study deer.

We investigated GonaCon's effects on reproductive status in male deer through necropsy observations and evaluation of blood serum in GonaCon-treated and noninjected control males. At necropsy in October 2007, we measured testicular circumferences to the nearest 0.5 cm and characterized the development of antlers (e.g., "buttons," "spikes," "2 × 2"). Later, we evaluated serum anti-GnRH antibody levels and testosterone concentrations via ELISAs. We then used 1-tailed, 2-sample t-tests ($\alpha = 0.05$) to compare mean testosterone levels and testicular circumferences of vaccinated and control males. All aspects of this research were approved by the Institutional Animal Care and Use Committee of the National Wildlife Research Center.

RESULTS

Adult Females

By June 2006, 8 of 32 GonaCon-treated and 2 of 15 control females had died or left the study site (Table 1). Field evaluations of udder condition were conducted during the summers of 2006 and 2007 by 2 independent observers. Because their conclusions were 100% in agreement when the same deer was studied ($n = 55$), the findings were considered conclusive when only one observer was able to

Table 1. Reproductive status of GonaCon-treated and control adult, female, white-tailed deer in New Jersey, USA, 2006–2007.

Treatment group	n (2005)	n (2006)[a]	Pregnant (2006)	Not pregnant (2006)	n (2007)[b]	Pregnant (2007)	Not pregnant (2007)
GonaCon	32	24	8 (33%)[c]	16 (67%)	23	13 (57%)[d]	10 (43%)
Control	15	13	12 (92%)[c]	1 (8%)	12	10 (83%)[d]	2 (17%)

[a] By Jun 2006, 8 GonaCon-treated and 2 control deer were no longer on the study area. Three deer (2 GonaCon-treated and 1 control) had been killed by collisions with motor vehicles, and 5 (4 treated and 1 control) deer had died of unknown causes. In addition, 2 treated deer had apparently left the study area.

[b] By Jun 2007, 2 additional deer (1 treated and 1 control) had died of unknown causes. The treated deer that died was 1 of the 2 treated deer (mentioned above) that had left the study area before summer 2006. The other treated deer that had left the study area could not be located in 2007. All 8 deer given booster vaccinations in Oct 2006 and Jun 2007 were included in the 2007 analysis (5 were pregnant).

[c] Rates of pregnancy were higher in control F than in GonaCon-treated F during 2006 (Fisher's exact test, $P < 0.001$).

[d] Rates of pregnancy were not higher in control F than in GonaCon-treated F during 2007 (Fisher's exact test, $P = 0.112$).

study a deer ($n = 14$). During summer 2006, 8 (33%) of the 24 remaining vaccinated females and 12 (92%) of the 13 control females lactated (Table 1). The pregnancy rate among control deer was nearly 3 times that of GonaCon-treated deer (Fisher's exact test, $P < 0.001$).

During summer 2007, 13 (57%) of 23 GonaCon-treated females and 10 (83%) of 12 control females lactated (Table 1). Necropsy observations in October 2007 confirmed the lactational status of these deer, except female number 14, who had stopped lactating by October. Although the pregnancy rate among control deer during 2007 was more than 45% higher than that of GonaCon-treated deer, the difference was not significant (Fisher's exact test, $P = 0.112$).

Anti-GnRH antibody titers ranged from 0 ($n = 2$) to 1:128,000 ($n = 5$) in GonaCon-treated adult females 2 years after treatment. Fifteen (83%) of the 18 vaccinated adult female deer we evaluated had titers $\geq$1:16,000, including 3 females that were given booster injections during the field study (deer no. 17 and 29 in Oct 2006, and deer no. 16 in Jun 2007, all of which had antibody titers of 1:128,000). All 12 control females had titers of 0.

Both ovaries were small and inactive in 8 (31%) of 26 adult deer at necropsy in October 2007; 7 of these 8 deer had been injected with GonaCon, and 6 of the 7 vaccinates had not lactated during 2006 or 2007. (Two of these 7 deer had received booster injections after summer 2006.) Ovaries of all but one control deer were normal in size and contained small follicles. Uterine size was within normal limits at necropsy in all 16 examined GonaCon-treated adult females and in 9 of 10 examined control adult females.

Fawns (Females, 8 Months Old)

Of the 4 female fawns treated in February 2006, 1 left the study site soon after vaccination and did not return. None of the other 3 lactated as a yearling in 2006. Two did not lactate as 2-year-olds in 2007; only 1 of these 2 deer (no. 76) was necropsied in 2007 and it had small, inactive ovaries, an enlarged uterus, and an anti-GnRH antibody titer of 1:128,000. The third deer (no. 78) lactated in 2007 and had normal ovaries with small follicles, a small, inactive uterus, and an antibody titer of 1:64,000.

Fawns (Females and Males, 3–4 Months Old)

Of the 8 female fawns treated in September–October 2006, 2 died of unknown causes before summer 2007. The remaining 6 females did not lactate as yearlings during summer 2007. Five of these 6 females were collected in October 2007, when they had anti-GnRH antibody titers ranging from 0 to 1:64,000. Necropsies of 4 of these deer revealed that ovaries were small and/or inactive at necropsy in 2 deer, and ovaries contained small follicles in 2 other deer. Two of these 4 deer had small, inactive uteri (one had small ovaries and one had normal ovaries with small follicles).

Seven of the 8 males treated as 3–4-month-old fawns survived to the end of the study and were collected and necropsied in October 2007. Testicular circumferences and serum testosterone concentrations did not differ between these males and control yearling males (circumferences: $t_5 = 1.75$, $P = 0.070$; testosterone: $t_7 = 0.01$, $P = 0.494$), or between these males and GonaCon-treated adult males (circumferences: $t_7 = -1.01$, $P = 0.172$; testosterone: $t_5 = -1.70$, $P = 0.073$).

Adult Males

Of 10 yearling and adult males treated during March 2007, 2 died of unknown causes and 2 left the study site before October 2007. One of the deer that left the site was observed with large, normal-appearing antlers during late June 2007. Four of the 6 males that remained on site had normal, hardened antlers, and testes of normal size (16.0–19.5 cm in circumference). In contrast, 2 others had antlers that were still in velvet and smaller testes (2-sample t-test, $t_3 = -10.55$, $P < 0.001$; testicular circumferences: 9.0 cm and 9.5 cm). Overall, however, mean testicular circumferences did not differ ($t_5 = -1.43$, $P = 0.102$) between males treated as adults in March 2007 and a group of control adults. Mean serum testosterone concentrations also did not differ ($t_9 = -0.13$, $P = 0.449$) between these 2 groups. Anti-GnRH antibody titers were consistently low ($\leq$1:8,000) in males that had hardened antlers, normal testes, and moderate testosterone levels, and they were consistently high ($\geq$1:32,000) in males that had antlers retaining velvet, small testes, and relatively low testosterone levels.

Control Males

Thirteen previously untreated males were collected for necropsy during October 2007 for comparison (see above) with GonaCon-treated males. Nine were adults with antlers normal in appearance and scrotal circumferences ranging from 15.5 cm to 20.0 cm. The other 4 males were yearlings with normal spiked ($n = 3$) or button ($n = 1$) antlers. All antlers lacked velvet and had hardened. Testicular circumferences in control yearlings ranged from 13.5 cm to 17.0 cm and did not differ ($t_3 = -1.42$, $P = 0.126$) from those of control adults. Serum testosterone concentrations were lower in control yearlings than adults ($t_7 = -2.55$, $P = 0.018$). Anti-GnRH antibody titers were not measured, but were assumed to be 0 in these noninjected males.

DISCUSSION

Induced Infertility in Female Deer

GonaCon Immunocontraceptive Vaccine induced temporary infertility in a large proportion of treated adult female white-tailed deer. The strength and duration of immune responses to immunocontraceptive treatment varied among treated individuals in this study and in previous studies (Miller et al. 2008*a*; Gionfriddo et al. 2009). In the current study, 67% of GonaCon-treated adult female white-tailed deer were infertile 1 year after vaccination, and 43% were infertile after 2 years. In a similar field study in Maryland, 88% of GonaCon-treated white-tailed females were infertile after 1 year, and 47% were infertile after 2 years (Gionfriddo et al. 2009). Captive adult female white-tailed deer ($n = 5$) treated with GonaCon at Pennsylvania State University remained infertile for 2 years, and 4 of 5 deer were infertile 5 years after treatment (Miller et al. 2008*a*). Six of 10 captive adult female black-tailed deer (*Odocoileus hemionus hemionus*) in Washington State, USA, were infertile for 3 years after

each received an injection of GonaCon vaccine (Perry et al. 2008).

Treatment of young (3–4-month-old) female fawns in our study did not induce a strong, sustained immune response required for contraception, a finding supported by Miller et al. (2008*b*). This result suggests that the immune systems of fawns may be insufficiently mature for immunocontraception, and that operational treatment of deer with GonaCon vaccine should focus on adult females. Nonetheless, experimental treatment of fawns to determine contraceptive efficacy is important because future wildlife contraceptive agents could be delivered orally, and treated bait may be consumed by deer of all ages.

The level of circulating anti-GnRH antibodies is a good indicator of the fertility status of an animal treated with an immunocontraceptive agent (McShea et al. 1997). Other than infertility, no side effects are associated with the presence of antibodies against GnRH (Delves 2002). In most treated animals, fertility is naturally regained after a decline in antibody titers (Keeling and Crighton 1984).

The immune response must reach and maintain a threshold level for contraception to occur (Adams and Adams 1990, Zeng et al. 2002). In white-tailed deer, infertility is normally associated with anti-GnRH antibody titers $\geq$1:64,000 (Miller et al. 2000, 2008*a*; Gionfriddo et al. 2009). In the current study, 27 months after vaccination, titers were $\geq$1:64,000 in 4 (29%) of 14 nonboosted GonaCon-treated adult females, all of which were infertile in 2006 and 2007. Of the 5 examined females that were vaccinated in September–October 2006 as 3–4-month-old fawns, 1 (20%) had a titer $\geq$1:64,000. Although these 5 deer were vaccinated too late in the 2006–2007 reproductive cycle to affect reproduction that year, they were likely too young to breed until (at least) autumn 2007. Both of the females that had been vaccinated at 8 months of age and remained alive on the study site had titers $\geq$1:64,000, although one of them was pregnant in 2007.

Severe overbrowsing was evident throughout the study area when the study began. Most deer captured in July 2005 were noticeably thin, with body masses estimated at 32–57 kg. Healthy adult female white-tailed deer in the northeastern United States generally weigh 41–96 kg (Whitaker and Hamilton 1998). Fat reserves in study deer at necropsy in 2007 were generally meager (Gionfriddo et al. 2011). The relationship between nutrition and immune response has long been recognized (Homsy et al. 1986, Chandra and Amorin 1992), and it is possible that stronger immune responses (and greater contraceptive efficacy) may have been achieved if study deer had been in better physical condition. As a practical matter, however, most overabundant deer populations that would be candidates for treatment with contraceptive agents would likely have experienced suboptimal nutrition.

Small and inactive ovaries and uteri, as observed in many of the GonaCon-treated females in this study, are commonly associated with GnRH immunocontraception and are indicators of successful contraception. GonaCon induces the production of anti-GnRH antibodies that bind to endogenous GnRH and prevent the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland. In the absence of LH and FSH, the gonads remain quiescent, production of sex hormones is sharply reduced, and the animal remains in a nonreproductive state.

The administration of booster vaccine injections to several of our adult female study deer provided an opportunity to evaluate the effect of such injections on contraceptive efficacy. For example, females numbered 17 and 29 were each given a 1.0-mL booster injection of GonaCon in October 2006 after lactating during summer 2006. Neither female lactated in 2007; both females had very high anti-GnRH antibody titers (1:128,000) when collected in October 2007, suggesting that the booster injections had stimulated strong immune responses. Deer number 28 followed a similar pattern. After her pregnancy in summer 2006, she was boosted in October and then did not lactate in 2007. Unfortunately her carcass was too badly decomposed when it was found in September 2007 to permit blood collection or necropsy examination. In contrast to these examples, a booster injection did not seem to be effective in deer number 5. She lactated in summer 2006, was boosted in October 2006, and lactated again in 2007. Her antibody titer was 1:32,000 in October 2007. Booster vaccine injections seem to increase or extend contraceptive efficacy in some, but not all, cases.

Responses of Male Deer to Vaccination

Although mean values for testicular circumferences and serum testosterone concentrations did not differ between GonaCon-treated and control males, vaccination with GonaCon affected reproductive parameters in some adult males (no. 81 and 82), which, 12 months after vaccination, had small testes, negligible testosterone concentrations, and antlers that retained velvet into October. In contrast, treated adult males 87, 88, and 90 had "normal" values for these parameters when compared with control adult males. Anti-GnRH antibody titers in males treated with GonaCon as 3–4-month-old fawns were similar to those of same-aged females and support the hypothesis that immunocontraception of young fawns may not be feasible.

In our study, deer were collected for necropsy in early October when testosterone concentrations normally increase as the rut approaches (Mirarchi et al. 1977). Treatment with GonaCon vaccine typically reduces the production of testosterone and growth of testes (Killian et al. 2005), and produces males that resemble morphological castrates. Thus it was not surprising that 2 treated deer had smaller testes and lower testosterone concentrations than untreated deer. Curtis et al. (2008) reported similar results for free-ranging male white-tailed deer treated with GnRH vaccines. We agree with Killian et al. (2005) and Curtis et al. (2008) that male deer should not be treated with GnRH vaccines because of potential social consequences for treated individuals.

MANAGEMENT IMPLICATIONS

Successful management of overabundant suburban deer populations requires an initial reduction in numbers to a desired

density, and then maintenance of that density (Merrill et al. 2006). Contraceptive efficacy rates demonstrated for GonaCon vaccine in New Jersey support the contention that the most efficient approach to managing overabundant suburban deer populations may be to combine culling (for initial population reduction) and contraception (for population stabilization; Bomford 1990, Hobbs et al. 2000). Once deer numbers have been reduced to a desired level, population stability can be achieved by preventing about 60–80% of the breeding females from reproducing each year (Garrott 1995, McCullough 1996). Such levels of infertility should be achievable through rigorous application of GonaCon vaccine. Timely reduction of overabundant deer populations using contraception alone, however, is not feasible at current efficacy rates.

The need for safe, effective, practical, and humane wildlife contraceptive agents to manage overabundant wildlife populations in situations where traditional management methods cannot be applied will continue to drive the development of contraceptive products. Immunocontraception of white-tailed deer shows promise as a tool for stabilizing demographically closed populations, but improvements may be needed if this technology is to be accepted and applied widely in real-world settings by natural resource professionals. Greater contraceptive efficacy (i.e., a higher proportion of treated animals rendered infertile and a longer duration of contraceptive effect) is a goal of ongoing research.

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IMMUNOCONTRACEPTION, GENETIC MANAGEMENT, AND DEMOGRAPHY OF FERAL HORSES ON FOUR EASTERN U.S. BARRIER ISLANDS

by

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IMMUNOCONTRACEPTION OF FERAL AND CAPTIVE HORSES USING AN ANTI-LHRH VACCINE[1]

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Abstract: We injected captive mares (n = 4 per treatment group) in 1988 with a microencapsulated anti-luteinizing hormone releasing hormone (LHRH) vaccine that either was precipitated on alum (AP) or emulsified with a triple adjuvant (TA). Control mares (n = 4 per group) received placebos containing vaccine without LHRH. The AP vaccine was less effective than the TA vaccine and was eliminated from further study. Mares treated with the TA vaccine produced elevated antibody titers against LHRH and demonstrated less follicular development during 11-13 weeks after treatment; all treated mares ovulated after LHRH antibody titers declined, and 3 mares that were bred produced at least 1 conceptus each. Treatment appeared only to delay onset of breeding in treated mares compared to controls. Mares that received the microencapsulated TA vaccine in 1988 produced slight elevations in antibody titers when the 1989 booster vaccines were adminstered remotely via biobullet. Little antibody production was observed in a third group of captive mares (n = 4) that received initial vaccine and a single booster administered via biobullet in 1989. Based on preliminary results from the corral study, we treated 21 feral mares with a series of 2-3 biobullets containing TA vaccine from 1988-1989. No differences in foal production or mortality were noted between treated and untreated feral mares. We suggest that our TA vaccine and other vaccines that require a number of boosters to maintain high antibody titers are not suitable

for use in field sterilization programs at their current level of development. We provide recommendations for future immunocontraception reseach and suggest long-acting sterilization techniques that could be implemented in feral horse populations until more efficient, reversible, remotely-delivered vaccines are developed.

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Key words: barrier islands, conservation biology, Equus caballus, feral horses, immunocontraception, immunosterilization, LHRH, biobullets.

A number of barrier islands along the U.S. Atlantic coast support feral livestock that forages on dune and marsh vegetation. Excessive grazing on sea oats (Uniola paniculata) and other dune-stabilizing vegetation can result in poorly developed frontal dunes and produce conditions that favor movement of sand landward from previously stable secondary dunes (Hillestad et al. 1975). Such dune changes can affect nesting habitat of loggerhead sea turtles (Caretta caretta) and deplete wetland habitat leeward of the dunes (Hillestad et al. 1975). Grazing and trampling by feral horses in salt marshs can alter primary production of smooth cordgrass (Spartina alterniflora), affect detritus production, reduce fiddler crab (Uca pugnax) densities, and change successional patterns (Ranwell 1961, Riemold et al. 1975, Turner 1988).

Feral cattle, sheep, and goats have been removed from most coastal islands. However, feral horses are maintained on Assateague Island, Virginia and Maryland (Chincoteague National Wildlife Refuge and Assateague Island National Seashore); Ocracoke Island, North Carolina (Cape Hatteras National Seashore); Shackleford Banks, North Carolina (Cape Lookout National Seashore); Carrot Island, North Carolina (Rachel Carson National Estuarine Research Reserve); and Cumberland Island, Georgia (Cumberland Island National Seashore). Currently, only the corralled population on Ocracoke Island and the privately owned population on the Chincoteague National Wildlife Refuge are managed.

Shooting, poisoning, and other lethal methods are banned on many public lands or may not be acceptable to the public to control feral horse herds. Corralling or relocating excess animals, as practiced with feral horses in the western U.S. and on the Chincoteague National Wildlife Refuge, is costly and may be highly stressful to the animals. Recently, fertility control has been suggested as an alternate, non-lethal method of feral horse regulation.

A male-based method of fertility control could be effective if dominant stallions retain herds for relatively long periods and if subordinate and bachelor males have limited access to harem females. Kirkpatrick et al. (1982) successfully reduced feral mare productivity in harems

controlled by stallions treated with microencapsulated testosterone propionate. Immobilized animals were injected with the treatment by hand; remote delivery, via dart, has not been successful (Kirkpatrick et al. 1990a). Vasectomy also has been used to control population growth rates in feral equids without affecting libido of the treated stallions (McCort 1980). In many horse populations, however, the effectiveness of male sterilization may be limited by unstable band composition and the potential for breeding by subordinate or bachelor males (Nelson 1980).

In most female mammals, reproductive processes dependent on a dynamic hormonal balance can be blocked by treatment with specific chemicals that affect release of reproductive hormones, ova fertilization, or embryo emplantation. In feral mares, silastic rods containing ethinylestradiol (EE_2) or EE_2 and progesterone reduced foal production over a 3-year period (Vevea et al. 1987, Plotka et al. 1989). Intraperitoneal implantation of the compounds, however, required animal immobilization and field surgery, which are costly and stress the animal. More recently, Kirkpatrick et al. (1990a) suppressed feral mare fertility for a year through active immunization against zonae pellucidae antigens. Vaccine was delivered remotely, via dart.

Active immunization against endogenous LHRH successfully blocked ovulation in laboratory rats and rabbits; one immunization was sufficient to stimulate

production of LHRH antibodies to LHRH, resulting in highly reduced or nondetectable levels of LH and FSH in the serum and anterior pituitary (Fraser 1975, Fraser and Gunn 1973, Fraser et al. 1975, Fraser and Baker 1977). Similar immunizations against LHRH before or at the onset of the breeding season prevented ovulation in sheep (Crighton and Foster 1977, Clarke et al. 1978, Adams and Adams 1986), gilts (Esbenshade and Britt 1985), and cattle (Johnson et al. 1988).

Domestic mares treated with LHRH conjugated to bovine serum albumin and emulsified in Freund's Complete Adjuvant (FCA) demonstrated reduced ovarian weights, number of follicles > 10 mm, and number of corpora lutea (Garza et al. 1986). Safir et al. (1987) treated domestic mares with LHRH conjugated to human serum albumin and emulsified in FCA. Three of 5 treated mares ovulated, despite receiving 4 boosters within 29 weeks after the study began. Schanbacher and Pratt (1985) administered a similar vaccine to a cryptorchid stallion, followed by 3 boosters within 2 months; antibody titers were sufficient to block testosterone production 4-6 months after the last booster.

None of the sterilization methods developed to date for feral horses meet the major criteria established by Turner and Kirkpatrick (1986) for an ideal fertility control method: seffective, safe, long-lasting but reversible, inexpensive, and delivered remotely. Our study was initiated to meet 2 major objectives: (1) to develop

and evaluate the immunological and physiological effects of selected contraceptive vaccines in captive mares, and (2) to evaluate the feasibility and effectiveness of these vaccines administered with a biobullet delivery system in a field situitation.

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METHODS

Study Areas

Corral studies were conducted at the Horse Research Center, University of Florida, Gainesville. Field studies were conducted on the feral horses of Cumberland Island, the largest and most southern of Georgia's barrier islands. Cumberland Island is about 28 km long, 0.8-4.8 km wide, and covers an area of 95.5 km^2. Eighty percent of the island is organized as the Cumberland Island National Seashore and includes a large area of federally designated wilderness.

Lenarz (1983) counted 144 horses on Cumberland Island in July 1981 and estimated that the population could increase 5-21% annually. A minimum of 154, 181, and 197 horses were counted in the March 1983, May 1985, and May 1987 censuses, respectively (Ambrose et al. 1983, Finley 1985, Koontz 1989). The population increased to an estimated 220 horses by September 1990 (Goodloe 1991).

Treatment Selection and Preparation

Our primary objective when evaluating potential immunocontraception methods was selection of a compound that provided long-term sterility and that could be delivered remotely via biobullet to enhance applicability and safety in field situations. Of secondary importance

was use of an adjuvant that did not cause adverse reactions at the injection site. Freund's Complete Adjuvant (FCA) has been used in most previous studies to enhance immune response to the antigen, but it is known to stimulate potentially life-threatening pyogenic abscesses that may affect survival of free-ranging animals.

The most promising technique we considered that met these requirements was active immunization against endogenous LHRH. Anti-LHRH vaccines, when conjugated to a variety of adjuvants, have successfully inhibited estrus and/or ovulation in domestic horses (Schanbacher and Pratt 1985, Garza et al. 1986, Safir et al. 1987) and a variety of other species (see Fraser 1985 for review). Our vaccine incorporated either alum or a lyophilizable triple adjuvant (100 mg each of monophosphoryl lipid A, trehalose dimycolate, and BCG cell wall skeleton; prepared by Ribi ImmunoChem Research, Inc., Hamilton, Mont.) to reduce the possibility of injection site abscesses and allow biobullet delivery.

Our immunogen was prepared with the carbodiimide reaction (Fraser et al. 1974) to conjugate LHRH to keyhole lempet hemocyanin (KLH) (prepared by Pitman-Moore, Inc., Terre Haute, Ind.). Two different vaccines were created by either precipitating the immunogen on alum (AP) or combining the immunogen in a 2% oil-in-water emulsion with the triple adjuvant components (TA). Two methods of vaccine delivery were used to treat corralled mares--hand

injection of microencapsulated (MC) vaccine in 1988 and biobullet (BB) implantation of vaccine in 1989. All feral horses were administered vaccine via biobullet.

Biobullets, which delivered a single-dose treatment that provided 15 mg LHRH immunogenic activity, were prepared by inserting the lyophilized vaccine into the tip of a 6.53-mm (0.25 caliber) hollow biodegradable bullet and sealing with a lactose plug (prepared by BallistiVet, Inc., Minneapolis, Minn.). Microencapsulated formulations were prepared by the solvent-evaporation process as described by Tice and Gilley (1985). The reservoir microcapsules were designed to release 1 vaccine dose immediately upon injection, 1 dose in 2-3 months, and a final dose in 6-9 months. Placebo treatments consisted of vaccine without LHRH. The microencapsulated vaccine exceeded the biobullets' weight capacity (500 mg) and was injected by hand.

Treatment and Sampling of Corralled Animals

1988.--Sixteen mares were assigned randomly to 1 of 4 experimental groups that were administered the following microencapsulated vaccines or placebos: (1) triple-adjuvanted LHRH-KLH treatment (MC88-TA-LHRH-KLH); (2) alum-precipitated LHRH-KLH treatment (MC88-AP-LHRH-KLH); (3) triple-adjuvanted KLH placebo (MC88-TA-KLH); and (4) alum-precipitated KLH placebo (MC88-AP-KLH). On 31 January 1988, each animal was injected at a single location in the

hindquarters with the microencapsulated, triple-dose formulation suspended in 3 ml of sterile injection vehicle.

1989.--On 13 April 1989, the 4 mares previously treated with MC88-TA-LHRH-KLH in 1988 received a single booster of vaccine, injected into the hindquarters via biobullet (BB89-MC88-TA-LHRH-KLH group). No placebo biobullets were administered to the control horses (MC88-TA-KLH group). In addition, a new treatment group (BB89-TA-LHRH-KLH) was established in 1989 to evaluate the effectiveness of biobullet administration of vaccine. Each of 4 mares (3 mares from the MC88-AP-KLH placebo group and 1 previously untreated mare) received a single biobullet containing TA-LHRH-KLH on 13 April 1989, followed by a booster bullet on 3 June 1989.

Sampling Procedure.--Blood samples were collected from all mares by venipuncture at about weekly intervals from 24 January-27 August 1988 and 13 April-14 July 1989. Samples were refrigerated until plasma could be separated and frozen. Plasma titers of LHRH antibodies were determined by indirect ELISA tests using LHRH-coated plates. Antibody titers in samples collected during the first 11 weeks after the 1988 treatments initially were assayed as serial dilutions to compare the AP and TA vaccines and select the most effective for use in the field portion of the study. All blood samples collected from TA-LHRH-KLH and TA-KLH mares during the 2 years of the study, including those samples tested previously, were analyzed for antibody titer

in a single run at the conclusion of the study to standardize results; titers were measured in units of optical density to allow more efficient statistical analysis of antibody data. Plasma concentrations of LH and FSH were quantified by radioimmunoassay using anti-ovine LH antisera and anti-equine FSH antisera, in conjunction with purified equine LH and FSH for radioiodination and standard preparation (Thompson et al. 1983a, 1983b). Insufficient blood was available from 1988 samples for hormone analysis.

From 5 June through 5 September 1988 and 6 May through 19 July 1989, mares were teased daily with a stallion to detect estrus and palpated rectally when estrus was detected to assess ovarian follicular growth and incidence of ovulation. Most mares were inseminated artificially or bred to stallions of known fertility upon display of estrus or detection of ovarian follicles $\geq$ 30 mm in diameter. Daily breeding continued until ovulation occurred, and all mares were checked for pregnancy with ultrasound echography daily beginning on Day 10 after ovulation. Conceptuses were flushed from the uterus on either Day 12 or Day 14 after ovulation was confirmed, and mares were administered 5 mg prostaglandin F2α (Lutalyse; Upjohn Co., Kalamazoo, Mich.) to regress the corpus luteum.

Treatment and Observation of Feral Horses

Treatment.--We conducted an initial survey of the Cumberland Island feral horse population in 1986 and 1987 to census the population, identify and age animals, and

determine pretreatment reproductive rates. Animals were identified by face and body markings and scar patterns. In addition, 10 feral mares, primarily on the northern half of the island, were immobilized between November 1986 and February 1987 (Goodloe 1991) and fitted with radio collars to assist in identification and location of herds. Ages assigned to animals in 1986 were based on body size, tail length, and muscle mass. Young horses (excluding known foals) were aged as yearlings if body size was small and the tail did not extend past the hocks. Larger, more muscular horses were considered to be 2 years old if the tail did not reach the hoofs.

Between 10 March and 2 May 1988, 21 mares were treated with biobullets containing single doses of TA-LHRH-KLH. Booster biobullets were administered to 20 of 21 mares between 19-26 July 1988 (1 mare was on private property during the treatment period), and to 19 of 21 mares between 3 February and 4 March 1989 (1 severely injured mare was not treated and another mare was not located during the treatment period).

Throughout the study, untreated mares thought to be $\geq$ 3 years old in 1987 were considered as controls but were not administered placebo biobullets. Nineteen mares treated with TA vaccine in 1988 and 1989 and 2 untreated control mares were administered unadjuvanted LHRH-KLH immunogen in 1987. However, identical treatments given to corralled mares in 1987 failed to stimulate detectable

levels of LHRH antibody or to reduce ovulation and productivity rates (Goodloe et al. 1991). Therefore, we included feral mares present on Cumberland Island in 1987 in pretreatment tallies of reproduction.

Observation after Treatment.--After initial surveys of the Cumberland Island population, we observed island feral horses intensively from March through September 1988 and March through August 1989 to document foal production and survival rates. Data also were collected during 0.5-2-week-periods in January, October, and December 1988 and February, November, and December 1989. Estimates of 1990 foal production were based on observations conducted in May and September 1990 and were augmented by analysis of fecal and urine samples collected from late July through December 1989, as described by Kirkpatrick et al. (1988, 1990c). Fecal and urine samples were collected immediately after deposition and subsequently frozen.

Information on total annual production and foal survival was collected through direct counts of live foals and searches for carcasses when foal death was suspected. We assumed that foals were born, but died before being tallied, if a mare had a fully distended udder at any point during the foaling season or appeared visibly pregnant up to a given time, particularly if a foal carcass was found in the herd's home range. Unproductive mares that were not located at frequent intervals during a given year were excluded from calculations of foal production, because the

possibility of foal death could not be eliminated. Mares that died during the study were included in counts of total foal productivity if a necropsy was conducted or if they were visibly pregnant at death; however, foals that died in utero were not included in mortality calculations. Date of foal birth was estimated as the midpoint between the dates when the mare was seen last without, and for the first time with, a foal; if this interval was > 2 weeks, then the foal was not included in estimates of mean date of birth. When foal death was suspected, date of birth was considered to be the last day the mare was observed with a full udder or the day when a fresh carcass was found. We observed a total of 21 treated and 39 untreated mares during the 4-year study (21 and 38 in 1987, 21 and 29 in 1988, 21 and 26 in 1989, and 20 and 23 in 1990.

Statistical Analysis

Antibody titers of corralled horses, measured in units of optical density (OD; from mares administered TA vaccines), were analyzed by repeated measures analysis of variance (Gill and Hafs 1971); the mean pretreatment values for each horse were designated as covariances in the model. Student's t-tests were used to compare mean date of first follicle $\geq$ 30 mm, mean date of first ovulation, and mean number of ovulations between treatment groups and the control group.

Differences between conceptus production in treated and untreated corralled mares and foal production and

survival in treated and untreated feral mares were evaluated by 2X2 contingency tables with Chi-square tests (Sokal and Rohlf 1969). Student's t-tests were used to compare mean dates of foal birth between treated and untreated groups. Statistical significance was indicated at $P \leq 0.05$.

RESULTS AND DISCUSSION

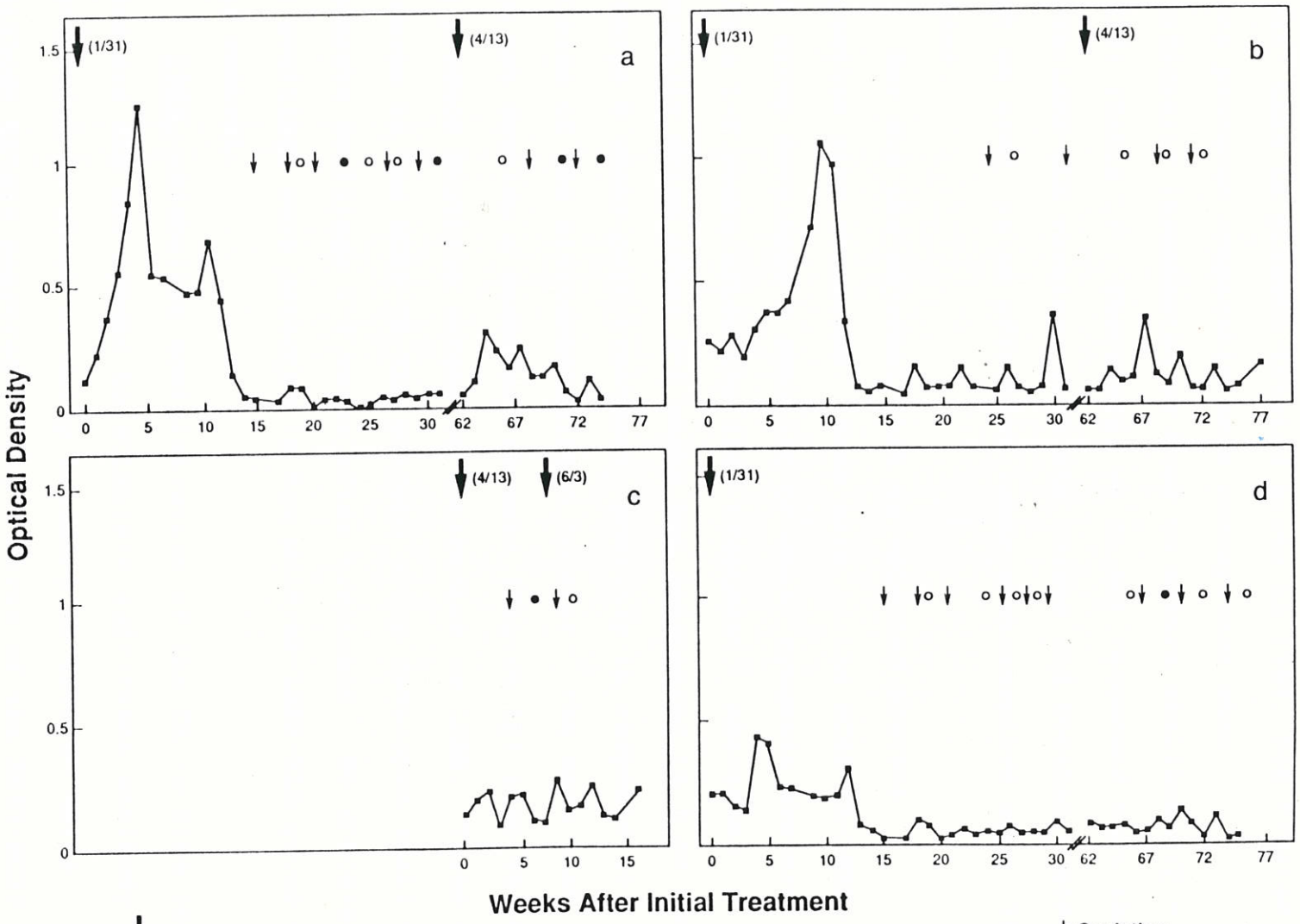
Corral Study

1988.--Preliminary results during the first 11 weeks after treatment indicated that the AP vaccine was less effective in stimulating LHRH antibody production than the TA vaccine. Antibody levels in mares treated with MC88-AP-LHRH-KLH rose to maximum titers of 1:200-1:300, which were only 2-6 times greater than levels produced by mares administered the MC-AP-KLH placebos. Peak titer levels (maximum titers of 1:300-1:3200) for the MC88-TA-LHRH-KLH group were 2-42 times higher than those produced by their controls (MC88-TA-KLH horses) during the same period. Three of the mares in the MC88-AP-LHRH-KLH group produced follicles $\geq$ 30 mm on average 50.3 days (SD = 22.5) after initial treatment, compared to a mean of 44.5 days (SD = 12.1) for MC88-AP-KLH control mares and 111.5 days (SD = 29.0) for mares treated with the TA-LHRH-KLH vaccine. The fourth mare in the MC88-AP-LHRH-KLH group first ovulated when the follicle was < 30 mm. Based on these preliminary results, the AP treatment was eliminated from the study, and all further efforts focused on the TA-LHRH-KLH vaccine.

Mares treated with MC88-TA-LHRH-KLH tended to produce higher antibody titers (expressed in units of optical density) than did mares administered placebos (P = 0.075) through Weeks 11-13 after treatment (Fig. 1a, b, and d). Differences in antibody production were not significant (P = 0.880) during Weeks 14-26 after treatment or when antibody production was compared over the entire 28 weeks of the 1988 study (0.121). Mean titers for the 4 MC88-TA-LHRH-KLH horses during the 13-week period after treatment were 0.351-0.726 OD (SE = 0.171; minimum - 0.041-0.084; maximum - 0.808-1.361). Three mares produced bi- or tri-phasic elevations of antibody levels > 0.69 OD at 1-7-week intervals (Fig. 1a), although the response of one was lower and more transitory than in the other animals. The fourth horse produced only a single surge of antibody at Week 9 (Fig. 1b). Similar variation in horse response to anti-LHRH vaccines was reported by Safir et al. (1987). Mean antibody titers for control mares during the first 13 weeks after treatment (Fig. 1d) ranged from 0.195-0.239 OD (SE = 0.018; minimum - 0.030-0.047; maximum - 0.414-0.524).

Despite the relatively high titers of LHRH antibodies after treatment, all mares in the MC88-TA-LHRH-KLH group cycled during the 1988 breeding season, and each of the 3 mares that were bred or artificially inseminated produced at least 1 conceptus. At best, our treatments only delayed breeding--the MC88-TA-LHRH-KLH mares tended to produce large follicles $\geq$ 30 mm later (P = 0.051) and to first

Figure 1. Antibody production by corralled mares treated with TA-LHRH-KLH vaccine in 1988 and 1989. Figure 1a and 1b represent mares treated with MC-TA-LHRH-KLH on 31 January 1988, followed by a booster administered via biobullet on 13 April 1989; Figure 1c represents a mare treated with single biobullet injection of TA-LHRH-KLH vaccine on 13 April and 3 June 1989; and Figure 1d represents a mare administered a microencapsulated placebo (MC-TA-KLH) on 31 January 1988 but no placebo in 1989. Large arrows annotated with dates indicate time of treatments, smaller arrows indicate ovulation dates, open circles indicate administration of 5 mg PGF, and closed circles indicate flushing of a conceptus.


Optical Density
(1/31)
(4/13)
a
b
(6/3)
c
d
0
0.5
1
1.5
0 5 10 15 20 25 30 62 67 72 77
Weeks After Initial Treatment
LHRH Vaccine
PGF Injection
Ovulation
Embryo flush

ovulate later (P = 0.060) than did control mares (Table 1). We detected fewer ovulations in the treated mares (P = 0.045) than in placebo-treated mares in 1988, but treated mares produced comparably more conceptuses when artificially inseminated or bred to mature stallions (P = 0.036) (Table 1). Therefore, we do not recommend that the MC-TA-LHRH-KLH vaccine, with the dosage and booster regime that we used, is an effective immunocontraceptive agent in horses.

Nor did the adjuvant that we selected prevent injection site abscesses. Each mare in the MC88-TA-LHRH-KLH group developed a swelling or small pyogenic abscess within 1-2 weeks after the 1988 treatment. Abscesses generally dried after 3-4 weeks but reappeared in 3 of the 4 mares 8 weeks post-treatment, possibly in response to release of the microencapsulated booster; the mare that did not develop a secondary abscess (Figure 1b) also failed to produce multiple surges of antibodies to LHRH. Three of 4 mares in the MC88-TA-KLH placebo group developed small to moderate swellings 1-2 weeks after treatment. No abscesses or swellings were detected in the MC88-AP-LHRH-KLH group or its control.

1989.--Biobullet administration of the TA vaccine and boosters did not appear to stimulate sufficient antibody production to block or delay breeding in treated mares. Antibody titers measured in the 4 mares of the BB89-MC88-TA-LHRH-KLH group after a booster treatment of TA vaccine

Table 1. Follicular development, ovulation, and production by corralled mares at the University of Florida that were treated in 1988 and 1989 with a TA-LHRH-KLH vaccine.

	1988[a]		1989[b]							
	MC88-TA-LHRH-KLH[c]		MC88-TA-KLH[d]		BB89-MC88-TA-LHRH-KLH[e]		MC88-TA-KLH[d]			
	X	SD	X	SD	X	SD	X	SD	X	SD
No. days, post treatment, for development of follicle > 30mm[f]	111.5	29.0	67.0	22.2	39.5	3.7	36.0	8.5	30.8	4.0
No. days, post-treatment, for first ovulation of year[g]	135.8	29.3	95.0	19.5	41.8	4.4	33.3	8.8	39.8	6.9
Total no. ovulations[h]	4.3	1.3	6.3	1.0	3.0	0.8	2.5	0.6	3.0	0.0
% breedings resulting in conceptus[i]	66.7		12.5		50.0		14.3		50.0	

[a] Means are based on data collected from 24 January through 27 August 1988.

[b] Means are based on data collected from 13 April through 14 July 1989.

[c] Mares were injected with a microencapsulated form of the TA-LHRH-KLH vaccine in January 1988 and administered a single booster via biobullet in April 1989.

[d] Control mares were injected with a microencapsulated placebo containing only the adjuvant and KLH in January 1988. No placebos were administered in 1989.

[e] Mares were administered biobullets containing single doses of TA-LHRH-KLH in April and June 1989.

[f] Differences between mares treated with BB89-MC88-TA-LHRH-KLH and MC88-TA-KLH were significant in 1989 ($P = 0.019$). Other differences were not significant (MC88-TA-LHRH-KLH vs. MC88-TA-KLH in 1988, $P = 0.051$; BB89-TA-LHRH-KLH vs. MC88-TA-KLH in 1989, $P = 0.308$).

[g] No differences in means were significant (MC88-TA-LHRH-KLH vs. MC88-TA-KLH in 1988, $P = 0.060$, BB89-MC88-TA-LHRH-KLH vs. MC88-TA-KLH, $P = 0.640$; BB89-TA-LHRH-KLH vs. MC88-TA-KLH in 1989, $P = 0.287$).

[h] Differences between mares treated with MC88-TA-LHRH-KLH and MC88-TA-KLH in 1988 were significant ($P = 0.045$). Other differences were not significant (BB89-MC88-TA-LHRH-KLH vs. MC88-TA-KLH in 1989, $P = 1.0$; BB89-TA-LHRH-KLH vs. MC88-TA-KLH in 1989, $P = 0.134$).

[i] Differences between mares treated with MC88-TA-LHRH-KLH and MC88-TA-KLH in 1988 were significant ($\underline{P}$ = 0.036). Differences among treatment groups in 1989 were not significant ($\underline{P}$ = 0.271).

via biobullet ranged from a maximum of 0.113-0.489 OD to a minimum of 0.028-0.160 OD (Fig. 1a and 1b), much lower than maximum titers the same horses produced in 1988 after treatment with microencapsulated TA vaccine (maximum - 0.808-1.361; minimum - 0.041-0.084). Little response to the TA vaccine was noted in the 4 animals that received initial biobullet treatment in April 1989 followed by a booster biobullet in June (BB89-TA-LHRH-KLH group; Fig. 1c). Maximum titer levels in the BB89-TA-LHRH-KLH animals ranged only as high as 0.112-0.278 OD; these titers were similar to values recorded for 3 of 4 control animals (maximum values of 0.102-0.183 OD; Fig. 1d). The other control mare, in contrast, produced 2 distinct surges of LHRH antibodies that exceeded 0.63 OD.

Overall, LHRH antibody titers produced by the 2 treatment groups did not differ ($P = 0.884$) from the MC88-TA-KLH control group. Mares in the BB89-MC88-TA-LHRH-KLH group produced follicles $\geq$ 30 mm later in the 1989 breeding season than did mares in the MC88-TA-KLH control group ($P = 0.019$) (Table 1). There was no difference between treatment groups and controls for date of first ovulation, number of ovulations, or percentage of mares that produced conceptuses ($P = 0.134-1.0$). Fluctuations of LH and FSH levels in all groups were similar to those described in other studies (Evans and Irvine 1975. Nett et al. 1976).

No abscesses were noted in either treatment group after biobullet delivery. Biobullet administration

appeared successful; entry wounds were observed on all corral mares treated with biobullets, and 64.3% of the mares bled freely after bullet entry.

Field Study

Our conclusion that the TA vaccine had little effect on ovulation and foal production, particularly when delivered by biobullet, was supported by results from the field study on Cumberland Island. Foal production did not differ (Table 2) between mares treated or not treated with TA vaccine during any of the 4 years of observation (1987, $\underline{P}$ = 0.268; 1988, $\underline{P}$ = 0.924; 1989, $\underline{P}$ = 0.258; 1990, $\underline{P}$ = 0.625). In addition, the proportion of productive mares within treated or untreated groups did not differ between years ($\underline{P}$ = 0.128-0.594; the contrast of untreated mares in 1987 vs. 1989 approached significance, $\underline{P}$ = 0.058).

Mortality of foals produced by treated mares in 1987 ($\underline{P}$ = 0.032) and 1988 ($\underline{P}$ = 0.089) was greater than for foals produced by untreated mares. However, more untreated mares (15 of 39; 38.5%) died during the 4-year study than did treated mares (2 of 21; 9.5%) ($\underline{P}$ = 0.037), particularly between September 1987 and March 1988. Mean date of foal production did not differ between treated and untreated mares within years or among years within groups ($\underline{P}$ = 0.125-0.732), indicating that treatment did not significantly delay onset of breeding.

Table 2. Annual production data for feral mares on Cumberland Island, Georgia, before and after treatment with a TA-LHRH-KLH vaccine administered via biobullet in 1988 and 1989. Only females ≥ 3 years old in 1987 are included in these totals.

Category	1987		1988		1989		1990[a]	
	Trt[b]	Con[c]	Trt	Con	Trt	Con	Trt	Con
No. mares	21	38	21	29	21	26	20	23
% productive mares[d]	71.4	55.3	76.2	72.4	61.9	76.9	75.0	65.2
Mean date of foal birth[e]	5/8	5/13	5/22	4/30	5/11	5/1	--	--
No. foals surviving 31 Aug.	8	17	7	16	8	13	10	11
No. foals dead before 31 Aug.	6	1	6	1	4	3	5	4
No. mares with full udder	0	0	2	4	1	1	0	0
No. mares visibly pregnant	0	2	1	0	0	0	0	0
Percent foal mortality[f, g]	42.9	15.0	56.2	23.8	38.5	23.5	33.3	26.7
No. fetuses found in utero	1	1	0	0	0	3	--	--

[a] Determination of production based, in part, on analysis of steroids in fecal and urine samples collected August-December 1989.

[b] Mares were treated with TA-LHRH-KLH via a biodegradable bullet in March-May 1988, and most received booster injections in July 1988 and February-March 1989.

[c] Control mares (no placebo administered).

[d] Differences between mares treated or not treated with TA vaccine were not significant during any year (1987, $P = 0.268$; 1988, $P = 0.924$; 1989, $P = 0.258$; 1990, $P = 0.625$), nor did the proportion of productive mares within treated or untreated groups differ between years ($P = 0.128$-0.594; the contrast of untreated mares in 1987 vs. 1989 approached significance, $P = 0.058$).

[e] Differences between treated and untreated mares within years or among years within groups were not significant ($P = 0.125$-0.732).

[f] Mortality rates of foals produced by treated mares in 1987 ($P = 0.032$) and 1988 ($P = 0.089$) were significantly different from mortality rates of foals produced by untreated mares. Other differences were not significant ($P > 0.05$).

[g] Only those foals that died before 31 August or that were assumed to have been produced by mares observed with full udder development or pregnant during the breeding season were included in estimates of total mortality.

MANAGEMENT RECOMMENDATIONS

We do not recommend that our TA vaccine should be recommended for use in field immunocontraception programs. Corralled mares treated with MC-TA-LHRH-KLH produced elevated anti-LHRH antibody titers that apparently blocked significant follicular development for 11-13 weeks, but all treated mares ovulated after antibody titers declined, and each mare that was bred produced a conceptus. These mares responded only minimally to biobullet boosters administered in 1989. Feral and captive mares that received initial treatment with TA vaccine via biobullet demonstrated no response to the vaccine, either in antibody production or reduced foal production. Our vaccine, therefore, failed to meet the 2 major criteria that we considered were necessary for a field sterilization program--provision of a compound that provided long-term sterility without the need for a number of booster inoculations and delivery of the vaccine effectively via biobullet.

We do not know why the TA vaccine administered via biobullet was less effective at stimulating antibody production than microencapsulated TA vaccine administered by hand. Failure of the majority of the bullets to implant due to poor shooting or inability to penetrate the skin does not seem probable. We observed blood at the implantation site of 57.6% of feral mares administered biobullets; blood was seen on the hindquarters of only 64.3% of the corral mares after treatment, although

biobullet entry holes were observed on all mares upon close examination. It is possible that vaccine was lost from some biobullets before implantation or was flushed from wounds when animals bled. However, the biobullet system has been used successfully by veterinarians for a number of years to deliver vaccines and other chemicals to domestic animals. In addition, it has been applied in field situations to vaccinate free-ranging elk (Cervus elaphus) and bison (Bison bison) against brucellosis (Herriges et al. 1990, Davis et al. 1991). We think that differences in antibody production stimulated by the microencapsulated TA vaccine in 1988 and the biobullet TA vaccine in 1989 were caused by other, vaccine-related factors.

The need for repeated boosters to maintain high antibody titers is a major limiting factor in the use of existing immunocontraception agents. Administration of boosters remotely requires accurate identification of previously treated animals and the ability to approach animals within the range of the delivery system. To avoid these problems, we think that future research should focus on immunization against proteins that are sufficiently large to stimulate "self-boosting" of the immune response when a treated animal is reexposed to the antigen naturally. Recent studies indicated that fertility in a number of species can be suppressed through immunization against proteins located on the acrosomal cap of spermatozoa. Natural boosting of the immune system

probably could be achieved with each successful mating (Talwar 1986, Alexander and Ackerman 1988).

Currently an effective, reversible sterilizing agent that can be delivered remotely without the need for repeated boosters is not available for use in field situations. Two techniques, however, have been tested that provide long-term sterility in equids--vasectomy of stallions (McCort 1980) and steroid implantation in mares (Vevea et al. 1987, Plotka et al 1989). Silastic rods containing EE_2 implanted in captive feral mares were 75% effective for at least 3 years (Plotka et al. 1989). The initial cost, manpower required, and animal stress incurred during immobilization would be higher than that for remotely delivered vaccines, but may be an acceptable tradeoff for long-term sterility in most treated mares.

At this time, sterilization of dominant stallions may be a more efficient method of population control, since fewer animals would need to be immobilized and treated than would be in a control program directed at mares. Concern has been expressed about the removal of permanently sterilized animals, particularly alpha males, from the gene pool (Nelson 1980). However, similar management practices on Chincoteague National Wildlife Refuge, where the majority of male foals are removed annually, have not reduced genetic variability within that feral population compared to other island horse populations or mainland breeds (Goodloe 1991). In addition, permanent corralling

of excess horses, which is the major management technique currently practiced in western states, also is genetically limiting but does not provide long-lasting population control.

Sterilization of the dominant stallions would reduce, but not eliminate, foal production in bands with treated males. Some mares probably will be bred by subordinate or bachelor males (Nelson 1980) or will move from 1 herd to another during the breeding season. Band fidelity, however, varies within and among feral horse populations (Nelson 1980, Berger 1986, Kirkpatrick et al. 1990a, Goodloe 1991), and this technique may provide relatively efficient population control in some areas until an effective, remotely delivered technique is developed.

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WILDLIFE RESEARCH

Reduction in growth rates of wild horse populations treated with the controlled-release immunocontraceptive PZP-22 in the western United States

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ABSTRACT

Context. Management of wild horses (*Equus caballus*) on public lands in the western United States by gathering and removing surplus animals has historically failed to keep up with annual population growth. Research has yielded several effective contraceptive agents but these agents have not been widely applied, especially on difficult-to-access horses occupying expansive ranges. **Aims.** This study examined wild horse population growth reduction associated with controlled-release PZP-22 immunocontraceptive vaccine treatments in two challenging herd management areas (HMA) in the western United States. **Methods.** Populations of wild horse herds at Cedar Mountain HMA, Utah (CM), and Sand Wash Basin HMA, Colorado (SWB), USA, were primed with PZP-22 in gathers conducted in 2008 and 2012 (CM only) and boosted with PZP-22 or native PZP administered by dart (SWB in 2010) or by hand (CM in 2012). The two herds were intensively observed between 2008 and 2015. Population size, mortality, reproductive rates and growth rates were calculated from exhaustive inventories of known individuals. **Key results.** Adult and foal mortality were low at both sites. Consequently, both reproductive rates and population growth rates were strongly predicted by a fertility control index, the product of proportion of mares treated and vaccine efficacy. Following the 2012 CM gather, at which 69% of the mares present were treated, only 20% of mares in the herd foaled, and population growth rate decreased by 74% relative to baseline levels, emphasising the importance of treating a large majority of mares. **Conclusions.** Contraception can significantly reduce rates of population growth in wild horse herds that occupy large areas and are challenging to access. Multiple-year efforts and recruitment of new mares into the treatment population beyond the initial application are needed to achieve a biologically significant impact. **Implications.** The strong quantitative relationship between proportion of mares treated and vaccine efficacy and population growth rates should allow managers to better forecast the effort and number of removals (if any) needed to cost-effectively regulate wild horse population numbers and protect the range ecosystem.

Keywords: feral horse, fertility control, population dynamics, population management, wild horse, wildlife contraception.

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Introduction

Management of free-roaming unowned horses and burros is a global challenge (Nimmo and Miller 2007; National Research Council 2013; Hobbs and Hinds 2018; Scasta *et al.* 2020). Among the concerns raised by abundant populations of wild horses and burros are undesirable ecological impacts (Nimmo and Miller 2007; Beever *et al.* 2008; deVillalobos and Zalba 2010; Davies and Boyd 2019), competition with livestock (Scasta *et al.* 2016; Danvir 2018), and the welfare of the animals themselves under harsh and changeable environmental conditions (Garrott 2018). Complicating these challenges are sharp divergences among stakeholders and members of the public in how wild horses and burros are perceived and valued (Nimmo and Miller 2007; Nimmo *et al.* 2007; Beever *et al.* 2018; Scasta *et al.* 2020). As a result, government agency actions to manage these populations

are frequently challenged publicly, in the press, and in court (Rikoon 2006; Nimmo and Miller 2007).

Population management options are limited and include direct and indirect lethal reduction by shooting or capture and transportation to slaughter, capture and removal for adoption or transfer to sanctuaries or other long-term holding facilities, and fertility control. In the USA, Australia, New Zealand, and other countries, killing wild horses or burros by any means to manage their populations is deeply unpopular with the public, especially with highly visible populations (Nimmo and Miller 2007; Scasta *et al.* 2020). Because of the high value that the public places on the lives of equids, often the only feasible solutions open to managers are removal for adoption or long-term holding and/or fertility control.

To manage wild horse and burro populations on US public lands, the Bureau of Land Management (BLM) and US Forest Service (USFS) have relied primarily on gathering, removing, and transporting wild horses and burros for adoption or long-term holding. This practice has been in place since shortly after the passage of the federal Wild Free-Roaming Horses and Burro Act in 1971 (USA; Boyles 1986; Garrott and Oli 2013; National Research Council 2013; Danvir 2018; Garrott 2018). The effectiveness of this approach to stabilising populations is undermined by the high population growth rates of wild horses and burros, which may even be accelerated by the gather-and-removal management strategy (National Research Council 2013). Annual population growth rates vary with environmental conditions and may be lower where predators are present, but most estimates for wild horses on US public lands range from 15% to 27%; estimates from removal data suggest system-wide growth rates to be about 15–20% (Garrott *et al.* 1991; Turner and Morrison 2001; Roelle *et al.* 2010; National Research Council 2013; Garrott 2018). Population growth rates in burros are less well known, but pregnancy rates are known to be high (Johnson *et al.* 1987; Wolfe *et al.* 1989). Linklater *et al.* (2004) estimated population growth rates for wild horses in the Kaimanawa Ranges, New Zealand, to be 9.8%, a bit lower than those reported for the USA.

The BLM began funding research into wildlife fertility control in the 1970s and has continued to do so to the present day (Kirkpatrick *et al.* 1982; Plotka *et al.* 1992; Turner *et al.* 1997, 2007). Since 1990, studies of contraceptives on wild horses have yielded more effective and longer-acting contraceptive agents (Massei and Cowan 2014; Roelle *et al.* 2017; Baker *et al.* 2018). Whereas the native PZP emulsion first tested on free-roaming horses at Assateague Island National Seashore in Maryland, USA, required two initial treatments and annual boosters (Kirkpatrick *et al.* 1990), the more recent PZP-22 preparation offers 1–2 years of effectiveness with a single initial treatment, with effectiveness extended three or more years by a single booster delivered 2–3 years after the initial treatment (Turner *et al.* 2007; Rutberg *et al.* 2017; Carey *et al.* 2019). Similar multi-year effectiveness with a single booster has been demonstrated in initial field tests of GonaCon™, and SpayVac® also shows promise of single-shot, multi-year effectiveness (Roelle *et al.* 2017; Baker *et al.* 2018).

While contraceptive agents were being developed and tested, other work modelled population effects of hypothetical contraceptives, either alone or in combination with other population-control strategies (Massei and Cowan 2014). Although employing diverse methods, most models concluded that combining removals and contraception reduces costs (especially if disposal of removed horses through adoption or holding is costly), lengthens gather cycles, and yields population levels that fluctuate less and remain closer to the target than do all-removal strategies (Garrott *et al.* 1992; Garrott 1995; Gross 2000; Hobbs *et al.* 2000; Bartholow 2007; de Seve and Griffin 2013; Garrott 2018). Incorporating contraception may also improve genetic outcomes relative to removals, especially if focused on younger individuals (which extends generation times and slows allele loss through genetic drift; Gross 2000). Several authors have recommended relying on removals to initially reduce the population to a target level, and then applying contraception to maintain population size (Hobbs *et al.* 2000; Massei and Cowan 2014; Garrott 2018; Hobbs and Hinds 2018). However, Bartholow (2007) suggested that maximum long-term cost reduction will be best achieved by pursuing multiple gather cycles of contraception and selective removals. Several studies have concluded that contraception will have little effect on population growth below a threshold proportion of mares treated (40–50%), and that a very high proportion of mares treated (60–90%) would be needed to stabilise populations in the absence of removals.

Empirical studies of the population effects of fertility control in wild horses are scarce (Massei and Cowan 2014). At Assateague, the population impacts of native PZP have been traced since the National Park Service (NPS) started applying it as a management tool in 1994. The Assateague horses showed an initial population stabilisation phase that lasted 10 years, followed by a slow decline that met progressively lower NPS population targets in 2008 and 2015 (Kirkpatrick and Turner 2008; National Park Service 2008, 2016). At Assateague, population control is enabled by the relative approachability of resident horses and their confinement on the narrow barrier island, which means that they can be reliably and repeatedly treated with PZP via dart and monitored regularly.

In western North America, wild horse herds vary widely in their size, density, spatial dispersion, detectability, and approachability in the field. Although horses in some BLM herd management areas are accessible for regular darting (e.g. Little Bookcliffs Wild Horse Range, CO, McCullough Peaks HMA, WY, and Pryor Mountains Wild Horse Range, WY/MT; Ransom *et al.* 2011), most are not. For these three sites, Ransom (2012) demonstrated that PZP treatments were associated with a reduction in mean population growth

from 15.6% pre-treatment to 8.2% post-treatment. Selection and number of horses treated at each site varied according to the site-specific management plans, with a mean of 32.2% treated across populations and across years. At the 234 703 ha Sheldon National Wildlife Refuge, NV, Collins and Kasbohm (2017) reported that, with annual helicopter roundups, the wild horse population was reduced by approximately 72% over 7 years, using a combination of removals, sterilisation of stallions with vasectomies or chemical epididymectomy, and surgical ovariectomy of mares by using colpotomy.

Annual helicopter round-ups are generally infeasible for BLM and USFS wild horse ranges, but longer-acting contraceptive vaccines such as PZP-22 and GonaCon^{TM} open possibilities for measurable population impacts on more difficult-to-access wild horse herds in North America and elsewhere (Garrott 1995; Hobbs *et al.* 2000; Bartholow 2007).

Extending the results on PZP-22 efficacy on individual mares reported by Rutberg *et al.* (2017), this paper reports the impacts of the management-level application of PZP-22 on the population dynamics of two wild horse herds occupying expansive herd management areas (HMAs) in the western USA, namely, Cedar Mountain HMA, Utah, and Sand Wash Basin HMA, Colorado.

Materials and methods

Study areas

Cedar Mountain herd-management area, Utah

Cedar Mountain HMA (CM) comprises 1666 km^2 located 80 km west of Salt Lake City and is part of the Great Salt Lake Desert and Great Basin Desert ecosystem. CM consists of alkali salt flats and playas, sagebrush and grassland benches and steep juniper woodland slopes. Elevation is approximately 1400–2460 m. Rainfall is 20–25 cm at lower elevations and 25-38 cm at upper elevations. Within CM lies the 402 km^2 Cedar Mountain Wilderness Area. In part because of the ban on vehicles in the wilderness area, there are few access roads within the mountain range, and only two passes traverse the range east–west. CM is bordered to the west and south by the US Army Dugway Proving Ground (DPG), part of which lies within the HMA boundaries. Downed or cut fences allow horses to access DPG, and some horses from the HMA utilise areas of DPG that are not part of the HMA. Cattle graze on CM, including parts of the wilderness area, approximately November–May.

Most water sources at CM consist of roadside cattle troughs, although there are also a few seeps and intermittent springs fed by spring runoff from snow that accumulates in the mountains. DPG water consists primarily of spring seeps. The CM population stays close to water sources, primarily during hot weather when water is scarce. Movement of DPG horses into off-HMA areas varies with water availability and season. The southern boundary line of DPG borders the Onaqui wild horse herd where cross-over is possible. CM horses also occasionally take advantage of open or downed fences to move off range to the east.

Horses in CM were wary of people on foot and in vehicles. DPG horses were more accustomed to vehicular traffic and could sometimes be observed roadside.

Appropriate management level (AML) for wild horses at CM is 190–390.

Sand Wash Basin herd-management area, Colorado

Sand Wash Basin HMA (SWB) comprises 638 km^2 in northwestern Colorado, 72 km west of Craig. The topography and vegetation encompass mesas and ridges interspersed with desert washes and salt desert shrub plant communities consisting of sagebrush/bunchgrass, saltbush and a small amount of juniper woodlands. Rainfall is approximately 18–30 cm yearly and elevation ranges from 1900 to 2600 m. The herd area is easily accessible with a network of dirt and primitive roads. Domestic sheep are grazed on the land in spring and fall.

Sand Wash Basin HMA incorporates a network of artificial water sources created for livestock use that are scattered throughout the range, along with intermittent springs and seeps. Horse movements are strongly influenced by water availability and can vary from season to season. Horses may enter and leave the HMA by jumping low fences or passing through southern range entrances lacking cattle guards, snow-covered cattle guards, and boundary gates when they are inadvertently left open. Domestic horses from a neighbouring ranch were observed occasionally on the HMA. Most were quickly removed, but one mare remained for up to 2 years.

Horses in SWB showed some initial wariness but became more approachable throughout the study as the HMA became increasingly popular with visitors and horses grew accustomed to human activity. AML for wild horses at SWB is 163–362.

Observations and demographics

Ground surveys to identify horses pre-gather (2008) and monitor population trends and contraceptive efficacy (2009–2015) were conducted on CM and SWB. Because 2008 pre-gather observations were limited and permission to conduct surveys on the CM HMA section of the Dugway Proving Grounds (DPG) was not obtained until July of 2009, pre-gather (2008) data are omitted from the results. Between 2010 and 2014, only 10 previously unobserved adults were identified at SWB (four in 2010 and six for the remaining 4 years of the study). Only five previously unobserved adults were identified at CM from 2010 to 2015 (four in 2010 and one in 2011). At both sites, new adults represented $<1\%$ of total horses observed, suggesting that our population inventories at both sites were substantially complete.

Observers averaged 990 field-hours per season (2008–2015) and 435 data-logging hours per season in CM and

711 field-hours per season and 350 data-logging hours per season in SWB (2008–2014). Time spent in the field depended on weather and road conditions. In the year prior to gathers (2008), 608 individual horses were identified at CM and 377 at SWB (Tables 1, 2). Observations of horses were concentrated at many of their main water sources, seeps and springs, as well as the various grass and sage landscapes in which they were located. Aside from the DPG groups, which mostly congregated during hot months, the CM horses were frequently observed in a large herd in which horses remained in their respective breeding groups. Elusive horses that were not consistently observed with other main groups of horses were identified and observed throughout the course of the study.

Field activities were performed from pre-dawn to twilight. In both herd areas, observations were made from vehicles, on foot, and at water sources. Infrared motion-detecting trail cameras were utilised during darting efforts in SWB to determine horse activity patterns and in CM to determine activity patterns, assist in individual identification, and monitor bait sites during baiting activities. To facilitate identification and relocation, CM was divided into four to six areas and SWB into six areas on the basis of water resources and landmarks. Observations were made weekly at each defined area and opportunistically to observe as many horses as possible. Individual horses and groups were recorded and photographed whenever sighted.

Horses were individually identified on the basis of their group associations, location, gender, age, colour, face and leg markings, unique identifiers, and mane and tail characteristics. Each positively identified individual was assigned a unique identification number, and for each study population, a horse-list inventory was updated weekly to reflect changes in population composition and group associations. Identification and association information was incorporated into summary spreadsheets and the Wild Horse Identification Management System (WHIMS; ©2010, Wildwise Solutions), which also catalogued photographs of individual horses.

Foals were matched to mares on the basis of nursing and other behavioural indicators of association. Population counts were finalised at the end of the observation season. CM estimates included horses that moved on and off the HMA into the part of DPG not included in the HMA, whereas SWB off-range populations were not reliably observed from year to year and therefore were not included in the population. Horses that cycled on and off range but were consistently observed were counted in the population.

Mortality was confirmed if a carcass was located or a euthanasia was reported by BLM personnel. Date of death was recorded if known. Carcasses and/or skeletal remains

Table 1. End of field season demographics and sex ratios for Cedar Mountain herd-management area, 2009–2015.

Year	Adult males	Yearling males	Male foals	Adult females	Yearling females	Female foals	Sex ratio, adults + yearlings	Sex ratio foals	Total foals	Total horses
2009	154	12	40	135	14	41	1.11	0.98	82[A]	397
2010	165	39	29	145	40	39	1.10	0.74	68	457
2011	199	29	56	176	36	53	1.08	1.06	109	549
2012	210	18	64	193	14	50	1.10	1.28	114	549
2013	220	65	19	205	48	24	1.13	0.79	43	581
2014	277	18	51	244	23	52	1.10	1.98	103	665
2015	285	52	58	257	50	72	1.10	0.81	130	774
Total			317			331	1.10	0.96	649	

[A]Includes one foal of unknown gender.

Table 2. End of field season demographics and sex ratios for Sand Wash Basin herd-management area, 2009–2014.

Year	Adult males	Yearling males	Male foals	Adult females	Yearling females	Female foals	Sex ratio adults + yearlings	Sex ratio foals	Total foals	Total horses
2009	88	3	22	78	0	36	1.17	0.61	58	227
2010	88	22	22	78	35	19	0.97	1.16	41	264
2011	106	22	20	112	19	23	0.98	0.87	43	302
2012	122	20	29	129	23	19	0.93	1.53	48	342
2013	140	29	39	150	19	30	1.00	1.30	69	407
2014	166	39	43	169	29	52	1.04	0.83	95	498
Total			175			179	1.02	0.98	354	

found were investigated for cause of death but did not always yield sufficient information to reach a conclusion. Generally, a horse was classified as 'missing' when it was not observed for that observation year. Dependent foals not observed with their mothers and older horses that had been observed in poor health were presumed dead if they went missing. Otherwise, unless there was specific knowledge to the contrary, horses were assumed to be dead or dispersed if, at the end of the field season, they had not been sighted on or adjacent to the HMA for at least 2 years.

Gathers and PZP treatments

Helicopter gathers took place at CM 9–17 December 2008 and 21–27 February 2012, and at SWB 17–22 October 2008. During the December 2008 CM gather, 447 horses were removed, and 70 mares were treated with PZP and released along with 55 stallions. In the February 2012 CM gather, 75 horses (including 69 yearlings) were removed, and 143 mares were treated with PZP and released along with 130 stallions. (An additional 19 study animals were removed from DPG during a 2012 gather at the adjacent Onaqui HMA immediately before the CM gather.) During the October 2008 SWB gather, 280 horses were removed, 62 mares were treated with PZP and released along with 55 stallions.

PZP treatments are described in detail in Rutberg *et al.* (2017). Mares gathered in 2008 were hand-injected in the left hip with 'PZP-22' primers, consisting of 100 μg PZP antigen in 0.5 mL phosphate buffer solution emulsified with 0.5 modified Freund's complete adjuvant (mFCA) plus 450 μg PZP and 900 μg QA-21 (Agenus, Lexington, MA, USA) incorporated into lactide–glycolide pellets engineered to release at 1, 3, and 12 months ($N = 70$ at CM and $N = 62$ at SWB). Mares gathered at CM in 2012 were injected with either PZP-22 primers if they had not been treated previously ($N = 85$) or, if they had been treated in 2008 ($N = 58$), boosters of PZP-22 (with Freund's incomplete adjuvant, FIA) or simple emulsions of PZP in FIA.

Darting horses was attempted by approaching on foot or vehicle, stalking, sitting behind vegetation or creating natural blinds where no permanent structures were allowed. PZP booster treatments were administered by dart at SWB between 11 September and 17 November 2010, by using Dan-Inject® CO2 Pistol (Dan-Inject Dart Guns, Austin, TX, USA) and PneuDart X-Caliber® rifles (Pneu-Dart Inc.) firing barbless PneuDart darts. Mares previously treated in 2008 received either PZP-22 boosters ($N = 34$) or PZP-FIA emulsion only ($N = 16$). In addition to the 2008 and 2010 treatments, BLM management at SWB re-boosted 23 previously treated mares with PZP/FIA emulsions (one was boosted twice because of misidentification) between 4 April and 9 May 2013.

Data analysis

For the purposes of analysis, treated mares were defined in general as a mare that had received a PZP treatment, primer or booster, within the prior 3.5 years. Because the CM and SWB horses typically mate in April–June and the gestation period of horses is approximately 11 months, mares treated in October (SWB) or December (CM) of 2008 would already have been pregnant for the 2009 foaling season at the time of treatment. Therefore, these horses were first considered as treated in 2010. Likewise, mares treated in February 2012 (CM) would also have been pregnant at the time of treatment and the effects of the 2012 treatments would have first appeared in 2013.

Annual population growth was defined as end-of-year population size for year ($T + 1$) divided by the end-of-year population size for year (T). The effect of the 2012 removals at CM was removed from the 2011–2012 population growth estimate by subtracting the number of animals gathered from the end-of-year 2011 estimate.

In preliminary analysis, linear modelling (Automatic Linear Modeling, SPSS Statistics 26), using proportion of mares treated, adult sex ratio, annual precipitation, and vaccine effectiveness to predict annual population increase identified the model including only a proportion of mares treated and effectiveness as the best fit on the basis of Akaike's information criterion adjusted for small sample sizes (AIC_C). Similar results were found in stepwise regression ($P = .05$ for inclusion), in which location (CM vs SWB), method of administration (hand versus dart), mare age (proportion of mares greater or equal to 4 years old), and adult sex ratio were excluded as predictors, leaving only proportion of mares treated and vaccine efficacy as significant predictors of annual population growth. Consequently, only the last two variables are used to predict annual population growth below.

Analysis was performed with Microsoft Excel and IBM–SPSS. Vaccine efficacy is defined as the proportion, $(P_{untreated} - P_{treated})/P_{untreated}$, where P = proportion of mares foaling in a given year (following Baker *et al.* 2018). For purposes of analysis, we defined a fertility control index (FCI) as the proportion of mares treated times vaccine efficacy. Linear regression was used to test proportion of mares treated and the FCI as predictors of population reproductive rate and annual population growth.

Results

Mortality and disappearances

Mortality was low at both sites. At CM, 23 foals died from 2009 to 2015, averaging 2.9% of foals born each year (range: 0–4.4%; Fig. 1*a*). Among 1 + -year-old horses, mortalities and disappearances averaged 2.7% per year (range: 1.9–4.8%), with 2 + year-old stallions representing 52 of 129 (40%) known deaths and disappearances. At SWB, 23 foals died from 2009 to 2014, averaging 6.1% of all foals born (range: 2.4–12.7%; Fig. 1*b*). There were 36 deaths and disappearances among horses 1 + years old, averaging 2.1% of all known individuals in those age classes (range:

0.6–3.9%). Of the 22 deaths and disappearances, 61% were 2+-year-old males.

Where cause of death could be determined, deaths were associated with injuries, broken legs, old age with deteriorating body condition, loss of access to water (including one group of CM horses that was trapped in a rancher's fenced pasture), lightning strikes, complications during foaling, and vehicle strikes. Aside from scheduled population management gathers, horses were removed infrequently; these included one orphaned foal and four horses that moved off the HMA at CM and one bachelor male that found its way into a corral on a private ranch adjacent to SWB.

Herd sex ratios

Adult sex ratios in CM were stable with a slight male bias throughout the study, whereas foal sex ratios varied from year to year but averaged very close to 1:1 (adult SR, 1.1 m:f; foal SR, 0.98 m:f; Table 1). At SWB, adult sex ratios were somewhat male biased following the 2008 gathers, but a female-biased foal crop in 2009 restored the adult sex ratio to about 1:1 and both foal and adult sex ratios averaged very close to 1:1 for the remainder of the study (adults, 1.02 m:f; foals, 0.98 m:f; Table 2).

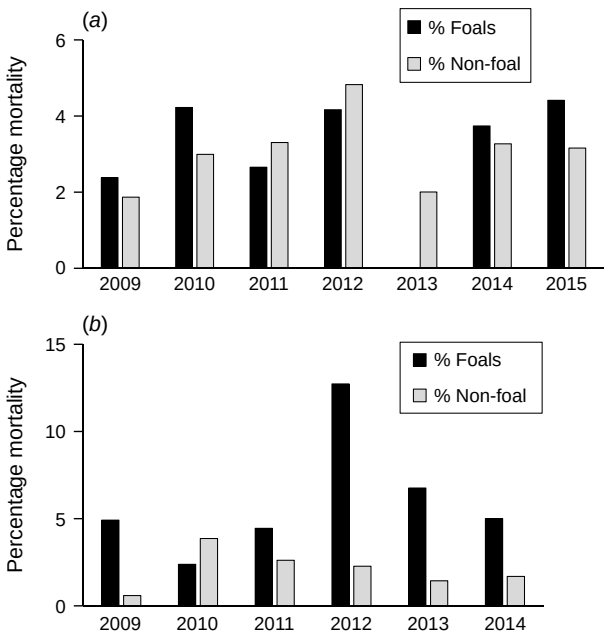


Fig. 1. Annual percentage mortality at (*a*) Cedar Mountain herd-management area, UT, 2009–2015, and (*b*) Sand Wash Basin herd-management area, CO, 2009–2014.

Application of contraception and herd reproductive rates

Efficacy of PZP-22 primers was lower than expected in 2008, with modest reduction of foaling rates in the initial 2 years after treatment at both sites (Table 3; Rutberg *et al.* 2017). Efficacy of the 2012 CM primers was high in the initial year after treatment (2013) but fell in 2014. Booster treatments were highly effective at both sites for at least 3 years after treatment. Of the 21 SWB mares initially treated in 2008, remotely boosted in 2010, and remotely boosted again in the spring of 2013, four (19%) foaled in 2014. Two mares treated only in 2008 and boosted in 2013 did not produce foals in 2014.

At CM, 51% of the mares known to be present in the population were hand-treated with PZP-22 in 2008 (including in the population 51 new mares identified and documented in 2009 on DPG after treatments had been administered). In February 2012, 69% of the mares known to be in the herd were treated, including 58 mares (92% of previously treated mares) that received boosters and 85 new mares that received initial injections of PZP-22. Both the primers and the boosters were highly effective that year, with only 11.4% of all treated mares foaling in 2013. The proportion of mares treated declined slowly after 2013 (Fig. 2*a*).

At SWB, 62 mares representing approximately 79% of the mares known to be present on the HMA at that time were hand-treated with PZP-22 in October 2008. By the time boosters were dart-delivered to a subset of 50 mares in autumn 2010, the herd had expanded, and treated mares represented only approximately 54% of adult SWB mares present. That proportion of treated mares continued to fall slowly through 2014 (Fig. 2*b*).

Overall, the proportion of mares treated following initial treatments averaged 51.4% at CM (2010–2015; range: 32.1–69.0%) and 46.6% at SWB (2010–2014; range: 30.6–76.9%).

Population foaling rates averaged 44.1% at CM (vs 61.6% pre-treatment (2009); range: 21.2–62.4%; Fig. 2*a*) and 49.2% at SWB (vs 78.2% pre-treatment (2009); range: 40.2–58.2%; Fig. 2*b*). In regression analysis with both study areas pooled,

Table 3. Summary of PZP-22 vaccine efficacy: foaling rates of treated and control mares (from Rutberg *et al.* 2017).

Treatment	Untreated controls (%)	Year 1 (%)	Year 2 (%)	Year 3 (%)
Cedar Mountains Primer 2008	63.9	26.1	55.9	–
Cedar Mountains Primer 2012	68.4	14.5	51.3	–
Sand Wash Basin Primer 2008	73.3	47.5	41.0	
Cedar Mountains Booster 2012	–	7.0	17.9	20.0
Sand Wash Basin Booster 2010	–	22.0	24.5	32.1

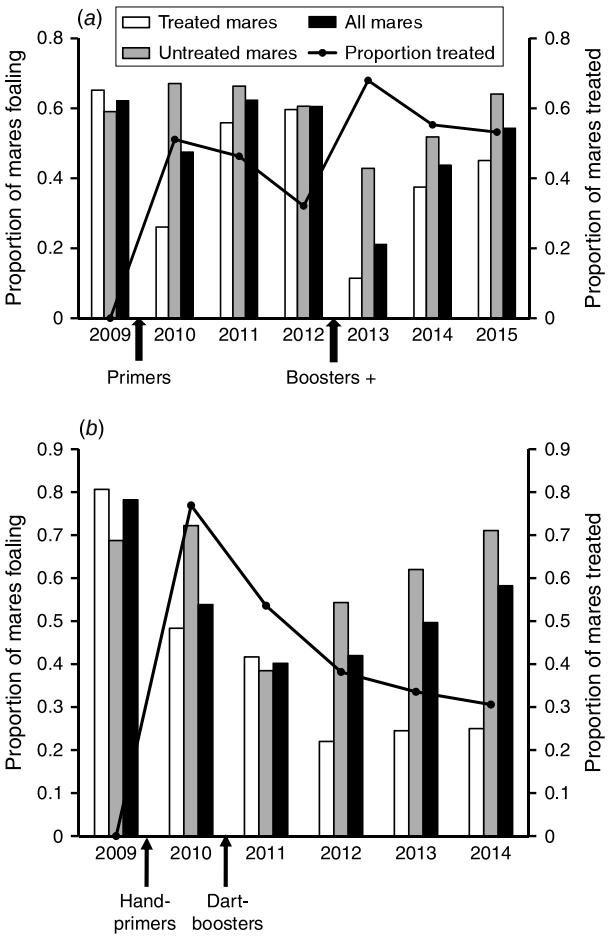


Fig. 2. Foaling rates and PZP treatments at (*a*) Cedar Mountains herd-management area, UT, 2009–2015, and (*b*) Sand Wash Basin herd-management area, CO, 2009–2014. Proportion of mares treated is defined as those mares that had received a PZP injection within 3.5 years.

the proportion of mares foaling was significantly lower when higher proportions of the mares were treated (Fig. 3*a*; $Y = -0.411X + 0.689$; $R^2 = 0.461$, $t = 3.07$, d.f. = 12, $P = 0.011$). Proportion of mares foaling in the two populations was more strongly predicted by the FCI, which also took into account vaccine efficacy (Fig. 3*b*; $Y = -0.841X + 0.675$; $R^2 = 0.756$, $t = 5.84$, d.f. = 12, $P < 0.001$).

Population growth

Including the effects of the removal at the 2012 gather, the CM population grew at an average annual rate of 11.8% from 2009 to 2015 (vs 1997–2000 rates of 24.4%; Fig. 4*a*). The lowest annual growth rate (5.8%, calculated from post-removal data and therefore not including the impact of the removals) was observed in 2013, one year after the 2012 administration of boosters to previously treated mares and of primers to new mares.

No animals were removed from the SWB herd after the gathers and removals were conducted in October 2008.

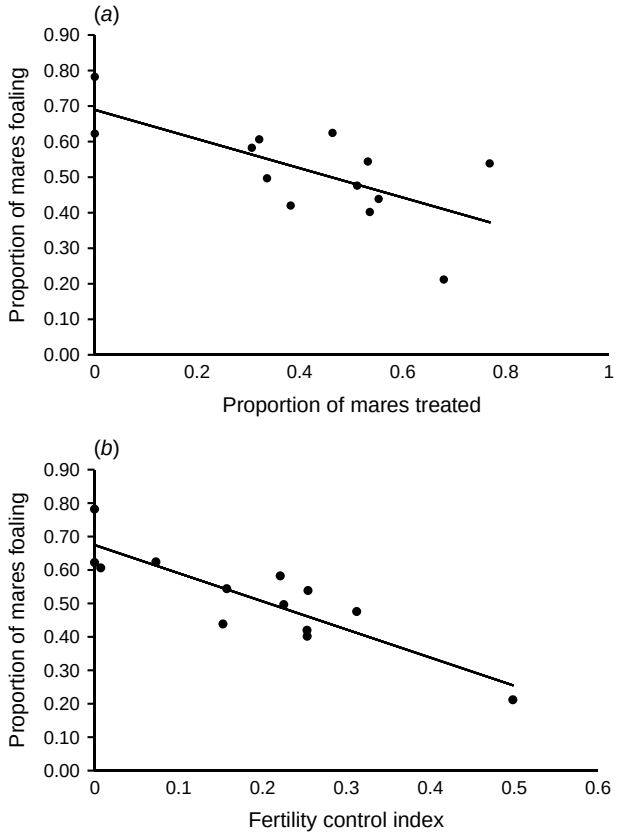


Fig. 3. Population foaling rate, both study sites pooled, versus (*a*) proportion of mares treated ($Y = -0.411X + 0.689$, $R^2 = 0.461$, $t = 3.07$, d.f. = 12, $P = 0.011$), and (*b*) fertility control index ($Y = -0.841 + 0.675$, $R^2 = 0.756$, $t = 5.84$, d.f. = 12, $P < 0.001$).

Between 2009 and 2014, population size grew at an average rate of 17.0% per year (compared with 2001–2004 annual growth of 22.0%), with the lowest rate of growth (13.3%) in 2012, the first year after the dart-delivered PZP boosters administered in fall 2010 took effect (Fig. 4*b*).

Because mortality and dispersal were low in both herds, population growth was mostly driven by foaling rate, and the predictors of population growth were similar to those of foaling rate. Proportion of mares treated significantly predicted annual population growth rate (Fig. 5*a*; $Y = -0.163X + 1.241$; $R^2 = 0.550$; $t = 3.80$, d.f. = 12, $P = 0.0025$), but population growth was better predicted by FCI (Fig. 5*b*; $Y = -0.300X + 1.229$; $R^2 = 0.734$; $t = 5.51$, d.f. = 12, $P \ll 0.001$).

Discussion

Independent corroboration of population inventories

Simultaneous double-count aerial surveys were independently conducted by the BLM and U.S. Geological Survey

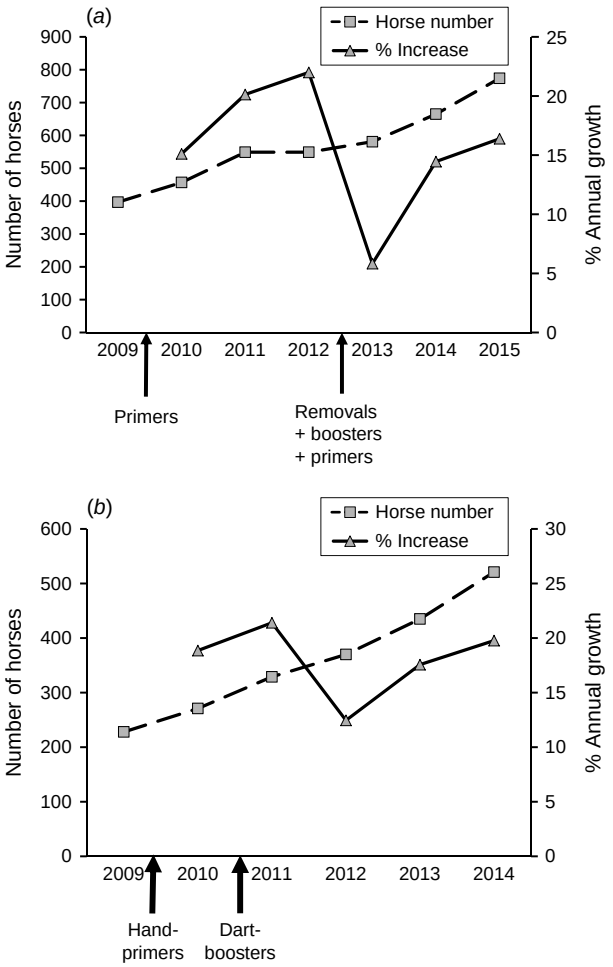


Fig. 4. Population size and growth rate at (*a*) Cedar Mountain herd-management area, UT, 2009–2015, and (*b*) Sand Wash Basin herd-management area, CO, 2009–2014. Arrows on *X*-axis indicate timing of when PZP treatments would take effect. To display the effects of contraception on growth rates more clearly, we calculated population growth rates in 2011–2012 at CM by subtracting the number of animals removed in 2012 from the 2011 baseline and used the post-gather 2012 numbers to calculate the rate of increase from 2012 to 2013. This produces the apparent discrepancy between absolute population size and population growth rate in 2011–2012.

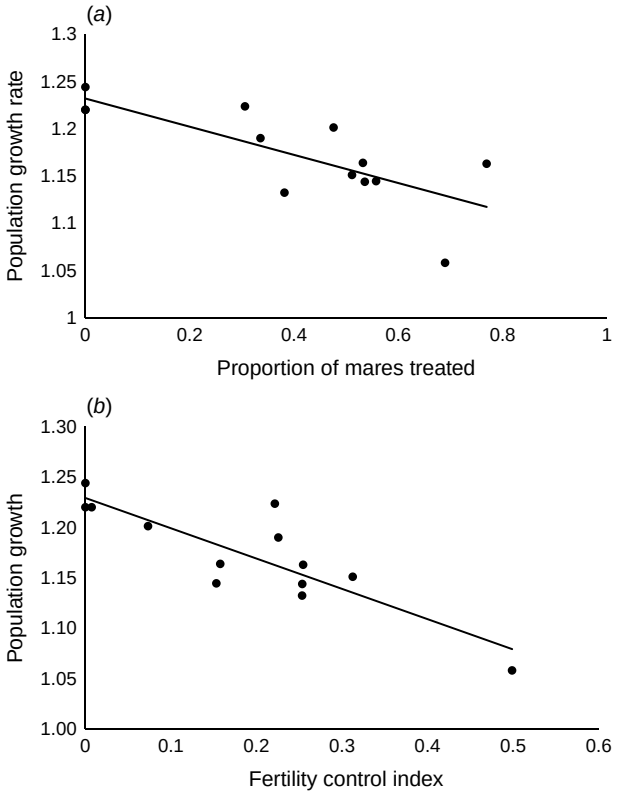


Fig. 5. Population growth rate and PZP treatments, both study sites pooled, versus (*a*) proportion of mares treated, and (*b*) fertility control index (FCI). Proportion of mares treated is defined as those mares that had received a PZP injection within 3.5 years. FCI is defined as the proportion of mares treated multiplied by vaccine efficacy.

post-gather on SWB and CM in February of 2009 (Lubow and Ransom 2016). Our SWB ground estimates fell within or close to the 95% CIs of the aerial survey estimates (aerial surveys, 133; 95% CI, 95–206; 128, 95% CI, 79–166; ground inventory, 169 adults and yearlings); low sightability associated with snow patches may in part account for the lower aerial survey count. Our 2009 CM end-of-year inventory number for adults and yearlings (315) plus the six adults known to have died or disappeared in 2009 (thus totalling 321) was very close to the CM aerial survey estimate (342) and well within the 95% CI (232–521). This corroboration adds confidence to our ground inventory numbers.

Population growth rates at SWB and CM

The regression of population growth rate versus FCI (fertility control index) permits a data-based prediction of what levels of population control will be obtained with a given proportion of mares treated and a given contraceptive agent efficacy (Fig. 5*b*). To achieve zero population growth in these two herds (annual growth = 1), the regression predicts FCI = 0.763, so that at 90% efficacy, for example, 85% of the mares would need to be treated to achieve ZPG.

This regression applies specifically to these two herds, in both of which reproduction and adult and foal survival are relatively high for wild horse herds generally (Garrott 1991; Linklater *et al.* 2004; Ransom 2012; National Research Council 2013), and movement of horses on and off range is modest. Herds with lower natural reproductive rates in untreated mares and lower survival will likely yield stronger population impacts for a given level of contraceptive effort, i.e. the regression line will be pushed downward. Mare age, which is known to influence reproductive rate (Linklater *et al.* 2004; Ransom *et al.* 2011; Rutberg *et al.* 2017), also is likely to influence the rate of population growth, despite the absence of statistically significant impacts in preliminary

modelling of population growth in this study. With those caveats, the regression should provide managers with a rough estimate of what effects on wild horse population growth a given level of contraceptive effort will provide.

The impact of contraception on population growth was most evident at CM, following treatments associated with the February 2012 gathers. In contrast to the 2008 gather, in which about half of the mares were gathered and treated, 69% of the mares remaining on the HMA following the 2012 gather received either a primer or a booster, and the booster proved to be more effective and long-lasting than the primer (Table 3; Rutberg *et al.* 2017). Population growth rate from 2012 (post-gather) to 2013 was only 5.8% in 2013 and remained slightly below the 15% threshold in 2014. This observation supports Bartholow's (2007) recommendation that managers think in terms of multiple cycles of contraceptive application.

The predictions of the impacts of contraception on wild horse population growth yielded by the current data-based relationship are generally in line with those of theoretical models (Garrott 1995; Gross 2000; Bartholow 2007). Empirically, Kirkpatrick and Turner (2008) reported that treating an average of 68% (range 52–95%) of mares annually stabilised and then reduced the population of wild horses at Assateague Island National Seashore, MD, although they effectively underestimated the proportion of females treated because the native PZP preparation they used showed some multi-year effectiveness among mares treated for three or more consecutive years.

Challenges to darting, monitoring, and management

Every free-roaming wild horse herd will yield its own specific management challenges.

In general, groups of horses at our sites tended to stay within their own established areas when water was plentiful. However, wild horses did move within and outside the HMAs in response to human activity, season, and especially water scarcity. Downed fences and other openings contributed to intermittent movements, such as, for example, allowing horses to move between CM HMA and adjacent Dugway Proving Grounds (DPG) to bordering water sources. Horses were also observed jumping low boundary fences; a neighbouring horse ranch that borders the western side of SWB would frequently find bachelor stallions inside their fences as domestic mares came into estrus during spring.

In 2008, when only about half the mares in the CM population were gathered, horses were scattered in small groups, and helicopter contractors struggled to capture them. Our inventory data also suggested that under these conditions, contractors underestimated the number of horses remaining on the range. CM gather efforts in 2012 were more successful in part because more horses were concentrated in open areas.

An additional risk of gathers was noted when contractors failed to return horses to the areas from which they were captured. Following the 2012 CM gather, eight horses became trapped inside a pasture without adequate resources (three died), six stallions disappeared, and three treated mares failed to return to their home range. These incidents account in part for the elevated non-foal mortality and disappearances observed in 2012.

Where feasible, remote darting of free-roaming animals is the safest, least expensive, and least invasive means to deliver immunocontraceptive vaccines. After initial administration of PZP-22 to mares gathered via helicopter round-up, remote darting of study subjects with PZP boosters was attempted at both CM and SWB. Success depended on wild horse wariness and gregariousness, the influence of water distribution on wild horse movements, vehicle access, and administrative restrictions placed on baiting and trapping (see also Naugle and Grams 2013). Remote darting at SWB was successful; at CM it was not.

Darting was unsuccessful at CM for multiple reasons. CM wild horses were generally unapproachable on foot, and the field environment was extremely challenging. Within the central 402 km^2 block of wilderness area inside CM, permanent structures such as blinds and trailers were prohibited or sharply restricted and vehicle movement was limited to a few designated roads. When, in late autumn 2010, the research team set up bait stations at CM to attract horses for trapping and darting with PZP boosters, horses failed to locate and habituate consistently to the bait sites before baiting was ended in November by a major snowstorm. In a follow-up baiting attempt in April, increased disturbance by range visitors and the wider availability of resources prevented horses from regularly visiting and concentrating at bait sites. Whereas concentration of horses in large groups in open areas helped helicopter contractors be more efficient (as noted above), such concentration discourages darting, and effectively confounded attempts to dart in CM in spring and summer of 2011.

Darting at SWB was also challenging; however, because SWB wild horses were less wary than were CM horses and more of the range is accessible by vehicle, darters ultimately succeeded by approaching horses on foot. However, unpredictability of water availability and horse movements hindered trapping at water sources, and access to SWB was inconsistent during winter months because significant precipitation quickly made roads impassable.

Management implications

This study is the first to demonstrate that the population growth rates of wild free-roaming horses in large herd-management areas (in the Western USA) can be substantially reduced by administration of contraceptives in the context of normal management activities. These studies also offer

managers a rough data-derived empirical guide to the magnitude of population impacts that can be expected when a given proportion of mares is treated with a contraceptive of known efficacy, whether it be PZP, GonaCon, or other agent. Application of data such as those presented here offers managers a useful approach to developing site-adapted projections of efficacy, cost, and outcome, and indicates what level of removals, if any, might be needed to supplement contraception to achieve population targets.

Strategies for administering contraceptive vaccines to wild horses will vary from site to site (Naugle and Grams 2013). In some herds, such as SWB, a majority of mares may be accessible and approachable enough to be treated via darting alone, whereas in others, gathers or bait-trapping (e.g. Carey *et al.* 2019) will be needed to ensure that a high enough proportion of mares will be treated to manage horses effectively.

Regardless of population targets, management goals, or means of access, one round of contraceptive treatments and/or removals will not yield effective management. Long-term planning, adaptive management, and persistence are necessary.

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Data availability. The data on which this paper are based are not publicly available at this time.

Conflicts of interest. The authors declare no conflicts of interest.

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An infanticide attempt by a free-roaming feral stallion (*Equus caballus*)

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Infanticide by adult males occurs in a variety of species. While infanticidal attacks have been documented in several equid species in captivity, it has never been witnessed in free-roaming feral horses. I report an infanticide attempt by a free-living feral stallion on a recently born female foal. The stallion picked up the foal by the shoulders, tossed it around twice and bit in on the neck several times. The dam of the foal charged the stallion and successfully protected her foal from additional attacks. The foal survived the attack and later weaned successfully. The stallion recently took over the band and was excluded as the sire through genetic analysis. While this type of attack is rare, this case lends support to the sexual selection hypothesis and further demonstrates that equids have evolved with the risk of infanticide. Furthermore, it shows that maternal protectiveness can be successful against attacks by infanticidal males.

Keywords: feral horse; maternal protectiveness; sexual selection hypothesis

1. INTRODUCTION

Infanticide, while rare, is a widely documented phenomenon in several mammal species including primates (Hrdy 1977; Crockett & Janson 2000; Watts *et al.* 2002), carnivores (Packer & Pusey 1983; Swenson *et al.* 1997) and rodents (Coulon *et al.* 1995; Vom Saal *et al.* 1995; Blumstein 2000). Male infanticide is generally considered to be an adaptive strategy whereby the interbirth interval is reduced, and the male can sire the subsequent offspring (Van Schaik 2000). Sexual selection infers a benefit to the reproduction of infanticidal males provided that only unrelated infants are killed, since infanticide is costly to males if related offspring are killed (Van Schaik 2000). Thus, recognition of paternity plays a key role in the evolution of this strategy.

With the exception of red deer (*Cervus elaphus*; Bartos & Madlefousek 1994) and the hippopotamus (*Hippopotamus amphibius*; Lewison 1998), the majority of cases of infanticide involving ungulates are reported in equids, including the horse (*Equus caballus*; Duncan 1982), Przewalski horse (*Equus przewalskii*; Ryder & Massena 1988; Feh & Munkhtuya 2008), mountain zebra (*Equus zebra*; Penzhorn 1984) and Burchelli's zebra (*Equus burchelli*; Pluhacek & Bartos 2000). Feral horses are unusual among ungulates in that they form stable harem-type groups year-round, consisting of one or more mares and usually one but sometimes several stallions (Linklater 2000). Band stallions are known to obtain high paternity within their band (Bowling & Touchberry 1990), but sneak copulations occur and mares change bands, which can result in paternity differences between foals (Miller 1981). In feral horses, infanticide has never been documented in the wild, but feticide may occur (Berger 1983). Feral mares have been shown to increase maternal protectiveness in response to paternity uncertainty (Cameron *et al.* 2003), which suggests that females have evolved to protect their foals from attacks against males. Here, I document a case of a resident band stallion attacking a young foal shortly after birth in a free-roaming situation.

2. MATERIAL AND METHODS

A feral horse population in the Virginia Mountain Range outside Reno, Nevada, was observed intensively from 2004 to 2008 for the purposes of another study. The total population fluctuated from over 1200 horses down to 1000 horses over the course of those years. The Nevada Department of Agriculture is responsible for managing the horses and the population size was and is still controlled by removals and contraception. The study included approximately 300 known individuals. Horses were habituated to human observers. The band in which the attack occurred was seen on a weekly basis to determine band membership and fidelity. At the time of the attack, the band consisted of two adult stallions, two adult mares, one juvenile male and one male foal. The band was being observed due to a recent birth of a foal and from that time, ad libitum observations were recorded until the end of the incident. All aggressive behaviours were documented and a description of these behaviours can be found in an equid ethogram (McDonnell & Haviland 1995).Genetic data were taken from faecal samples from the foal, dam and band stallion. Paternity was assessed using 12 polymorphic horse-specific microsatellites.

3. RESULTS

During routine behavioural observations on 12 April 2005, an infanticide attempt was witnessed. The band was passed at 15.40, while observing another band. At that time, the foal was not born. At 16.14, while passing the band again, the mare had recently given birth. A new female foal was covered with mucous and lying on the ground next to her dam. The foal was estimated to be no more than 1 hour old when the attack occurred and had not attempted to stand up. The rest of the band, including the dominant band stallion (stallion A), were all resting near the mare and newborn foal. At 16.20, a stallion (stallion B) from a nearby band called out and approached the mare. She immediately gave a head threat towards stallion B. The foal attempted unsuccessfully to stand up. The band stallion (stallion A) lunged towards the mare and foal and immediately bit the foal on the neck several times. Stallion A subsequently picked the foal up by the shoulders twice and shook it around several times. The foal was dropped to the ground and subsequently bitten and kicked by the front legs of stallion A several times. The attack lasted approximately 1 min. During the attack, the mare defended her foal by charging, biting and kicking stallion A, which led to the end of the attack. After the attack, stallion A had several aggressive interactions with stallion B and continued to try to herd the mare away from the area. The mare continued to charge and head threat stallion A when he came near her and she remained in her position over the foal. Stallion B called out again, which led to

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another aggressive interaction between stallion A and the mare. While the foal appeared to be in shock, there was no visible major damage, but she suffered minor superficial wounds. The foal stood up at 17.39 and nursed at 17.47, and at that time the observations ended. The band was observed over the course of the next year and the foal successfully weaned.

The attacker (stallion A) was excluded as the father of the foal through microsatellite DNA analysis.

4. DISCUSSION

The attack was unmistakably an infanticide attempt as it was similar to other reported attacks in equids (Duncan 1982; Feh & Munkhtuya 2008). Most infanticidal attacks by stallions occur before the foal reaches one month (Pluhacek & Bartos 2000) and in this case, the foal was recently born. Infanticide imposes direct fitness consequences on the parents of the offspring, especially the mother. Such costs include decreased inclusive fitness, energy expenditure and the loss of investment (Ebensperger 1998). Females should adopt counterstrategies against infanticide in order to maximize their lifetime fitness (Ebensperger 1998). These counterstrategies include fighting back, having close associations with males for infant protection, prolonged receptivity, territoriality, avoidance and termination of infant care (Ebensperger 1998). In horses, mares will defend against infanticidal attacks (Feist & McCullough 1975; Ryder & Massena 1988) by generally positioning themselves between their foal and another approaching horse, even if no direct threat is apparent (Crowell-Davis & Houpt 1986). This protective behaviour was witnessed during the attack, as the mare defended her foal by charging and biting the stallion. She also tried to position herself between the stallion and foal and stood over the foal after the incident. All of these actions appeared to stop the attack and prevent further assaults.

There were several factors that possibly led up to this attack. First, genetic analysis confirmed that the band stallion was not the sire. Second, the band stallion had recently taken over the band and shared the band with another adult stallion. Owing to these factors, there was a high level of paternity uncertainty that may have influenced the decision to attack. Little is known about how males detect paternity, but evidence in primates suggests that males detect paternity by consort time, phenotypic matching or the amount of time spent within the troop during the mating season (Buchan *et al.* 2003). In this case, the male did not have a consortship with the female, nor did he spend anytime with this female during the previous mating season. The uncertainty of paternity has been postulated as proximate cause of an infanticide attempt (Borries *et al.* 1999) and is believed to be the major cause in this particular case.

Recently, infanticide in equids was suggested to be in response to human intervention (human disturbance hypothesis), as most cases have been associated with unnatural environments and introductions of foreign males (Feh & Munkhtuya 2008). While humans may influence infanticide attacks, most documented cases have also been associated with foreign males. For example, in equids, attacks end after males and females have been housed together for longer periods of time (Ryder & Massena 1988), suggesting that the unnatural conditions do not explain infanticide. In addition, many infanticidal events in the wild are associated with contact with foreign males, and attacks are generally attributed to these males. In this case, the male was relatively foreign in the band and several factors increased the level of uncertainty of paternity (i.e. multi-stallion band, recent takeover, no consortship before the foal was born). In addition, the similarity of attacks between equid species, maternal protectiveness and genetic data all suggest that equids have evolved with and respond to infanticide risk. Furthermore, infanticide has been postulated as a main explanation for primate social structure and the evolution of year-round male–female associations (Kappeler 1997; Van Schaik & Kappeler 1997). Feral horses have similar social structure to primates and male harassment plays a primary role in social structure (Linklater *et al.* 1999).

This was the only attack witnessed during 3 years of behavioural observations. Several studies suggest that feticide may be more adaptive in horses (Berger 1983), and there are some suggestions that infanticide is low risk in equids (Feh & Munkhtuya 2008). However, several foals go missing and are found dead every year in all populations, even when no predators are present. While these deaths could be due to numerous factors, infanticide is a possible cause of some deaths. Furthermore, foals not sired by the band stallion have lower survival rates to weaning (M. E. Gray 2008, unpublished data). All of this makes a witnessed event and possible causes important to document. To my knowledge, this is the first documentation of this type of attack in feral horses in a free-living situation and lends support to the role of infanticide in social structure.

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University of Nevada, Reno

The Influence of Reproduction and Fertility Manipulation on the Social Behavior of Feral Horses (*Equus caballus*)

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in
Ecology, Evolution and Conservation Biology

by

Meeghan E. Gray

Dr. Mary Peacock/Dissertation Advisor

May, 2009

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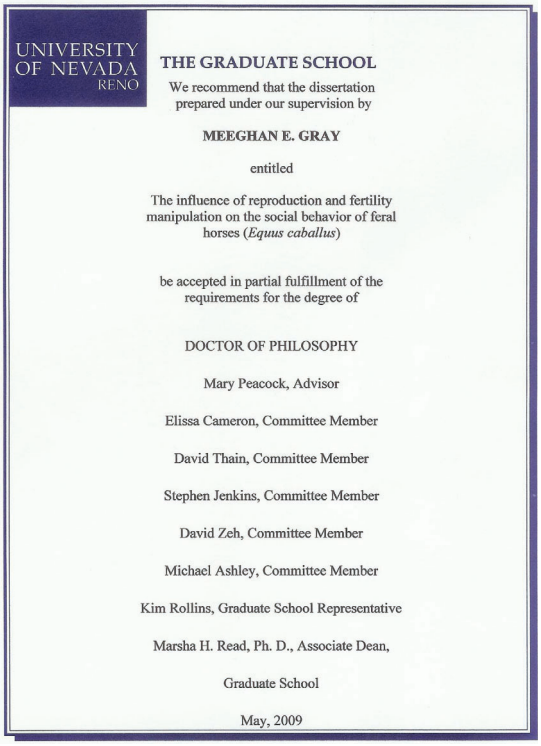
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ABSTRACT

Sex and reproduction influence mating systems and both are important for maintaining long-term associations between males and females. The mating decisions of females can have consequences for their reproductive success as females decide who to mate with and how often to mate. Females can mate with several males, but risk increasing chances of infanticide by having an offspring not sired by the behavioral father. Sexual interactions can also have social consequences, whereby females may engage in consortships that result in protection from harassment by other males. Thus, both sex and reproduction have important functions in the maintenance of male-female bonds in many mammalian species. Feral horses exhibit a rare social structure among ungulates in that they stay in year-round bands with several females and usually one male. Bands function to decrease harassment by other males and females in stable bands have higher reproductive success by forming consortships with harem stallions. Paternity analysis confirmed that most mares remain loyal to the band stallion and these foals have higher survival than those born in a band without their sire, indicating the importance of band fidelity for infanticide risk. I found females that changed bands suffered higher aggression rates, spent more time traveling and avoided the band stallion, further demonstrating the costs of male harassment. The socio-sexual behavior of females has been postulated as the proximate cause for the long term associations between mares and stallions. I found that fertility manipulation with two contraceptive formulations did not change male-female relationships, sexual behaviors or band fidelity. In addition, lack of foal production did not alter band fidelity. The two contraceptive formulations worked for multiple years with one injection, but were not as efficacious as other tested formulations. However, the

lower efficacy may be explained by the high intensity of research, resulting in high detection of contraceptive failure. Furthermore, there were individual differences in responsiveness as there was more contraceptive failure when females were in poor body condition. A meta-analysis of other studies on contraceptives confirmed that behavioral side effects occur after contraceptive treatment in a variety of species, suggesting the importance of experimental studies on the behavior and welfare of contracepted individuals.

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INTRODUCTION



"The wind of heaven is that which blows between a horse's ears."
Arabian proverb

Photo caption: G Band, subordinate stallion (DBS)

INTRODUCTION

Selection pressures on group living mammals have been traditionally linked with resource distribution and predation (Krause and Ruxton 2002) and yet social factors may have a stronger influence on the formation and maintenance of groups (Clutton-Brock et al. 1992). There is increasing evidence that male harassment and infanticide risk are important influences that determine social structure, suggesting that group living may have benefits beyond predation risk and availability of resources (Williams et al. 2004, Muller et al. 2007, Cappozzo et al. 2008). For males, living in stable groups with females can increase mating opportunities and subsequent paternity of offspring (e.g., Huck et al. 2005, Kappeler and Schäffler 2008). Females benefit from lower rates of harassment, lowered risk of infanticide, and higher body condition, all of which can increase their reproductive success (Linklater et al. 1999, van Schaik and Kappeler 1997).

Within groups, the mating strategies of females can have implications not only for their own reproductive success, but also for the males living in their groups. For example, females can form consortships with males during fertile stages of estrous and throughout pregnancy and offspring dependency (Dixson 1998). Females can benefit from these associations by decreased harassment from other males and increasing their offspring survival (Smuts and Smuts 1993, Dixson 1998, Linklater et al. 1999). The maintenance of these associations has been linked to affiliative and sexual behaviors. However, females may mate with other males both within and outside the group, which can decrease offspring survival, particularly through infanticide (van Schaik 2000).

Hypotheses concerning the role of social factors in group formation and maintenance have been tested primarily in primates and carnivores (Packer and Pusey

1984, van Schaik 2000) and have yet to be tested in wild ungulates with similar social systems. In addition, it is unknown if the manipulation of fertility impacts sexual behavior and social behavior of females and ultimately the fidelity of these long term associations.

Feral horses as a model

Feral horses are ideal for testing hypotheses on the influence of social factors (e.g., male harassment, paternity, mating, and pregnancy) on social behavior and group fidelity. They are unusual among ungulates in that they live in harem bands and maintain stable year-round social relationships over long time periods (Klingel 1979, Berger 1986, Linklater 2000). Harassment by males has been proposed as a factor that strongly influences feral horse social structure, with one of the primary roles of a harem stallion being to minimize harassment by non-band males (Linklater et al. 1999). Since band stallions defend their females from other males, dominant males are expected to achieve high paternity of offspring within their band. Thus, if paternity is high within a band, infanticide should be rare and only occur when a male can shorten the time to a female's next estrous cycle. The rarity of infanticide does not imply absence of these events or that it is not a strong selective force since infanticide has been reported in all equids [horse, *Equus caballus* (Duncan 1982); Przewalski horse, *Equus przewalskii* (Ryder and Massena 1988; Feh and Munkhtuya 2008); mountain zebra, *Equus zebra* (Penzhorn 1984); and Burchell's zebra, *Equus burchelli* (Pluháček and Bartoš 2000, 2005)], and mares are more protective of foals in situations where paternity is uncertain (Linklater et al. 1999, Cameron et al. 2003). Under such circumstances, females are expected to be

more protective of foals not sired by the band stallion. While changes in female behavior are known to occur when paternity is uncertain, there have been no studies that directly link actual paternity with these behaviors and how paternity is correlated with offspring survival.

Feral horses are also ideal to test predictions about the importance of reproduction and fertility on the social behavior of females and band fidelity. Females often stay in bands for long periods of time or for most of their lives after natal dispersal (Klingel 1979, Berger 1986, Linklater 2000). There appears to be strong selection for mate fidelity and band loyalty by females so as to decrease harassment by other males because females that change bands have higher parasite loads, lower body condition, and higher harassment rates (Linklater et al. 1999). The social structure of long term harem bands is thought to be supported by the socio-sexual behavior of mares (Asa 1986). Female horses are unlike most ungulates in that mares display sexual behavior throughout the year (Asa 1986). Thus, sexual behavior, regardless of whether mares become pregnant, may be important to the maintenance of band stability. As a result, the manipulation of fertility of females may have important behavioral and social consequences. Recently, contraception has been proposed as a viable, alternative management strategy to removals for feral horse populations. The manipulation of fertility is a way to test the role of sex and reproduction in male-female consortships and band fidelity.

My study investigated the influence of social factors on the stability and maintenance of harem bands by asking the following questions:

1) Is paternity reliable in stable harems bands, do females change maternal behavior in response to the paternity of their offspring and does paternity influence foal survival?

2) If sexual interactions and reproduction are important for maintaining mare-stallion relationships, what happens to band fidelity and consortships when fertility is manipulated?

3) Does fertility manipulation alter behavior in other species?

Study population

The feral horse population was located in the Virginia Range (145,000 ha) in Storey County, outside of Reno, Nevada (Figure 1). Since the mountain range was declared “wild horse” free by the US Bureau of Land Management in the 1980’s and is private land, the horses are legally defined as estray horses by Nevada statute and are managed by the Nevada Department of Agriculture. Currently, the total population is about 1400 horses, but the study population consisted of 300 individuals including 74 mares from 49 bands that were intensively observed for the duration of the study (2005-2008). The study population was located in the northwest section of the mountain range.

This study was initiated in June 2005 when females were treated with two different contraceptive formulations and were followed until August 2007. We used two different formulations that decrease fertility in significantly different ways. First, SpayVac® is a porcine zona pellucida (PZP) formulation that acts to block fertilization and allows for continued and sometimes increased cycling (Brown et al. 1997, Fraker et al. 2002, Killian et al. 2008). In contrast, GonaCon-B™ is a gonadotropin-releasing hormone (GnRH) vaccine that acts to block cycling altogether by significantly reducing the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Miller et al. 2000, Killian et al. 2006, 2008). During the course of the study, horses were

easily indentified by coat color and white markings and adult mares were freeze branded with a unique mark. Most bands habituated to human observers, making behavioral observations easy. Lastly, the study was done blind, so all behavioral observations took place before contraceptive treatment and paternity status of offspring was revealed.

The Study

I was interested in the social factors involved in harem band formation and maintenance, especially offspring paternity and the influence of mating behavior and pregnancy in maintaining long term associations between males and females. I investigated how social behavior can be changed by fertility manipulation and paternity of offspring. My dissertation is organized into five separate papers intended for publication. Consequently, there is some repetition among chapters, particularly in the methods sections.

In the first chapter, I look at how often females have offspring sired by the band stallion and under what circumstances females seek extra-band copulations. I also investigate if females are more protective of foals sired by an extra-band stallion and if foal survival is related to paternity. Previous research indicates that females may be more protective of foals when paternity is uncertain (Cameron et al. 2003), but linking such behaviors to actual paternity has not been done previously. Furthermore, band stability and the tenure of consortships have been shown to increase reproductive success of females (Kaseda et al. 1995, Linklater et al. 1999) and here I investigate additional benefits of not only staying in a band, but having a foal sired by the band stallion. In

addition, I document the first reported case of an infanticide attempt in feral horses and investigate the circumstances under which this attack occurred (Chapter 2).

The next chapter examines how fertility can be manipulated with contraceptive treatment. I compare two contraceptive treatments, SpayVac® and GonaCon-B™, to controls in order to test if these formulations reduce foaling rates in females. Previous studies have shown that these formulations can last up to five years with one injection (Killian et al. 2008), but this is the first test in free-roaming feral horses. I also show how efficacy can be influenced by body condition at time of treatment and discuss some issues with methodologies for evaluating efficacy rates in horses. Since concerns have been raised about the potential side effects of contraceptive treatment, I investigate how treatments could impact foal sex ratio and foal survival in mares for which the contraception failed. Previous studies have raised theoretical concerns about the side effects of contraception (e.g., Nettles 1997). However, the potential role of contraception as an experimental tool to manipulate fertility has been largely overlooked. Therefore, in Chapter 4 I consider whether contraceptive treatment results in changes in physiology, ecology and particularly behavior by reviewing the literature on contraceptive side effects and performing a meta-analysis. I then consider the utility of contraception as an experimental tool.

In Chapter 5 I examine how contraceptive treatment can impact the behavior of mares in relation to band stability and consortships with stallions. Few studies have investigated behavioral side effects of contraceptive treatment, especially in wild social mammals (but see Nuñez et al. 2009). Consortships are important to reproductive success of mares and are thought to be maintained by copulations and other sexual interactions

(Asa 1986, Kaseda et al. 1995, Linklater et al. 1999). Furthermore, mares show receptive behaviors during anestrus and during the nonbreeding season, which have been proposed to maintain stallion interest and strengthen the bonds between males and females year-round. I look at how fertility manipulation can change sexual interactions and social behavior of mares and how both could influence band stability.

Finally, I discuss behavioral consequences of contraceptive treatment and paternity of offspring in feral horses. I further discuss the management implications of contraceptive treatment in feral horses.

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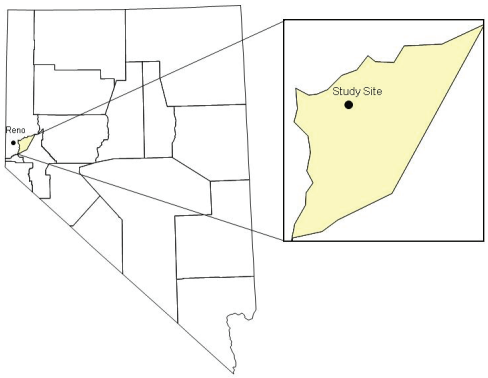


Figure 1. Location of the study site in the Virginia Range in Storey County southeast of Reno, Nevada.

CHAPTER 1

Low occurrence of extra-band copulations and parental behavior in response to actual paternity in free-ranging feral horses



“Maternity is a matter of fact; paternity is a matter of opinion.”
American Proverb

Photo caption: Thunder band; band stallion and mare (1/M) mating

Abstract: A growing number of studies conducted on diverse taxa have shown that extra-pair/group paternity is higher than what would be predicted from behavioral observations alone. While the benefits outweigh the costs for females to mate with multiple males in many situations, this often results in offspring not sired by the behavioral father, which could influence offspring survival, especially in social mammals. Feral horses (*Equus caballus*) maintain stable social relationships over several years, usually with one stallion defending a harem band of unrelated mares against other males. Sneak copulations by subordinate males have been observed and mares sometimes change bands, both of which can result in foals sired by males other than the socially dominant band stallion. We measured mare fidelity in free-ranging feral horses in 23 bands, with 51 foals over 4 foaling seasons and tested offspring paternity against parental behaviors. Genetic samples were obtained from both blood and feces. We used 12 polymorphic microsatellite loci and the program CERVUS 2.0 to determine and exclude potential sires. The majority of mares remained stable in the band with the sire of their foal resulting in most foals being sired by the band stallion. Most offspring not sired by the band stallion were the result of mares changing bands and not extra-band copulations. Foals born in a band without their sire had lower survival and suffered more aggression from stallions. Mothers were significantly more protective of foals not sired by the dominant stallion. These findings show the benefits of band stability to the reproductive success of mares and appear to support the importance of harassment and infanticide risk in equid social structure.

Introduction

Selection theory predicts that individuals should attempt to maximize lifetime reproductive success in terms of both offspring survival and numbers, but optimal strategies could vary markedly between the sexes, because the costs of producing gametes differ between males and females (Andersson 1994). For males, gametes are generally cheap and easy to produce (Bateman 1948, Trivers 1972). Consequently, males are predicted to maximize their reproductive success by mating with as many fertile females as possible (Andersson 1994). This occurs directly through male-male competition by either controlling females or excluding other males from territories or indirectly by controlling access to critical resources (Emlen and Oring 1977). Conversely, female gametes are more costly, limiting the number of offspring that females can produce (Bateman 1948, Trivers 1972), with a single mating being sufficient to produce an offspring (Trivers 1972). Furthermore, in mammals, females are the primary caregivers due to the constraints of lactation. Therefore, since females invest heavily in individual offspring, they should be the choosier sex in mate selection and select high quality males to maximize their reproductive success (Andersson 1994). Such sexual selection leads to male and female reproductive tactics that differ in ways that create conflict over who to mate with, how often to mate, and levels of parental investment (Andersson 1994).

Female choosiness does not imply that females will not seek additional matings from males other than the behavioral father or dominant group male since they might receive benefits by mating with the highest quality male (Jennions and Petrie 2000). However, the costs of sneak copulations to females can be substantial due to increased

risks of sexually transmitted diseases, aggression, and increased predation risk due to conspicuousness (Daly 1978, Arnqvist and Rowe 2005). Nonetheless, numerous studies have shown that there are potential advantages to females who seek extra-pair/group matings including direct benefits such as nuptial gifts (e.g., Wedell 1997), increased parental care (Nakamura 1998), confusion of paternity (Hrdy 1979, van Schaik et al. 2000), or increased litter size (Dewsbury 1984), as well as indirect benefits. Indirect benefits appear to explain a large proportion of cases, and include genetic advantages that increase offspring performance, diversity or viability (e.g., Andersson 1994, Endo and Doi 2002, Cohas et al. 2007, Suter et al. 2007, Dreiss et al. 2008), inbreeding avoidance (Tregenza and Wedell 2002), guarding against male infertility (Preston and Stevenson 2001), and genetic compatibility (Zeh and Zeh 2001, reviewed in Stockley 2003). While the benefits often outweigh the costs of mating with multiple males, this often results in offspring not sired by the behavioral father, which could influence offspring survival, especially in social mammals and particularly through infanticide (van Schaik 2000).

Several mammalian species have long lasting social bonds in which several females and males remain in groups beyond the mating season. Males secure matings by defending females (female-defense polygyny) or resources to attract females (resource-defense polygyny) (Emlen and Oring 1977). In these groups, male reproductive success is usually heavily skewed in favor of a few, often dominant males, although other males do get some mating opportunities (e.g., Huck et al. 2005, Brauch et al. 2008, Kappeler and Schäffler 2008). If females mate with males from other groups or subordinates within the group then males may lose opportunities to maximize reproductive success, or even expend valuable resources on females raising offspring sired by other males, thus

creating another conflict between the sexes. The most extreme form of conflict can result in the death of an offspring (infanticide, van Schaik 2000). Males from many species kill young conspecifics that are not genetically related to them (e.g., lions, Packer and Pusey 1984; primates, reviewed in van Schaik 2000; rodents, Cicirello and Wolff 1990, Coulon et al. 1995, reviewed in Blumstein 2000; equids, reviewed in Gray 2009). Males will benefit from infanticide only if certainty of paternity is high, so as to minimize the risk of killing their own offspring, and if the female becomes reproductively active earlier without dependent offspring (Packer and Pusey 1984; Sommer 1994, Borries 1997, Borries et al. 1999). Females however, would benefit from raising their current offspring regardless of paternity, and have adopted counter-strategies to protect offspring from such infanticidal attacks (Ebensperger 1998). Females then benefit from tactics to maximize protection against males, or confuse males about paternity (Hrdy 1979, van Schaik and Kappeler 1997, van Schaik et al. 2000). Hypotheses concerning mating strategies, paternity, infanticide, and female protectiveness have been tested primarily in primates and carnivores (Packer and Pusey 1984, van Schaik 2000), yet many of these hypotheses have yet to be tested in wild ungulates with similar social systems.

Feral horses (*Equus caballus*) are female-defense polygynous mammals that maintain stable year-round social relationships over long time periods (Feist and McCullough 1976, Klingel 1979, Berger1986, Linklater 2000). They group in harem bands with usually one, but sometimes several males defending a harem of unrelated females and their offspring from other males in the population (Feist and McCullough 1976, Klingel 1979, Berger1986, Linklater 2000). The adult sex ratio is usually even, so males without harems join ephemeral bachelor groups (Berger 1986, Khalil and Kaseda

1998). All male and female offspring disperse from their natal band, usually before reproductive age; therefore inbreeding remains low is most populations (Feist and McCullough 1975, Klingel 1979, Berger 1986, Monard et al. 1996). Females are limited to one foal per year and typically give birth in the spring (Feist and McCullough 1975, Keiper and Houpt 1984, Crowell-Davis and Houpt 1986). Since band stallions defend their females from other males, dominant males are expected to achieve high paternity of offspring within their band. Previous studies have shown that band stallions appear to attain a high fraction of paternity within their bands, but these results were only based on blood typing and do not have the level of accuracy of genetic methodologies present today (Bowling and Touchberry 1990, Feh 1990). These improved methodologies for determining paternity, specifically microsatellites, have shown that females from a wide variety of taxa engage in extra-pair/group copulations (e.g., Griffith et al. 2002, Lane et al. 2008, Munshi-South 2007). In feral horses, sneak copulations by bachelors and subordinates are observed, and mares sometimes change bands, all of which can result in foals sired by males other than the dominant band stallion (Miller 1981).

Since paternity is expected to be high within a band, and bands stay together for multiple reproductive seasons, paternity should be relatively predictable. Under such circumstances, infanticide would be favored if foal-killing enhanced the mother's return to fertility. Infanticide has been reported in all equids [horse, *Equus caballus* (Duncan 1982); Przewalski horse, *Equus przewalskii* (Ryder and Massena 1988; Feh and Munkhtuya 2008); mountain zebra, *Equus zebra* (Penzhorn 1984); and Burchell's zebra, *Equus burchelli* (Pluháček and Bartoš 2000, 2005)], including feral horses (Gray 2009). In addition, mares are more protective when paternity is uncertain, such as in multi-

stallion bands (Linklater et al. 1999, Cameron et al. 2003), with mares positioning themselves between their foals and other horses and actively defending their foals against attacks (Crowell-Davis and Houpt 1986). Stallions are also more protective of foals that are born within their bands than they are of other foals (Berger 1986, Boyd 1991), suggesting that both males and females assess paternity and change their behaviors accordingly. While changes in female behavior are known to occur when paternity is uncertain, there have been no studies that directly link actual paternity with these behaviors.

The aim of this study was to assess male reproductive success in free-ranging feral horses over 4 foaling seasons using DNA from fecal samples. We therefore asked (1) how often band stallions sire offspring in their bands, (2) what is the rate of mate fidelity by females, (3) what is the frequency of occurrence of extra-band copulations and (4) under what circumstances do females seek extra-band copulations. In horses, some of the major hypotheses that predict extra-band mating would not apply (e.g., decreased harassment, confusion of paternity, inbreeding avoidance, Wolff and MacDonald 2004). Therefore, we predict a low occurrence of extra-band copulations since bands are stable over long periods of time and both harassment and infanticide by foreign males would be extremely costly to females (Linklater et al. 1999). Sexual harassment influences feral horse social structure, with one of the primary roles of harem stallions being to minimize harassment by non-band males (Linklater et al. 1999). In addition, as females disperse from their natal bands, the risk of inbreeding is low. Under all of these circumstances, we predict the costs would be high and outweigh the benefits of this behavior in females. We hypothesize that sneak copulations may occur when females are seeking increased

male quality, thus most sneak copulations would occur with other band stallions as opposed to bachelors.

In addition to quantifying male reproductive success, we tested whether stallion aggression and harassment toward foals and mare protectiveness of foals were related to paternity, and tested whether there was any difference in foal survival in relation to whether the resident stallion was their father. We predicted that foals not sired by the band stallion would have an increased risk of infanticide, suffer higher rates of aggression by the band stallion, and have lower overall survival rates. We also predicted that females would increase their protective behavior as a counter-strategy to infanticide risk by maintaining more contact with their foals not sired by the dominant stallion. Here we present results of the first study investigating paternity with microsatellites in feral horses and how both maternal and paternal behaviors are linked to actual paternity.

Methods

Study Site

The study was conducted in the Virginia Range centered at 39°26'N and 119°30'W, located southwest of Reno, Nevada. The range is approximately 145,000 hectares and is mostly privately-owned land. The population size of the free-roaming horses was around 1400 individuals, and these horses were managed by the Nevada Department of Agriculture. Other ungulates in the range included mule deer (*Odocoileus hemionus*) and pronghorn (*Antilocapra americana*). The mountain range is dominated by sagebrush (*Artemesia tridentata*) at lower elevations. Other common species in this community include rabbitbrush (*Chrysothamnus nauseosus*), bitterbrush (*Purshia*

tridentata), bluegrass (*Poa secunda*), cheatgrass (*Bromus tectorum*), blue mustard (*Chorispora tenella*) and fiddleneck (*Amsinckia intermedia*). Jeffrey pine (*Pinus jeffreyi*), singleleaf pinyon (*Pinus monophylla*) and Utah juniper (*Juniperus osteosperma*) are found in higher elevations on top of ridges.

Subjects and Sampling Regime

We gathered horses (N=222) into purpose-built yards with the aid of helicopters by the Nevada Department of Agriculture in June 2005 for the purposes of a contraception trial. All females were individually freeze-branded with a unique code for future individual identification and blood was taken from every adult horse. At that time, most adult horses were aged using tooth eruption and wear patterns (Tutt 1968), which were used to classify mares into age categories. We classified mares from 3-5 years old as young since mares do not reach their full reproductive potential until the age of 5 (Berger 1986), and in our study, no mare younger than three gave birth to a foal. We classified mares from 6-8 years as medium age and because aging becomes inaccurate for wild horses 10 years and older (Richardson et al. 1995), we classified all females 9 and older in the old category.

We collected behavioral data on 23 focal bands that were located on a weekly to monthly basis from 2005-2007. Bands were encountered randomly and behavioral observations were evenly distributed during daytime hours. Behavioral sampling periods lasted for up to one hour or until the focal animal moved irretrievably from view. Adults were identified by individual freeze brands or by coat color and unique white markings on the face and legs. Observations were done from a vehicle or on foot within an

approachable distance to habituated bands or with binoculars from greater distances. Mares with foals born in 2006 and 2007 foaling seasons were studied to maximize information known about each foal. Behavioral data were collected during the period of offspring dependency until foals were weaned, died, or the end of the study. The sex of each foal was recorded shortly after birth by sighting external genitalia and the type of band the foal was born in (single stallion vs. multi-stallion) was recorded.

Behavioral Data Collection

At the beginning of each sampling period, we recorded band membership in order to track any changes in female movements and foal survival. If females changed bands during the study, their new band was recorded and behavioral observations were taken with the new band. Mares were classified as band changers only if they left their band for more than 1 day and stayed with a new band. If females came back to their original band after leaving for less than one day, they were considered stable members of the band. The time of band change was also recorded in order to determine if the mare left the band while pregnant or lactating. We determined foal survival up to weaning age and a foal was determined to be dead if it disappeared before age one, since they rarely disperse so young (Linklater 2000).

During the behavioral sampling period, we focal sampled mares and stallions to determine changes in maternal and paternal behavior (Altmann 1974). In order to measure maternal protectiveness, every approach and leave by mares and their foals within a distance of two adult body lengths was recorded. A two adult body length distance is considered close contact and is similar to the criterion used in other studies

that have measured mother-infant spatial relationships and protectiveness (e.g., Linklater et al. 1999, Cameron et al. 2003, Heitor and Vicente 2008). The percentage of approaches and leaves was then transformed into a contact maintenance score as a measure of protective behavior (Hinde and Atkinson 1970). This score was calculated from the percentage of approaches towards the foal due to the mare minus the percentage of leaves from the foal due to the mare:

$$\%A_M\text{-}\%L_M = [A_M/(A_M+A_F] - [L_M/(L_M+L_F)]$$

where A_M is the number of approaches by the mare, A_F is the number of approaches of the foal, L_M is the number of leaves by the mare and L_F is the number of leaves by the foal (Hinde and Atkinson 1970). The resultant score varies from +1 to -1, with a score closer to +1 indicating more effort by the mare to in maintain contact with her foal. In order to determine changes in nutritional investment, we recorded all nursing episodes as well as the individual who ended the episode (Cameron and Linklater 2000). For changes in stallion behavior, we measured all aggression directed towards the foal by the stallion. These behaviors included kicks, kick threats, head threats, bites, charges, and bite threats (see McDonnell and Haviland 1995 for ethogram). These data are presented as average aggressive interactions per hour.

Fecal Sample Collection and DNA Extraction Procedure

Fecal samples were collected on an opportunistic basis from known individuals during behavioral sampling. To avoid cross contamination all fecal samples were collected while wearing gloves and individual sterile swabs were used to collect the mucus on the exterior of each fecal bolus. The mucus has been shown to contain the

highest number of cells that have been shed from the intestinal lining during defecation (Wehausen et al. 2004). We placed swabs in collection tubes filled with Buffer ASL (lysis buffer, Qiagen QIAamp DNA Stool Mini Kit). Additional fecal samples that were not swabbed in the field were frozen, and genomic DNA was extracted at a later time by removing and placing a small amount (approximately 2 mg) of the surface of the pellet into the ASL buffer. DNA extraction was carried out following the QIAamp DNA Stool Mini Kit manufacturer's protocol with minor modifications as per Wehausen et al. (2004) as follows: 1) after the addition of ethanol, the samples were left for 1 hour to maximize precipitation of DNA and 2) the elution buffer (AE) was heated to 70ºC and incubated in the spin columns for three minutes.

DNA from blood samples taken during the round-up was extracted using Qiagen Tissue and Blood kit according to the manufacturer's specifications. After extraction, all samples were stored at -20ºC until further processing. DNA from blood samples was isolated and analyzed separately from that of the fecal samples. Consistency of genotype scoring for DNA from fecal samples was assessed for multiple individuals for whom both blood and fecal samples were available.

PCR Conditions

Twelve horse-specific nuclear polymorphic microsatellite loci were chosen from the literature (Marklund et al. 1994, Tallmadge et al. 1999, Tozaki et al. 2000a, 2000b, 2001; Table 1). We chose loci that could be combined in multiplex PCR reactions and that produced a small amplicon, which are more reliably amplified from fecal DNA (Marklund et al. 1994, Tallmadge et al. 1999, Tozaki et al. 2000a, 2000b, 2001; Table 1).

We ordered all forward microsatellite primers with M13 tail extension to the 5'end to allow for an economic method to label the PCR product with a corresponding fluorescent dye, as suggested by Schuelke (2000). This protocol uses 4 different M13 tails, each 5' end labeled with a different dye: NED (taaaacgacggccagtgc), PET (ggaaacagctatgacca), VIC (gcggataacaatttcacacagg), or FAM (tttcccagtcacgacgttg) to allow for multiplex reactions. All of the reverse primers were pigtailed by adding (gtttcttt) to the 5' end. We chose all primers to have an optimal annealing temperature from 60-63 °C. A touch down annealing temperature was used in the PCR parameters starting at 65°C and ending at 55 °C. In the early cycles of the PCR (65-58°C), the horse specific primers anneal, and in the later cycles (55 °C), the fluorescently labeled M13 primers anneal to label the products. We optimized PCR reactions to include 4 loci per reaction. Optimal PCR conditions were slightly different for blood samples than for fecal samples as detailed.

For the blood samples, we prepared a multiplex primer cocktail to give a final primer concentration of 0.05 µM for each tailed forward primer, 0.5 µM for each reverse primer, and 0.1 µM for each labeled M13 primer in a 16 µl reaction. PCRs consisted of 8 µl Qiagen Multiplex PCR kit master mix and 50 ng of genomic DNA where possible. PCR cycle parameters included a 15 minute hot start at 95°C, followed by 41 cycles of 95°C for 30 seconds, a touch-down annealing temperature for 90 seconds, and 72 °C for 30 seconds. The annealing temperature had 7 cycles with an annealing temperature of 65 °C, 7 cycles at 61 °C, 7 cycles at 58 °C, and 20 cycles at 55 °C in which the first 21 cycles were amplifying the horse DNA template and the final 20 cycles were amplifying the labeled M13 tail.

We carried out PCRs for the fecal samples in a separate laboratory in order to avoid cross contamination from blood samples. To increase the signal received from the fecal DNA, we modified the tailed protocol to include 2 PCR reactions per sample, such that the amplification of the template DNA was done to completion and then a second PCR reaction was carried out to fluorescently label the product. Four µl of fecal DNA extract, 7.5 µl Qiagen Multiplex PCR kit master mix, and 0.125 µM for each tailed forward and 1.25 µM for each reverse primer were used in the first reactions with a final reaction volume of 10 µl with addition of molecular grade water. PCR cycle parameters included a 15 minute hot start at 94°C followed by 35 cycles of 95°C for 30 seconds, a touch down annealing temperature for 90 seconds, 72°C for 30 seconds followed by a final extension of 62°C for 30 minutes. Annealing temperature started at 65°C and was reduced by 0.2°C each cycle for 35 cycles, ending at 58°C. One µL of this PCR product was used as a template for a second reaction with 0.53 µM for each fluorescently labeled M13 primers as a forward primer, 1.33 µM for each equine specific reverse primer, 7.5 µl Qiagen Multiplex PCR kit master mix with a final reaction volume of 15 µl by addition of molecular grade water. PCR cycle parameters for the second reaction included a 15 minute hot start at 94°C followed by 25 cycles of 94°C for 30 sec, 55°C for 90 sec, 72°C for 30 sec , followed by a final extension of 62°C for 30 minutes.

We diluted all PCR products 1:100 to 1:400, depending on the intensity of allele peaks during test runs. One µl of diluted product was added to 19 µl of HiDye Formamide with Liz (500) size standard. Fragment analysis was carried out on an ABI Prism 3730 DNA analyzer by the University of Nevada, Reno Genomics Center. We used ABI GeneMapper 3.7 software to score and bin all alleles per locus.

To ensure accurate paternity assignment, we collected at least three different fecal samples per foal, mare and stallion. This has been shown to reduce problems of low DNA quantity and poor extract quality found in fecal samples and increase the probability of reliably genotyping each individual (Frantzen et al. 1998, Taberlet et al. 1999, Goossens et al. 2000). At least 2 of the 3 fecal samples had to match in order for an allele call to be made (Taberlet et al. 1999). If samples did not match, we extracted another sample and rerun for the same individual. The two most common alleles were considered the true alleles. Individuals that did not consistently genotype were excluded from the data set.

Paternity Analysis

We used program CERVUS 2.0 to perform a parentage likelihood analysis for each foal (Marshall et al. 1998). In addition to paternity assignment, the program calculates deviations from Hardy-Weinberg equilibrium, observed and expected heterozygosities, and null allele frequencies (Table 1). To determine paternity, CERVUS 2.0 uses maximum likelihood analysis and calculates the log-likelihood of each candidate parent against random individuals. This method assigns a LOD score, with the highest LOD score representing the most likely father. The LOD score is obtained by taking the natural log of the overall likelihood ratio. The difference between the LOD score of the putative father and that of the next likely candidate is called as delta (Δ). The significance of delta values was assessed using paternity simulation of 10,000 cycles at an estimated 1% genotyping error rate and paternity was assigned with strict (95%) confidence levels (Marshall et al. 1998). Each foal had a known mother and was tested against all candidate males sampled for this study. We used the software GIMLET version 1.3.3

(Valière 2002) to compute the probability of identity (P_{ID}), which is the probability that two individuals randomly selected from a population contain the same genotype at multiple loci (Waits et al. 2001).

Statistical Analyses

To test differences in maternal behavior, we used a multivariate analysis of variance with paternity status (sired vs. not sired by band stallion), group size, sex of foal, and age of mother as predictor variables of contact maintenance between mares and foals and nutritional investment. For females that had foals in both paternity categories (sired and not sired by the band stallion), we used a paired t-test to examine differences in protective behavior. We used a Mann-Whitney test to examine differences in stallion aggression toward related and nonrelated offspring. The difference in survival to weaning based on paternity status was analyzed with a chi-squared. We considered $P<0.05$ as statistically significant and $0.05<P<1.10$ as a nonsignificant trend.

Results

Genetic Characteristics

A total of 51 foals, 33 mares, and 26 stallions from 23 bands were successfully genotyped and had sufficient alleles to make accurate paternity assessment. An additional 48 stallions from other bands and bachelors were also included. The average observed heterozygosity for the entire population was 0.673 based on all loci. The average allelic richness for each locus was 6.17 (Table 1). The genetic characteristics are summarized for each band in Table 2. There were no significant deviations from Hardy-Weinberg

equilibrium and no issues with null alleles (Table 1, 2). The probability of identity value for this subpopulation was 2.72E-10.

Band Composition

Most of the bands in the study had one adult stallion (19/23). One band had two stallions and three had three stallions. The average group size of all bands was 3.50 adults (range 2-6) and 2.19 adult females (range 1-5). Most mares (23/33, 70%) were found in the same band at the beginning and end of the study.

Mating Strategies

Females followed three different reproductive strategies: (1) females mated with the band stallion and stayed in the band while they raised their foal (N=31); (2) mares immigrated to a new band during pregnancy or during offspring dependency (N=12); and (3) females mated with males other than their band stallion (N=4) (Table 3). Four additional mares had an unknown band status at the beginning of the study, so could not be classified into any of the reproductive strategies. However, they were most likely band changers since three of these mares had foals sired by another band stallion. Most females mated with the band stallion and stayed in the band while they raised their foal (31/51 foals), resulting in stallions siring 61% of the foals in their bands (Table 3).

While most females remained in their same band, the next most common pattern was band changing. Seven of the12 mares that changed bands did so as a result of the roundup in 2005, so the cause of band changing may have been artificially induced. However, five of these seven mares stayed in the same band they changed into after the

roundup suggesting that stability in bands occurs even after a disturbance. The remaining five mares changed bands without human disturbance. Three of these were young, but the sample size was too low to detect any consistent reasons for females changing bands. Of the other two mares, one was old, but left a band with 2 stallions for a band with one stallion, and the other mare left her band for a group of 5 males, but later returned to her original band. Of the four females who engaged in extra-band copulation, the fathers were not identified among the sampled band or bachelor stallions.

In multi-stallion bands, mares followed the same strategies as described above. In the Fire band, two mares had two foals and neither was sired by any of the band stallions. In the O band, the female had two foals, one of which was sired by the dominant stallion and the other by none of the stallions. In the Y band, two females had a total of three foals. The first female had two foals both sired by the dominant stallion. The other female had a foal sired by the second ranked male, but changed to another multi-stallion band (Y/2 band) while she was pregnant and was young at the time of dispersal.

Maternal Behavior and Foal Survival

The only significant predictor of maternal protectiveness was paternity status of foals, as mothers were more protective of foals not sired by the band stallion, and maintained contact with their foals more often than those mares with offspring whose father was the dominant stallion ($F_{1,18}=6.323$, $P=0.022$, Figure 1). In order to control for the age of the foal, we looked at the contact maintenance scores of foals less than 200 days old, and the trend was the same with paternity status as the only significant predictor of protectiveness ($F_{1,14}=7.485$, $P=0.016$). For females that had foals sired and not sired by

the band stallion (N=3), the same trend of protectiveness was seen (paired t-test, t =7.83, $P=0.016$). For foals not sired by the band stallion, there was no difference in level of protectiveness based on whether they were born in the band or changed with their mothers ($F_{1,5}=0.113$, $P=0.750$). There was no difference in nursing episodes ended by the mother regardless of paternity status ($F_{1,23}=0.812$, $P=0.377$), suggesting that nutritional investment did not vary but protectiveness did.

The paternity status of foals had a significant impact on survival to weaning as foals born in the band with their sire had a higher chance of survival to weaning than foals born in bands without their sire ($\chi^2_1=4.006$, $P=0.045$, Figure 2). In addition, foals not sired by the band stallion had different survival rates depending on the timing of the band change. Foals that changed bands with their mothers had higher survival (6/8 foals survived) than foals born in a band without their sire as a result of mothers that changed bands while pregnant or had engaged in extra-band copulations (2/11 foals survived) ($\chi^2_1=6.134$, $P=0.013$). There was a nonsignificant trend for stallions to be more aggressive towards foals sired by another male (Mann Whitney test: $W=430.5$, $N_1=21$, $N_2=15$, $P=0.091$; Figure 3).

Discussion

Female Strategies

Our results are similar to blood-typing studies that showed that band stallions sire 85% of offspring in stable bands (Feh 1990, Kaseda and Khalil 1996). The majority of mares remained stable in one band and had a foal sired by the band stallion. However, we also found some bands have even lower rates of paternity and supported that up to 40%

of all foals in a band are not sired by the band stallion (Ashley 2000). Extra-band paternity could be due to either temporary band changes or to sneak copulations. We found that the majority of extra-band paternity was attributable to females who immigrated to new bands. Since this behavior persists, it is fundamental to understand under what circumstances females will change bands. Most of these females were young and most likely their band changes can be attributed to the dispersal process. Young mares disperse from their natal bands when they become reproductively mature (Monard et al.1996). However, during the dispersal process, mares commonly have low band fidelity and change bands up to the age of five until they eventually stay in a single band (Linklater and Cameron *in press*). This instability or lack of fidelity of young females is likely the cause of our results. Older mares that changed bands were changing into bands with fewer stallions, but since few older females engaged in this behavior; it is difficult to make conclusions about this behavior.

Most females do not seem to regularly seek extra-band copulations, but immigrate during pregnancy or during offspring dependency. The low rate of active extra-band copulations suggests that this behavior may be too costly for females as we predicted. The main cost would be losing their foal to infanticide, which has been shown to occur in feral horses, and is related to paternity (Gray 2009). In addition, females that solicit extra band copulations risk increased harassment by stallions. Male harassment is costly to mares, and has been shown to decrease body condition scores and reproductive success in females (Linklater et al. 1999). However, since some females engage in this mating behavior, there might be some benefits. Inbreeding avoidance is a possible benefit, but given that mares disperse from their natal bands we did not expect females to seek extra-

band matings in order to reduce inbreeding. Furthermore, there is evidence that both fathers and step fathers will not copulate with young females in their bands, suggesting active inbreeding avoidance (Berger 1986, Berger and Cunningham 1987). Another possible explanation for why females seek these copulations is to mate with males of higher quality, as seen in other species (reviewed in Jennions and Petrie 2000). However, a previous study suggested that extra-band paternity could be attributed to either other harem stallions or bachelors, which questions the importance of male quality (Ashley 2000). Unfortunately, we could not identify the males in the few documented cases of extra-band copulations. This behavior warrants further investigation.

Maternal behavior

Since infanticide occurs in equids and an attempt has been documented in feral horses (Gray 2009), females that adopt counter strategies including increased protectiveness have an advantage (Agrell et al. 1998). Protective behavior by mothers increases in the presence of non-paternal males in many species and these behaviors can decrease attacks (lions: Packer and Pusey 1983; rodents: Wolff 1985, vom Saal et al. 1995, Ebensperger 1998; primates: Collins et al. 1984, Fairbanks and McGuire 1987, Maestripieri 1995). Female feral horses are known to increase their level of protectiveness of their foals in situations in which paternity is uncertain (e.g., multistallion bands, after a band change; Cameron et al. 2003). However, it was not known if females changed their protectiveness in response to actual paternity or just the uncertainty of paternity. We found that these mares, regardless of the breeding strategy, were more protective of foals not sired by the band stallion. Furthermore, females that

changed bands with their foal or while pregnant but had a foal the subsequent year sired by the band stallion all showed the same pattern, becoming less protective toward the foals sired in the band. While the sample size was small (N=3), these data suggest that females are responding to paternity and not just general stallion aggressiveness, especially since stallions were not more aggressive towards these mares. In bands where paternity is uncertain due to presence of other males (e.g., multistallion bands), females were more protective of their offspring, even when the sire was present. This may be due to higher rates of harassment (Cameron et al. 2003, Linklater et al. 1999), but perhaps also due to an increased risk of infanticide (Cameron et al. 2003). Mares may use cues such as band changing, lack of mating, or lack of consortship with the band stallion to determine the paternity of their foals, but this behavior warrants further investigation. While maternal protectiveness changed in response to paternity, survival also varied with these different strategies, suggesting that males may also assess paternity.

Stallion Behavior and Foal Survival

Paternity is relatively predictable and, with the exception of band changes, foals are almost always sired by the band stallion. While most band stallions are successful at securing paternity of the foals in their band, there are some circumstances in which paternity is less certain. Infanticide occurs in equids and is usually attributed to stallions that did not father an offspring (Duncan 1982, Penzhorn 1984, Ryder and Massena 1988, Pluháček and Bartoš 2000, Feh and Munkhtuya 2008, Gray 2009). While there was only one witnessed infanticide attempt in this population (Gray 2009), this does not indicate that these attacks do not exist, only that they are rarely documented (as in other species;

Pusey and Packer 1994). Infanticidal males can increase their reproductive success by decreasing the time needed for females to come into estrus again (van Schaik 2000). Mares that lose foals have higher foaling rates in the subsequent year (Cameron et al. 2003), thus infanticidal males would be advantaged to attack when a foal is young in order to return a mare to estrus. We found that the strategies of mares dramatically changed the risk of foal death. Almost all foals died that were born in a band without their sire, but most foals survived that immigrated with their mothers to a new band, suggesting that being born in band without their sire increases their risk of infanticide. Our data lend support to the importance of the timing of the infanticidal attack and the potential benefits of it. Most documented attacks occur early in the foal's life, usually before 2 months of age (Pluháček and Bartoš 2005), and the benefit to return the mare to estrus and increase her subsequent reproductive success would only occur early in the foal's dependency. Here we provide more evidence that infanticidal attacks may not be advantageous when foals are older, and this is demonstrated in their higher survival rates.

Stallions may assess paternity through phenotypic matching, but it is more likely they use simple cues, similar to females, such as consort time or presence during the breeding season. Regardless, there was a trend for stallions to be more aggressive toward these foals and for these foals to have lower survival. The results suggest that stallions may also be assessing paternity and changing their behaviors accordingly.

Conclusions

Infanticide and male harassment may be more important determinants of social structure than resource acquisition (Kappeler 1997, van Schaik and Kappeler 1997,

Linklater et al. 1999). In horses, band stability has been shown to benefit mares in a variety of ways including lower parasite levels, higher reproductive success and lower harassment rates (Kaseda et al. 1995, Linklater et al. 1999). We have further demonstrated that staying in a band and raising a foal sired by the band stallion has additional benefits. First, these foals have higher rates of survival to weaning and suffer lower rates of stallion aggression. Second, mares expend less effort in maintaining contact with their foals. Protective behavior is costly and has been correlated with both lower body condition scores and reproductive success (Cameron et al. 2003). Therefore, raising a foal in the band with its sire is good for both current and future reproductive success. Furthermore, the majority of mares that were forced to change bands due to the round-up were stable afterwards and never changed bands suggesting that band stability occurs even after a disturbance. We provide further evidence for the benefits of having long tenure in a band and it appears most mares are engaged in this strategy, rather than band changing or seeking extra-band copulations.

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Table 1. Summary of 12 microsatellite loci used for genetic paternity analysis in feral horses, including the number of alleles per locus (A), the number of genotyped individuals (N), observed (H_O) and expected (H_E) heterozygosities, null allele frequencies (NA), and estimates of exclusion probability for paternity assignment. Values derived from CERVUS 2.0 (Marshall et al. 1998). Primers were multiplexed into 3 different panels, with loci TKY 223, TKY276, TKY 284, TKY 286 in the first panel, COR 61, COR 72, HTG 7, TKY 312 in the second panel and COR 68, COR 71, HTG 15, TKY 282 in the third panel.

Locus	A	N	H_O	H_E	NA	Exclusion Probability	Reference
TKY223	6	167	0.665	0.627	-0.037	0.362	Tozaki et al. 2000a
TKY276	5	162	0.623	0.634	0.007	0.364	Tozaki et al. 2000b
TKY284	8	162	0.623	0.663	0.028	0.402	Tozaki et al. 2000b
TKY286	4	154	0.662	0.652	-0.011	0.364	Tozaki et al. 2000b
COR61	9	163	0.748	0.774	0.015	0.567	Tallmadge et al. 1999
COR72	6	165	0.727	0.686	-0.036	0.435	Tallmadge et al. 1999
HTG7	4	163	0.712	0.663	-0.037	0.417	Marklund et al. 1994
TKY312	7	161	0.770	0.733	-0.026	0.496	Tozaki et al. 2001
COR68	5	163	0.601	0.594	-0.008	0.366	Tallmadge et al. 1999
COR71	8	157	0.758	0.788	0.018	0.578	Tallmadge et al. 1999
HTG15	6	162	0.568	0.532	-0.043	0.303	Marklund et al. 1994
TKY282	6	157	0.713	0.715	0.000	0.453	Tozaki et al. 2000b

Table 2. Summary of genetic characteristics based on each individual band including the number of alleles per locus (A), the number of genotyped individuals in each band (N), observed (H_O) and expected (H_E) heterozygosities. Each band is displayed vertically and each locus is displayed horizontally. Bachelors and other band stallions are listed in the "unk" column.

Locus		A	B	C	E	I	N	O	Q	U	V	Y	Z
TKY223	N	5	7	6	7	4	3	6	3	4	10	8	4
	A	2	2	3	3	1	2	3	2	2	3	4	3
	He	0.45	0.52	0.65	0.52	0.00	0.50	0.65	0.50	0.42	0.55	0.57	0.46
	Ho	0.60	0.57	1.00	0.71	0.00	1.00	0.33	0.67	0.50	0.80	0.63	0.50
TKY276	N	5	7	6	7	4	3	5	2	4	10	8	4
	A	4	3	3	3	3	3	3	2	3	2	3	2
	He	0.80	0.69	0.65	0.46	0.58	0.67	0.60	0.50	0.63	0.52	0.63	0.58
	Ho	1.00	0.71	0.33	0.57	0.75	0.33	1.00	0.50	1.00	0.50	0.75	0.25
TKY286	N	5	7	4	7	4	2	4	3	4	10	8	4
	A	3	3	2	3	3	2	4	3	3	2	3	3
	He	0.68	0.58	0.42	0.70	0.67	0.50	0.79	0.67	0.58	0.52	0.69	0.67
	Ho	1.00	0.86	0.50	0.71	0.75	1.00	0.75	1.00	0.75	0.50	0.75	0.75
TKY284	N	5	7	5	7	4	3	6	3	3	10	8	4
	A	3	3	3	3	2	1	3	1	3	3	4	3
	He	0.63	0.66	0.50	0.62	0.50	0.00	0.63	0.00	0.83	0.63	0.52	0.58
	Ho	0.60	0.57	0.60	0.57	0.00	0.00	0.50	0.00	0.67	0.70	0.50	0.75
COR61	N	5	7	6	7	4	3	6	3	4	10	8	4
	A	5	6	6	5	3	4	4	1	3	4	5	4
	He	0.75	0.81	0.85	0.71	0.46	0.92	0.75	0.00	0.67	0.71	0.78	0.75
	Ho	0.80	0.71	0.83	0.57	0.50	0.67	0.67	0.00	0.75	1.00	0.88	1.00
COR72	N	5	7	6	6	4	3	6	3	4	10	8	4
	A	3	3	3	4	3	3	3	2	4	4	3	2
	He	0.68	0.67	0.53	0.73	0.75	0.83	0.43	0.50	0.71	0.73	0.70	0.50
	Ho	0.80	0.71	0.67	1.00	0.75	0.33	0.50	1.00	1.00	0.80	0.75	1.00

HTG7	N	5	6	6	6	4	2	5	3	4	10	8	4
	A	2	3	4	3	2	1	4	2	4	3	3	4
	He	0.35	0.43	0.72	0.70	0.50	0.00	0.75	0.33	0.71	0.49	0.65	0.63
	Ho	0.40	0.50	0.67	0.50	0.75	0.00	1.00	0.33	1.00	0.50	0.88	0.75
TKY312	N	5	7	5	7	4	3	6	3	4	9	8	4
	A	3	7	4	4	4	3	4	2	3	4	4	4
	He	0.60	0.88	0.75	0.73	0.67	0.67	0.72	0.50	0.71	0.75	0.73	0.75
	Ho	1.00	1.00	1.00	0.57	0.50	1.00	1.00	0.67	1.00	0.78	0.88	0.75
COR68	N	5	7	5	7	4	3	6	2	4	10	8	4
	A	3	4	2	3	3	3	3	2	3	4	3	2
	He	0.63	0.57	0.20	0.41	0.46	0.67	0.72	0.50	0.46	0.71	0.42	0.25
	Ho	0.60	0.57	0.20	0.14	0.50	1.00	0.67	0.50	0.50	0.80	0.50	0.25
COR71	N	5	7	5	7	4	2	5	3	4	10	8	4
	A	4	4	4	3	5	2	6	3	3	6	4	4
	He	0.70	0.77	0.73	0.50	0.79	0.50	0.85	0.67	0.75	0.78	0.66	0.75
	Ho	0.80	0.86	0.80	0.29	0.75	0.50	0.80	1.00	0.75	0.80	0.38	0.75
HTG15	N	5	7	5	7	4	3	6	3	4	10	8	3
	A	3	3	4	3	2	3	3	2	3	3	4	2
	He	0.50	0.54	0.68	0.63	0.25	0.75	0.70	0.33	0.63	0.54	0.35	0.33
	Ho	0.60	0.57	1.00	1.00	0.25	0.67	0.83	0.33	1.00	0.60	0.38	0.33
TKY282	N	5	6	4	6	4	3	6	3	4	10	7	4
	A	3	4	3	4	3	2	3	4	3	4	3	3
	He	0.70	0.58	0.58	0.68	0.67	0.67	0.60	0.83	0.58	0.60	0.62	0.63
	Ho	1.00	0.33	0.75	0.83	1.00	0.00	0.83	0.67	0.75	0.70	0.71	0.50

Locus		IC	NA	Af	As	Ant	Thunder	Sun	Fire	H3	SA	Unk
TKY223	N	9	3	8	4	5	7	7	7	3	3	33
	A	3	3	3	2	2	3	3	5	4	3	5
	He	0.59	0.58	0.48	0.50	0.45	0.67	0.66	0.66	0.83	0.58	0.71
	Ho	0.88	0.67	0.63	0.75	0.60	0.43	0.71	0.71	1.00	0.67	0.70
TKY276	N	8	3	8	4	5	7	7	6	3	3	34
	A	3	2	4	3	3	2	2	2	2	3	5
	He	0.70	0.50	0.67	0.67	0.38	0.52	0.38	0.53	0.33	0.67	0.66
	Ho	0.71	0.67	0.75	0.25	0.40	0.57	0.14	0.50	0.33	1.00	0.68
TKY286	N	8	3	8	4	5	7	6	7	2	2	32
	A	3	2	3	3	3	3	3	4	2	1	3
	He	0.59	0.33	0.60	0.75	0.65	0.69	0.67	0.70	0.50	0.00	0.66
	Ho	0.57	0.33	0.75	0.50	0.40	0.86	0.67	0.57	0.50	0.00	0.63
TKY284	N	8	3	8	4	5	7	7	7	3	3	32
	A	3	3	4	3	3	4	4	4	3	3	5
	He	0.67	0.75	0.69	0.58	0.65	0.74	0.73	0.66	0.67	0.67	0.69
	Ho	0.71	1.00	0.50	0.75	0.40	1.00	0.57	0.71	1.00	1.00	0.63
COR61	N	8	3	8	3	5	7	7	7	2	3	32
	A	5	2	4	4	4	5	5	5	3	2	6
	He	0.78	0.33	0.52	0.83	0.78	0.82	0.80	0.67	0.75	0.50	0.72
	Ho	0.57	0.33	0.50	1.00	1.00	0.86	1.00	0.71	1.00	0.67	0.75
COR72	N	7	3	8	4	5	7	7	7	3	3	34
	A	3	4	4	3	3	3	4	3	3	2	6
	He	0.46	0.75	0.73	0.63	0.70	0.46	0.74	0.55	0.67	0.50	0.71
	Ho	0.67	1.00	0.88	1.00	0.80	0.57	0.86	0.43	1.00	0.67	0.53
HTG7	N	9	3	8	4	5	7	7	7	3	3	34
	A	3	3	3	3	3	4	4	4	3	3	4
	He	0.66	0.67	0.55	0.63	0.68	0.81	0.74	0.71	0.67	0.67	0.73

	Ho	0.75	1.00	0.88	1.00	0.80	0.71	1.00	0.71	1.00	1.00	0.65
TKY312	N	9	3	8	4	3	7	7	7	3	3	32
	A	3	2	5	3	2	4	3	3	3	3	6
	He	0.49	0.67	0.79	0.63	0.33	0.79	0.57	0.67	0.67	0.58	0.76
	Ho	0.75	0.33	1.00	0.50	0.33	0.71	0.71	0.71	1.00	0.67	0.78
COR68	N	8	3	8	4	5	7	7	7	3	3	33
	A	4	3	4	3	3	4	3	3	3	1	5
	He	0.60	0.75	0.74	0.71	0.55	0.63	0.55	0.38	0.75	0.00	0.67
	Ho	0.43	0.67	0.88	0.75	0.20	1.00	0.43	0.43	0.67	0.00	0.79
COR71	N	8	3	8	4	5	7	6	7	3	3	33
	A	4	2	4	5	4	5	3	4	3	2	8
	He	0.73	0.50	0.69	0.75	0.65	0.83	0.53	0.82	0.58	0.50	0.80
	Ho	0.86	0.67	0.75	1.00	0.60	1.00	0.67	0.57	0.67	0.67	0.91
HTG15	N	8	3	8	4	5	7	6	6	3	3	34
	A	5	2	2	3	3	2	2	3	2	2	4
	He	0.65	0.50	0.13	0.46	0.53	0.52	0.30	0.52	0.50	0.33	0.52
	Ho	0.71	0.67	0.13	0.50	0.40	0.57	0.33	0.67	0.67	0.33	0.50
TKY282	N	7	3	8	4	5	7	7	6	3	3	34
	A	4	3	4	3	3	3	3	4	4	3	5
	He	0.58	0.75	0.63	0.67	0.63	0.70	0.63	0.78	0.75	0.58	0.74
	Ho	0.33	0.67	0.88	1.00	0.60	0.86	0.86	0.67	1.00	0.67	0.68

Table 3. Genetic data from all successfully genotyped foals. LOD scored and delta values were derived from CERVUS 2.0. The most likely father was the band stallion, another band stallion, bachelor, or unidentified male. Band stallion indicates the foal was born in the band with its true sire and other band stallion indicates the foal was sired by a different band stallion. Unidentified males were males whose DNA was taken during the round-up but they could not be identified in the field as bachelors or band stallions. All of these males were old age category. Confidence levels were set at 95% in order to determine the most likely father.

Band	Female	Foal	LOD Score	Delta	Most Likely Father	Changed Bands
A	BM	RF07	5.90	3.48	Band Stallion	No
A	W-	CHF07	8.68	5.11	Band Stallion	No
Africa	E6	CHF06	8.02	5.97	Band Stallion	No
Africa	E6	BF08	9.94	9.05	Band Stallion	No
Africa	M6	RF05	4.95	1.58	Band Stallion	No
Africa	M6	BF07	6.02	2.38	Band Stallion	No
Antarctica	3/7	DBF07	5.84	3.75	Bachelor	Yes
Antarctica	7/W	DBF07	6.40	2.82	Band Stallion	No
Asia	-/3	DBF07	4.83	0.45	Band Stallion	No
Asia	-/3	DBF06	2.82	0.01	Unidentified Male	Yes
B	1/3	BF05	0.75	0.08	Unidentified Male	Yes
B	6M	BF06	3.40	1.22	Band Stallion	No
B	6M	BF08	5.59	1.41	Band Stallion	No
B	6M	BF05	1.78	0.21	Unidentified Male	Yes
C	LE	BF06	6.04	3.11	Other Band Stallion	Unknown
C	ML	RF06	1.86	0.39	Unidentified Male	Unknown
C	ML	RF05	7.19	4.44	Unidentified Male	Yes
E	3L	CHF05	4.72	1.40	Unidentified Male	Yes
E	BM	BF07	1.51	1.21	Band Stallion	No
E	BM	BF06	4.05	0.42	Unidentified Male	Unknown
E	BM	BF05	2.76	0.30	Unidentified Male	Yes
Fire	7/3	BF07	2.39	0.01	Unidentified Male	No
Fire	7/3	CHF05	4.92	4.16	Unidentified Male	Yes
Fire	GM	BF07	4.84	1.22	Unidentified Male	No
H/3	L/3	RF08	5.29	1.40	Band Stallion	No
I	-3	RF07	9.10	3.26	Band Stallion	No
I	-3	CHF05	3.23	2.63	Unidentified Male	Yes
IC	1M	BF07	4.06	4.06	Band Stallion	No
IC	1M	BF06	1.79	1.68	Unidentified Male	No
N	-/E	BF05	3.98	0.49	Other Band Stallion	Unknown
NA	37	RF07	2.71	0.59	Band Stallion	No
O	3-	BF07	5.05	2.34	Band Stallion[a]	No

O	3-	BF06	2.46	1.66	Unidentified Male	No
Q	E9	DBF05	5.72	2.40	Unidentified Male	Yes
SA	7W	DBF05	5.66	2.92	Band Stallion	No
Sun	L3	PF07	10.70	9.76	Band Stallion	No
Sun	M/7	CHF05	10.00	8.21	Band Stallion	No
Sun	M/7	RF06	8.54	4.37	Band Stallion	No
Sun	M/7	RF07	5.27	2.81	Band Stallion	No
Thunder	1/M	BF07	7.51	2.69	Band Stallion	No
Thunder	1/M	BF08	7.94	3.35	Band Stallion	No
Thunder	13	BF08	7.90	7.06	Band Stallion	No
U	LW	BF07	5.88	3.09	Previous Band Stallion	Yes
V	1/W	RF04	3.09	0.75	Band Stallion	No
V	1/W	DBF05	4.05	1.20	Band Stallion	No
V	3/1	RF05	5.72	3.66	Band Stallion	No
V	3/1	CHF04	6.89	5.11	Band Stallion	No
Y	E/-	BF05	5.45	1.71	Band Stallion[a]	No
Y	E/-	BF06	5.66	3.90	Band Stallion[a]	No
Y/2	M/-	BF07	7.13	3.04	Previous Band Stallion	Yes
Z/2	-/M	RF07	4.91	1.72	Band Stallion	No

a Females in multi stallion bands where one of the stallions was the sire

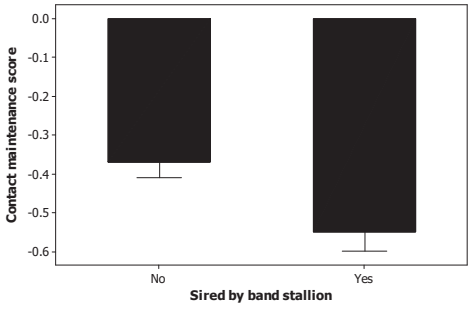


Figure 1. The average contact maintenance scores for all mares and foals based on paternity status of foals. Scores closer to zero represent the dam maintaining more contact with the foal.

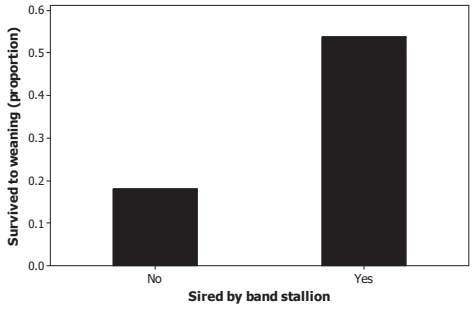


Figure 2. The proportion of foals that survived to weaning age based on whether they were born in the band with their true sire (N=26) or another stallion (N=11).

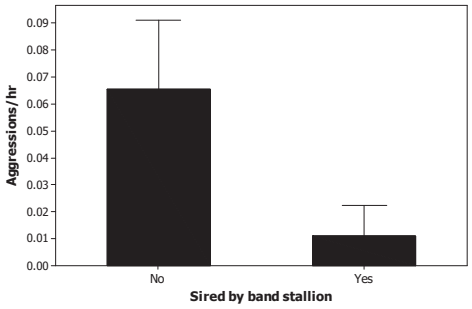


Figure 3. The average aggressions/per hour directed towards foals by band stallions based on paternity status of foals.

CHAPTER 2

An infanticide attempt by a free-roaming feral stallion (*Equus caballus*)



"You cannot love a thing without wanting to fight for it."
Gilbert K. Chesterton

Photo caption: Asia band: Mare (-/3) grazing close to her newborn foal.

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Abstract: Infanticide by adult males occurs in a variety of species. While infanticidal attacks have been documented in several equid species in captivity, it has never been witnessed in free-roaming feral horses. I report an infanticide attempt by a free-living feral stallion on a recently born female foal. The stallion picked up the foal by the shoulders, tossed it around twice and bit in on the neck several times. The dam of the foal charged the stallion and successfully protected her foal from additional attacks. The foal survived the attack and later weaned successfully. The stallion recently took over the band and was excluded as the sire through genetic analysis. While this type of attack is rare, this case lends support to the sexual selection hypothesis and further demonstrates that equids have evolved with the risk of infanticide. Furthermore, it shows that maternal protectiveness can be successful against attacks by infanticidal males.

Introduction

Infanticide, while rare, is a widely documented phenomenon in several mammal species including primates (Hrdy 1977; Crockett & Janson 2000; Watts *et al.* 2002), carnivores (Packer & Pusey 1983; Swenson *et al.* 1997), and rodents (Coulon *et al.* 1995; Vom Saal *et al.* 1995; Blumstein 2000). Male infanticide is generally considered to be an adaptive strategy whereby the interbirth interval is reduced, and the male can sire the subsequent offspring (van Schaik 2000). Sexual selection implies a benefit to the reproduction of infanticidal males provided that only unrelated infants are killed, since infanticide is costly to males if related offspring are killed (van Schaik 2000). Thus, recognition of paternity plays a key role in the evolution of this strategy.

With the exception of red deer (*Cervus elephus*) (Bartoš & Madlefousek 1994) and the hippopotamus (*Hippopotamus amphibus*) (Lewison 1998), the majority of cases of infanticide involving ungulates are reported in equids, including the horse (*Equus caballus*) (Duncan 1982), Przewalski horse (*Equus przewalskii*) (Ryder & Massena 1988; Feh & Munkhtuya 2008), mountain zebra (*Equus zebra*) (Penzhorn 1984), and Burchell's zebra (*Equus burchelli*) (Pluháček & Bartoš 2000). Feral horses are unusual among ungulates in that they form stable harem-type groups year-round consisting of one or more mares and usually one but sometimes several stallions (Linklater 2000). Band stallions are known to obtain high paternity within their band (Bowling & Touchberry 1990), but sneak copulations occur and mares change bands, which can result in paternity differences between foals (Miller 1981). In feral horses, infanticide has never been documented in the wild, but feticide may occur (Berger 1983). Feral mares have been shown to increase maternal protectiveness in response to paternity uncertainty (Cameron

et al. 2003), which suggests that females have evolved to protect their foals from attacks by males. Here, I document a case of a resident band stallion attacking a young foal shortly after birth in a free-roaming situation.

Methods

A feral horse population in the Virginia Range outside Reno, Nevada was observed intensively from 2004-2008 for the purposes of another study. The total population fluctuated from over 1200 horses down to 1000 horses over the course of those years. The Nevada Department of Agriculture is responsible for managing the horses and the population size was and is still controlled by removals and contraception. The study included approximately 300 known individuals. Horses were habituated to human observers. The band in which the attack occurred was seen on a weekly basis to determine band membership and fidelity. At the time of the attack, the band consisted of 2 adult stallions, 2 adult mares, one juvenile male, and one male foal. The band was being observed due to a recent birth of a foal and from that time, ad libitum observations were recorded until the end of the incident. All aggressive behaviours were documented and a description of these behaviours can be found in an equid ethogram (McDonnell & Haviland 1995).Genetic data were taken from fecal samples from the foal, dam, and band stallion. Paternity was assessed using 12 polymorphic horse-specific microsatellites.

Results

During routine behavioural observations on April 12, 2005, an infanticide attempt was witnessed. The band was passed at 15:40, while observing another band. At that

time, the foal was not born. At 16:14, while passing the band again, the mare had recently given birth. A new female foal was covered with mucous and lying on the ground next to her dam. The foal was estimated to be no more than 1 hour old when the attack occurred and had not attempted to stand up. The rest of the band, including the dominant band stallion (stallion A), were all resting near the mare and newborn foal. At 16:20, a stallion (stallion B) from a nearby band called out and approached the mare. She immediately gave a head threat towards stallion B. The foal attempted unsuccessfully to stand up. The band stallion (stallion A) lunged towards the mare and foal and immediately bit the foal on the neck several times. Stallion A subsequently picked the foal up by the shoulders twice and shook it around several times. The foal was dropped to the ground and subsequently bitten and kicked by the front legs of stallion A several times. The attack lasted approximately 1 minute. During the attack, the mare defended her foal by charging, biting, and kicking stallion A, which led to the end of the attack. After the attack, stallion A had several aggressive interactions with stallion B and continued to try to herd the mare away from the area. The mare continued to charge and head threat stallion A when he came near her and she remained in her position over the foal. Stallion B called out again, which led to another aggressive interaction between stallion A and the mare. While the foal appeared to be in shock, there was no visible major damage, but she suffered minor superficial wounds. The foal stood up at 17:39 and nursed at 17:47, and at that time the observations ended. The band was observed over the course of the next year and the foal successfully weaned.

The attacker (stallion A) was excluded as the father of the foal through microsatellite DNA analysis.

Discussion

The attack was unmistakably an infanticide attempt as it was similar to other reported attacks in equids (Duncan 1982; Feh & Munkhtuya 2008). Most infanticidal attacks by stallions occur before the foal reaches one month (Pluháček & Bartoš 2000) and in this case, the foal was recently born. Infanticide imposes direct fitness consequences on the parents of the offspring, especially the mother. Such costs include decreased inclusive fitness, energy expenditure, and the loss of investment (Ebensperger 1998). Females should adopt counterstrategies against infanticide in order to maximize their lifetime fitness (Ebensperger 1998). These counterstrategies include fighting back, having close associations with males for infant protection, prolonged receptivity, territoriality, avoidance, and termination of infant care (Ebensperger 1998). In horses, mares will defend against infanticidal attacks (Feist & McCullough 1975; Ryder & Massena 1988) by generally positioning themselves between their foal and another approaching horse, even if no direct threat is apparent (Crowell-Davis & Houpt 1986). This protective behaviour was witnessed during the attack, as the mare defended her foal by charging and biting the stallion. She also tried to position herself between the stallion and foal and stood over the foal after the incident. All of these actions appeared to stop the attack and prevent further assaults.

There were several factors that possibly led to this attack. First, genetic analysis confirmed the band stallion was not the sire. Second, the band stallion had recently taken over the band and shared the band with another adult stallion. Because of these factors, there was a high level of paternity uncertainty which may have influenced the decision to attack. Little is known about how males detect paternity, but evidence in primates

suggests that males detect paternity by consort time, phenotypic matching, or the amount of time spent within the troop during the mating season (Buchan *et al.* 2003). In this case, the male did not have a consortship with the female, nor did he spend any time with this female during the previous mating season. The uncertainty of paternity has been postulated as a proximate cause of an infanticide attempt (Borries at al. 1999), and is believed to be the major cause in this particular case.

Recently, infanticide in equids was suggested to be in response to human intervention (human disturbance hypothesis), as most cases have been associated with unnatural environments and introductions of foreign males (Feh & Munkhtuya 2008). While humans may influence infanticide attempts, most documented cases have also been associated with foreign males. For example, in equids, attacks end after males and females have been housed together for longer periods of time (Ryder & Massena 1988), suggesting that the unnatural conditions do not explain infanticide. In addition, many infanticidal events in the wild are associated with contact with foreign males, and attacks are generally attributed to these males. In this case, the male was relatively foreign in the band and several factors increased the level of uncertainty of paternity (i.e. multi-stallion band, recent takeover, no consortship before foal was born). In addition, the similarity of attacks between equid species, maternal protectiveness, and genetic data all suggest that equids have evolved with and respond to infanticide risk. Furthermore, infanticide has been postulated as a main explanation for primate social structure and the evolution of year-round male-female associations (Kappeler 1997; van Schaik & Kappeler 1997). Feral horses have similar social structure to primates and male harassment plays a primary role in social structure (Linklater *et al.* 1999).

This was the only attack witnessed during 3 years of behavioural observations. Several studies suggest that feticide may be more adaptive in horses (Berger 1983), and there are some suggestions that infanticide is low risk in equids (Feh & Munkhtuya 2008). However, several foals disappear and are found dead every year in all populations, even when no predators are present. While these deaths could be due to numerous factors, infanticide is a possible cause of some deaths. Furthermore, foals not sired by the band stallion have lower survival rates to weaning (*unpublished data*). All of this makes a witnessed event and possible causes that much more important to document. To my knowledge, this is the first documentation of this type of attack in feral horses in a free-living situation and lends support to the role of infanticide in social structure.

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CHAPTER 3

Multi-year fertility reduction by two contraceptive formulations in free-roaming feral horses: Is body condition important when evaluating efficacy?



“Contraceptives should be used on all conceivable occasions.”
Spike Milligan

Photo caption: Antarctica band: Mare (3/7) right before birth of 2007 foal

Abstract: Feral horses (*Equus caballus*) continue to increase in numbers in the western United States. Past management techniques have used the round-up and adopt-out method, but contraception is becoming increasingly tested and used to reduce fertility in wildlife populations. To date, multi-year fertility reduction with one vaccination has not been demonstrated in free-roaming feral horses. We tested two contraceptive formulations, a porcine zona pellucida (PZP) formulation SpayVac®, and a gonadotropin releasing hormone (GnRH) formulation GonaCon-B™, to determine efficacy in a population of free-roaming feral horses near of Reno, Nevada. Females were treated in June 2005 and monitored year round until the summer of 2008. For mares treated with GonaCon-B™, the average reproductive success from 2006-2008 was 39%, 42% and 31% respectively. SpayVac® treated mares showed average reproductive success of 36%, 56%, and 44% respectively. During the same seasons, 61%, 67% and 76% of control females foaled. Linear mixed model analysis revealed that both contraceptive treatments significantly reduced fertility in the combined 3 years. However, several treated females in both treatment groups had foals and our fertility rates were much higher than reported in other studies. Our lower efficacy rates may have been due to our intensive sampling efforts that increased our ability to observe foals more often than researchers in other studies who only surveyed a few times per year. However, when the treatments worked on individuals, they worked for several years. Most of the treated females were never fertile or fertile one year compared with control females were fertile every year or 2 of 3 years, suggesting that contraceptive treatments worked better on certain individuals. We found that body condition at time of treatment had a significant impact on reducing fertility as females in good condition showed reduced fertility

compared to controls, but females in poor condition did not. We found no significant effects from contraceptive treatment on the sex ratio of foals, birthing season or foal survival. The results of this study demonstrate that both vaccines are capable of significantly reducing fertility for several years without boosters and may aid in population control of feral horses, but body condition at time of treatment may be an important, yet overlooked factor when evaluating efficacy.

Introduction

Anthropogenic disturbance of natural ecosystems by removal of predators and introduction of invasive species, has led to the overabundance of many species to the detriment of both the environment and other wildlife species (e.g., white tailed deer; Warren 1997, feral horses; Wagner 1983, Canadian geese; Conover and Chasko 1985). Traditionally, populations of overabundant wildlife were controlled by manipulating mortality by removing individuals permanently from the population by lethal control or permanent removals. However, mortality manipulation can cause increased population growth (Garrott and Taylor 1990, Kirkpatrick and Turner 1991), changes to animal movements (Henderson et al. 2001) and public objections (Jonker et al. 2006, Stout et al. 1997, Lauber et al. 2007). Consequently, non-lethal methods of control are becoming increasingly advocated when traditional culling methods are impractical or undesirable (Kirkpatrick 2007). Non-lethal population management usually involves manipulating fertility rather than mortality.

Contraception has become a widely used nonlethal technique to reduce fertility in managed species and is usually considered a more humane long-term approach to population control because of its reversibility (Oogjes 1997, Kirkpatrick 2007). In particular, immunocontraception, which uses an animal's own immune system to inhibit fertility, has been tested extensively in many wildlife species to inhibit pregnancy by blocking fertilization or estrous cycling with the use of GnRH immunizations [e.g., elk (*Cervus elaphus*): Baker et al. 2002, Conner et al. 2007; feral swine (*Sus scrofa*): Killian et al. 2006; white-tailed deer (*Odocoileus virginianus*): Miller et al. 2000a], porcine zona pellucida [PZP, e.g., black bears (*Ursus americanus*): Lane et al. 2007; burros (*Equus*

asinus): Turner et al. 1996; elephants (*Loxodonta africana*): Fayrer-Hosken et al. 2000, Delsink et al. 2002; elk (*Cervus elaphus*): Kirkpatrick et al. 1996; feral horses (*Equus caballus*): Kirkpatrick et al. 1992, Turner et al. 1997, 2001, 2007; white-tailed deer (*Odocoileus virginianus*): Miller et al. 2000b, Naugle et al. 2002, Walter et al. 2002, Rutberg et al. 2004], or progesterone implants [e.g., gray kangaroos (*Macropus giganteus*): Nave et al. 2002, Herbert et al. 2005; tammar wallabies (*Macropus eugenii*): Hynes et al. 2007]. While biologically effective, contraceptive formulations also need to be practical for management, using a single dose and having minimal side effects.

Feral horses (*Equus caballus*), federally managed under the Free-Roaming Wild Horse and Burro Protection Act of 1971, continue to overpopulate rangelands in the western United States where they can negatively impact grasslands, soils and other wildlife species (Rogers 1990, Beever and Brussard 2000, Levin et al. 2002, Beever and Brussard 2004, Zalba and Cozzani 2004, Beever and Herrick 2006). Despite this trend, lethal control is illegal and largely unacceptable to the public. The Bureau of Land Management (BLM) is responsible for maintaining sufficient numbers of horses in order to preserve genetic diversity while balancing rangeland capacity. Past management techniques have focused on the "round-up and adopt-out" method, but there have been concerns about the efficacy of these programs and their impact on animal welfare (Kirkpatrick 2007). While these management techniques have initially reduced numbers, feral horse populations continue to grow, and adoption rates are declining, resulting in a surplus in holding facilities. Consequently, new ways to manage free-living horses effectively and humanely are increasingly urgent. Contraceptive formulations have been extensively researched in free-roaming horses (PZP; Kirkpatrick et al. 1990, 1996, Turner

et al. 1997, 2001, 2002, 2007 and GnRH; Killian et al. 2008) and show promise for population control that may be more cost effective than removals over the long term (Bartholow 2007). However to date, there is no formulation that lasts for several years without the need for boosters, and there is limited data on the behavioral and physiological consequences of contraceptive treatment in feral horses.

Concerns have been raised over the potential side effects of contraceptive treatments (see Nettles 1997), yet there has been little research conducted that addresses side effects in comparison to efficacy studies in many species (see Gray and Cameron *in review*). The manipulation of fertility will most likely impact the physiology and behavior of treated individuals, which can in turn impact other individuals in the population (Gray and Cameron *in review*). Most research on side effects has focused on breeding behavior and body condition of treated females. For example, several studies have shown that contraceptives alter breeding behavior of females resulting in a lengthened breeding season and later offspring birth dates (McShea et al. 1997; Heilmann et al. 1998; Miller et al. 2000b). Several studies have also reported increased body condition of treated females in wild species (McShea et al. 1997, Turner and Kirkpatrick 2002). While increases in body condition can be desirable, this could impact offspring sex ratios as females that experience increased condition tend to have more sons (see Cameron 2004). Another potential side-effect that has yet to be tested is the unintended selection of immunocompromised individuals. Because immunocontraception relies upon a competent immune system, animals with more expansive immune systems would have greater reductions in fertility than those with poor immune systems (Nettles 1997, Cooper and Herbert 2001, Cooper and Larsen 2006). Over time, this could lead to the selection of

non-responders, eventually making the treatment ineffective (Nettles 1997, Cooper and Herbert 2001, Cooper and Larsen 2006). While there is a potential for this to occur over a long time, it is not known if the variation in response to contraception is related to immune system function (Cooper and Larsen 2006). Regardless of treatment, side effects should be studied on managed species in order to minimize impacts on behavior, physiology and population health.

We simultaneously tested efficacy of alternative contraceptive formulations in free-roaming feral horses in the Virginia Range in Nevada. We used two different formulations which were developed to reduce fertility for multiple years from one injection and without the need for revaccination. The first formulation, SpayVac®, is a liposome encapsulated porcine zona pellucida (PZP) that allows for continued cycling and acts to prevent pregnancy by blocking fertilization, with a single dose lasting for several years (Brown et al. 1997). This contrasts with other PZP formulations that require multiple doses to achieve several years of infertility (Turner et al. 2001, 2002). It has been successfully used in fallow deer (Fraker et al. 2002), white-tailed deer (Hernandez et al. 2006), hooded seals (Brown et al. 1997), and captive feral horses (Killian et al. 2008). The second formulation, GonaCon-B™, is a gonadotropin-releasing hormone (GnRH) vaccine that combines GnRH with a mollusk protein. The antibodies resulting from this vaccine bind with endogenous GnRH (Miller et al. 2008). The resultant product is too large to diffuse through the hypothalamo-hypophyseal portal system into the anterior pituitary, and prevents GnRH from binding to pituitary receptors (Miller et al. 2008). Therefore, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are not produced in high quantities and do not act on the gonads, thereby preventing

ovulation (Talwar 1985). This product takes GnRH out of circulation, but does not alter the hormone itself (Miller et al. 2008) and does not impact other hypothalamic hormones (Awoniyi et al. 1993). This formulation has been proven to work in several ungulates including white-tailed deer (Miller et al. 2000a), bison (Miller et al. 2004), feral swine (Killian et al. 2006), wild boar (Massei et al. 2008), and captive feral horses (Killian et al. 2008). Each formulation alone would not elicit a large or long-term response, thus AdjuVac™, an oil based adjuvant, was used to increase the immune response. Here we present efficacy data for 3 foaling seasons (2006-2008) for contraceptive formulations that have never been field tested in free-roaming feral horses, using intensive observations of individuals supplemented by hormonal analysis of pregnancy.

In addition to efficacy, we looked at potential side effects of contraceptive treatment. We tested if contraception impacted foal survival, birth sex ratio, and birthing season. We also tested if body condition at time of treatment had an impact on the reduction in fertility to determine if immunocontraceptive efficacy is dependent on individual quality as a potential indicator of immune system health.

Methods

Study site and animals

The study was conducted in the Virginia Range centered at 39°26'N and 119°30'W, located southwest of Reno, Nevada. The range is approximately 145,000 hectares and is mostly private land, with a small percentage developed for houses and industry. The mountain range consists of sagebrush communities (*Artemesia tridentata*) with groups of pine (*Pinus jeffreyi, Pinus monophylla*) and juniper (*Juniperus*

osteosperma) in higher elevations. The range has several other common plant species including bluegrass (*Poa secunda*), rabbitbrush (*Chrysothamnus nauseosus*), bitterbrush (*Purshia tridentata*), and cheatgrass (*Bromus tectorum*). There are several natural and artificial water sources, including a spring that runs year-round and is used by the study population. Other than feral horses, other ungulates in the range included mule deer (*Odocoileus hemionus*) and pronghorn (*Antilocapra americana*). The only predators in the range were mountain lions (*Puma concolor*), bobcats (*Lynx rufus*) and coyotes (*Canis latrans*).

The feral horses in this population are classified as estray horses since they range on private property and are therefore not protected under the Wild Horse and Burro Protection Act. Consequently, they are legally defined as feral animals, and the Nevada Department of Agriculture is responsible for the management of the population. The population size has fluctuated from over 1200 in 2004 to around 1400 in 2009, and is currently being managed with the use of both removals and contraception. The long term management goal is to continue to decrease and stabilize population growth.

Formulation types and treatment protocol

We selected a treatment site for both ease of capture, the large number of horses present in the valley, and permission from private land owners. Horses were rounded up with a helicopter on June 15th and 16th 2005. Horses were held in purpose-built yards and restrained in a hydraulic squeeze chute for treatment. We treated every female over one year of age with 1 cc intramuscular dose in the neck of 1 of 4 treatments: a) GonaCon-B™, a GnRH immunization (N=20), b) SpayVac®, a PZP formulation (N=20), c) control

consisting of adjuvant only (ADJ) (N=19), or d) control consisting of no treatment (N=18), but every other capture aspect was equivalent. After testing for differences in efficacy rates between adjuvant treated mares and those that received no injection, we grouped these together as the control group. All of the treatments contained the same adjuvant. Treatments were provided by the National Wildlife Research Council. Finally, the treatment group to which each mare belonged was kept blind from the field observer.

Mares ranged in age from 1 to 20 years. All females were aged by tooth eruption and wear patterns (after Tutt 1968), examined for overall health, had a blood sample taken and were freeze branded with unique brands on the left side of the rump. We used body condition scores on the date of treatment to visually estimate fat deposits around the neck, ribs and rump using an 11 point scale from 0-5, with 0.5 gradations (Carroll and Huntingdon 1988, Rudman and Keiper 1990). We used these scores as a measurement of energy reserves, which are correlated with fat percentage in horses (Henneke et al. 1983). We classified females into 3 categories of either good condition (score of 3 or greater), average condition (score of 2.5) and poor condition (score of 2 and below). A total of 77 females were treated over the two day period. All horses were immediately released after treatment. A small group of females (N=9) were treated in May of 2005. These horses were captured by a feed trap and processed using the same protocol. All females were monitored for pregnancy until summer 2008. All handling and treatment procedures were done by and under the supervision of veterinarians from the Nevada Department of Agriculture and were approved by the University of Nevada, Reno Animal Care and Use Committee.

Data Collection

We monitored mares on at least weekly basis from the ground to determine fertility rates until August 2007 and during the spring and summer of 2008. Efficacy of treatment was determined if a foal was born at any time during the year or if a female was noticeably pregnant, judged conservatively by an enlarged belly and swollen teats. If females were not seen until after the foaling season and no foal was present, they were recorded as unknown and excluded from the analysis.

Fecal Sampling and Steroid Hormone Assay

To supplement visual observations, we collected fecal samples opportunistically from October to May to determine pregnancy of mares that may have lost a foal before any visual observations took place. Fecal samples were stored in -20°C freezers until extraction. We used the extraction protocol of Asa et al. (2001). Samples were thawed and approximately 0.5 g of wet fecal material was placed in a vial with 5 ml of modified phosphate-saline buffer (Shideler et al. 1993) and shaken overnight at room temperature. Samples were centrifuged for one hour and the supernatant was stored at -80°C until the assay was conducted. The remaining fecal material was dried in a 100°C oven overnight and weighed the next day. Hormone values are based on the dry weight of the fecal samples and are presented as ng/g of dried feces.

Fecal progesterone assays were run at the Saint Louis Zoo and have been used to verify reproductive status of females in many species (Asa et al. 1996, Asa et al. 2001, Munson et al. 2001). We analyzed extracted fecal samples for progesterone with commercially available radioimmunoassay kits (Progesterone Coat-a-Count, Siemens

Medical Solutions Diagnostics, Los Angeles, CA, USA). We used stripped calf serum to prepare all standards and to dilute all fecal sample extracts. Fecal samples were run against a calibration curve of progesterone from stripped calf serum. Extraction buffer was added to tubes containing standards and calf serum was added to fecal extracts to equalize the matrices of the samples and standards. All samples, standards and quality controls were run in duplicate.

Statistical Analysis

We determined the effectiveness of contraceptive treatment by comparing the proportion of fertile females in each treatment group to control females across the combined foaling seasons. Therefore, efficacy rates are presented as fertility rates of each treatment group. We classified females into three categories of reproductive status. The first group consisted of mares that were seen with a foal or visibly pregnant and the second group consisted of females that tested pregnant through fecal progesterone. Both of these groups were considered contraceptive failures and combined for analyses. The third group comprised females in which contraception was considered successful, i.e., mares that did not foal and did not test positive for pregnancy. If any female did not meet these criteria (e.g., not seen on a regular basis or no fecal samples) they were excluded from the analysis. Therefore, fertility rates include females that were observed with a foal, pregnant, or tested pregnant with fecal progesterone. We used a linear mixed model analysis with restricted maximum likelihood estimation to determine treatment effects on fertility rates. We used subjects as a random variable repeated across three foaling seasons. A chi-squared test was used to test differences between foal sex ratios,

seasonality of births and foal survival. We defined the foaling season to include April, May and June (Kirkpatrick and Turner 2003). Results are shown as means ± standard errors where appropriate.

Results

Efficacy

There was no difference in foaling rates between control mares and adjuvant only mares across the 3 years (2006: t =-0.138, P=0.891, 2007: t=0.244, P=0.809, 2008: t=1.185, P=0.246; Table 1), thus they were combined for all further analyses. Treatment with contraception significantly reduced fertility for all combined foaling seasons ($F_{2,72}$=6.489, P=0.003). Post hoc comparisons revealed that both contraceptive treatments significantly differed from control mares (GonaCon-B™: t_{75}=-3.452, P=0.001, SpayVac®: t_{68}=-2.135, P=0.036). Overall, treated mares foaled less often in the 3 years, but the biggest difference was found in 2008. For individual females with known reproductive status for all three foaling seasons, most treated females were never fertile over the three years, or fertile for one year, whereas the majority of control females was fertile every year or two out of the three years (Figure 1).

We supplemented fertility data with four additional mares that were not seen on a regular basis, but had fecal samples tested for progesterone levels. We found two females that were not pregnant (both <50 ng/g progesterone) and two females that were pregnant (>1000 ng/g progesterone) (Asa et al. 2001). Pregnant mares had mean fecal progesterone of 2242 ± 258 ng/g and non-pregnant females averaged 109 ± 11.5 ng/g. Fecal progesterone was biologically verified correctly on 17/19 mares, but revealed that two

treated mares were pregnant in the first year, but lost their foal. Neither of these mares looked visibly pregnant and would have been counted as not pregnant. One mare (GonaCon-B™ treated) lost her foal in October and the other mare (SpayVac® treated) lost her foal sometime between March and April. The GonaCon-B™ treated mare may have already been pregnant when she was treated, but these data indicate that intensive visual and fecal sampling is necessary to get an accurate picture of mare fertility.

Body Condition

The average body condition score of all females at treatment was 2.38 ± 0.048. There was no difference in the mean body condition between treated (GonaCon-B™, 2.40 ± 0.09 and SpayVac®, 2.32 ± 0.10) and control females (2.40 ± 0.07) ($F_{1,71}$=0.28, P=0.754). However, body condition at the time of treatment significantly impacted efficacy in the 2007 and 2008 foaling seasons. While mares in good condition treated with either formulation had the expected reduction in fertility rates (41% fertile) compared to control females (75% fertile; χ^2_1=4.432, P=0.035), there was no reduction in fertility between contraception treated (60% fertile) and control females (55% fertile) in poor condition (χ^2_1=0.064, P=0.801). The same trend was seen in 2008 as good condition treated females had lower fertility rates (21%) than controls in good condition (76%) (χ^2_1=11.056, P=0.001), but there was no difference for poor condition females (55% fertile treated and 75% fertile control females, χ^2_1=0.786, P=0.375). In addition, there was a trend in the last two foaling seasons for treated females in good condition at time of treatment to have lower fertility rates than treated females in poor condition at time of

treatment ($F_{1,32}$=3.123, P=0.087). There was no difference in control mares based on condition at time of treatment during those same seasons ($F_{1,30}$=0.934, P=0.342).

Side effects of Treatment

In order to examine impacts of contraception on foals, we compared fertile treated mares (contraceptive failures) to control mares. There was no difference in the seasonality of births across the first two years between these treated mares and controls (χ^2_1 = 0.406, P=0.524), with the majority of all foals being born in April (40%) and May (52%) (Figure 2). Among treated mares, 88% foaled during April, May, and June and for control mares, 94% foaled during this time period. There was no treatment effect on foal survival (χ^2_3=0.002, P=0.969). There was also no difference in the foal sex ratio between treatments across three foaling seasons (χ^2_3=0.939, P=0.816), as 48% of foals were males from treated mares and 50% from control mares.

Discussion

Free-living horse populations continue to present a management problem in the western United States, and past management methods are increasingly unfeasible or ineffective. Consequently, contraceptive treatments have become more widely used to reduce herd numbers and population growth, especially with the development of a contraceptive agent which provides multi-year contraception after a single dose (Killian et al. 2008). We show that free-living horse fertility rates were decreased by contraceptive formulations with the use of a single vaccination. Females treated with either GonaCon-B™ or SpayVac® had lower fertility rates every foaling season than

control females. Furthermore, both treatments consistently reduced fertility within individual females throughout the three years.

While we found a reduction in fertility, we did not have as high efficacy rates as other free-living horse studies that have shown efficacy >90% during the first year (Kirkpatrick et al. 1990, Turner et al. 2002, Turner et al. 2007). Although there may have been problems with the treatment method, this seems unlikely since all mares received the full dose while in the squeeze chute. Alternatively, we monitored our horses more intensively than other studies by locating horses on a weekly to monthly basis, and used conservative estimates of pregnancy and foal production since horses may abort fetuses throughout the year or may lose foals shortly after birth (Keiper and Houpt 1984, Lucas et al. 1991). If individuals are not closely monitored, these events would be missed and would increase the appearance of contraceptive efficacy. In our study, mares that were only seen late in the foaling season without a foal were excluded from the analysis since it was not possible to determine if they had lost their foal or had not foaled at all. In addition, intensive monitoring and fecal hormone evaluation enabled us to identify treated females that were pregnant and lost their foal, which would have been recorded as effective contraception in a less intensive study. These techniques contrast with these of other researchers who only sample a few times per year, but use absence of offspring as an indicator that the contraceptive worked (Kirkpatrick et al. 1990, Rutberg et al. 2004, Turner et al. 1997, 2007). Furthermore, some of these studies have shown a similar trend with fecal hormone sampling and foaling data providing different results (e.g., mares pregnant from fecal hormone analysis, but show no visible signs) (Turner et al. 1997,

2007). This suggests that visual estimations a few times per year do not give an accurate representation of contraceptive efficacy.

Body condition

We found significant variation in contraceptive efficacy, explaining some of the inter-individual variation in responsiveness to immunocontraception. Fertility rates were lower in females that were in good condition at treatment, while mares in poor condition foaled at the same rate as control mares. Furthermore, Killian et al. 2008 showed high efficacy in captive feral horses using the same treatments on the same species from the same population we studied. The captive mares had a much better response to the treatment and showed higher levels of fertility reduction for the first 2 years post treatment [e.g.,100% and 83% for SpayVac® treated mares and 94% and 60% for GonaCon treated mares (Killian et al. 2008)]. All of these captive mares were in good condition (3 or better) at the time of treatment (Thain, personal communication). Captive populations are typically in better condition and have access to better-quality nutrition and could therefore respond better to contraceptive treatment. Furthermore, studies have demonstrated variation in response to different contraceptive formulations and adjuvants both among and within species (Garrott et al. 1998, Fayrer-Hosken et al. 2002, Frank et al. 2005). However, there have been no studies that investigated differences in individual phenotypes as a possible explanation for this variation. One such explanation could be body condition.

Individuals in better body condition, or with more energy reserves, generally have better immune systems and are able to respond better to foreign bodies (Chandra 1996,

Alonso-Alvarez and Tella 2001, Demas et al. 2003, Ujvari and Madsen 2006, Houston et al. 2007, Vicente et al. 2007, Corbin et al. 2008). Both contraceptive treatments we tested use the individual's own immune system to elicit a response that blocks critical aspects of reproduction. Theoretical concerns have been raised over the idea that over time, immunocontraception treatment leads to the removal of individuals with good immune systems from the population; essentially selecting for immunocompromised individuals that do not respond to immunocontraception and potentially have lower disease resistance (Nettles 1997, Cooper and Herbert 2001, Cooper and Larsen 2006). While our study is too short to evaluate the long-term impacts of contraception on immunocompetence, we did find consistent variation in relation to body condition at treatment, which may be an indicator for immune health. Our results therefore suggest that further research is required on inter-individual variation in responsiveness, particularly in relation to body condition and health at treatment, and indicate that long-term impacts of population health, particularly immunocompetence, could occur.

Impacts on foals

Feral horse populations differ in the birthing season, but the majority of foals are born in the spring (Feist and McCullough 1975, Keiper and Houpt 1984, Berger 1986). Foal survival is related to season of birth, as foals are more likely to survive when they are born in the spring than in the winter. Because contraception can cause out of season births, which can impact survival, we tested if treated mares foaled out of season. Previous studies have shown that PZP treatments can lead to increased estrus cycling during the nonbreeding seasons (McShea et al. 1997; Miller et al. 2000b, Curtis et al.

2002). These side effects can result in offspring born out of season leading to reduced survival (e.g., white-tailed deer, McShea et al.1997). We found no evidence to suggest that foals were born out of the normal birthing season to any treated females. A majority of the foals were born in the spring months of April and May to both treated and control females and this is consistent with other feral horse populations. Our data on foaling dates are consistent with other studies that investigated seasonality of foal births of treated individuals (see Kirkpatrick and Turner 2003). These data suggest that contraceptive treatment does not impact birthing seasons in feral horses or foal survival.

Contraceptive treatment has been shown to increase body condition of mares (Turner and Kirkpatrick 2002). These changes to body condition can impact the sex of the foal as females that experience an increase in body condition at time of conception have more males and females that experience a decrease in body condition have more females (Cameron and Linklater 2007). In our study, we found no evidence that the sex ratio of foals changed due to contraceptive treatment. Since treated females in poorer condition were more likely to foal, we expected some of these females to have more daughters, but there was no difference between treatment groups. It would be interesting to continue to monitor sex ratios in mares after the contraceptive has ceased to be effective, since mares that have not foaled for several years should be in better body condition (e.g., Turner and Kirkpatrick 2003), which could lead to an increase in male births (e.g. Monard et al. 1997, Cameron and Linklater 2000, Cameron and Linklater 2007).

Summary

Our findings demonstrate that new contraceptive formulations are able to reduce fertility in females for several years. Only one shot was necessary to deliver the contraceptive vaccine, and would be beneficial to managers because it eliminates the need for several round-ups. We conclude that these formulations would be useful for managing feral horse populations, but models are needed to determine how many females should be treated in order to reduce or stabilize the population. In addition, our results indicate that body condition may be an important but overlooked factor in efficacy of treatment and sampling methods are important when studying efficacy rates in wild populations.

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Table 1. Fertility rates of treated and control females for three foaling seasons. The number of females seen in each group is in parentheses. Both treatments significantly reduced fertility in the combined three foaling seasons ($P<0.01$).

Treatment	2006 (N)	2007 (N)	2008 (N)
Control (no injection)	60% (15)	69% (13)	86% (14)
Adjuvant only	63% (16)	65% (20)	67% (15)
SpayVac®	36% (14)	56% (16)	44% (16)
GonaCon-B™	39% (18)	42% (12)	31% (16)

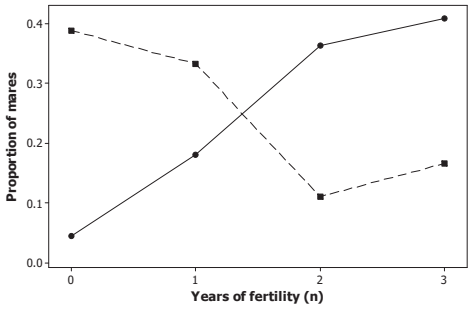


Figure 1. The proportion of females that were never fertile (0), fertile one year (1), fertile 2/3 years (2), or fertile all 3 years (3). All females had a known fertility status in all three foaling seasons within treatment and control groups. The dashed line with squares represents treated mares combined (N=18) and the solid line with circles represents control females (N=22).

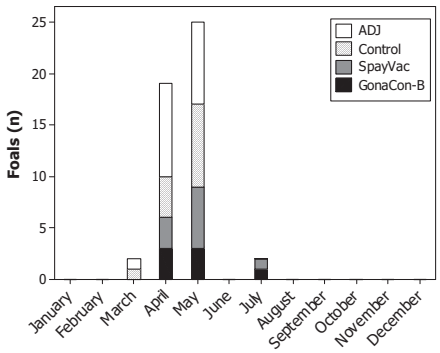


Figure 2. The number of foals born in each month of the year. Data pooled over two foaling seasons (2006 and 2007) and only from foals with known birth dates.

CHAPTER 4

Does contraceptive treatment in wildlife result in side effects? A review of quantitative and anecdotal evidence



"There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after."
J.R.R. Tolkien

Photo caption: Fire band in the spring of 2006

Author's note: Chapter four has been submitted to the journal Reproduction and is presented in the style of this journal.

Abstract: The efficacy of contraceptive treatments has been extensively tested, and several formulations are effective at reducing fertility in a range of species. However, these formulations should minimally impact the behaviour of individuals and populations before a contraceptive is used for population manipulation, but these effects have received less attention. Potential side effects have been identified theoretically and we reviewed published studies that have investigated side effects on behaviour, physiology or population dynamics, which provided mixed results. Physiological side effects were most prevalent. Most studies reported a lack of secondary effects, but these reports were usually based on qualitative data or anecdotes. A meta-analysis on quantitative studies of side effects showed that secondary effects consistently occur across all categories and all contraceptive types. This contrasts with the qualitative studies, suggesting that anecdotal reports are insufficient to investigate secondary impacts of contraceptive treatment. We conclude that more research is needed to address fundamental questions about secondary effects of contraceptive treatment and experiments are fundamental to conclusions. In addition, researchers are missing a vital opportunity to test the roles of reproduction, sex and fertility in wildlife species.

Introduction

Fertility control for population management of wildlife and zoo species has some advantages over other control methods, such as lethal control (Kirkpatrick 2007). For example, culling reduces population size, but also increases population growth rates, counteracting the goals of most population control operations (Garrott *et al.* 1991). Furthermore, there are ethical concerns about culling (Littin *et al.* 2004) such that public acceptance of lethal control is typically low when other management options are viable (e.g. Stout *et al.* 1997). However, for contraception to be successful for population control it must not only be safe, effective and long-acting, but also have minimal impact on the social organisation, behaviour, and ecology of populations with treated individuals (Castle & Dean 1996). Contraceptive technologies are becoming increasingly effective, safe and long-lasting (Kirkpatrick & Turner 2007), but despite several decades of contraceptive treatment, little research has considered secondary impacts, except in domestic animals. Fertility control is used to manage farm, domestic and recreational animals (D'Occhio 1993) and provides a viable and reversible alternative to de-sexing (e.g. cats, Ivanova *et al.* 1995; Gorman *et al.* 2002), but only if the animals behave similarly before and after treatment. In wildlife, the alternative is often lethal control which raises a plethora of other legal and logistical issues (e.g. Kirkpatrick 2007). Nonetheless, the side effects of contraception need to be understood for fertility control to be an effective management tool, even if alternative management practices have more extreme side effects (see Kirkpatrick 2007).

Contraceptive formulations either prevent ovulation, fertilization or implantation, or terminate pregnancy. Immunocontraception uses an animal's own immune system to

prevent reproduction. The body produces antibodies in response to the injection of a foreign body, and the antibodies then interfere with critical events in reproduction. Several different types of immunocontraceptive formulations have been developed, including those against ova (Sacco 1987), sperm (Primakoff *et al.* 1988) and gonadotropins (Moudgal *et al.* 1986). The most commonly used formulation is porcine zona pellucida (PZP), which stimulates the production of antibodies to zona pellucida (ZP) when injected into the host (Aitken *et al.* 1984). These antibodies prevent sperm binding to the ZP sperm receptors, thereby preventing fertilization (Sacco *et al.* 1984). Infertility results, but females continue to cycle. Since the structure of these receptors is conserved, PZP inhibits fertility in a range of mammalian taxa (Wood *et al.* 1981; Mahi-Brown *et al.* 1985; Sacco 1987; Kirkpatrick *et al.* 1996). Gonadotropin releasing-hormone (GnRH) formulations, both agonists and active immunizations, result in a cessation of the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Treated females thereby enter a variable length anestrous period (Talwar 1985). These GnRH formulations have been widely used in domesticated livestock as a practical application to improve carcass quality and to reduce aggressive behaviour (i.e. Huxsoll *et al.* 1998; Kiyma *et al.* 2000). To a lesser extent they have been used for fertility control in both males and females of wildlife species (e.g. Miller *et al.* 2000a). Other common types of contraceptive agents include (1) melengestrol acetate (MGA), a progesterone-like drug that suppresses LH, preventing ovulation, but allowing development of large follicles and endogenous estradiol, and (2) progesterone analogs that inhibit ovulation and prevent implantation, both of which have been used successfully in several zoo species (reviewed in Patton *et al.* 2007). Surgical sterilization is also an option in some species, but presents

some practical issues associated with surgery (i.e. catching, anesthesia, irreversibility, morbidity, mortality) that make it unsuitable for most wildlife species.

The potential side effects of these contraceptive treatments have been considered theoretically, but few have been tested empirically. Nettles (1997) reviewed potential side effects, which included physiological effects, behavioural effects, population impacts and evolutionary impacts, and more recently suggested side effects include evolutionary effects (Magiafoglou *et al.* 2003; Cooper & Larsen 2006) and impacts on disease transmission rates (Tuyttens & MacDonald 1998; Caley & Ramsey 2001; Miller *et al.* 2004). The diversity of contraceptive agents means that there is potential for a variety of secondary impacts. Here we review the literature to determine 1) potential impacts on individuals and populations, 2) which impacts have been documented and 3) which impacts have been empirically tested. Finally, we consider the implications of the side effects of contraception for addressing fundamental theoretical questions, particularly in behavioural ecology.

Materials and Methods

We searched ISI Web of Science (1975-2007) for 'immunocontraception' and 'contraception'. Additional searches were made to narrow the papers to those with the terms 'side effects' and 'secondary effects', and we further searched the literature cited section of each paper to find more studies that were not identified in the initial search. Only papers that included applications to mammalian wildlife, zoo, or livestock species were used in the review, and humans were excluded. We use the terms secondary effects and side effects interchangeably throughout the paper and define these terms as any

effects resulting from contraceptive treatment but are not related to the actual contraceptive action, and could be either positive or negative.

Initially we reviewed the theoretically suggested side effects. We then compiled all the studies that had addressed secondary effects either directly or during the course of a study on efficacy. Papers that only researched efficacy or impacts of contraception on fertility directly were not included. A total of 212 papers were found that investigated secondary effects or reported side effects noticed during research on efficacy (the full list is presented as Appendix 1). The papers fell into three categories; 1) physiological effects, 2) behavioural effects on the individuals, and 3) population-level effects, and we use these three categories hereafter. From these papers, we considered sex, species, sample size, length of study, type of effect investigated and if that effect had an impact on the animals. We then compared the theoretical effects with the empirical evidence to determine which effects seemed to be supported, or not supported, and in which areas more research is required.

Meta-analysis

From the reviewed papers, we analyzed studies that empirically tested if side-effects were found in treated wildlife populations through a meta-analysis in order to estimate the magnitude of the experimental effect (effect size). We only included wildlife populations and excluded wildlife in zoos if the contraceptive treatment was used intentionally to change behaviour (i.e. decreasing aggressions or sexual activity), so as to not skew the analysis towards side-effects. In addition, in an effort to be conservative, livestock papers were excluded since 87% of these studies reported side-effects and most

of these papers used the contraception to make a change. A criticism of every meta-analysis is the possibility of publication bias (file-drawer problem), in which only statistically significantly papers are published (see Gurevitch and Hedges 1999). Therefore, we made every attempt to remove publishing bias by the previously described methods. In addition, since our original search included papers that did not experimentally test for secondary effects but reported that they found none, we included those papers if a test statistic could be computed to help reduce this bias. For the meta-analysis we used p-values and sample size reported in each study in order to obtain a combined effect size (r). Effect sizes were weighted for sample size and indicate the strength of associations across the selected studies. The corresponding $Z_{weighted}$ value provides the significance of the difference between treated (contracepted) and control groups. In addition, we calculated a χ^2 statistic to test for heterogeneity of results across the selected studies. A random effect model was used in order to account for differences in both effects size and sampling error (Gurevitch and Hedges 1999). A total of 54 papers were included that empirically tested secondary effects from contraceptive treatment. From those papers, all measures were treated as separate statistics. If there was not sufficient evidence to determine p-values and sample sizes, the papers (or statistics) were excluded from the analysis. An overall analysis was done, in which all experimental studies were included, regardless of treatment type and study classification. The separate analyses were done to test for differences within each study category (physiology, behaviour and population-level effects) and contraceptive type (GnRH, progesterone, PZP, and surgery). The meta-analysis was conducted using META, v 5.3 (Schwarzer 1989).

Results

Trends in publications

The reviewed studies emphasized pest animals and agriculturally important species. Several orders have been investigated, but studies concentrated on Artiodactyla (particularly deer and domestic cows), Perissodactyla (mainly horses), and Carnivora (particularly felids) (Appendix Table 1). There was an increase in published papers from 1975-2002 (Fig. 1). These publication trends indicate increasing interest in side effects with a peak in 2002 when a special issue of Reproduction Supplement was published. After this supplement, the rate of publication returned to a consistent pre-2001 level.

Most studies investigated the impacts of female contraceptives (Table 1), which is consistent with efficacy studies in wildlife which usually target females. Physiological responses were addressed in a high percentage of the papers (85%) for both males and females. While physiological impacts are important, behavioural and population-level effects may be more relevant to the management of wild populations. Approximately 35% of the papers addressed individual behaviour and 11% addressed population-level effects. Roughly 30% of the papers addressed more than one category. There was a sex bias with 90% of the papers on males considering physiological side effects (particularly carcass quality), compared to 83% in females (usually impacts on reproductive organs). Behavioural measures were also investigated more in males (Table 1). Only one paper investigated population-level effects in males, most likely because females are treated more often than males in wildlife populations (Table 1).

Documented secondary effects

Several secondary effects are theoretically possible, and these are summarized in Table 2. Only a few hypothesized impacts have not been investigated, with most being studied at least once. However, few studies produced consistent results, even among studies using the same formulations. The main impacts are summarized below.

Physiology

How does contraception impact internal organs?

The impact of contraception on internal organs has received the most attention, with a total of 135 studies published. Changes in ovarian structure and function were found in most studies (83%, Table 2), which is not surprising since contraceptive formulations act directly on reproductive functioning, and so changes in reproductive organ histology and function are more likely than other impacts. For example, intrauterine devices (IUD's) increase inflammation of the uterus (Daels & Hughes 1995), while ovarian function is altered by both GnRH treatment (McNeilly & Fraser 1987; Gong *et al.* 1995; D'Occhio & Kinder 1995; Maclellan *et al.* 1997) and PZP treatment (Dunbar *et al.* 1989; Kirkpatrick *et al.* 1992; Kitchener *et al.* 2002; Curtis *et al.* 2007). In addition to alterations in ovarian functions, MGA implants can increase mineralization and cystic hyperplasia in captive felids (Munson *et al.* 2002). Consequently, changes to the ovaries or female reproductive tract were consistently found for all contraceptive treatments.

In domesticated livestock, GnRH treatment has been used to improve carcass characteristics by increasing lean meat (Huxsoll *et al.* 1998), feeding efficiency (Cook *et*

al. 2001) and live weight (Bonneau *et al.* 1994; Zeng *et al.* 2002), and treatment usually resulted in positive or no effects on carcass traits. Milk production did not seem to be influenced by contraception in the few studies in which it has been investigated (Mattos *et al.* 2001; Nave *et al.* 2000), although there was some evidence of mammary hyperplasia in cats treated with progesterone (Loretti *et al.* 2005)

In addition to effects on internal organs, several studies (64%, Table 2) found inflammation or abscesses at the injection site. These usually healed quickly with no long-term health effects. Overall, most of the treatments showed little to no impacts on internal organs other than the ovaries (or testes) and some of the effects were considered positive.

Does contraception alter body condition?

Without the costs of lactation and gestation, females should hypothetically have improved condition and health, possibly leading to increased longevity and lifespan (Williams 1966). The impacts could extend beyond treated females. For example, untreated males may lose condition in the presence of contracepted females due to prolonged breeding activities (Ji *et al.* 2000). Some studies have confirmed that treated females are in better condition than untreated conspecifics (26% of reviewed studies), but the majority found no difference, or that differences did not last (Table 2). For example, PZP treatment increased female condition in both feral horses (*Equus caballus*) and white-tailed deer (*Odocoileus virginianus*) in some studies (McShea *et al.* 1997; Turner and Kirkpatrick 2002), but not in others (Walter *et al.* 2003). Generally, females treated with GnRH show no changes in weight or condition (Miller *et al.* 1997; D'Occhio *et al.*

2002; Herbert *et al.* 2005; Herbert *et al.* 2006; Rubion *et al.* 2006). Finally, both longevity and offspring survival could be influenced by contraceptive treatment (Table 2). Furthermore, untreated females may have more successful reproduction due to reduced competition for resources. The impacts of these factors on population dynamics can counteract the desired impact of the contraceptive treatment in reducing population size (see Williams *et al.* 2007). Such population-level effects are discussed later.

Are some contraceptive agents reversible?

For contraceptive treatment to be an effective management tool, it usually needs to be reversible (Kirkpatrick & Turner 1991). A long term study of feral horses (*Equus caballus*) showed that PZP was reversible even when females were treated for several years (Kirkpatrick & Turner 2002). However some females appeared not to return to full fertility after long-term PZP treatment and similar side effects were shown with progesterone implants in marsupials (*Macropus giganteus*) (Nave *et al.* 2000) and GnRH treatments in deer (*Odocoileus virginianus*) (e.g. Miller *et al.* 2000a). Consequently, most wildlife contraceptives are reversible, or have minimal impact after prolonged use.

Individual Behaviour

Do activity patterns change in treated individuals?

Most studies suggest that activity patterns were unchanged after contraceptive treatment (80%, Table 2). With PZP treatment, no changes were detected in activity budgets of elk (*Cervus elaphus*), horses (*Equus caballus*), elephants (*Loxodonta africana*), and white-tailed deer (*Odocoileus virginianus*) females (McShea *et al.* 1997;

Heilmann *et al.* 1998; Powell 1999; Delsink *et al.* 2002), although the increased number of estrus cycles resulted in PZP treated white-tailed does (*Odocoileus virginianus*) being more active than control females (McShea *et al.* 1997). GnRH did not change activity budgets in treated kangaroo females (*Macropus giganteus*) (Woodward *et al.* 2006), nor did progesterone implants in kangaroos (*Macropus giganteus*) (Poiani *et al.* 2002), or fruit bats (*Pteropus rodricensis*) (Hayes *et al.* 1996). However, MGA treatment caused baboons (*Papio hamadryas*) to decrease their time foraging (Portugal & Asa 1995). Overall, few studies report changes in activity patterns after contraceptive treatment.

Does rank and dominance change in treated females?

Rank and dominance are important in social mammals (Ellis 1995). By changing the levels of hormones or cycling patterns, females could become more or less aggressive, which could have implications for behaviour, social structure and welfare. Such side effects have been used by captive managers (e.g. GnRH to control aggressive behaviour, Patton *et al.* 2001), but the few studies on wildlife have produced mixed results (50% showed changes Table 2). In captive felids, females treated with PZP had increased levels of irritability, masculine behaviour, and aggression (Harrenstien *et al.* 2004), and in white-tailed deer (*Odocoileus virginianus*), treated females associated less frequently with conspecifics (Bertrand *et al.* 1996). MGA treatment in tamarins (*Leontopithecus chrysomelas*) caused a decrease in affiliative and sexual interactions, with a subsequent increase in aggression toward other females (De Vleeschouwer *et al.* 2000; De Vleeschouwer *et al.* 2003). Pioani *et al.* (2002) found that progesterone treatment altered the amount of time males spent with females, as males preferred to be in

groups with untreated females. In elephants (*Loxondonta africana*), the matriarch remained in place after treatment with PZP (Delsink *et al.* 2002), but was affected by hormonal treatments (Fayrer-Hosken *et al.* 2000). PZP treatment did not change the spatial relationship between females and males in horse harem bands (*Equus caballus*) (Powell 1999), although all females had been treated with PZP at some point, confounding the conclusions. Finally, surgical sterilization had little impact on coyote (*Canis latrans*) pair bond maintenance or territorial behaviour (Bromley & Gese 2001), and no changes in social hierarchy were observed in brushtail possums (*Trichosurus vulpecula*) with GnRH treatment (Jolly *et al.* 1996).

Unfortunately, many of the studies reporting no change in social structure have either used opportunistic observations of social groups, rather than quantitative techniques, or have been of short duration. Consequently, the results are inconclusive, and studies that specifically and intensively investigate behavioural changes at a fine level over a long time period are urgently needed.

Are home ranges and movement patterns altered?

If sexual behaviour is altered in treated females, movement patterns could also change. PZP treatment resulted in no difference in space use or home range size in elephants (*Loxodonta africana*) and white-tailed deer (*Odocoileus virginianus*) (Delsink *et al.* 2002; Hernandez *et al.* 2006), similar to surgically sterilized foxes (*Vulpes vulpes*) (Saunders *et al.* 2002). In contrast, surgically sterilized female rats (*Rattus argentiventer*) had larger ranges than both control and hormonally sterilized females (Jacob & Matulessy 2004). In addition, hormonally sterilized female rats abandoned burrows more

often, suggesting a change in dominance or social status, although overall habitat use was not changed (Jacob & Matulessy 2004). Male home range size may also be impacted, as found in brushtail possums (*Trichosurus vulpecula*) (Ramsey 2007). Sterilization resulted in no difference in territory size, overlap or territorial behaviour between controls and treated coyote packs (*Canis latrans*) (Bromley & Gese 2001). Consequently, most studies suggest that contraception had minimal impact on home range size and movement patterns, but there was some evidence that males alter their movement patterns to be in groups of untreated females, or with treated females during the nonbreeding season (Poiani *et al.* 2002).

Do sexual interactions and breeding behaviour change?

Changing the fertility of individual females could impact sexual interactions, especially in PZP-treated individuals because the number of estrous cycles is increased (i.e. McShea *et al.* 1997; Heilmann *et al.* 1998; Miller *et al.* 2000b; Miller *et al.* 2000c; Curtis *et al.* 2002). There was some evidence that breeding behaviour changed in treated females (Table 2), and this varied with contraceptive type. Does (*Odocoileus virginianus*) treated with GnRH immunizations had fewer estrus events (Miller *et al.* 2000a), but female wapiti (*Cervus elaphus*) showed no difference in copulatory behaviours (Baker *et al.* 2004). Treatment with progesterone analogs showed similar results to GnRH, but more males were seen in groups of control females (Poiani *et al.* 2002). Ji *et al.* (2000) suggested that males were attracted to areas with sterilized females, potentially increasing male mortality due to decreased body condition. In contrast, with PZP treatment, females remained sexually active after the end of the breeding season and showed an increase in

estrus events (McShea *et al.* 1997; Heilmann *et al.* 1998; Miller *et al.* 2000b; Miller *et al.* 2000c). Although changes in breeding behaviour appeared to be related to contraceptive formulations, 64% of the studies found changes in estrus and breeding behaviour (Table 2), suggesting that these changes should be expected.

Does contraception impact the overall welfare of individuals?

The reviewed impacts on physiology and behaviour suggest that contraception had little impact on overall welfare of individuals, particularly compared to the alternative options (see Kirkpatrick 2007). While most studies found some changes in physiology or behaviour, these changes may not impact the individual enough to affect its welfare, as most studies found no changes to activity, movements or rank of treated females. However, these conclusions are based largely on qualitative and anecdotal studies. In addition, some studies showed that changes could occur to non-treated animals in the population (e.g. males), which needs to be considered in future research.

POPULATION-LEVEL EFFECTS

Do survival, abundance, and age class distribution change in treated populations?

Contraception reduces the costs of reproduction, and could therefore result in increased body condition and longer lifespan, thereby altering population dynamics. Furthermore, the offspring of untreated females may have less competition for resources, enhancing their survival and working against the goals of the management operation. Few studies had the longitudinal data required to investigate increased survival, but 75% found an increase in longevity or survival in individuals. For example, PZP treated feral

horse mares (*Equus caballus*) lived longer, resulting in a new age class (>25 years) not present before treatment (Turner & Kirkpatrick 2002). Therefore, while contraception may limit offspring production, these benefits may be offset by decreased mortality in older individuals, negating the population effects of contraceptive treatment. In addition, both adults and juveniles had increased survival in surgically sterilized rabbits (*Oryctolagus cuniculus*) (Williams *et al.* 2007), which meant that the majority of females must be sterilized to reduce population size (Williams *et al.* 2007). Furthermore, non-treated females show improved breeding success (Chambers *et al.* 1999), which also counteracted the effects of contraception for reducing population growth rates. Finally, while the sex ratio of offspring could change due to increased maternal condition (e.g. Cameron 2004), this has yet to be investigated, although Ji *et al.* (2000) recorded a change in population sex ratios, probably as a result of male dispersal. Overall, contraceptive treatment appears to increase longevity in treated females and, in some cases, non-treated individuals.

Is the mating season extended?

Mating behaviour can demand high levels of energy for both sexes. In species with a discrete breeding season, the lengthening of the season could impact the survival and welfare of treated females and nontreated males. Most studies showed a change in the annual breeding season where it was investigated (78%, Table 2). Treatment with PZP increased breeding behaviour, caused later fawning dates, and increased sexual activity outside the normal breeding season in several studies (McShea *et al.* 1997; Heilmann *et al.* 1998; Miller *et al.* 2000b). However, no differences were reported in

populations with females treated with GnRH (Miller *et al.* 2000a; Baker *et al.* 2002; Baker *et al.* 2004; Woodward *et al.* 2006; Conner *et al.* 2007), or progesterone (Poiani *et al.* 2002). Differences in the actions of these formulations and their contrasting effects on cycling probably account for the differences in the impacts on the breeding season. Consequently, the type of contraceptive treatment should be considered for species with a discrete breeding and birthing season.

Does contraception decrease disease transmission?

If contraception decreases sexual interactions, there may be a decrease in overall social interactions which could decrease the level of disease transmission of certain diseases. This decrease would be advantageous in certain pest species, such as the brushtail possum (*Trichosurus vulpecula*), which have a high population size, and are carriers of economically or ecologically important diseases, such as bovine tuberculosis (Caley & Ramsey 2001; Ramsey 2007). A potential decrease of brucellosis transmission is predicted in bison (*Bison bison*) with GnRH treatment (Miller *et al.* 2004), as anestrous females would be unable to spread the disease. However, PZP treatment, in which females continue to cycle, could have the opposite effect as there could be an increase in sexual interactions. While there has been little research, most studies concur that disease transmission rates remain unchanged with contraceptive treatment (Kerr *et al.* 1998; Williams *et al.* 2007). However, the potential to reduce disease transmission exists (Ramsey 2007) and studies on rabbits (*Oryctolagus cuniculus*) showed a decrease in flea abundance (Twigg *et al.* 2000; Williams *et al.* 2007); possibly indicating a decrease in overall interactions between individuals. More research is clearly required.

Meta-Analysis

Of the 212 reviewed papers, 54 (25%) experimentally tested the secondary effects of contraception and reported their results in sufficient detail to be included in the meta-analysis. From our qualitative review, most papers concluded no significant secondary effects were found. However, the quantitative studies produced contrasting results. From the 339 test statistics from 54 papers, 52% found side effects from contraception and an additional 8% showed a non-significant trend suggesting side effects. The support varied between contraceptive types and between secondary effect types, with progesterone treatments and physiological studies having the most side effects (Fig. 2). Despite this variation, an overall meta-analysis confirmed that side effects consistently occurred where investigated as there was a significant relationship for secondary effects, with high predictive power and relatively large effect size, and no significant heterogeneity of data (Table 3). Therefore, contraceptive treatment resulted in changes to the physiology and behaviour of individuals, and impacted populations with treated individuals.

An interesting contrast thereby arises in that quantitative studies showed that side effects were consistently found, whereas qualitative studies and anecdotal reports suggest that side effects were generally absent. While a publication bias is possible, most studies were predominantly publishing results on efficacy, such that data on side effects would not influence publication potential. Consequently, more studies specifically investigating secondary effects are required to quantify the impacts of contraceptive treatment on physiology, behaviour and population dynamics.

Discussion

Future Directions

While there have been an increasing number of studies of side effects, much is still unknown. Unfortunately, many studies were not designed to investigate the side effects and many conclusions were based on small sample sizes, anecdotal reports, or lacked a true control. In this review, sample sizes, when reported, ranged from 1-353 for treated individuals and 0-230 for control individuals, with an average sample size of 31.7 ± 49.0 (median 17) treated individuals and 18.2 ± 30.0 (median 9) for controls, indicating high variability among studies. Most studies were of relatively short duration, with an average duration of 1.8 ± 2.4 years; although data collection was often conducted in short field periods of a few days or weeks over the course of the entire study, probably resulting in an overestimate of average study length. Most studies failed to address changes over a long period of time.

Conclusions based on data from studies designed to investigate efficacy may not be methodologically suitable for testing for behavioural or ecological effects. Many studies were designed to test efficacy, and the side effects reported were based only on short-term observations. Many studies on efficacy concluded “no change” for side effects based on anecdotal observations, but these studies did not directly investigate the physiology, behaviour or ecology during the study, limiting the conclusions. This contrasts with the meta-analysis further questioning the validity of qualitative studies reporting “no change”. While the meta-analysis does not account for the severity of the effect, all of the major contraceptive treatments resulted in side effects (Table 3). Thus, these qualitative studies represent an interesting foundation, but future research requires

more detailed behavioural and ecological protocols, larger sample sizes, and longer study durations.

Evolutionary level

No evolutionary level questions have been investigated, but are most relevant for future impacts (Table 2). Two main impacts of immunocontraception have been proposed; 1) genetic resistance could evolve, similar to the response seen to other biological control agents (Magiafloglou *et al*. 2003), and 2) immunocontraceptives could be selecting for immuno-compromised individuals since those treated females that do breed have a lower immune response to the contraceptive (Nettles 1997; Cooper & Herbert 2001). These aspects require long-term research which has not been possible to date. Nonetheless, given the potential implications of either of these changes, research is urgently required in this field.

Conclusions

While physiological effects have been well-documented, there is less consistency in studies investigating behavioural or ecological side effects, compounded by the tendency for studies to be designed to investigate efficacy specifically and side effects only opportunistically. Such studies often lack appropriate controls, sampling methodology or time scales. Those studies that have specifically investigated side effects consistently find them. While contraception is supported as a humane treatment, research has not extended much beyond the scope of management concerns.

Both physiological and behavioural effects can impact population dynamics. The goal of contraceptive treatment is to manipulate population dynamics, and consequently it is imperative to understand population-level effects, some of which compromise the effectiveness of contraceptive treatment (such as through increased breeding in untreated females or increased survival of offspring), while others could enhance effectiveness (such as a male biased sex ratio). To be an effective tool, we need to understand these impacts. Furthermore, most research examined short term effects and research is needed that addresses questions beyond one estrous cycle or breeding season. For example, no studies looked at how genetic diversity or immunity could change over time.

Finally, contraception provides a powerful tool to address fundamental questions in biology but such applications have not been realized to date. By manipulating fertility we can address questions regarding, for example, the costs of reproduction, importance of copulation in maintaining social structure and parental investment strategies. This would enable us to simultaneously address management questions, questions about contraceptive actions and secondary effects, and questions in basic biology, thereby maximizing the utility of contraception research.

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Table 1. A comparison of the 212 reviewed papers (%) based on sex and category.

	Total Papers	Behaviour	Physiology	Population
Female (N=145)	68	30	83	14
Male (N=50)	24	46	90	2
Both (N=17)	8	41	88	12

Table 2. Theoretical side effects of contraceptives, and the number of studies investigating these side effects. References are in Appendix Table 2.

Side-effect	Theoretical	Effect	No effect
Physiological			
Harmful effects on pregnant animals	*Nettles 1997*		
Stillbirth		8	1
Abnormal offspring		2	1
Inhibition of parturition or dystocia	*Nettles 1997*	2	0
Changes in ovarian structure or function	*Nettles 1997*	52	11
Changes in lactation or mammary glands	*Nettles 1997*	1	4
Changes in secondary sex characteristics	*Seal 1991, Nettles 1997*	0	0
Changes in bodyweight or condition	*Nettles 1997*		
Increased in treated females		9	26
Decreased in males		1	0
Toxicity	*Nettles 1997*	0	0
Interference with diagnostic tests	*Nettles 1997*	1	0
Abscesses or inflammatory reactions	*Nettles 1997*	14	8
Individual behaviour			
Changes in behaviour	*Nettles 1997*		
Sexual activity		9	5
Increased movement		2	1
Activity patterns		2	8
Increased irritability or aggression		3	0
Social disruption, change in status, territory loss		5	5
Population-level			
Changes in sex ratio	*Nettles 1997*		
Due to immigration		1	0
Offspring sex ratios		0	0
Changes in annual breeding season	*Nettles 1997*	7	2
Ecological alterations	*Nettles 1997*		
Home range or territory size		3	4
Increased immigration or emigration		0	1
Change in habitat use		1	1
Increased longevity or survival		6	2
Increased survival of offspring		3	2
Impact on fertility of young	*Nettles 1997*	0	1
Improved breeding of non-treated females		1	0
Disease transmission rates	*Tuyttens & MacDonald 1998; Caley & Ramsey 2001; Miller et al. 2004*	1	2
Evolutionary level			
Genetic resistance	*Magiafoglou et al. 2003*	0	0
Genetic changes influencing disease resistance or immune function	*Nettles 1997; Cooper & Larsen 2006*	0	0

Table 3. Meta-analysis of reviewed papers on wildlife species when contraceptive treatment was investigated for secondary effects.

Measure	$n_{studies}$	$n_{statistics}$	$n_{datapoints}$	$Z_{weighted}$	effect size (r)	Heterogeneity (x^2)	p value
All studies	54	339	9471	19.4002[a]	0.1993	338.1099	0.4881
Physiology	29	91	2679	11.1917[a]	0.2126	102.8470	0.1673
Behaviour	27	171	3872	14.8088[a]	0.238	156.9311	0.7553
Population	15	77	2920	8.9712[a]	0.3041	75.6087	0.4911
PZP	16	79	3842	10.8354[a]	0.1748	76.9119	0.5136
GnRH	8	51	1149	8.2056[a]	0.2421	35.5526	0.9388
Progesterone	16	96	2655	9.3193[a]	0.1809	81.8345	0.8300
Surgery	13	121	2055	17.6090[a]	0.3884	126.4755	0.3251

[a]p<0.0001

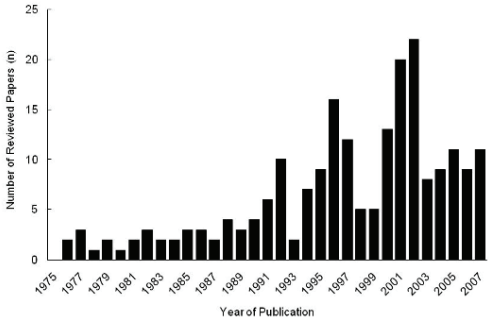


Figure 1. Publication trends of papers that investigated or reported on secondary effects of contraceptive agents from 1975-2007.

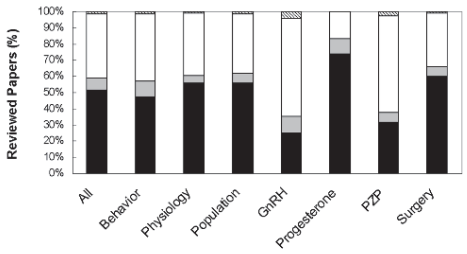


Figure 2. Wildlife studies that empirically tested if contraceptive treatment produced side effects that showed significant support (■), non-significant support (▨), no significant support (□), and significant support for secondary effects not found (▧).

Appendix 1. Bibliography of all reviewed papers that investigated side effects of contraception.

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Appendix Table 1. The entire set of papers that published results on secondary effects, based on the species researched. GnRH = either immunization or agonists, IUD's = intrauterine devices, LH = luteinizing hormone agonists, MGA = melengestrol acetate, Progesterone = hormone treatments that contain progesterone, PZP = porcine zona pellucida.

Order	Common Name	Scientific Name	Contraceptive Type	References
Artiodactyla	Addax	*Addax nasomaculatus*	MGA	*Hall-Woods et al. 2007*
Artiodactyla	Antelope	*Antilopa cervicapra*	MGA	*Raphael et al. 2003*
Artiodactyla	Axis Deer	*Cervus axis*	MGA, PZP	*Kirkpatrick et al. 1996; Raphael et al. 2003*
Artiodactyla	Banteng	*Bos javanicus*	PZP	*Kirkpatrick et al. 1995*
Artiodactyla	Barasingha	*Cervus duvauceli*	MGA	*Raphael et al. 2003*
Artiodactyla	Domestic Cow	*Bos taurus*	GnRH, IUD, LH, MGA, Surgery	*Jeffcoate et al. 1982; Robertson et al. 1982; Adams & Adams 1990; MacMillan & Thatcher 1991; Lobley et al. 1992; Finnerty et al. 1994; D'Occhio & Kinder 1995; Gong et al. 1995; Prendiville et al. 1995a; Prendiville et al. 1995b; Adams et al. 1996; Bergfeld et al. 1996; Finnerty et al. 1996; Gong et al. 1996; Johnson et al. 1996; Bell et al. 1997; Jago et al. 1997; Maclellan et al. 1997; Popp et al. 1997; Turin et al. 1997; Hussell et al. 1998; Cook et al. 2000; Cook et al. 2001; Fordyce et al. 2001; Mattos et al. 2001; Aissat et al. 2002; D'Occhio et al. 2002; Imwalle et al. 2002; Geary et al.2006*
Artiodactyla	Domestic Goat	*Capra hircus*	GnRH	*Godfrey et al. 1996*
Artiodactyla	Domestic Pig	*Sus scrofa*	GnRH, LH	*Falvo et al. 1986; Bonneau et al. 1994; Meloen et al. 1994; Schneider et al. 1998; Brussow et al. 2001; Dunshea et al. 2001; Zeng et al. 2002*
Artiodactyla	Domestic Sheep	*Ovis aries*	GnRH, LH, Surgery	*Clarke et al. 1978; Jeffcoate et al. 1982; Schanbacher 1982; Dobson 1985; McNeilly et al. 1986; McNeilly & Fraser 1987; Brown et al. 1994; Jewell 1997; Fahmy et al. 1999; Kiyma et al. 2000; Ferro et al. 2004; Stoops et al. 2006*
Artiodactyla	Elk	*Cervus elaphus*	GnRH, LH, PZP	*Lincoln 1987; Ataja et al. 1992; Freudenberger et al. 1993; Kirkpatrick et al. 1996; Heilmann et al. 1998; Baker et al. 2002; Baker et al. 2005; Conner et al. 2007*
Artiodactyla	Fallow Deer	*Dama dama*	MGA, PZP	*Fraker et al. 2002; Wilson et al. 2002; Deigert et al. 2003*
Artiodactyla	Feral Pig	*Sus scrofa*	PZP	*Killian et al. 2005*
Artiodactyla	Formosan sika deer	*Cervus nippon*	MGA, PZP	*Kirkpatrick et al. 1996; Raphael et al. 2003*

Artiodactyla	Gazelle	*Gazella dorcas*	GnRH	*Penfold et al. 2002*
Artiodactyla	Gerenuk	*Litocranius walleri*	GnRH	*Penfold et al. 2002*
Artiodactyla	Giraffe	*Giraffa camelopardalis*	GnRH	*Patton et al. 2006*
Artiodactyla	Himalayan tahr	*Hemitragus jemlahhicus*	PZP	*Kirkpatrick et al. 1996*
Artiodactyla	Hippopotamus	*Hippopotamus amphibious*	MGA, Progesterone	*Wheaton et al. 2007*
Artiodactyla	Mule deer	*Odocoileus hemionus*	GnRH, Progesterone	*Jacobsen et al. 1995; Baker et al. 2004*
Artiodactyla	Oryx	*Oryx dammah*	GnRH	*Penfold et al. 2002*
Artiodactyla	Oryx (Arabian)	*Oryx leucoryx*	MGA	*Hall-Woods et al. 2007*
Artiodactyla	Oryx (Fringed-ear)	*Oryx gazella*	MGA	*Patton et al. 2001*
Artiodactyla	Reeves muntjac	*Muntiacus reevesi*	PZP	*Kirkpatrick et al. 1996*
Artiodactyla	Sambar Deer	*Cervus unicolor*	MGA, PZP	*Kirkpatrick et al. 1996; Raphael et al. 2003*
Artiodactyla	White-tailed deer	*Odocoileus virginianus*	GnRH, MGA, Progesterone, Prostaglandins, PZP	*Roughton 1978; Turner et al. 1992; White et al. 1994; Bertrand et al. 1996;Turner et al. 1996b; McShea et al. 1997; Becker et al. 1999; Miller et al. 2000a; Miller et al. 2000b; Miller et al. 2000c; Miller et al. 2001; Waddell et al. 2001; Curtis et al. 2002; Naugle et al. 2002; Walter et al. 2003; Hernandez et al. 2006; Curtis et al. 2007*
Carnivora	African Wild Dog	*Lycaon pictus*	GnRH	*Bertschinger et al. 2001; Bertschinger et al. 2002*
Carnivora	Black-footed cat	*Felis nigripes*	GnRH, MGA	*Bertschinger et al. 2001; Munson et al. 2002*
Carnivora	Bobcat	*Felis rufus*	MGA, PZP	*Kazensky et al. 1998; Munson et al. 2002; Harrenstien et al. 2004*
Carnivora	Brown Bear	*Ursus arctos*	Anti-progestins	*Goritz et al. 2001*
Carnivora	Bush Dog	*Speothos venaticus*	GnRH	*Bertschinger et al. 2001*
Carnivora	Canada lynx	*Felis canadensis*	MGA, PZP	*Kazensky et al. 1998;Harrenstien et al. 2004*
Carnivora	Caracal	*Lynx caracal*	MGA	*Munson et al. 2002*
Carnivora	Cheetah	*Acinonyx jubatus*	GnRH, MGA	*Kazensky et al. 1998; Bertschinger et al. 2001; Bertschinger et al. 2002; Munson et al. 2002; Bertschinger et al. 2006*
Carnivora	Clouded leopard	*Neofelis nebulosa*	MGA	*Munson et al. 2002*
Carnivora	Cougar	*Felis concolor*	MGA, PZP	*Kazensky et al. 1998; Munson et al. 2002; Harrenstien et al. 2004*

Carnivora	Coyote	*Canis latrans*	Surgery	*Bromley & Gese 2001a; Bromley & Gese 2001b*
Carnivora	Dog	*Canis lupus*	GnRH, MGA, PZP	*Sokolowski & Van Ravenswaay 1976; Goyings et al. 1997; Mahi-Brown et al. 1985; Mahi-Brown et al. 1988; Mircu et al. 2001; Trigg et al. 2001; Junaidi et al. 2003; Sabuer et al. 2003; Ferro et al. 2004; Rubion et al. 2006*
Carnivora	Fishing cat	*Felis viverrinus*	MGA	*Munson et al. 2002*
Carnivora	Fox (Fennec)	*Vulpes zerda*	GnRH	*Bertschinger et al. 2001*
Carnivora	Geoffroy's cat	*Felis geoffroyi*	MGA	*Kazensky et al. 1998; Munson et al. 2002*
Carnivora	House Cat	*Felis catus*	Bisdiamines, GnRH, Progesterone, PZP	*Baldwin et al. 1994; Looper et al. 2001; Gorman et al. 2002; Munson et al. 2004; Levy et al. 2004; Levy et al. 2005; Loretti et al. 2005; Munson et al. 2005; Robbins et al. 2004*
Carnivora	Jaguar	*Panthera onca*	MGA, PZP	*Kazensky et al. 1998; Munson et al. 2002; Harrenstien et al. 2004*
Carnivora	Jaguarundi	*Felis yagouarondi*	MGA	*Munson et al. 2002*
Carnivora	Jungle cat	*Felis chaus*	MGA	*Kazensky et al. 1998; Munson et al. 2002*
Carnivora	Leopard	*Panthera pardus*	GnRH, MGA, PZP	*Kazensky et al. 1998; Bertschinger et al. 2001; Bertschinger et al. 2002; Munson et al. 2002; Harrenstien et al. 2004*
Carnivora	Leopard cat	*Prionailurus bengalensis*	MGA	*Munson et al. 2002*
Carnivora	Lion	*Panthera leo*	GnRH, MGA, Progesterone, PZP	*Seal et al. 1976; Black et al. 1979; Kazensky et al. 1998; Bertschinger et al. 2001; Bertschinger et al. 2002; Munson et al. 2002; Harrenstien et al. 2004*
Carnivora	Lynx	*Lynx lynx*	MGA, PZP	*Munson et al. 2002; Harrenstien et al. 2004*
Carnivora	Margay	*Felis wiedii*	MGA	*Kazensky et al. 1998; Munson et al. 2002*
Carnivora	Ocelot	*Felis pardalis*	MGA	*Kazensky et al. 1998; Munson et al. 2002*
Carnivora	Red Fox	*Vulpes vulpes*	Surgery	*Saunders et al. 2002*
Carnivora	S& cat	*Felis margarita*	MGA	*Munson et al. 2002*
Carnivora	Serval	*Felis serval*	MGA, PZP	*Kazensky et al. 1998; Munson et al. 2002; Harrenstien et al. 2004*
Carnivora	Snow leopard	*Panthera uncia*	MGA, PZP	*Kazensky et al. 1998; Munson et al. 2002; Harrenstien et al. 2004*
Carnivora	Tiger	*Panthera tigris*	MGA, PZP	*Black et al. 1979; Linnehan & Edwards 1991; Kazensky et al. 1998; Munson et al. 2002; Harrenstien et al. 2004*
Carnivora	Tigrina	*Felis tigrina*	MGA	*Kazensky et al. 1998; Munson et al. 2002*
Carnivora	Wild cat	*Felis silvestris*	MGA	*Munson et al. 2002*
Carnivora	Wolf (gray)	*Canis lupus*	Bisdiamine, GnRH	*Asa et al. 1996; Bertschinger et al. 2001*
Carnivora	Wolf (red)	*Canis rufus*	GnRH	*Bertschinger et al. 2001*

Chiroptera	Rodrigues Fruit Bat	*Pteropus rodricensis*	MGA	*Hayes et al. 1996*
Diprotodontia	Brushtail Possum	*Trichosurus vulpecula*	GnRH, Surgery	*Jolly & Spurr 1996; Jolly et al. 1996; Ji et al. 2000; Ramsey 2005; Eymann et al. 2007; Ramsey 2007*
Diprotodontia	Eastern Grey Kangaroo	*Macropus giganteus*	GnRH, Progesterone	*Nave et al. 2002; Poiani et al. 2002; Herbert et al.2006; Woodward et al. 2006*
Diprotodontia	Tammar Wallaby	*Macropus eugenii*	GnRH, Progesterone, PZP	*Short et al. 1985; Nave et al. 2000; Kitchener et al. 2002; Herbert et al. 2004a; Herbert et al. 2004b; Herbert et al. 2005; Hynes et al. 2007*
Lagomorpha	Rabbit	*Oryctolagus cuniculus*	GnRH, PZP, Surgery	*Wood et al. 1981; Skinner et al. 1984; Sehgal et al. 1989; Bhatnagar et al. 1992; Jones et al. 1992; Kerr et al. 1998; Kumar et al. 2000; Twigg et al. 2000; Williams et al. 2007*
Mustelidae	Sea Otter	*Enhydra lutris*	GnRH	*Bertschinger et al. 2001*
Perissodactyla	Burro	*Equus asinus*	PZP	*Turner et al. 1996a*
Perissodactyla	Horse (Domestic)	*Equus caballus*	GnRH, IUD, PZP	*Garza et al. 1986; Liu et al. 1989; Montovan et al. 1990; Boyle et al. 1991; Daels & Hughes 1995; Tshewang et al. 1997; Watson et al. 2000; Hinojosa et al. 2001; Malmgren et al. 2001; Dalin et al. 2002; Turkstra et al. 2005*
Perissodactyla	Horse (Wild)	*Equus caballus*	PZP, Progesterone	*Plotka et al. 1988; Liu et al. 1989; Kirkpatrick et al. 1990; Kirkpatrick et al. 1992; Powell 1999; Powell & Monfort 2001; Kirkpatrick et al. 1992; Kirkpatrick et al. 1996; Turner et al. 1997; Turner et al. 2001; Kirkpatrick & Turner 2002; Turner & Kirkpatrick 2002; Kirkpatrick & Turner 2003; Lyda et al.2005; Kirkpatrick & Turner 2007*
Primates	Baboon	*Papio hamadryas*	MGA, Progesterone, PZP	*Dunbar et al. 1989; Portugal & Asa 1995; Plowman et al. 2005*
Primates	Chimpanzee	*Pan troglodytes*	IUD, MGA, Progesterone, Surgery	*Porteous et al. 1994; Bettinger et al. 1997; Hoffman et al. 2002; Bourry et al. 2005*
Primates	Common Marmoset	*Callithrix jacchus*	LH, MGA, PZP	*Hodges & Hearn 1977; Paterson et al. 1992; Paterson et al. 1996; Mohle et al. 1999*
Primates	Cynomolgus Monkey	*Macaca fascicularis*	MPA, PZP	*Zumpe & Michael 1988; Zumpe et al. 1991; Zumpe et al. 1992; Gulyas et al. 1993*
Primates	Goeldi's Marmoset	*Callimico goeldii*	MGA	*Murnane et al. 1996*

Primates	Golden-headed lion tamarin	*Leontopithecus chrysomelas*	MGA	*De Vleeschouwer et al. 2000a; De Vleeschouwer et al. 2000b; De Vleeschouwer et al. 2003*
Primates	Golden Lion Tamarin	*Leontopithecus rosalia*	MGA	*Wood et al. 2001*
Primates	Japanese Monkey	*Macaca fuscata*	IUD	*Shimizu et al. 1991*
Primates	Lemur (black)	*Eulemur macaco*	Progesterone	*Asa et al. 2007*
Primates	Rhesus Monkey	*Macaca mulatta*	Anti-progestin, LH	*Balmaceda et al. 1984; Borman et al. 2003*
Primates	Squirrel Monkey	*Saimiri sciureus*	MGA, PZP	*Sacco et al. 1983; Sacco et al. 1991; Murnane et al. 1996*
Primates	Stumptailed Macaque	*Macaca arctoides*	LH	*Kerr-Wilson et al. 1981*
Proboscidea	African elephant	*Loxodonta africana*	PZP	*Fayrer-Hosken et al. 2000; Delsink et al. 2002; Delsink et al. 2007*
Rodentia	Hamster	*Mesocricetus auratus*	PZP	*Hasegawa et al. 1992*
Rodentia	House mouse	*Mus musculus*	PZP, Surgery	*Rhim et al. 1992; Lou et al. 1995; Chambers et al. 1999; Ferro et al. 2004*
Rodentia	Norway rat	*Rattus norvegicus*	GnRH, LH, Progesterone, PZP	*Gwatkin et al. 1977; Okon et al. 1980; Bhasin et al. 1988; Giri et al. 1990; Awonyi et al. 1993; Duckett et al. 1997; Miller et al. 1997; Kumar et al. 2000; Ferro et al. 2004; Vargas et al. 2005*
Rodentia	Ricefield rat	*Rattus argentiventer*	Surgery	*Jacob & Matulessy 2004*

Appendix Table 2. Summary of findings of studies that have investigated female contraceptives in relation to potential secondary effects.

Side effect	Theoretical	Effects	No effects
Physiological			
Harmful effects on pregnant animals	*Nettles 1997*		
Stillbirth		*Hodges & Hearn 1977; Gulyas et al. 1983; Kirkpatrick et al. 1996; De Vleeschouwer et al. 2000a; Bertschinger et al. 2001; Goritz et al. 2001; Wood et al. 2001; Raphael et al. 2003*	*Fayrer-Hosken et al. 2000*
Abnormal offspring		*Wood et al. 1981; Kirkpatrick et al. 1996*	*Lyda et al. 2005*
Inhibition of parturition or dystocia	*Nettles 1997*	*Goyings et al. 1977; Miller at al. 2001*	
Changes in ovarian structure or function	*Nettles 1997*	*Sokolowski & VanRavenswaay 1976; Goyings et al. 1977; Hodges & Hearn 1977; Clarke et al. 1978; Okon et al. 1980; Wood et al. 1981; Gulyas et al. 1983; Sacco et al. 1983; Skinner et al. 1984; Dobson 1985; Mahi-Brown et al. 1985; Short et al. 1985; Garza et al. 1986; McNeilly et al. 1986; McNeilly & Fraser 1987; Mahi-Brown et al. 1988; Dunbar et al. 1989; Sehgal et al. 1989; Adams & Adams 1990; Montovan et al. 1990; MacMillan & Thatcher 1991; Sacco et al. 1991; Bhatnagar et al. 1992; Hasegawa et al. 1992; Jones et al. 1992; Kirkpatrick et al. 1992; Paterson et al. 1992; Rhim et al. 1992; D'Occhio & Kinder 1995; Daels & Hughes 1995; Gong et al. 1995; Kirkpatrick et al. 1995; Prendiville et al. 1995a; Bergfeld et al. 1996; Gong et al. 1996; Paterson et al. 1996; Tshewang et al. 1997; Turin et al. 1997; Killian et al. 2006; Maclellan et al. 1997; Kazensky et al. 1998; Watson et al. 2000; Brussow et al. 2001; Goritz et al. 2001; Mircu et al. 2001;Dalin et al. 2002; Kitchener et al. 2002; Munson et al. 2005; Geary et al. 2006; Stoops et al. 2006; Curtis et al. 2007*	*Gwatkin et al. 1977; Kerr-Wilson et al. 1981; Liu et al. 1989; Porteous et al. 1994; Lou et al. 1995; Black et al. 1997; McShea et al. 1997; Fraker et al. 2002; Robbins et al. 2004; Levy et al. 2005; Hynes et al. 2007*
Changes in lactation or mammary glands	*Nettles 1997*	*Loretti et al. 2005*	*Roughton 1979; Baldwin et al. 1994; Nave et al. 2000; Mattos et al. 2001*
Changes in secondary sex characteristics	*Seal 1991, Nettles 1997*		
Changes in bodyweight or condition	*Nettles 1997*		
Increased in treated females		*Portugal & Asa 1995; Hayes et al. 1996; Bettinger et al. 1997; McShea et al. 1997; Turin et al. 1997; Mohle et al. 1999; Looper et al. 2001; Turner & Kirkpatrick 2002; Bourry et al. 2005*	*Seal 1976; Roughton 1979; Prendiville et al. 1995a; Prendiville et al. 1995b; Jolly et al. 1996; Miller et al. 1997; Tshewang et al. 1997; Ji et al. 2000; Miller et al. 2001; Bertschinger et al. 2001; Baker et al. 2002; Bertschinger et al. 2002; D'Occhio et al. 2002; Fraker et al. 2002; Nave et al. 2002; Zeng et al. 2002; Walter et al. 2003;Bourry et al. 2005; Herbert et al. 2005; Herbert et al. 2006; Rubion et al. 2006; Asa et al. 2007; Conner et al. 2007; Eymann et al. 2007; Hall-Woods et. al 2007; Wheaton et al. 2007*
Decreased in males		*Ji et al. 2000*	
Toxicity	*Nettles 1997*		
Interference with diagnostic tests	*Nettles 1997*	*Kirkpatrick et al. 1995*	
Abscesses or inflammatory reactions	*Nettles 1997*	*Mahi-Brown et al. 1985; Plotka et al. 1998; Becker et al. 1999; Kirkpatrick et al. 1990; Turner*	*Sokolowski & VanRavenswaay 1976; Turner et al. 1997; Trigg et*

		et al. 1992; Jacobsen et al. 2005; Kirkpatrick et al. 1995; Turner et al. 1996b; Warren et al. 1997; Harrenstien et al. 2004; Lyda et al. 2005; Munson et al. 2005; Curtis et al. 2007; Delsink et al. 2007*	*al. 2001; Turner et al. 2001; Fraker et al. 2002; Deigert et al. 2003; Baker et al. 2005; Levy et al. 2005*
Individual behavior			
Changes in behavior	*Nettles 1997*		
Sexual activity		*Turner et al. 1996b; Heilmann et al. 1998; Miller et al. 2000a; Miller et al. 2000b; Miller et al. 2000c; Curtis et al. 2002; Fraker et al. 2002; Baker et al. 2004; Woodward et al. 2006*	*Porteous et al. 1994; Turner et al. 1996a; Baker et al. 2002; Bourry et al. 2005; Hernandez et al. 2006*
Increased movement		*McShea et al. 1994* ; McShea et al. 1997*	*Hernandez et al. 2006*
Activity patterns		*Portugal & Asa 1995; McShea et al. 1997*	*Hayes et al. 1996; Heilmann et al. 1998; Powell 1999; Powell & Monfort 2001; Delsink et al. 2002; Poiani et al. 2002; Woodward et al. 2006; Conner et al. 2007*
Increased irritability or aggression		*De Vleeschouwer et al. 2000b; De Vleeschouwer et al. 2003; Harrenstien et al. 2004*	
Social disruption, change in status, territory loss		*Bertrand et al. 1996; Fayrer-Hosken et al. 2000; Poiani et al. 2002; Saunders et al. 2002; Jacob & Matulessy 2004*	*Jolly & Spurr 1996; Jolly et al. 1996; Heilmann et al. 1998; Bromley & Gese 2001a; Delsink et al. 2007*
Population-level			
Changes in sex ratio	*Nettles 1997*		
Due to immigration or emigration		*Ji et al. 2000*	
Offspring sex ratios			
Changes in annual breeding season	*Nettles 1997*	*Kirkpatrick et al. 1992; McShea et al. 1997; Warren et al. 1997*; Heilmann et al. 1998; Ji et al. 2000; Miller et al. 2000b; Miller et al. 2000c*	*Miller et al. 2000a; Conner et al. 2007*
Ecological alterations	*Nettles 1997*		
Home range or territory size		*Saunders et al. 2002; Jacob & Matulessy 2004; Ramsey 2007*	*Bertrand et al. 1996; Delsink et al. 2002; Saunders et al. 2002; Hernandez et al. 2006*
Increased immigration			*Saunders et al. 2002*

Change in habitat use		*Poiani et al. 2002*	*Jacob & Matulessy 2004*
Increased longevity or survival		*Twigg et al. 2000; Bromley & Gese 2001a; Turner & Kirkpatrick 2002; Ramsey 2005; Kirkpatrick & Turner 2007; Williams et al. 2007*	*Saunders et al. 2002; Jacob & Matulessy 2004*
Increased survival of offspring		*Twigg et al. 2000; Turner & Kirkpatrick 2002; Williams et al. 2007*	*Kirkpatrick & Turner 2002; Kirkpatrick & Turner 2003*
Impact on fertility of young	*Nettles 1997*		*Kirkpatrick & Turner 2002*
Improved breeding of non-treated females		*Chambers et al. 1999*	
Disease transmission rates	*Tyttens & MacDonald 1998; Caley & Ramsey 2001; Miller et al. 2004*	*Ramsey 2007*	*Kerr et al. 1998; Williams et al. 2007*
Evolutionary level			
Genetic resistance	*Magiafoglou et al. 2003*		
Genetic changes influencing disease resistance or immune function	*Nettles 1997; Cooper & Larsen 2006*		

*Cited in Nettles (1997)

CHAPTER 5

The role of sex in social life: lack of behavioral consequences of contraceptive treatment in feral horses



“The common thread that binds nearly all animal species seems to be that males are willing to abandon all sense and decorum, even to risk their lives, in the frantic quest for sex.”
Randy Thornhill & Craig T. Palmer

Photo caption: Unknown band; mare and foal allogrooming

Abstract: Both sex and reproduction play important roles in the life of social mammals and are often associated with social bonding and cohesion. Consortships are long lasting bonds between males and females and sex functions to maintain these bonds. Females mating within social groups can increase social relationships, decrease harassment, and potentially increase offspring survival suggesting that copulation has important implications for female fitness. The manipulation of sexual behavior may have undesirable consequences for females that live in social groups. Feral horses live in stable year-round harem bands with one to several stallions and adult females with offspring. They are polygynous breeders, but females often stay in bands for long periods of time or for most of their lives. Long tenure in bands by females is correlated with an increase in body condition and reproductive fitness. We manipulated fertility in feral horse mares by using two contraceptive formulations, SpayVac® and GonaCon-B™, that have opposite effects on cycling (increase in estrous cycling vs. reduced cycling) but similar effects on fertility (both prevent pregnancy). We analyzed several behavior measures using linear mixed models to determine if contraceptive treatments affect female behavior. Sexual interactions did not vary among treatment groups and we found few changes in behavior of treated mares related to the mare-stallion relationship. Treated mares did not change bands more often than controls. However, mares that changed bands, regardless of treatment, suffered more aggression, spent more time traveling, and avoided the harem stallion more than females in single stallion bands. While we found few changes in behavior of treated mares, we caution that these results may not reflect all contraceptive formulations and other horse populations. In addition, we saw no breakdown in the consortships between stallions and treated mares and it is likely that sexual interactions,

rather than foal production, are sufficient to maintain these social bonds in feral horse systems on a short time scale.

Introduction

Stable group living is found in many mammalian species (Krause and Ruxton 2002). Traditionally, the driving force behind sociality was related to resource distribution and predation risk (Alexander 1974, Bertram 1978, Wrangham 1980, Clark and Mangel 1986, Chapman et al. 1995, Janson and Goldsmith 1995, Hill and Dunbar 1998, Hass and Valenzuela 2002, Krause and Ruxton 2002). However, social explanations have also been proposed (e.g., sexual coercion, male harassment, infanticide risk) as strong factors that drive the evolution of these mating systems (Clutton-Brock et al. 1992, Smuts and Smuts 1993, Clutton-Brock and Parker 1995, Kappeler 1997). A growing body of literature has demonstrated costs to females associated with male harassment (e.g., infanticide, injuries, mortality, constraints on mating preferences) and how that harassment influences social structure of many mammals [chimpanzees (*Pan troglodytes*) (Williams et al. 2004), New Zealand sea lions (*Phocarctos hookeri*) (Chilvers et al. 2005), American sea lions (*Otaria flavescens*) (Cassini and Fernández-Juricic 2003, Cappozzo et al. 2008), feral horses (*Equus caballus*) (Linklater et al. 1999, Cameron et al. 2003) feral sheep (*Ovis aries*) (Reale et al. 1996) orangutans (*Pongo pygmaeus*) (Fox 2002), quail (*Cortunix japonica*) (Persaud and Galef 2003)]. Moreover, stable group membership has been shown to increase reproductive success of females, lower risk of infanticide, and increase male reproductive success, suggesting that group living has additional benefits beyond lowering predation risk and increasing availability of resources [primates (Kappeler 1997, van Schaik and Kappeler 1997), equids (Kaseda et al. 1995, Linklater et al. 1999, Cameron et al. 2003)].

Within groups, males and females may form bonds known as consortships that can vary from temporary associations to long term bonds (Carpenter 1942, Dixson 1998). Consortships are maintained through social interactions, particularly affiliative behaviors like grooming, remaining in close proximity, and sexual interactions (Bercovitch 1995). They require mutual effort in maintaining close proximity and communication between males and females (Bercovitch 1995). While the overall function of these associations is debated, both sexes appear to benefit. Males benefit by potentially increasing paternity through mate guarding or monopolization (Szykman et al. 2001, Engelhardt et al. 2006, Brauch et al. 2008). Females can benefit from these associations by decreased harassment from other males and increasing their offspring survival, both of which can increase reproductive success (Smuts and Smuts 1993, Dixson 1998, Linklater et al. 1999, Fox 2002). Therefore, the function of these bonds is associated with the production of offspring, and their maintenance is associated with sexual interactions, suggesting that both reproduction and fertility play key roles in the cohesion of these social bonds.

Feral horses are unusual among ungulates in that they live in stable year-round harem bands with one to several stallions and adult females with offspring (Linklater 2000). Females often stay in bands for long periods of time or for most of their lives after natal dispersal (Klingel 1979, Berger 1986, Linklater 2000). These long term relationships between stallions and mares have been previously described as consortships (Kaseda et al. 1995). There is evidence of strong selection for mate fidelity and band loyalty so as to decrease harassment by other males (Linklater et al. 1999). Females that change bands have higher parasite loads, lower body condition, higher harassment rates and lower reproductive success, suggesting that band stability is adaptive for females

(Linklater et al. 1999). In addition, the longevity of band fidelity, or long term consortships, is correlated with an increase in body condition and reproductive fitness in mares (Kaseda et al. 1995). Females that maintain long term bonds with the same male avoid aggression associated with changing bands and increase their reproductive success (Kaseda et al. 1995).

The unusual social structure of long term harem bands is thought to be supported by the socio-sexual behavior of mares (Asa 1986). Female horses are unlike most mammals because they display sexual behavior throughout the year, even during pregnancy, the anovulatory period and after an ovariectomy (Asa 1986, Crowell-Davis 2007). These sexual behaviors have been proposed to maintain interest and stimulate loyalty with stallions and to be a possible explanation for the unusual ungulate mating system (Asa 1986, Linklater et al. 1999). Thus, sexual behavior, regardless of the whether mares become pregnant, is important to the social behavior of mares and the maintenance of band stability. As a result, the manipulation of fertility of females may have important behavioral and social consequences.

Female contraception has been extensively used in many wildlife species to reduce fertility (see Chapter 3). While it is used as an important management tool, the manipulation of fertility in females can also offer insights into sexual and social behavior. Several studies have examined efficacy of contraception, but in comparison, little research has been conducted on the behavioral side effects (reviewed in Chapter 4). While most of the literature shows mixed support for secondary effects of treatment, several contraceptive formulations have been shown to impact behavior (Chapter 4). We studied how contraceptive treatment could impact fertility and mating behavior of mares.

We used two different formulations that have significantly different ways of decreasing fertility. First, SpayVac® is a porcine zona pellucida (PZP) formulation that acts to block fertilization and allows for continued, and sometimes an increase in cycling (Brown et al. 1997, Fraker et al. 2002, Killian et al. 2008). In contrast, GonaCon-B™ is a gonadotropin-releasing hormone (GnRH) immunization that acts to block cycling altogether by significantly reducing the production of LH and FSH (Miller et al. 2000a, Killian et al. 2006, 2008). Both formulations work to reduce pregnancy, but do so in ways that allow us to predict how sexual interactions, or lack thereof, can impact female behavior and social life and if absence of foaling can also cause breakdown in social structure.

Several factors suggest that changes in behavior and band stability would occur in mares treated with contraceptives. First, the socio-sexual behavior of mares implies an important function of social structure and altering fertility could impact consort relationships and band stability. Second, several studies have found changes in behavior with both contraceptive formulations in other species. For example, PZP is a widely used contraceptive in wildlife species and has been shown to prolong breeding behavior and breeding seasons and delay birthing dates in ungulates (McShea et al. 1997, Heilmann et al. 1998, Miller et al. 2000b, 2000c, Curtis et al. 2002). In addition, increased aggression and irritability have been reported with PZP treatment in captive female felids (Harrenstien et al. 2004). Studies that investigated GnRH contraceptive formulations found decreased sexual behavior in females (Miller et al. 2000a), while others have found no changes in sexual behavior (Baker et al. 2002, 2004, Conner et al. 2007). One study showed that male kangaroos preferred to be closer to groups of control kangaroo females

rather than GnRH-treated females, suggesting that sexual interactions may have a function in group formation and fidelity (Poiani et al. 2002). Third, while there is a lack of research that has investigated changes in behavior of feral horses, the few studies that have been conducted have resulted in mixed outcomes, with one study showing no difference in female behavior within an estrous cycle (Powell 1999) and a recent study showing significant changes in female behavior and group fidelity during the nonbreeding season (Nuñez et al. 2009). In addition, both of these studies only used PZP formulations, and a GnRH formulation has not been investigated in feral horses to date. Lastly, since group maintenance and function have been linked to the production of offspring, it is possible that when females do not produce offspring, groups may eventually start to break down.

The current study was initiated in June 2005 when females were treated with two different contraceptive formulations and then followed until August 2007 to look for differences in mare-stallion relationships. By manipulating fertility, we tested the importance of sexual behavior to social behavior in mares, the importance of offspring production and how both could impact band loyalty over time. We looked for differences in behavior in both the breeding and nonbreeding season. First, we tested if foal production was necessary for band cohesion. Then, we investigated how sexual interactions influenced female behavior and impacted consortships. We predicted that SpayVac® (PZP) would increase harassment due to increased sexual activity, which would lead to more band changes, especially in the nonbreeding season. Conversely, since GnRH formulations mimic a more natural cycling pattern (e.g., most mares are pregnant year round and do not cycle), we expected less harassment in the nonbreeding

season, but increased harassment in the breeding season since we expected there would be few sexual interactions with the stallion. While we initially expected an absence of sexual behavior in GonaCon-B™ treated mates, there is some evidence that GonaCon-B™ does not impact FSH, therefore females produce small amounts of estrogen that can stimulate estrus behavior (Killian et al. 2008). Thus, we predicted a significant reduction, rather than absence, of breeding behavior in these females. We tested whether contraception impacted individual behavior of mares, and whether contraceptive treatment impacted consort relationships with the band stallion. We specifically looked at aggressive interactions, sexual interactions, affiliations, spatial distancing, and contact maintenance with the band stallion. We then tested if alterations to sexual behavior and lack of offspring production would impact band fidelity of treated females.

Methods

Study Subjects

The feral horse study population lives in the Virginia Range, east of Reno, Nevada. The mountain range is approximately 145,000 hectares. While the total population is currently around 1400 individuals, the study population consisted of 300 individuals including stallions, mares, juveniles and foals. Feral horses were gathered in June 2005 and females were treated with contraceptive formulations; 1) SpayVac®, a PZP formulation, 2) GonaCon-B™, a GnRH immunization. Control mares consisted of females that received only the adjuvant or no injection. We aged females by tooth eruption patterns (Tutt 1968) and classified into the following age categories: 1-5 (young), 6-8 (middle age), and >9 years (old). We branded all treated females with

individual brands on their left hindquarter, and followed for the next 2 years. Details of treatment protocols can be found in Chapter 3. All treatment procedures were carried out under the supervision of veterinarians from the Nevada Department of Agriculture.

Sampling Regime

Behavioral observations were carried out by one person who was unaware of the treatment status of each female. Behavioral observations began in July 2005 and ended in August 2007. We located bands randomly and observations were evenly distributed during daytime hours. A total of 44 bands were located on a weekly-monthly basis, with 36 having a single stallion and 8 having more than one stallion. Once a band was located, we recorded the GPS location and behavioral observations took place for 30-60 minutes or until the focal animals moved out of view. Samples shorter than 30 minutes were not used in analysis. Observations took place on foot near habituated bands or in a truck from farther distances with the aid of binoculars. Horses were identified by brands or unique coat color and white markings on the face and legs. Branded mares were considered focal individuals and no more than two focal mares were sampled during the observation period (Altmann 1974).

Activity, Aggression and Social Behavior

In order to determine changes in activity, we recorded the focal mare's activity every four minutes. The four minute time interval is similar to that used in other horse studies (Duncan et al. 1984, Crowell-Davis 1986, Cameron and Linklater 2000, Linklater et al. 1999) and enhances the independence of data (Rollinson et al. 1956). Most

activities could be classified into grazing, resting (standing or lying), alert, travel (walk, trot, canter), social (play, affiliative behaviors, sexual behaviors), and not seen (individual out of view or activity could not be determined).

To determine changes in the spatial relationship with the band stallion, we recorded the distance from the focal mare to the stallion in adult body lengths at four minute intervals. Only samples in which at least one member of the dyad was active were included in the analysis. Distances that were less than one body length were recorded as 0.5 body lengths. Spatial distances were transformed into average distance to stallion. In addition, we recorded the nearest neighbors within a two adult body length distance from each focal mare every four minutes. These data were transformed into the percentage of time a female was within two body lengths of the stallion. A two body length distance is considered a close distance and a measure of association (Linklater et al. 1999). We recorded all approaches and leaves within a two body length boundary of the focal mare and stallion on an all-occurrence basis (Altmann 1974). These data were transformed into an index that measures contact maintenance as described by Hinde and Atkinson 1970. Briefly, this score is calculated from the percentage of approaches by the mare towards the stallion minus the percentage of leaves from the stallion by the mare. The resultant score varies from +1 to -1, with a score closer to -1 indicating that the mare was avoiding the stallion (Hinde and Atkinson 1970). Therefore, the spatial relationship between focal mares and stallions was measured as the average body length distance, the percentage of time they were within two body lengths of each other, the percentage of approaches by mares and the contact maintenance scores between focal mares and stallions.

We recorded all social interactions between the focal mare and stallion on an all-occurrence basis. The initiator of the interaction was recorded as well as whether the interaction was aggressive or affiliative. Aggressive interactions include head threats, bites, bite threats, kicks, and kick threats (McDonnell and Haviland 1995). Only aggressive interactions between adult horses were included in the analysis. Sexual interactions included naso (nose to nose sniffing), general sniffing, stallion calls directed towards the focal mare, rubbing by the stallion, genital sniffing, tail lifting from the mare, mounting, and copulation. Both affiliative behaviors and aggression were transformed into average rates of interaction per hour of observation.

Estimating Band Changes

At the beginning of each sampling period, we recorded band membership, which included observations on births, deaths and dispersals. We classified a band change as immigration into a new band that lasted for more than a day. The amount of band changing was calculated as the number of band changes divided by the total days the mare was observed in each season. The mares that changed bands more than once during the course of the study were classified as maverick mares or band changers (Linklater et al. 1999).

Seasonal Designations

Previous studies have shown that behavioral effects of contraceptive treatment differ between breeding and nonbreeding seasons (McShea et al. 1997, Nunez et al. 2009). Because we expected that these effects could occur, all behavioral data were

subdivided into breeding and nonbreeding seasons. We defined the breeding seasons to encompass 30 days before the first foal was born to 30 days after the last foal was born in the foaling season in order to include the peak of sexual activity. This designation resulted in March-July being the breeding season and the nonbreeding season encompassed the rest of the year. During the course of the study, two breeding seasons and two nonbreeding seasons were studied.

Statistical Analyses

A nested analysis to examine differences among bands could not be conducted due to low sample size. Therefore, we tested for behavioral differences based on contraceptive treatment with linear mixed models with restricted maximum likelihood estimation (Bolker et al. 2009). Mares were treated as individual replicates, but their band was treated as a random effect to account for variation among bands. The fixed predictor variables were treatment (control, GonaCon-B™, SpayVac®), band type (single stallion, multistallion, maverick) and age (young, medium, old age). Band type was classified by the number of stallions in the band and if females were habitual band changers (maverick) as these different strategies impact female behavior and reproductive success (Linklater et al. 1999). We combined behavioral data from both breeding seasons and both nonbreeding seasons and females were considered individual samples for each season. A female must have been sampled more than once to be included in the analyses and those females only sampled once were excluded from the analyses. Treated mares that foaled or were pregnant (e.g., contraceptive failures) were excluded from the analysis. We ran all models in SPSS 14.0 (SPSS 2005).

We analyzed band changing data with an analysis of variance using treatment type and age category as predictor variables. Data were normalized by log transformation. All band changes during the first nonbreeding season were excluded because these changes may have been due to the effects of the 2005 roundup. The rest of the seasons (breeding seasons 1 and 2, nonbreeding season 2) were analyzed separately. Data are shown as mean ± standard error. We considered statistical significance as $P<0.05$ and trends as $0.05<P<0.10$.

Results

Behavioral Data Characteristics

A total of 716.38 hours of observation took place during both nonbreeding seasons combined and 558.70 hours for both breeding seasons combined for the mares included in the analysis. A total of 44 bands and 64 mares were used in these analyses. There were no differences in the average hours observed among treatment groups, age classes, and band types within the combined nonbreeding and combined breeding seasons (Table 1). There was no difference in observation hours among treatment groups within each age class for both the nonbreeding (χ^2_4=4.743, P=0.315) and the breeding season (χ^2_4=5.124, P=0.275).

Activity

There was no difference among treatment groups in time spent grazing, resting or traveling during the breeding (graze: $F_{2,58}$=0.063, P=0.939; rest: $F_{2,57}$=0.117, P=0.890; travel: $F_{2,49}$=0.234, P=0.793) and nonbreeding season (graze: $F_{2,80}$=0.444, P=0.643; rest:

$F_{2,78}$=1.094, P=0.340; travel: $F_{2,74}$=0.694, P=0.503). There was a trend for band type to interact with activity as maverick mares traveled more than mares in single stallion bands in the breeding season ($F_{2,29}$=2.804, P=0.077) and mares in multistallion bands traveled more than mares in single stallion bands ($F_{2,35}$=3.403, P=0.045; Figure 1).

Sexual Interactions

There were no differences in sexual interactions among the treatment groups both within the breeding season ($F_{2,45}$=0.538, P=0.588) and outside the breeding season ($F_{2,79}$=0.746, P=0.478) (Figure 2). A total of 25 copulations were witnessed during the course of the study with 13 control mares, 11 SpayVac® mares, and 1 GonaCon-B™ mare. Most of the copulations were seen during the breeding season (N=20). During the nonbreeding season, there was no evidence that SpayVac® mares cycled more and had more sexual interactions than the control mares. The one copulation observed with the GonaCon-B™ mare was seen in the nonbreeding season.

Stallion-Mare Relationships

In both the breeding and nonbreeding seasons, there was no overall treatment effect on the distance of the mare to the stallion (breeding: $F_{2,58}$=0.502, P=0.608; nonbreeding: $F_{2,76}$=0.213, P=0.809), the percentage of time within two body lengths of the stallion (breeding: $F_{2,51}$=0.085, P=0.918; nonbreeding: $F_{2,74}$=1.723, P=0.186), the percentage of approaches by the mare (breeding: $F_{2,56}$=0.237, P=0.790; nonbreeding: $F_{2,67}$=0.344, P=0.710) and the contact maintenance score with the stallion (breeding: $F_{2,56}$=0.537, P=0.587; nonbreeding: $F_{2,67}$=1.597, P=0.210). However, post hoc

comparisons suggested that SpayVac® mares spent less time within two body lengths of the band stallions than control mares in the nonbreeding season (t_{74}=1.675, P=0.098, Figure 3).

There was trend for band type to affect the percentage of approaches by mares (breeding: $F_{2,39}$=2.911, P=0.066, nonbreeding: $F_{2,67}$=2.390, P=0.099), as maverick mares significantly approached stallions less than mares in single stallion bands (breeding: t=2.386, df=48, P=0.021; nonbreeding: t_{67}=2.134, P=0.036, Figure 4). In the breeding season, maverick mares had lower contact maintenance scores compared to mares in single stallion bands (t_{47}=2.132, P=0.038) and mares in multistallion bands (t_{43}=1.804, P=0.078), indicating that maverick mares avoided stallions (Figure 5).

Aggression

There was no overall treatment effect on the amount of aggression a mare received from the rest of the group (breeding: $F_{2,44}$=1.527, P=0.229, nonbreeding: $F_{2,68}$=0.194, P=0.824) or the amount of aggression given by a mare towards other adults in the band (breeding: $F_{2,61}$=0.341, P=0.713, nonbreeding: $F_{2,74}$=0.507, P=0.604). However, the GonaCon-B™ mares tended to receive more aggression than SpayVac® mares during the breeding season (t_{46}=1.735, P=0.089) (Figure 6).

There was an overall band type effect on aggression received in the breeding season ($F_{2,27}$=5.047, P=0.014) as maverick mares received more aggressive behaviors than mares in single stallion bands (t_{30}=2.589, P=0.015) and multistallion bands (t_{31}=2.589, P=0.004).

Band Changing

There was no difference in band changing based on treatment status in any season (first breeding season: $F_{2,32}$=1.40, P=0.262, second nonbreeding season: $F_{2,35}$=1.40, P=0.262, second breeding season: $F_{2,36}$=0.05, P=0.948) (Figure 7).

Discussion

The Role of Sex

Sexual interactions have been associated with social bonding and cohesion, and are thought to maintain male-female bonds, suggesting the importance of sexual interactions in the social life of many group living mammals. The socio-sexual behavior of mares has been hypothesized to play a major role in the social life of feral horses and potentially maintains the stability of bands throughout the year and over long periods of time (Asa 1986). The control of feral horse populations with contraception presents the unique opportunity to manipulate fertility and test predictions about sex, reproduction and group living. Contrary to most of our predictions, contraceptive treatment had minimal impact on female social behavior with regards to the relationship with the band stallion. However, most of our predictions were based on expected changes in sexual behavior. Previous studies on PZP treatment demonstrated changes in sexual behaviors in other species as treated females showed an increase in sexual activity (McShea et al. 1997, Heilmann et al. 1998, Miller et al. 2000b, 2000c, Curtis et al. 2002). We found no difference in the amount of sexual behavior or actual copulations between SpayVac® mares and controls. It is possible that since horses can display sexual behavior year round (Asa 1986), an increase in estrous cycling in SpayVac® mares may not be observed.

Recently a study showed that PZP treated mares showed an increase in sexual behavior compared to controls and had lower band fidelity (Nuñez et al. 2009). We found no evidence that SpayVac® mares had increased cycling during the nonbreeding season or changed bands more frequently. The difference between these results may be due to a difference in PZP formulations, as our formulation SpayVac® may not increase estrous cycling (Killian et al. 2008). Therefore, treatment with PZP that does not increase estrous cycling may not have harmful side effects suggesting that the cycling, not the PZP, causes changes in behavior and band fidelity. The discrepancy in results may also be due to a difference in methods. It was not clear whether the investigators took band variation into account, and all controls from their study would be considered old aged females by our methodology, all of which can confound results (see Nunez et al. 2009).

In contrast, we expected a significant reduction of all breeding behavior in GonaCon-B™ mares (Miller et al. 2000a, Killian et al. 2006, 2008). Again, we detected no difference in sexual behavior between GonaCon-B™ mares and controls. Our results are similar to those of other studies that investigated GnRH treatment in other species in which females showed no difference in sexual behavior compared to controls (Baker et al. 2002, 2004, Conner et al. 2007). GonaCon-B™ did not suppress reproductive behaviors of mares and it is unknown why these females still seem to be receptive to stallions. In a captive study, GonaCon-B™ mares had plasma estrodial concentrations similar to control mares, contrary to what we expected because GonaCon-B™ works to reduce hormone levels that control estrous cycling (see Killian et al. 2008). However, there is evidence that follicle-stimulating hormone-releasing hormone (FSHRH) is secreted by the anterior pituitary and would cause some follicular development (McCann

et al. 1993, Yu et al. 1997, Padmanabhan and McNeilly 2001). GonaCon-B™ works to inhibit the LH surge needed for ovulation, but would not inhibit FSHRH, thus some estrodial would be produced from follicular development. This suggests that these mares may have enough circulating estrodial to exhibit estrous-like behavior, but do not completely cycle and therefore do not become pregnant. While these results are still inconclusive, it would appear that these mares continue to act like untreated mares and engage in sexual interactions. Since sexual interactions did not change in treated individuals, it is possible that other social behaviors will not change enough to disrupt consortships and band fidelity on a short time scale. The development of a FSHRH agonist and its use in conjunction with a GnRH agonist may help elucidate the role of sex in social life.

The Role of Foals

While sex maintains consortships, the function of these social bonds is linked to reproduction as consorting with a male has been shown to decrease male harassment (Linklater et al. 1999) and decrease the chance of infanticide (Kaseda et al. 1995, Gray 2009). Sexual interactions occurred in all treatment groups, even without reproduction, suggesting that consortships can be maintained even without the production of offspring. We found that treated females without foals did not change bands more often than control mares. This indicates that sex alone, and not necessarily reproduction is important to maintaining these social bonds. These results do not imply that reproduction is irrelevant to consortships or group living in horses, only that these relationships are maintained in

the short term without foals. It is possible that an absence of foaling for several years would cause the social units to break down, but this requires a longer study.

Mare-Stallion Relationships

Based on our behavioral measurements, contraceptive treatment had little impact on mare-stallion relationships. Most treated females maintained a similar spatial relationship with the band stallion, except for SpayVac® mares that spent less time within two body lengths of the stallion in the nonbreeding season (Figure 3).While this may indicate some avoidance by these mares, none of the other behavioral measurements showed any trends. Over time, if SpayVac® mares start to avoid stallions, they may begin to change bands more often than controls, but these effects would take time to elucidate.

The only difference found in GonaCon-B™ treated mares was aggressive interactions in the breeding season compared to SpayVac® mares. Since this formulation works at the hormone level, it is likely that changes to hormone levels (e.g., estrogen, progesterone) could impact aggressive and submissive behavior. The influence of sex hormones on aggressive behavior in females has largely been ignored compared to research on males and research shows mixed results depending on species (Floody 1983, Debold and Miczek 1984, Kapusta 1998, Woodley and Moore 1999, Kohlert and Meisel 2001). However, research on horses has shown that mares during diestrous are more aggressive towards stallions when circulating progesterone is high (Crowell-Davis 2007), suggesting that lower progesterone levels might decrease aggressive behavior. In addition, observations of captive GonaCon-B™ mares showed that these mares were

more submissive and easier to work with, thus we expected these mares in the wild to receive more aggression. We only found this trend when they were compared to SpayVac® mares. There is some evidence that GonaCon-B™ may decrease aggressiveness in females and over time, these females may suffer higher rates of harassment, which could influence band changing. Ultimately, we expected behavioral changes to the mare-stallion relationship would lead to changes in band fidelity. Again, we found no evidence that band changing was impacted by contraceptive treatment.

Behavioral Consequences of Band Changing

Stallion harassment has been shown to decrease the health and reproductive success of mares (Linklater et al. 1999). Our results confirmed that band loyalty and the presence of more than one stallion had a greater influence on female behavior, than contraceptive treatment. Regardless of treatment, mares that changed bands suffered higher rates of aggression and spent more time traveling than mares that were loyal to bands. We also found that maverick mares approached stallions less often and had lower contact maintenance scores with stallions, which would indicate that these mares avoid stallions during the breeding season (Figure 4, 5), most likely due to increased harassment rates. These data support the importance of long term band fidelity in mares.

Management Implications

Both of these contraceptive formulations offer the benefit of a single shot for multi-year infertility, which contrasts to other formulations currently being used to treat wildlife species. Few studies have investigated behavioral side effects of contraceptive

treatment, and most of those studies focused on short term effects in captive or semi-captive species (McShea et al. 1997, Heilmann et al. 1998, Miller et al. 2000b, 2000c, Baker et al. 2002, 2004, Massei et al. 2008). While we found no major behavioral changes, we caution that these results were based on observations for only 2 years post treatment and sample sizes for treatment groups were low in the analysis of the data. The low efficacy rates were not expected (see Chapter 3); therefore many of our treated females were excluded due to an ineffective treatment. This lowered our sample size only in our treatment groups which resulted in an unbalanced design and possibly not enough power to detect changes. It is also possible that major changes take more time to occur.

There is potential that these particular formulations may have minimal impacts on horses. Our results suggests that at least initially, treated females have little change in behavior and do not have lower band fidelity because sexual behaviors appear to be the same in treated and control mares. These contraceptive formulations could be used for management application with little behavioral consequences in the short term. However, more long term longitudinal studies are required on other formulations in order to determine the effects of contraceptive treatment over a long time period.

Conclusions

While consortships are thought to be maintained by sexual interactions, band formation has also been linked to male harassment and infanticide avoidance. Thus, mares in single stallion bands have better reproductive success and overall health than mares in multistallion bands or mares that continually change bands. Fertility manipulation was hypothesized to impact mare-stallion relationships since sex is deemed

important to band stability and group maintenance is associated with offspring. These manipulations allow for separation of impacts of sexual interactions and reproduction. We found no evidence that behavioral changes occurred and this is likely because sexual behaviors did not change. We showed that without changes to sexual behavior, changes to social behavior or structure should not be expected in feral horses. We confirmed that the mating strategy of mares (band type) is an important determinant of female social behavior and mare-stallion relationships. There was no breakdown in the consortships between stallions and mares and it is likely that sexual interactions, rather than foal production, are sufficient to maintain these social bonds in feral horse systems on a short time scale.

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Table 1. The mean hours observed for mares in each treatment group, age category and band type in both the breeding and nonbreeding season.

Breeding Season Treatment	N	Mean	SE	F	*P*
Control	27	12.33	2.29	$F_{2,47} = 0.44$	0.645
GonaCon-B™(GnRH)	9	9.07	1.42		
SpayVac® (PZP)	14	10.31	2.03		
Age					
Young (<5 yrs)	21	9.11	1.20	$F_{2,47} = 2.47$	0.095
Medium (6-8 yrs)	9	17.39	4.01		
Old (>9yrs)	20	10.54	2.55		
Band Type					
Single Stallion	41	12.21	1.63	$F_{2,47} = 1.29$	0.284
Multistallion	5	6.67	1.55		
Maverick	4	6.22	1.49		

Nonbreeding Season Treatment	N	Mean	SE	F	*P*
Control	32	12.66	2.07	$F_{2,57} = 0.48$	0.662
GonaCon-B™(GnRH)	13	13.08	4.10		
SpayVac® (PZP)	15	9.42	1.90		
Age					
Young (<5 yrs)	23	8.86	1.61	$F_{2,56} = 1.07$	0.349
Medium (6-8 yrs)	9	13.61	1.95		
Old (>9yrs)	27	12.93	2.68		
Band Type					
Single Stallion	49	12.53	1.71	$F_{2,57} = 0.55$	0.580
Multistallion	7	7.64	1.67		
Maverick	4	12.20	7.00		

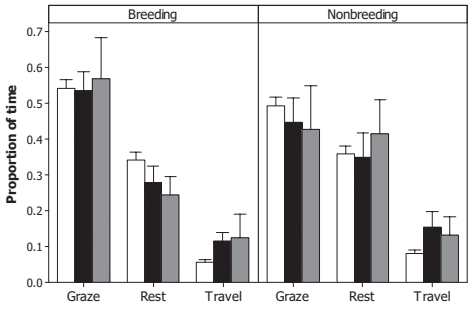


Figure 1. The proportion of time devoted by mares in single stallion bands (□), mares in multistallion bands (■), and band changers (mavericks) (▨) to grazing, resting and traveling. Time spent traveling differed between maverick mares and mares in single stallion bands in the breeding season ($P<0.10$) and between mares in multistallion bands and mares in single stallion bands ($P<0.05$).

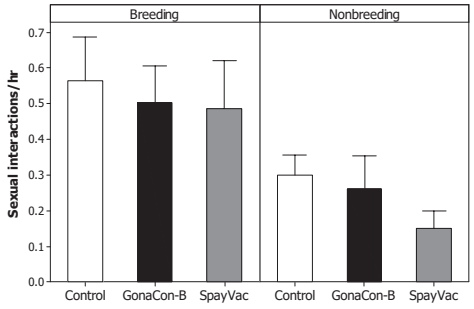


Figure 2. The total number of sexual interactions in the breeding and nonbreeding seasons for each treatment group. There was no difference among treatment groups in either season ($P>0.05$).

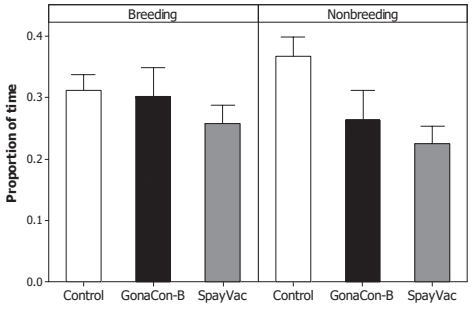


Figure 3. The proportion of time a mare was within two body lengths of the harem stallion based on treatment category. SpayVac® mares spent less time within two body lengths of the band stallions than control mares in the nonbreeding season ($P<0.10$).

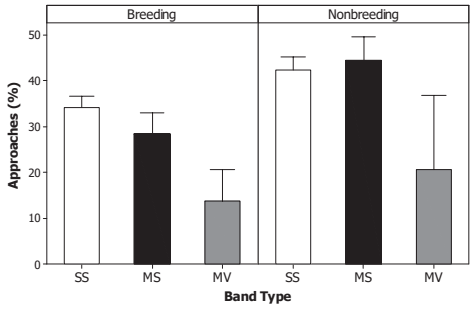


Figure 4. The percentage of approaches between the mare and stallion done by the mares in single stallion bands (SS) (□), mares in multistallion bands (MS) (■), and mavericks (band changers) (MV) (▩). Maverick mares significantly approached stallions less than mares in single stallion bands in both seasons (P<0.05).

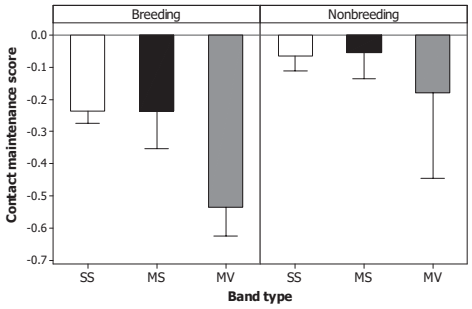


Figure 5. The contact maintenance scores between band stallions and mares in single stallion bands (SS) (□), multistallion bands (MS) (■), and mavericks (MV) (▩). Scores closer to -1 indicate avoidance by mares. Maverick mares had lower contact maintenance scores in the breeding season compared to mares in single stallion bands (P<0.05) and multistallion bands (P<0.10).

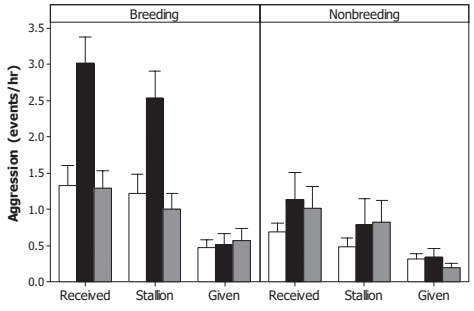


Figure 6. The rate of aggression received by other members in the band (received), received by the band stallion (stallion) and given towards other members of the band (given) for control mares (□), GonaCon-B™ mares (■), and SpayVac® mares (▩). Values differed between GonaCon-B™ mares and SpayVac® mares during the breedingseason (P<0.10).

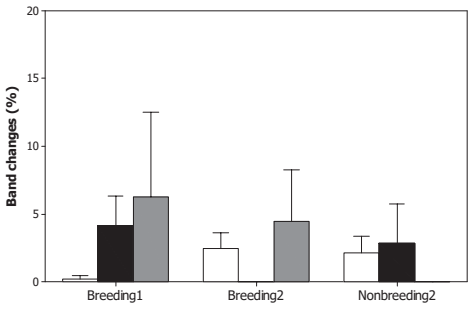


Figure 7. The percentage of band changes observed in the first breeding season, the second breeding season and the second nonbreeding season in control mares (□), GonaCon-B™ mares (■), and SpayVac® mares (▩).

DISCUSSION



"...I heard a neigh. Oh, such a brisk and melodious neigh as that was! My very heart leaped with delight at the sound."

Nathaniel Hawthorne

Photo Caption: Four mares from the V band grazing in winter.

DISCUSSION

Feral horses are ideal for testing hypotheses on group living, mating strategies and social behavior. Unlike other social mammals, feral horses require some management, often in the form of contraception, which sets up experimental manipulations that allow testing of the roles of sex and reproduction in the social behavior of females and band fidelity.

Male harassment and infanticide have been proposed as major influences on mating systems and male-female associations (Kappeler 1997, van Schaik and Kappeler 1997). There is increasing evidence that females join groups to avoid harassment from other males and potentially increase the protection of their offspring (e.g., Williams et al. 2004, Muller et al. 2007, Cappozzo et al. 2008). This is especially true in feral horses, where females that join and remain loyal in bands have higher reproductive success than mares with lower fidelity (Kaseda et al. 1995, Linklater et al. 1999). In my first chapter, I explored these influences in the context of paternity of offspring. Females may choose to seek extra-group copulations, but can suffer severe consequences, such as infanticide (van Schaik 2000). I found that in feral horses extra-band copulations were rare and most females were loyal to their band stallion. Most of the foals not sired by the band stallion were the result of band changing. Females that remained stable in their bands had higher offspring survival than those that had foals sired by an extra-band stallion, further supporting the idea of band stability as an adaptive strategy. In addition, almost all foals died that were born in a band without their sire, but most foals survived that immigrated with their mothers to a new band, suggesting that being born in band without their sire increases the risk of infanticide. Females were also more protective of foals not sired by

the band stallion, suggesting that females assess paternity and alter their behavior accordingly. Furthermore, I documented the first infanticide attack in free-roaming feral horses and showed that the attacker was not the genetic sire, but also that the mother was successful at ending the attack by protecting her foal. Given these results, I demonstrated that infanticide is a risk that females assess and attempt to minimize by increasing their protectiveness. However, most females are loyal to their band stallion, suggesting that infanticide risk is high. Furthermore, mares that changed bands remained loyal thereafter, further supporting strong selection for long term stable associations. I showed that infanticide risk may be an important determinant of this mating system and confirmed the majority of females engage in the strategy of band loyalty, rather than band changing or seeking extra-band copulations.

Most horse populations require some form of management. The increasing use of contraceptives provides potential experiments for testing the importance of reproduction and fertility in consortships. I worked with two contraceptive formulations, initially looking at efficacy. Neither of these formulations had been tested in free-roaming horses previously. I demonstrated that these contraceptive formulations can reduce fertility for multiple years with the use of one injection. I expected the efficacy rates to be as high as 90-100%, but instead they were 60-70%. Several factors could explain some of this lowered level of efficacy. First, the horses in the study were monitored on a weekly basis, which is much more intensive than other studies that use a few surveys per year, which allowed me to observe early foal loss. I also tested all mares for pregnancy using fecal progesterone levels and found that 2/19 mares that showed no visible signs of pregnancy were actually pregnant, further demonstrating the importance of constant monitoring of

wild populations for accurate reporting of efficacy. Second, I looked at body condition at time of treatment and found that there were more contraceptive failures in females that were in poor condition at time of treatment. My results indicate that body condition may be an important but overlooked factor in efficacy of treatment, but this requires further investigation. I found no evidence that the contraceptives impacted foal sex, foal survival or timing of birth in females that were contraceptive failures, which supports previous research on other feral horse populations (Kirkpatrick and Turner 2003). I also investigated whether side effects occurred in other species treated with contraceptives. A meta-analysis confirmed that side effects are found when they are investigated, including changes to behavior. This result was contrary to conclusions made from papers that suggested that there were no side effects, but did not quantitatively investigate their claims and often lacked appropriate controls and sampling methodology. Most of the side effects were minor, including some physiological changes to the reproductive system, but some were more serious, including changes to breeding behavior, activity, and the mating season (McShea et al. 1997, Heilmann et al. 1998, Curtis et al. 2002). If species are going to be managed by contraception, then continued research is necessary to understand population-level effects of wildlife species. Contraception provides a powerful tool to address fundamental questions in biology, such as the costs of reproduction, importance of copulation in maintaining social structure and parental investment strategies. I then investigated if contraception would impact the behavior of the females due to lack of reproduction and the alteration of sexual activity and if these factors ultimately lead to changes in band fidelity.

Contrary to most of my predictions, contraceptive treatment did not impact interactions between mares and stallions or band fidelity. Sexual interactions were the same among treatment groups, thus consortships were maintained even without the production of offspring. There was no difference in the mare-stallion relationship in treated mares and these females did not change bands more often than controls. However, these changes may take time to occur, and my study did not extend past two years post-treatment. I did find that mating strategy and band type had a greater impact on female behavior. Females that changed bands suffered from more aggression, spent more time traveling, and avoided the stallion, all of which confirms previous research suggesting the importance of band stability for female reproductive success and the costs of male harassment (Linklater et al. 1999).

Managers should consider both contraceptive type and the species before they begin treatment of individuals in order to minimize changes to behavior and social structure. While I found no change in social behavior and consortships, it is possible that these changes take time to occur. It is also possible that the formulations that I used had little impact on behavior. Numerous studies that investigated changes to behavior have found them, thus it is important to monitor treated individuals for changes in behavior and welfare.

In conclusion, I conducted a study on social behavior in feral horses, with an emphasis on the influence of paternity and fertility manipulation. I found that most females remain stable in a band and have a foal sired by the band stallion. For females that did not have a foal sired by the band stallion, they were more protective, but their foals had a lower survival rate. I also found that contraception may not be as efficacious

as some report, and intensive monitoring should be done to get more accurate measurements of efficacy. In addition, consortships can be maintained for the short term without the requirement of foal production as long as sexual activity remains unchanged.

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Corrigendum

Multi-year fertility reduction in free-roaming feral horses with single-injection immunocontraceptive formulations

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The authors wish to alert readers to the following error.

We mistakenly used the term SpayVac® to describe the PZP preparation tested in our manuscript. SpayVac® is a trade name that refers to a PZP formulation made by Immunovaccine Technologies (IVT) with an IVT DepoVax® liposome technology. SpayVac® does not include the adjuvant AdjuVac™. The PZP tested in our study was prepared by IVT without the liposomes and sent to the USDA/APHIS National Wildlife Research Center (NWRC), where the final formulation was made as a water-in-oil emulsion of the PZP with NWRC's AdjuVac™. The formulation we evaluated was not a conjugate, as stated in the manuscript.

In our article we reported that 'Killian *et al.* (2008) showed higher efficacy rates in captive feral horses by using the same treatments on the same species from the same population as we studied,' and hypothesised that this might have been due to differences in condition between captive and wild mares. However, we now acknowledge that the difference in PZP preparations may have contributed to the difference in efficacy rate between these studies.

We accept responsibility for this mistake and apologise to readers and Immunovaccine Technologies.

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Killian, G., Thain, D., Diehl, N. K., Rhyan, J., and Miller, L. (2008). Four-year contraception rates of mares treated with single-injection porcine zona pellucida and GnRH vaccines and intrauterine devices. *Wildlife Research* **35**, 531–539. doi:10.1071/WR07134

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Multi-year fertility reduction in free-roaming feral horses with single-injection immunocontraceptive formulations

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Abstract

Context. Contraception is increasingly used as a management technique to reduce fertility in wildlife populations; however, the feasibility of contraceptive formulations has been limited until recently because they have required multiple treatments to achieve prolonged infertility.

Aims. We tested the efficacy and evaluated potential side effects of two contraceptive formulations, a porcine zona pellucida (PZP) formulation, SpayVac® and a gonadotrophin-releasing hormone (GnRH) formulation GonaCon-B™, in a population of free-roaming feral horses (*Equus caballus*). Both formulations were developed to provide several years of infertility with one injection.

Methods. Females were treated in June 2005 with either GonaCon-B ($n = 24$), SpayVac ($n = 20$), adjuvant only ($n = 22$), or received no injection ($n = 18$). Females were monitored for fertility status year round for 3 years after treatment.

Key results. Both contraceptive treatments significantly reduced fertility for 3 years. Fertility rates for GonaCon-B mares were 39%, 42% and 31%, respectively, and 37%, 50% and 44% for SpayVac mares. During the same seasons, 61%, 67% and 76% of control females were fertile. We found no significant effects from contraceptive treatment on the sex ratio of foals, birthing season or foal survival.

Conclusions. These results demonstrated that both vaccines are capable of significantly reducing fertility for several years without boosters.

Implications. Contraceptive vaccines examined in the present study represent a useful tool for the management of feral horses, because of their being efficacious for 3 years in the absence of booster immunisations.

Additional keywords: field study, GnRH vaccine, immunocontraception, population control, PZP vaccine, side effects, wild horses.

Introduction

The overabundance of wildlife is an ongoing problem, with many pest species increasing to numbers that become detrimental to both the environment and other wildlife species (e.g. elephants (*Loxodonta africana*): Whyte *et al*. 1998; white-tailed deer (*Odocoileus virginianus*): Warren 1997; feral horses (*Equus caballus*): Wagner 1983; Canadian geese (*Branta canadensis*): Conover and Chasko 1985). Traditionally, populations of overabundant wildlife were controlled by lethal control or permanent removals. However, mortality manipulation can cause increased population growth as a result of compensatory growth (Garrott and Taylor 1990; Kirkpatrick and Turner 1991), changes to animal movements (Henderson *et al*. 2000) and public objections (Stout *et al*. 1997; Lauber *et al*. 2007). Consequently, non-lethal methods of control, usually involving fertility manipulations, are becoming increasingly advocated when traditional culling methods are impractical or undesirable (Kirkpatrick 2007).

Contraception has become a relatively widely used non-lethal technique to manage overabundant wildlife populations and is usually considered a more humane and politically acceptable long-term approach to population control because animals remain in the wild and most treatments are reversible (Oogjes 1997; Kirkpatrick 2007). In particular, immunocontraception, which uses an animal's own immune system to inhibit fertility, has been tested extensively in many wildlife species to prevent pregnancy, particularly formulations that block oestrous cycling or fertilisation through GnRH immunisation (Miller *et al*. 2000*a*; Baker *et al*. 2002; Killian *et al*. 2006; Conner *et al*. 2007) or treatment with porcine zona pellucida (PZP) (Kirkpatrick *et al*. 1992, 1996; Turner *et al*. 1996, 1997, 2001, 2007; Fayrer-Hosken *et al*. 2000; Miller *et al*. 2000*b*; Delsink *et al*. 2002; Naugle *et al*.

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2002; Walter *et al*. 2002; Rutberg *et al*. 2004; Lane *et al*. 2007). While biologically effective, contraceptive formulations also need to be practical and cost-effective for management, preferably using only a single dose.

In the USA, feral horses are federally managed and protected on public lands under the *Wild Free-Roaming Horse and Burro Act of 1971* and continue to overpopulate rangelands where they can have a negative impact on grasslands, riparian areas, soils and other wildlife species (Rogers 1990; Beever and Brussard 2000, 2004; Levin *et al*. 2002; Zalba and Cozzani 2004; Beever and Herrick 2006). Despite this trend, lethal control is largely unacceptable to the public. Past-management techniques have focused on the 'round-up and adopt-out' method, although there have been concerns about the efficacy of these programs and their impact on animal welfare (Kirkpatrick 2007). Contraceptive formulations have been extensively researched in feral horses (PZP; Kirkpatrick *et al*. 1990, 1996; Turner *et al*. 1997, 2001, 2002, 2007) and show promise for population control that may be more cost effective than removals over the long-term (Bartholow 2007). However, there is a need for a formulation that lasts for more than 1 year without the need for boosters or multiple injections so as to decrease the costs and stress associated with multiple gathers.

Concerns have been raised over the potential side effects of contraceptive treatments (see Nettles 1997), yet there has been little research conducted that addresses side effects in comparison to efficacy studies (Gray and Cameron 2010). Most research on side effects has focused on breeding behaviour and body condition of treated females. For example, several studies have shown that contraceptives alter breeding behaviour of females, which results in a lengthened breeding season and later offspring birth dates (McShea *et al*. 1997; Heilmann *et al*. 1998; Miller *et al*. 2000*b*). However, a study on PZP showed no difference in the month that foals were born to females where contraception was waning (Kirkpatrick and Turner 2003). Several studies have also reported increased body condition of treated females in wild species (McShea *et al*. 1997, Turner and Kirkpatrick 2002). Although increases to body condition can be desirable, they could have an impact on offspring sex ratios because females that experience increased condition tend to have more sons (Cameron 2004). Regardless of treatment, side effects should be studied on managed species to minimise impacts to behaviour, physiology and population health.

We tested efficacy of contraceptive formulations in free-roaming feral horses in the Virginia Range in Nevada. We used two different formulations that were developed to reduce fertility over multiple years following one vaccination. The first formulation, SpayVac®, is a liposome-encapsulated porcine zona pellucida (PZP) that acts to prevent pregnancy by blocking fertilisation by targeting the zona pellucida of the ovum. A single dose can last for several years (Brown *et al*. 1997). This contrasts with other PZP formulations that require multiple doses to achieve multiple years of infertility (Turner *et al*. 2001, 2002), although a vaccination that lasts between 2 and 3 years has been developed recently (Turner *et al*. 2007). SpayVac has been successfully used as a single-dose, multi-year contraceptive in fallow deer (*Dama dama*) (Fraker *et al*. 2002), white-tailed deer (Hernandez *et al*. 2006), grey seals (*Halichoerus grypus*) (Brown *et al*. 1997) and captive feral horses (Killian *et al*. 2008). The second formulation, GonaCon-B™, is a gonadotrophin-releasing hormone (GnRH) vaccine that consists of synthetic GnRH conjugated with a blue mollusk protein. Once injected, antibodies are produced and bind to endogenous GnRH. The antibody–GnRH complex is too large to diffuse from the blood into the anterior pituitary, resulting in a significant reduction in the production of follicle-stimulating hormone (FSH) and luteinising hormone (LH), thereby preventing ovulation (Miller *et al*. 2008). This formulation has been effective in white-tailed deer (Miller *et al*. 2000*a*), bison (*Bison bison*) (Miller *et al*. 2004), feral swine (*Sus scrofa*) (Killian *et al*. 2006; Massei *et al*. 2008) and captive feral horses (Killian *et al*. 2008). Both formulations alone would not elicit a large or long-term response, thus AdjuVac™, an oil-based adjuvant (a non-specific immune stimulant), was used to increase the immune response. Our main objectives were to test two contraceptive formulations in free-roaming horses to determine (1) the effectiveness of these contraceptives to induce infertility up to 3 years after administration of a single dose and (2) potential side effects on foal survival, birth sex ratio and birthing season.

Materials and methods

Study site and animals

The study was conducted in the Virginia Range, south-west of Reno, Nevada, USA. The range is ~145 000 ha of private land, with a small percentage developed for houses and industry. The mountain range consists of sagebrush (*Artemesia tridentata*) communities, with groups of pine (*Pinus jeffreyi*, *Pinus monophylla*) and juniper (*Juniperus osteosperma*) at higher elevations. Other common plant species include bluegrass (*Poa secunda*), rabbitbrush (*Chrysothamnus nauseosus*), bitterbrush (*Purshia tridentata*) and cheatgrass (*Bromus tectorum*). In addition to feral horses, other ungulates in the range included mule deer (*Odocoileus hemionus*) and pronghorn (*Antilocapra americana*). The predators in the range were mountain lions (*Puma concolor*), bobcats (*Lynx rufus*) and coyotes (*Canis latrans*).

The population of feral horses is classified as estray by the Nevada Department of Agriculture because they range on private property and are therefore not protected under the *Wild Free-Roaming Horse and Burro Act of 1971*. Consequently, they are legally defined as feral livestock, and the Nevada Department of Agriculture is responsible for the management of the population. The population size has fluctuated from >1200 in 2004 to ~1400 in 2009, and is currently being managed with the use of both removals and contraception. The long-term management goal is to decrease population size and stabilise population growth.

Vaccine types and treatment protocol

We selected a treatment site for both ease of capture, the large number of horses present in the valley and permission from private land owners. Horses were rounded up with a helicopter on 15 and 16 June 2005. Horses were held in purpose-built yards and restrained in a hydraulic squeeze chute for treatment. We treated every female >1 year of age with 1-mL intramuscular dose in the left side of the neck in the serratus ventralis muscle with one of the following four treatments: (1) GonaCon-B (NWRC, Fort Collins, CO, USA) ($n = 20$), (2) SpayVac (ImmunoVaccine

Technologies (IVT), Halifax, Nova Scotia, Canada) ($n = 20$), (3) control, consisting of adjuvant only (AdjuVac) (NWRC) ($n = 19$) or (4) control, consisting of no injection ($n = 18$), with every other capture aspect being equivalent. Both GonaCon-B (1000 μg of conjugate) and SpayVac (400 μg of conjugate) were prepared as a water-in-oil emulsion in AdjuVac.

All females were aged by tooth eruption and wear patterns (after Tutt 1968), examined for overall health status and freeze-branded for permanent identification on the left side of the rump. In total, 77 females ranging in age from 1 to 20 years were treated systematically over the 2-day period, allowing for random distribution of treatment across all age categories. All horses were immediately released after treatment. In addition to the 77 mares, a small group of females ($n = 7$) were treated with GonaCon-B ($n = 4$) or adjuvant only ($n = 3$) in May of 2005 in a different part of the range. These horses were captured by a feed trap and processed by using the same protocol. They were grouped with the rest of the mares in all of the analyses. All handling and treatment procedures were carried out by and under the supervision of veterinarians from the Nevada Department of Agriculture and were approved by the University of Nevada, Reno Animal Care and Use Committee (Protocol #A06/07-47).

Field observations

We monitored mares from the ground at least weekly to determine fertility rates until December 2008. The treatment group to which each mare belonged was not known to the field observer. Mares were judged fertile if a foal was born at any time during the year or if a female was noticeably pregnant, judged conservatively by an enlarged belly and swollen teats. If females were not seen until after the foaling season and no foal was present, they were recorded as unknown and excluded from the analysis. To examine impacts of contraception on foals, we compared fertile treated mares (contraceptive failures) to control mares. During field observations, we would record the birth of any foal, the sex of the foal and foal survival to weaning.

Faecal sampling and steroid hormone assay

To supplement visual observations, we collected faecal samples opportunistically from August to May yearly to determine pregnancy of mares that may have lost a foal before being visibly pregnant. The behaviour of the horses was monitored throughout the year and when a horse defecated during the observation period, the time and horse identity were recorded. The faecal sample was collected once collection would not disturb the horses. Only samples with 100% certainty of which horse they came from were collected to be as conservative as possible. The number of faecal samples collected from an individual ranged from 1 to 32.

Faecal samples were stored in –20°C freezers until extraction. We used the extraction protocol of Asa *et al*. (2001). Samples were thawed and ~0.5 g of wet faecal material was placed in a vial with 5 mL of modified phosphate–saline buffer (Shideler *et al*. 1993) and shaken overnight at room temperature. Samples were centrifuged for 1 h and the supernatant was stored at −80°C until the assay was conducted. The remaining faecal material was dried in a 100°C oven overnight and weighed the next day. Hormone values are based on the dry weight of the faecal samples and are presented as ng g^{-1} of dried faeces.

Faecal progesterone assays were run at the Saint Louis Zoo and have been used to verify reproductive status of females in many species (Asa *et al*. 1996, 2001; Munson *et al*. 2001). We analysed extracted faecal samples for progesterone metabolites with a commercially available radioimmunoassay kit (Progesterone Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The progesterone antibody in this kit cross-reacted with multiple progesterone metabolites: progesterone (100%), 17α-hydroxyprogesterone (3.4%), 5α-pregnan-3,20-dione (9.0%) and 5β-pregnan-3,20-dione (3.2%). We used stripped calf serum to prepare all standards and to dilute all faecal-sample extracts. Faecal samples were run against a calibration curve of progesterone from stripped calf serum. Extraction buffer was added to tubes containing standards and calf serum was added to faecal extracts to equalise the matrices of the samples and standards. All samples, standards and quality controls were run in duplicate.

The inter-assay coefficients of variation were 19%, 23%, 28.5% for low-, medium- and high-quality control standards for seven different assays. The assays were validated for faecal extracts by demonstrating parallelism between serial dilutions of faecal samples (from 1 : 2 to 1 : 16) to the standard curve. The displacement curves were parallel to the standard curve, with correlation coefficients $r^2 > 0.99$. Recovery of known amounts of hormone added to faecal supernatants (diluted in calf serum) at several different concentrations was 92.3 ± 3%. We biologically verified faecal progesterone samples from 19 mares by comparing their progesterone levels to our observations of their pregnancy and foaling status.

Statistical analysis

We determined the efficacy of contraceptive treatment by comparing the proportion of fertile females in each treatment group with control females across the combined foaling seasons. Females were classified as being either fertile or infertile on the basis of visual monitoring of reproductive status or faecal progesterone levels. If any female was not seen on a regular basis or had no faecal samples collected, they were excluded from the analysis. We used a linear mixed-model analysis with restricted maximum likelihood estimation to determine treatment effects on fertility rates. We used individual identification as a random variable repeated across three foaling seasons. A chi-square test was used to test for differences between foal sex ratios, seasonality of births and foal survival. We defined the foaling season to include April, May and June (Kirkpatrick and Turner 2003). Results are shown as means ± standard errors where appropriate.

Results

Efficacy

At the time of treatment, there were no differences in the age among all treatment groups ($F_{3,79} = 0.48$, $P = 0.694$). There was no difference in fertility rates between control mares and adjuvant only mares across the three years ($F_{1,30} = 0.737$, $P = 0.397$; Table 1), and so they were combined for all further analyses. Treatment with contraception significantly reduced fertility

Table 1. Fertility rates (%) of treated and control females for three foaling seasons

Both treatments significantly ($P < 0.01$) reduced fertility for the combined three foaling seasons. The number of mares observed in each season is given in parentheses

Treatment	Percentage fertile mares		
	2006	2007	2008
Control (no injection)	60 (15)	69 (13)	86 (14)
Adjuvant only	63 (16)	65 (20)	67 (15)
SpayVac®	37 (14)	50 (16)	44 (16)
GonaCon-B™	39 (18)	42 (12)	31 (16)

for all combined foaling seasons ($F_{2,72} = 5.811$, $P = 0.005$). Post hoc comparisons revealed that both contraceptive treatments significantly differed from control mares for all foaling seasons combined (GonaCon-B: $t_{75} = -3.138$, $P = 0.002$, SpayVac: $t_{68} = -2.314$, $P = 0.024$).

We supplemented fertility data with those from four additional mares that were not seen on a regular basis, but had faecal samples tested for progesterone metabolite levels from October through March. We found two females that were probably not pregnant (both <50 ng g^{-1} progesterone) and two females that were probably pregnant (both >1800 ng g^{-1} progesterone) (Asa *et al*. 2001). We confirmed our visual observations of fertility status with faecal progesterone levels in 17 out of 19 mares (control: $n = 10$; treated: $n = 7$) from faecal samples that ranged from 8 to 24 samples per female. Pregnant mares had mean faecal progesterone of 2426 ± 228 ng g^{-1} and non-pregnant females averaged 105 ± 10 ng g^{-1}. The other two mares, neither of which looked visibly pregnant although they had faecal progesterone levels that indicated pregnancy, were one SpayVac-treated mare that lost her foal between March and April 2006 (likely post partum), and one GonaCon-B-treated mare who lost her foetus in October 2005. The cause of these losses is unknown.

Side effects of treatment

During our monitoring efforts, we found no abscesses or inflammation at the injection sites in the mares observed after treatment. There was no difference in the seasonality of births across the 3 years between these treated mares and controls ($\chi^2_1 = 0.630$, $P = 0.427$), with the majority of all foals being born in April (46%) and May (46%) (Fig. 1). There was no treatment effect on foal survival ($\chi^2_3 = 0.002$, $P = 0.969$). There was also no difference in the foal sex ratio between treatments across three foaling seasons ($\chi^2_3 = 0.939$, $P = 0.816$), with 48% and 50% of the foals being male in the treated and control mares, respectively.

Discussion

As with many wildlife species, free-living horse populations continue to present a management problem, and past management methods are increasingly unfeasible or ineffective. Consequently, contraceptive treatments have become more widely tested to reduce herd numbers and population growth, especially with the development of a contraceptive agent that provides multi-year contraception

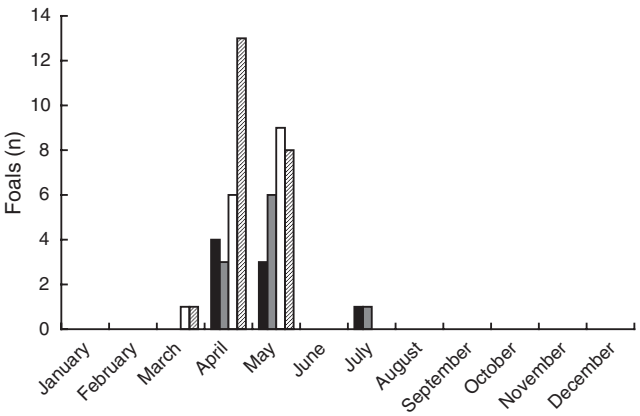


Fig. 1. The number of foals born in each month of the year from control mares (open), adjuvant only mares (striped), SpayVac® mares (grey) and GonaCon-B™ mares (black). Data pooled over three foaling seasons and only from foals with known birth dates.

after a single dose (Turner *et al*. 2007; Killian *et al*. 2008). We show that free-living horse fertility rates were decreased by contraceptive formulations, with the use of single-injection vaccinations. Both GonaCon-B and SpayVac reduced fertility rates in every foaling season, suggesting that both formulations were successful at preventing pregnancy for multiple years. In addition, both treatments consistently reduced fertility within individual females throughout the 3 years. Our results indicated that long-term contraception of free-roaming horses is possible with both GonaCon-B and SpayVac, providing viable alternatives for long-term population control in feral horses. Furthermore, both GonaCon-B and SpayVac have been shown to be effective in several other wildlife species (Brown *et al*. 1997; Miller *et al*. 2000*a*, 2004; Fraker *et al*. 2002; Hernandez *et al*. 2006; Killian *et al*. 2006; Massei *et al*. 2008). Although we report fertility rates for only 3 years after treatment, we expect, on the basis of our captive trials (Killian *et al*. 2008), that treated mares will continue to be effectively contracepted for the following 2 years and we are continuing to monitor these females for the duration of that time.

Although we found a reduction in fertility, our efficacy was lower than in most previous studies of contraception in free-living horses (Kirkpatrick *et al*. 1990; Turner *et al*. 2002, 2007). Although this may have been due to the treatment method, this seems unlikely because all mares received the full dose while in the squeeze chute. It is likely that in the first foaling season (2006) some mares were already pregnant when we treated them in 2005, thus reducing the appearance of efficacy for the first season because they were already pregnant and these formulations are not supposed to interfere with pregnancy. This would not explain our lower efficacy rates in the second and third season. It is possible that our lower efficacy rates are due to our sampling techniques. We monitored our horses more intensively than in other studies by locating horses at least weekly and used highly conservative estimates of pregnancy and foal production because horses may abort foetuses throughout the year or may lose foals shortly after birth (Keiper and Houpt 1984; Lucas *et al*. 1991). If individuals are not closely monitored, these events would be missed and would increase the appearance of contraceptive

efficacy and also fertility in control females. In our study, mares that were only seen late in the foaling season without a foal were excluded from the analysis because it was not possible to determine whether they had lost their foal or had not foaled at all. The inclusion of these mares would significantly boost our efficacy rates. This suggests that constant monitoring of individuals may give a more accurate representation of fertility rates.

Our lower efficacy rates may have resulted from inter-individual differences in responsiveness to the treatment. We noticed that certain mares consistently responded to the treatment, whereas others did not (e.g. they were pregnant or foaled). Variation in response to different contraceptive formulations and adjuvants has been demonstrated (Garrott *et al*. 1998; Fayrer-Hosken *et al*. 2002; Frank *et al*. 2005), although the cause of that variation has not been investigated. Killian *et al*. (2008) showed higher efficacy rates in captive feral horses by using the same treatments on the same species from the same population as we studied. The captive mares had a much better response to the treatment and showed higher levels of fertility reduction for the first 2 years after treatment (e.g. 100% and 83% for SpayVac-treated mares and 94% and 60% for GonaCon-treated mares (Killian *et al*. 2008)). We hypothesise that this may be due to differences in condition between captive and wild mares because captive mares are all in good body condition and have access to better-quality nutrition, which may increase immune functioning compared with animals in poor condition (Houston *et al*. 2007).

Impacts on foals

Another limitation of contraceptive treatment is the potential for side effects (Nettles 1997). We investigated how contraceptive treatment could affect foals born to females in which the contraception failed. Feral-horse populations differ in the timing of their birthing season, although the majority of foals are born in the spring (Feist and McCullough 1975; Keiper and Houpt 1984; Berger 1986). Foal survival is related to the season of birth, with foals being more likely to survive when they are born in the spring than in the winter. Previous studies have shown that PZP treatments can lead to increased oestrous cycling during the non-breeding seasons (McShea *et al*. 1997; Miller *et al*. 2000*b*; Curtis *et al*. 2002). These side effects can result in offspring born out of season leading to reduced survival (e.g. white-tailed deer, McShea *et al*. 1997). Although we found two foals born in July, most foals were born in the spring months of April and May to both treated and control females and this is consistent with other feral-horse populations (Kirkpatrick and Turner 2003). These data suggest that contraceptive treatment does not significantly affect the timing of foal births or foal survival in feral horses.

Contraceptive treatment has been shown to increase the body condition of mares (Turner and Kirkpatrick 2002). These changes to body condition can have an impact on the sex of the foal because females that experience an increase in body condition at the time of conception have more males (Cameron and Linklater 2007). In our study, we found no evidence that the sex ratio of foals changed as a result of a failure of contraceptive treatment. It would be interesting to continue to monitor sex ratios in mares after the contraceptive has ceased to be effective, because mares that have not foaled for several years should be in better body condition (e.g. Turner and Kirkpatrick 2002), which could lead to an increase in male births (e.g. Monard *et al*. 1997; Cameron and Linklater 2000, 2007).

Summary

A current drawback of most contraceptive vaccines is the need for annual inoculations and, therefore, the need for multiple gathers. For many wildlife managers, this issue alone makes contraception an impractical option. Fertility control of pest or invasive species has become increasingly important for management, especially when lethal control is unacceptable or impractical. Our findings demonstrate that new contraceptive formulations are able to reduce fertility in females for several years without the need for boosters. We conclude that these formulations would be useful for managing feral-horse populations, and also other species that require long-term population control because they reduce the costs and stress associated with multiple treatments. We also emphasise the importance of sampling methods when studying efficacy rates in wild populations to get an accurate representation of fertility rates in treated and control individuals.

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REPRODUCTION
REVIEW

Does contraceptive treatment in wildlife result in side effects? A review of quantitative and anecdotal evidence

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Abstract

The efficacy of contraceptive treatments has been extensively tested, and several formulations are effective at reducing fertility in a range of species. However, these formulations should minimally impact the behavior of individuals and populations before a contraceptive is used for population manipulation, but these effects have received less attention. Potential side effects have been identified theoretically and we reviewed published studies that have investigated side effects on behavior and physiology of individuals or population-level effects, which provided mixed results. Physiological side effects were most prevalent. Most studies reported a lack of secondary effects, but were usually based on qualitative data or anecdotes. A meta-analysis on quantitative studies of side effects showed that secondary effects consistently occur across all categories and all contraceptive types. This contrasts with the qualitative studies, suggesting that anecdotal reports are insufficient to investigate secondary impacts of contraceptive treatment. We conclude that more research is needed to address fundamental questions about secondary effects of contraceptive treatment and experiments are fundamental to conclusions. In addition, researchers are missing a vital opportunity to use contraceptives as an experimental tool to test the influence of reproduction, sex and fertility on the behavior of wildlife species.

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Introduction

Fertility control for population management of wildlife and zoo species has some advantages over other control methods, such as lethal control (Kirkpatrick 2007). For example, culling reduces population size, but also increases population growth rates, counteracting the goals of most population control operations (Garrott *et al*. 1991). Furthermore, there are ethical concerns about culling (Littin *et al*. 2004) such that public acceptance of lethal control is typically low when other management options are viable (e.g. Stout *et al*. 1997). However, for contraception to be successful for population control it must not only be safe, effective and long-acting, but also have minimal impact on the social organization, behavior, and ecology of populations with treated individuals (Castle & Dean 1996). Contraceptive technologies are becoming increasingly effective, safe and long-lasting (Kirkpatrick & Turner 2007), but despite several decades of contraceptive treatment, little research has considered secondary impacts, except in domestic animals. Fertility control is used to manage farm, domestic and recreational animals (D'Occhio 1993) and provides a viable and reversible alternative to de-sexing (e.g. cats, Ivanova *et al*. 1995, Gorman *et al*. 2002), but only if the animals behave similarly before and after treatment. In wildlife, the alternative is often lethal control which raises a plethora of other legal and logistical issues (e.g. Kirkpatrick 2007). Nonetheless, the side effects of contraception need to be understood for fertility control to be an effective management tool, even if alternative management practices have more extreme side effects (see Kirkpatrick 2007).

Contraceptive formulations either prevent ovulation, fertilization or implantation, or terminate pregnancy. Immunocontraception uses an animal's own immune system to prevent reproduction. The body produces antibodies in response to the injection of a foreign body, and the antibodies then interfere with critical events in reproduction. Several different types of immunocontraceptive formulations have been developed, including those against ova (Sacco 1987), sperm (Primakoff *et al*. 1988), and gonadotropins (Moudgal *et al*. 1986). The most commonly used formulation is porcine zona pellucida (PZP), which stimulates the production of antibodies to zona pellucida (ZP) when injected into the host (Aitken *et al*. 1984). These antibodies prevent sperm binding to the ZP sperm receptors, thereby preventing fertilization (Sacco *et al*. 1984). Infertility results, but females continue to cycle. Since the structure of these

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receptors is conserved, PZP inhibits fertility in a range of mammalian taxa (Wood *et al*. 1981, Mahi-Brown *et al*. 1985, Sacco 1987, Kirkpatrick *et al*. 1996). GNRH formulations, both agonists and active immunizations, result in a reduction of the release of FSH and LH. Treated females thereby enter a variable length anestrous period (Talwar 1985). These GNRH formulations have been widely used in domesticated livestock as a practical application to improve carcass quality and to reduce aggressive behavior (i.e. Huxsoll *et al*. 1998, Kiyma *et al*. 2000). To a lesser extent they have been used for fertility control in both males and females of wildlife species (e.g. Miller *et al*. 2000*a*). Other common types of contraceptive agents include 1) melengestrol acetate (MGA), a progesterone-like drug that suppresses LH, preventing ovulation, but allowing development of large follicles and endogenous estradiol, and 2) progesterone analogs that inhibit ovulation and/or prevent implantation, both of which have been used successfully in several zoo species (reviewed in Patton *et al*. (2007)). Surgical sterilization is also an option in some species, but presents some practical issues associated with surgery (i.e. catching, anesthesia, irreversibility, morbidity, and mortality) that make it unsuitable for most wildlife species.

The potential side effects of these contraceptive treatments have been considered theoretically, but few have been tested empirically. Nettles (1997) reviewed potential side effects, which included physiological effects, behavioral effects, population impacts and evolutionary impacts, and more recently suggested side effects include evolutionary effects (Magiafoglou *et al*. 2003, Cooper & Larsen 2006) and impacts on disease transmission rates (Tuyttens & MacDonald 1998, Caley & Ramsey 2001, Miller *et al*. 2004). The diversity of contraceptive agents means that there is potential for a variety of secondary impacts. Here we review the literature to determine: 1) potential impacts on individuals and populations, 2) which impacts have been documented, and 3) which impacts have been empirically tested. Finally, we consider the implications of the side effects of contraception for addressing fundamental theoretical questions, particularly in behavioral ecology.

Results

Trends in publications

The reviewed studies emphasized pest animals and agriculturally important species. Several orders have been investigated, but studies concentrated on Artiodactyla (particularly deer and domestic cows), Perissodactyla (mainly horses), and Carnivora (particularly felids) (Supplementary Table 1, see section on supplementary data given at the end of this article). There was an overall increase in published papers from 1975 to 2002 (Fig. 1). These publication trends indicate

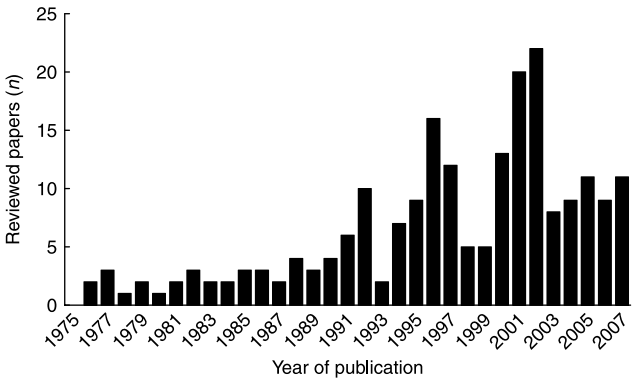


Figure 1 Publication trends of papers ($n=212$) that investigated or reported on secondary effects of contraceptive agents from 1975 to 2007.

increasing interest in side effects with a peak in 2002 when a special issue of *Reproduction Supplement* (vol 60) was published. After this supplement, the rate of publication returned to a consistent pre-2001 level.

Most studies investigated the impacts of female contraceptives (Table 1), which is consistent with efficacy studies in wildlife which usually target females. Physiological responses were addressed in a high percentage of the papers (85%) for both males and females. While physiological impacts are important, behavioral and population-level effects may be more relevant to the management of wild populations. Approximately 35% of the papers addressed individual behavior and 11% addressed population-level effects. Roughly 1/3 of the papers (30%) addressed more than one category. Only one paper investigated population-level effects in males, most likely because females are treated more often than males in wildlife populations (Table 1). Most of the research in the reviewed papers was done in captivity (84%) and the majority of studies that investigated changes in behavior of contracepted individuals were conducted in captivity (76%).

In this review, sample sizes, when reported, ranged from 1 to 353 for treated individuals and 0–230 for control individuals, with an average sample size of 31.7 ± 49.0 (median 17) treated individuals and 18.2 ± 30.0 (median 9) for controls, indicating high variability among studies. Most studies were of relatively

Table 1 A comparison of the 212 reviewed papers (%) based on sex and category that were included in the review. Total papers refers to the combined set of papers only based on sex, while the rest of the categories are specific to each sex.

	Total papers	Behavior	Physiology	Population
Female ($n=145$)	68	30	83	14
Male ($n=50$)	24	46	90	2
Both ($n=17$)	8	41	88	12

short duration, with an average duration of 1.8±2.4 years; although data collection was often conducted in short field periods of a few days or weeks over the course of the entire study, probably resulting in an overestimate of average study length.

Documented secondary effects

Several secondary effects are theoretically possible, and these are summarized in Table 2. Only a few hypothesized impacts have not been investigated, with most being studied at least once. However, few studies produced consistent results, even among studies using the same formulations. The main impacts are summarized below.

Physiology

How does contraception impact internal organs?

The impact of contraception on internal organs has received the most attention, with a total of 134 studies published. Changes in ovarian structure and function were found in most studies (82%, Table 2), which is not surprising since contraceptive formulations act directly on reproductive functioning, and so changes in reproductive organ histology and function are more likely than other impacts. Most changes that occurred to the reproductive tract were changes to follicle number, weight and size with the use of GNRH formulations (McNeilly & Fraser 1987, D'Occhio & Kinder 1995, Gong *et al.* 1995, Maclellan *et al.* 1997) and PZP

Table 2 Theoretical side effects of contraceptives and the number of studies that investigated these side effects. Both experimental and anecdotal papers are included in this table.

Side-effect	Theoretical	*Effect (*n*)	No effect (*n*)
Physiological			
Harmful effects on pregnant animals	Nettles (1997)		
Stillbirth		8	1
Abnormal offspring		2	1
Inhibition of parturition or dystocia	Nettles (1997)	2	0
Changes in ovarian structure or function	Nettles (1997)		
Associated with contraceptive mechanism		17	11
Associated with pathological changes		34	
Changes in lactation or mammary glands	Nettles (1997)	1	4
Changes in secondary sex characteristics	Seal (1991) and Nettles (1997)	0	0
Changes in bodyweight or condition	Nettles (1997)		
Increased in treated females		9	26
Decreased in males		1	0
Toxicity	Nettles (1997)	0	0
Interference with diagnostic tests	Nettles (1997)	1	0
Abscesses or inflammatory reactions	Nettles (1997)	14	8
Individual behavior			
Changes in behavior	Nettles (1997)		
Sexual activity		9	5
Increased movement		2	1
Activity patterns		2	8
Increased irritability or aggression		3	0
Social disruption, change in status, territory loss		5	5
Population-level			
Changes in sex ratio	Nettles (1997)		
Due to immigration		1	0
Offspring sex ratios		0	0
Changes in annual breeding season	Nettles (1997)	7	2
Ecological alterations	Nettles (1997)		
Home range or territory size		3	4
Increased immigration or emigration		0	1
Change in habitat use		1	1
Increased longevity or survival		6	2
Increased survival of offspring		3	2
Impact on fertility of young	Nettles (1997)	0	1
Improved breeding of non-treated females		1	0
Disease transmission rates	Tuyttens & MacDonald (1998), Caley & Ramsey (2001) and Miller *et al.* (2004)	1	2
Evolutionary level			
Genetic resistance	Magiafoglou *et al.* (2003)	0	0
Genetic changes influencing disease resistance or immune function	Nettles (1997) and Cooper & Larsen (2006)	0	0

*References are in Supplementary Table 2, see section on supplementary data given at the end of this article.

treatment (Dunbar *et al*. 1989, Kirkpatrick *et al*. 1992, Kitchener *et al*. 2002, Curtis *et al*. 2007). However, more serious side effects have been reported including increased inflammation of the uterus by intrauterine devices (Daels & Hughes 1995) and alterations to ovarian function, oophoritis and cyst formation with PZP treatment (Mahi-Brown *et al*. 1988, Sehgal *et al*. 1989, Rhim *et al*. 1992, Stoops *et al*. 2006, Curtis *et al*. 2007). In addition, MGA implants can increase mineralization and cystic hyperplasia in felids (Munson *et al*. 2002) and cause endometrial hyperplasia (Sokolowski & VanRavenswaay 1976). Consequently, changes to the ovaries or female reproductive tract were consistently found for all contraceptive treatments.

In domesticated livestock, GNRH immunization has been used to improve carcass characteristics by increasing lean meat (Huxsoll *et al*. 1998), feeding efficiency (Cook *et al*. 2001) and live weight (Bonneau *et al*. 1994, Zeng *et al*. 2002), and treatment usually resulted in positive effects on carcass traits, or no effect. Milk production did not seem to be influenced by contraception in the few studies in which it has been investigated (GNRH agonist: Mattos *et al*. 2001; progesterone: Nave *et al*. 2000), although there was some evidence of mammary hyperplasia in cats treated with progesterone (progestin: Loretti *et al*. 2005).

In addition to effects on internal organs, several studies (64%, Table 2) found inflammation or abscesses at the injection site. The majority of these papers examined inflammations due to injectable contraceptive formulations ($n=18$) and most found some inflammation at the injection site (67%). Most of these papers ($n=14$) investigated the impacts of PZP treatment and the majority found injection site reactions (71%). However, the injection site reactions were often associated with the type of adjuvant (e.g. Freund's Complete Adjuvant) used in association with contraceptive treatment, and these effects have been reduced with the use of modified adjuvants (Deigert *et al*. 2003). These injection site reactions usually healed quickly with no long-term health effects. Overall, most of the treatments showed little to no impacts on internal organs other than the ovaries (or testes) and some of the effects were considered positive.

Does contraception alter body condition?

Without the costs of lactation and gestation, females should hypothetically have improved condition and health, possibly leading to increased longevity and lifespan (Williams 1966). These impacts could extend beyond treated females. For example, untreated males may lose condition in the presence of contracepted females due to prolonged breeding activities (Ji *et al*. 2000). Some studies have confirmed that treated females are in better condition than untreated conspecifics (26% of reviewed studies), but the majority found no difference, or that differences did not last (Table 2). For example, PZP treatment increased female condition in both feral horses and white-tailed deer in some studies (McShea *et al*. 1997, Turner & Kirkpatrick 2002), but not in others (Walter *et al*. 2003). Generally, females treated with GNRH show no changes in weight or condition (Miller *et al*. 1997, D'Occhio *et al*. 2002, Herbert *et al*. 2005, 2006, Rubion *et al*. 2006). Finally, both longevity and offspring survival could theoretically be influenced by contraceptive treatment because untreated females may have more successful reproduction due to reduced competition for resources. The impacts of these factors on population dynamics can counteract the desired impact of the contraceptive treatment in reducing population size (see Williams *et al*. 2007) and such population-level effects are discussed later.

Are some contraceptive agents reversible?

For contraceptive treatment to be an effective management tool, it usually needs to be reversible (Kirkpatrick & Turner 1991). A long term study of feral horses showed that PZP was reversible even when females were treated for several years (Kirkpatrick & Turner 2002). However some females appeared not to return to full fertility after long-term PZP treatment and similar side effects were seen with GNRH treatments in deer (e.g. Miller *et al*. 2000*a*). Consequently, most wildlife contraceptives are reversible, or have minimal impact after prolonged use.

Individual behavior

Do activity patterns change in treated individuals?

Most studies suggest that activity patterns were unchanged after contraceptive treatment (80%, Table 2). With PZP treatment, no changes were detected in activity budgets of elk, horses, elephants, and white-tailed deer females (McShea *et al*. 1997, Heilmann *et al*. 1998, Powell 1999, Delsink *et al*. 2002), although the increased number of estrous cycles resulted in PZP treated white-tailed does being more active than control females (McShea *et al*. 1997). GNRH did not change activity budgets in treated kangaroo females (Woodward *et al*. 2006), nor did progesterone implants in kangaroos (Poiani *et al*. 2002), or fruit bats (Hayes *et al*. 1996). However, MGA treatment caused baboons to decrease their time foraging (Portugal & Asa 1995). Overall, few studies reported changes in activity patterns after contraceptive treatment.

Does rank and dominance change in treated females?

Rank and dominance are important in social mammals (Ellis 1995). By changing the levels of hormones or cycling patterns, females could become more or less

aggressive, which could have implications for behavior, social structure and welfare. Such side effects have been used by captive managers (e.g. GNRH to control aggressive behavior, Patton *et al*. 2001), but the few studies on wildlife have produced mixed results (50% showed changes Table 2). In captive felids, females treated with PZP had increased levels of irritability, masculine behavior, and aggression (Harrenstien *et al*. 2004), and in white-tailed deer, treated females associated less frequently with conspecifics (Bertrand *et al*. 1996). MGA treatment in tamarins caused a decrease in affiliative and sexual interactions, with a subsequent increase in aggression towards other females (De Vleeschouwer *et al*. 2000, 2003). Poiani *et al*. (2002) found that progesterone treatment altered the amount of time males spent with females, as males preferred to be in groups with untreated females. In elephants, the matriarch remained in place after treatment with PZP (Delsink *et al*. 2002), but changes occurred with hormonal treatments (unpublished study described in Fayrer-Hosken *et al*. (2000)). PZP treatment did not change the spatial relationship between females and males in horse harem bands (Powell 1999), although all females had been treated with PZP at some point, confounding the conclusions. Finally, surgical sterilization had little impact on coyote pair bond maintenance or territorial behavior (Bromley & Gese 2001), and no changes in social hierarchy were observed in brushtail possums with GNRH treatment (Jolly *et al*. 1996).

Unfortunately, many of the studies reporting no change in social structure have either used opportunistic observations of social groups, rather than quantitative techniques, or have been of short duration. Consequently, the results are inconclusive, and studies that specifically and intensively investigate behavioral changes at a fine level over a long time period are urgently needed.

Are home ranges and movement patterns altered?

If sexual behavior is altered in treated females, movement patterns could also change. PZP treatment resulted in no difference in space use or home range size in elephants and white-tailed deer (Delsink *et al*. 2002, Hernandez *et al*. 2006), similar to surgically sterilized foxes (Saunders *et al*. 2002). In contrast, surgically sterilized female rats had larger ranges than both control and hormonally sterilized females (Jacob & Matulessy 2004). In addition, hormonally sterilized female rats abandoned burrows more often, suggesting a change in dominance or social status, although overall habitat use was not changed (Jacob & Matulessy 2004). Male home range size may also be impacted, as found in brushtail possums (Ramsey 2007). Sterilization resulted in no difference in territory size, overlap or territorial behavior between controls and treated coyote packs (Bromley & Gese 2001). Consequently, most studies suggest that contraception had minimal impact on home range size and movement patterns, but there was some evidence that male kangaroos alter their movement patterns to be in groups of untreated females, or with treated females during the nonbreeding season (Poiani *et al*. 2002).

Do sexual interactions and breeding behavior change?

Changing the fertility of individual females could impact sexual interactions, especially in PZP-treated individuals because the number of estrous cycles is increased (i.e. McShea *et al*. 1997, Heilmann *et al*. 1998, Miller *et al*. 2000*b*, 2000*c*, Curtis *et al*. 2002). There was some evidence that breeding behavior changed in treated females (Table 2), and this varied with contraceptive type. Does treated with GNRH agonists had fewer estrus events (Miller *et al*. 2000*a*), but female wapiti showed no difference in copulatory behaviors (Baker *et al*. 2004). Treatment with progesterone analogs showed similar results to GNRH, but more males were seen in groups of control females (Poiani *et al*. 2002). Ji *et al*. (2000) suggested that males were attracted to areas with sterilized females, potentially increasing male mortality. In contrast, with PZP treatment, females remained sexually active after the end of the breeding season and showed an increase in estrus events (McShea *et al*. 1997, Heilmann *et al*. 1998, Miller *et al*. 2000*b*, 2000*c*). Although changes in breeding behavior appeared to be related to contraceptive formulations, 64% of the studies found changes in estrus and breeding behavior (Table 2), suggesting that these changes should be expected.

Does contraception impact the overall welfare of individuals?

Animal welfare refers to the systematic concern for the well-being of animals. The assessment of welfare involves the examination of both emotional and physical states (Fraser 1995). The reviewed impacts on physiology and behavior suggest that contraception had little impact on overall welfare of individuals, particularly compared to the alternative options (see Kirkpatrick (2007)). While most studies found some changes in physiology or behavior, these changes may not impact the individual enough to affect their well-being, as most studies found no changes to activity, movements or rank of treated females. However, these conclusions are based largely on qualitative and anecdotal studies and there were no studies that specifically set out to address welfare of individuals. While welfare can be difficult to measure, there are many ways to objectively evaluate welfare (Dawkins 2001, Scott *et al*. 2003). For example, measuring stress levels in both treated and control individuals could give an assessment of overall well-being, yet none of the reviewed studies took a systematic approach to address this issue. In addition, some studies

indicated that changes could occur to non-treated animals in the population (e.g. males), which needs to be considered in future research. Furthermore, since most of these studies were conducted in captivity, the applicability to free-ranging populations may be limited since conditions in captivity do not necessarily mimic natural conditions. Given all of these issues, the assessment of welfare implications of contraceptive treatment is a research priority.

Population-level effects

Do survival, abundance, and age class distribution change in treated populations?

Contraception reduces the costs of reproduction, and could therefore result in increased body condition and longer lifespan, thereby altering population dynamics. Furthermore, the offspring of untreated females may have less competition for resources, enhancing their survival and working against the goals of the management operation. Few studies had the longitudinal data required to investigate increased survival, but 75% found an increase in longevity or survival in individuals. For example, PZP treated feral horse mares lived longer, resulting in a new age class (>25 years) not present before treatment (Turner & Kirkpatrick 2002). Therefore, while contraception may limit offspring production, these benefits may be offset by decreased mortality in older individuals, negating the population level effects of contraceptive treatment. In addition, both adults and juveniles had increased survival in surgically sterilized rabbits (Williams *et al*. 2007), which meant that the majority of females must be sterilized to reduce population size (Williams *et al*. 2007). Furthermore, non-treated females showed improved breeding success (Chambers *et al*. 1999), which also counteracted the reduction of population growth rates by contraception. Finally, while the sex ratio of offspring could change due to increased maternal condition (e.g. Cameron 2004), this has yet to be investigated, although Ji *et al*. (2000) recorded a change in population sex ratios in brushtail possums, probably as a result of male dispersal. Overall, contraceptive treatment appears to increase longevity in treated females and, in some cases, non-treated individuals.

Does contraception decrease disease transmission?

If contraception decreases sexual interactions, there may be a decrease in overall social interactions which could decrease the level of disease transmission. Diseases could also be decreased in a treated population simply from lower densities of animals and healthier conditions (see body condition section). This decrease would be advantageous in certain pest species, such as the brushtail possum, which have a high population size, and are carriers of economically or ecologically important diseases, such as bovine tuberculosis (Caley & Ramsey 2001, Ramsey 2007). In addition, a potential decrease of brucellosis transmission is predicted in bison with GNRH treatment (Miller *et al*. 2004), as anestrous females would be unable to spread the disease due to lack of offspring production. Both of these circumstances would result in a positive side effect of contraceptive treatment. Alternatively, if sexual interactions increase, such as in the case of PZP treatment, there could be a potential increase in the transmission of certain diseases. While there has been little research that addresses these effects, most studies concur that disease transmission rates remain unchanged with contraceptive treatment (Kerr *et al*. 1998, Williams *et al*. 2007). However, the potential to reduce disease transmission exists (Ramsey 2007) and studies on rabbits showed a decrease in flea abundance (Twigg *et al*. 2000, Williams *et al*. 2007); possibly indicating a decrease in overall interactions between individuals. More research is clearly required.

Is the mating season extended?

Mating behavior can demand high levels of energy for both sexes. In species with a discrete breeding season, the lengthening of the season could impact the survival and welfare of treated females and nontreated males. Most studies showed a change in the annual breeding season where it was investigated (78%, Table 2). Treatment with PZP increased breeding behavior, caused later fawning dates, and increased sexual activity outside the normal breeding season in several studies (McShea *et al*. 1997, Heilmann *et al*. 1998, Miller *et al*. 2000*b*). However, no differences were reported in populations with females treated with GNRH (Miller *et al*. 2000*a*, Baker *et al*. 2002, 2004, Woodward *et al*. 2006, Conner *et al*. 2007), or progesterone (Poiani *et al*. 2002). Differences in the actions of these formulations and their contrasting effects on cycling probably account for the differences in the impacts on the breeding season. Consequently, the type of contraceptive treatment should be considered for species with a discrete breeding and birthing season. These side effects could have animal welfare implications as these changes could lead to a reduction in the survival of treated females, untreated males and offspring.

Meta-analysis

Of the 212 reviewed papers, 54 (25%) experimentally tested the secondary effects of contraception and reported their results in sufficient detail to be included in the meta-analysis. Studies were classified as experimental if the authors described an experiment with controls, and used statistical analysis of their results. We did not attempt to judge the robustness of their experimental design since we could not always judge

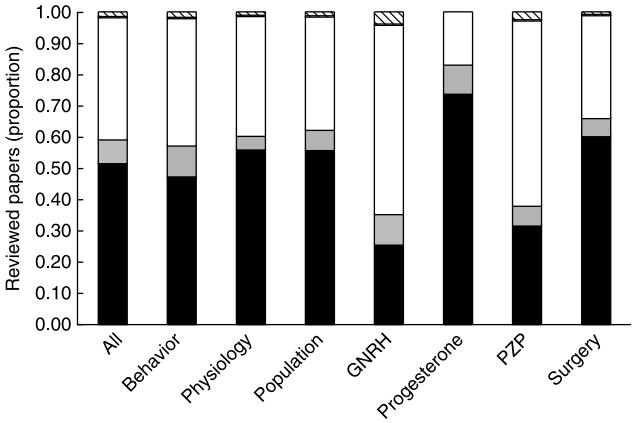


Figure 2 Wildlife studies that empirically tested ($n=54$) if contraceptive treatment produced side effects that either showed significant support (■) or non-significant trend to support (▣), or no significant variation (□), or significant support in the opposite direction to that predicted (▧).

from methodological descriptions whether these represented true experiments with randomized subjects and appropriate controls. Although the quality of experiments may vary, they provide results suitable for meta-analysis, and enable the detection of general trends. From our qualitative review, most papers concluded no significant secondary effects were found. However, the quantitative studies produced contrasting results. From the 339 test statistics from the 54 papers, 52% found side effects from contraception and an additional 8% showed a non-significant trend suggesting side effects. The support varied between contraceptive types and between secondary effect types, with progesterone treatments and physiological studies reporting the most side effects (Fig. 2). Despite this variation, all meta-analyses yielded highly significant z values for the difference between treated and control groups and confirmed that side effects consistently occurred where investigated regardless of study category or treatment type (Table 3). The magnitude of the overall effect size was medium, as r ranged from 0.17 to 0.39. Furthermore, there was no significant heterogeneity of effect sizes within analyses, suggesting that studies were measuring the same thing and coming to the conclusion that side effects occur when investigated. Therefore, contraceptive treatment resulted in changes to the physiology and behavior of individuals, and impacted populations with treated individuals.

An interesting contrast thereby arises in which quantitative studies showed that side effects were consistently found, whereas qualitative studies and anecdotal reports suggest that side effects were generally absent. While a publication bias is possible, most studies were predominantly publishing results on efficacy, such that data on side effects would not influence publication potential. Consequently, more studies specifically investigating secondary effects are required to quantify the impacts of contraceptive treatment on physiology, behavior and population dynamics.

Discussion

Future directions

While there have been an increasing number of studies on side effects, much is still unknown. Unfortunately, many of the reviewed studies were not designed to investigate the side effects and many conclusions were based on small sample sizes, anecdotal reports, or lacked true controls. Conclusions based on data from studies designed to investigate efficacy may not be methodologically suitable for testing for behavioral or ecological effects. Many studies were designed to test efficacy, and the side effects reported were based only on short-term observations. Many studies on efficacy concluded 'no change' for side effects based on anecdotal observations, but these studies did not directly investigate the physiology, behavior or ecology during the study, limiting the conclusions. This contrasts with the meta-analysis further questioning the validity of qualitative studies reporting 'no change'. While the meta-analysis does not account for the severity of the effect, all of the major contraceptive treatments resulted in side effects (Table 3). Thus, these qualitative studies represent an interesting foundation, but future research

Table 3 Meta-analysis of reviewed papers on wildlife species when contraceptive treatment was investigated for secondary effects ($n=54$). All data are generated from Meta 5.3 including effect size (difference between treated and control groups among all studies), $Z_{weighted}$ (the significance of the effect size), heterogeneity (difference of measurements among studies) and the P value of the heterogeneity.

Measure	$n_{studies}$	$n_{statistics}$	$n_{datapoints}$	Effect size (r)	$Z_{weighted}$	Heterogeneity (x^2)	P value
All studies	54	339	9471	0.1993	19.4002[a]	338.1099	0.4881
Physiology	29	91	2679	0.2126	11.1917[a]	102.8470	0.1673
Behavior	27	171	3872	0.2380	14.8088[a]	156.9311	0.7553
Population	15	77	2920	0.3041	8.9712[a]	75.6087	0.4911
PZP	16	79	3842	0.1748	10.8354[a]	76.9119	0.5136
GNRH	8	51	1149	0.2421	8.2056[a]	35.5526	0.9388
Progesterone	16	96	2655	0.1809	9.3193[a]	81.8345	0.8300
Surgery	13	121	2055	0.3884	17.6090[a]	126.4755	0.3251

[a]All z values were significant; $P<0.0001$.

requires more detailed behavioral and ecological protocols, larger sample sizes, and longer study durations.

Evolutionary level

No evolutionary level questions have been investigated, but are most relevant for future impacts (Table 2). Two main impacts of immunocontraception have been proposed; 1) genetic resistance could evolve, similar to the response seen to other biological control agents (Magiafoglou *et al*. 2003), and 2) immunocontraceptives could be selecting for immuno-compromised individuals since those treated females that do breed have a lower immune response to the contraceptive (Nettles 1997, Cooper & Herbert 2001). These aspects require long-term research which has not been possible to date. Nonetheless, given the potential implications of either of these changes, research is urgently required in this field.

Conclusions

While physiological effects have been well-documented, there is less consistency in studies investigating behavioral or ecological side effects, compounded by the tendency for studies to be designed to investigate efficacy specifically and side effects only opportunistically. Such studies often lack appropriate controls, sampling methodology or time scales. Those studies that have specifically investigated side effects consistently find them. While contraception is currently supported as a humane treatment by many managers, researchers and advocacy groups, research in this field has not extended much beyond the scope of management concerns.

Both physiological and behavioral effects can impact population dynamics. The goal of contraceptive treatment is to manipulate population dynamics, and consequently it is imperative to understand population-level effects, some of which compromise the effectiveness of contraceptive treatment (such as through increased breeding in untreated females or increased survival of offspring), while others could enhance effectiveness (such as a male biased sex ratio). To be an effective tool, we need to understand these impacts. Furthermore, most research examined short term effects and research is needed that addresses questions beyond one estrous cycle or breeding season. For example, no study investigated how genetic diversity or immunity could change over time. In addition, most studies, including behavioral studies, were done in captivity, which often limits the application to wildlife species, further suggesting the need for long term experimental studies on wildlife populations.

Finally, contraception provides a powerful tool to address fundamental questions in biology but such applications have not been realized to date. By manipulating fertility we can address questions regarding, for example, the costs of reproduction, importance of copulation in maintaining social structure and parental investment strategies. This would enable us to simultaneously address management questions, questions about contraceptive actions and secondary effects, and questions in basic biology, thereby maximizing the utility of contraception research.

Materials and Methods

We searched ISI Web of Science (1975–2007) for 'immunocontraception' and 'contraception'. Additional searches were made to narrow the papers to those with the terms 'side effects' and 'secondary effects', and we further searched the literature from the cited section of each paper to find more studies that were not identified in the initial search. Only papers that included applications to mammalian wildlife, zoo, or livestock species were used in the review, and humans were excluded. Papers that only researched efficacy or impacts of contraception on fertility directly were not included.

Initially we reviewed the theoretically suggested side effects. We then compiled all the studies that had addressed secondary effects either directly or during the course of a study on efficacy. We defined a side effect as an unintended or unwanted secondary effect and use the terms side effect and secondary effect interchangeably. Both of these terms refer to unintended side effects, but could be positive, neutral or negative. We considered changes to ovarian structure as side effects as both changes induced by pathology or association with the mechanism of contraceptive action. Pathological changes would include cyst formation, hyperplasia, abnormal tissue formation, as well as changes that occurred from a contraceptive treatment where the intended purpose was not associated with ovarian function (e.g. block fertilization). This resulted in the classification of most papers that researched GNRH formulations as changes associated with the mechanism of contraceptive action since GNRH formulations directly act on hormones that regulate cycling and oocyte development. This definition also resulted in papers that investigated PZP being classified under pathological changes. In some of these cases the side effects are absolutely expected, but nonetheless they are secondary to the main contraceptive action and therefore considered side effects.

A total of 212 papers were found that investigated secondary effects or reported side effects noticed during research on efficacy (the full list is presented as Supplementary Appendix 1, see section on supplementary data given at end of this article). The papers fell into three categories; 1) physiological effects, 2) behavioral effects on the individuals, and 3) population-level effects, which we use hereafter. From those papers, we considered sex, species, sample sizes, length of study, type of effect investigated and if that effect had an impact on the animals. We then compared the theoretical effects with the empirical evidence to determine which effects seemed to be supported, or not supported, and in which areas more research is required.

Meta-analysis

From the reviewed papers, we analyzed studies that empirically tested if side effects were found in treated wildlife populations through a meta-analysis in order to estimate the magnitude of the experimental effect (effect size). We only included wildlife populations and excluded wildlife in zoos if the contraceptive treatment was used intentionally to change behavior (i.e. decreasing aggression or sexual activity), so as not to skew the analysis towards side effects. In addition, in an effort to be conservative, livestock papers were excluded since 87% of these studies reported side effects and most of these papers used the contraception to make a change. Every meta-analysis suffers from the possibility of publication bias; therefore every attempt was made to remove publishing bias by the previously described methods. In addition, our search results included papers that primarily investigated efficacy but reported on side effects in order to reduce this bias.

For the meta-analysis we used the *P* values and sample size reported in each study in order to obtain a combined effect size (*r*). Effect sizes were weighted for sample size and indicate the strength of association across the selected studies. The corresponding $Z_{weighted}$ value provides the significance of the difference between treated (contracepted) and control groups. In addition, we calculated a χ^2 statistic to test for heterogeneity of results across the selected studies. A random effect model was used in order to account for differences in both effect size and sampling error (Gurevitch & Hedges 1999). A total of 54 papers were included that empirically tested secondary effects from contraceptive treatment. From those papers, all measures were treated as separate statistics. If there was not sufficient evidence to determine *P* values and sample sizes, the papers (or statistics) were excluded from the analysis. An overall analysis was done, in which all experimental studies were included, regardless of treatment type and study classification. We then conducted separate analyses to test for differences within each side effect category (physiology, behavior and population-level effects) and contraceptive type (GNRH, progesterone, PZP, and surgery). The meta-analysis was conducted using META, v 5.3 (Schwarzer 1989).

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-08-0456.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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A dynamic simulation model for evaluating effects of removal and contraception on genetic variation and demography of Pryor Mountain wild horses

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Abstract

I used an individual-based model to simulate population dynamics and changes in genetic variation of horses in the Pryor Mountain Wild Horse Range under a range of management strategies. Management strategies included population objectives of 90 to 180 horses and removal and/or contraception of animals of random, young, or old ages. Populations controlled only by removals fluctuated widely in size and generally exceeded objectives by >20%. Populations controlled by contraceptives remained within 3% of objective. Over a 200-year period, populations were very likely to lose alleles with an initial frequency of <3%, which included nearly 30% of alleles in the population. Allelic diversity was lost faster than population-level heterozygosity. Removal or contraceptive treatment of older animals hastened the loss of genetic variation compared to treatment of random-aged or young animals. Rate of loss of genetic variation was directly related to generation interval, and differences in loss of genetic variation were greater between treatments of old and young animals than between populations that differed in size by a factor of 2. These results apply to management of many long-lived species in harvested populations and they illustrate the importance of actions that modify age structure and generation interval. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Contraception; Genetic variation; Infertility; Population control; Simulation model

1. Introduction

About 150 horses inhabited the Pryor Mountain Wild Horse Range (PMWHR) in 1998, close to the estimated range carrying capacity (BLM, 1997). The small size of this population concerned those mandated to sustain a viable and healthy population, because genetic diversity rapidly disappears in small populations (Wright, 1931). The Pryor horse population has the reproductive potential to double in size every 4–5 years and attain a size that poses risks to habitats and to populations of mountain sheep and deer that also inhabit the PMWHR (Garrott and Taylor, 1990). Management of the Pryor horse population is illustrative of the general predicament faced by agencies with a mandate to control an isolated population characterized by high reproductive potential, long life span, and limited area of available habitat. In these situations, management plans must balance measures to minimize risk of long-term habitat degradation while maximizing long-term retention of genetic variation and the likelihood of population persistence. There are thus conflicting needs to minimize population size to control habitat degradation while maximizing population size to conserve genetic resources.

Feral horses in the United States are protected by the Wild Free-roaming Horse and Burro Act of 1971 and management of most feral horse populations falls under the jurisdiction of the Bureau of Land Management (BLM, 1997). Wild horse populations in the western United States have exhibited growth rates that frequently exceed 15% per year (Garrott et al., 1991a) and they can rapidly achieve population sizes that raise concerns about long-term habitat damage. A total of about 35,000 horses inhabit ranges on public lands in the western United States, but only 25% of the 186 wild

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horse populations under active management have a population objective of greater than 150 horses (BLM, 1997). The small size of these populations raised concerns about long-term maintenance of genetic variation and emphasized the need to identify methods to control population size while retaining genetic variation.

Wild horse populations have been controlled primarily through periodic gathers and removals. Programs were developed to place wild horses in private ownership, but this has proven to be exceedingly expensive (> $16 million in 1995; BLM, 1997) and it has become impossible to place all horses acquired by removal programs. As a result, there is great interest in the potential use of contraceptives to reduce fertility of horses and other overabundant species (Sturtevant, 1970; Hoffman and Wright, 1990; Garrott et al., 1991b; Holden, 1992; Eagle et al., 1992; Fayrer-Hosken et al., 1997). Use of contraceptives could provide opportunities for a variety of new management scenarios, including permanent infertility, delayed reproduction, or permitting mares to reproduce once and then preventing further breeding. Simulations of horses population dynamics have shown that a relatively high level of infertility would likely be needed to maintain a low growth rate in horse populations (Garrott, 1991), but no analyses have specifically considered the consequences of alternative management actions on long-term persistence of genetic variation in horses or similar mammals.

Management strategies to control growth rates of abundant populations by removal or contraception must focus on three decisions: How many animals should there be? What sex and age of animals are to be removed or made infertile? How often are animals removed or treated? The model described below was developed to address these questions by simulating removals only, contraceptives only, or a combination of removal and contraception. The model was specifically applied to the Pryor wild horse population to evaluate a set of realistic management alternatives. Results from the simulations lend insight to the consequences of management actions used or considered for control of overabundant species including bison, deer, elk, elephants, mountain goats, and some carnivores (Hoffman and Wright, 1990; Bradley, 1994; Fayrer-Hosken et al., 1997; Kirkpatrick et al., 1997). To preserve genetic variation, guidelines for managers almost always emphasize actions that increase overall population size or reduce reproductive variance. I offer analyses that clearly demonstrate the importance of age-related management actions and show that actions that increase (or decrease) generation time of long-lived species can have an effect equal to doubling population size. These results are important because it is frequently much easier to modify age structure than increase population size.

2. Method

I developed an individual-based population model to simulate the dynamics of the PMWHR horse population. The model operated on a yearly time step and it simulated the processes of breeding, recruitment, removal, contraception, natural mortality, and aging. Each individual in the population was explicitly represented and the sex, age, genetic constitution, number of mates, and reproductive status of all animals was followed from birth to death. Model outputs included sex and age of each individual in the population, number of mates (by age) of males and females, and the frequency of all alleles.

2.1. Model processes and parameter estimates

Wild horses on the PMWHR have high rates of survival and reproduction (Garrott and Taylor, 1990; Singer et al., 1998), consistent with observations of wild horse populations throughout the western United States (Berger, 1986; Garrott et al., 1991a). Estimates of fecundity and survival were derived from observations of the Pryor population, emphasizing data subsequent to management changes that occurred about 1990 (Garrott and Taylor, 1990; Singer et al., 1998). Horses in the PMWHR have been intensively studied since the 1980s and about 95% of all horses are individually known and repeatedly observed each year. Age-specific survival rates are thus known with a high degree of confidence up to about 15 years of age. Relatively few horses have lived longer than 15 years and these older horses make up a small proportion of the population. No density dependent regulation of survival or reproduction was included in the model because the horse population has been maintained at a size well below estimates of range carrying capacity and evidence for density dependence is lacking. Recent analyses found a significant correlation between growth rate and population size, but neither recruitment nor survival varied significantly with population size ($P > 0.10$; Singer et al., 1998).

Breeding was simulated by comparing a uniform random deviate to the probability of recruitment for each mare, and then selecting a mate. Age-specific breeding success of wild stallions was used to simulate paternity (Berger, 1986, p. 200). It is difficult to determine mating success in the field and tenuous to extrapolate results to another site. However, sensitivity analysis showed that model results were insensitive to age-specific breeding success, but were more sensitive to the probability that a male bred during its lifetime. Estimates of breeding success (Berger, 1986; Singer et al., 1998) show that that virtually all prime-aged stallions breed. The likelihood that a particular stallion mated with a female was determined by the number of breeding-age males in the

population and the age-specific probability of breeding. Every male in the population was assigned the appropriate age-specific probability of breeding (Table 1) and the cumulative breeding probabilities were placed in a vector with a length equal to the total number of breeding-age stallions. For each female that bred, a uniform random deviate between 0 and the sum of breeding probabilities was generated and compared to values in the vector. The male that corresponded to the value of the deviate was chosen as the mate for that female. All matings were independent. Factors that determined harem formation, size, and retention were too poorly known to include in the model, but harem interchange rates in the Pryor horse population are so high (Singer et al., 1998) that the age-specific algorithm was probably sufficient for this situation. If only harem-holding stallions mated, simulation results reported here will have overestimated retention of genetic variation.

After the parents of an offspring where determined, Mendelian inheritance was simulated by randomly selecting one allele from each parent at 10 independent loci. At the beginning of each set of simulations, individuals were assigned alleles in the frequency in which they occurred in the Pryor population (G. Cothran, pers. comm.). Each simulation began with a total of 41 alleles distributed over 10 loci; two to 13 different alleles occurred at each locus with an initial frequency of 0.0035 to 0.844.

2.2. Population objective, removal and contraception treatments

The current management plan for the PMWHR includes a population objective of 95 horses, excluding foals (BLM, 1992). A population objective, or appropriate management level (AML), is the optimum number of horses that remain after a removal event in order to maintain a "thriving ecological balance". Foals are usually excluded in determining the AML, and in this paper foals were always excluded from counts used to determine compliance with a population objective or to categorize a population size. Simulations included an AML of 90 horses, and increases in AML to 120, 150, and 180 horses. These objectives might be accommodated by supplemental feeding, acquisition of additional habitat, or other strategies. No specific means to attain a particular objective is implied.

Removals and/or contraceptive treatments of wild horses were simulated by applying rules based on current population size, post-treatment population objective, sex and age of animals to be treated, the minimum number of horses in each sex/age class that were to be unaffected by the treatment, and for removals, the length of time since a previous removal. To determine the annual control treatment, the population was first compared to size thresholds used to categorize the population as small, normal, or large. If the population size was less than or equal to the lower threshold, it was categorized as small. If the population size was greater than the lower threshold and less than or equal to the higher threshold, it was categorized as normal. If greater than the upper threshold it was considered large.

Population size could alter both the timing and magnitude of treatment. It is expensive to gather and remove wild horses and managers seek to minimize the frequency of these events (Boyles, 1986; BLM, 1997). The model therefore simulated a removal event only when the population size had grown to a sufficient size. Removals or contraceptive treatments always occurred when the population size was categorized as large, regardless of how long it had been since a previous treatment. Removals also occurred when the population size was categorized as normal and a threshold number of years had passed since the previous removal. This time threshold was set at 3 years for removal-only treatments and 5 years for removal-and-contraceptive treatments. For removal-only treatments, the threshold between a small and normal population size was set at 150% of the population objective and the threshold between a normal and large population size was set at 200% of the population objective. A removal never occurred when the population size was categorized as small.

Several variables determined which animals were removed from the population. The first variable was the

Table 1
Demographic parameters used to simulate wild horse dynamics

Males			Females		
Age	Survival	Probability of mating	Age	Survival	Probability of recruiting
1–4	0.98	0.00	1–2	0.98	0.00
5–6	0.98	0.10	3–4	0.98	0.50
7–8	0.98	0.26	5–16	0.98	0.60
9–16	0.98	0.64	17–22	0.75	0.47
17–19	0.75	0.50	23–24	0.50	0.47
20–22	0.62	0.25	25	0.00	0.00
23–24	0.50	0.25			
25	0.00	0.00			

population objective, which was the number of horses (excluding foals) remaining after a removal was completed. Removal treatments were categorized as young, old, or random for the age of horses emphasized in the treatment. Random animal treatments selected horses randomly with regard to sex or age until the population objective was reached. For old animal treatments, the oldest horses in the population were selected first, leaving a few males and females in each multi-year age class. Similarly, young treatments first removed the youngest animals, again leaving a few males and females in each multi-year age class. Males and females were removed to leave a slightly female-biased sex ratio, consistent with observations of other wild horse populations and prior BLM removals (Berger, 1986, p. 91).

The proportion of mares treated with contraceptives varied with population size. For each contraception-only treatment, the level of contraception was initially calibrated to achieve a stable population size. However, demographic stochasticity often resulted in populations that either grew or declined. In these situations, the rate of application of contraceptives was increased or reduced until the population declined or increased to a size that fell within the normal range. For populations regulated by contraceptives, populations were categorized as normal when they were 85 to 110% of the population objective. Contraceptive treatments were administered every year and treated mares remained infertile for one year. As with removals, females were selected for contraceptive treatments based on age, using rules that selected breeding-age females randomly, or that resulted in treatment of the oldest or youngest mares first.

Simulations that used both infertility and removal employed a constant rate of application of contraceptives and a threshold population size for determining the frequency of harvest. The rate of contraception was initially calibrated to result in removal of about 50 horses every 5 years.

2.3. Genetic analyses

Observed heterozygosity [H_o; Eq. (1)], expected heterozygosity [H_e; Eq. (2)], and the inbreeding coefficient [F_{is}; Eq. (3)] were calculated following Hartl and Clark (1997), where N is the number of loci.

$$H_o = \frac{\sum_{n=1}^{N} \frac{\text{heterozygotes at locus}_n}{\text{number of individuals}}}{N} \quad (1)$$

$$H_e = \frac{\sum_{n=1}^{N} \left(1 - \sum_{k=1}^{\infty} (\text{freq. of allele k})^2\right)}{N} \quad (2)$$

$$F_{is} = 1 - \frac{H_o}{H_e} \quad (3)$$

In the absence of agency-mandated objectives for conserving genetic diversity, changes in genetic variation were evaluated relative to a goal to achieve a 90% probability of conserving 90% of selectively neutral genetic variation for 200 years. This is a slightly less ambitious objective than suggested by Soule et al. (1986). The frequency of each allele at each locus was recorded and used to evaluate rate of loss of alleles.

2.4. Initial conditions, simulation procedures, and evaluation of results

All simulations started with an initial population of 147 horses that matched the sex and age structure of the PMWHR horse population in fall 1998. The initial population consisted of 65 males aged 1 to 22 years and 82 females aged 1 to 17 years. Treatments were evaluated from results of 500 simulations that each lasted 200 years.

A variety of output files were written to permit evaluation of the causes of differences between treatments. Each year, the age structure of the population, the age and sex of individuals removed, and the age of females treated with contraceptives was recorded. Summary files included data on age-specific fecundity and lifetime reproductive success of males and females. These outputs were used to construct life tables of simulated populations, from which standard demographic statistics such as growth rate and generation time were calculated (Carey, 1993). Annual growth rates for removal treatments accommodated removals by adding the number of horses removed back to the population, such that

$$\lambda_t = \frac{N_t}{N_{t-1} + R_{t-1}} \quad (4)$$

where λ_t is the apparent annual growth rate at year t, N is the total number of animals in the given year, and R_{t-1} is the number of animals removed from the population between year $t-1$ and t. The initial age structure led to instability for the first 10–25 years in simulations of populations regulated by contraceptives, and analyses of rates of contraception and average population processes excluded data from the first 30 years of each simulation.

3. Results

3.1. Treatment levels and demographic responses

Removal and contraceptive treatments resulted in population dynamics that were fundamentally different. Populations controlled by removals increased rapidly and declined precipitously when a removal occurred. In

contrast, populations controlled by contraceptives remained relatively stable. As a result of these dynamics, the average size of populations controlled by removals was 18–27% greater than the AML, whereas populations controlled by contraception remained within 3% of the AML (Fig. 1; dark bars referring to horses 1 year and older). Populations controlled by a combination of contraception and removal were on average 18–5% greater than the AML for population objectives of 90–180, respectively. The coefficient of variation of population size for populations controlled by removal averaged 17%, compared to 8% for populations controlled by contraceptives alone and 11% for populations controlled by contraceptives and removals.

Average annual growth rate (λ) of populations controlled by removals was 1.15 when young horses were removed, 1.16 when old horses were removed, and 1.17 for removal of random-aged horses. Population objective had no influence on average growth rate and the maximum difference in growth rate within a removal treatment (old, young, or random) was 0.004. For populations controlled by contraception and removal of younger horses, λ varied with population objective and ranged from 1.05 to 1.09 for simulated populations with objectives from 180 to 90, respectively. For these treatments, differences in λ were driven by differences in the application rate of contraceptives (Table 2), which were adjusted to achieve a removal of about 50 horses every 5 years.

To achieve a stable population with contraceptive-only treatments, an average of 78, 73, or 74% of all mares 2 years or older were rendered infertile for treatments of young, old, or random-aged mares (Table 2). Depending on population objective, this resulted in

Table 2
Percent and number of horses 1 year or older affected by simulated contraception treatments of the Pryor wild horse population, and the average number of foals in the population

	Population objective	Percent treated	Number treated (mean±1 S.D.)	Average number of foals
Contraception-only treatments				
Young age	90	78	32±7.6	5.7
	120	78	44±8.9	7.7
	150	78	54±10.1	9.6
	180	78	65±11.2	11.5
Old age	90	73	31±6.4	5.8
	120	74	43±8.8	7.9
	150	73	52±8.5	9.7
	180	74	62±9.4	11.6
Random age	90	74	32±8.1	5.9
	120	74	43±8.8	7.9
	150	74	54±9.9	10.0
	180	74	64±10.9	12.0
Contraception and removal treatments				
Young age	90	50	25±4.7	17.2
	120	58	37±5.2	18.3
	150	61	46±6.0	20.1
	180	64	57±6.6	21.5

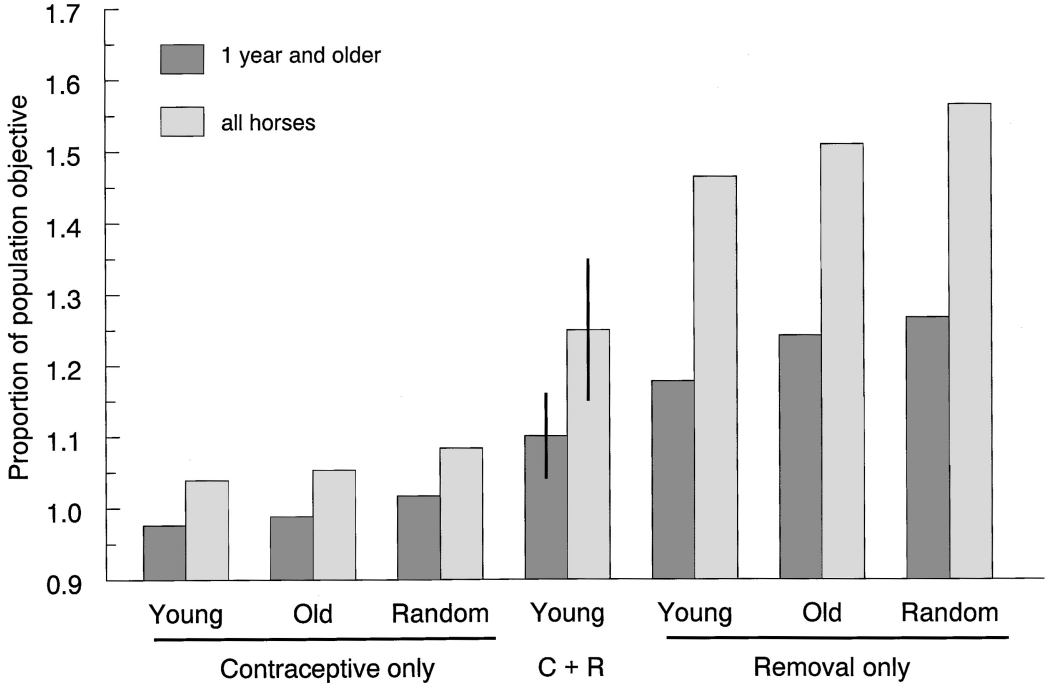


Fig. 1. Average size of populations with (dark bars) and without foals (light bars) as a proportion of population objective; C + R = control using both contraceptives and removals. Population size as a proportion of the population objective varied with the size of the population objective for only C + R treatments; vertical lines show the largest values (for an objective of 90) and smallest value (for an objective of 180).

treatment of an average of 32 to 65 mares per year. For treatments that combined both contraceptives and periodic removals, an average of 50, 58, 61 or 64% of breeding-age mares were rendered infertile each year for population objectives of 90, 120, 150, and 180 horses, respectively. These levels of infertility required treating, on average, 25 to 57 mares each year. When simulated populations were controlled by a combination of removals and contraception, more than 90% of all removals took place at a 5 or 6 year interval regardless of population objective. About 55 horses (range of means = 53–58) were culled from the population each time a removal occurred (Table 3).

For removal-only treatments, more than 90% of removals occurred at three year intervals regardless of population objective or the age of horses removed. Each time a removal occurred, an average about 35% of all horses were removed (range of means = 33 to 37%; Table 3). The number of animals removed was directly related to growth rates; the lowest rate of removal was for treatments of younger horses and the greatest was for random removals (Table 3). Removal of a random selection of horses required harvesting about 20% more horses than when younger horses were removed.

Removals and infertility treatments resulted in populations that had drastically different age structures, but differences in age structure were due entirely to the removal or contraceptive treatment and not to population objective. Within a removal or contraceptive treatment, the proportion of the population in a particular age class remained constant across all population objectives simulated. All simulated removal treatments resulted in age structures characteristic of growing populations, while contraception resulted in the flat age structure characteristic of a stable population (Fig. 2). The average number of foals in the population at the time of a removal varied from 20 to 67 depending on AML and treatment (Table 3). Because foals were not counted towards the AML, populations with a large number of foals in the year of a removal far exceeded the AML in the subsequent year. This occurred because survival rates were high for both foals and adults, and thus the number of foals was far in excess of the number of animals dying from natural causes.

Contraception of older mares or removal of older horses resulted in much shorter generation times than treatments that removed younger females or that delayed reproduction. Average generation time of mares varied by more than a factor of 3, from an average of 4.7 years (contraception of older mares) to a maximum of 16.8 years (contraception of younger mares; Fig. 3). Differences in generation time for males varied far less than that of females, from a minimum of 10.4 years to a maximum of 12.6 years (Fig. 3). Generation time for males was the same for all treatments except for removals of random or old horses; these treatments resulted in shorter generation intervals. For both sexes, differences related to population objectives were small compared to those between treatments that affected different-aged segments of the population.

Table 3
Simulated removal treatments for the Pryor horse population[a]

Age treated	Population objective	Number removed per event (mean±S.D.)	Number removed per year	Proportion removed per event	Harvest interval (years)	Foals at time of harvest
Contraception and removal treatments						
Young age	90	58±10.7	12	0.35	5	20.1
	120	54±9.7	11	0.28	5	20.8
	150	54±10.0	11	0.24	5	22.5
	180	53±9.8	11	0.21	5	23.8
Removal-only treatments						
Young age	90	61±8.3	20	0.34	3	30.2
	120	81±9.7	27	0.33	3	40.2
	150	100±11.1	33	0.33	3	49.9
	180	119±13.1	40	0.33	3	59.8
Old age	90	65±8.2	22	0.35	3	31.9
	120	87±9.9	29	0.35	3	42.6
	150	108±11.1	36	0.35	3	52.8
	180	129±12.4	43	0.35	3	63.3
Random age	90	72±7.7	24	0.37	3	33.7
	120	96±9.0	32	0.37	3	44.9
	150	120±10.3	40	0.37	3	55.9
	180	144±11.4	48	0.37	3	67.1

[a] Mean percent and number of horses 1 year and older removed, and the average number of foals at the time of a removal.

3.2. Effects of treatments on heterozygosity

Initial H_o averaged 0.433 (range of means = 0.432–0.434; range of individual simulations = 0.388–0.479). After 200 years, mean H_o varied from a low of 0.301 (70% of initial H_o), for control by removal of old animals with a population objective of 90, to a high of 0.413 (95% of initial H_o) for populations controlled by contraception and removal of younger mares with a population objective of 180 (Fig. 4). The method of control and age of animal treated or removed had a greater effect on retention or loss of H_o than did population size over the ranges simulated. Treatments that affected older individuals uniformly resulted in more rapid declines in H_o than treatments of young animals. More than 10% of all simulated combinations of population objective and control strategy failed to retain 90% of initial H_o. Using H_o as a measure of genetic diversity, these simulated control strategies thus failed to meet the objective to achieve a 90% probability of retaining 90% of neutrally selective diversity for 200 years.

Inbreeding coefficients (F_{is}) after 200 years of simulation averaged 0.092 (range of means = 0.086–0.096) across all treatments and population objectives. F_{is} tended to be lower for small population sizes, a trend especially pronounced for populations with objectives of 90 and 120 horses. This counterintuitive trend probably resulted from the greater loss of rare alleles in smaller populations, which also reduced H_e in these scenarios (see below).

The use of allele frequencies from the Pryor population resulted in populations in which 34% of all alleles had an initial frequency of 0.05 or less and in which 12% of all alleles occurred at a frequency of 0.02 or less. With an AML of 90, all simulated control strategies resulted in an average loss of more than 25% of alleles (Fig. 5). Almost half (48%) of all alleles failed to persist

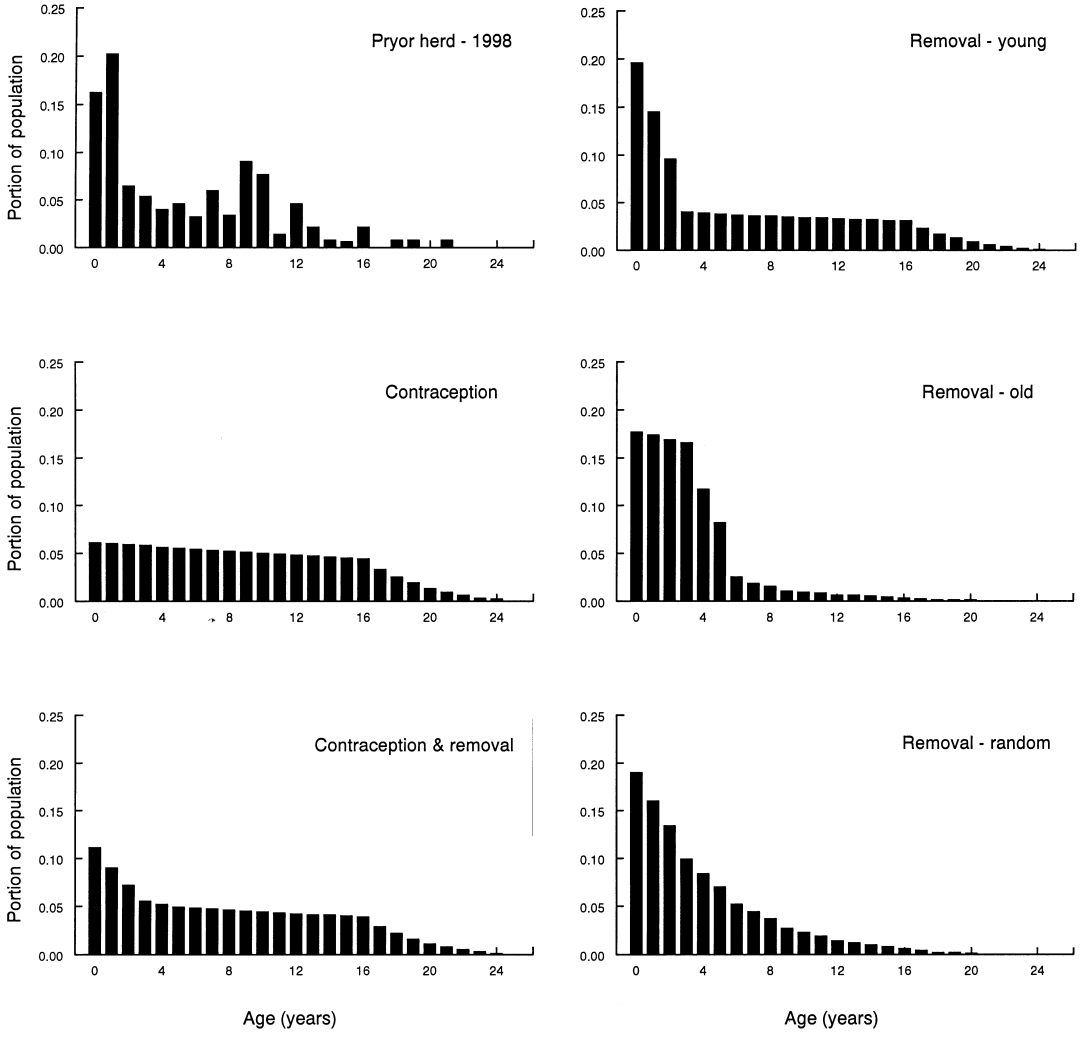


Fig. 2. Age structure of the initial population and simulated populations controlled by contraception, removal, or a combination of both. Age structure of the population was the same regardless of population objective or ages of mares rendered infertile.

for 200 years when old animals were removed from herds with a population objective of 90. Among treatments simulated, contraception of young animals or a combination of contraception and removal of young animals resulted in the greatest persistence of alleles, and an average of 83% of alleles persisted for 200 years under these treatments. These treatments had a 90% chance of retaining 76% of the initial alleles for 200 years. The lower 10th percentile of observations for other treatments included retention of as few as 47% of the initial alleles.

Rates of loss of alleles were highly and non-linearly related to allele frequency. Alleles with an initial frequency of less than about 0.025 tended to be lost very rapid during the first 50 years of simulations. The absolute rate of loss of alleles with initial frequencies greater than about 0.025 tended remain constant throughout the 200 year simulations (Fig. 6).

4. Discussion

4.1. Population control

Simulation results clearly identified contrasting effects of population control by contraception or removals. Compared to removals, infertility treatments reduced variance in population size and resulted in lower standing populations. Because the average sizes of populations managed by infertility were about 25% smaller than those managed by removals, objectives for populations controlled by infertility could be increased to reflect these differences in average size, further enhancing the retention of genetic variation.

Existing chemical contraceptive treatments are effective for a limited period, and they require repeated applications to maintain infertility. Short-duration contraceptives have been used to successfully control growth of horses on Cumberland Island, Georgia and Assateague Island, VA (Kirkpatrick et al., 1997) and they may be suitable for other populations where individuals are easily approached, such as the Pryor herd. A very intensive, and probably very expensive, effort would be required to use short-duration contraceptives as the primary means to control a free-ranging horse population. These treatments are not likely to be successfully applied to widely dispersed populations, but the advantages of contraceptives over removals has stimulated active and promising research that will likely lead to longer-acting contraceptives that can be applied via a "biobullet" or other remote means (Muller et al., 1997). Infertility treatments remain a high priority because they can be far less expensive than removal and adoption programs and they generally receive widespread public support (Garrott et al., 1992; BLM, 1997).

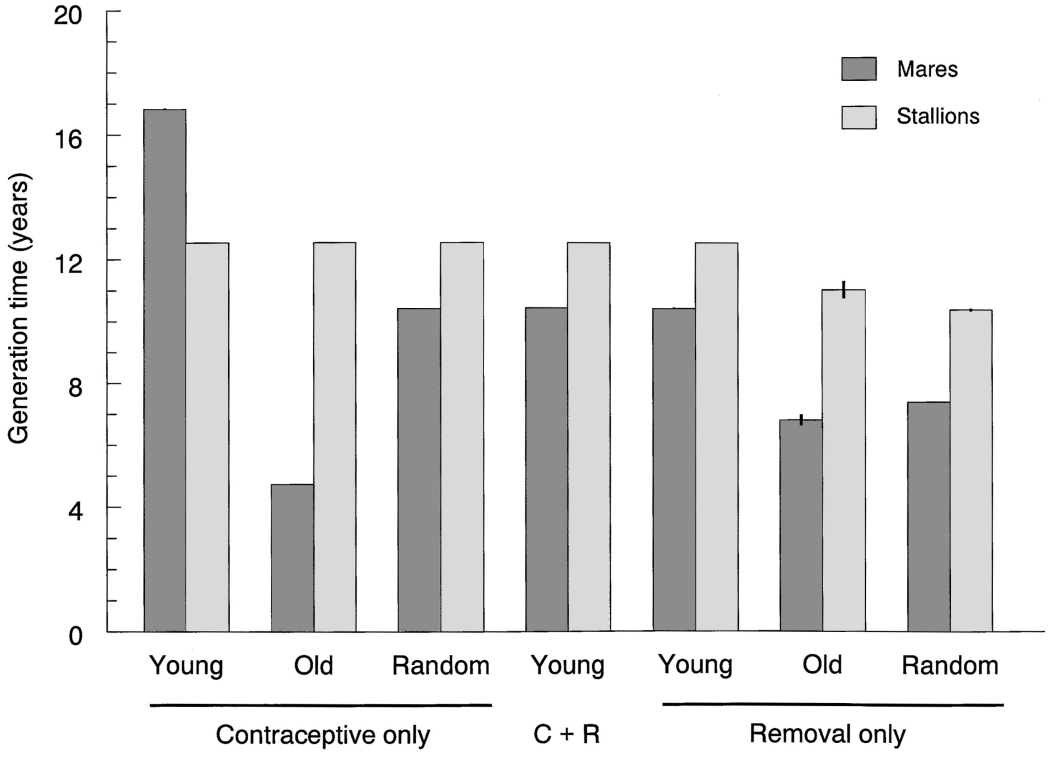


Fig. 3. Generation time of mares (dark bars) and stallions (light bars) in simulated populations controlled by contraceptives only, contraceptives and removal (C + R), or removal only of young, old, or random-age horses. Vertical lines are±1 S.D. of average from populations with management objectives of 90 to 180 horses.

Regardless of the control methods used, intensive management will be necessary to restrict the size of populations with high growth potentials. Contraceptives could eliminate or reduce the need to remove and place wild horses, but a complete reliance on contraceptives can lead to other problems. Contraceptive-only treatments provide no mechanism for controlling the sex ratio of a population, which may become highly skewed, and they provide a rather weak means for controlling populations which have exceeded a size objective. The most flexible and realistic program is thus likely to combine the advantages of infertility and removals. Contraceptives can be used to slow growth rates, while removals provide a means to modify sex ratio or rapidly bring a population into compliance with a size objective.

4.2. Genetic considerations — alternatives to increasing population size?

Simulation results emphasized the importance of age structure and demography on processes that govern the rate of loss of genetic variation. Recommendations for preserving genetic variation have focused on increasing population size, and few papers even consider other strategies that may dramatically affect long-term persistence of genetic diversity. This oversight is not due to misunderstanding among geneticists (Hedrick and Miller, 1992, Fig. 5; Lande, 1995, Fig. 14.4), but rather a lack of appreciation among wildlife mangers and biologists of the important roles of generation time and effective population size in maintaining genetic variation (but see Ryman et al., 1981). Age-specific treat-

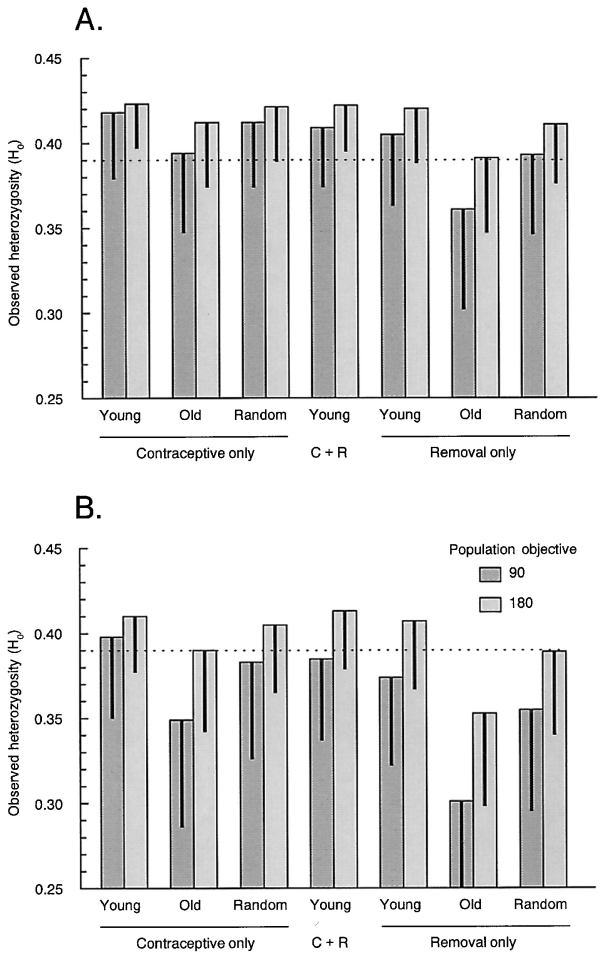


Fig. 4. Observed heterozygosity after 100 years (A) and 200 years (B) in simulated populations controlled by contraceptives only, contraceptives and removal (C + R), or removal only of young, old, or a selection of random-age individuals, for population objectives of 90 (dark bars) or 180 (light bars). Vertical lines descend to the 10th percentile of observations; the horizontal line is at 90% of initial heterozygosity. Initial heterozygosity was 0.433.

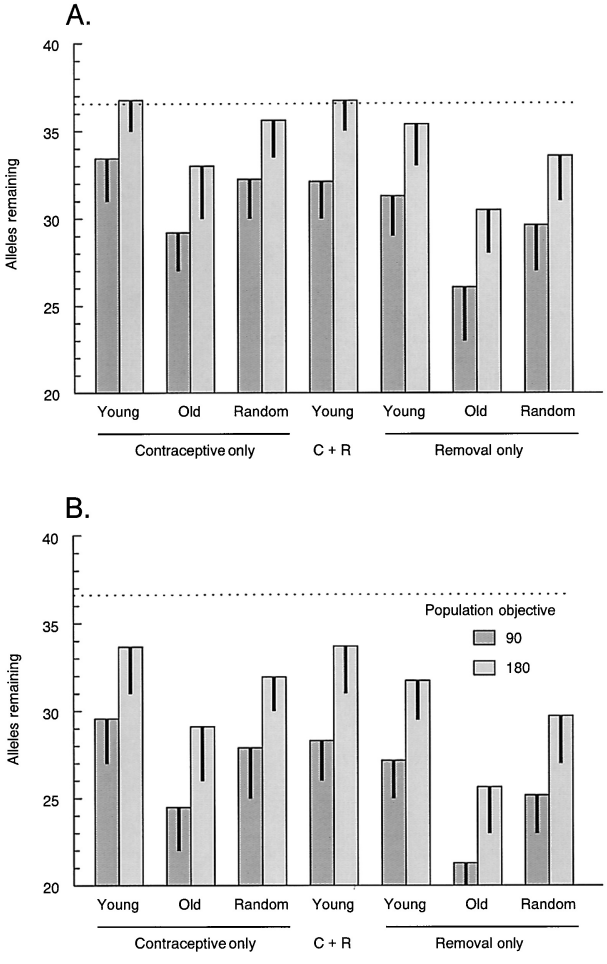


Fig. 5. Number of alleles persisting in simulated populations controlled by contraceptives only, contraceptives and removal (C + R), or removal only of young, old, or a selection of random-age individuals, after 100 years (A) and 200 years (B), for population objectives of 90 (dark bars) or 180 (light bars) horses. Vertical lines descend to the 10th percentile of observations; the horizontal line illustrates 90% of the number of alleleles that were present at the start of simulations.

ments may offer viable alternatives to increases in population size for long-lived species subject to management control. Dramatic differences in the rate of loss of genetic variation between treatments that removed young or old animals are especially pertinent to evaluating management plans because many large mammals of conservation interest are still harvested, either for sport alone or as part of a community-based conservation plan. Both types of harvest usually emphasize removal of older animals from the populations. Consistent harvest of older individuals can have a large effect on retention of genetic variation even when population size exceeds those simulated here by an order of magnitude (Ryman et al., 1981).

The use of multiple measures of genetic variation revealed that under these situations allelic diversity was a much more sensitive indicator of changes in genetic variation than was H_o. Both measures indicated a high probability ($>10\%$) of losing more than 10% of initial variation, even using the best management option simulated. Additional simulations of the contraceptive-and-removal strategy revealed that a population objective of about 500 was necessary to achieve a 90% probability of retaining 90% of alleles, and an objective of 250 was necessary to achieve a 90% probability of retaining 90% of initial observed heterozygosity. It is impossible to implement management plans that achieve levels of contraception or removals as precise as those simulated, thus rates of loss of genetic variation observed under field conditions will vary from those simulated. For treatments of young animals, observed rates of loss of variation will be greater than simulated, while the opposite will likely result from treatments of older animals.

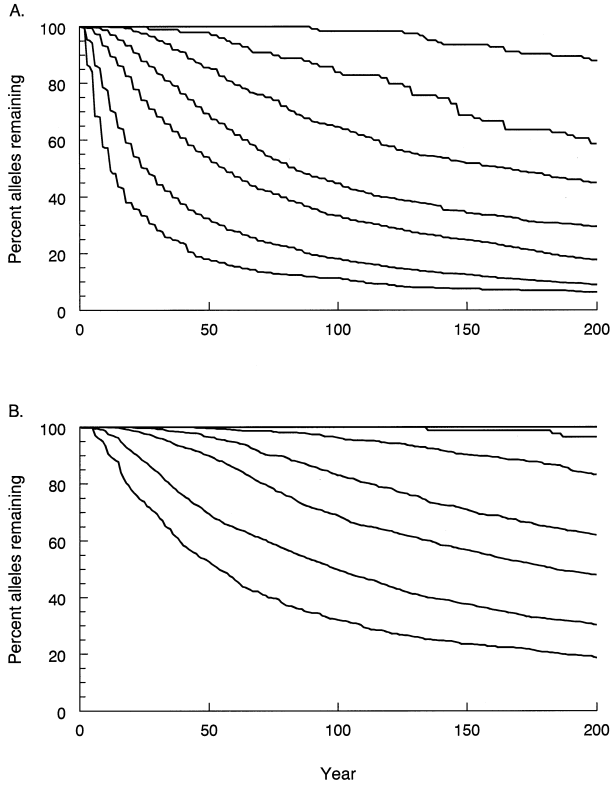


Fig. 6. Persistence of alleles with different initial frequencies over 200 years of simulation. A. Populations controlled by removal of random-aged horses with a population objective of 90. B. Population controlled by contraceptives and removal with a population objective of 150. From the bottom of the plots, lines pertain to alleles that occurred with initial frequencies of 0.005, 0.01, 0.02, 0.05, 0.10, 0.15, and 0.20.

Projected losses of genetic variation were strongly influenced by the frequency distribution of alleles used to initialize simulations. The Pryor population exhibited an unusually high level of heterozygosity (Wooten and Smith, 1985) and it contained many rare alleles. If this population had been isolated for an extended period, many of the rarer alleles would already have been lost and the effects of population size or age of treated animals would have been less. This leads to a rather surprising conclusion: as existing genetic variation declines, fewer individuals are needed to retain a high proportion of the existing variation. This can occur because rare alleles will have already been lost from the population, and those alleles that occur at a relative high frequency are lost slowly (Fig. 6). In natural populations the level of genetic variation will reflect processes that increase and decrease natural variation. Mutations increase variation, but they occur at a slow rate and new variation must be maintained to contribute to long-term heterozygosity. On the other hand, genetic drift results in loss of variation.

In this model, changes in genetic variation resulted only from genetic drift. While the inclusion of mutations would have resulted in lower rates of loss of variation, genetic drift is by far the dominant process leading to changes in gene frequencies in small populations (Nunney and Campbell, 1993; Lynch, 1996). This occurs for two main reasons. First, sampling errors are exacerbated in small populations, and second, the proportion of genetic variation that is selectively neutral increases as population size declines. Selection tends to be far less important in small populations because a much larger selection coefficient (selective advantage) is necessary for selection to act in a deterministic manner as population size declines. For quantitative characters, this situation arises from the distribution of selective forces over a large number of loci, thus selection on any one locus is small enough to be overwhelmed by random genetic drift (Lynch, 1996). A consequence of the dominance of random drift is that an allele with strongly negative effects can become fixed in the population. In large populations, a small selective disadvantage may be sufficient to reduce the frequency of a deleterious allele, whereas this is unlikely to occur in small populations.

Several factors not included in the simulation analysis could result in more rapid loss of genetic variation than these results suggest. Periodic catastrophes could lead to temporary reductions in population size. The Pryor population suffered high winter loss in 1977–1978 (Garrott and Taylor, 1990), and catastrophes are thought to be an important factor in other large mammal populations (Young, 1994, 1999; Ebb and Boyce, 1999). Other factors that may be important and that require further investigation include density dependence, compensatory reproduction, and the role of environmental stochasticity. All of these factors could increase the rate of loss of genetic variation. The results of these simulations should thus be interpreted conservatively if they are used to guide management decisions.

These simulations illustrated several important genetic and demographic concepts. For managed populations, actions that delay the age of reproduction and increase generation interval will reduce the loss of genetic variation. Allelic diversity can be a much more sensitive index to loss of genetic variation than population-level heterozygosity, especially when there are many alleles at a locus. Compared to periodic removals, use of contraceptives under simulated conditions resulted in smaller populations and a much less variation in population size. Management plans that use contraceptives to control the rate of growth and that employ occasional removals to control sex ratio and reduce population size could emphasize the advantages of both approaches, while avoiding the most undesirable characteristics. Overall, these results support use of management strategies that rely less on removal and more on infertility treatments to reduce long-term loss of genetic variation.

Acknowledgements

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Wildlife population management: are contraceptive vaccines a feasible proposition?

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1. ABSTRACT

To minimize human-animal conflicts for habitation and burden of zoonotic diseases, it is imperative to develop new strategies for wildlife population management. In this direction, contraceptive vaccines eliciting immune response against hormones/ proteins critical for reproduction have emerged as one of the promising options. Contraceptive vaccines based on neutralization of gonadotropin releasing hormone (GnRH) have been used for inhibition of fertility in various species such as wild horses, white-tailed deer, pigs, cats, dogs etc. It has been used for immunocastration of male pigs to improve meat quality. However, additional safety studies of GnRH vaccine will be needed in light of presence of its receptor at extra-pituitary sites. Native porcine zona pellucida (PZP)-based contraceptive vaccines have shown their utility in the management of the population of both captive and free-ranging wild horses and white-tailed deer. Long-term use of the PZP-based contraceptive vaccines has also demonstrated their safety. Ideally single injection of the contraceptive vaccine should elicit long lasting immune response and desired contraceptive efficacy, which will require development of novel vaccine delivery platforms and more potent adjuvants.

2. INTRODUCTION

The uncontrolled increase in population of free-ranging animal species such as elephants in Africa, kangaroos in Australia, feral horses and white-tailed deer in USA, street dogs and cats in several developing countries, and monkeys in India etc is leading to increasing conflicts for habitation between humans and wildlife species. Further, wild as well as domesticated animals may act as vectors for viruses, bacteria or parasites and thus pose a major risk to human health (1). There is an increase in the emerging infectious diseases in humans in last 3 decades and 70% of these are zoonotic in nature (2, 3). As an example, dogs are the main vectors that maintain rabies virus circulation within human communities. Human mortality from rabies has been estimated to be 55,000 deaths per year and 90% of deaths occur in the developing world (4). In India, approximately, 20,000 people die from rabies per year and 96% of these are infected by dogs (5, 6). Almost 1.8. million people annually receive post-exposure prophylaxis against rabies following bite or exposure to rabid or suspected rabid animal. The global threat posed by zoonoses (transmitted by bats and palm civets) is also illustrated by the 2003 outbreak of severe acute

respiratory syndrome coronavirus (SARS-CoV), which emerged in Hong Kong but affected 37 countries leading to over 8,000 cases of infection and approximately 800 deaths (7). Currently, there is concern about Middle East respiratory syndrome coronavirus (MERS-CoV), which emerged in Saudi Arabia in 2012 and is suspected to be zoonotic (initially transmitted by dromedary camels) in nature with over 1,100 cases of infection and 440 deaths, including travel related cases worldwide (8).

Wildlife managers have often used lethal means such as culling to control wildlife population; however, growing public concern over animal welfare issues and new legislative laws banning culling in several countries make such an approach increasingly unacceptable. Substantially reducing the number of abundant species may have indirect effects on the population of other species. For instance, in United Kingdom culling of Eurasian badgers (*Meles meles*) in an effort to reduce the incidence of bovine tuberculosis in cattle herd led to an increase in the population of red fox (*Vulpes vulpes*) (9). It has also been observed that repeated badger culling in the same area allow badgers from neighbouring area to re-colonize culled areas leading to increased prevalence of *M. bovis* infection in badger (10). Culling also leads to disturbance in the social group of animals leading to increased movement and thus instead of reducing transmission of zoonotic diseases it results in their increase (11). Thus, culling as a measure to control wildlife population and transmission of infectious disease has proven to be inefficient (12, 13). Translocation of animals from overpopulated areas to low population areas or in a confined environment has also been used as a measure for population control of wildlife in a particular territory, to minimise human-animal conflicts, and to reduce the burden of infection in the host wild species or its transmission to other animals including humans (14, 15). However, relocation of animals to new environment may lead to both physical and mental stress, disturbances in social culture, including adequate accessibility of food.

Surgical sterilization such as spaying of female dogs and castration of male dogs is used by few countries to control their population. Newly castrated male dogs may temporarily suffer from testosterone withdrawal leading to irritability and lethargy, a phenomenon known as the “irritable male syndrome” (16). Other potential complications with surgical castration include haemorrhage and excessive swelling, especially if the spermatic cords are cut but not crushed (17). Ovariohysterectomy of female dogs may lead to complications such as haemorrhage, ovarian remnant syndrome, stump pyometra, stump granuloma, fistula draining tracts and estrogen responsive urinary incontinence (17). To control monkey (*Rhesus macaque*) population in Himachal Pradesh, India, surgical sterilization of males by thermo-cauteric coagulative vasectomy and of females by endoscopic thermo-cauteric tubectomy has been used (unpublished observations). However, long term side-effects of such a surgical sterilization program to control population of monkeys needs further investigation. Surgical sterilization by and large is more socially accepted than culling, though it is relatively expensive and required specialized facilities and staff. In addition to surgical sterilization, chemical sterilization by intra-testicular injection of zinc gluconate (Neutersol, Addison Biological Laboratory Inc.), which was approved in 2003 by the US Food and Drug Administration (FDA), that causes sclerosis of the testes and sterility has been used for controlling fertility of dogs and cats (18–20). In addition to zinc gluconate, intra-testicular injection of calcium chloride in dogs also led to dose-dependent decrease in sperm count (21). The low cost and ease of use of chemical sterilants as compared to surgical sterilization will be useful for their application at field level; however, additional field trials are needed to ascertain their safety and efficacy.

Administration of synthetic progestins such as megestrol acetate, melengestrol acetate, and levonorgestrel has been used as contraceptives for dogs and cats leading to inhibition of oestrous (For review see 22). This approach is suitable for confined companion animals or animals in zoo but unsuitable for free-roaming animals. Administration of melengestrol acetate as implants induced infertility for up to 2 years. Due to uterine pathology, long term administration of melengestrol acetate is not recommended (23). Levonorgestrel implant has also shown to be effective for prevention of fertility in tammar wallabies (*Macropus eugenii*), grey kangaroos (*Macropus giganteus*) and koala (*Phascolarctos cinereus*) females (24–26). In addition to synthetic progestins, gonadotropin releasing hormone (GnRH) agonists such as deslorelin (Suprelorin, Virbac) and azagly-nafarelin (Gonazon, Intervet International B.V.) have been used to inhibit fertility in dogs and tammar wallabies (22, 23, 27). For population management of free-ranging wildlife, hormonal contraceptive may not be a very good option due to high cost, difficulty in delivery, health risk in pregnant animals and changes in social behaviour. In addition, immunocontraception as one of the potential humane options for wildlife population management is being considered favourably by various agencies. In this article, various contraceptive vaccines that are being used for the wildlife population management will be reviewed. The efficacy and safety aspects of contraceptive vaccines will also be discussed. The current limitations for contraceptive vaccines and the plausible solutions will also be described.

3. CONTRACEPTIVE VACCINES

The basic principle of contraceptive vaccine is to generate either humoral and/or cell mediated immune response against hormones/proteins that are

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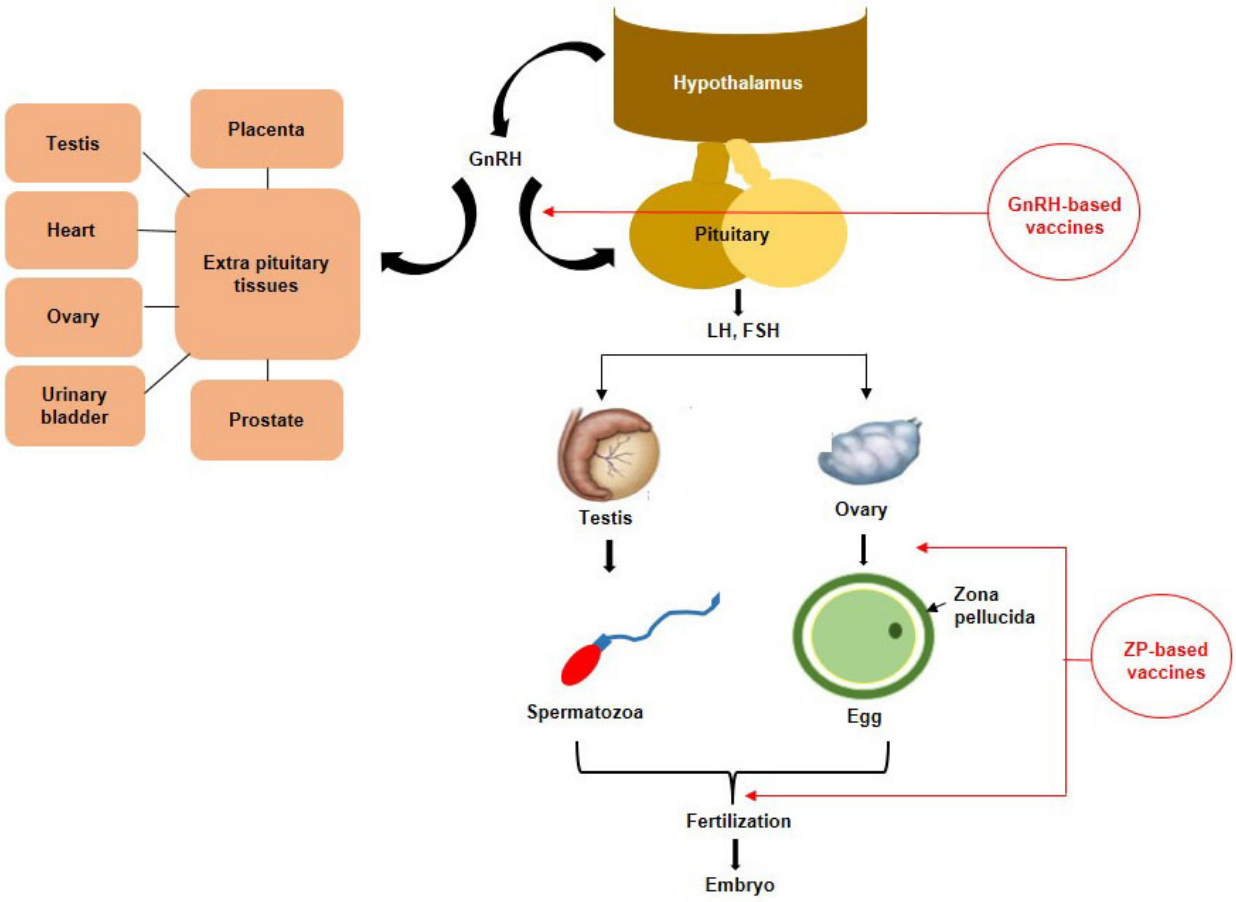


Figure 1. Schematic representation of the processes targeted by the contraceptive vaccines meant for wildlife population management. Possible sites have been shown for the action of GnRH- and ZP-based contraceptive vaccines. Antibodies against GnRH will inhibit production of LH and FSH from anterior pituitary thereby leading to inhibition of spermatogenesis in males and ovarian follicular development and ovulation in females. The anti-GnRH antibodies may also act on other extra-pituitary sites such as placenta, testis, ovary, heart, prostate, urinary bladder etc. Antibodies against ZP curtail fertility by either inhibiting folliculogenesis or fertilization or both.

critical for reproduction, which will lead to interference in their biological activity resulting in inhibition of fertility. Basically, contraceptive vaccines can be broadly categorised as those i) inhibit production of gamete (spermatozoa, egg); ii) inhibit gamete functions such as fertilization; and iii) inhibit gamete outcome. GnRH primarily synthesized and secreted by the hypothalamus (though extra-hypothalamic sites for its secretion have been reported) acts on the anterior pituitary and in turn regulate the production of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Both, LH and FSH secreted by pituitary, in turn acts on testes and ovaries leading to production of sperm and oocytes, respectively. Thus neutralization of GnRH, LH and FSH or blocking LH/FSH receptor by generating specific antibodies may lead to inhibition of gamete production. Generation of immune response against spermatozoa- and/or egg-specific proteins will interfere in their respective functions thereby leading to inhibition of fertilization. Post-fertilization, the embryo synthesize and secrete human chorionic gonadotropin (hCG), which has also been used as target for development of contraceptive vaccine for women (28). In the context of wildlife population management, contraceptive vaccines based on GnRH and zona pellucida (ZP) have been tried at the field level. Figure 1 illustrates the basic principle and steps at which the GnRH and ZP-based contraceptive vaccines will act thereby leading to inhibition of fertility. In the present review, both GnRH- and ZP-based contraceptive vaccines will be briefly discussed with respect to their contraceptive efficacy and safety. In addition, the current limitations with respect to contraceptive vaccine delivery in free-ranging wildlife and plausible solutions will be discussed.

4. GnRH-BASED CONTRACEPTIVE VACCINES

GnRH, a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2) is primarily synthesized and secreted by the hypothalamus (29), though its presence and/or secretion in other tissues such as ovary, testis,

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Table 1. List of commercially available GnRH-based contraceptive vaccines and their intended use

GnRH-based vaccines	Name of the company	Observations	References
GonaCon™	National Wildlife Research Centre, USA	• Provided multi-year contraception in female white-tailed deer • Single immunization achieved 80–100% contraception in female elk • Immunized prairie dogs showed high contraception • Showed inhibition of fertility in both male and female cats	33, 35–38
Improvac®	Pfizer Animal Health, Australia	• Effective in reducing sexual and aggressive behaviour in pigs • Improvement of meat quality in male pigs • Suppressed testosterone concentration, number of sperm and sexual behaviour in boars	39–42
Equity™	CSL, West Ryde, NSW, Australia	• Suppressed testicular function in elephant • Suppressed testicular function and sexual behaviour in stallion	44, 45
Bopriva®	Pfizer Animal Health, Parkville, Australia	• Decreased testicular development, serum testosterone and physical activity in bulls • Suppressed testicular growth and testosterone secretion in male calf • Inhibit fertility in boars and dairy cattles	46–49
Improvest®	Zoetis, Kalamazoo, MI, USA	• Castration of male pigs • Suppress heat in female gilts	50, 51
Repro-BLOC™	Amplicon Vaccine LLC, Pullman, WA, USA	• Reduced uterine size, vascularity and suppressed ovarian steriodogenesis in elephant	52

prostrate and placenta has also been reported (30, 31). To develop contraceptive vaccine, GnRH being a self-peptide hormone, its conjugation with various carrier proteins such as tetanus toxoid (TT), diphtheria toxoid (DT), keyhole limpet hemocyanin (KLH), blue protein from mollusc (*Concholepas concholepas*), ovalbumin etc have been made to provide T-cell help to enable generation of antibodies against GnRH. GnRH-based contraceptive vaccines work in both males and females as neutralization of its biological activity inhibit production of sperm in males and eggs in female. After establishing proof-of-concept of GnRH-based contraceptive vaccines in laboratory experimental animals including non-human primates, currently there are at least six commercial vaccines available (Table 1).

GonaCon™ developed by National Wildlife Research Center, USA is comprised of synthetic GnRH coupled to KLH (more recently with blue protein from mollusc) and supplied with AdjuVac™ (diluted version of Jones disease vaccine containing killed mycobacteria and oil adjuvant). GonaCon™ has been approved by the Environment Protection Agency (EPA), USA for fertility regulation in white-tailed deer in urban/suburban areas and subsequently for wild horses (*Equus caballus*) and burros (*Equus asinus*) (32). Single injection of GonaCon™ induced infertility ranging from 2 to 5 years in white-tailed deer as a measure to control their population (33). It also successfully blocked the cyclic activity and ovulation in captive wild mares (34). To reduce the potential of ecological damage and spread of brucellosis, the contraceptive efficacy of GonaCon™ has also been demonstrated in female elk (*Cervus elaphus*) (35). The contraceptive potential of GonaCon™ in black-tailed prairie dogs has also been shown (36). The contraceptive efficacy ranging from 5 months to 5 years after single injection of GonaCon™ in female cats has also been reported, which depends on the immune response generated in the respective cat (37, 38). In general, the vaccine was more effective in female cats as compared to male cats (37).

Another commercial GnRH-based contraceptive vaccine, Improvac®, consists of synthetic incomplete analogue of gonadotropin-releasing factor (GnRF) linked to a carrier protein to make it immunogenic. Immunization of cross-bred Iberian female pigs with Improvac® led to reduction in the incidence of standing oestrous, serum progesterone levels, and development of uterus and ovaries (39). Immunization led to long-lasting immunity of at least 20 months after third injection (39). Immunization of male pigs with Improvac® led to a significant reduction in their sexual and aggressive behaviour as observed by reduction in mounting, fighting, pushing, head butting and tail manipulation (40). Pre- and post-pubertal rams and boars accumulates androgen derivatives namely androstenone and skatole in their adipose tissues, which gives an unpleasant odour to meat. To overcome this taint, surgical sterilization of male pigs is routinely done. Vaccination of boars with Improvac® contraceptive vaccine has shown to reduce the pig meat taint (41). Further, it has been shown that immunization of male pigs with Improvac® led to improvement in the lean meat content as compared to surgically castrated pigs (42). Immunization of adult ewes (White Alpine sheep) with Improvac® led to suppression of cyclicity concomitant with reduction in progesterone levels (43).

Immunization of a male Asian elephant with combination of two commercial GnRH-based contraceptive vaccines i.e. Improvac® and Equity™, led

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to a decrease in serum testosterone concentrations, testicles diameter, penile atrophy and weight gain (44). After 1 year of initial treatment, no spermatozoa were observed in the semen suggesting that GnRH vaccine may be a useful non-invasive method of contraception for Asian elephants (44). Immunization of adult stallion with commercially available GnRH-based vaccine, Equity™, using three injections schedule led to suppression of testosterone levels, reduction in semen quality and changes in sexual behaviour, the extend of which was variable among the immunized animals (45).

Bopriva® is one of the commercially available GnRH-based vaccine, developed by Pfizer Animal Health, Parkville, Australia, which induces antibodies against GnRF. Active immunization of pubertal bulls with this vaccine led to decrease in testosterone levels in blood, testicular development and physical activity, leaving the body weight gain unaffected (46). Further, two injections of Bopriva® in the peripubertal bull also suppressed the testicular growth and blood testosterone concentration for at least 10 weeks after the booster injection (47). Bopriva® was also effective in suppression of testicular functions including sperm production in boars (48). Immunization of female dairy cattles (cyclic Swiss Fleckvieh cows) with Bopriva® showed decrease in progesterone levels without affecting estrogen levels, suppression of oestrud and impaired folliculogenesis (49).

Another commercial vaccine based on GnRH, Improvest® (Zoetis, Kalamazoo, MI, USA), has been used for castration of male pigs and improvement in the meat quality (50). Female gilts receiving two injections of Improvest® showed better growth performance and suppression of heat (51).

Repro-BLOC™ (Amplicon Vaccine, LLC, Pullman, WA, USA) is a recombinant ovalbumin-GnRH fusion protein vaccine developed for use in cattle, although there is limited data on its efficacy. Multiple injections (primary followed by 4 booster) of Repro-BLOC™ in a 59-year old Asian elephant (*Elephas maximus*) led to suppression of ovarian steriodogenesis for over a year and reduction in uterine size and vascularity (52).

4.1. Safety of GnRH-based contraceptive vaccine

GnRH-based vaccines have shown successful contraceptive efficacy in both male and female domestic horses, without any serious adverse effects (34, 45, 53–54). Suppression of oestrous and unwanted oestrud behaviour by GnRH-based vaccines (53, 54) may be desirable in domestic species including cats and dogs. However, inhibition in the production of reproductive steroid hormones (progesterone in females and testosterone in males) by GnRH-based vaccines may have impact on the complex evolutionary social organization of horses, elephants and captive exotic species in zoos, which needs to be investigated more carefully (55).

There was no significant haematological or endocrinological changes in the two groups of mongreal dogs, when either immunized with Improvac® or surgically castrated, suggesting that immunological castration may provide a safer alternative (56). No adverse effects on body weight or blood chemistry were observed in black-tailed prairie dogs immunized with GonaCon™ (36). It has also been reported that immunization with Improvac® can improve the welfare of male pigs as it was able to inhibit sexual and aggressive behaviour (40). The vaccine was well tolerated in the pigs and no injection site reactions were observed (41). However, in few female cats immunized with GonaCon™, non-painful late-onset granulomas were observed at the injection site (38). A study demonstrated that simultaneous administration of anti-GnRH (GonaCon™) vaccine along with rabies vaccine had no adverse impact on the seroconversion against rabies virus antigen suggesting that GnRH vaccination can be used along with rabies immunization program (57).

It is imperative to take into consideration that use of GnRH-based vaccine in pregnant animals may lead to abortion, if LH is critical to maintain the corpus luteum throughout during gestation such as in bison. GnRH receptors have been described at several extra-pituitary sites such as ovary, testes, placenta, prostrate including urinary bladder and heart (30, 31, 58, 59). These observations suggest that more carefully monitored field trials of GnRH-based contraceptives vaccines should be undertaken to rule out any adverse effect on other physiological processes than reproduction.

5. CONTRACEPTIVE VACCINES BASED ON ZONA PELLUCIDA GLYCOPROTEINS

In mammals, egg is surrounded by an extracellular translucent glycoproteinaceous matrix known as zona pellucida (ZP). It acts as a relatively species-specific ‘docking site’ for binding of the spermatozoa to the oocyte, induces acrosomal exocytosis in zona-bound spermatozoa, prevents polyspermy, and plays an important role in the protection of a pre-implanted blastocyst. Due to their critical role in reproduction, ZP glycoproteins have been used as candidate antigens for contraception via immunological intervention. Zona matrix is composed of either 3 or 4 glycoproteins. In mice, it is composed of 3 glycoproteins namely ZP glycoprotein -1 (ZP1), -2 (ZP2), and -3 (ZP3), whereas pig and canine ZP matrix is also composed of 3 glycoproteins but instead of ZP1, ZP glycoprotein-4 (ZP4) is present. In non-human primates and humans, ZP matrix is composed of 4 glycoproteins that are ZP1, ZP2, ZP3,

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Table 2. Sequence homology of porcine ZP2, ZP3 and ZP4 with other species at amino acid level

Animal Species	Percentage sequence homology with porcine		
	ZP2	ZP3	ZP4
Mouse (*Mus musculus*)	55	66	50[1]
Dog (*Canis lupus*)	70	75	72
Horse (*Equus caballus*)	72	77	75
Monkey (*Macaca radiata*)	63	72	67[1]
Bison (*Bison bison*)	78	88	77[1]
Rat (*Rattus norvegicus*)	54	65	60
African Bush Elephant (*Loxodonta africana*)	65	73	65
Brushtail Possum (*Trichosurus vulpecula*)	51	50	56[1]

[1]Instead of ZP4, ZP1 is present

and ZP4. Structure and functions of all the four ZP glycoproteins during fertilization from various species has been investigated by various groups, which has been reviewed elsewhere (60). Characterization and sequencing of ZP glycoproteins from various species revealed that their basic structure is evolutionary conserved but have variable degree of amino acid sequence identity. For example porcine ZP3 has 66% identity at amino acid level with mouse ZP3 and 75% with canine ZP3. The extent of amino acid sequence identity of various porcine ZP glycoproteins with their respective homologues from other species is listed in Table 2. This property of ZP proteins has made heterologous immunization as a feasible proposition. It is further strengthened by the observations that polyclonal antibodies generated against porcine heat solubilised isolated zona pellucida (SIZP) cross-react immunologically with ZP of human, squirrel monkey, rabbit, rat, and mouse (61). Due to easy accessibility of porcine ovaries from abattoirs, contraceptive vaccine based on porcine ZP preparations became the antigen of choice.

5.1. Native porcine zona pellucida (PZP)-based contraceptive vaccine

Contraceptive potential of either native porcine SIZP (PZP) or its purified component (ZP3) has been demonstrated in female rabbits (62), bitches (63), non-human primates (64, 65), domestic and captive wild horses (66), captive white-tailed deer (67) and a variety of zoo animals (68). The field trial of native PZP-based contraceptive vaccine for the management of wild horses population on Assateague Island National Seashore, a barrier island off the coast of Maryland, USA demonstrated its contraceptive efficacy (69). The vaccine was delivered remotely using dart gun. Inhibition of fertility in wild horses could be maintained by giving annual booster of PZP vaccine. It was shown that third consecutive annual booster of porcine ZP led to 79% efficacy in preventing pregnancies in mares (70). Smithsonian Institute's Conservation and Research Center at Front Royal, VA, USA initiated the first field trials of PZP-based contraceptive vaccine in white-tailed deer inhabitating Fire Island National Seashore, NY, USA (71). Deer were captured, tagged, given primary injection of PZP vaccine, and then released. Subsequent booster injections were given remotely by using dart gun. Between 1993 and 1999, twinning rates among individually known vaccine treated adult female deer decreased by 78.9.% from pre-treatment rates (72). The contraceptive efficacy of PZP vaccine was also demonstrated in free-ranging African elephants (*Loxodonta africana*) in the Kruger Park, South Africa (73). Thus PZP-based contraceptive vaccine has been successfully used for the management of the population of wild horses (34, 66, 69, 70, 74, 75, 76), urban deer (71, 72, 77, 78), wapti (79), and African elephants (73, 80). Keeping in view the above, currently three commercial vaccines based on PZP are available as listed in Table 3.

5.2. Safety of PZP-based contraceptive vaccine

Ovarian specific expression of zona proteins and lack of cross-reactivity of antibodies generated against zona proteins with other tissues and protein hormones (81, 82) is of an advantage of PZP-based vaccines as compared to GnRH-based contraceptive vaccine. Zona pellucida-based contraceptive vaccine primarily works by either inhibiting fertilization and/or folliculogenesis (Figure 1). Potential changes in ovarian pathology represented by follicular atresia along with sometime depletion of primordial follicles pool in rabbits (62), dogs (63), sheep (83) immunized with porcine zona proteins was one of the main concern for the application of PZP-based contraceptive vaccine for wildlife population management. However, no changes in ovarian histology were found in PZP-based contraceptive vaccine immunized horses and deer (66, 71). Inhibition of fertility was reversible in the captive deer immunized with PZP-vaccine after one to four years of immunization (67). In another study,

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Table 3. List of commercially available porcine ZP-based vaccines and their applications

Commercial PZP-based vaccines	Name of the company	Applications	References
Spayvac®	ImmunoVaccineTechnologies™, Halifax, Nova Scotia, Canada	• Showed 100% contraceptive efficacy the first year then 83% the next 3 years in wild horses • Showed 85–90% infertility in female deer	34, 78
ZonaStat-H	Humane Society of the United States, Washington, DC, USA	• Only PZP vaccine approved by the Environmental Protection Agency (EPA) for use in female wild horses and burros • Suppressed fertility by 86% in domestic and wild horses	76
PZP-22	University of Iowa, School of Pharmacy, Iowa City, USA	• Provided two years of infertility in feral horses	74, 76

no changes in ovarian histology of PZP immunized deer were reported. However, breeding season was extended by 1–2 months (71). PZP-vaccinated deer were more active and gained significantly more weight by summer following immunization than untreated deer, presumably because of avoidance of lactation, but by the following fall, all weight differences disappeared (84). Ovarian eosinophilic oophoritis was reported in PZP immunized deer, which; however, was not statistically different as compared to unimmunized group (85). Injection site abscesses were reported only in 0.5.% of PZP immunized deer, but all of them revealed the formation of granuloma at the injection site without any complication (72, 85). In elephants immunized with PZP-based contraceptive vaccine, examination of the ovaries and uteri by ultrasound revealed no adverse changes (73, 80). However, there may be species-specific differences in the ovarian histopathology subsequent to PZP-based contraceptive vaccine immunization, which should be investigated in detail in the respective target species.

Long-term treatment of wild horses with PZP-based contraceptive vaccine did not lead to any permanent or significant changes in ovarian endocrine parameters and oestrous cyclicity as monitored by urinary steroid metabolites (70). It had no adverse outcome when used in pregnant mares. The contraceptive effect was reversible (86). Increased body conditions and longevity were observed in PZP-immunized horses (87). There was no significant change in the social organization or behaviour of PZP-immunized wild horses (88). However, there is some concern over injection site reactions (55). It is likely that the remote delivery of PZP-based contraceptive vaccine by dart may result in surface bacteria and debris being pushed into the injection site resulting in injection site reactions (55).

Few significant changes in blood chemistry such as levels of urea and creatine were observed in PZP immunized deer but none of these were associated with any physiological abnormalities (85, 89). PZP immunized Dall sheep (*Ovis dalli*) and domestic goats (*Capra hircus*) also did not reveal any significant changes in blood chemistry (90).

5.3. Recombinant zona proteins as candidates for contraceptive vaccine

Due to limited availability of native porcine zona proteins from pig oocytes and to circumvent the apprehension of their probable contamination by other ovarian-associated proteins, recombinant zona proteins have also been used as candidate immunogens. Immunization of female baboons (*Papio anubis*) with *E. coli*-expressed recombinant bonnet monkey (*Macaca radiata*) ZP4 (previously designated as ZPB) coupled with diphtheria toxoid (DT) led to reversible block of fertility (91). However, immunization of female bonnet monkeys with the above immunogen led to inhibition of fertility which was not reversible. Histology of the ovaries from immunized monkeys revealed follicular atresia (92). Active immunization of female marmosets (*Callithrix jacchus*) with mammalian-expressed recombinant human ZP3 also led to inhibition of fertility associated with ovarian pathology characterized by depletion of primordial follicle pool (93). Immunization of cynomolgus monkeys (*Macaca fascularis*) and baboons (*Papio cynocephalus*) with mammalian expressed recombinant human ZP2, ZP3 and ZP4 respectively showed higher contraceptive efficacy in animals immunized with ZP4 (94). These studies in non-human primates showed the potential of recombinant zona proteins as candidate immunogens for the development of contraceptive vaccine.

In order to control the population of street dogs and thereby reduce the burden of rabies infection, it was demonstrated that non-descript female dogs immunized with *E. coli*-expressed recombinant dog ZP3 conjugated to DT failed to conceive (95). Ovarian histology of the immunized dogs revealed degenerative changes in the ZP matrix and follicular atresia. Subsequently, to avoid chemical conjugation of recombinant dog ZP3 with DT and to obtain recombinant protein without His_6-tag, it has been expressed in *E. coli* as a fusion protein with promiscuous T cell epitope of tetanus toxoid (TT, amino acid residues 830–844) with dilysine linker (TT-KK-ZP3) (96). Immunization of female mice with recombinant TT-KK-ZP3 led to significant reduction in fertility, which was associated with antibody titres (96). In Australia and

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New Zealand, the potential of recombinant brushtail possum (*Trichosurus vulpecula*) ZP3 protein to control the fertility of Koalas (*Phascolarctos cinereus*) and Eastern Grey Kangaroos (*Marcopus giganteus*) has also been explored (97, 98).

In light of limited availability of native PZP-based contraceptive vaccine, recombinant porcine ZP3 and ZP4 have also been expressed in *E. coli* and their contraceptive efficacy was evaluated (99). Immunization of female mice with recombinant porcine ZP3 and ZP4 led to significant inhibition of fertility and decrease in litter size as compared to the adjuvanted control (99). Interestingly, *E. coli*-expressed recombinant porcine ZP3 and ZP4 boosted the antibody response in female mice primed with porcine native SIZP, suggesting that recombinant proteins can be used as booster doses thereby enabling wider coverage of immunization program in light of limited supply of native PZP-based contraceptive vaccine (99). Contraceptive efficacy of recombinant porcine ZP3 and ZP4 vis-à-vis PZP vaccine has also been evaluated in pony mares. Extended anoestrous was observed in 86% of the PZP immunized mares as compared to 14% in recombinant porcine ZP3/ZP4 immunized group, which correlated with basal serum oestradiol and progesterone levels (100). All mares resumed cyclicity by 10 months post-treatment. None of the PZP-immunized mare conceived whereas all the unimmunized mares became pregnant. Out of 7 pony mares immunized with recombinant porcine ZP3/ZP4, 3 failed to conceive. These experiments suggest the potential of recombinant proteins for controlling fertility in mares, which; however, need further investigations (100).

6. VACCINE DELIVERY: MAJOR CHALLENGE IN THE APPLICATION OF CONTRACEPTIVE VACCINES FOR WILDLIFE POPULATION MANAGEMENT

In the context of free-ranging wildlife population management, it will be ideal, if single injection of contraceptive vaccine generates adequate immune response leading to inhibition of fertility for several years or even permanent sterility. Though it has been shown that single injection of GnRH-based contraceptive vaccine may induce infertility ranging from 2–5 year in white-tailed deer (33), by and large booster doses of the contraceptive vaccine are required to achieve desirable contraceptive efficacy and duration of infertility. It will necessitate development of sensitive tracking technologies to distinguish unvaccinated animals from those that have been vaccinated and among vaccinated animals to distinguish how many prior booster dose(s) have been delivered. Additionally, it will be imperative to develop more potent adjuvants and novel vaccine delivery platforms.

6.1. Remote vaccine delivery by either dart gun or oral bait

It is an expensive and cumbersome exercise to capture the free-ranging animals to deliver the contraceptive vaccine by intramuscular route. Immunization of free-ranging large animals such as horses and white-tailed deer by remote delivery of contraceptive vaccine by dart-gun has been very useful (69, 72). However, vaccine delivery by dart gun may not be a feasible proposition in small free-ranging animals such as monkeys etc, as vaccine dart may hit some sensitive part of the body and thereby leading to injury. Another strategy to deliver vaccines may be to use oral baits. In the second half of the 20th Century, oral rabies vaccines in the form of different types of baits incorporating different strains of live replication-competent rabies virus strains were developed with an aim to eliminate rabies infection circulating in European wildlife in particular foxes (101). The oral baits were dropped by helicopters in the forests inhabitated by wild foxes. Within the past 30 years, the overall incidence of rabies in Europe has decreased by approximately 80% and it has been completely eliminated from Western and Central Europe (www.who-rabies-bulletin.org). However, delivery of contraceptive vaccine as oral baits for the management of free-ranging wildlife population may be a difficult proposition as it will have adverse consequences on the fertility of other animal species, who may also consume these baits beside the target species. Contraceptive vaccines as oral baits may still be a feasible proposition for captive wildlife, where the individual animal is fed under supervision.

6.2. Edible vaccine

Plants can be engineered to produce foreign proteins in large amount which can be fed to the animals thereby eliciting appropriate immune response. As a proof-of-principle, it was shown that genetically modified potatoes (by inserting gene encoding the heat labile enterotoxin unit B from enterotoxigenic *E. coli* bacteria) when fed to mice as well as humans led to generation of immune response against the heat labile enterotoxin unit B antigen (102, 103). In an effort to control the population of possums in New Zealand, attempts are being made to develop genetically modified carrots expressing possum ZP3 (104). However, the outcome of these efforts is not as yet published.

6.3. Virus-like particles and bacterial ghosts

Virus-like particles (VLPs) are essentially non-infective viruses, which comprised of self-assembled viral envelop proteins without the genetic material. VLPs have unique property to display multiple copies of small foreign peptides on its surface, without losing the self assembly property of

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the envelop protein, thereby leading to potent immune response against the foreign peptide (105). Their size typically falls in nanoparticles range and is stable and versatile in nature (106). VLPs offer excellent adjuvant characteristics which can induce innate and adaptive immune responses. As a matter of fact, VLPs have shown to enhance immunogenicity of peptides and proteins which otherwise are weakly immunogenic. There are various VLP vaccines that have been evaluated such as HPV L1 VLP, norovirus VLP vaccine and chikungunya (106). Johnson grass mosaic virus (JGMV) coat protein self-assemble to form rod-shaped VLPs and can be engineered to express on its surface foreign peptides (107). Immunization of mice with JGMV coat protein-based VPLs expressing a fusion peptide comprising of mouse ZP3 and spermatozoa-specific YLP_{12} peptide separated by diglycine spacer led to sub-fertility in the immunized animals (108). The basic problem with VLPs as a platform to deliver contraceptive vaccine is that in addition to antibodies generated against the foreign peptide/protein, a more potent antibody response is also generated against the envelop/coat protein of the virus.

Bacterial ghost, made-up of non-living bacterial cell without genetic component, has also been effectively used as platform to deliver antigens for eliciting immune response. Immunization of female brushtail possum with bacterial ghost expressing either N-terminal (41–316 amino acid residues) or C-terminal (308–636 amino acid residues) fragments of possum ZP2 fused to maltose-binding protein in *E. coli* NM522 strain led to the generation of both humoral and cell-mediated immune responses (109). Animals immunized with C-terminal fragment of ZP2 showed significant reduction in fertility (109). Bacterial ghost expressing brushtail possum ZP3 has also been prepared by using bacteriophage phiX174 lysis gene E in *E. coli* NM522 and their immunogenicity demonstrated in homologous animal model (110).

6.4. Live-vector based contraceptive vaccines

By using reverse genetic approach, attenuated *Salmonella typhimurium*-expressing mouse ZP3 was developed and delivered in mice through oral route. Immunized animals showed reduction in fertility (111). Mimovirus expressing B cell epitope of spermatozoa specific protein, eppin, has also been developed (112). Intranasal immunization of male mice with the above mimovirus led to generation of anti-eppin IgA antibodies in the genital tract. Mating of immunized males with unvaccinated females showed reduction in the fertility. Interestingly, immunization with the above antigen did not lead to any histopathological changes in the testes or their function (112).

Host-specific live vectors expressing various zona proteins have also been evaluated for their contraceptive potential. For example, mice infected with recombinant ectromelia virus (a natural pathogen that causes mouse pox) expressing mouse ZP3 showed infertility ranging from 5 to 9 months that was associated with disruption of ovarian folliculogenesis (113). Immunization of female rabbits with recombinant myxoma virus expressing either rabbit ZP3 or ZP4 also showed infertility accompanied with ovarian pathology (114, 115). Infection of mice with recombinant cytomegalovirus (mouse-specific β herpes virus) expressing mouse ZP3 led to permanent infertility, primarily due to induction of the ovarian autoimmune pathology leading to progressive depletion of follicles (116). Basic concept of developing host-specific live vector-based contraceptive vaccine was to release these in the environment so that the recombinant virus gets transmitted from one animal to another thereby leading to effective management of pests such as rats and rabbits. It will save the task of individually capturing the animal, followed by vaccination and releasing the immunized animals in its natural habitat. The overall concept was good; however, it was observed that recombinant viruses have lower infectivity as compared with the wild type virus, which will eventually reduce the contraceptive efficacy of live vector-based contraceptive vaccine (117, 118). Another major concern was about the consequences on the population of other species, if by chance the recombinant virus loses its host specificity (117). Keeping in view the above, it is less likely that host specific live-vector-based contraceptive vaccine will be used in near future for population management of free-ranging pests.

6.5. Microparticles for contraceptive vaccine delivery

One of the exciting option to deliver the contraceptive vaccine is to use inert nanoparticles or microparticles which themselves may not be immunogenic but provide sustained immune response against an entrapped antigen for a long period. It is primarily due to the slow release of the antigen from such particles. Liposomes comprising of phospholipids and cholesterol is one such option. Grey seals *(halichoerus grypus*) immunized with porcine ZP entrapped in liposomes, decreased fertility in 90% of the immunized animals over a 5-year period (119). Antigen entrapped in biodegradable and biocompatible polymeric particles of poly-lactide (PLA) and poly(lactic-co-glycolic acid) (PLGA) have been used to reduce the number of injections and to generate long-lasting immune response. Immunogenicity of PLA based microparticles have been shown to improve by the use of permissible adjuvant like alum (120). Incorporation of CpG (small stretches of synthetic oligodeoxy nucleotides corresponding to unmethylated DNA of bacterial origin) motif can also enhance immune response of microparticles (121).

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In general, nanoparticles favour generation of cell mediated immune response whereas microparticles favour humoral immune response (122). It has been shown that polymer particle-entrapped vaccines can elicit memory antibody response from single-point immunization (123). Therefore, a microsphere particles-based formulation supplemented with suitable adjuvant(s) is expected to reduce the number of doses of the vaccine required to achieve the desired immune response and efficacy. In addition to PLA/PLGA polymer, microparticles of water-soluble polyphosphazenes have also been used successfully as antigen delivery platform. Indeed, it was shown that microparticles of poly(di(sodiumcarboxylatoethylphen oxy) phosphazene) entrapping pertussis toxoid, CpG motif and synthetic cationic innate defense regulator peptide 1002 elicited effective immune response against *Bordetella pertussis* in mice (124). It has been shown that hCG entrapped in PLA/PLGA microparticles showed antibody response following one injection which was comparable with four injections of free hCG in rats (125). In the microparticle immunized group, antibody titres reached at peak on day 56 and maintained a steady state till day 84. Hence, it seems that contraceptive candidate proteins entrapped in PLA/PLGA based microparticles supplemented with appropriate adjuvant may lead to long-lasting immune response with lesser number of injections and thereby making single dose contraceptive vaccine a reality in future.

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Bottlenecked but long-lived: high genetic diversity retained in white-tailed eagles upon recovery from population decline

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Most of the white-tailed eagle (*Haliaeetus albicilla*) populations in Europe experienced dramatic declines during the twentieth century. However, owing to intense conservation actions and the ban of DDT and other persistent pollutants, populations are currently recovering. We show that despite passing through demographic bottlenecks, white-tailed eagle populations have retained significant levels of genetic diversity. Both genetic and ringing data indicate that migration between populations has not been a major factor for the maintenance of genetic variability. We argue that the long generation time of eagles has acted as an intrinsic buffer against loss of genetic diversity, leading to a shorter effective time of the experienced bottleneck. Notably, conservation actions taken in several small sub-populations have ensured the preservation of a larger proportion of the total genetic diversity than if conservation had focused on the population stronghold in Norway. For conservation programmes targeting other endangered, long-lived species, our results highlight the possibility for local retention of high genetic diversity in isolated remnant populations.

Keywords: bottleneck; conservation genetics; generation time; microsatellites; mitochondrial DNA; single large or several small

1. INTRODUCTION

The white-tailed eagle (*Haliaeetus albicilla*) is used as a major flagship and umbrella species for conservation work throughout large parts of Europe. The species was once widely distributed across the continent (Helander & Stjernberg 2003), but direct persecution and low reproductive success as a result of environmental pollutants led to dramatic declines during the twentieth century (Bijleveld 1974; Helander *et al.* 2002). By the 1970s, many local populations in Europe had gone extinct and most surviving sub-populations barely reached one or a few tens of breeding pairs (figure 1). However, a stronghold remained along the Norwegian Atlantic coast with at least 700–800 breeding pairs (Folkestad 2003), corresponding to more than 70% of the northern and central European population (Helander *et al.* 2003). The population along the Norwegian coast utilized relatively uncontaminated food resources from the northern Atlantic and was thus largely saved from the detrimental effects of harmful pollutants (Helander *et al.* 1982). On the other hand, around the heavily polluted Baltic Sea, white-tailed eagles suffered strongly reduced breeding success (Helander *et al.* 1982).

With the ban in the 1970s of DDT, PCB and other detrimental chemicals, European white-tailed eagle populations soon started to recover, a process that is still ongoing (figure 1). For instance, the number of territorial pairs in southern and central Sweden increased from about 40 in 1974 to 250 in 2000 (Helander 2003*a*), and in Estonia from 10–15 between 1965 and 1975 to 116 in 2002 (Volke & Randla 2003). This population expansion appears to have been based mainly on local recruitment, since almost no inter-population exchange of breeders has been recorded from ringing data. In particular, the large Norwegian population does not seem to have acted as a major founding source for recovering populations; none of the more than 3000 eagles colour-ringed in Norway has so far been found breeding outside Norway (Folkestad unpublished data). Further, among more than 150 ringed breeding birds in Sweden, all but a few individuals from the nearby Finnish coast were offspring of resident pairs (Helander 2003*b*). Similarly, most of the few recorded emigrants from the German eagle population settled to breed nearby in Poland (Köppen 2003). Given this apparent trend of local recruitment, current populations might have been depleted of genetic diversity by the recent demographic declines.

Loss of genetic diversity can reduce both short-term viability (e.g. Saccheri *et al.* 1998) and long-term adaptability (Frankham 2005). To assess the genetic impact of population declines during the twentieth century, we therefore studied the genetic variability at mitochondrial DNA (mtDNA) control region sequences and autosomal microsatellite markers in north and central European white-tailed eagle populations.

2. MATERIAL AND METHODS

Several populations from northern and central Europe were sampled, yielding a total number of 218 presumably unrelated individuals: Estonia (EST, $n=13$ samples), Germany (GER, $n=18$), Swedish Lapland (LAP, $n=24$), southern and central Sweden (SWE, $n=117$), Kola peninsula, Russia (KOLA, $n=10$) and Norway (NOR, $n=36$). Most of the samples consisted of blood, but nine samples were moulted feathers that had been collected in the field.

Polymerase chain reaction (PCR) of 26 autosomal microsatellite loci was performed (details are given in Hailer *et al.* (2005) and the electronic supplementary material). In order to directly compare

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the amount of genetic variation (expected and observed heterozygosity, H_E and H_O, and the mean number of alleles per locus, N_A) between population samples, we standardized for sample size by employing a bootstrapping procedure (see electronic supplementary material). We tested for deviation from Hardy–Weinberg equilibrium (HWE) and genotypic differentiation using GENEPOP 3.4 (Raymond & Rousset 1995). GENETIX 4.05.2 (Belkhir *et al.* 2004) was used to calculate F_{ST} and its 95% confidence interval. Assignment tests were carried out using GENECLASS 2.0.d (Piry *et al.* 2004) and demographic simulations were performed in BOTTLESIM (Kuo & Janzen 2004). For the latter, population size was held constant at 300 actively reproducing pairs for 10 years, followed by a bottleneck of 30 reproducing pairs for 40 years (see electronic supplementary material for details). The program BOTTLENECK (Cornuet & Luikart 1996) was used to detect possible traces of population bottlenecks on the allele frequency spectrum, applying the recommended settings and tests for microsatellites (two-phased model: 90% one-step mutations and 10% multi-step changes; evaluation with standardized differences and Wilcoxon tests).

A part of the mtDNA control region was amplified and sequenced using the primers *HalHVR1F* (5′-CCCCCCCTATG-TATTATTGT-3′) and *HalHVR1R* (5′-TCTCAGTGAAGAGC-GAGAGA-3′) developed for *H. albicilla*, yielding a total of 500 bp for analysis. Similar to the microsatellite analyses, we used a bootstrapping procedure to correct diversity estimates for sample size (see the electronic supplementary material). DnaSP 4.10 (Rozas *et al.* 2003) was used to determine Tajima's *D* (Tajima 1989) and G_{ST}, an estimator of genetic differentiation among populations, using haplotype identity as the underlying statistic. Sequences obtained have been placed in the EMBL database (accession numbers AM156933–AM156934 and AM156936–AM156943).

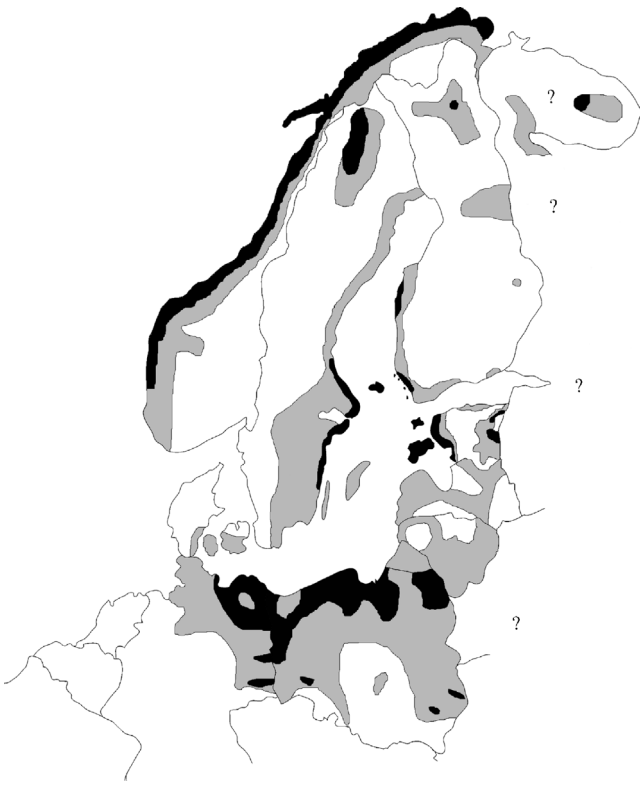


Figure 1. Distribution of the white-tailed eagle in north–central Europe. Black shading indicates regions where the species survived the mid-twentieth century population crash, light grey shading refers to regions re-colonized in the phase of population recovery since 1980.

3. RESULTS

A total of 139 alleles were observed across the 26 microsatellite markers. Significant heterozygote deficit was detected in the SWE, LAP and NOR populations. However, the signal disappeared when the locus *Hal 10* was excluded; we take this as an indication that this locus exhibits a null allele with restricted geographical distribution and that the populations otherwise do not deviate significantly from HWE.

Observed and expected heterozygosities estimated from data corrected for inter-population variation in sample size were in the range of 0.41–0.51 for all populations (table 1). Similarly, the mean number of alleles per locus did not vary much among populations, being in the range of 2.9–3.5. Importantly, there was no evidence for the large Norwegian population to show higher levels of genetic diversity ($H_E=0.48$, $H_O=0.49$, $N_A=3.1$), as compared to the other populations that had experienced a significant demographic bottleneck. Further, no population showed significant allele frequency shifts ($p>0.05$) which are expected after strong demographic bottlenecks.

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4. DISCUSSION

In summary, our data suggest that effective dispersal between populations has not been common during the past decades. We conclude that sustained genetic

Table 1. Genetic diversity at microsatellite loci. Number of samples analysed, unbiased expected (H_E) and observed (H_O) heterozygosity, mean number of alleles per locus (N_A) and their inter-locus standard deviations. For comparison between populations, standardized diversity values are also given.

population	sample size	H_E (s.d.)	corrected H_E	H_O (s.d.)	corrected H_O	N_A (s.d.)	corrected N_A
Norway	36	0.49 (0.05)	0.48	0.49 (0.02)	0.49	3.9 (2.3)	3.1
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Kola peninsula[a]	10	0.52 (0.05)	—	0.51 (0.03)	—	3.8 (2.0)	—
Lapland	24	0.51 (0.05)	0.49	0.49 (0.02)	0.49	4.2 (2.3)	3.4
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[a]diversity statistics of the remaining populations were standardized to sample size of $n=10$, corresponding to the number for Kola peninsula.

Table 2. Genetic diversity at mitochondrial control region sequences. The number of analysed eagle territories (maternal lineages) is denoted by n, and N denotes the number of unique haplotypes found in each population. To correct for unequal sample sizes among populations, the mean number of alleles encountered in 100 bootstrap samples of 10 individuals each, and the standard deviation of these values, is presented.

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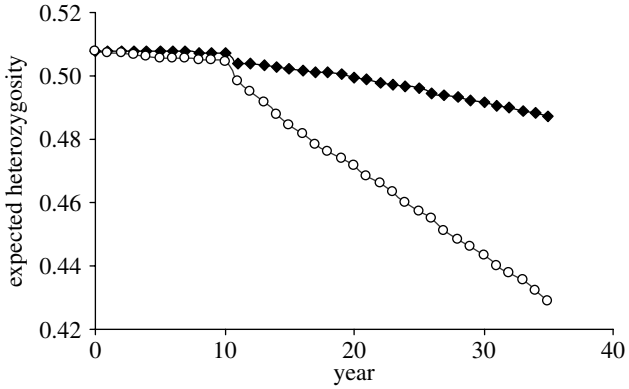


Figure 2. Loss of genetic diversity (as measured by expected heterozygosity) in a bottleneck of 30 reproducing pairs and overlapping generations. Filled diamonds and open circles consider an individual lifespan of 17 years and 1 year, respectively. The simulation was started at a population size corresponding to 300 reproducing pairs (years 0–10).

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Starting in the late 1960s, several countries in Europe developed conservation programmes to protect eagles (Helander 1990). Nest site protection enabled fertile pairs to raise fledging young. Carcasses free of contaminants were laid out in winter and, as a result, juvenile survival was increased to 80% (Helander 2003*b*). Our results, therefore, indicate that these conservation efforts have led to the preservation of ample genetic diversity in local eagle populations. Moreover, conservation of eagles at local scales has resulted in the preservation of more total genetic diversity than if all conservation efforts had been centred on the coastal Norwegian population.

There has been an intense debate among conservation biologists about reserve design (the SLOSS debate—single large or several small; reviewed in Kingsland 2002). This debate was directly linked to the relative importance of local conservation versus a focus on population strongholds. Our results illustrate the high conservation value of local populations for species with a long generation time. A number of other species of conservation concern also have long generation times (e.g. raptors, turtles, large mammals and orchids) and for many of these significant genetic diversity may still exist in local populations. For the white-tailed eagle, protection of the local populations did not arrive too late.

The sampling of blood for this study was coordinated by the Swedish Society for Nature Conservation/Project Sea Eagle. We thank Kurt Elmqvist and Robert Franzén for assistance with the sampling of blood in the field in Sweden, Andreas Mertens for contributing feather samples, Torsten Stjernberg for comments on the distribution map and Mia Olsson for assistance in the lab. Deborah Dawson and Ruth Tingay kindly provided their markers prior to publication. The editor and two anonymous reviewers are thanked for constructive comments. This work was financially supported by Alvin's foundation, the Sven and Lilli Lawski foundation and the Knut and Alice Wallenberg foundation (to F.H.). H.E. is a Royal Swedish Academy of Sciences Research Fellow supported by a grant from Knut and the Alice Wallenberg foundation.

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Bottlenecked but long-lived: high genetic diversity retained in white-tailed eagles upon recovery from population decline

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Most of the white-tailed eagle (*Haliaeetus albicilla*) populations in Europe experienced dramatic declines during the twentieth century. However, owing to intense conservation actions and the ban of DDT and other persistent pollutants, populations are currently recovering. We show that despite passing through demographic bottlenecks, white-tailed eagle populations have retained significant levels of genetic diversity. Both genetic and ringing data indicate that migration between populations has not been a major factor for the maintenance of genetic variability. We argue that the long generation time of eagles has acted as an intrinsic buffer against loss of genetic diversity, leading to a shorter effective time of the experienced bottleneck. Notably, conservation actions taken in several small sub-populations have ensured the preservation of a larger proportion of the total genetic diversity than if conservation had focused on the population stronghold in Norway. For conservation programmes targeting other endangered, long-lived species, our results highlight the possibility for local retention of high genetic diversity in isolated remnant populations.

Keywords: bottleneck; conservation genetics; generation time; microsatellites; mitochondrial DNA; single large or several small

1. INTRODUCTION

The white-tailed eagle (*Haliaeetus albicilla*) is used as a major flagship and umbrella species for conservation work throughout large parts of Europe. The species was once widely distributed across the continent (Helander & Stjernberg 2003), but direct persecution and low reproductive success as a result of environmental pollutants led to dramatic declines during the twentieth century (Bijleveld 1974; Helander *et al.* 2002). By the 1970s, many local populations in Europe had gone extinct and most surviving sub-populations barely reached one or a few tens of breeding pairs (figure 1). However, a stronghold remained along the Norwegian Atlantic coast with at least 700–800 breeding pairs (Folkestad 2003), corresponding to more than 70% of the northern and central European population (Helander *et al.* 2003). The population along the Norwegian coast utilized relatively uncontaminated food resources from the northern Atlantic and was thus largely saved from the detrimental effects of harmful pollutants (Helander *et al.* 1982). On the other hand, around the heavily polluted Baltic Sea, white-tailed eagles suffered strongly reduced breeding success (Helander *et al.* 1982).

With the ban in the 1970s of DDT, PCB and other detrimental chemicals, European white-tailed eagle populations soon started to recover, a process that is still ongoing (figure 1). For instance, the number of territorial pairs in southern and central Sweden increased from about 40 in 1974 to 250 in 2000 (Helander 2003*a*), and in Estonia from 10–15 between 1965 and 1975 to 116 in 2002 (Volke & Randla 2003). This population expansion appears to have been based mainly on local recruitment, since almost no inter-population exchange of breeders has been recorded from ringing data. In particular, the large Norwegian population does not seem to have acted as a major founding source for recovering populations; none of the more than 3000 eagles colour-ringed in Norway has so far been found breeding outside Norway (Folkestad unpublished data). Further, among more than 150 ringed breeding birds in Sweden, all but a few individuals from the nearby Finnish coast were offspring of resident pairs (Helander 2003*b*). Similarly, most of the few recorded emigrants from the German eagle population settled to breed nearby in Poland (Köppen 2003). Given this apparent trend of local recruitment, current populations might have been depleted of genetic diversity by the recent demographic declines.

Loss of genetic diversity can reduce both short-term viability (e.g. Saccheri *et al.* 1998) and long-term adaptability (Frankham 2005). To assess the genetic impact of population declines during the twentieth century, we therefore studied the genetic variability at mitochondrial DNA (mtDNA) control region sequences and autosomal microsatellite markers in north and central European white-tailed eagle populations.

2. MATERIAL AND METHODS

Several populations from northern and central Europe were sampled, yielding a total number of 218 presumably unrelated individuals: Estonia (EST, $n=13$ samples), Germany (GER, $n=18$), Swedish Lapland (LAP, $n=24$), southern and central Sweden (SWE, $n=117$), Kola peninsula, Russia (KOLA, $n=10$) and Norway (NOR, $n=36$). Most of the samples consisted of blood, but nine samples were moulted feathers that had been collected in the field.

Polymerase chain reaction (PCR) of 26 autosomal microsatellite loci was performed (details are given in Hailer *et al.* (2005) and the electronic supplementary material). In order to directly compare

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the amount of genetic variation (expected and observed heterozygosity, H_E and H_O, and the mean number of alleles per locus, N_A) between population samples, we standardized for sample size by employing a bootstrapping procedure (see electronic supplementary material). We tested for deviation from Hardy–Weinberg equilibrium (HWE) and genotypic differentiation using GENEPOP 3.4 (Raymond & Rousset 1995). GENETIX 4.05.2 (Belkhir *et al.* 2004) was used to calculate F_{ST} and its 95% confidence interval. Assignment tests were carried out using GENECLASS 2.0.d (Piry *et al.* 2004) and demographic simulations were performed in BOTTLESIM (Kuo & Janzen 2004). For the latter, population size was held constant at 300 actively reproducing pairs for 10 years, followed by a bottleneck of 30 reproducing pairs for 40 years (see electronic supplementary material for details). The program BOTTLENECK (Cornuet & Luikart 1996) was used to detect possible traces of population bottlenecks on the allele frequency spectrum, applying the recommended settings and tests for microsatellites (two-phased model: 90% one-step mutations and 10% multi-step changes; evaluation with standardized differences and Wilcoxon tests).

A part of the mtDNA control region was amplified and sequenced using the primers *HalHVR1F* (5′-CCCCCCCTATG-TATTATTGT-3′) and *HalHVR1R* (5′-TCTCAGTGAAGAGC-GAGAGA-3′) developed for *H. albicilla*, yielding a total of 500 bp for analysis. Similar to the microsatellite analyses, we used a bootstrapping procedure to correct diversity estimates for sample size (see the electronic supplementary material). DnaSP 4.10 (Rozas *et al.* 2003) was used to determine Tajima's *D* (Tajima 1989) and G_{ST}, an estimator of genetic differentiation among populations, using haplotype identity as the underlying statistic. Sequences obtained have been placed in the EMBL database (accession numbers AM156933–AM156934 and AM156936–AM156943).

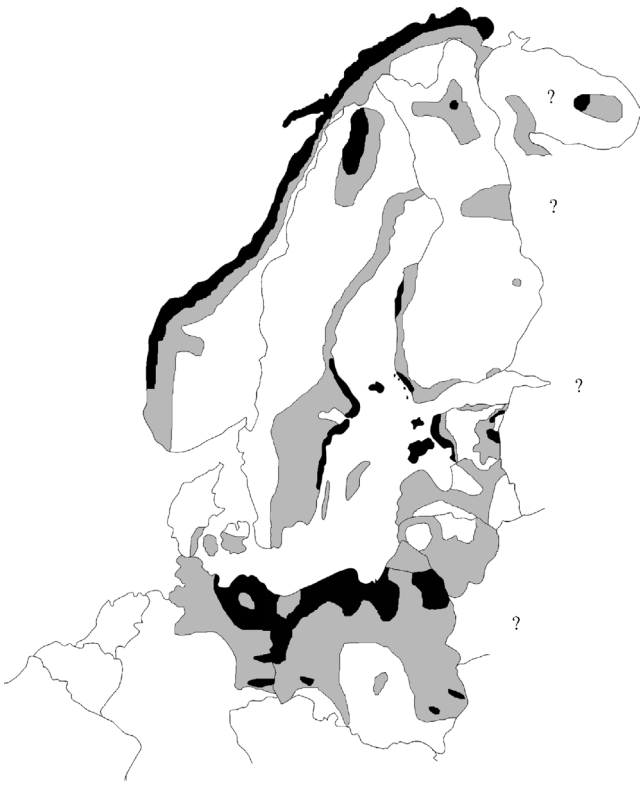


Figure 1. Distribution of the white-tailed eagle in north–central Europe. Black shading indicates regions where the species survived the mid-twentieth century population crash, light grey shading refers to regions re-colonized in the phase of population recovery since 1980.

3. RESULTS

A total of 139 alleles were observed across the 26 microsatellite markers. Significant heterozygote deficit was detected in the SWE, LAP and NOR populations. However, the signal disappeared when the locus *Hal 10* was excluded; we take this as an indication that this locus exhibits a null allele with restricted geographical distribution and that the populations otherwise do not deviate significantly from HWE.

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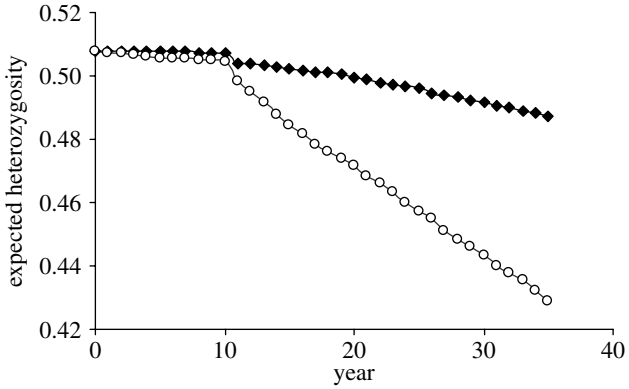


Figure 2. Loss of genetic diversity (as measured by expected heterozygosity) in a bottleneck of 30 reproducing pairs and overlapping generations. Filled diamonds and open circles consider an individual lifespan of 17 years and 1 year, respectively. The simulation was started at a population size corresponding to 300 reproducing pairs (years 0–10).

diversity in extant north–central European white-tailed eagle populations has a predominantly local origin, although some immigration may have contributed to local gene pools. We suggest that the long generation time of white-tailed eagles (average life span is around 17 years; Helander 2003*b*; Struwe-Juhl 2003) has minimized genetic drift and acted as an intrinsic buffer against rapid loss of genetic diversity. When a randomly mating population passes through a bottleneck, reduction of genetic diversity depends on population size and time according to $H_t=H_0(1-1/(2N_e))^t$; H_0 and H_t being the past and present heterozygosity, respectively, N_e the effective population size and t the time measured in number of generations. A bottleneck lasting about 20–30 years is equivalent to about two white-tailed eagle generations. Simulation of a bottleneck comprising 30 reproductive pairs (which corresponds to the situation in several European populations during the 1970s; see §1) shows that only about 4% of the original heterozygosity is expected to be lost in an eagle population during that time (figure 2). By contrast, for an organism with a generation time of 1 year, the corresponding loss of genetic diversity is considerably higher (16%; figure 2). Our results are compatible with the relatively high levels of genetic diversity seen in remnant and bottlenecked populations of other long-lived species, e.g. black rhinoceros (Swart *et al.* 1994) and greater one-horned rhinoceros (Dinerstein & McCracken 1990).

Starting in the late 1960s, several countries in Europe developed conservation programmes to protect eagles (Helander 1990). Nest site protection enabled fertile pairs to raise fledging young. Carcasses free of contaminants were laid out in winter and, as a result, juvenile survival was increased to 80% (Helander 2003*b*). Our results, therefore, indicate that these conservation efforts have led to the preservation of ample genetic diversity in local eagle populations. Moreover, conservation of eagles at local scales has resulted in the preservation of more total genetic diversity than if all conservation efforts had been centred on the coastal Norwegian population.

There has been an intense debate among conservation biologists about reserve design (the SLOSS debate—single large or several small; reviewed in Kingsland 2002). This debate was directly linked to the relative importance of local conservation versus a focus on population strongholds. Our results illustrate the high conservation value of local populations for species with a long generation time. A number of other species of conservation concern also have long generation times (e.g. raptors, turtles, large mammals and orchids) and for many of these significant genetic diversity may still exist in local populations. For the white-tailed eagle, protection of the local populations did not arrive too late.

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Review

Non-surgical sterilisation methods may offer a sustainable solution to feral horse (*Equus caballus*) overpopulation

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Abstract. Feral horses are a significant pest species in many parts of the world, contributing to land erosion, weed dispersal and the loss of native flora and fauna. There is an urgent need to modify feral horse management strategies to achieve public acceptance and long-term population control. One way to achieve this is by using non-surgical methods of sterilisation, which are suitable in the context of this mobile and long-lived species. In this review we consider the benefits of implementing novel mechanisms designed to elicit a state of permanent sterility (including redox cycling to generate oxidative stress in the gonad, random peptide phage display to target non-renewable germ cells and the generation of autoantibodies against proteins essential for conception via covalent modification) compared with that of traditional immunocontraceptive approaches. The need for a better understanding of mare folliculogenesis and conception factors, including maternal recognition of pregnancy, is also reviewed because they hold considerable potential in providing a non-surgical mechanism for sterilisation. In conclusion, the authors contend that non-surgical measures that are single shot and irreversible may provide a sustainable and effective strategy for feral horse control.

Additional keywords: fertility, immunocontraception, oxidative stress, primordial germ cells, spermatozoa.

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Introduction

Feral horses (*Equus caballus*) are a significant pest species in many parts of the world, particularly in the US, regions of Europe, Australia and New Zealand (Long 2003). The large, hard-hooved herbivore is responsible for considerable environmental damage (Dyring 1990; Andreoni 1998; English 2000), the spread of disease (Dobbie *et al.* 1993; Campbell *et al.* 2002; Cheng and Currie 2005; Dawson *et al.* 2006) and an indirect decline in native flora and fauna populations (Dobbie *et al.* 1993; Symanski 1994; Levin *et al.* 2002; Beever and Brussard 2004; Zalba and Cozzani 2004; Beever and Herrick 2006). A lack of predators and favourable environmental conditions can allow feral horse numbers to rise by 20% per annum (Eberhardt *et al.* 1982; Dobbie *et al.* 1993; Dawson *et al.* 2006; National Research Council 2013). In Australia, the population of feral horses is in excess of 600 000 brumbies, notably the largest feral horse population in the world (Berman and Jarman 1987; Clement *et al.* 1990; Dawson *et al.* 2006; Nimmo and Miller 2007). Integrated pest management strategies for feral horses include trapping and mustering, where animals are euthanased and sold for meat or trained and used as stock horses (Rolls 1969; Warren *et al.* 1993; Symanski 1994; Csurhes *et al.* 2009; Garrott and Oli 2013). However, the most extensively practiced method for population control is culling in the form of aerial shooting (English 2000; Dawson *et al.* 2006; English 2006). Although the requirements to be a shooter are high, the method remains contentious (Symanski 1994; Chapple 2005; Bradshaw *et al.* 2007; Nimmo and Miller 2007). Aerial shooting of feral horses is largely unaccepted by the public because free-ranging horses are often considered a cultural icon and an important part of national heritage (Berman 1991; Bomford and O'Brien 1997; Chapple 2005; Dawson *et al.* 2006; Bradshaw *et al.* 2007; Nimmo and Miller 2007; Killian *et al.* 2008). Furthermore, most control methods are laborious, expensive and ineffective, markedly failing to reduce the population growth rate of this pest species (Dobbie *et al.* 1993; Symanski 1994; Dawson *et al.* 2006; Killian *et al.* 2008; National Research Council 2013). Legal restrictions have been used to ban aerial shooting (English 2000; Chapple 2005), which has created an urgent need to implement secondary control strategies that are publicly accepted and offer a sustainable solution to feral horse overpopulation.

Considerable progress has been made in the area of immunocontraceptive development (Fagerstone *et al.* 2010; Gray and

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Table 1. Summary of contraceptive options currently available and emerging for feral horse management
For each contraceptive, the duration of effect, efficacy and the sex to which the method pertains are listed. GnRH, gonadotrophin-releasing hormone; ZP, zona pellucida

Target	Contraceptive	Stage of development	Sex	No. doses	Contraceptive coverage	Efficacy (%)	References
GnRH	Equity	Established	Male and female	2	1 year	100	Elhay *et al.* (2007), Janett *et al.* (2009)
	Improvac	Established	Female	2	1–2 years	100	Imboden *et al.* (2006), Botha *et al.* (2008)
	GonaCon	Established	Male and female	1	1–4 years	40–94	Killian *et al.* (2006), Killian *et al.* (2008), Miller *et al.* (2008), Gray *et al.* (2010)
ZP	SpayVac	Established	Female	1	≥3 years	83–100	Powell and Monfort (2001), Killian *et al.* (2006), Killian *et al.* (2008), Turner *et al.* (2008), Gray *et al.* (2010), Bechert *et al.* (2013), Mask *et al.* (2015)
Proteins found on primordial follicles, spermatogonial stem cells, Sertoli cells	Phage peptides	Emerging	Male and female	1	Lifelong (intended)	–	Eidne *et al.* (2000), Meloen *et al.* (2000), Aitken (2006), Samoylova *et al.* (2010, 2012, 2012, 2015)
Proteins found on spermatozoa, oocyte and blastocyst	Aldehyde-adducted proteins	Future	Male and female	1	Lifelong (intended)	–	Dixit *et al.* (1975), Bohring and Krause (2003), Frank *et al.* (2005), Klein *et al.* (2010), Wang *et al.* (2012)

Cameron 2010; Kirkpatrick *et al.* 2011; Massei and Cowan 2014), but a sustainable and effective method for free-ranging populations has yet to be implemented successfully (Bomford and O'Brien 1997; English 2000; Dawson *et al.* 2006; Hinds 2006; Killian *et al.* 2008; Powers *et al.* 2011; National Research Council 2013). This is due, at least in part, to the need for frequent readministration. One way to overcome this is by using non-surgical methods of sterilisation, which are suitable in the context of this mobile and long-lived species (Dell'Omo and Palmery 2002; National Research Council 2013; Amory *et al.* 2014; Aitken 2015; Naz and Saver 2016). The success of such a formulation is likely predicated on its ability to induce a state of permanent and irreversible sterility. It is highly desirable that the sterilant is cell and species specific and capable of affecting both sexes uniformly to cause permanent sterility after a single application. Herein we briefly discuss fertility control vaccines currently available and review novel approaches that remove the need for readministration (Table 1). Novel methods of non-surgical sterilisation may prove preferable in the context of integrated management approaches to feral horse populations.

Fertility control currently used for feral horse management

Compared with human and domestic animal contraception, the implementation of effective fertility control strategies for feral horses encompasses several unique challenges (Killian *et al.* 2008; Kirkpatrick *et al.* 2011). Issues surrounding specificity, reversibility, methods of delivery and variability in response have restricted the widespread adoption of fertility control strategies as part of integrated management approaches (Stout and Colenbrander 2004). Notwithstanding these limitations, at least two contraceptive methods based on targeting critical aspects of gonadal regulation or gamete interaction have shown promise for feral horse management (Fig. 1).

The first gonadotrophin-releasing hormone (GnRH) immunisation in horses was conducted by Schanbacher and Pratt (1985) to assess its effect on cryptorchidism in stallions. The ensuing three decades saw a proliferation of GnRH-targeting strategies implemented for fertility control (Stout and Colenbrander 2004; Fagerstone *et al.* 2010), but many methods were largely discounted for free-ranging species in favour of vaccination regimens incorporating formulations of the GnRH peptide (or a conjugated form of the hormone; Miller *et al.* 2008; Powers *et al.* 2011, 2014). Pertinent for both sexes, GnRH formulations assessed in stallions have been shown to affect sperm motility acutely before resulting in a complete loss of libido, disrupted spermatogenesis and reduction in testis size, measured 2 weeks after the second immunisation (Turkstra *et al.* 2005).

Several commercial GnRH vaccines are currently in production, including Improvac (CSL), Equity (Pfizer Animal Health) and GonaCon (United States Department of Agriculture National Wildlife Research Centre). In studies by Imboden *et al.* (2006), two doses of Improvac in the mare permitted a contraceptive coverage of 23 weeks, but this coincided with an undesirable reaction to the adjuvant formulated into this commercial vaccine. Subsequent studies using Improvac reported no adverse reaction (Botha *et al.* 2008). Equity, a vaccine created specifically for horse contraception, generated better coverage (at least 1 year) with no adverse reactions to the immunisation (Elhay *et al.* 2007). Finally, 4 years after GonaCon delivery, 40% of mares experienced a continued contraceptive effect (Killian *et al.* 2008).

In the US, these vaccines are not commercially available, leading Donovan *et al.* (2013) to instead test a commercially available canine GnRH in mares. The findings of that study revealed the vaccine inhibited ovarian function, but also altered reproductive behaviours that are integral to the maintenance of the complex social structure of herd animals such as horses.

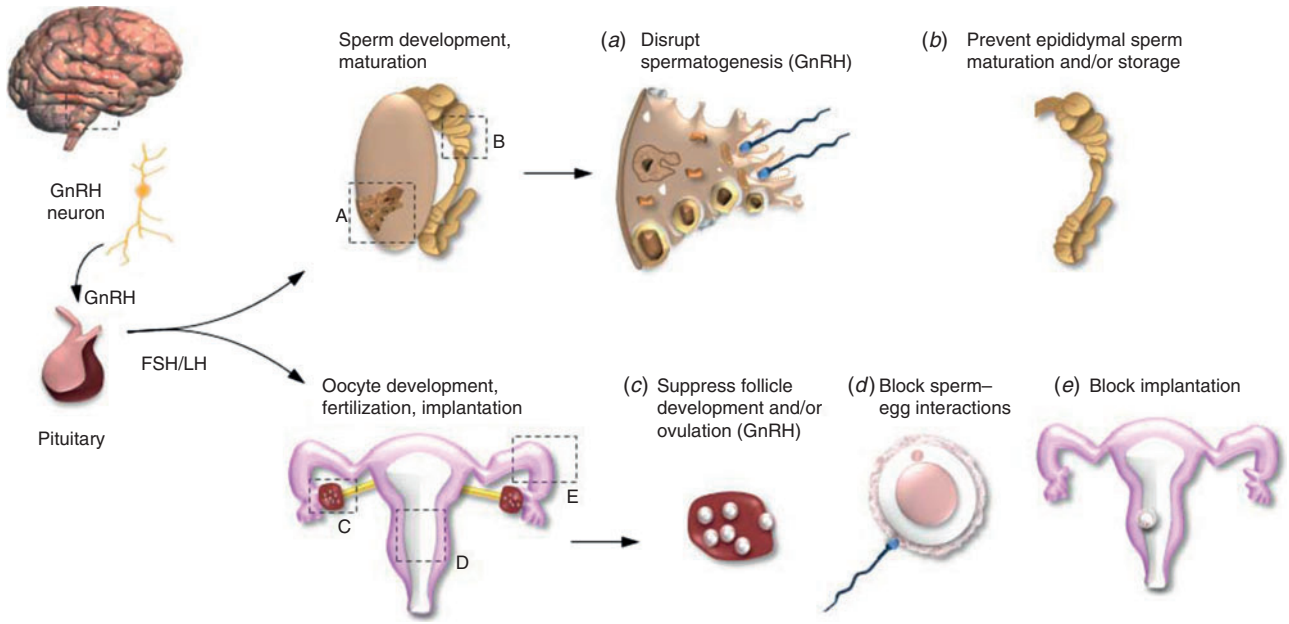


Fig. 1. Target sites for contraceptive intervention. In the male reproductive system, fertility control methods are used to disrupt spermatogenesis (gonadotrophin-releasing hormone (GnRH); A) or prevent epididymal sperm maturation and/or storage (B). In the female reproductive system, fertility control methods suppress follicle development and/or ovulation (C), block sperm–egg interactions (D) or block implantation (E).

Kisspeptin has also been investigated because it plays an important role in initiating GnRH secretion, but research is currently in its infancy and it is difficult to investigate in the horse (Decourt *et al.* 2008). Altering normal GnRH activity through the use of agonists has also proven quite difficult in mares (Fitzgerald *et al.* 1993) due to internalisation of GnRH and downregulation of GnRH receptors (Kutzler and Wood 2006; Finch *et al.* 2009). Additional issues are that these vaccines are neither tissue nor species specific (Kirkpatrick *et al.* 2011; Samoylova *et al.* 2012), and booster immunisations are required to maintain effective antibody titres (Herbert and Trigg 2005). A more promising approach has centred on the development of anti-zona pellucida (ZP) vaccines incorporating native formulations of whole ZP that have been harvested from the pig, a tissue resource that is readily available from abattoirs (Kutzler and Wood 2006; McLaughlin and Aitken 2011). Such vaccines exploit the high degree of species conservation among the ZP proteins and have been used on over 80 species, with variable degrees of efficacy (Kirkpatrick *et al.* 2011). This may be due to the unique differences in the number and localisation of ZP glycoproteins between species (Mugnier *et al.* 2009). The anti-ZP antibodies generated following immunisation bind antigenic sites on the outer surface of the ZP and thereby competitively inhibit sperm binding or prevent sperm accessing target ligands through a process of steric hindrance (Aitken and Richardson 1981; Liu *et al.* 1989).

Liu *et al.* (1989) first demonstrated the potential applicability of anti-porcine (p) ZP as an immunocontraceptive in captive mares following four injections of the vaccine. Subsequently, its efficacy in controlling feral horse populations was assessed in field trials targeting a herd of free-ranging horses inhabiting Assateague Island in the US (Kirkpatrick *et al.* 1990). That study demonstrated that remote delivery of the anti-pZP vaccine could successfully impede fertility of both pregnant and non-pregnant mares without affecting foetal health. These novel findings stimulated a series of elegant secondary studies assessing reversibility (Kirkpatrick and Turner 2002; Feh 2012), time to population decline (Kirkpatrick and Turner 2008), duration of contraceptive effect (Turner *et al.* 2008; Ransom *et al.* 2011) and changes to social behaviours (Nuñez *et al.* 2009, 2010; Madosky *et al.* 2010; Ransom *et al.* 2010). Contraceptive studies were conducted predominantly using a pelleted pZP vaccine called PZP-22, before registration of ZonaStat-H by the Humane Society of the United States as a contraceptive for free-ranging horses (Mask *et al.* 2015). However, the most recent commercially available pZP vaccine is SpayVac (IVT), a formulation that has demonstrated continued fertility control in feral horses for at least 3 years after a single administration (Killian *et al.* 2008; Bechert *et al.* 2013). Although directed against ZP glycoprotein 3 (ZP3), SpayVac is regarded as less pure than either PZP-22 or ZonaStat-H, potentially increasing its antigenicity and making it the best candidate for field application (National Research Council 2013; Mask *et al.* 2015).

In studies that have sought to directly compare the efficacy of anti-pZP vaccination with that of anti-GnRH for feral horse control, the former has generally proven more favourable (Bechert *et al.* 2013; National Research Council 2013). Two studies by Killian *et al.* (2006, 2008) found higher rates of infertility (83%) in mares 3 years after a single administration of SpayVac, compared with only 60% infertility after treatment with GonaCon. Notwithstanding these promising data, studies of anti-pZP have reported females exhibiting prolonged reproductive behaviours (Nuñez *et al.* 2010; Ransom *et al.* 2010), as well as decreased band fidelity, which significantly affects overall

harem stability (Nuñez *et al.* 2009; Madosky *et al.* 2010). Although such atypical behaviour may be a common feature of immunocontraceptive strategies (National Research Council 2013; Ransom *et al.* 2013), targeting the conserved molecular structure of the mammalian ZP proteins does impose additional limitations with regard to a lack of species specificity (Samoylova *et al.* 2012). Furthermore, the vaccine is only useful as a female contraceptive and the immunological response varies between individual animals treated (Kirkpatrick *et al.* 2012). Following SpayVac administration to eight mares, Mask *et al.* (2015) reported that although all produced elevated anti-ZP antibody titres, one mare was still able to conceive, inferring antibody titres are not a stand-alone representation of fertility cessation.

Additional consequences of anti-pZP immunisation tend to vary between species (Barber and Fayrer-Hosken 2000; Prasad *et al.* 2000), but include interference with follicle maturation and ovulation as well as primordial follicle depletion (Skinner *et al.* 1984; Paterson *et al.* 1998; Tung *et al.* 2002; Bechert *et al.* 2013). In feral horses, ovarian pathology was reported following 3 years of immunocontraception with anti-pZP (Powell and Monfort 2001). The mechanisms underpinning the induction of such pathologies warrant further investigation because they may provide insight into the development of a novel permanent fertility control agent that persists over the lifetime of the animal and thus removes the need for readministration.

Non-surgical sterilisation technologies

Induction of reproductive senescence

Most contraceptive strategies for animal population control require readministration (Asa *et al.* 2010) because they target regenerative proteins or secretory products, making the immune response a very inefficient process. Prolonging the efficacy of vaccines by lengthening the time to readministration or removing the need for readministration completely requires targeting non-renewable germ cells, and is a critically important consideration for long-term, sustainable feral horse control. Primordial follicles in the ovary and spermatogonial stem cells and Sertoli cells in the testis are essential for the expression of normal fertility. These cells represent ideal targets for fertility control in that they are terminally differentiated cell types that cannot be replenished by the reproductive system (Aitken and Curry 2011). Thus, once these cells have been depleted, the reproductive potential of the animal is lost (Meirow and Nugent 2001; Aitken *et al.* 2004; Sobinoff *et al.* 2010).

A variety of cellular insults have been identified that are capable of inducing germ cell depletion and hence infertility, including autoimmune disease, exposure to radiation, xenobiotics and other cytotoxic agents (Aitken *et al.* 2004; Silva *et al.* 2014). These insults generally lead to the production of free radicals, such as reactive oxygen species (ROS), and thus exploit the sensitivity of the developing gamete to a state of oxidative stress (Hoyer *et al.* 2001; Hanoux *et al.* 2007). The process of redox cycling has been well documented for a variety of xenobiotics, such as pesticides, insecticides, flame retardants, polycyclic aromatic hydrocarbons and dioxins (Hoyer *et al.* 2001; Aitken *et al.* 2004; Aitken 2008; Aitken and De Iuliis 2010). The generation of superoxide radicals begins a cascade of events leading to the formation of ROS, lipid peroxidation and subsequent electrophile generation (Gutierrez 2000; Aitken *et al.* 2012). Reagents responsible for redox cycling and the generation of oxidative stress could make ideal contraceptive agents (Aitken and Baker 2006), particularly in the case of horse spermatozoa, because they contain high levels of polyunsaturated fatty acids, rendering them highly susceptible to electrophile accumulation and DNA damage (Aitken *et al.* 2012).

Oxidative stress associated with excessive ROS generation and the induction of lipid peroxidation is an acknowledged cause of spontaneous infertility in human males (Aitken and Clarkson 1987; Moazamian *et al.* 2015). Similarly, in the female reproductive system, numerous studies have examined free radical production through redox cycling leading to oxidative stress capable of depleting the germ cell pool (Meirow and Nugent 2001; McLaughlin *et al.* 2003; Pujianto *et al.* 2010; Sobinoff *et al.* 2010; Kim *et al.* 2013). As a specific example, menadione, a redox active naphthoquinone, causes widespread oxidative stress in male and female gametes leading to significant DNA damage and loss of functionality (De Iuliis *et al.* 2006; Sobinoff *et al.* 2010; Aitken *et al.* 2013).

Such proof-of-concept studies provide evidence that a localised state of oxidative stress within the gonad can cause infertility. One technology that may prove amenable to selectively delivering this form of cellular insult to the gonad is that of random peptide phage display (Meloen *et al.* 2000). Initially introduced in a contraceptive context by Eidne *et al.* (2000), this technology was used to generate peptides against the sperm surface that bound to the equatorial segment and interfered with sperm–oocyte fusion. Subsequently, random peptide phage display technology was used in dogs (Samoylova *et al.* 2010) and pigs (Samoylova *et al.* 2012) and succeeded in identifying key sperm proteins that mediate adhesion to the ZP in these species. Similar techniques have also been used to increase the immunogenicity of GnRH (Sabeur *et al.* 2003), which has low antibody response due to its prevalence in the body (Samoylov *et al.* 2012). Although these reports highlight the usefulness of using targeting peptides to directly disrupt normal cellular signalling and affect fertility, there is also the opportunity to couple these peptides with redox cycling xenobiotics to ensure apoptosis of the target cells and non-surgical sterilisation (Aitken 2006). This strategy relies on iterative rounds of selection to identify bacteriophage expressing peptide inserts that bind to the target cell types (e.g. the pregranulosa cells and oocyte that make up primordial follicles in the ovary, and spermatogonial stem cells and Sertoli cells in the testis) with a high degree of affinity and specificity (Eidne *et al.* 2000; Samoylova *et al.* 2012). These peptides are then conjugated to xenobiotics engineered in a manner that permits their delivery only in the immediate vicinity of the target cell, thereby ensuring both the efficacy and safety of the approach (Humphrys and Lapidge 2008). Substantial progress with this peptide technology has been achieved in recent years, enabling xenobiotics to be coupled to phage peptides in such a way that they can be targeted directly to the gonad with a single intraperitoneal (i.p.) injection. For example, in studies by Amory *et al.* (2014), an FSHβ–melphalan peptide conjugate suppressed normal spermatogenesis in mice.

Another mechanism to recruit non-renewable germs cells is via local activation of signalling molecules within the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway (Reddy *et al.* 2008; McLaughlin and McIver 2009; Sobinoff *et al.* 2012). During folliculogenesis, activation of the PI3K/AKT/mTOR pathway determines the rate of primordial follicle activation, mediated by phosphatase and tensin homologue (PTEN) phosphorylation (Keating *et al.* 2009; Sobinoff *et al.* 2010; Kim *et al.* 2013). Uncontrolled activation of the PI3K/AKT/mTOR pathway has been postulated as a main cause for premature ovarian failure. In a study by Adhikari *et al.* (2012), bisperoxovanadium, a known PTEN inhibitor, directly disrupted primordial follicle senescence by activating the PI3K/AKT/mTOR pathway. Exposure to 4-vinylcyclohexene diepoxide also delivered this effect, depleting ovarian follicle number in murine studies (Hoyer *et al.* 2001; Mayer *et al.* 2002; Dyer *et al.* 2013). Importantly, unregulated activation of this pathway has been associated with a concomitant increase in ROS production (Kim *et al.* 2013). Delivery of a local PTEN inhibitor could be achieved using peptides targets, but it would first be necessary to ensure gonadal signalling processes that are well characterised in the mouse model are conserved and function in the same manner in the horse. Indeed, little is known about horse gametogenesis compared with other species and, although many mechanisms are highly conserved, underpinning the mechanisms behind both spermato- and folliculo- genesis is crucial to the development of these technologies.

Generation of autoantibodies

A vaccine's efficacy is underpinned by its ability to present a foreign antigen to the body in such a way that it elicits an immune response that leaves the animal with lifelong immunity (Muller *et al.* 1997; Tung *et al.* 1999; Hardy *et al.* 2006). However, in the case of autoimmunity, events can occur whereby the immune system will fail to maintain tolerance and mount an immune response against self-antigens. Indeed, ovarian autoantibodies have been cited as responsible for reproductive failure in several cases, eliciting autoimmune oophoritis and premature ovarian failure (Garza and Tung 1995; Forges *et al.* 2004; Bakalov *et al.* 2005; Altuntas *et al.* 2006; Silva *et al.* 2014). Similarly, with regard to males, autoantibodies against sperm proteins are often generated in the male if the blood–testis barrier is breached (e.g. vasectomy; Bohring and Krause 2003). Spermatozoa are unique in that they are generated after immunological tolerance has been established in the body. Therefore, these cells have value as targets for fertility regulation because they are potentially antigenic (Alexander and Anderson 1979). In a study by Bohring and Krause (2003), the presence of anti-sperm antibodies was found to interfere with factors essential for fertilisation, including sperm morphology, sperm–oolemma binding ability and acrosome integrity. A variety of proteins have been investigated for their importance in reproductive processes and it is becoming increasingly evident that the development of these immune responses involves the targeting of a multitude of proteins, rather than singular entities, that are essential in sperm–egg binding and fertilisation (Nixon *et al.* 2007). Although oocyte- and ovarian-specific proteins are difficult to identify, many sperm-specific antigens have been discovered (Bohring and Krause 2003). An extensive overview of promising protein targets for immunocontraception in the horse can be found elsewhere (Swegen and Aitken 2016).

For feral horse control, the potential to identify antigens present on terminally differentiated cells allows for the development of innovative contraceptives with lifelong effectiveness. However, we are currently faced with a challenge of not only defining the precise subset of proteins involved in sperm–egg binding, but also delivering them to the immune system in such a manner that will elicit an immune response significant enough to cause infertility. Therefore, a method that can identify proteins essential for conception holds considerable promise. In a study by Wang *et al.* (2012), proteins were found to generate immune responses once they had been covalently modified as a result of becoming alkylated by electrophilic lipid aldehydes, such as 4-hydroxynonenal (4HNE) and malondialdehyde. In particular, Wang *et al.* (2012) found that aldehyde-modified proteins were capable of eliciting autoantibody formation and thus contribute to autoimmune disease pathogenesis. In this instance, autoimmunity arises because immunological tolerance to important protein antigens has been compromised (McLaughlin and Aitken 2011). By altering protein composition in such a way as to break tolerance, aldehyde-modified proteins are capable of eliciting an increase in immunogenicity that could result in a long-term contraceptive effect and even permanent sterility. This principle was established many years ago in dogs, where alkylation of sperm proteins using α-chlorohydrin caused lesions in the testis inhibiting spermatogenesis (Dixit *et al.* 1975). The concept of using aldehyde-modified sperm proteins as immunogens for long-term feral horse fertility control is promising given the evidence that pathological autoimmunity can be elicited in instances where proteins have become modified as a consequence of oxidative damage or adduction with small molecular mass metabolites, such as 4HNE or homocysteine thiolactone (Jakubowski 2010; Aitken *et al.* 2012; Gibb *et al.* 2014). We propose that aldehyde adducts on what are otherwise unknown proteins could be exploited to identify key proteins involved in conception and break immune tolerance to induce a state of autoimmunity in horses.

Maternal recognition of pregnancy

In the early stages of pregnancy, a process commonly referred to as 'maternal recognition of pregnancy' is initiated, whereby the conceptus secretes messages to promote the continued production of progesterone by the corpus luteum (CL), which is essential for supporting early embryonic development and survival (Bazer *et al.* 1994; Aitken 2002; McLaughlin *et al.* 2003; Gupta and Bansal 2010). In many animals, the maternal recognition of pregnancy is well understood. For example in ruminants, mononucleate cells of the trophectoderm release large amounts of interferon-τ (IFNT), which suppresses prostaglandin (PG) $F_{2\alpha}$ release from the endometrium (Roberts *et al.* 1996; Spencer and Bazer 2004). In comparison, the pig trophoblast produces oestrogen as it elongates in the uterine horn, which counteracts the effects of $PGF_{2\alpha}$ on the CL (Bazer *et al.* 1994; Klein *et al.* 2010; Swegen and Aitken 2016). However, the trophoblast of the horse

conceptus is unable to elongate due to internal turgor pressure (Allen and Wilsher 2009). Instead, $PGF_{2\alpha}$ and PGE_2 stimulate peristaltic contractions of the myometrium, allowing the embryo to travel around the uterus 12–14 times per day, sending signals to prevent luteolysis (Roberts *et al.* 1996; Stout and Allen 2001; Allen and Wilsher 2009; Klein *et al.* 2010). Many studies have tried to determine the nature of the factors emanating from the conceptus; however, the exact repertoire of signalling molecules released by the horse conceptus and their mechanisms of action remain to be fully resolved (Allen 1982; Bazer *et al.* 1994; Roberts *et al.* 1996; Allen and Wilsher 2009; Ealy *et al.* 2010; Klein *et al.* 2010; Swegen and Aitken 2016). A study by Ealy *et al.* (2010) provides strong evidence to suggest that the unknown signal, whatever it may be, likely targets the enzyme PGG/H synthase 2 to prevent luteolysis.

Using the technologies mentioned previously, it may be possible to increase the immunogenicity of as yet unknown proteins found on the blastocyst surface, perform immunisations and determine reproductive outcomes. This technology has been assessed previously, whereby peptides were developed to mimic trophoblast epitopes and their antigenicity tested by assessing their binding affinity to autoantibodies (Frank *et al.* 2005). Rather than interrupting conception, this approach inhibited the implantation of a successfully fertilised embryo. This later stage intervention alleviates any concern for changes to social dynamics and behaviours, but public acceptance of such an approach will need to be assessed to ensure its feasibility as a contraceptive for feral horses (Fagerstone *et al.* 2010). Using novel phage panning and aldehyde-modification technologies, we have the opportunity to identify proteins essential for the horse maternal recognition of pregnancy and exploit these to prevent embryo implantation, if and when fertilisation occurs.

Non-surgical sterilisation implementation and feasibility

Research into the molecular mechanisms governing mammalian reproduction has provided unprecedented insight into how reproductive processes may be compromised for the purpose of achieving long-term fertility control in feral horses. Indeed, the use of non-surgical sterilisation offers significant advantages in that it is cheaper to use than alternative methods, respects animal welfare and can be more effective than culling (Hobbs *et al.* 2000; Turner *et al.* 2001). However, incorporating non-surgical methods into our existing armoury of integrated pest management strategies faces several challenges, including stringent assessment of feral horse numbers and contraceptive suitability based on changes to reproductive cycling, animal behaviour and disruption to social dynamics between horses (Kirkpatrick and Turner 2008; Zabek *et al.* 2014*a*). As highly social animals, it remains imperative that we understand how management strategies including non-surgical sterilisation techniques may alter feral horse social structure (Madosky *et al.* 2010). It is difficult to ascertain whether compensatory factors, such as increased survival, may occur after the burden of fertility is removed from target populations (Warren *et al.* 1993; Ransom *et al.* 2014), as it has done following previous immunocontraceptive delivery (Turner and Kirkpatrick 2002; Kirkpatrick and Turner 2007), or whether selective pressure will manipulate the long-term effect of a particular contraceptive agent (Turner and Kirkpatrick 2002; Kirkpatrick and Turner 2008). However, this has not been the case in previous studies (Ransom *et al.* 2014) and changes to population demographics and social hierarchy issues have been reviewed extensively (Berman 1991; Dobbie *et al.* 1993; Fagerstone 2002; Asa and Porton 2005; Kirkpatrick *et al.* 2011; Massei and Cowan 2014).

Perhaps one of the greatest obstacles to the implementation of an efficacious contraceptive strategy is the development of a suitable delivery method that is both practical and cost-effective (Bomford and O'Brien 1997; Lapidge *et al.* 2010). Remote delivery has been thoroughly investigated for its applicability, regarded as an ideal method for reducing labour costs and minimising stress experienced by animals (Kirkpatrick *et al.* 1990, 1996, 2011). However, an in-depth review by the National Research Council (2013) describes the shortcomings of this technique, concluding that it is unlikely to be suitable for feral horses owing to the complexities associated with successful administration (Garrott 1995; Nimmo *et al.* 2007). Indeed, remote delivery also poses an environmental contamination concern, because most darts fall out of the animal and are unrecovered (Warren *et al.* 1993). Oral baiting may ultimately be the only effective delivery mechanism for free-ranging horse populations (Killian *et al.* 2006; Humphrys and Lapidge 2008; Samoylova *et al.* 2012), but it is seldom discussed for incorporation into feral horse management strategies. Peptide technology certainly meets all the requirements for oral delivery under field conditions in that it is species specific, has shown oral activity and is environmentally safe (Humphrys and Lapidge 2008). Although oral baiting mitigates the problems that apply to remote delivery, it is not without its own limitations. For example, previous work has shown that baiting is complicated by the behavioural characteristics of feral horses, which exhibit a natural tendency to avoid unknown roughage, such as hay, even when presented to them in periods of drought (Hampson *et al.* 2011). In certain instances, bait delivery may alter normal behaviour, where animals associate bait with helicopters and avoid that particular form of roughage in the future (Kirkpatrick 2007). It is therefore important to integrate suitable bait systems that are enticing to horses and are likely to be actively consumed. An important consideration for the manufacturer of such a baiting system is that both the active agent and the oral bait itself are long lasting and capable of resisting exposure to fluctuations in temperature and humidity (Humphrys and Lapidge 2008). In this regard, it is anticipated that a large block would be more appropriate than pellets, because a block would expose less surface area to the environment and be more resistant to desiccation. In pilot studies in Australia, this bait structure has been successfully implemented as part of a pest management strategy for feral deer and goats (Hunt *et al.* 2014). Although results are promising, they dictate the contraceptive agent used in the bait will have to elicit a species specific, or at least species restricted, response to avoid unintentional effects on non-target domestic and native species. Consideration of the use of adjuvants will also need to be addressed, as will the possibility of being able to deliver a contraceptive that does not contain an adjuvant and thus eliminate adverse reactions (Kirkpatrick 2007). One further consideration is that the contraceptive agent is entirely consumed within the

target species and not prone to bioaccumulation, should the animal be collected at a later point and meat sold for pet consumption (Warren *et al.* 1993; Garrott 1995).

A non-surgical sterilisation method allowing lifelong coverage for feral horses would be incredibly valuable to industry stakeholders, but it must be ensured that the contraceptive agent is not passed from the animal in waste and does not taint agricultural land and water systems. The feasibility of any new management plan is also predicated on in-depth field studies into population demographics and distribution. There is still much to learn about feral horse ecology, which has been impeded by the remote habitats in which the feral horses typically reside (Zabek *et al.* 2014*b*). Traditional immunocontraceptive strategies have primarily focused on reducing female fertility, which is appropriate in the context of feral horse herd structure. However, the opportunity to impede the fertility of both sexes simultaneously is a feature that has not been offered previously, and poses the greatest opportunity for successful control (Dell'Omo and Palmery 2002). Such studies will likely be complemented by simulated modelling approaches to inform the level of contraceptive efficacy needed to achieve the desired reduction in horse numbers and the most suitable timing for introduction of the contraceptive into the population (Garrott 1995; Hone 1999; Hobbs *et al.* 2000; Ballou *et al.* 2008). Early work by Bomford and O'Brien (1997) highlighted the merits of implementing a non-surgical sterilisation method following periods of severe drought when feral horse numbers have declined and when the density of animals is likely to increase around watering holes. This was also suggested by Berman (1991) and the National Research Council (2013). Integrated feral horse management beginning with a population decline followed by intensive delivery of a non-surgical sterilant to both sexes is likely to prove the most successful in achieving milestones for feral horse control.

Contraceptive approaches are likely to continue to need public and political approval (Powers *et al.* 2014), a major obstacle that limits the application of current methods that rely on culling via aerial shooting (Nimmo and Miller 2007). It is likely that at the time of administration animals will be pregnant. Therefore, it is important to ensure contraceptives administered mid-gestation will cover females against future pregnancy, but not affect the health of the current foetus (Powers *et al.* 2011).

Feral horse population control is a long-term project, where integrated pest management strategies incorporating non-surgical sterilisation methods will require adequate funding and resource investment before a significant population reduction is seen. Previous contraceptive application in other species has been very costly, so it is important to develop new, cost-effective and sustainable technologies for implementation in population management strategies. Feral horse management is not a short-term issue and thus long-term solutions, such as non-surgical sterilisation, will likely have important roles in containing future population growth.

Conclusion

Current control methods for feral horses meet with considerable public criticism, which has provided the impetus to explore novel contraceptive strategies. Non-surgical methods of sterilisation offer an alternative and sustainable approach based on endocrine disruption of gamete development or immunological targeting of gamete interaction. Herein we provide a review of novel mechanisms designed to elicit a state of permanent sterility, including redox cycling to generate oxidative stress in the gonad, random peptide phage display to target non-renewable germ cells and generation of autoantibodies against proteins essential for conception via modification following aldehyde alkylation. Such approaches have shown considerable promise in a laboratory setting, but successful application in free-ranging, long-lived species such as the feral horse presents several unique obstacles. No single management strategy will be a panacea for feral horse control; management strategies will need to be revised frequently and incorporated together to achieve this long-term goal.

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Review

Is Wildlife Fertility Control Always Humane?

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Simple Summary: There are various fertility control methods (modalities) currently available that aim to reduce the abundance of problematic free-ranging mammalian wildlife. Here, we propose that dissimilarities in the mechanism of action indicate these methods produce great variation in animal welfare outcomes. We present a framework to assist managers in minimising animal welfare risks.

Abstract: Investigation of fertility control techniques to reduce reproductive rates in wildlife populations has been the source of much research. Techniques targeting wildlife fertility have been diverse. Most research into fertility control methods has focused upon efficacy, with few studies rigorously assessing animal welfare beyond opportunistic anecdote. However, fertility control techniques represent several very different mechanisms of action (modalities), each with their own different animal welfare risks. We provide a review of the mechanisms of action for fertility control methods, and consider the role of manipulation of reproductive hormones ("endocrine suppression") for the long-term ability of animals to behave normally. We consider the potential welfare costs of animal manipulation techniques that are required to administer fertility treatments, including capture, restraint, surgery and drug delivery, and the requirement for repeated administration within the lifetime of an animal. We challenge the assumption that fertility control modalities generate similar and desirable animal welfare outcomes, and we argue that knowledge of reproductive physiology and behaviour should be more adeptly applied to wild animal management decisions. We encourage wildlife managers to carefully assess long-term behavioural risks, associated animal handling techniques, and the importance of positive welfare states when selecting fertility control methods as a means of population control.

Keywords: behaviour; capture; endocrinology; fertility; immunocontraception; physiology; reproduction; sterilization; welfare; wildlife

1. Introduction

Animal welfare has been steadily gaining importance and society now recognises it has an obligation to consider the welfare of all animals, including free-ranging species that are perceived to be overabundant or pests. As community and ethical opposition to the killing of animals has risen, especially for charismatic species (e.g., feral horses (*Equus caballus*); [1]), more focus has been devoted to non-lethal methods, particularly fertility control [2]. Fertility control methods have often been grouped together and described using terms such as "humane" [3–5], or "benign" [6,7]. These are not universal opinions though, as some authors have urged consideration of potential animal welfare impacts [8,9], including recognition that the welfare consequences of different methods may vary considerably [10].

Despite the importance of animal welfare, there are other aspects of fertility control methods that cannot be ignored. Efficacy will always need to be considered because if a method is chosen that has a reduced welfare impact on the individual, but does not achieve its animal management goals, then arguably doing nothing at all would have resulted in better animal welfare outcomes [11]. The overall welfare impacts of fertility control techniques should also be considered alongside the impacts of other control tools that may be considered as an alternative [12]. In any context, once an "optimal" decision has been made, it should not be blindly extrapolated to all species for all circumstances. With this in mind, there are large gaps in the published literature as most research has focused on the outcomes of fertility control programs in terms of reproductive output (its efficacy). Much less research has examined how each method induces decreased reproductive output, and at what cost to the animal. This evolution of research into wildlife fertility control methods appears to be tracking the same course that investigations into the use of lethal toxins for wildlife control followed, where efficacy studies preceded assessments of welfare impacts [13].

Aims of This Review

Once it has been decided that a fertility control program will be employed, there is considerable uncertainty when choosing a specific fertility control product or technique. Several authors have attempted to address the broad side-effects associated with fertility control of wildlife, including animal welfare impacts [9,14,15], but the relative risks of each fertility control method has so far escaped critical review. However, the American Zoo and Aquarium (AZA) Contraception Advisory Group has provided several articles examining the relative risks of fertility control modalities relevant to collection animals (e.g., [16]). Equivalent critical reviews have been performed for the manipulation of reproductive hormones in human medicine [17], highlighting that divergent properties of fertility control methods are related to dissimilarities in mechanisms of action, and ultimately produce variable clinical consequences.

This review attempts to demonstrate the differences between broad categories of fertility control techniques, with a view to facilitating future selection of practices. Fertility control has been applied to non-mammalian wildlife (such as birds; [18]) but this review will restrict its examination to mammalian

wildlife. The range of fertility control approaches used to manage mammalian wildlife is discussed, with a focus on mechanisms of action, the importance of maintaining natural behaviour and the need to assess all stages of animal manipulation involved in administering required treatments. We suggest principles to advance the scientific rigour behind the selection of fertility control techniques toward a "best practice" model to maximise animal welfare outcomes.

The central aim of this study is to specifically provide a framework for assessing animal welfare risks for various fertility control methods for mammalian wildlife. It cannot be disputed that efficacy and financial costs are important considerations when management decisions are being made, however, these issues have been thoroughly addressed by other reviews [2] and so will not be discussed in detail in this study. The aims of this study are not to compare fertility control with lethal methods, and not to assess side-effects of fertility control methods as a whole. This review explicitly examines animal welfare risks specific to each class of fertility control method and presents a framework for selecting fertility control methods that minimise animal welfare risks.

2. Fertility Control Methods Currently Used

A wide diversity of methods has been applied to wildlife fertility control over several decades. Table 1 lists the most commonly applied fertility control approaches in free-ranging mammals. Fertility control methods vary greatly in the mechanism of action of the treatment applied, as well as the administration method used to deliver them to animals (e.g., surgery *versus* remote injection or "darting"). Table 1 also illustrates that within individual species (e.g., white-tailed deer (*Odocoileus virginianus*)), multiple fertility control methods, representing several mechanisms of action, have been employed. These methods, and many more, have previously been reviewed in detail [2,15]. Several approaches to classifying the various fertility control methods have been suggested (e.g., physical *versus* chemical), but given the inextricable association between any method's mechanism of action and its effect, we argue that classification according to mechanism of action is the most logical approach.

2.1. Mechanisms of Action

Owing to the diversity of fertility control techniques currently available (Table 1; [2]), an appreciation of welfare impacts is not possible unless focus is first placed upon mechanisms of action. "Mechanistic" approaches have been central to rationalising and standardising the use of other wildlife management techniques. For example, rationalisation of a vast array of trade and colloquial names has been possible for toxins [13] and kill-traps [19] that are subjected to welfare assessment. All effects are produced by a mechanism of action, and dissimilarities in these processes in fertility control need to be understood to avoid misunderstanding [20]. For this reason we recommend the use of the term fertility control modalities to highlight the importance of considering how a method works and the long term welfare outcome at the individual animal level, rather than simply considering efficacy at a population level.

Table 1. Wildlife fertility control modalities that have been trialled, categorised according to their mechanism of action.

Class	Technique	Route	Description	Duration of Effect	Examples of Products	Sex Sterilized	Example Application	
							Species	Reference
Endocrine suppression	Castration (gonadectomy)	Surgical	Removal of testicles	Permanent	N/A	Male	Eastern grey kangaroos (*Macopus giganteus*)	[21]
	Ovariectomy/ovariohysterectomy (gonadectomy)	Surgical	Removal of ovaries +/− uterus	Permanent	N/A	Female	Brushtail possums (*Trichosurus vulpecula*)	[22]
	GnRH agonism	Implant	Binds to GnRH receptors	1–2 years	Deslorelin® Suplorelin®	Female Male	Tammar wallabies (*Macropud eugenii*)	[23,24]
	GnRH immunocontraception	Injection	Induced immunity to GnRH	>3 years	GonaCon®	Female Male	Wild boar (*Sus scrofa*)	[25]
	Synthetic testosterone	Injection	Suppressed spermatogenesis	Temporary	N/A	Male	Wild horses (*Equus caballus*)	[26]
	Synthetic progestins	Implant	Prevention of ovulation	3–5 years	Norplant®	Female	Koalas (*Phascolarctos cinereus*)	[27]
	Synthetic prostaglandin	Implant/biobullet	Pregnancy termination	Temporary	N/A	Female	White-tailed deer (*Odocoileus virginianus*)	[28]
	Synthetic oestrogen	Oral bait	Prevention of implantation	Temporary	N/A	Female	White-tailed deer (*O. virginianus*)	[29]
	Synthetic oestrogen	Implant	Prevention of implantation	Temporary	N/A	Female	White-tailed deer (*O. virginianus*)	[30]
Physical non-endocrine	Vasectomy	Surgical	Ligation of vas deferens	Permanent	N/A	Male	Eastern grey kangaroos (*M. giganteus*)	[21]
	Epididymectomy	Surgical	Removal of epididymis	Permanent	N/A	Male	Feral goats (*Capra hircus*)	[31]
	Tubal ligation	Surgical	Ligation of oviducts	Permanent	N/A	Female	White-tailed deer (*O. virginianus*)	[32]
	Oviduct "webbing"	Surgical	Removal of oviducts	Permanent	N/A	Female	Feral cattle (*Bos taurus*)	[33]
	Intra-testicular injection	Injection	Sclerosis of testicles	Permanent	Neutersol®	Male	American black bears (*Ursus americanus*)	[34]
	Intra-uterine devices	Implant	Endometrium irritation	Temporary	N/A	Female	Wild horses (*E. caballus*)	[35]
Chemical non-endocrine	Zona pellucida immunocontraception	Injection	Induced immunity to ovum proteins	>3 years	ZonaStat-H® SpayVac®	Female	Tule elk (*Cervus elaphus nannodes*)	[36]

2.2. Broad Classes of Fertility Control Modalities

In order to clarify the modalities for each fertility control agent, the following broad classes should be adopted: endocrine suppression methods, physical non-endocrine methods, and chemical non-endocrine methods (Table 1).

"Endocrine suppression" [37,38] (or endocrine disruption) methods alter the actions of naturally occurring reproductive hormones. Endocrine processes may be targeted centrally, depressing all reproductive hormonal activity (e.g., gonadotrophin-releasing-hormone (GnRH) agonists) or peripherally (e.g., progestin antagonists). Such is the complex nature of reproductive endocrine feedback processes that all supplementation, agonism, antagonism, or removal of endocrine hormones will ultimately lead to the suppression of natural reproductive hormone effects. Hence, we refer to all processes that affect reproductive hormones as inducing hormonal incompetence [39]. There are four main modalities in this category. Firstly, disruption can be achieved through the surgical removal of gonads (gonadectomy). Secondly, disruption can be achieved through the supply of exogenous (synthetic or externally derived) versions of naturally occurring reproductive hormones (*i.e.*, testosterone, oestrogen, progestins; Table 1) that interfere with normal feedback control and secretion of endogenous hormones. This method is commonly utilised in human contraception [17,40,41]. Thirdly, endocrine disruption can be achieved by agonism or antagonism of naturally occurring (endogenous) reproductive hormones (e.g., GnRH), via the administration of chemicals that actively compete with hormone binding sites. Finally, immunocontraception can be used to induce an autoimmune response through vaccination against naturally occurring reproductive hormones (e.g., GnRH).

Physical non-endocrine methods rely on physical (rather than chemical) means to disrupt the normal functioning of the reproductive system, and do not alter naturally occurring reproductive hormone levels. These methods encompass surgical techniques that *occlude* reproductive passages (but do not remove endocrine reproductive organs) or the use of implants that physically irritate the female reproductive tract (e.g., non-hormonal intrauterine devices; [35]). Surgical techniques that occlude reproductive passages but leave endocrine reproductive function intact include modalities that ligate (oviduct ligation, vasectomy) or remove (oviduct removal, epididymectomy) reproductive passages, but not gonads (Table 1). It is important to note that traditional surgical techniques used for fertility control in companion animals remove gonads ("gonadectomy"), including castration and ovariectomy/ovariohysterectomy (Table 1), and render reproductive hormone patterns nonfunctional [42,43]. We therefore classify these methods as inducing endocrine suppression despite their application through physical means (surgery).

Chemical non-endocrine methods rely on inducing an autoimmune response through vaccination against naturally occurring ovum proteins, most commonly the zona pellucida (ZP) [44], to prevent fertilisation of ova. This is achieved in the same way that a vaccine is used to mount an immune response against a pathogenic microbe (e.g., virus or bacterium) except that the immune response is being directed towards the animal's own ovum proteins. Once the animal's immune system disrupts the capacity for an ovum to be fertilised, affected females are rendered infertile irrespective of how many times they are mated by a male [44]. Similarities in the effects of physical and chemical non-endocrine modalities have been demonstrated by the use of physical occlusion surgeries to model the effect of ovum protein immunocontraception in epidemiological studies [45].

This first principles approach of classifying fertility control methods according to modalities should enable more meaningful comparisons and improved assessments of welfare risks at an individual animal level. This approach should also help to clarify some misconceptions arising from the oversimplification that has been used in the past to describe classes of methods (e.g., [46]). We therefore advocate a focus on modalities, rather than colloquialisms or trade names [47], when considering the implications for individual animals for any chosen treatment.

2.3. Best Practice Approaches

For some time, many contentious lethal (e.g., shooting, toxins) and non-lethal (e.g., marking) wildlife management methods have been closely scrutinised, with strong focus placed upon animal welfare impacts, rather than efficacy in isolation [19,48]. Quantitative assessment methods have allowed ranking of humaneness for many lethal wildlife control methods [12], in combination with the development of "best practice" approaches (e.g., [49]). In contrast, animal welfare assessment of fertility control methods has not been evaluated with the same level of scientific rigour and much uncertainty remains regarding animal welfare impacts [50]. As identified in reviews [9], most research into welfare risks has used qualitative, anecdotal evidence, and/or presented opportunistic, qualitative data lacking appropriate controls, sampling methodology or time scales. For these reasons, reviews have identified the assessment of welfare risks for fertility control as a research priority [9]. In the absence of quantitative humaneness assessment templates for fertility control modalities, "best practice" standards have not been developed and methods are still largely chosen on an *ad hoc* basis, and there remains much variability in how modalities are chosen and applied.

3. Animal Welfare Risks

It is proposed that any animal treatment can have short-term adverse effects on welfare (for example those caused by an injection) followed by long-term effects, which may be very different [51]. Both should be assessed. At the same time, modern approaches to animal welfare propose that avoiding negative welfare states (harms) should be considered in addition to the provision of opportunities for pleasurable experiences (such as social play, courtship or parenting [52,53]). This approach dictates that animals need positive experiences, in addition to minimised suffering [52,54]. Below we present a list of all of the animal welfare risks associated with fertility control, from administration (short term) to the response to treatment (long term) and include those imposing negative, and restricting positive welfare outcomes, respectively.

3.1. Short-Term (Administration) Negative Animal Welfare State Risks

3.1.1. Animal Capture, Restraint, Anaesthesia, Marking and Surgery

Most stages of wildlife management techniques that involve animal manipulation are likely to impose welfare costs. This is recognised for lethal wildlife control techniques, where animal welfare assessment frameworks require consideration of stages of animal manipulation prior to the killing method [12]. Some wildlife fertility control programs require animals to be captured and handled [55] or undergo anaesthesia and surgery [21] while other techniques allow animals to be darted (remotely treated)

in situ [56]. Classes of fertility control methods that currently require animal capture are shown in Table 2. These restraint procedures, required to administer a fertility treatment, may impose animal welfare costs that are in addition to the physiological and behavioural effects of the fertility control procedure or agent.

Table 2. Animal welfare risks from administration techniques used to implement common currently used fertility control modalities in mammalian wildlife. All potential stages of animal manipulation are shown to demonstrate techniques that require multiple stages of animal manipulation and those that do not.

Mechanism	Technique	Effect of Administration					
		Capture	Anaesthesia	Surgery	Remote Administration	Multiple Doses *	Frequency of Treatment
Chemical non-endocrine	ZP immunocontraception	−	−	−	+	±	3 years
Physical non-endocrine	Vasectomy	+	+	+	−	−	Permanent
	Tubal ligation	+	+	+	−	−	Permanent
Endocrine suppression	GnRH immunocontraception	−	−	−	+	−	3 years
	GnRH agonism	±	−	−	±	−	1–2 years
	Synthetic progestins	±	−	−	±		3–5 years
	Castration	+	+	+	−	−	Permanent
	Ovariohysterectomy	+	+	+	−	−	Permanent

* 2 doses are often required to ensure the efficacy of a single treatment; ± Can be administered via remote delivery or via capture, restraint and implantation.

In contrast, other fertility control programs have recognised the potential animal welfare costs of administration procedures and have chosen methods that allow for remote delivery of a single dose of an agent [57], effectively minimising stress other than that associated with potential darting injuries [58] or injection site reactions [59]. With the use of many modalities, for example, following remote drug delivery (darting) and surgical procedures, there is a requirement for visual marking of treated animals [60,61]. Marking in itself has been demonstrated to impose animal welfare impacts in many wildlife species [48].

3.1.2. Repeated Treatments

There are variations in the durations of action for different fertility control approaches. Some methods (e.g., surgical intervention) produce permanent infertility while some exogenous endocrine methods may affect fertility for less than one year (e.g., exogenous prostaglandins used to terminate pregnancy; [28]). Some chemical fertility control methods will require repeated administration every 1−2 years (e.g., GnRH agonists; [24]) or require booster administration 1−4 weeks after initial treatment (e.g., older ZP vaccines; [60]). All repeat treatments will impose additional animal welfare costs, as animals will need to be captured/injected several times. Some programs addressed the requirement for booster doses for older ZP vaccines by maintaining wild animals in captivity between injections, a process that can be highly stressful for free-ranging species [62]. For long-lived species (>5 years), multiple rounds of administration may be required to maintain infertility in treated animals [63]. An additional consideration is the aversion behaviour of wild animals after previous exposure to a stressful method.

For example, studies requiring animals to be re-darted [60] or re-trapped [64] have observed greater difficulty in re-administration of a treatment.

3.1.3. Injection or Implantation Site Local Inflammatory Reactions

Many remotely delivered immunocontraceptive modalities, being adjuvanted vaccines that rely on stimulating the immune system, have been shown to induce granulomatous inflammation at the site of injection [59,65]. Some studies have suggested that the welfare impact of such reactions is likely to be similar to the use of any adjuvanted vaccine. Likewise, many forms of chemical implants have demonstrated similar local inflammation effects associated with their administration [66]. The incidence of such adverse reactions, and their impact on welfare in free ranging species may be unknown, given the limited monitoring of many animals post-administration.

3.2. Long-Term (Treatment) Negative Welfare States Risks

3.2.1. Physiological Changes

Significant or prolonged physiological changes may be considered an animal welfare concern as they may alter an animal's ability to perform natural behaviour [67] and/or prevent adaptation to environmental challenges. Physiological responses may affect the animal directly, in terms of increased metabolic rate or redistribution of body reserves, or indirectly, in terms of loss of fitness, such as loss of appetite or decreased immunity [68]. All animals have the capacity to make behavioural changes in response to stimuli but marked physiological changes may restrict the capacity of animals to express the full range of natural behaviour [69]. Physiological changes have been demonstrated for several fertility control modalities, particularly those that derive their effect from endocrine suppression. Many studies on endocrine suppression methods have demonstrated weight gain in treated animals [25,27,70], but have considered the change to be positive or neutral, from a health perspective, without considering the effect of natural behaviour for a free-ranging species required to thermoregulate, forage, escape predators, compete with cohort animals or migrate. Immature male animals subjected to endocrine suppression generally do not grow as large as untreated males, as seen in white-tailed deer (*O. virginianus*; [71]) and tammar wallabies (*Macropus eugenii*; [66]). Endocrine suppression in immature male tammar wallabies (*M. eugenii*) also resulted in increased fat deposition [66]. Changes have also been demonstrated from endocrine suppression modalities to processes such as antler shedding in deer species [72]. Especially when endocrine suppression modalities are applied to juvenile animals, sex-specific phenotypes often fail to develop and male animals can become "feminized". This alteration in natural development means that these impacts are permanent [66,72]. Such altered physiology will likely reduce the capacity of such animals to express a full range of normal behaviour, and adapt to environmental changes.

3.2.2. Altered Behaviour

The importance of natural behaviour, and the ability of animals to experience positive welfare states, is a central tenet of modern animal welfare science [54]. Behavioural repertoires indicating satisfactory welfare should include the expression of normal patterns of maintenance and social behaviour, such as grooming, rest and play [52]. Deprivation of normal behaviour may compromise welfare by limiting

an animal's opportunities for positive experiences [52]. Changes in behaviour can be difficult to measure, but the importance of maintaining natural behaviour has been recognised for wildlife fertility control [73]. Of the few studies that have explicitly assessed behavioural changes in free-ranging wildlife (e.g., [74]), changes have been demonstrated to feeding, resting and dispersal behaviour.

For example, behavioural effects in free-ranging feral horses (*E. caballus*) subjected to endocrine suppression (GnRH immunocontraception) included observations that treated animals fed less, rested more, and travelled less [75]. Endocrine suppression in immature captive male tammar wallabies (*M. eugenii*) resulted in decreased agonistic (related to fighting) behaviour with intact males, lower dominance, and higher sexual interest from intact males [66]. Other behavioural changes are less predictable. For example, it has been demonstrated that male eastern grey kangaroos (*Macropus giganteus*) prefer to be associated with females that have not been subjected to endocrine suppression [76]. On the basis of such non-intuitive findings in an inherently complex field, we hypothesise that the real effect of altered social structures and disrupted social cohesiveness is not yet fully understood.

3.2.3. Extended Breeding Seasons

Beyond any welfare impact suffered by individual animals subjected to fertility control treatment, there are also often non-intuitive welfare costs to other animals of the same species, through social interactions. Specifically, fertility control modalities that prevent pregnancy occurring without preventing oestrus behaviour (physical non-endocrine and chemical non-endocrine methods) often induce a prolonged breeding season. This phenomenon has been observed for white-tailed deer (*O. virginianus*; [4,77,78]) and feral horses (*E. caballus*; [79]). The prolongation of natural breeding seasons can represent animal welfare risks for treated and untreated animals, specifically males. For example, the study of Ji *et al.* [80] demonstrates a pronounced impact on the body condition and welfare of male brushtail possums (*Trichosurus vulpecula*) with the extension of a breeding season through the presence of sterile, but hormonally competent, females. The average body condition of these males was significantly poorer than that of males in control areas. Similar effects have been observed in male deer [81]. It has been argued that extension of reproductive cycling may have important social consequences for feral horses (*E. caballus*; [79]) and African bush elephants (*Loxodonta africana*; [82]). In addition, increases in the cumulative number of mating attempts (social contacts) with non-endocrine modalities may increase infectious disease transmission risks [45].

3.3. Long-Term (Treatment) Positive Welfare States Risks

3.3.1. Deprivation of Parental Behaviour in Nurturing Species

Despite the central intended effects of fertility control agents in preventing the birth of offspring, and hence the opportunity for animals to express parental behaviour, the loss of this positive welfare state has not commonly been cited as a welfare concern for treated wildlife. In comparison, the welfare risks of deprivation of this natural behaviour have been recognised in zoo animals. The European Association of Zoos and Aquaria [83] stated: "limiting the opportunity to breed in species which display nurturing parental behaviour, by definition, reduces an individual animal's opportunity to express one of the most

important and complex sets of natural behaviour and can thus lead to a decrease in welfare". Contention surrounding the importance of this deprivation has been at the heart of many discussions of ethical practices in zoos worldwide [4,84,85].

The impact of this animal welfare risk is not easily demonstrated with empirical evidence. However, deprivation of freedom to express natural behaviour is universally considered an animal welfare concern [54] as it alters an animal's nature or "telos" [67]. Preventing such freedoms may affect animal wellbeing by reducing their ability to seek and gain pleasurable experiences and/or denying individuals the satisfaction of desires or preferences [86]. However, it must be noted that there are also welfare costs associated with reproduction, involving energetic costs and mortality risks that animals must trade-off with reproductive success [87]. Maternal behaviour in particular is complex, involving birth, nursing, and care of young. This loss of an important natural behavioural repertoire to many nurturing mammalian species warrants much greater discussion and consideration in the wildlife management sector.

3.3.2. Deprivation of Courtship and Mating Behaviour

Another positive welfare state that may be deprived of animals is the freedom to express natural reproductive behaviour, including courtship and mating. Endocrine suppression modalities often reduce or eliminate courtship and mating behaviour [81], through their mechanism of action targeting endocrine processes centrally, and depressing all reproductive hormonal activity [88]. For example, white tailed deer (*O. virginianus*) males subjected to an endocrine suppression modality (GnRH immunocontraception) display very few mating attempts with untreated (hormonally competent) females [78]. Courtship and mating behaviour are considered essential components of natural behaviour for many free-ranging mammal species and the deprivation of this natural behaviour must also be considered an important animal welfare impact, whether or not it is experienced as deprivation given the lack of reproductive hormonal stimulation for the behaviour to occur.

4. Fertility Control in Other Species

Many fertility control modalities currently applied in wildlife management were initially developed to meet the economic and emotional needs of owners of domesticated animals, such as companion or production animals. Some endocrine suppression modalities (e.g., GnRH immunocontraception) were developed for livestock species to intentionally alter physiology (to influence meat quality) and behaviour (to reduce aggression) to benefit production, rather than to prevent reproduction [89]. Fertility control of wildlife was initially performed as an extension of the practices used by veterinarians for domestic animal control and ownership, primarily gonadectomy [42,61]. However, gonadectomy is not a universally supported practice for companion animals, and the considerable welfare costs associated with the profound endocrine suppression it induces has seen its use considered unethical or even illegal in some countries (e.g., Sweden, Norway) [42,90]. The justification of the need for routine gonadectomy of companion animals is not uniform between countries, and these inconsistent views are based on value differences [42].

In livestock management, hormonally incompetent animals have been viewed as "modified" by many scientists, with concerns about the welfare of treated animals as well as human health from consuming treated animals [51]. Many studies of domestic animals subjected to endocrine suppression modalities

have demonstrated (often desired) physiological and behavioural changes, when observation methods have been intensive and long-term [89]. Despite the paucity of similar studies examining long-term changes in wildlife species, it would be naïve to assume that similar welfare costs would be unexpected in free-ranging animals that were similarly treated. It is worth considering if modalities that were originally considered humane in domestic animals should also be considered to be humane in wildlife.

5. Improving Assessment of Animal Welfare Risks

Animal welfare is complex and guidelines for how it should be assessed have evolved over time [91,92]. Modern approaches require that both negative and positive welfare states are considered [53], and that measures used to assess welfare states include both physiology ("health") and behavioural measures, as well as measures of mental state [93]. Fertility control studies have primarily focused on efficacy (reproductive output) in the short term but many studies have explicitly or opportunistically assessed health side-effects [9]. Few studies have assessed broader animal welfare impacts beyond short-term health effects, such as the impact on animal wellbeing when reproductive and social behaviour patterns are disrupted in response to altered physiology, or deprivation of positive welfare states.

5.1. Welfare Is More than Just Health

Although animal welfare is a complex entity, the Five Domains model was proposed over 20 years ago as a conceptual framework to simplify welfare considerations for research animals [94], but it has since been extended to incorporate positive welfare states [92]. The model proposes four physical/functional domains (nutrition, environment, health, and behaviour) and the fifth domain is mental state [94].

The majority of studies that have addressed animal welfare during fertility control programs have effectively restricted their assessments to domain 3 of the model; health, as typically only measures of injury rate, the presence of disease, and body condition have been examined [59]. The high frequency with which local reactions to chemical modalities at injection/insertion sites are described in the literature [9] suggest focus on this in the past has been disproportionally high given the low intensity and duration of suffering involved. However, it has been recognised for more than a decade that assessment of animal welfare must be based on a wide range of measures in addition to health indices [95]. Community debate about intensive livestock farming has demonstrated clearly the difference between concern for animal health and welfare. While intensively raised pigs and chickens demonstrate very high health outcomes (body condition, absence of parasitism *etc.*), their welfare is considered by a large majority of the general public to be severely impaired through impedance of natural behavior, and an absence of positive experiences, that is imposed by small, restrictive housing [69,96,97].

For the majority of wildlife fertility control studies, the length of time that any side-effects have been monitored for has been restricted to short time frames (*i.e.*, 2–3 years [55,77]). However, there have also been longer term studies performed [78]. The potential for, and occurrence of, long-term behavioural changes not detected by short-term studies, has been demonstrated for marine mammal marking techniques [48], and terrestrial chemical capture techniques (bear species; [98]). These studies have urged researchers to look beyond short-term health approaches for holistic assessment of welfare. While the "short-term health" approach is deemed appropriate for assessing lethal methods where death

is expected to be an acutely reached endpoint [19], it is less appropriate for non-lethal methods where duration of suffering may be chronic (years). Long-term welfare risks clearly exist for all fertility control modalities, but until evidence exists to the contrary it should be assumed that the magnitude of the impact of these effects on welfare could be significant.

5.2. Maintenance of Natural Behaviour in Free-Ranging Animals

Similar approaches have been used for fertility control in domestic and free-ranging animals, but there are important differences in society's perception of our duty of care for animals in each context. There is broad acceptance that the maximisation of health should be the focus of animal welfare for the keeping of companion animals [90], while the focus of stewardship for wildlife centres on the maintenance of natural behaviour. For this reason, practices such as supplementary feeding and veterinary treatment of wildlife populations have been consistently discouraged [99–101], For a discussion of the role of ethics in wildlife management decisions related to health and welfare, see the study of Rolston [102].

Many fertility control methods that do affect behaviour and physiology (e.g., altered fat deposition; [103]) for companion animals are considered acceptable (by some scientists [42]) because human owners take full responsibility for the care of these animals. Decreasing a companion animal's survival skills, such as the ability to hunt, survive adverse climatic conditions and defend itself against another competitor are deemed acceptable by society because humans can continue to provide food, shelter and protection for these animals. "Nuisance behaviour" in companion animals [104] such as fighting, roaming, territory marking, and oestrus calling, that are prevented or reduced by endocrine suppression, are all examples of natural behaviour essential for the welfare of free-ranging animals. This distinction between the behavioural needs of companion and free-ranging animals has not been adequately recognised by authors studying species that may exist in either state (e.g., feral/domestic cats (*F. catus*); [103,104]). We accept that the management of some free-ranging species blurs the line between domestic and wild animals (e.g., zoo animals; [84], or animals partially confined in fenced urban reserves; [21]). For these contexts, preservation of natural behaviour may be argued to be ethically less important if animals are not required to escape predation, or extensively forage. This consideration is particularly important given that many studies attempting to assess welfare impacts of fertility control modalities have provided *ad libitum* access to resources (water and feed) for study animals [105,106]. In some contexts where animals are not considered clearly domestic *or* wild (e.g., "street dogs" [42,61]), some management programs have explicitly aimed to modify natural behaviour when it creates conflict with human values (e.g., aggression leading to attacks on humans [61]).

The same logic should not apply for all wild animals, as the duty of care provided by humans often ends for managed wildlife at the point of release from restraint or administration of treatment (e.g., [107]). For this reason in feral horse (*E. caballus*) fertility control, approaches that impact upon androgenic (male) behaviour [26] are no longer considered humane or appropriate. In another context, it has been argued that the only fertility control options that should be considered for coyotes (*Canis latrans*) should be those that allow the animals to remain hormonally competent, such is the importance of the endocrine system for the maintenance of natural behaviour [108]. We argue that the preservation of hormonal competence is important to maximise the welfare of wild animals, due

to the profound effects of endocrine suppression on important behaviour such as mating, aggression and dominance.

6. Case Studies

To illustrate the dissimilarities in approaches that have been taken to fertility control in different species and jurisdictions, we present two case studies. The first describes refinement of modalities used for feral horses (*E. caballus*) in North America and the second describes the relative lack of refinement in modalities considered suitable for Eastern grey kangaroos (*M. giganteus*) in Australia.

6.1. Case Study: Feral Horses (E. caballus) in North America

Fertility control modalities for feral horses have been investigated for several decades in the USA [26,109]. While a range of modalities were used in the past (e.g., exogenous testosterone; [26], intrauterine devices; [35]; Table 1), more recently, remotely delivered GnRH immunocontraception [110] and particularly ZP immunocontraception [62,74,79,111], have become the focus. Importantly, it was recognised decades ago that the short-term welfare risks associated with immobilisation and capture necessitated that fertility control modalities were administered remotely [56]. Immunocontraception vaccines may be administered via remote delivery, removing the need for capture, anaesthesia or surgery, but the requirement for booster administration with older ZP vaccines led some authors to maintain horses in captivity between treatments [62]. However, ZP vaccines also often lead to extended breeding seasons, and therefore there are accompanying increased energetic costs and injury risks from increased mating attempts [112]. The focus on ZP immunocontraception developed from a formal process initiated by the Bureau of Land Management (BLM) [111] in the USA to identify the most humane and practical fertility manipulation options. This departure from *ad hoc* trials of many different methods has seen progress towards adoption of "best practice" methods.

6.2. Case Study: Eastern Grey Kangaroos (M. giganteus) in Australia

Many different fertility control modalities have been trialed for easily captured eastern grey kangaroos in Australia [20]. The fertility control modalities that have been applied to kangaroos and their accompanying welfare risks have recently been reviewed [113]. Much recent research has focused on the use of a range of endocrine suppression modalities. GnRH agonists [24,105], and synthetic progestins [20,63,114], have been the subject of several studies. Chemical non-endocrine (ZP immunocontraception; [115]) and physical non-endocrine (vasectomy; [21]) modalities have also been investigated less frequently. However, eastern grey kangaroos are still managed with physical endocrine suppression modalities (castration; [21]) that are rarely applied to other wildlife species. In addition, modalities that have been delivered via remote injection in other contexts (e.g., GnRH agonists; [116]) are often delivered to eastern grey kangaroos as implants, following capture, restraint and anaesthesia ([105]). There have been no formal attempts to standardise modalities (but see [113]), and no apparent progress toward development of "best practice" guidelines for kangaroo fertility control in Australia, and so *ad hoc* methods continue to be used without careful consideration of animal welfare impacts.

The management of Australian eastern grey kangaroos contrasts strongly with the management of American feral horses. In the USA, management goals have included minimising animal welfare impacts of fertility control on wildlife, particularly feral horses [4], by using methods that only rely on darting animals. In Australia, marsupials are still regularly caught, and subjected to anaesthesia and surgery to administer fertility control treatments that, when compared to ZP immunocontraception, have greater effects on their natural behaviour and physiology [21,63,66]. In Australia, similar modalities have not only been used in several kangaroo species [24], but also wallaby species [66,117] and koalas (*Phascolarctos cinereus*) [27,115,118].

These case studies have shown the different approaches taken towards fertility control in two wildlife contexts and highlight the need for increased consideration for the welfare risks of current practices. For this reason, we propose a framework to better enable researchers and managers to understand the animal welfare risks of any fertility control modality and to improve the balance between meeting animal interests with management needs.

7. Suggested Future Improvements

Given the several decades over which wildlife fertility control research has been performed, we contend that sufficient information exists to move from a *carte blanche* approach towards a "best practice" approach for refining methods to minimise animal welfare impacts. It is our contention that methods that induce endocrine suppression, and requiring repeated or multi-stage interventions, are likely to represent poorer animal welfare outcomes than those methods that do not (Table 3). We suggest where duration is similar, the modality inducing the least changes to natural behaviour and physiology will pose the least welfare risk. Likewise for administration risks, the total welfare risk should be considered as the cumulative total of the invasiveness of the procedure, and the duration and frequency of the procedure. For this reason, we propose that welfare risks associated with long-term treatment effects are considered a higher welfare priority than short-term administration risks.

We contend that the deprivation of parental behaviour is an unavoidable welfare cost common to all fertility control modalities. Beyond this cost, several studies have identified that the modalities that minimise welfare risks prevent pregnancy but do not disrupt endocrine function, thus ensuring that reproductive/social behaviour changes are minimised [39,79]. However, with the availability and marketing of modalities that induce endocrine suppression but have appealing ease of administration benefits (e.g., GonaCon™), many researchers have opted to induce endocrine suppression in treated animals despite the existence of alternative modalities that do not (e.g., ZP immunocontraception). We recognise that GnRH vaccines offer increased efficacy and convenience through their effectiveness against both sexes, single injection administration, and lack of extended breeding seasons. However, these efficacy advantages should be balanced against clearly increased welfare costs for a method that causes changes to natural behaviour and physiology [89].

Table 3. Suggested principles for assessing cumulative animal welfare impacts for wildlife fertility control modalities. Methods are listed from top (least animal welfare impact) to bottom (most impact). For example, administration methods requiring capture, anaesthesia, and surgery are given a +++ rating. ZP = zona pellucida. GnRH = gonadotrophin releasing hormone.

Mechanism	Technique	Impact of Treatment	Impact of Administration
Chemical non-endocrine	ZP immunocontraception	+	+
Physical non-endocrine	Vasectomy	+	+++
	Tubal ligation	+	+++
Endocrine suppression	Synthetic progestins	++	++
	GnRH agonism	++	++
	GnRH immunocontraception	+++	+
	Gonadectomy	+++	+++

We suggest that methods requiring capture, anaesthesia, and/or surgery, or frequently repeated administration (in long-lived species) represent poorer animal welfare outcomes when compared to remotely deliverable methods (Table 3). Given the above animal welfare risks associated with treatment (long-term) and administration (short-term), we contend that remotely deliverable non-endocrine vaccines (*i.e.*, ZP immunocontraception) are likely to be the most humane fertility manipulation methods currently available (Table 3). While welfare impacts are high for administration of physical non-endocrine modalities (vasectomy, tubal ligation), repeated treatments are not required and hormonal competence is maintained. For all non-endocrine modalities, important positive welfare states (courtship, mating) are maintained. However, the effects of extended breeding season associated with all non-endocrine modalities suggests that species specific traits need to be considered to avoid welfare impacts relating to large increases in mating attempts (e.g., [80]) or the birth of offspring in non-favourable seasons [78].

On the same mechanistic basis, we contend that gonadectomy represents the least humane of the currently available fertility control technique for wildlife. Gonadectomy requires capture, often major (abdominal) surgery, anaesthesia, and induces complete and irreversible reproductive endocrine suppression, and accompanying permanent changes in natural behaviour and physiology [42,90]. We suggest that the welfare impacts associated with gonadectomy render it appropriate only for domestic animals where a human duty of care is provided, and where behavioural changes are desired (e.g., [119]). GnRH immunocontraception and agonism both induce partial suppression of hypothalamic-pituitary-gonadal axis function [106], but the effects are not as profound as those of gonadectomy. GnRH immunocontraception is likely to pose the lowest welfare risks of all endocrine suppression modalities due to its' favourable administration method (Table 3). GnRH agonism and exogenous progestin modalities both induce endocrine suppression and require repeated administration (sometimes via remote delivery but often requiring capture), but not major surgery (Table 3). However, there has been progress toward developing remote delivery systems for both progestin implants [56] and GnRH agonists [116], reducing the short-term welfare risks associated with their administration.

Suggested Principles for Selection of Fertility Control Methods

In an attempt to improve the present approach to the choice of fertility approaches, some authors have suggested the use of formal criteria for the selection of methods that are appropriate for field applications [2]. While we support the criteria approach of Massei and Cowan [2] for assessing efficacy, we suggest that more holistic scientific rigour should be applied to assessing welfare impacts. We present Table 3 as a framework for assessing the welfare pros and cons of commonly used modalities. The challenging nature of making welfare decisions does not justify averting from the central tenets of scientific logic: observation, hypothesis and experiment. Many decisions on the choice of fertility control modalities have been *carte blanche* and this represents the antithesis of scientific reasoning. We have attempted to address the short-term and long-term animal welfare risks, as per Broom [51], in a framework using a weighting approach (as per Dubois and Fraser [101]). Table 3 summarises the parameters that we consider most important for selecting control methods, and we suggest the following principles for selecting the most humane fertility control methods for wildlife populations:

(1) It should be recognised that all fertility control methods impose animal welfare impacts. The deprivation of the opportunity to display parental behaviour in nurturing species is an animal welfare impact,
(2) Methods should be chosen that pose the lowest cumulative animal welfare cost, including capture, administration and frequency of treatment,
(3) All domains of animal welfare should be considered, including behaviour and the opportunity for animals to have positive experiences,
(4) Methods that maintain hormonal competence, thus promoting natural behaviour, should be chosen over methods that induce endocrine suppression, and
(5) Where uncertainty exists regarding welfare impacts, fertility control modalities should not necessarily be chosen over alternative lethal or non-lethal methods.

Any decisions regarding wildlife management will involve seeking a balance between the interests of the wildlife and other stakeholders. The suggested use of our framework in ethical decision-making has some unavoidable subjectivity relating to the difficulty in comparing various types of risks (e.g., long duration-low intensity welfare impacts with short duration-high intensity welfare impacts), which value-based ranking systems (e.g., [12]) may address. However, we contend that the proposed weighting approach will be more informative for wildlife managers than a list of comparative features without commentary. In addition, the weighting approach has been widely applied for welfare studies lacking comprehensive empirical evidence of all aspects (e.g., [101]).We present this framework as a provocation to wildlife researchers and wildlife managers to improve the rigour with which they assess management options, and to broaden their understanding of animal welfare.

We recognise that the range of species and contexts for which wildlife fertility control is desired makes the proposal of a single preferred method impossible [40]. We accept that efficacy, economic costs, ecological side-effects, and other factors are also important considerations for fertility control programs [2]. We present this framework to help with objective assessment of animal welfare, and to aid managers in achieving balance when weighing welfare impacts against other management considerations. It is an accepted reality that methods representing optimal animal welfare outcomes

may not be appropriate for all management contexts. We contend that understanding the complexities of both reproductive physiology and animal welfare science should be improved in wildlife management. Claims that fertility control modalities pose "no undesirable physiological or behavioural side-effects" from limited observations [105] are unrealistic and display a poor understanding of animal welfare. Likewise, the lack of consideration for the importance of depriving animals of positive experiences when comparing fertility control to other management options requires re-consideration. The lack of standardisation of wildlife fertility control modalities in many contexts is indicative of the enduring misconception that non-lethal reproductive manipulation is synonymous with benign outcomes. We contend that wildlife fertility control should not always be assumed to be humane.

8. Conclusions

Efforts to apply fertility control to wildlife have now been performed for over 30 years but there has been little standardisation of procedures over this time. Research into this area has primarily focused on the important aspect of efficacy but this has meant that the associated impacts on animal welfare have only seldom been addressed. Consequently, there is a growing need for more meaningful interchange between animal welfare scientists, wildlife biologists and wildlife managers [120]. Understanding the mechanisms of fertility control modalities and the resultant impacts on natural behaviour are important and have been under-appreciated. Broader understanding of modern animal welfare science and the importance of positive welfare indicators are required for wildlife managers. We contend that endocrine suppression in free-ranging animals is an undesirable outcome because of its long-lasting alterations to natural behaviour and physiology. Not all fertility control modalities induce endocrine suppression and these methods that do not should be treated preferentially. Likewise, administration methods that require capture, surgery, or frequently repeated treatment are similarly undesirable. Animal welfare impacts should be recognised and carefully balanced against existing management goals to ensure continuous improvement in wildlife fertility control methods.

Author Contributions

Jordan Hampton and Teresa Collins were largely responsible for the generating of ideas and writing of the manuscript. Tim Hyndman and Anne Barnes provided input and associated literature on specific topics, and reviewed the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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BEHAVIORAL RESPONSE OF FREE-RANGING ELK TREATED WITH AN IMMUNOCONTRACEPTIVE VACCINE

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Abstract: Most wildlife contraceptive studies to date have evaluated treatment efficacy on individuals, but little research has evaluated the potential behavioral effects associated with inhibiting conception in polyestrous species. During autumn 1993 and 1994, we compared the behavior of 10 free-ranging, radiocollared cow elk (*Cervus elaphus*) vaccinated with porcine zona pellucida (PZP) immunocontraceptive vaccine with 10 unvaccinated cow elk. We tested the hypothesis that breeding behavior would be prolonged in PZP-treated cows due to repeated estrus cycling. Sexual interaction rates of treatment and control cows were similar during the normal breeding season ($P = 0.96$) but differed significantly during the postbreeding season ($P < 0.001$), when almost no sexual interactions were observed in control cows. Postbreeding season interaction rates in treated cows remained at the level observed during the breeding season. We did not detect any significant changes in activity patterns or social structure of the elk during the postbreeding season as a consequence of the apparent PZP-induced cycling ($P > 0.50$). It is uncertain whether the results of this study apply to possible effects of PZP treatment for management-level applications where a large proportion of the females would be treated. We recommend population-level experiments to further our understanding of the effects of contraceptive technologies on the behavior and dynamics of free-ranging ungulate herds.

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Key words: breeding, *Cervus elaphus*, estrus, immunocontraception, porcine zona pellucida, PZP, reproduction, Washington.

The manipulation of reproduction in ungulates has been explored by biologists and physiologists for nearly 30 years (Kirkpatrick and Turner 1985). Several female-oriented contraceptive technologies have potential applications to ungulate populations; however, most recent investigations have focused on immunocontraception by zona pellucida (ZP) vaccines (Liu et al. 1989, Kirkpatrick et al. 1990, Turner et al. 1992). Immunocontraception attempts to stimulate the target animal's immune system to interfere with some critical reproductive event (Glass and Hanson 1974). In the case of ZP vaccines, the immunological response prevents the sperm from penetrating the acellular glycoprotein layer surrounding the mammalian oocyte (zona pellucida), thus blocking fertilization (Sacco et al. 1984, Liu et al. 1989). Porcine ZP vaccine has proven an effective contraceptive for a variety of ungulates, including feral horses (*Equus caballus*), Przewalski's horses (*E. przewalski*), sika deer (*Cervus nippon*), sambar deer (*C. unicolor*), axis deer (*C. axis*), and white-tailed deer (*Odocoileus virginianus*; Kirkpatrick and Turner 1991). The vaccine also has been tested on captive North American elk, inhibiting reproduction in 7 of 8 treated cows (Kirkpatrick et al. 1996). Although numerous studies have evaluated the efficacy of contraceptive technologies on individual captive or free-ranging animals, little attention has been given to behavioral changes potentially caused by reproductive inhibition (Garrott 1995). Because ZP vaccines block fertilization, treated females may continue estrous cycles beyond the normal breeding season, which normally concludes once most females have been fertilized (Morrison 1960, Guinness et al. 1971). If the breeding season is extended in this manner for elk, the normal postbreeding activity budgets of both cows and bulls may be affected because of prolonged rutting activities, which, in turn, could have energetic and demographic consequences (Flook 1970).

We performed an experiment in a free-rang-

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ing population to investigate the potential behavioral consequences of blocking conception in elk. Our objectives were as follows: (1) to determine if blocking conception through immunocontraception would result in extending sexual interactions beyond the normal breeding season, and (2) to determine if normal activity budgets were affected if prolonged breeding behavior was detected.

STUDY AREA

Our study was conducted on and near the 330-km^2 Fitzner-Eberhardt Arid Lands Ecology (ALE) Reserve located in southcentral Washington (46°23′N, 119°32′W). The study area is classified as interior cold desert, with arid, hot summers and moderately cold winters. The ALE Reserve is a remnant of the shrub–steppe ecosystem (Daubenmire 1970) and is dominated by big sagebrush (*Artemesia tridentata*) with an understory of bluebunch wheatgrass (*Pseudoregnaria spicata*) or Sandberg's bluegrass (*Poa sandbergii*). Detailed descriptions of the study area are provided by Rogers and Rickard (1988) and Hinds and Rogers (1991).

The elk population inhabiting the Reserve was apparently established during winter 1972 by natural colonization (Rickard et al. 1977). The herd is nonmigratory and isolated from the nearest elk population by over 80 km. The rate of increase displayed by the population is among the highest recorded for the species (McCorquodale et al. 1988, Eberhardt et al. 1996). Aerial counts of the population increased from 190 to 257 animals during this study.

METHODS

Treatment and Observation

In March 1993, we immobilized 20 cow elk by darting from helicopters with 3.5 mg of Carfentanil citrate (Wildlife Pharmaceuticals, Fort Collins, Colorado, USA). Target animals were randomly selected from elk groups encountered. One upper canine was extracted for aging via cementum annuli, and pregnancy status was determined by rectal palpation (Greer and Hawkins 1967). We reversed the effect of the immobilizing drug with 325 mg of naltrexone Hcl (Wildlife Pharmaceuticals, Fort Collins, Colorado, USA). Prior to immobilization, we performed a Bernoulli trial to determine if each consecutively captured cow received a PZP vaccine or would serve as a control. We continued this random draw until 10 animals were assigned to the vaccination group or the control group, with the balance of the 20 captured animals assigned to the remaining treatment. The PZP vaccine was prepared from porcine ovaries as described by Liu et al. (1989) and stored at −5°C until used in the field. The inoculation was prepared as an emulsion of 0.5 cc vaccine (equivalent to approximately 5,000 zonae or 64.3 μg of protein) in phosphate buffer and 0.5 cc of Freund's Complete Adjuvant. We mixed the 2 vaccine components in the field using 100 plunger strokes of 2 5-cc glass syringes joined with a double-hubbed needle. The emulsified vaccine-adjuvant mix was then administered to treatment elk via intramuscular injection in the rear leg (Liu et al. 1989). We fitted white butyl radiocollars with distinct symbols on treatment animals and placed similar yellow collars on control animals.

We planned to administer an initial dose of PZP vaccine to treatment animals and a placebo containing only adjuvant to control animals at the time elk were immobilized for radiocollaring. However, our adjuvant supply was depleted after inoculating 5 treatment and 5 control cows; hence, the remaining 10 cow elk were marked with the appropriate radiocollars and released. We later discovered that the glass syringes were manufactured poorly, which allowed fluid to leak behind the plunger. This leakage caused larger volumes to be drawn into the syringes than indicated by the graduations, which depleted our adjuvant supply prematurely and possibly reduced the amount of vaccine administered to each animal by an unknown amount; the problem was rectified for all subsequent treatments. In August 1993, we remotely treated the 10 designated treatment animals with PZP vaccine administered via darting from helicopters. The 5 cows initially treated in March received a booster vaccination of 0.5 mL of PZP 5000 and Freund's Incomplete Adjuvant, and the remaining 5 treatment cows received an initial dose of PZP 5000 and Freund's Complete Adjuvant. In August 1994, all 10 treatment cows were administered booster vaccinations via darting from helicopters. We attempted to evaluate the calving status of all experimental animals during 1994 and 1995 by relocating each elk weekly during June and July; however, animals occupying private agricultural lands adjacent to the ALE Reserve were inaccessible. We used nursing and social grooming

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as indicators that a calf was the offspring of a radiocollared cow.

We collected behavior data from all radiocollared elk from September through late November 1993 and 1994. After this time, cow–calf associations became difficult to determine because of their aggregation into larger winter herds (McCorquodale 1987). We attempted to sample the behavior of each cow twice weekly. The order and frequency with which animals were sampled was determined via a restricted randomization design, which attempted to balance observations as much as possible among all experimental animals throughout the field season.

Once a breeding group containing the desired instrumented animal(s) was located via radiotelemetry, the observer, depending on environmental conditions and available cover, attempted to approach the group undetected to within 150–1,000 m. We conducted observations with the aid of a 15–60-× spotting scope and a field computer. Three types of data were collected during each observation session: breeding group attributes, focal animal behavior records, and an all-occurrences log of predefined, stereotyped behavioral events. We began a new observation session if any new animals moved into the social group; thus, the duration of watching a group may be longer than an individual observation session. We terminated <10% of the observation sessions because of animal disturbance by the observer.

We used focal animal sampling with an instantaneous recording method to sample activity states of all experimental animals in a breeding group at 2-min intervals (Altmann 1974, Martin and Bateson 1986). Four behavior states were identified. Elk were classified as feeding when standing with head lowered into the vegetation. Idling was recorded when an elk was upright and not feeding, or in a prone position. Moving was recorded when a female was actively locomoting, and not visible was recorded when an elk was not visible to the observer.

The observer temporarily interrupted focal animal sampling and immediately recorded into the all-occurrences log any predefined sexual interactions between a bull and experimental cow (Altmann 1974). We considered 2 behavioral events as sexual interactions: sniff–lick (Clutton-Brock et al. 1982) and mounting. A sniff–lick event was recorded when a bull muzzled or licked the perineum region of a cow or smelled fresh cow urine in the specific area where a cow was previously bedded or had just urinated. We recorded mountings as described by Struhsaker (1967).

Statistical Analyses

Because the PZP vaccine blocks fertilization, we expected treated females to cycle beyond the normal breeding season. We tested 2 null hypotheses: (1) there would be no difference in the rate of sexual interactions displayed by treatment and control cows following the normal breeding season, and (2) there would be no difference between treatment and control cows in the proportions of activity states following the normal breeding season. We assumed there would be no difference in sexual interaction rates or activity patterns during the normal breeding season between treatment and control cows; however, we tested these assumptions as secondary hypotheses.

To test hypotheses, we categorized the observation sessions as occurring during either the normal breeding period or the postbreeding period. These periods were defined by evaluating temporal patterns in the rate of bull sexual searches toward control cows. The onset of sexual interactions toward control cows established the start of the normal breeding season; we subjectively defined the end of the normal breeding season based on a decline of interactions.

We could not consistently identify individual experimental animals during observation sessions because of obstruction or distortion of collar markings by heat waves, vegetation, and other animals. The white-and-yellow collars of treatment and control animals, however, could always be distinguished, and thus provided reliable discrimination of the 2 groups. Thus, our experimental unit for analyses was the subgroup of treated or control cows in each breeding group observed. To test if blocking conception through immunocontraception resulted in extended breeding behaviors, we calculated sexual interaction rates as acts per animal per hour for both treated and control cow subgroups. We used activity–state proportions to test whether PZP treatment affected normal activity budgets. To test these hypotheses, we selected an appropriate data model and adjusted means by the principle of least squares (SAS Institute 1982). To test our specific hypotheses, we used pairwise comparisons of adjusted means for treatment and control cows during the normal and

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postbreeding periods (SAS Institute 1982). We transformed the response variable to natural logs if residual plots indicated a problem with homogeneity of variance.

Because our sampling unit was the observation session of a breeding group, the continuous covariates of breeding group composition (number of cows and adult bulls, number of treatment and control cows), total group size, and the categorical covariate of time of day (morning, 0500–1030; midday, 1031–1500; late day, 1500–2000) may have affected each of the 4 response variables measured. Exploratory model building was used to determine if any of these potential covariates were significant. We determined significant covariates for each of the 4 data models by performing a backwards elimination of covariates from saturated models with P-values < 0.05. Once significant covariates for each of the models were determined, we initiated hypothesis testing by using a regression approach with the main effects of treatment condition, breeding period, and year included in the data model, as well as the covariates that were significant in each model. We included year as a main effect because differences in vaccine treatments between years potentially resulted in differences in contraceptive efficacy, and hence behavioral responses. We included all possible 2- and 3-way interaction terms that included the main effects. To test our hypotheses, we used the adjusted means of the treatment × period interaction term and performed pairwise comparison on these adjusted means with a P-value < 0.05 considered as significantly different.

If both treated and control subgroups were present in the same observation session, we used both response rates with the attribute data for that session. Thus, our observations are not strictly independent. To help protect against Type I or Type II errors, we also performed analyses of the difference in rates on the subset of data where both control and treatment animals were present during the same observation sessions. This has the advantage of reducing the variation from individual experimental units and provided confirmation of conclusions derived from the analysis of the entire dataset (Johnson and Bhattacharyya 1987).

RESULTS

Experimental cows ranged in age from 1 to 14 years ($\bar{x}$ = 4.17, SD = 3.35), and 19 of the 20 cows were determined pregnant at capture. One control animal, aged 14, was found dead 2 weeks after radiocollaring, apparently because of complications from the immobilizing drugs. One treatment and 1 control cow were harvested during the 1994 hunting season. Following the 1993 treatment, 20% (1 of 5) of the cows that received the 2-dose treatment and 60% (3 of 5) of the cows that received the 1-dose treatment were observed with a calf, compared with 33% (2 of 6) of control cows being observed with a calf. Following the 1994 treatment, 0% (n = 9) of the treated cows were observed with a calf, compared to 100% (n = 7) of the control cows being observed with a calf.

We analyzed 81 observation sessions during 1993 that included 123 animal-hours (1 animal observed for 1 hr) of observation on treatment animals and 65 on control animals. In 1994, we performed 58 observation sessions including 60 animal-hours of observation on treatment animals and 33 animal-hours on control animals. During the study, the length of observation sessions ranged from 0.13 to 1.12 hr ($\bar{x}$ = 0.59, SD = 0.23). Total group size ranged from 2 to 136 animals ($\bar{x}$ = 29.4, SD = 24.0), with a range of 1–23 adult bulls ($\bar{x}$ = 3.7, SD = 4.1) and 1–61 cows ($\bar{x}$ = 15.2, SD = 11.4). The number of treated cows in an observation session ranged from 0 to 7 ($\bar{x}$ = 2.2, SD = 1.3), and the number of control cows ranged from 0 to 6 ($\bar{x}$ = 1.1, SD = 1.0). There were 86 observation sessions where both treatment and control cows were present in a social group, 38 observation sessions with only treatment cows present, and 15 observations sessions with only control cows present. We observed the first sexual interaction in both years on September 17 and a marked decline in sexual interactions of control cows by October 13. Thus, observation sessions occurring during this period were considered "breeding period" sessions, whereas later sessions were categorized as "postbreeding period" observations.

To test our primary hypothesis, we used sexual interaction rate as the response variable; the response variable was transformed because the untransformed data did not meet the assumption of homogeneity of variance as indicated by residual plots. We detected no difference in rate of sexual interaction between years ($F_{1,220}$ = 0.31, P = 0.53); so, data from both years were pooled. Number of control cows present was a significant covariate ($F_{1,220}$ = 5.79, P = 0.02),

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Table 1. Pairwise comparison of sexual interaction rates (events per hr observation) and proportion of time spent in various actives for PZP-treated and control cow elk during the breeding (17 Sep–13 Oct) and postbreeding season (14 Oct–28 Nov), 1993 and 1994. Values are adjusted means with standard errors reported in parentheses (SAS Institute 1982).

Behavior	Season breeding		Postbreeding season	
	Control	PZP-treated	Control	PZP-treated
Sexual interaction rate	0.58 (0.06)	0.57 (0.06)	0.01[a] (0.07)	0.57 (0.07)
Proportion time feeding	0.40 (0.03)	0.42 (0.03)	0.34 (0.04)	0.36 (0.04)
Proportion time idling	0.47 (0.03)	0.48 (0.03)	0.49 (0.04)	0.47 (0.04)
Proportion time moving	0.11 (0.02)	0.08 (0.02)	0.14 (0.02)	0.13 (0.02)

[a] Significant difference (t-test, $P \leq 0.05$). All other tests were not significant ($P > 0.05$).

and there was a treatment × period interaction for adjusted means of the transformed sexual interaction rate ($F_{1,220} = 19.00$, $P < 0.001$). During the normal breeding period, there was no difference in the frequency of sexual interactions of treatment and control cows (t-test, $P = 0.96$); however, during the postbreeding period, sexual interaction rates for treatment cows were different than control cows (t-test, $P < 0.001$; Table 1). The postbreeding interaction rate for treated cows was similar to the rates observed for both experimental groups during the breeding season. We obtained similar results using only the subset of observation sessions where both treatment and control cows were present in the observed group.

The residual plots for all 3 activity category models (feeding, idling, moving) indicated constant variance on the error term; hence, these data were not transformed. Year was not an influential covariate for any of the models; thus, data from both years were pooled. The only significant covariate detected in the feeding model was time of day ($F_{1,219} = 44.51$, $P < 0.001$). There was no treatment × period interaction for adjusted means of proportion of time spent feeding, idling, or moving ($F_{1,215-219} = 0.01–0.26$, $Ps = 0.60–0.91$). There were no differences in the proportion of time treatment and control cows spent feeding, idling, or moving during either the breeding (t-tests: $Ps = 0.28–0.85$) or postbreeding season (t-tests: $Ps = 0.64–0.73$).

DISCUSSION

This study was not designed to test the contraceptive efficacy of PZP vaccine; from the results of a study with captive animals (Kirkpatrick et al. 1996), we assumed that the treatment would block conception. Failure to detect any calves associated with treated cows in the second year of the study, while all control cows calved, suggests high contraceptive efficacy, although sample sizes are too small to warrant either statistical tests or strong conclusions. We attribute the relatively high proportion of treated cows associated with a calf in spring 1993 to the problem of the syringe leakage of the adjuvant-vaccine emulsion beyond the plunger during the initial year of treatment. Because we did not detect differences in the measured behavioral parameters between years, and because PZP-treated cows during the initial year of the study continued sexual interaction rates during the postbreeding season that were comparable to breeding season rates, the data strongly suggest the vaccination treatment the first year was indeed effective for at least the first 1–2 estrus cycles. This interpretation is supported by results of a recently completed study of antibody response of PZP-treated captive elk that indicates the elk immune system is particularly sensitive to challenge with the PZP vaccine; antibody responses are essentially maximal from a single injection (R. A. Garrott, unpublished data). Although most elk in the field experiment probably received less than the planned dose of PZP-adjuvant per injection during the first year of the study, all animals received 2 injections (1 in March when they were first radiocollared and a booster via darting from helicopter a month prior to the breeding season). These data suggest that even animals that may have received less than target doses during the initial year of treatment probably incurred at least a short-term elevation of antibody titers for several months posttreatment. A similar phenomenon has been documented for white-tailed deer. Investigators reported delayed parturition dates for does treated with a single dose of vaccine, which indicated elevation of antibody levels during at least the first estrous cycle (McShea et al. 1997).

Sexual interaction rates of treatment and control cows were similar during the normal breed-

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ing season but differed significantly during the postbreeding season. We observed almost no sexual interactions in control cows during the postbreeding season, but interaction rates in treated cows remained at the level observed during the breeding season. We interpret the elevated postseason sexual interaction rates as evidence of recurrent estrous cycling of PZP-treated cows because of a failure to conceive during the normal breeding season. Repeated cycling and resultant sexual interactions, however, did not appear to disrupt normal activity patterns of the treated cows, because we failed to detect any difference in the proportion of time treatment and control cows spent in feeding, idling, and moving during the postbreeding period. Hence, the treatment-induced postbreeding activity appeared to have no bioenergetic consequences for the cows. These results are very similar to those obtained by McShea et al. (1997) for captive white-tailed deer treated with various PZP vaccine regimes.

MANAGEMENT IMPLICATIONS

Results of this study suggest that the behavioral and biological consequences of recurrent estrus are not overly deleterious when only a small proportion of the females in a population is treated with PZP vaccine. Effective management regimes, however, will in all likelihood require treatment of a relatively high proportion of the females in a population (Garrott et al. 1992, Hone 1992). Recurrent estrous cycling in large numbers of females may stimulate bulls to maintain extended breeding behaviors such as bugling, harem formation, and defense, as well as antagonism with conspecifics. The mechanisms by which estrous cycling terminates are unknown (Lincoln 1985); hence, the potential duration of such an extended breeding season is uncertain. When our postbreeding observations terminated in late November, the rate of sexual interactions of treated cows was similar to the rates observed for the normal breeding period, which demonstrates extended breeding activity for a minimum of 6 weeks. Breeding could extend well beyond the period monitored in this study because both bulls and cows are capable of remaining reproductively active until March, which is effectively 5 months beyond normal breeding activities (Haigh and Hudson 1993).

Possible consequences of extended breeding could include disruption of normal postbreeding activity and spatial distribution patterns of both cows and bulls. Major disruptions of normal behavioral patterns are probably not a desirable side effect of contraceptive treatment in many situations. Behavioral modifications may also result in demographic consequences such as increased vulnerability to human harvest and higher overwinter mortality of breeding bulls because of greater depletion of body reserves from extended breeding (Flook 1970, Bowyer 1981). Cow activity patterns also could be affected if harem associations are maintained, which could increase energy expenditures, decrease foraging efficiency, or both. Such potential effects, however, may be offset by a decrease in energetic requirements of late gestation and lactation because of the failure of treated cows to conceive. Repeated estrous cycling also raises the potential of cows conceiving and giving birth months after the normal calving season, if PZP antibody levels fall below the threshold needed for effective contraception. Hence, repeated estrous could result in serious disruption, asynchrony, or both in breeding and birthing seasons, similar to that demonstrated by computer simulation of male-oriented contraception in feral horse populations (Garrott and Siniff 1992).

All of these possible scenarios are speculative, and their legitimacy and significance cannot be evaluated with small-scale, individual-based experiments such as this study. These questions, like those related to practical, contraceptive-based management of populations, can only be addressed with carefully designed and implemented population-level experiments where a large proportion of the animals are treated and adequate pre- and posttreatment data are available (Garrott 1995). Although logistic constraints and a lack of individually identifiable bull elk required that this study focus only on cow behavior, we recommend that investigators performing population-level experiments also devote considerable effort to evaluating the potential behavioral consequences of PZP treatment on bulls, because bioenergetic effects may be particularly pronounced in this segment of the population.

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LITTERFALL: AN OVERLOOKED FOOD SOURCE FOR WINTERING WHITE-TAILED DEER

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Abstract: Litterfall in mature forest stands may be a significant food resource for white-tailed deer (*Odocoileus virginianus*) wintering in northern regions, but has received little study. To determine forage production, we measured biomass of litterfall in unharvested stands of 3 wintering areas, and biomass of browse from deciduous trees and shrubs in unharvested stands and harvested patches of the same wintering areas during January–March 1994. Mean biomass of northern white-cedar (*Thuja occidentalis*), eastern hemlock (*Tsuga canadensis*), arboreal lichens (*Usnea* spp. and *Evernia* spp.), and hardwood leaves in unharvested stands was 18.4 kg/ha (SE = 4.80). At mean digestible energy (DE) levels of 1.5 and 1.7 kcal/g dry matter (DM), biomass of hardwood browse and litter biomass did not differ ($P > 0.15$) between harvested patches and unharvested stands. At greater mean levels of digestible energy (1.9 and 2.1 kcal/g DM), biomass was greater ($P < 0.05$) in unharvested stands than harvested patches because of the relatively high DE content of litterfall. We concluded that litterfall in unharvested stands was high relative to the availability of more commonly measured browses (e.g., understory trees and shrubs), and may be more palatable and of greater nutritional value. However, availability of hardwood browse during winter may be more predictable than litterfall.

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Key words: deer wintering area, forage, lichen, litterfall, Maine, nutrition, *Odocoileus virginianus,* white-tailed deer.

Wintering areas are critical habitat for white-tailed deer in northern regions because of reduced snow depths and enhanced thermal cover on these conifer-dominated sites (Telfer 1967, Ozoga 1968). However, the mature conifer canopy also reduces production of hardwood browse in the understory (Blouch 1984), and preferred browses such as northern white-cedar and eastern hemlock are typically unavailable because of overbrowsing (Frelich and Lorimer 1985, Verme and Johnston 1986). In addition, most browse species readily available in wintering areas are relatively low in nutritional value (Robbins and Moen 1975, Mautz et al. 1976).

There is some evidence that litterfall may be an important winter food for white-tailed deer, but litterfall has rarely been included in studies of food availability. Crawford (1982) and Shively (1989) reported that tame white-tailed deer ate eastern hemlock shoots that collected on the snow surface following periods of strong winds. Stevenson and Rochelle (1984) and Hodgman and Bowyer (1985) found that black-tailed deer (*O. hemionus columbianus*) and white-tailed deer consume considerable amounts of arboreal lichens during winter. Arboreal lichens have a substantially greater digestibility than other common winter foods (Robbins 1987) and may be an important winter food resource.

We quantified litterfall deposited during winter in forest stands typically used by deer in Maine wintering areas. To evaluate the potential significance of litterfall to wintering deer, we also compared biomass and nutritional quality

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Applications of GnRH in the control and management of fertility in female animals☆

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Abstract

Gonadotropin releasing hormone (GnRH) has long been recognized as a potential target for the control and management of fertility in female animals. Attempts to apply GnRH-based technology to manage fertility have focussed on the development of GnRH agonists, antagonists and vaccines. All of these methods have potential, but the widespread application of these technologies has been limited to date.

The greatest advance in the use of GnRH-based technology for long-term fertility control in recent years has been the development and commercialization of depot formulations that release GnRH agonists for periods of up to 1 year. These products have a broad range of potential applications in production and domestic animal management.

The further development and commercialization of GnRH vaccines has been hampered by the variability of response between individual animals. The need to use adjuvant and multiple boosters also make this a less attractive option than the current GnRH agonist technology. However, GnRH vaccines have the advantage that they do not induce the initial stimulatory response that follows GnRH agonist administration. GnRH antagonists and GnRH-toxin conjugates show promise but are in an earlier phase of development. To date, no depot or long-acting formulations of antagonists have been developed. GnRH-toxin conjugates have yet to achieve permanent sterilization, but further dose-response trials may advance this approach.
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Keywords: GnRH; Female contraception; Dosage; Drug delivery

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1. Introduction

GnRH is a desirable target for contraception because it acts through specific, high affinity receptors on gonadotropes. Traditionally, there are three different ways that GnRH can be employed to inhibit reproduction by direct suppression of the pituitary-gonadal axis at the level of the gonadotrope: (1) chronic administration of GnRH agonists causing down regulation of GnRH receptors and desensitization of pituitary gonadotropes; (2) immunization against GnRH resulting in the neutralization of GnRH in the hypophyseal portal blood by antibodies; and (3) the use of GnRH antagonists to block pituitary GnRH receptors to occupancy by endogenous GnRH (Fraser, 1982). A more recent phenomenon has been the development of GnRH-toxin conjugates that disrupt pituitary gonadotropes (Sabeur et al., 2003).

Long-term fertility control agents based on GnRH are of interest for the management of reproduction in humans, domestic animals and wildlife. Control of reproductive activity in farm animals is an important management issue from both a behavioral and fertility standpoint, as is the case with domestic dogs and cats. Historically, fertility control has been achieved through surgical sterilization. However, in some species the permanent nature of these procedures is a disadvantage. Other disadvantages include the associated trauma, production setbacks and potential death (D'Occhio, 1993). Fertility control has also become a popular concept for the management of highly valuable but overabundant wildlife species. Here the aim is to decrease population size by reducing the fertility of the population. Since GnRH is highly conserved in mammals, fertility control technology founded on this hormone has a wide range of applications in different species.

2. GnRH agonists

2.1. GnRH agonists and pituitary down regulation

GnRH agonists are peptides that are similar to GnRH but are modified at sites of enzymatic degradation of GnRH. This increases resistance to peptidases and enhances receptor-binding affinity, with GnRH agonists having a longer half-life in circulation. Substitution of a D-amino acid instead of the glycine at position 6 and replacement of the C-terminal glycinamide residue with an ethylamide group produces agonists that are up to 200 times more potent than native GnRH (for review see Padula, this volume; Karten and Rivier, 1986). GnRH agonists were originally developed to treat infertility but paradoxically inhibited reproduction because the importance of pulsatile administration had not been realized (Schriock, 1989). This discovery led to numerous indications for the use of GnRH agonists today, including contraception and the treatment of hormone-dependent diseases.

GnRH agonist treatment initially induces a large increase in LH and FSH concentrations (the acute phase), which can last for several days, followed by a return to basal levels (D'Occhio et al., 2000). Continued exposure abolishes pulsatile secretion of LH as a result of down regulation of GnRH receptors on gonadotrope cells (Hazum and Conn, 1988) and an uncoupling of second messenger systems (Huckle and Conn, 1988). This results in a decrease in the synthesis of LH and FSH, which appears to be effected specifically through

Table 1
Structure, potency, dose and duration of slow release GnRH agonist formulations

Trade name	Agonist	Structure	Relative potency[a]	Route and formulation	Dose (mg)	Duration (month)	Reference
Zoladex®	Goserelin	[D-Ser(But)^6, AzaGly^{10}]GnRH		SC depot polymer implant	3.6	1	Fraser (1988)
Zoladex LA	Goserelin				10.8	3	Fraser (1988)
Suprefact®	Buserelin	[D-Ser(But)^6, Pro^{9}Net]GnRH	20	SC depot polymer implant	3–6	3	Fraser, (1988)
Lupron Depot®	Leuprolide	[D-Leu^6, Pro^{9}Net]GnRH	15	IM microspheres	3.75–30	1, 3 or 4	Periti et al. (2002)
Viadur®	Leuprolide			SC osmotic pump implant		12	Fowler et al. (2000)
Decapeptyl C.R.	Tryptorelin	[D-Trp^6]GnRH	100	Microspheres	7.5	1	Schneider et al. (1998)
Suprelorin®	Deslorelin	[D-Trp^6, Pro^{9}Net]GnRH	114	IM depot implant	6	6	Trigg et al. (2001)
Not yet named	Histrelin	[D-His(Bzl)^6, Pro^{9}Net]GnRH	210	SC hydrogel implant	50–60	12	Schlegel et al. (2001)

SC, subcutaneous; IM, intramuscular.
[a] In vitro potency relative to GnRH.

suppression of production of β-subunit mRNA (Aspden et al., 1997). These conditions affect normal pituitary function such that the pituitary response to endogenous GnRH is impaired. The end-point of these changes is a decline in the concentration of gonadal steroids. In females this is accompanied by an inhibition of follicular development as FSH secretion is suppressed below the threshold required for follicular development and inhibition of the oestrogen induced positive feedback mechanism and ovulation (reviewed by Fraser, 1993). A lack of pulsatile secretion of LH is maintained as long as the GnRH agonist is present above a threshold in the circulation (D'Occhio and Aspden, 1996). The chronic effects of GnRH are further reviewed (this volume).

2.2. Delivery

While the potential fertility control applications of GnRH agonists have been recognized for almost three decades, successful development of a commercial product for animals has been limited by failure to develop a suitable delivery system (Trigg et al., 2001). Recent development of bio-compatible GnRH agonist formulations which are easy to use, cost effective and release sufficient quantities of GnRH agonist over long periods of time are now overcoming the previous impediments to long-term fertility control applications (Table 1). The mode of delivery ranges from smooth microspheres (20–30 μm diameter) suspended in vehicle, which are administered by subcutaneous or intramuscular injection, to depot polymer implants that are injected subcutaneously. The majority of these formulations have been developed for the treatment of GnRH-dependent diseases in humans and have a lifespan of 1–3 months. To date only one product has been developed specifically for long-term (6 month) fertility control of domestic animals. This product, with the trade name Suprelorin, releases the GnRH agonist deslorelin from a matrix consisting predominantly of low-melting point lipids and biological surfactant (Peptech Animal Health Sydney Trigg et al., 2001). It has been approved for sale in male dogs in New Zealand and Australia in a 6 month duration form, and a 12 month product is in advanced development for both sexes.

2.3. Long-term fertility control

Administration of GnRH agonist formulations can achieve long-term reversible suppression of the pituitary–ovarian axis in a wide range of species (Table 2). Treatment of cats with implants containing 6 and 12 mg of deslorelin suppressed oestradiol secretion for periods of at least 14 months in 80% of animals (Munson et al., 2001). Comparable levels of response to the same peptide have been observed in bitches (Trigg et al., 2001). Large-scale trials of similarly formulated deslorelin in female cattle have demonstrated that implants containing higher doses (12 mg) can inhibit reproduction for >300 days in 100% of animals and >400 days in 90% of animals ($n = 99$) (D'Occhio et al., 2002). These figures demonstrate the extremely high contraceptive success rate achieved using these products. The long duration of release of agonists like deslorelin (1 year) suits livestock production schedules and domestic pet veterinary regimes.

One of the advantages of GnRH agonists is that contraception is effected after a single injection without the need for use of adjuvants. Treatment with agonists has so far proved to be safe, with no serious side effects. Occasionally animals will develop a local reaction at

Table 2
Contraceptive success and duration following treatment with slow-release GnRH agonist formulations in six mammalian species

Species	Agonist	Dose (mg)	*N*	Oestrus induction	Infertile (%)	Duration (mean ± S.E.M.) (range) (days)[a]	Reference
Cats	Suprelorin	6	*C* = 5 *T* = 5	Yes[b]	100[b]	333 ± 39 (240–420)	Munson et al. (2001)
Cats	Suprelorin	12	*C* = 5 *T* = 5	Yes[b]	100[b]	330 ± 31 (225–420)	Munson et al. (2001)
Dogs	Suprelorin	3 6 12	*T* = 8 *T* = 8 *T* = 9 *C* = 10	Yes (50%)	100[c,d]	321 ± 42[c,d] 438 ± 105 465 ± 51 165 ± 15	Trigg et al. (2001)
Heifers	Suprelorin	8 8 12	*T* = 76 *T* = 84 *T* = 99 *C* = 59	?	97[e,f] 100[e,f] 100[e,f]	231 ± 3 (*n* = 9) 244 ± 13 (*n* = 8) 336 ± 3 (*n* = 20)	(D'Occhio et al., 2002)
Heifers	Buserelin	6 12	*T* = 5 *T* = 5	?	100[c]	48.4 ± 3.8 87.4 ± 17.4	D'Occhio et al. (1996)
Heifers	Suprelorin	5 10	*T* = 8 *T* = 9	?	88[c] 100[c]	203 ± 26 (111–280) 170 ± 28 (70–280)	D'Occhio et al. (1996)
Wapiti	Leuprolide	32.5	*T* = 4 *C* = 5	?	100[c,f]	225	Baker et al. (2002)
Cheetahs	Suprelorin	6 12	*T* = 8 *T* = 8	Yes (31%)	100 100	>485c[c,d] >640[c,d]	Bertschinger et al. (2001) Bertschinger et al. (2002)
Wild dogs	Suprelorin	6	*T* = 6	?	83	365[c]	Bertschinger et al. (2001)

N, number of treated (*T*) and control (*C*) animals; >, trial ongoing and animals still to breed; *n*, refers to the number of animals the mean was calculated from (i.e. calculated on the first animals to resume cycling).
[a] Single values represent the earliest resumption of reproductive activity in the group.
[b] Determination of fertility/oestrus based on plasma oestradiol.
[c] Determination of fertility/oestrus based on plasma progesterone.
[d] Determination of fertility/oestrus based on vaginal cytology.
[e] Determination of fertility/oestrus based on ovarian histology.
[f] Determination of fertility/oestrus based on pregnancy/birth.

the injection site, e.g. in cats minimal oedema developed at the site of implant insertion for 3–5 days (Munson et al., 2001), but these effects were transient and not widespread. In dogs, there was no evidence of inflammation or oedema following treatment of 55 animals (Trigg et al., 2001), and subsequent treatment of 387 dogs (T. E. Trigg, unpublished observations).

The results of numerous studies suggest that a longer duration of fertility control can be achieved using higher doses of agonist within similar formulations. In male dogs there was a positive relationship between the dose of agonist (mg kg^{-1}) and the duration of suppression

(Trigg et al., 2001). Similar relationships have been observed in heifers (D'Occhio et al., 2002). The reason(s) for this dose-dependent increase in duration are not clearly understood. It is possible that a higher starting dose of agonist in a slow release system means that the release rate can be maintained above a theoretical critical threshold for down regulation for a longer period of time. It is also possible that the pituitary takes longer to recover from a higher dose. The exact nature of the dose-response relationship for GnRH agonist-induced pituitary down-regulation requires further investigation, as it does not always hold true for all species (Table 2).

The initial stimulatory gonadotropin response to GnRH agonist treatment can induce oestrus in some species. In cats this was characterised by an initial oestrus-like increase in faecal oestradiol concentrations in all animals (Munson et al., 2001). In dogs, oestrus was generally induced within 4–8 days when plasma progesterone concentrations were $<5\ \text{ng ml}^{-1}$ (Trigg et al., 2001). This agonist-induced oestrus can be suppressed in bitches by progestin treatment initiated before agonist administration ($2\ \text{mg progestin kg}^{-1}$ body weight orally for 1 week before and 1 week after) (Wright et al., 2001). This dose needs further titration and other progestin products should be analyzed. Trials should also be conducted on a range of species, as simultaneous progestin and GnRH agonist treatment in wild carnivores did not prevent agonist-induced oestrus (Bertschinger et al., 2001). Although agonist-induced oestrus has been observed in many species, not all animals which display oestrus behaviour will allow mating, e.g. female cheetahs and lionesses (Bertschinger et al., 2001).

There can be a wide range of variation between individuals in the interval to resumption of oestrous cycles during GnRH agonist treatment. This variability may be the result of genetic variations in the sensitivity of individual animals to GnRH-induced down-regulation. As the variability is often greatest at lower doses it may represent a dose-response relationship where a particular dose may be sub-threshold for some individual animals. An alternative hypothesis is that there may be some variability in the implant manufacture process.

There also appears to be a proportion of animals that continue to cycle during GnRH agonist treatment, i.e. 'non-responders'. The proportion of these animals can be difficult to determine in larger scale trials that do not regularly monitor progesterone concentrations or ovarian cycles, e.g. if you get a much shorter duration of action in an individual, is this 'non-response' or is this an earlier return to oestrous cycles for a different reason? There is sufficient data to determine that non-responders are present at a very small percentage in some populations (e.g. heifers, wild dogs). The basis of this variability is probably genetic, as repeated treatment of animals and administration of higher doses does not seem to elicit a contraceptive response (Herbert, unpublished data from kangaroo trials).

3. Immunization against GnRH

Active immunization against GnRH creates an immunological barrier between the hypothalamus and the anterior pituitary gland. Antibodies bind to GnRH in the hypothalamo-hypophyseal portal circulation, which prevents GnRH from binding with receptors on pituitary gonadotropes. This results in suppression of gonadotropin secretion, inhibiting follicular development and ovulation, as well as reproductive behavior. Because

Table 3
Contraceptive success and duration following immunization against GnRH in four mammalian species

Species	Antigen	Adjuvant	Booster(s) (wppv)	*N*	% immune response	% infertile	Infertility duration	Reference
Heifers	GnRH-KLH	Freund's	8	*C* = 9 *T* = 30	100[b]	80[c,d]	>28 weeks	Adams and Adams (1990)
Heifers	GnRH-OVA (Vaxstrate®)	DEAE	16	*C* = 200 *T* = 400	?	82[d]	>207 days	Hoskinson et al. (1990)
Chinese pigs	GnRH-OVA	Specol	8	*C* = 12 *T* = 12	100[b]	83[c,d]	>18 weeks	Zeng et al. (2002)
White-tailed deer	GnRH-KLH	Freund's	4, 52	*C* = 4 *T* = 8	100[b]	88[e]	Insufficient data	Miller et al. (2000)
White-tailed deer	GnRH-OVA	DEAE-dextran	4, 8, 12, 41	*C* = 4 *T* = 4	100	50[c]	<60 weeks	Becker et al. (1999))
Mares	GnRH-BSA	Equimune®	3, 6, 10	*C* = 1 *T* = 3	100[b]	66[c,d]	13–15 months	Dalin et al. (2002)
Dogs	GnRH-KLH	Threonyl-MDP	3, 6	*T* = 8 (sc) *T* = 8 (im)	100	0[c] 50[c]	14 weeks >10 weeks	Gonzalez et al. (1989)
Cats	IPS-21[a](10 μg) IPS-21(100 μg)		4, 96 4, 96	*T* = 5 *T* = 5	100 100	100[c] 100[c]	>2 years >2 years	Robins (2002)

wppv, weeks post primary vaccination; *N*, number of treated (*T*) and control (*C*) animals.
[a] Recombinant vaccine.
[b] Immune response significantly lower in non-responders.
[c] Determination of fertility based on plasma progesterone.
[d] Determination of fertility based on uterine and ovarian histology.
[e] Determination of fertility based on births.

GnRH is a weak antigen (due to its low molecular weight) and also a 'self' hormone, it needs to be conjugated to a large carrier in order to elicit an immune response. Common carriers include ovalbumin (OVA), keyhole limpet hemocyanin (KLH), tetanus toxoid (TT) and diptheria toxoid (DT) (Talwar, 1997).

Immunization against GnRH was initially used to gain a further understanding of the role of GnRH in mammalian reproduction. However, scientists soon realized the potential therapeutic applications of this approach for both human and domestic animal medicine. Active immunization against GnRH has been investigated in a range of domestic animals and wildlife species (Table 3). Contraceptive success rates typically range from 50 to 90%, with occasional 100% success rate in some small trials. Long-term fertility control (i.e. ∼12 months) can be achieved with this approach, but usually requires multiple vaccinations. The duration of contraception is typically quoted from the time of primary vaccination, which can make comparison with other "single-shot" methods misleading.

Immunization against GnRH has the advantage that it does not have the initial stimulatory acute phase that GnRH agonists have, which thereby avoids the problem of treatment-induced oestrus/ovulation. However, this immunocontraception approach introduces a range of other problems.

The primary problem with immunization against GnRH (or indeed all other 'self' antigens) is the highly variable response between individual animals. In almost all trials it is evident that non-responders are present within the population. These are animals that do not have a contraceptive response to the vaccine. In many cases reproductive function is maintained despite an immunological response to the vaccine. When Chinese female pigs (Zeng et al., 2002), feedlot heifers (Adams and Adams, 1990) and mares (Dalin et al., 2002) were immunised against GnRH, the non-responders mounted immune responses that were significantly lower than those successfully immunocontracepted. This suggests there is a critical immunological threshold that must be attained to adequately neutralize GnRH (Adams and Adams, 1990; Zeng et al., 2002). In the case of feedlot heifers the variability was unlikely to be the result of insufficient dose of immunogen as the number of non-responders was evenly spread across three different dosage groups (Adams and Adams, 1990). It is widely thought that the variation in immune response between individual animals is a result of genetic variation (Cooper and Herbert, 2001). If this genetic foundation of non-response is heritable, the number of non-responders will increase with successive generations, particularly if there is high selective pressure (e.g. if non-responders are the only individuals to breed). Under this scenario the lifespan of these products would be severely curtailed.

Another drawback is that most immunization regimes require repeated secondary immunizations to ensure the production and maintenance of high anti-GnRH titres. In some cases it may take up to three immunizations to see a significant antibody response compared with control animals (e.g. mares; Garza et al., 1986). The need for repeated immunizations can be costly, time consuming and impractical, particularly in large-scale farming operations and free-range wildlife situations. Development of single-injection controlled-release vaccines of 1 year duration could overcome this drawback (Turner Jr. et al., 2002), but they are difficult to successfully develop.

Currently there is a need to use an adjuvant to obtain sufficient immunogenicity to inhibit reproduction. In a study of white-tailed deer, the use of diethylaminoethyl (DEAE)-

dextran as the adjuvant caused local reactions ranging from inflammation, minor alopecia and erythema to dry necrosis, sloughing of the skin and purulent lesions (Becker et al., 1999). In heifers, transient injection site reactions to DEAE were noted in 30% of animals (Hoskinson et al., 1990). Mares immunised against GnRH–BSA with Equimune® adjuvant showed a range of reactions to the booster ranging from fever to oedematous swelling and front leg lameness (Dalin et al., 2002). These reactions at the injection site are not uncommon with adjuvants and animal ethics committees and regulatory bodies throughout the world either do not allow their use or are fazing out the use of some adjuvants, in particular Freund's adjuvant, because of animal welfare concerns. These negative effects can be reduced in some species by reducing the amount of adjuvant used (Dalin et al., 2002), altering the route of administration (e.g. subcutaneous instead of intramuscular) and by using alternative adjuvants (Leenaars and Hendriksen, 1998; Leenaars et al., 1998). Some of these alternative adjuvants (e.g. Specol) cause fewer pathological changes than Freund's adjuvant.

Other concerns about immunization against GnRH and other types of immunocontraception relate to the potential for unknown side-effects. The unforseen consequences of provoking an autoimmune reaction are a concern that may limit the development of a contraceptive vaccine for women (Baird, 2000). In addition, there is concern that as animals with a poor immune response are less likely to be affected than other animals, immunocontraception may artificially select for immunocompromised animals which are more susceptible to diseases (Oogjes, 1997).

The concerns outlined above are likely to affect the successful commercialization of GnRH vaccines. Other impediments include the high cost of antigen, which results from a low efficiency of conjugation. In addition, the fact that these vaccines will be active in humans means that there is also a risk of self-inoculation. This risk is greater with a vaccine than with a GnRH agonist implant. The first GnRH vaccine that was released commercially for prevention of pregnancy in animals (Vaxstrate®, Peptech Animal Health Pty Limited, North Ryde) is no longer on the market due to the need for two injections.

Recent advances in GnRH vaccine technology have included the development of a recombinant vaccine. The antigen consists of eight copies of GnRH linked to each end of a proprietary carrier protein, making it greater than 50 kDa, which is large enough to elicit an immune response (reviewed by Rhodes and Moldave, 2002). This vaccine has successfully inhibited reproduction in 100% ($n = 10$) of prepubertal female cats for periods in excess of 2 years. The vaccine was also effective in 75% ($n = 4$) of male cats following two immunizations (Robins, 2002).

4. GnRH antagonists

GnRH antagonists act on the same receptors as native GnRH. When administered, they bind to GnRH receptors in the pituitary but are devoid of stimulatory activity. Receptor occupancy by GnRH antagonists blocks the receptor to occupancy by endogenous GnRH causing an immediate suppression of gonadotropin release (Conn and Crowley, 1994; Schalley, 1999). From the early 1970s an increasing number of GnRH antagonists have been developed. The development process involves a stepwise introduction of hydrophobic residues

that block proteolysis, increase GnRH receptor affinity and increase the pharmacokinetics of the molecule (Fraser, 1988). Initially GnRH antagonists exhibited significant histamine-releasing ability which almost halted their further development (Conn and Crowley, 1994).

GnRH antagonists have successfully been employed to inhibit the LH surge and ovulation in gilts (Brussow et al., 2001) and they have been used to help determine the endocrine control of follicular development in a range of species (e.g. mares; Watson et al., 2000). While GnRH antagonists have the advantage that they do not provoke the initial stimulatory effect of agonists and their effects are immediate, the high dose required to prevent oestrous cycles and the failure to develop an efficient controlled-release system has reduced their practicality. Until an efficient delivery system can be developed, the use of these products is likely to be limited to short-term treatment, e.g. down-regulation of the pituitary during IVF treatment regimes.

5. GnRH-toxin conjugates

The approach of coupling a protein synthesis inhibitor (usually cytotoxic) to GnRH is a more recent approach to long-term fertility control. Because GnRH receptors are highly specific a variety of cytotoxic agents could be used in an attempt to disrupt pituitary gonadotropes by conjugating them to GnRH or GnRH analogues. Death of the gonadotrope will occur when the gonadotropes internalise the GnRH conjugate as part of the normal process of receptor deactivation. Following a sufficient reduction in the number of gonadotrope cells, there will be inadequate production of LH and FSH to maintain reproductive function (Nett and Jarosz, 2002). A recent study has attempted to inhibit reproductive function in male dogs with the use of GnRH conjugated to pokeweed antiviral protein (PAP) (Sabeur et al., 2003). GnRH–PAP caused a reduction in basal LH and testosterone concentrations and testes size. By week 20 post-treatment basal LH and testosterone concentrations showed evidence of recovery (Sabeur et al., 2003). The resumption of reproductive activity may be the result of insufficient dose, or it may be the result of undifferentiated stem cells in the pituitary.

6. Conclusions

GnRH agonists and vaccines are the only two GnRH-based fertility control methods that have successfully been used in a wide range of domestic animal species and are approaching the commercialization stage. There are now many commercially available GnRH agonist formulations, but most are designed for human use and may therefore be hard to access for animals and/or be prohibitively expensive. There is now one GnRH agonist formulation that has been specifically developed for domestic animals (Suprelorin®) and this is approved in two countries and commercialisation worldwide is proceeding.

The impetus for the development of GnRH-based fertility control has been founded on the potential applications in domestic animals and humans, but a new and emerging field has been the use of these products to control overabundant wildlife. The fact that research and development of this technology has been funded from the more lucrative fields of

human medicine and production animal health has made the transfer of this technology to wildlife relatively cheap (i.e. compared to the development of species specific zona pellucida vaccines for example). The highly conserved nature of the role of GnRH in reproduction has allowed easy transfer of this technology to other species.

GnRH agonists have a much higher contraceptive success rate and less negative side effects than immunization against GnRH in both domestic animals and wildlife. The high variability of response to GnRH vaccines is a problem that may eventually see the pursuit of such 'self' vaccines abandoned. Attempts to further develop GnRH antagonist and GnRH-toxin technology may provide useful products with a range of potential applications.

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EFFECTS OF FERTILITY CONTROL ON POPULATIONS OF UNGULATES: GENERAL, STAGE-STRUCTURED MODELS

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Abstract: Regulating the abundance of ungulate populations using hunting can prevent populations from reaching levels that cause harm to natural and human dominated systems. However, there are an increasing number of cases where hunting is infeasible, and in such cases, fertility control has been widely advocated as an alternative means for controlling populations. Here, we develop simple analytical models offering general insight into the feasibility of using fertility control to regulate the abundance of ungulates. The models are structured in stages to represent variation in the duration of effect of fertility control agents. Analysis of these models offers several predictions, amenable to testing in field studies. (1) More than 50% of fertile females will need to be maintained infertile to achieve meaningful reductions in ungulate numbers even when fertility rates are low. (2) The relationship between the proportion of females maintained infertile and the steady state density is highly nonlinear. This means that small errors in estimating levels of infertility can lead to large errors in achieved density. It also means that managers should expect to see little change in steady-state density across a broad range of delivery rates. (3) The efficacy of fertility control as a management technique depends strongly on the persistence of the effect of the fertility control agent and the ability of managers to recognize previously treated animals. (4) Fertility control using long-lived agents can be more efficient than culling in regulating ungulate numbers. (5) Treating small populations with irreversible agents magnifies the likelihood of population extinction relative to treatment by culling. As with all techniques, managing population fertility must extend from a sound understanding of the influence of management actions on the state and dynamics of the population.

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Controlling the abundance of ungulates by regulating harvest is one of the prevailing activities of wildlife managers throughout North America. Regulated harvest offers a variety of benefits to society, including recreation for citizens and revenue for agencies and private enterprise. An additional benefit is a reduction in animal numbers, which if left unchecked, can cause significant harm to natural and human-dominated systems (Jewell and Holt 1981, Diamond 1992, Garrott et al. 1993, McCullough et al. 1997).

Although harvest can be an effective method for manipulating ungulate numbers, there are a growing number of situations where it is not feasible. The rapid expansion of urban and suburban areas during the last decade has brought populations of ungulates in close proximity to high densities of people (Adams 1994, McCullough et al. 1997). This proximity restricts the use of hunting as a management tool because it poses unacceptable risks to human safe-

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ty (Decker and Connelly 1989, Brush and Ehrenfeld 1991, McAnnich 1993, Wright 1993), and because urban residents often oppose hunting animals on ethical grounds (Decker and Connelly 1989, McCullough et al. 1997). In addition, there are many conservation areas, national parks prominent among them, where hunting is proscribed by law and policy (Leopold et al. 1963, Wright 1993, Porter et al. 1994, Wagner et al. 1995, Frost et al. 1997, Porter and Underwood 1999). In all of these situations, culling animals to regulate populations has significant liabilities, and as a result, wildlife managers are seeking innovative alternatives to lethal methods of population control (Kirkpatrick and Turner 1985, Bomford 1990, Brush and Ehrenfeld 1991, Diamond 1992, Garrott et al. 1993, McCullough et al. 1997).

Fertility control has been widely advocated as an alternative to culling (reviewed by Kirkpatrick and Turner 1985, Bomford 1990, Tuyttens and Macdonald 1998). Field and laboratory studies have evaluated efficacy of delivery of contraceptives to ungulates (Jacobsen et al. 1995, McCallum 1996, DeNicola et al. 1997, Fayrer Hosken et al. 1997, Kirkpatrick et al. 1997, Nielsen et al. 1997, Turner et al. 1997), and models have been developed to represent effects of fertility control on the population dynamics of individual species (Garrott and Siniff 1992, McAninch 1993, Swihart and DeNicola 1993, Boone and Wiegert 1994, Seagle and Close 1996). However, despite these efforts, a broad understanding of effects of fertility control on ungulate populations has failed to emerge. The absence of such understanding retards efforts to apply fertility control as a method to control ungulate numbers.

Here, we develop a general theory of regulation of ungulate populations using fertility control agents. Specifically, our objective was to develop simple analytical and simulation models offering general insight into the following questions. (1) What proportion of females in a population must be infertile to maintain the population at target densities? (2) How does fertility control compare with culling as a means to regulate ungulate populations? (3) How does duration of efficacy of contraceptives influence the number of animals that must be treated to achieve target population levels? (4) To what extent does timing of delivery (pre- or post-breeding) influence the ability of contraceptives to regulate ungulate numbers? (5) Do assumptions on the operation of density dependence modify the insights gained above? (6) Is fertility control a "safe" method for regulating small populations vulnerable to stochastic, local extinction? We provide a general mathematical basis for screening the feasibility of fertility control before developing detailed, species-specific models or conducting extensive field applications.

METHODS

Base Model

Because our purpose was to develop general insights, we used a model that was as simple as possible (Levins 1966, Starfield et al. 1990, Starfield and Bleloch 1991, Starfield 1997). The model needed to be sufficiently detailed to mimic the behavior of ungulate populations in a reasonable way, but it also needed to avoid detail peripheral to the questions posed above. To meet this challenge, we began with a simple formulation and incrementally added detail needed to meet our objectives.

Our starting point was a stage-structured model representing species where adult survivorship is high and where density dependence operates primarily on recruitment, characteristics that are particularly faithful to the life histories of many species of ungulates (McCullough 1979, Houston 1982, Skogland 1990, Bartmann et al. 1992, Gaillard et al. 1993, Putman et al. 1996). The model was formulated as

$$F_{t+1} = F_t(S + m - \beta(F_t - H_t)) - H_t, \quad (1)$$

where F_t is the density of females at time t, S is the annual per-capita survival rate of females, m is the maximum number of breeding females annually recruited to the population per breeding female, β is the linear slope of the relationship between recruitment of females to breeding age and female density, and H_t is the number of females culled at time t. The model predicts the preharvest population density of females using 3 parameters (S, m, β) and a single decision variable, H_t. Although the model represents a single stage explicitly (adult females), it implicitly represents sexually immature juveniles via the recruitment term. The explicit juvenile stage drops out of equation (1) via model reduction (Caswell 1989:100).

The model is based on several simplifying assumptions. First, we assume that the probability of survival of adults does not change with age. Thus, we treat longevity as the outcome of a

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series of independent Bernoulli trials rather than being fixed at a specific age (Gurney and Nisbet 1998:80). Culling is assumed to occur after breeding, but before recruits are born. We assume that density dependence is linear and operates entirely on recruitment. That is, the number of new breeding females recruited to the population is directly proportionate to the postharvest population density of females. Effects of nonlinear density dependence will be included as part of the analysis of the model.

It is possible to formulate the model using an explicit parameter for the density at which recruitment = 0 (i.e., $K_r = m/\beta$). Alternatively, we can represent effects of density dependence using a slope (β) and intercept (m) for the relationship between density and recruitment. We used this approach because we believe these parameters offer useful biological interpretations; the parameter β represents the "strength" of density dependence and the parameter m represents the maximum possible recruitment rate. Both of these representations prove useful when interpreting model analysis.

Although this model is extremely simple, it can represent the dynamics of ungulate populations with reasonable fidelity (Fig. 1). A more detailed model would achieve higher levels of realism, but only at the expense of reduced clarity and generality.

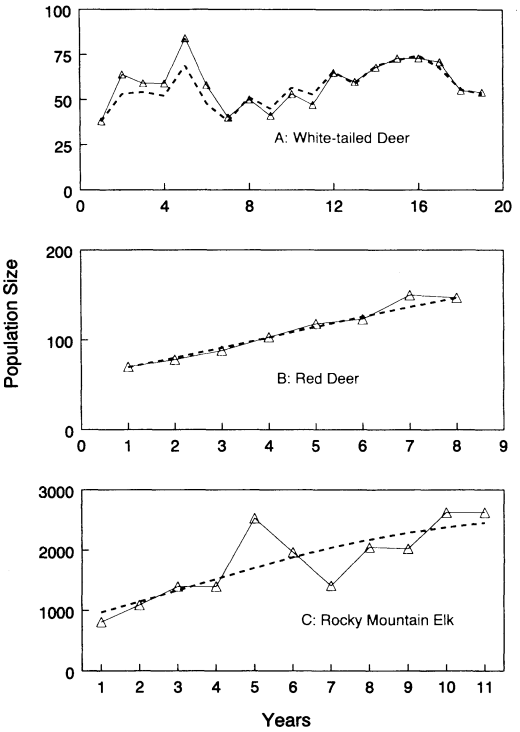


Fig. 1. Comparison of model projections (dashed line) with observed trajectories of ungulate populations (triangles). Model parameters were estimated by minimizing the squared difference between observations and predictions. A close fit between observations and predictions allows the conclusion that the model contains sufficient detail to reasonably represent trajectories of ungulate populations if its parameters are properly estimated. Data are from [A]—McCullough (1979: Table 3.2), [B]—Clutton-Brock et al. (1982), [C]—Houston 1982.

Model Development

We modified equation (1) to represent effects of fertility control as an alternative to culling. Node diagrams and the matrix form of the model are shown in Fig. 2. Here, we summarize our modifications using finite difference equations.

We defined the number of animals harvested annually as

$$H = cF_t. \quad (2)$$

Thus, c is the per capita rate of culling, the number of breeding females culled annually per breeding female in the population. It follows that the effects of culling can be represented in the model as

$$F_{t+1} = F_t(1 - c)(S + m - \beta(1 - c)F_t). \quad (3)$$

We will refer to equation 3 as the culling model.

To allow comparison with fertility control, we redefined c as the per capita rate of delivery of contraceptives. There are now 2 stages that we wish to track in the population; females that are fertile (i.e., F_t) and those that are infertile (i.e., I_t). We define fertile animals as those that are capable of breeding and carrying a fetus to term. We define infertile animals as those that cannot successfully breed. When the effect of the contraceptive persists for a single breeding season, then the dynamics of the fertile (F_t) and infertile stages (I_t) can be represented as

$$F_{t+1} = F_t(1 - c)(S + m - \beta N_t) + (1 - c)I_t, \text{ and} \quad (4)$$

$$I_{t+1} = F_t c(S + m - \beta N_t) + I_t Sc, \quad (5)$$

where

$$N_t = F_t + I_t. \quad (6)$$

However, if the effects of the contraceptive persist for more than 1 breeding season, then we must account for the time since the animal was treated. This accounting can be accomplished

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by adding stages to the model. If the duration of the contraceptive is finite, then the dynamics of the fertile and infertile stages can be represented as

$$F_{t+1} = F_t(1-c)(S + m - \beta N_t) + (1-c)I_{\tau_t} \quad (7)$$

$$I_{1_{t+1}} = F_t c(S + m - \beta N_t) + I_{\tau_t} Sc \quad (8)$$

$$I_{2_{t+1}} = I_{1_t} S, \quad (9)$$

$$I_{3_{t+1}} = I_{2_t} S, \quad (10)$$

$$I_{4_{t+1}} = I_{3_t} S, \quad (11)$$

$$I_{\tau_{t+1}} = I_{\tau-1_t} S, \quad \text{and} \quad (12)$$

$$N_t = F_t + \sum_{i=1}^{\tau} I_{i_t}, \quad (13)$$

where τ = the duration of the contraceptive in number of breeding seasons. Thus, the number of stages in the model is $\tau + 1$, 1 stage for the breeding females and 1 stage for each year that animals are infertile following treatment. The total population of females is the number of animals summed across stages (equation 13). We will refer to equations 7–13 as the fixed duration model.

It is possible to develop agents that will render animals infertile for life (reviewed by Muller et al. 1997, Tuyttens and Macdonald 1998). To represent effects of such agents, only 2 stages need to be tracked (fertile and infertile animals), and the dynamics of these stages are connected only by the rate of addition of animals to the infertile pool and the effect of total density on recruitment. Thus, the dynamics of the population are given by

$$F_{t+1} = F_t(1 - c)(S + m - \beta N_t), \quad (14)$$

$$I_{t+1} = F_t c(S + m - \beta N_t), \quad \text{and} \quad (15)$$

$$N_t = F_t + I_t. \quad (16)$$

We will refer to equations 14–16 as the lifetime duration model.

The fixed and lifetime duration models depend on the same set of assumptions described for the culling model. However, these models require the additional assumptions that treatment is applied immediately before breeding occurs and that managers can differentiate between animals that have been treated and those that have not been treated. We will examine the consequences of these assumptions as part of model analysis. Time of census in the model is

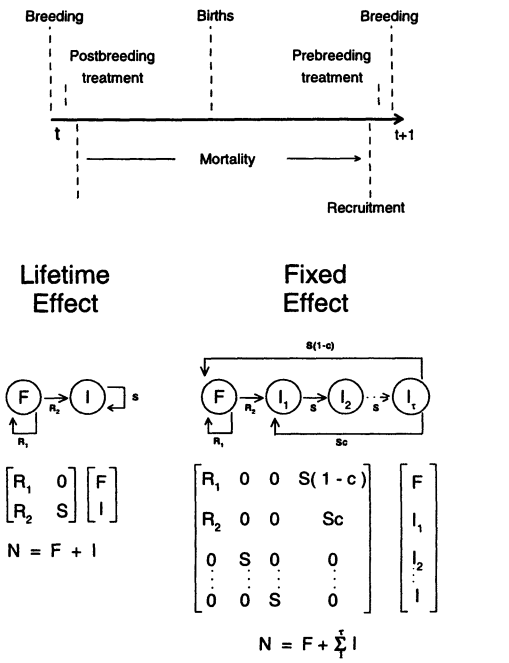


Fig. 2. Schematic of model structure and assumptions. The upper panel depicts assumptions on timing of events represented in the model. The central panel shows the structure of the model in node and matrix formats. The lower panel shows representations of assumptions about timing of delivery and the operation of density dependence.

assumed to occur annually, immediately after breeding (Fig. 2), and all fertile animals are assumed to be bred at the time of census. We chose breeding rather than births as the point of reference for census because the state of the animal that influences model behavior is its fertility rather than its age.

Model Analysis

Deterministic Analysis.—We used standard matrix techniques and algebraic manipulations (see Appendix I for details) to derive several quantities of interest. These included: K_e, the equilibrium density of animals in the absence of fertility control or culling; K_c, the equilibrium

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density of animals as a function of delivery or culling rate; c^*, the delivery rate required to maintain a target population density; P, the proportion of the population that must be infertile to maintain a target population density; and n^*, the number of animals that must be treated or culled annually to maintain a target population density. To examine the effects of timing of delivery on model predictions, we changed model formulations to represent delivery after breeding (Fig. 2). In this case, delivery of contraceptives did not prevent birth by treated animals during the current time step (because treated animals were assumed to be pregnant at the time of delivery) but did prevent births during the subsequent time step (Fig. 2). To examine effects of nonlinearity in density dependence, we reformulated the model (Fig. 2) so that recruitment remained relatively constant at low population density and then declined as threshold densities were reached (Bartmann et al. 1992). To examine consequences of relaxing assumptions on known fertility status of animals, we developed an estimator to show how many animals must be treated to achieve a treatment target, given unknown fertility status. This estimator deals with the multinomial case of sampling with replacement, and estimates necessary treatment rates, assuming that animals could be treated more than once.

Stochastic Analysis.—We performed Monte Carlo simulations to examine the relative ability of culling and fertility control to regulate animal numbers near a target density. These simulations were organized in 4 model scenarios that included 2 types of management and 2 sources of stochastic variation. Management types include static and adaptive. Static management applied the same per capita rate of delivery annually, a rate that would achieve a target population density if applied accurately. Adaptive management changed the delivery or culling rate in response to differences between realized density and an acceptable range of target densities. The acceptable range was defined as the target ±25%. If the population exceeded the target by ≥25%, then the culling or delivery rate was increased by 10% until the population returned to acceptable levels. If the population dropped below the target by ≥25%, then culling or fertility control was discontinued until the population recovered to a density within the acceptable range.

In addition to 2 types of management, model scenarios included 2 sources of stochastic variation; process and observer. Process variation was included to mimic density independent effects of the environment on population dynamics. To represent this variation, adult survival varied according to a log-normal distribution with mean 0.9 and variance 0.1. We varied the effect of density on recruitment (i.e., β) using a normal distribution with a mean of 0.009 and variance 0.01. To assure that stochastic effects on mortality and recruitment were correlated, we derived them from the same normally distributed variate at each time step. Observer variation was included to mimic the error in estimates of population size that are typical in managing ungulate populations. To represent observer variation, delivery and culling rates were based on an estimate of the current population density derived by sampling from a normal distribution with a mean equal to the model density at time t (F_t) and a standard deviation equal to 15% of the mean. Initial conditions for population density were set at the target density. Initial conditions for population composition (i.e., fertile–infertile stages) were set at levels equivalent to the deterministic steady-state at that target density. We conducted 1,000 model runs of 50 years each for 2 target densities, set as percentages (25 and 75%) of the steady state that would occur in the deterministic model when culling or fertility control was not applied (i.e., K_e). We applied 4 methods of population control: culling, and fertility control with the duration of treatment equal to 1 year, 3 years, and lifetime. For each set of model runs, we recorded the total number of animals treated or culled, the overall variance in population density, and the number of years that the population was outside the acceptable ranges. We also recorded the frequency of extinction and the persistence time of the population.

Procedures for Analysis and Plotting.—Analytical results were obtained using Maple V (Waterloo Maple, Waterloo, Ontario, Canada). All analytical results were checked for accuracy using simulation of difference equations with Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA). Monte Carlo simulation experiments were conducted using Visual Basic for Applications running under Excel. For the purpose of illustrating analytical results in plots, we chose parameter values that are reasonable for white-tailed deer (*Odocoileus virginianus*; $m = 0.6$, $S = 0.9$, $\beta = 0.005$) and that

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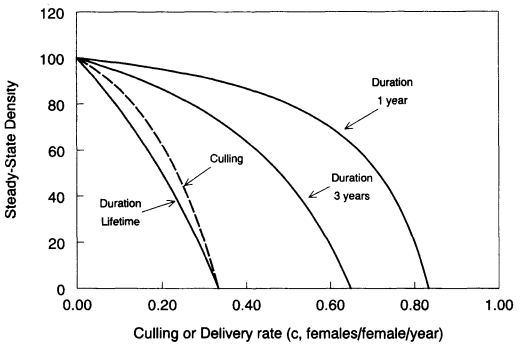


Fig. 3. Model predictions (equation 18) of steady-state density as a function of culling or delivery rate using parameter values $m = 0.6$, $S = 0.9$, $\beta = 0.005$. Different lines show differences due to variation in duration of efficacy of contraceptives.

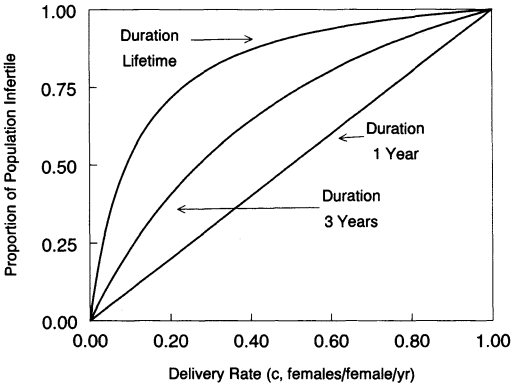


Fig. 4. Model predictions (equation 19) of effects of delivery rate on the proportion of the female population that is infertile. Different lines show differences due to variation in duration of efficacy of contraceptives. Parameter values are $m = 0.6$, $S = 0.9$, $\beta = 0.005$.

produced an equilibrium value of population density = 100 in the absence of any treatment or culling. Because density can scale to any unit area, our plots can be viewed in absolute or relative terms. For example, $N = 90$ can be accurately interpreted as an absolute density (e.g., 90 animals per unit area) or as a percentage of steady state population density that would be achieved in the absence of treatment (e.g., 90% of K_e).

RESULTS

Equilibrium Population Density and Composition

In the absence of treatment or culling (i.e., $c = 0$), all models predicted that the population would reach a steady state density, K_e,

$$K_e = \frac{S + m - 1}{\beta}. \quad (17)$$

As the rate of treatment with contraceptives increases, models predicted that steady state density declines in a nonlinear fashion (Fig. 3) according to

$$K_c = \frac{S + m + S^{\tau}c - Sc - cm - 1}{(1 - c)\beta}. \quad (18)$$

In the lifetime case, the S^{τ}_c term drops out of equation (18).

Fixed-effect models predicted a nonlinear, threshold relationship between equilibrium density and treatment rates. We found a broad range of treatment rates that exert relatively weak effects on steady state density, particularly for short-duration models (Fig. 3). Increasing duration of fertility control tended to make this relationship more linear (Fig. 3).

Fixed-effect models illustrated that when the duration of the contraceptive is 1 breeding season, the proportion of the population that is infertile (P) is equal to the treatment rate (i.e., $P = c$). However, when effects persist beyond a single season, infertile animals accumulate in the population treated annually, and as a result, $P > c$ (Fig. 4). Analysis of the model (Appendix A) suggests that the relationship between P and c depends on the duration of the effect of the contraceptive and the adult survival rate, i.e.,

$$P = \frac{(1 - S^{\tau})c}{1 + Sc - S - S^{\tau}c}, \quad (19)$$

but does not depend on birth rate (m) or the strength of density dependence (β). In the lifetime case, this simplifies to

$$P = \frac{c}{1 - S + Sc}. \quad (20)$$

The relationship between target density (N^*) and the proportion of the population that is infertile (P) is identical for all fertility control models (Fig. 5),

$$N^* = \frac{S + m - Pm - 1}{\beta(1 - P)}. \quad (21)$$

Equation 21 shows that increasing dependence of recruitment on density amplifies the effect of fertility control on the steady state population size. That is, achieving a given target density will require a smaller proportion of infertility in populations where density-dependent effects

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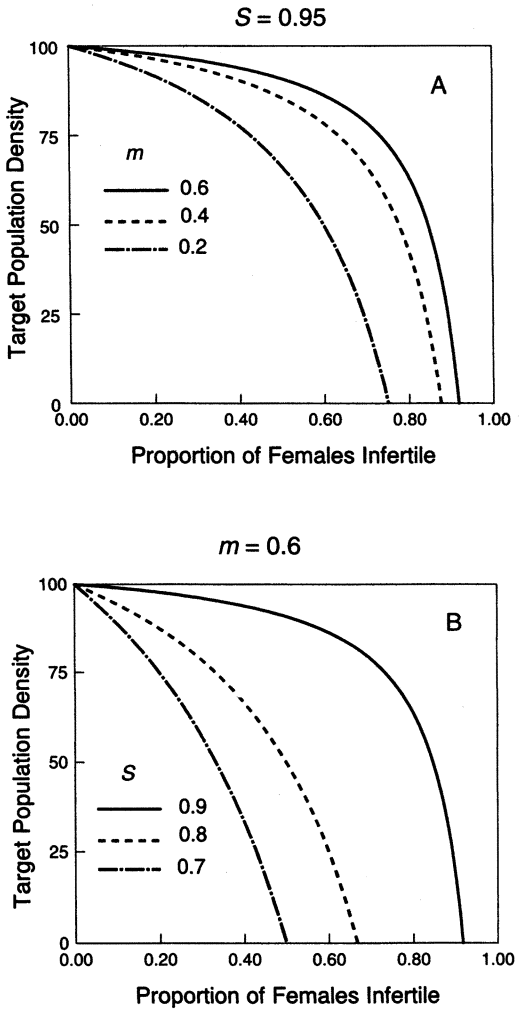


Fig. 5. Model predictions (equation 21) of the proportion of the females that must be infertile to achieve a target population density assuming a constant ecological carrying capacity of K_e = 100. Panel A shows this proportion with adult female survival rate held constant (S = 0.95) and maximum recruitment rate varying. Panel B shows this proportion with maximum recruitment rate held constant (m = 0.6) and survival rate varying.

are stronger than in populations where such effects are weak.

Population managers need a relatively easy way to estimate the proportion of the population that would need to be maintained infertile to achieve a given target density. This can be obtained as follows. By rearranging equation 21, we find P as a function of the target density:

$$P = 1 - \frac{1 - S}{m - \beta N^*}. \quad (22)$$

This result simply says that for any steady-state population density, N^*, the proportion of the population that is fertile (i.e., $1 - P$) must equal the adult per capita mortality rate divided by the per capita recruitment rate (i.e., females per female per year). When this is true, the number of animals recruited will equal the number that die.

Note that the duration of the contraceptive determines the rate of delivery that must be applied each year to achieve a given level of population infertility, (equation 19), but the duration does not affect the level of infertility required to achieve a given steady-state density (equation 22). Equation 22 also illustrates that the necessary proportion infertile will increase with increasing adult survival and maximum recruitment. It will decline with increasing "strength" of density dependence.

Equation 22 requires an estimate of β, which can be difficult to obtain. However, it is possible to derive an expression for P that does not depend on estimating β. Defining Δ as a proportion of the steady-state density (i.e., $N^* = \Delta K_e$) and substituting the right hand side of

$$\Delta K_e = \Delta\left(\frac{S + m - 1}{\beta}\right) \quad (23)$$

for N^* in equation 22, we obtain an expression for the proportion of animals that must be infertile to achieve a target density specified as a proportion of the untreated steady state, i.e.,

$$P = 1 - \frac{1 - S}{m + \Delta(1 - S - m)}. \quad (24)$$

Thus, when $\Delta = 1$, there is no reduction in density; when $\Delta = 0$, the population goes extinct. Equation 24 is an important result because it provides a general rule of thumb for estimating the effort required to regulate a population using fertility control without knowing the strength of density dependence. This rule requires estimates of only 2 parameters (adult female survival rate and maximum recruitment rate of females) and a decision on the targeted reduction, Δ.

Vital Rates and Population Composition at Equilibrium

Equation 24 also reveals that a relatively large proportion of the population must be infertile in order to achieve meaningful reductions in density (Fig. 5). When adult survival is high (>95%),

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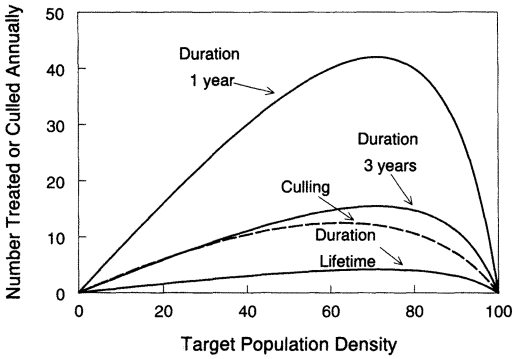


Fig. 6. Model predictions (equations 25–28) of the number of animals that would need to be treated or culled to maintain a population at the specified target density. Parameter values are $m = 0.6$, $S = 0.9$, $\beta = 0.005$. Different lines show differences due to variation in duration of efficacy of contraceptives.

it is reasonable to assume that >60% of the breeding females will need to be infertile to achieve meaningful reductions in population density, even for species with very low intrinsic rates of increase (e.g., $m = 0.2$, Fig. 5). The shape of the relationship between the proportion of the population that is infertile and steady-state density is more sensitive to differences in adult survival than to differences in maximum rates of recruitment. This relationship remains strongly nonlinear as long as adult survival is high. However, as adult survival declines, the relationship approaches linearity (Fig. 5).

Number of Animals Treated or Culled at Equilibrium

When effects of fertility control agents persist for only 1 year, then the proportion of the population that is infertile equals the delivery rate. Moreover, in this case, the number of animals that must be treated the first year to achieve a given target is the same as the effort required in subsequent years. However, when regulating populations with fertility control agents that persist for >1 breeding season, there are 2 types of effort required: the effort required to achieve a specified level of infertility in the population, and the effort required to maintain that level of infertility. Here, we examine this maintenance effort and compare it with culling.

In populations regulated with culling, the number of animals harvested annually (n) to maintain a given target density, N^*, at steady state is simply

$$n = c^*N^*, \quad (25)$$

where c^* is the culling rate needed to maintain a population at the target density. For the culling model, c^* is given by

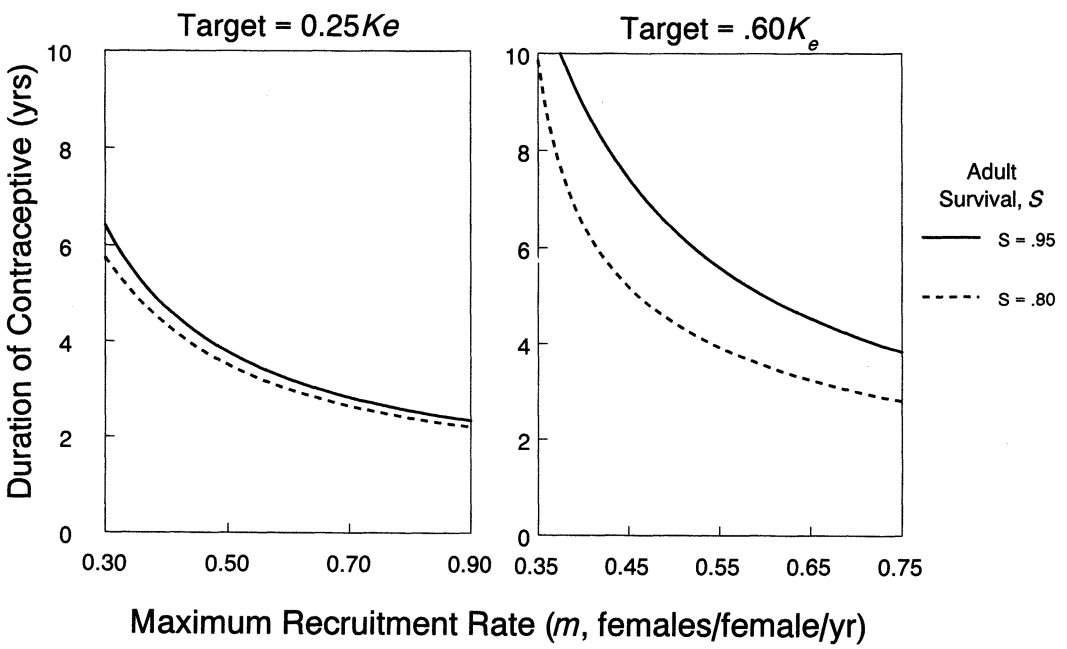


Fig. 7. Model predictions of the duration of contraceptive delivered to an animal needed to cause the same effect on population growth as culling the animal (equations 25 and 28).

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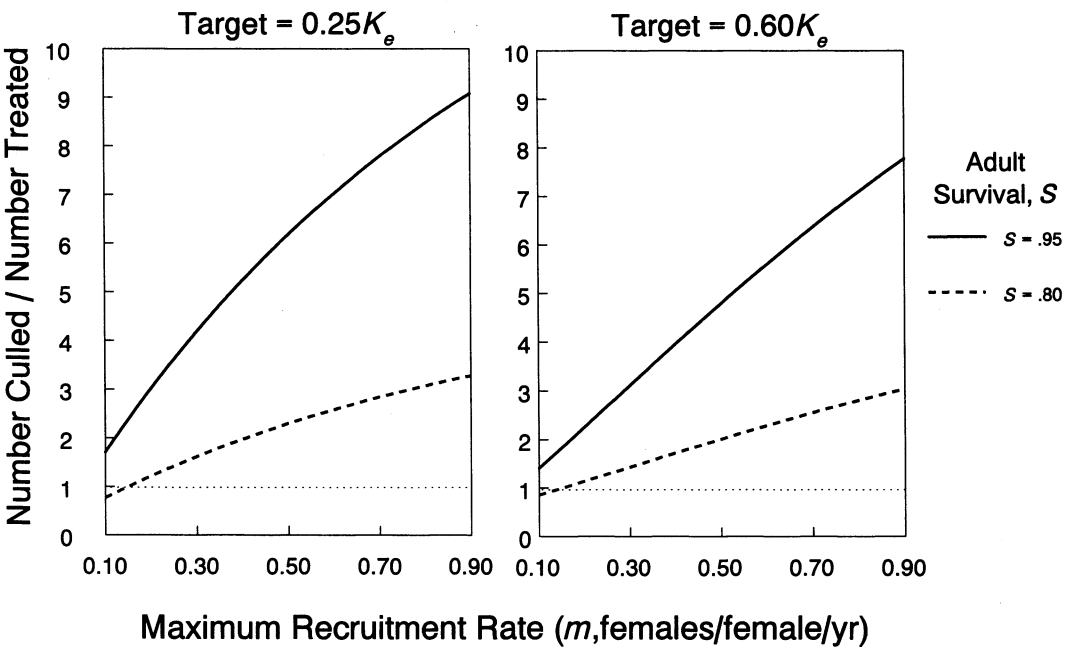


Fig. 8. Model predictions of the ratio of the number of animals that would need to be culled relative to the number treated with contraceptives to maintain steady states at target densities. The horizontal dotted line shows where the number culled equals the number treated (equations 25–28).

$$c^* = \left(2N^*\beta - S - m + \sqrt{S^2 + 2Sm + m^2 - 4N^*\beta}\right)/(2N^*\beta). \quad (26)$$

In populations regulated with fertility control, there are 3 cases relevant to the number of animals treated annually (n) to maintain a given target density. In the case of fixed duration agents lasting a single year, the delivery rate at equilibrium (c^*) is the same as the proportion of the population that is infertile, so using equation 22, we calculate the number treated as

$$n = c^*N^* = PN^* = \left(1 - \frac{1 - S}{m - \beta N^*}\right)N^*. \quad (27)$$

In the case of agents with duration >1 year and greater than a lifetime, the number that must be treated annually is

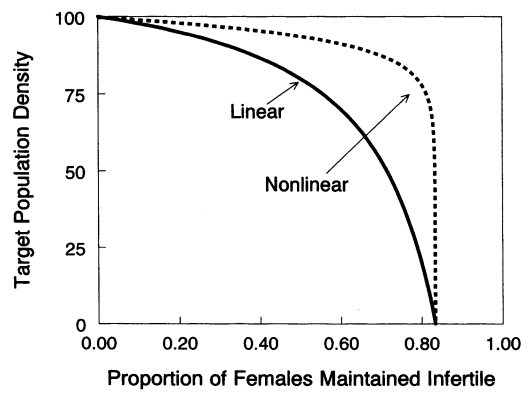


Fig. 9. Comparison of model predictions of the relationship between the target density and the proportion of the population that is infertile, assuming linear (solid line) and nonlinear (dashed line) representations of the effects of density dependence on recruitment.

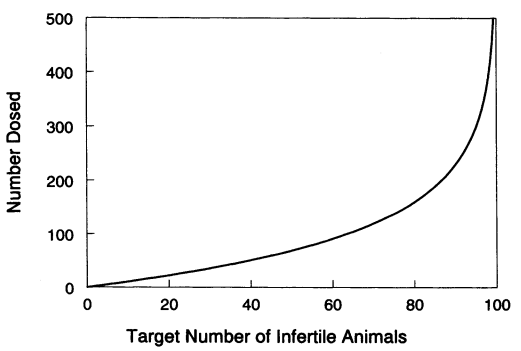


Fig. 10. Predictions of the number of animals that would need to be dosed to achieve a given level of infertility in a population, assuming animals are not marked and are subject to treatment more than once. The axes can be interpreted as a number of individuals in a population of 100, or as percentages.

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Table 1. Results of 1,000 model runs of 50-year duration with stochastic variation. Static management applied a constant, per capita culling or delivery rate needed to achieve the target density. Adaptive management adjusted the culling or delivery rate to keep the population within ±25% of the target density. Process variation mimicked stochastic environmental effects on adult survival and juvenile recruitment. Observer variation mimicked uncertainty in estimating population size. Control methods included culling and 4 contraceptives differing in duration, compared with the no management case (i.e., "Natural"). Results include average population size (*N*) and its coefficient of variation. We also report percentage of runs for which population density dropped below 1 (% extinct) and mean time to extinction averaged across model runs (persistence time in yr).

Management type	Variation type	Control method (duration of contraceptive)	Target density							
			25				75			
			N	CV	% extinct	Persistence time	*N*	CV	% extinct	Persistence time
Static	Process	Culling	22.1	51.3	0.5	49.9	69.1	31.7	0.2	49.9
		1 yr	23.2	46.3	0.2	49.6	71.3	28.4	0.1	50.0
		3 yr	23.3	49.3	2.6	49.5	71.8	28.8	0.1	50.0
		Lifetime	19.7	61.3	37.6	41.7	69.2	33.4	3.2	49.3
		Natural	105.5	25.2	0.0	50.0	108.0	23.0	0.0	50.0
Static	Process and observer	Culling	21.6	65.4	1.3	49.4	68.7	32.2	0.0	50.0
		1 yr	29.9	56.2	0.5	49.8	71.2	31.0	0.0	50.0
		3 yr	23.3	85.1	12.3	45.0	72.3	41.8	1.3	48.7
		Lifetime	17.6	87.1	40.1	34.2	69.2	34.7	3.8	49.2
		Natural	105.4	25.7	0.0	50.0	108.1	23.0	0.0	50.0
Adaptive	Process	Culling	27.3	37.2	0.0	50.0	75.8	50.6	0.2	49.9
		1 yr	28.9	29.4	0.5	49.9	79.0	29.2	0.0	50.0
		3 yr	31.0	35.8	0.6	49.8	79.0	43.4	0.0	49.9
		Lifetime	22.7	76.7	10.1	37.7	74.2	37.5	1.0	49.6
		Natural	105.5	25.7	0.0	50.0	108.1	29.4	0.0	50.0
Adaptive	Process and observer	Culling	27.3	36.6	2.5	49.5	75.7	31.3	0.2	49.9
		1 yr	31.8	37.0	1.0	49.8	80.5	29.2	0.0	50.0
		3 yr	34.9	56.44	9.5	47.8	84.5	43.4	5.9	48.7
		Lifetime	27.0	62.26	29.4	44.8	75.2	37.6	1.4	49.7
		Natural	103.4	34.23	0.1	50.0	108.9	29.4	0.0	50.0

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Table 2. Modeling estimates of proportion of the target sex that must be infertile to achieve target population objectives. Assumed annual growth rate in absence of treatment = 1.49.

Species	Population objective	% infertile	Reference
White-tailed deer	Negative growth rate[1]	90%	(Swihart and DeNicola 1993)
Feral horses	Reduce population from 600 to range of 300–600	68–82% depending on treatment regime	(Garroutt et al. 1992)
White-tailed deer	Maintain at ½ carrying capacity	80%	(Boone and Wiegert 1994)
Feral horses	Reduce growth rate (i.e., lambda) to 1	90–95%	(Garrott and Siniff 1992)
White-tailed deer	Reduce population size	>50%	(Seagle and Close 1996)
White-tailed deer	Maintain at ½ carrying capacity	96%	(Nielsen et al. 1997)

$$n = c^*(1 - P)N^*(S + m - \beta N^*) + c^*\rho N^*, \quad (28)$$

where c^* is the treatment rate required to achieve a given target density, N^*P is the portion of the population that is infertile at time t as a result of applying c^*, and ρ is the proportion of the population that becomes fertile during $t \rightarrow t + 1$ given a treatment rate = c^*. The components of equation 28 are as follows. The variable P is given by equation 22. The equilibrium treatment rate is

$$c^* = \frac{S + m - 1 - \beta N^*}{S + m - S^\tau - \beta N^*}. \quad (29)$$

The proportion of the population that will become fertile during a time step is

$$\rho = \frac{S^{\tau-1}(1 - S)c^*}{1 + Sc - S - S^\tau}. \quad (30)$$

In the lifetime duration case, the S terms with exponents drop out of the expression for c^* and $\rho = 0$.

Equations 25 and 28 allow comparison of the effort required to control populations using culling or fertility control. Because these equations predict the number of animals that must be treated or culled to maintain populations below K_e (Fig. 6), they are analogous to classical production functions, also known as yield curves (Roughgarden 1997).

Effect of Duration of Contraception

We asked the question, "On average, how long must the effect of a contraceptive persist in an animal to exert the same effect on population-level production as culling that animal?" To address this question, we chose a broad range of parameter values to represent diverse life-history characteristics of ungulates, and applied those values to equations 25 and 28. Models suggested that for a broad range of assumptions about survival and maximum recruitment, delivery of a contraceptive to an individual animal would have the same effect as culling that animal if the effects of the contraceptive persisted for 2–5 years (Fig. 7). Briefer duration is needed when adult survival rates are low. Increasing strength of density dependence reduces the required duration; weakening it increases the required duration.

When contraception persists for the lifetime of the animal, models predicted that in most cases, the effort required to regulate a population at a specified density using fertility control will be less than the effort required by culling (Fig. 8). Exceptions to this result occur when adult survival is low and recruitment is low, that is, when populations grow slowly (Fig. 8). For species with high rates of adult survival and high recruitment rates, the number of animals culled could be almost an order of magnitude greater than those treated with contraceptives at the same target density (Fig. 8). Thus, if we define efficiency of control in terms of the number of animals that must be culled or treated annually to maintain a population at a specified target, then culling will be more efficient than lifetime duration contraception only for slowly growing populations. In all other cases, models suggest that treatment with lifetime effect contraceptives is likely to be more efficient than culling. However, it must be understood that these estimates of effort specify the number of animals that must be treated annually to maintain a steady state population with a proportion infertile, P (equation 22). These are not estimates of the effort required to achieve P in the pretreatment population.

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Consequences of Changing Model Assumptions

Assumed Pre-breeding Delivery.—In all results above, we assumed that contraceptives were delivered to females before breeding occurs. It is possible to change the model to represent delivery after breeding by assuming that treated animals give birth during the current time step but are infertile during the subsequent one (Fig. 2). An important result emerged from this analysis. We found that when contraceptives are delivered postbreeding, there is a fundamental limit to the magnitude of the reduction in population density that can occur using fertility control,

$$N^* \geq \frac{m - 1}{\beta}, \tag{31}$$

or, solving in terms of a proportionate reduction,

$$\Delta \leq \frac{m - 1}{S + m - 1}. \tag{32}$$

The right-hand-side of equation 32 gives the steady-state density of females that occurs when the delivery rate = 100%. At this density, each fertile female produces exactly 1 female offspring surviving to reproductive age and the proportion of fertile females in the population equals the adult mortality rate.

Equations 31 and 32 are important because they reveal that it may not be possible to achieve meaningful regulation of populations where contraceptives are delivered after animals have been bred, even when the entire population of fertile females is treated. If animals can breed at 1 year of age, then a cohort of animals always escapes the postbreeding treatment, and this escape may allow populations to grow to levels that are close to K_e, if recruitment rates are sufficiently high. Equations 31 and 32 raise serious doubt about the efficacy of fertility control as a means of regulating populations whenever yearlings contribute significantly to breeding and contraceptives are delivered after they are bred.

Assumed Linear Density Dependence.—Changing the form of the relationship representing density dependence from linear to nonlinear did not change the qualitative results obtained above (Fig. 2). The composition of the population at equilibrium (i.e., equations 19 and 20) does not depend on the form of density-dependent relationships. However, the relationship between equilibrium population size and the proportion of the population infertile changed when we changed the formulation for density dependence. In the case of nonlinear density dependence,

$$N^* = \frac{\ln\left(\frac{S + m - Pm - 1}{1 - S}\right) + \alpha}{\theta}, \tag{33}$$

where α and θ are parameters controlling the shape of the nonlinear relationship between recruitment and density.

Note that this expression does not depend on the duration of the effect of the contraceptive, as we saw earlier for the linear case. Plotting equation 33 shows that nonlinearity in density dependence can sharpen the threshold relationship between N^* and P (Fig. 9). In the neighborhood of this threshold, small errors in estimating the proportion of the population that is infertile can lead to very large errors in equilibrium population size. Nonlinear density dependence amplifies the consequences of such errors. Nonlinearity in density dependence also expands the range of levels of infertility in the population (i.e., P) over which we expect virtually no change in equilibrium density (Fig. 9).

Assumed Knowledge of Fertility Status.—One of the assumptions of our base model is that managers can differentiate between fertile and infertile animals when delivering fertility control treatments. There are cases where this assumption is reasonable, for example, when treated animals can be visually marked, or when it is possible to achieve the desired delivery rates by treating young of the year. However, there will be many cases where the fertility status of animals is unknown. This means that some animals are likely to be treated more than once, thereby increasing the number of "doses" of fertility control agents that must be delivered to achieve a target delivery rate.

This is essentially a problem in multinomial sampling with replacement. That is, after an animal is given a dose of contraceptives, it is released into the population unmarked, and is subject to "resampling" a later time. Because it is unmarked, its fertility status is unknown, and it will be redosed if it is resampled. We are interested in estimating the number of doses (d) that must be delivered in order to treat a target number of animals (η) in a population of size

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N. We first consider the case where the duration of contraceptives is a single breeding season and all infertile animals must be dosed annually to maintain infertility. In this case, it can be shown (Appendix B) that the number of animals (d) that must be dosed to expect η animals to be infertile at the end of the treatment campaign can be estimated as

$$d = \frac{\ln\left(\frac{N-\eta}{N}\right)}{\ln\left(\frac{N-1}{N}\right)}. \quad (34)$$

Alternatively, when the duration of contraceptives persists for more than a year, there will be some animals in the population that will remain infertile and do not need to be retreated. Define I as the number of animals in the population that are infertile at the start of a treatment campaign and that remain infertile during the subsequent year. In this case the number of animals that must be dosed to expect that η additional animals are infertile is given by

$$d = \frac{\ln\left(\frac{N-I-\eta}{N-I}\right)}{\ln\left(\frac{N-1}{N}\right)}. \quad (35)$$

Equations 34 and 35 portray the large inefficiencies associated with treating a significant proportion of a population whenever treated animals are not easily distinguished from untreated ones (Fig. 10). For example, a population of 100 fertile animals would require dosing 160 animals to reasonably expect 80 infertile animals in the population after treatment (Fig. 10). A population of 100 animals, 80 of which are infertile at the time of delivery, would require dosing 69 animals to expect that only 10 additional animals would be infertile during the subsequent year. This result illustrates that the feasibility of treating populations with fertility control agents depends in a fundamental way on knowing the fertility status of individual animals at the time of treatment. In the absence of such knowledge, achieving meaningful reductions in steady state density could require delivering doses in excess of the total number of females in the population.

Stochastic Models

We compared the ability of different control regimes to maintain populations at target densities in the face of environmental stochasticity and error in estimation of population size. For all model runs, simulations of fixed-effect fertility control with single year duration showed the lowest coefficients of variation around target population densities, lowest likelihood of extinction, and longest persistence times (Table 1). Results from the culling model resembled those of single-year duration fertility control, but tended to show slightly higher variability and slightly greater probability of extinction. Lifetime effect fertility control models predicted the highest levels of variability and greatest chance of extinction. In the extreme case, extinctions occurred in >40% of all model runs for lifetime effect representing static application of fertility control with process and observer variation to populations at 25% of K_e (Table 1). Population variability and indicators of extinction risk for models of fertility control with 3-year duration showed effects intermediate to the culling model and the lifetime model. For all models, simulations of adaptive management scenarios increased variability in population density (as measured by coefficients of variation), but reduced probability of extinction and increased persistence time.

DISCUSSION

Relationships to Earlier Models

The models we offer differ in 4 fundamental ways from previous modeling efforts directed at understanding effects of fertility control on vertebrates (Garrott 1991, Garrott and Siniff 1992, Swihart and DeNicola 1993, Boone and Wiegert 1994, Seagle and Close 1996, Pech et al. 1997, White et al. 1997). First, our models are structured in stages where each stage represents the time since a female was treated. Earlier models have been structured by animal age, irrespective of time since delivery. As a result, previous efforts failed to consider effects of duration of contraceptives or the interaction of duration with other model parameters. Stage structure allows us to offer the first analysis of the consequences of variation in the duration of fertility control agents. As a result, we are able to show that animals with long lifetimes (i.e., high adult survival rates) can be effectively controlled with long duration contraceptives, even

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when they have relatively low fertility rates. This contrasts with earlier efforts that argued that r-selected species with short lives and high fertility were most appropriate targets for regulation by controlling fertility (e.g., Hone 1992).

Second, we offer closed-form solutions for relationships among delivery rate, proportion of the population infertile, number of animals treated, and target population densities. In the past, estimating these relationships demanded laborious trial and error using simulations (Garrott et al. 1992, Boone and Wiegert 1994, Seagle and Close 1996). Unlike previous results, our models enjoy analytical solutions that provide a rapid and general way to approximate the effort required to stabilize a population using fertility control, without developing detailed simulation models.

Third, our models are the first to consider effects of different fertility control regimes on risks of extinction of small populations. We demonstrate that small populations regulated with long-lived contraceptives will be vulnerable to stochastic extinction.

And finally, we are the first to explicitly consider the inefficiencies associated with resampling unmarked animals when delivering fertility control agents. We show that failing to know the fertility status of individuals can create large inefficiencies in achieving desired levels of infertility in real-world populations.

Although our models differ in important ways from earlier ones, we also reinforce previous findings. Similar to other modeling results, we found that more than half of the breeding females must be infertile to achieve meaningful reductions in population density (Fig. 5). It is true that a smaller infertile fraction may be required for species with low adult survival and low recruitment rates (Fig. 5), but these are not usually the species that cause problems of overabundance. These general results are consistent with several, more detailed studies of effects of fertility control on ungulates (Table 2), as well as, field tests in small mammals (McCallum 1996). It follows from these results that there may be situations where it is logistically impossible to treat a sufficient portion of the population to achieve population objectives. This may explain, in part, why field studies have failed to demonstrate that fertility control can maintain populations at lower densities (McCullough et al. 1997). We should not expect any change in steady state when the proportion of the population treated is small (McCullough et al. 1997).

Our models reveal that this problem is exacerbated by the nonlinear relationship between the proportion of the population treated and the steady-state density (Fig. 4). That is, there is a broad range of levels of infertility producing essentially no change in population equilibrium. This relationship could make it very difficult to assess the effect of different levels of population infertility in field studies because widely differing levels of infertility could result in very similar steady-state densities. Similar nonlinear relationships have been identified in other modeling efforts (Boone and Wiegert 1994, McCallum 1996, Seagle and Close 1996). This problem is magnified in populations where the fertility status of animals is not known (e.g., equations 34 and 35, Fig. 10). In this case, the number of doses that would need to be delivered to achieve a meaningful reduction in population density could easily exceed the total number of animals in the population.

Efficiency of Fertility Control Relative to Culling

There is broad disagreement about the relative efficiency of lethal and nonlethal means of controlling animal abundance. It has been argued that fertility control is more efficient than culling because infertile animals remain in the population, maintaining density-dependent feedback to recruitment and survival (Knipling 1959, Sturtevant 1970, Knipling and McGuire 1972). In contrast, others contend that there are virtually no circumstances in which fertility control will be more efficient than lethal methods (Bomford 1990, Garrott 1995, Nielsen et al. 1997, Swinton et al. 1997). Some workers even assert it is not possible to reduce population numbers with nonlethal control (Seal 1991).

The models we offer can resolve these differences of opinion. There are 2 types of efficiency in population regulation. The first type assesses the amount of time required to bring an overabundant population to an acceptable lower density. There is no question that culling is more efficient than fertility control when efficiency is defined in these terms. Using lethal methods, it is theoretically possible to bring a population to a desired lower density in 1 season of effort. In contrast, the maximum rate of reduction of a population regulated by fertility control is simply $1 - S$, the natural adult mor-

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tality rate. Assuming that all fertile females are treated ($c = 1$), our models show that the annual growth rate of the population (λ) is S. Because $S < 1$, the population will decline when $c = 1$, but it may decline very slowly if the adult survival rate is high.

In contrast, it is also possible to define efficiency in terms of the number of animals that must be treated annually to maintain a population at a specified target density. By defining efficiency as the number of animals that must be treated, we deliberately ignore the time required to treat them or the monetary expense of treatment. These costs are highly variable and situation specific. Thus to achieve generality, we will restrict our discussion to the number of animals that must be treated or culled and will allow the reader to apply cost estimates to weight efficiency in a case-specific fashion.

When efficiency is defined in terms of the number that must be treated annually, our models reveal that long-duration fertility control agents can be more efficient than lethal methods if the fertility status of treated animals is known. Although this result may be surprising at first glance, a small thought experiment reveals the intuitive basis for our result. Assume that you have 100 breeding animals with a survival rate of 0.9. On average, each of these animals produces a single female offspring every year. Nine of these offspring must enter the population to offset the losses of adults who die annually. It follows that 91 recruits must be culled to maintain the population at 100 animals. Now, presume instead that the population is stabilized by controlling fertility. Ninety animals are sterilized, leaving 10 fertile. One of the fertile animals dies each year after reproducing. Thus, there are 10 fertile offspring, replacing 9 infertile deaths and 1 fertile death. Only 9 of the recruits must be sterilized to maintain a level of infertility in the population = 90%. However, this result does not hold for short-duration agents. In this case, the 90 fertile adults and 90 recruits must be treated to maintain 90% infertility in the population.

Obviously, this is a grand oversimplification of population dynamics, but it illustrates the fundamental logic underpinning the efficiency of fertility control relative to culling. The number of females that must be treated or culled to maintain a given density depends on the difference between the total number of births and the total number of natural deaths. When a large proportion of long-lived species is sterile, this difference will be likely smaller than in fertile populations that are culled because very few animals will be reproducing. Reducing the duration of the fertility control agent increases the number that must be treated annually until this number eventually exceeds the number culled.

MANAGEMENT IMPLICATIONS

We can provide 4 general insights for implementing fertility control relative to culling. First, the efficacy of fertility control as a means to regulate populations pivots on the ability to develop agents with persistent effects. Second, we also point out that the expense of fertility control will never compete favorably with the revenue that can be provided by licensed hunting, regardless of the duration of contraceptives. Fertility control will only be cost-effective for situations where recreational hunting isn't feasible and where population control can only be achieved by professional treatment, lethal or nonlethal. Third, the feasibility of achieving target levels of infertility in the population will require knowledge of fertility status of individuals. In cases where this status is not known, the inefficiencies associated with redosing animals can increase the number of animals that must be treated by several fold. Finally, the greatest efficiency can be obtained by combining culling with contraception such that an initial reduction in animal numbers is maintained by annual treatment of marked animals with long-acting fertility control agents.

The reason that lifetime duration fertility control can be an efficient method for maintaining a population at target densities also explains why the approach is difficult to implement initially and why it poses grave risks to the persistence of small, closed populations. Our models suggest that it is relatively easy to maintain a population near a given steady state, once a sufficient number of animals have been sterilized. Achieving this initial level of infertility in the population will often be a formidable task, however, and accomplishing that task means that the breeding population will be small. Such breeding populations are subject to stochastic risks of loss of genetic variability and even extinction, as our models illustrated. It follows that while fertility control may not affect survival of individuals, it can easily be lethal to populations.

This creates a genuine dilemma for manag-

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ers. Small, closed populations are the most likely candidates for regulation by fertility control. This is simply because delivery of fertility control agents to a sufficient number of animals is most likely to be logistically feasible when populations are small and bounded in space. However, these populations are also the most prone to extinction if managers make mistakes in applying fertility control agents. The nonlinearity in many of the relationships we observed magnifies the likelihood of such errors. It follows that although fertility control can be an efficient means of regulating animal numbers, that efficiency creates risk for the population manager. As with all other techniques, managing population fertility must be grounded in a fundamental understanding of the state of the population and the role of management in influencing its dynamics. This simple fact reinforces the conclusions of Garrott (1995) that careful field studies applying control techniques over long time periods are needed to thoroughly understand effects of fertility control.

We offer a suite of predictions that can be usefully tested in field studies. Our models provide a sketch of the dynamics of populations regulated by fertility control. Offering a sketch rather than a blueprint requires that we avoid many details. Understanding those details will no doubt prove necessary to minimize unpleasant surprises in managing fertility of ungulate populations (Caughley et al. 1992, Nettles 1997). However, by offering general rather than detailed models, we provide broad insights useful as a starting point for future studies.

We identify 2 urgent needs for future work on fertility control agents. Development of techniques that render animals infertile with a single dose offer a promising alternative to culling in situations where it is feasible to mark animals to know their fertility status. Thus, work at the level of the individual animal should emphasize increasing the duration of effect of fertility control agents. Studies at the level of the population must demonstrate that fertility control can achieve meaningful reductions in rates of increase in the field. Although models like ours can suggest situations where fertility control could be efficacious in controlling abundance of ungulates, such efficacy remains in doubt until field studies demonstrate such control in real-world populations.

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APPENDIX A

Example Derivation of Deterministic Results

We used a standard series of steps to analyze all models. Here, we illustrate those steps using fixed-effect model, assuming prebreeding delivery of contraceptives. We begin with a state vector $[F\ I_1\ I_2\ I_3]$ for the case where duration of fertility control persists for 3 years. The corresponding projection matrix is

$$\begin{bmatrix} (1-c)(S+m-\beta N) & 0 & 0 & S(1-c) \\ c(S+m-\beta N) & 0 & 0 & Sc \\ 0 & S & 0 & 0 \\ 0 & 0 & S & 0 \end{bmatrix} \quad (36)$$

We obtain the characteristic polynomial of the matrix:

$$0 = \lambda^4 - \lambda S^3 c - S\lambda^3 - m\lambda^3 + \beta N\lambda^3 + Sc\lambda^3 + cm\lambda^3 - c\beta N\lambda^3. \quad (37)$$

By iterating over several values of τ, we observe a general series in characteristic polynomials for all $(\tau + 1 \times \tau + 1)$ matrices:

$$0 = \lambda^{\tau+1} - \lambda S^\tau c - S\lambda^\tau - m\lambda^\tau + \beta N\lambda^\tau + Sc\lambda^\tau + cm\lambda^\tau - c\beta N\lambda^\tau. \quad (38)$$

When the population is at equilibrium, $\lambda = 1$. Therefore, to find a general expression for the equilibrium density as a function of delivery rate we set $\lambda = 1$ and solve equation 38 for N, obtaining:

$$N^* = \frac{S + m + S^\tau c - 1 - Sc - cm}{\beta(1-c)}. \quad (39)$$

Solving for c, we find the delivery rate (c^*) needed to achieve a specified target density (N^*),

$$c^* = \frac{S + m - N^*\beta - 1}{S + m - N^*\beta - S^\tau}. \quad (40)$$

To find the composition of the population (i.e., the proportion of each stage of infertility in the population) we obtain the eigenvectors of several projection matrices corresponding to different values of τ. For each matrix, we extract the first element ($v[1]$) of the normalized vector (corresponding to an eigenvalue of 1), which gives the proportion of the population that is fertile,

$$v[1] = \frac{1-c}{(1-c)/c + 1 + S + S^2 + S^3 \ldots + S^\tau}. \quad (41)$$

To find the infertile proportion (P), we subtract $v[1]$ from 1 and simplify,

$$P = \frac{(1 - S^\tau)c}{1 + Sc - S - S^\tau c}. \quad (42)$$

In a similar way, we observe the last normalized element of the eigenvector for several values of τ and find that the proportion of the population that is in the final stage of infertility (ρ) is given by a series which simplifies to

$$\rho = v[\tau + 1] = \frac{(1-S)S^{\tau-1}c}{1 + Sc - S - S^\tau c}. \quad (43)$$

We then derive a general expression for the proportion of the population (P) that must be infertile to achieve a target density N^*. We substitute the right hand side of equation for c in equation 42, and after some algebra, obtain

$$P = 1 - \frac{1-S}{m - N^*\beta}. \quad (44)$$

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To remove β from the denominator, we observe from equation 39 that the equilibrium density in the absence of fertility control (i.e., $c = 0$) is

$$K = \frac{S + m - 1}{\beta}. \quad (45)$$

Defining Δ as the proportional reduction in population density below K (i.e., $N^* = \Delta K$), it follows that

$$N^* = \Delta K = \frac{\Delta(S + m - 1)}{\beta}. \quad (46)$$

Substituting the right hand side of equation 46 for N^* in equation 44,

$$P = 1 - \frac{1 - S}{m + \Delta(1 - S - m)}. \quad (47)$$

To derive an expression for the number of animals treated (n) to maintain a target density, we observe from the projection matrix and the state vector that the number of animals treated at equilibrium is

$$n = c^*F_t(S + m - \beta N^*) + c^*I_{t_\tau}, \quad (48)$$

which is simply the treatment rate multiplied by the steady state number of fertile females at time t plus the number of females that are recruited plus the number that regain fertility during $t \rightarrow t + 1$. Substituting $(1 - P)N^*$ for F_t and ρN^* for I_{t_τ} we have

$$n = c^* (1 - P)N^* (S + m - \beta N^*) + c^* \rho N^*, \quad (49)$$

Substituting the right-hand-side of equation 40 for c^*, rhs equation 44 for P and rhs equation 43 for ρ provides the needed expression for n in terms of the decision variable N^* and the parameters m, S, and β.

APPENDIX B

Derivation of an Unbiased Estimator for the Number of Doses that must be Delivered to a Population of Unknown Fertility to Achieve a Target Level of Infertility

We assume that a simple random sample of d animals is selected with replacement from a population of N animals. All of the animals in the sample are dosed. At the beginning of the sampling effort, the population contains I infertile animals. In order to establish the results shown in equations 34 and 35 we note that equation 35 is a special case of equation 36 where $I = 0$. We define t_i as an indicator random variable that equals 0 if the *ith* fertile animal is still not dosed after d selected animals are treated and equals 1 if it has been dosed at least once for $i = 1, 2 \ldots, N - I$. We define a as the number of additional infertile animals that are added to the population after d doses are delivered,

$$a = \sum_{i=1}^{N-I} t_i. \quad (50)$$

The expected number of additional infertile animals is given as

$$\eta = E(a) = \sum_{i=1}^{N-I} E(t_i) = (N - I)E(t_i). \quad (51)$$

Define $P(t_I = 1)$ as the probability that $t_i = 1$; then,

$$E(t_i) = P(t_i = 1) = 1 - P(t_i = 0) = 1 - \left(\frac{N - 1}{N}\right)^d. \quad (52)$$

Thus,

$$\eta = (N - I)\left(1 - \left(\frac{N - 1}{N}\right)^d\right). \quad (53)$$

Solving equation 53 for d gives equation 35. The variance of a can also be easily determined.

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Extrapituitary Action of Gonadotropin-Releasing Hormone: Direct Inhibition of Ovarian Steroidogenesis

Abstract. *Gonadotropin-releasing hormone (GnRH) and its agonistic analogs inhibited the follicle-stimulating hormone (FSH)-induced increase of estrogen and progesterone production in vitro by rat ovarian granulosa cells. Likewise, GnRH analogs inhibited FSH-induced changes in ovarian function in hypophysectomized rats in vivo. These results indicate that GnRH, in addition to its well-known gonadotropin-releasing action in the pituitary, exerts a direct inhibition of ovarian steroidogenesis.*

Gonadotropin-releasing hormone (GnRH) stimulates pituitary gonadotropin production (*1*). However, pharmacological doses of GnRH and its agonists have been shown to inhibit a variety of female reproductive functions, such as ovarian steroidogenesis, ovulation, ovum transport, ovum implantation, pregnancy, and ovarian-dependent mammary tumorigenesis (*2*). The mechanism by which GnRH inhibits reproductive function is unknown. Although it is generally believed that the inhibitory effects of GnRH are mediated at the pituitary level, the possibility that GnRH may act at the level of the gonads is not excluded. We now report that GnRH and its agonistic analogs act independently of the pituitary to inhibit gonadal function: blockage of the gonadotropin-induced increases in ovarian steroidogenesis in vitro and antagonism of the follicle-stimulating hormone (FSH)-induced changes in ovarian function in hypophysectomized rats in vivo.

Follicle-stimulating hormone stimulates production of estrogen and progestin in primary cultures of ovarian granulosa cells (*3*). The effect of GnRH on these FSH actions was investigated. Granulosa cells from immature intact (23 to 25 days of age) and hypophysectomized diethylstilbestrol-treated (27 to 30 days of age, 5 days after operation) rats were cultured for 2 days (*4*). In the presence of estrogen synthetase (also called aromatase) substrate ($10^{-7}M$ androstenedione), purified FSH (100 ng/ml; Papkoff G4-150C, FSH potency equal to 50 NIH-FSH-S1 standard units per milligram) stimulated estrogen production by 40- to 60-fold. Concomitant treatment with GnRH or GnRH agonists ([D-Leu^6-(N$^\alpha$Me)Leu^7]GnRH, analog 1; des-Gly^{10}-[D-Try^6, Pro^9-NHEt]GnRH, analog 2) (*5*, *6*) inhibited FSH-stimulated increase in estrogen production (Fig. 1A). Similarly, GnRH and its agonists inhibited the FSH-induced increase in progesterone production by the granulosa cells (Fig. 1B). These results indicate that GnRH exerts a direct inhibition on ovarian steroidogenesis.

The in vivo effects of GnRH agonists on ovarian function were also examined in hypophysectomized estrogen-treated rats. Four days after hypophysectomy, immature estrogen-treated female rats were injected subcutaneously, twice daily, for 2 days with 100 μg of ovine FSH (NIH-FSH-S12) or the same amount of FSH plus GnRH analogs (50 μg of analog 1 per 0.1 ml of saline; 10 μg of analog 2 per 0.1 ml of saline). The ovarian weight and steroidogenic potential were determined 12 hours after the last injection. The FSH induced a significant increase in ovarian weight (control, 12.2 ± 1.1 mg, N = 11; FSH treated, 29.0 ± 2.0 mg, N = 8). In contrast, concomitant treatment with GnRH agonists inhibited the FSH-induced increase in ovarian weight (FSH plus analog 1, 15.6 ± 2.0 mg, N = 8; FSH plus analog 2, 14.7 ± 1.3 mg, N = 8). In vivo FSH treatment for 2 days induced a substantial increase in aromatase activity in ovarian granulosa cells. Granulosa cells were harvested from control and treated animals and incubated for 3 hours in the presence of a saturating dose of estrogen synthetase substrate ($10^{-7}M$ Δ^4-androstenedione). The estrogen production was stimulated from an undetectable level in granulosa cells of control animals to 1.65 ng per 10^5 granulosa cells in FSH-treated animals. Concomitant treatment with GnRH agonists inhibited the FSH-induced increase in estrogen production (FSH plus analog 1, 0.29 ng per 10^5 cells; FSH plus analog 2, 0.25 ng per 10^5 cells). The results indicate that treatment in vivo with GnRH agonists in hypophysectomized animals interferes with the FSH action at the ovarian level.

Our results demonstrate that GnRH and its agonists exert extrapituitary action as shown by their ability to inhibit ovarian function directly. This finding may serve as the basis for explaining the inhibitory effect of GnRH on reproductive functions. Thus, in addition to their well-known releasing action on the pituitary gonadotrophs, pharmacological doses of GnRH and its agonists could decrease ovarian estrogen production by directly interfering with normal ovarian function. A GnRH-induced decrease in estrogen production offers an expla-

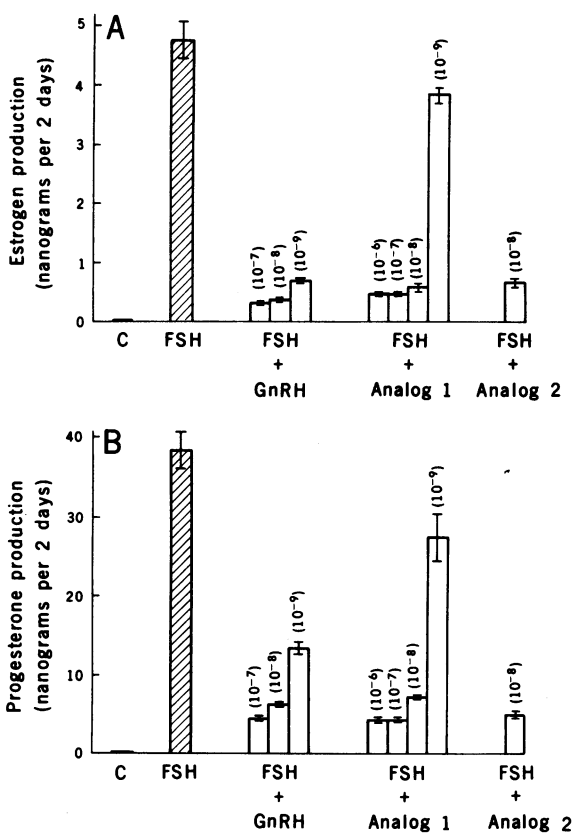


Fig. 1. Effect of GnRH and its agonistic analogs on FSH-induced increase in steroid production by ovarian granulosa cells. (A) Estrogen production; (B) progesterone production. Ovarian granulosa cells were obtained from hypophysectomized estrogen-treated immature female rats and cultured for 2 days in the presence of $10^{-7}M$ androstenedione. Ovine FSH (100 ng/ml), with or without GnRH or its agonists, was added to the media at the beginning of the culture. Estrogen and progesterone content in the media after 2 days culture was determined by radioimmunoassay. Similar results were obtained with granulosa cells from intact immature rats. Each data point represents mean ± standard deviation of four to six separate cultures. The numbers inside the parentheses represent the final concentration of GnRH or analogs in culture medium.

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nation for the reported blocking effect of GnRH on various estrogen-dependent processes, including ovulation, ovum transport, ovum implantation, and mammary tumorigenesis. Moreover, the inhibitory effect of GnRH and agonists on FSH-induced progesterone production in cultured granulosa cells could offer a possible explanation for the interference by GnRH and its agonists in such a progesterone-dependent process as pregnancy. Our results, however, do not exclude the possibility that administration in vivo of pharmacological doses of GnRH in intact animals may also cause imbalances in pituitary gonadotropin production that also result in the inhibition of various reproductive functions (*7*).

The mechanisms by which GnRH exerts inhibition on ovarian granulosa cells are not known. Although GnRH receptors have been identified in the anterior pituitary by radiotracer binding technique and by immunocytochemical methods (*8*), their tissue specificity has not been demonstrated. Bernardo *et al.* (*9*) have reported putative GnRH binding sites in the mouse adrenal cells. It is possible that ovarian granulosa cells may have GnRH receptors and that the inhibitory effect of GnRH that we observed is mediated through hormone-receptor interactions.

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5. Abbreviations are Leu, leucine; Gly, glycine; Try, tryptophan; Pro, proline; NHEt, ethylamide; and Me, methyl.
6. The potencies of the GnRH analog 1 and analog 2 relative to native GnRH, as determined by their ability to cause luteinizing hormone release in cultured pituitary cells, are approximately 6- and 144-fold, respectively.
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10. We thank L. Tucker and C. Fabics for their technical assistance; Dr. S. S. C. Yen for his interest; Drs. N. Ling and J. Rivier for providing the GnRH analogs; and Dr. H. R. Papkoff and the NIAMDD Pituitary Hormone Distribution Program for providing FSH preparations. Supported by NIH grant 1-R01-CA 21867 and Rockefeller Foundation grant RF-75029.

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Periodicity of Deoxyribonuclease I Digestion of Chromatin

Abstract. *Two methods have been used to measure the single-strand lengths of the DNA fragments produced by deoxyribonuclease I digestion of chromatin. The average lengths obtained are multiples of about 10.4 bases, significantly different from the value of 10 previously reported. This periodicity in fragment lengths is closely related to the periodicity of the DNA double helix in chromatin, but the two values need not be exactly the same.*

The first level of condensation of DNA in chromatin is brought about by its interaction with histones to form nucleosomes, the elementary subunits of the structure. For a detailed understanding of how the DNA is folded, it is necessary to know both the path of the double helix and its periodicity, or the number of base pairs per turn, in the nucleosome. X-ray crystallographic studies indicate a path in which the DNA is wrapped twice around the histones (*1*). This appears to conflict with measurements on closed, circular DNA extracted from SV 40 chromatin showing nearer one superhelical turn (*2*). The conflict can be resolved by postulating a change in the periodicity of the DNA as it is folded into a nucleosome (*1*). A decrease of only about 5 percent is required, and so before firm conclusions can be drawn it is necessary to know the relevant parameters as accurately as possible.

Noll (*3*) has suggested that the perio-

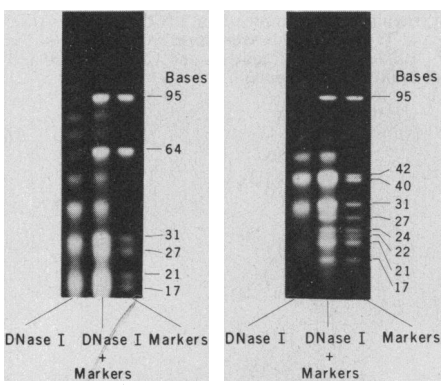


Fig. 1 (left). Comparison of DNase I and marker fragments in polyacrylamide gels. DNase I fragments were prepared by DNase I digestion of rat liver nuclei and extraction of the DNA (*3*). Marker fragments were prepared as described in Table 1. All fragments were labeled with ^{32}P as follows. DNA (40 μg/ml) in 50 m*M* sodium acetate (*p*H 4.6) and 1 m*M* EDTA was boiled for 1 minute and treated with spleen acid phosphomonoesterase B (*16*) (0.1 unit per milliliter) for 2 hours at 37°C. The *p*H was then raised to 8.0 by the addition of tris base to 85 m*M* and the mixture was boiled for 1 minute, supplemented with $MgCl_2$ (10 m*M*), 2-mercaptoethanol (15 m*M*), and γ-^{32}P-labeled ATP (tenfold excess over 5′-OH termini), and treated with polynucleotide kinase (20 unit/ml) (New England Biolabs) for 1 hour at 37°C. The labeled DNA was purified by filtration through Sephadex G-25 in a mixture of 0.1*M* NaCl, 10 m*M* tris-HCl (*p*H 7.5), and 1 m*M* EDTA and subjected to electrophoresis in 12 percent (left panel) and 20 percent (right panel) polyacrylamide–98 percent formamide gels (*17*). Negatives of autoradiograms of the gels are shown.

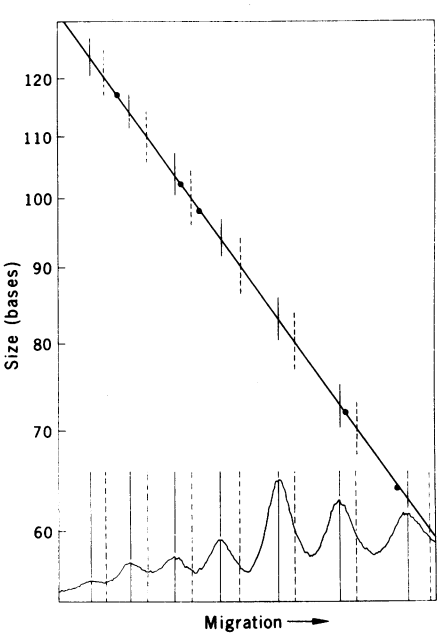


Fig. 2 (right). Size determination of DNase I bands. Densitometer traces of lanes in left panel of Fig. 1 containing DNase I (bottom) and marker fragments were aligned. The sizes and distances of migration of the marker fragments (filled circles) were used to construct a calibration curve from which the sizes of the DNase I fragments were derived. The distances of migration of the DNase I fragments (solid vertical lines) are contrasted with those expected if the fragments were multiples of 10.0 bases (dashed vertical lines).

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Extrapituitary action of gonadotropin-releasing hormone: direct inhibition ovarian steroidogenesis
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Influence of immunization against GnRH on cycling activity and estrous behaviour in the mare

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Arbeit unter der Leitung von Dr. F. Janett

Influence of immunization against GnRH on reproductive cyclicity and estrous behavior in the mare

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Influence of immunization against GnRH on reproductive cyclicity and estrous behavior in the mare

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Abstract

The aim of this study was to evaluate the effect of active immunization against GnRH on ovarian activity, plasma progesterone and estradiol concentrations and on estrous behavior in adult mares. Eighteen cyclic mares were randomly divided into a treatment and control group. Nine mares were immunized twice with 2 mL (400 μg GnRH-protein conjugate) of a GnRH-vaccine (Improvac®, CSL Limited, Australia) administered intramuscularly, 4 weeks apart. Control mares received the same amount of saline solution. Ovaries and uterus of all mares were examined weekly by ultrasonography from 3 weeks before to 60 weeks after the first immunization. Thereafter, vaccinated mares were evaluated monthly until 100 weeks after the first vaccination. In addition, mares were teased with a stallion for assessment of estrous behavior and blood was collected for progesterone, estradiol-17β and GnRH antibody titer determination. Results demonstrate that vaccination against GnRH significantly (P<0.05) influenced all parameters, except estradiol-17β concentration. All vaccinated mares ceased reproductive cyclicity (plasma progesterone <1 ng/mL, follicles <3 cm) within 8 weeks after the first injection and ovarian activity remained suppressed for a minimum of 23 weeks. Five mares resumed cyclicity (follicles >3 cm, progesterone >1 ng/mL) while 3 mares showed only follicular activity (follicles >3 cm) and 1 mare remained completely suppressed for the entire duration of the study. In spite of ovarian suppression, 4 mares expressed sporadic and 1 mare continuous estrous behavior. All 9 control mares showed regular development of large follicles and associated periods of estrous behavior during the breeding season. During the winter months, 6 of them became acyclic, whereas the other 3 continued showing reproductive cyclicity and estrous

behavior. In conclusion, reproductive cyclicity in adult mares can be successfully suppressed by immunization against GnRH but the timing of resumption of cyclicity is highly variable and estrous behavior may occur in spite of ovarian suppression.

1. Introduction

The suppression of sexual behavior in mares has long been an issue for both veterinarians and horse owners. Undesirable behavioral traits, especially during estrus, can make a mare difficult to handle and may have detrimental effects on performance. There are numerous methods for managing this problem. One of the most wide spread is the use of progesterone or progestins to artificially simulate diestrus [1]. However, the possibility of a negative effect on the future breeding prospects after repeated use in prepubertal fillies has been reported [2] and the short duration of action of this hormone, necessitating repeated administration, make its use costly and impractical. More drastic means of suppressing estrous behavior are breeding a mare and accepting the ensuing break in the mares' sporting career, or terminating the pregnancy after maternal recognition and maintenance of the primary Corpus luteum (CL) [3], which poses ethical problems. Ovariectomy is a further alternative, though not always effective, as some mares can continue to show estrous signs [4-6], furthermore it carries the disadvantage of surgical risk and permanently eliminating a mare's reproductive potential. Other methods of reversible suppression of sexual activity that warrant being mentioned are the administration of GnRH antagonists or agonists [7]. The antagonist antarelix has been shown to reliably postpone ovulation after administration and increase the inter-ovulatory interval [8,9]. Treatment with high doses of agonists can effectively desensitize the pituitary to GnRH in various species [10]. Horses are on the whole less sensitive to down-regulation, however, prolonged or high dose administration can also result in increased inter-ovulatory intervals [11,12] and in some individuals even in a temporary cessation of ovarian activity [13,14]. As all these treatments require frequent administration and generally result in only a short-term effect with highly individual responses, their use for behavioral control is limited.

The manipulation of the reproductive activity of both males and females through the use of a GnRH vaccine has been extensively studied in many species, including humans and with a wide variety of objectives. In farm animals, this method offers an alternative to surgical castration for minimizing sexual and aggressive behavior, suppressing reproductive function and preventing pregnancy [15-18]. In wild animals, vaccination against GnRH is considered an effective method for population control [19,20]. Its application in human medicine is as a treatment for prostate cancer [21]. In the field of

equine reproduction the first report of GnRH vaccination was also as a possible alternative to surgical castration in either cryptorchid [22] or normal males [23,24]. The principal aims were the evaluation of its effectiveness at inhibiting sexual behavior and libido while safeguarding the reproductive potential of an animal. The first studies in mares [25,26] were aimed primarily at investigating the effect of GnRH vaccination on gonadotropin secretion. Observations of follicular activity and estrous behavior were not a major part of the experimental design and mares were ovariectomized [25] or the study was terminated [26] before the mares returned to normal ovarian cyclicity; neither study concentrated on the possibility of using this method as an alternative to ovariectomy. Two further studies have been reported [27,28]: one documented effective suppression of ovarian function and estrous behavior along with a subsequent reversal of these effects in six 2-year-old fillies [27]; the second study on mature mares showed a greater individual variation and less reliability of the vaccination [28] .

The aim of the present experiment was to investigate the influence of active immunization against GnRH on reproductive cyclicity, hormone levels and antibody titers in a relatively large group of mature mares. This included evaluation of its influence on the ovaries and uterus, the duration of action, effects on estrous behavior and adverse reactions to the vaccine.

2. Materials and methods

2.1. Experimental design

Eighteen barren light-horse mares, aged between 3 and 17 years, (mean age 7.8 years) were used for the experiment. They had variable reproductive histories, but none had been bred within the last 2 years. All mares showed normal reproductive cyclicity with typical signs of behavioral estrus prior to treatment. The mares were randomly allocated to a control and a treatment group of nine mares each (mean age 7.2 and 8.4 years respectively). The treatment consisted of two vaccinations four weeks apart using a commercially available anti-GnRH vaccine (Improvac®, CSL Limited, Australia) developed in Australia to control boar taint in pigs. Improvac® contains a synthetic analogue to GnRH linked to a carrier protein and accompanied by an aqueous adjuvant (200 μg GnRH-protein conjugate per mL). The dosage used for both initial and booster vaccination was 400μg GnRH-protein conjugate (2 mL). Control animals received an equivalent volume of saline solution. All injections were applied intramuscularly into the left side of the neck. The experiment started at the end of March and initial vaccination took place at the end of April. Adverse effects were monitored by twice daily clinical examination with particular regard to body temperature, signs of tissue reaction at the site of injection and stiffness of the neck. Mares were examined for 3 days after vaccination or until all reactions had subsided.

2.2. Ovarian activity, uterine changes and estrous behavior

The mares' genital tracts were examined by means of transrectal ultrasonography with a 5 MHz linear array probe (Aloka Echocamera SSD-500, Aloka Holding Europe, Zug, Switzerland). At each scanning session, the ovaries were measured in two perpendicular dimensions. The number and diameters of all follicles larger than 1 cm in diameter were recorded and assigned to one of 3 categories: small follicles, <2 cm in diameter; medium follicles, 2-3 cm in diameter and large follicles, >3 cm in diameter. The diameter of the largest follicle (to the nearest 0.5 cm) present on either ovary was recorded separately. The presence of a CL was also noted. The amount of edema in the uterine wall was scored as 1 (homogeneous in appearance, non edematous), 2

(mildly heterogenous, slightly edematous) or 3 (“wagon wheel” appearance, highly edematous) [29].

Mares without a clearly visible CL were teased with a stallion after each scanning session. Four characteristic estrous signs were noted: tail raising, receptivity to the stallion, clitoral eversion and urination. Excessive kicking, flattening of ears and total lack of interest in the stallion were judged as negative signs. For a mare to be considered as showing typical estrus, at least 3 of 4 positive signs and no negative signs had to be observed. Over the course of 64 weeks (3 weeks before to 60 weeks after the primary immunization) mares were examined at weekly intervals (period 1). Mares in the treatment group that had not yet returned to normal reproductive cyclicity after week 60 were examined monthly until they showed a resumption of ovulation (period 2). Examinations were discontinued after a maximum of 100 weeks.

2.3. Definition of reproductive activity

Mares were considered to be acyclic when no large follicles were recorded, no CL was visible and progesterone values were lower than 1 ng/mL for at least 4 consecutive weeks. If the criteria were fulfilled only during short periods of 1 to 3 weeks, these were regarded as periods of temporary follicular quiescence. After an acyclic period, mares were judged as having resumed follicular activity when large follicles (>3 cm diameter) were recorded. When the mares with follicular activity showed a rise in progesterone (plasma progesterone concentration >1 ng/ml) indicating ovulation and formation of a functional CL, they were judged as having resumed ovulatory activity. The period of ovarian suppression was defined as the number of weeks between cessation of all cyclic activity and resumption of follicular activity (no less than 4 weeks).

2.4. Hormone analysis and GnRH antibody titers

Blood samples (EDTA) were collected at each examination by jugular venipuncture, immediately centrifuged (4000 x *g*, 10 min) and the plasma frozen (-20°C) until analysis. Progesterone and estradiol-17ß concentrations were measured using electrochemiluminescence immunoassay (Elecsys 2010, Roche Diagnostics, Basel, Switzerland). The detection limit of the assay was 0.03 ng/mL for progesterone and 3.1

pg/mL for estradiol-17ß. Inter- and intraassay coefficients of variation for progesterone were 2.8% and 2.2% respectively and for estradiol-17ß 6.5% and 2.5% respectively.
Antibody titers against GnRH were determined using a radio ligand binding assay as described previously [30]. Results are presented as percentage of total I^{125}-labelled GnRH bound at a plasma dilution of 1:40.

2.5. Statistical analysis

Statistical analysis was performed using the Statview 5.0 software program (SAS Institute, Switzerland). A multivariate analysis of variance (ANOVA) was carried out to assess the effects of treatment and time on ovarian and uterine parameters as well as on plasma progesterone and estradiol concentrations. Differences between the groups in weekly parameter means were compared with the unpaired *t*-test. Values were considered to be statistically significant at $P<0.05$.

3. Results

3.1. Ovarian and uterine changes

Means (± S.E.M.) of all parameters measured in the treatment and the control group from week 1 to 60 are presented in Table 1.

Table 1
Mean (± S.E.M.) of reproductive activity parameters examined weekly during 60 weeks in GnRH immunized (Improvac®) and control mares (n=9, each) and the statistical significance of the influence of treatment and time of examination

Parameter	GnRH immunized	Control	Treatment (*P*)	Time (*P*)
Number of small follicles	4.9 ± 0.20	6.7 ± 0.20	<0.0001	<0.0001
Number of medium follicles	0.8 ± 0.10	3.0 ± 0.10	<0.0001	0.0004
Number of large follicles	0.1 ± 0.02	0.8 ± 0.05	<0.0001	0.0228
Diameter of largest follicle (cm)	1.5 ± 0.04	2.8 ± 0.05	<0.0001	<0.0001
Ovarian diameter (cm)	4.2 ± 0.03	5.3 ± 0.03	<0.0001	<0.0001
Number of CL	0.2 ± 0.02	0.7 ± 0.03	<0.0001	<0.0001
Uterine edema score [a)]	1.3 ± 0.03	1.7 ± 0.03	<0.0001	0.0113
Progesterone (ng/mL)	1.2 ± 0.17	6.8 ± 0.36	<0.0001	<0.0001
Estradiol-17β (pg/mL)	24.4 ± 0.44	24.8 ± 0.35	0.216	<0.0001

[a)] 1: homogenous, 2: slightly heterogenous, mildly edematous, 3: wagon wheel appearance, highly edematous

Vaccination against GnRH significantly (*P*<0.05) influenced all parameters, except plasma estradiol-17β concentration. Fig. 1 shows the mean number of large follicles (a), mean diameter of the largest follicle (b) and mean ovarian diameter (c) in treated and control mares from 3 weeks before until 60 weeks after first immunization.

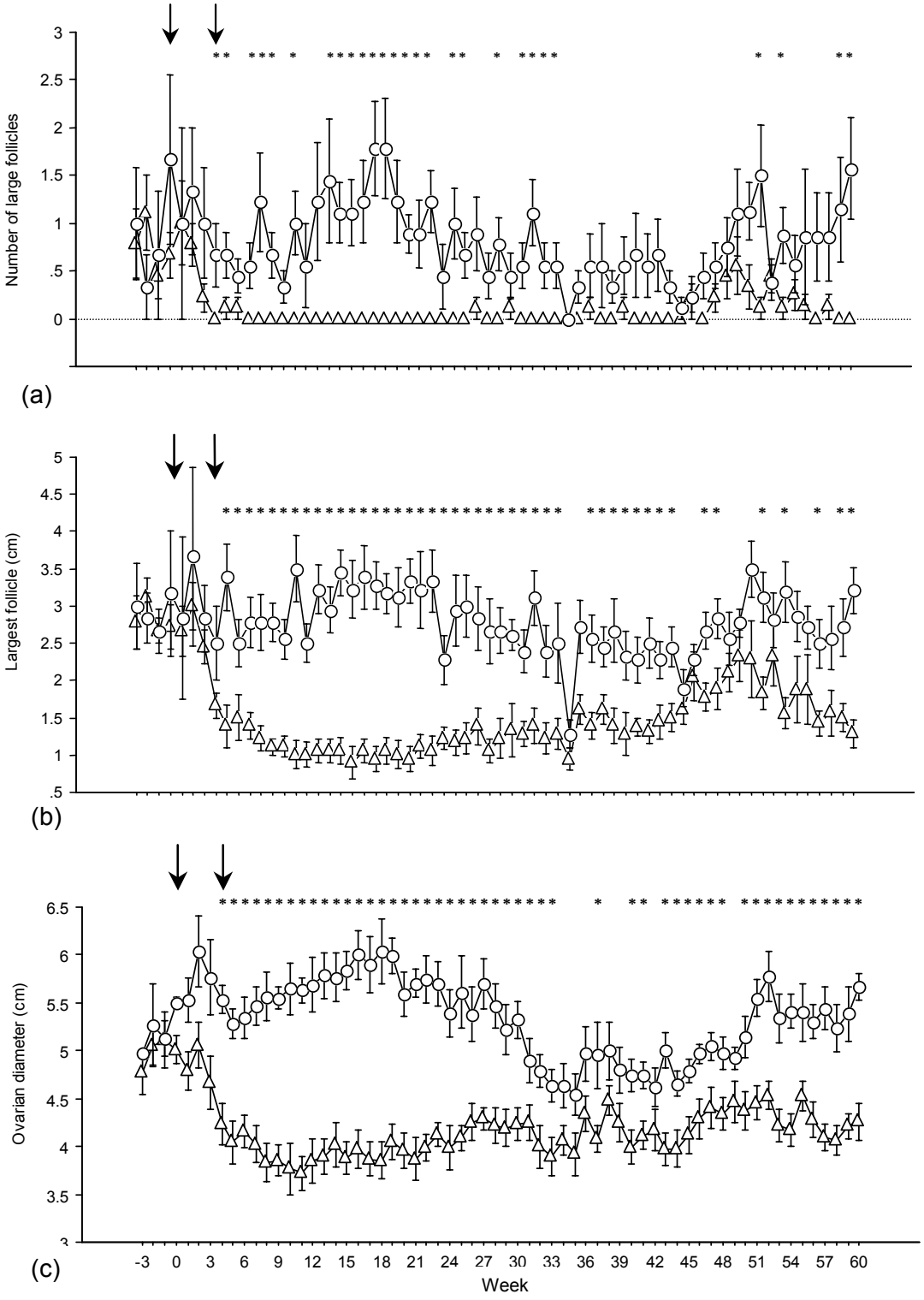


Fig. 1. Mean (±S.E.M.) number of large follicles (a), diameter of the largest follicle (b) and ovarian diameter (c) in vaccinated (△) and control (○) mares before and after immunization (↓). Asterisks indicate a significant difference (P<0.05, unpaired t-test) between groups.

Before the first vaccination there were no significant differences between the groups. The mean number of large follicles (Fig. 1a) in vaccinated mares decreased rapidly after the first vaccination and large follicles were completely absent from weeks 7 to 26. The mean number of large follicles remained below 1 until the end of period 1. These changes were also reflected by the mean diameters of the largest follicle (Fig. 1b), which in vaccinated mares dropped below 2 cm in week 4 and remained between 1 and 2 cm for 41 consecutive weeks. Mean diameters of the largest follicle in control mares only dropped below 2 cm in weeks 35 and 45. The difference between the groups was significant ($P<0.05$) during weeks 5 to 35. Mean ovarian diameters were also significantly ($P<0.05$) lower in vaccinated mares from week 4 to 33 (Fig. 1c).

Fig. 1 shows that mares in the control group continued normal cyclic activity with regular development of large follicles. As the study progressed into the winter months (weeks 32 to 46), most of the control mares gradually entered seasonal anestrus, showing reduced follicular activity and ovarian diameter. The weekly differences between the 2 groups were no longer significant. The period of ovarian suppression was accompanied by an equivalent period in which no CL were observed. In both treatment and control groups, the reappearance of a CL was preceded by a notable increase in follicular activity and indicated return to cyclic activity.

Uterine changes mirrored the changes in ovarian activity. The vaccinated mares had little ultrasonographically detectable uterine edema from the time when ovarian activity ceased until follicular activity recommenced. In control mares, uterine scores of 2 and 3 were always observed at regular 2 to 4 week intervals, except during the winter months.

3.2. Plasma progesterone and estradiol-17β concentrations

Fig. 2a shows the mean concentrations of plasma progesterone in treated and control mares from 3 weeks before to 60 weeks after first vaccination. Before treatment, the mean progesterone values in mares of both groups fluctuated between 1.7 and 11.7 ng/mL. After initial vaccination, the mean plasma progesterone concentration in the treated group fell below 1 ng/mL in week 4 and remained at this level until week 28. From week 28 small episodic increases in mean progesterone concentration were observed, which became more pronounced towards the end of period 1. In control

mares, mean plasma concentration of progesterone rose to 15.4 ng/mL during the breeding season and declined during winter months (weeks 32 to 46), remaining below 2.5 ng/mL from week 37 to 44 (January and February). Values rose again to 17 ng/mL as the study progressed into the new breeding season. Significant differences (P<0.05) between groups were present continuously during weeks 4 to 30 and at single weeks during the following breeding season.

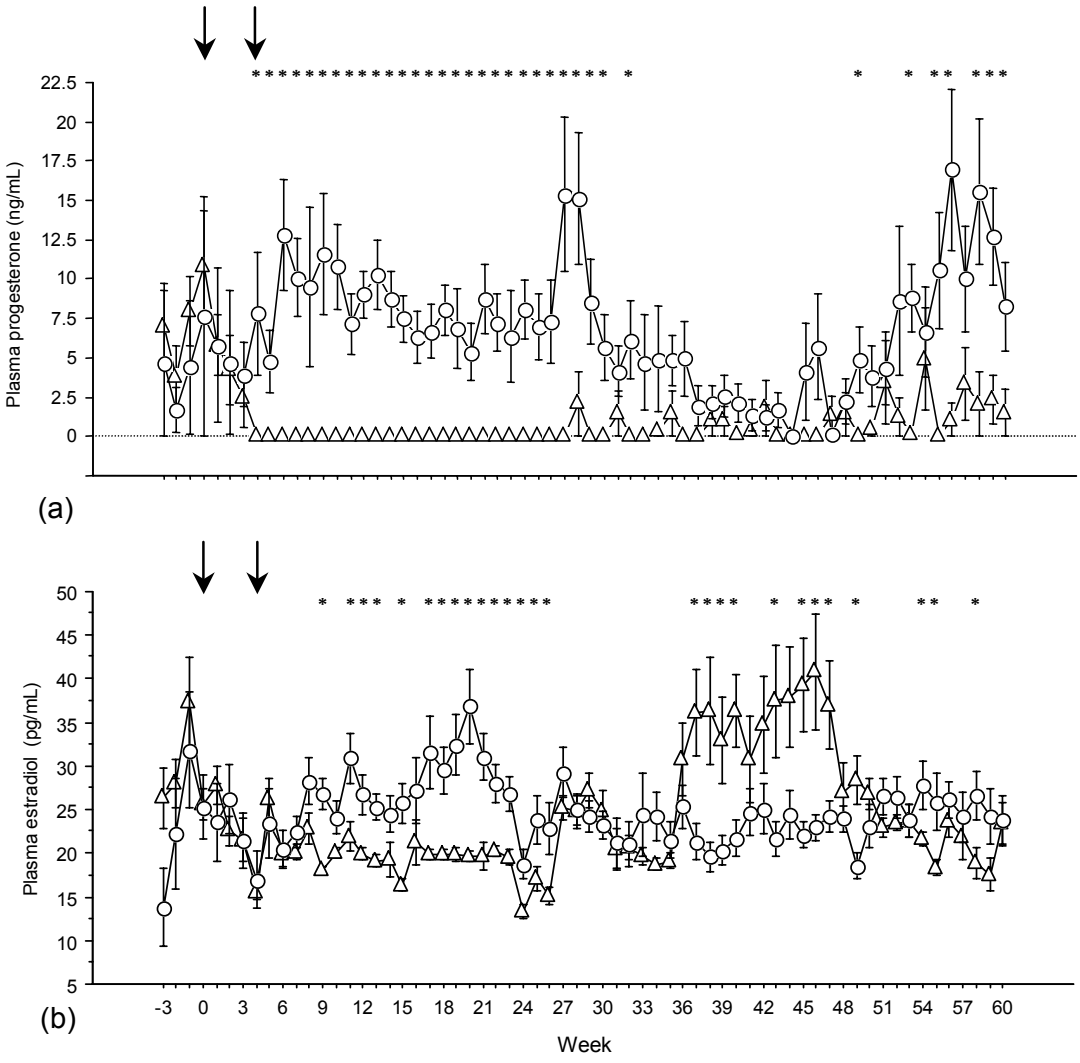


Fig. 2. Mean (±S.E.M.) plasma progesterone (a) and estradiol-17β (b) concentrations in vaccinated (△) and control (○) mares before and after immunization (↓). Asterisks indicate a significant difference (P<0.05, unpaired t-test) between groups.

Changes in mean concentrations of plasma estradiol-17β during period 1 are shown in Fig. 2b. Before treatment, mares of both groups had mean plasma estradiol-17β values fluctuating between 13.8 and 37.3 pg/mL. After the first vaccination the mean estradiol-17β concentrations in treated mares declined and fluctuated between 13.4 and 27.8 pg/mL until week 35. From week 36 to 47 (January to March) a clear increase in mean plasma estradiol-17β concentrations occurred in treated mares with values up to 40.9 pg/mL. Thereafter estradiol-17β concentrations rapidly decreased to levels observed before week 36. In control mares mean plasma estradiol-17β concentrations varied between 17.0 and 36.9 pg/mL during weeks 1 to 60. Significant differences (P<0.05) between groups were mainly observed between week 9 to 26 and 36 to 50.

3.3. Duration of ovarian suppression and effect on estrous behavior

All 9 immunized mares ceased cycling within 8 weeks of the first vaccination and the minimum period of ovarian suppression was 23 weeks. During period 1, three mares resumed both follicular (weeks 27, 46, 53) and ovulatory activity (weeks 28, 51, 54). In period 2, another two mares resumed both follicular (weeks 80, 96) and ovulatory activity (weeks 84, 100). Of the 4 treated mares that did not resume ovulatory activity during the study, 3 resumed follicular activity at weeks 48, 64 and 100. Of these, one showed regular development of large follicles for over 50 weeks without a progesterone rise, and one was destroyed for reasons unrelated to the study shortly after resumption of large follicle development. One mare remained completely suppressed for the entire duration of the study.

Six of 9 control mares became acyclic during weeks 33 and 41 (December to January) and cyclic activity was reestablished between weeks 45 and 56 (March to May). The other three control mares never fulfilled the criteria of acyclicity. Fig. 3 shows the plasma progesterone concentration together with the diameter of the largest follicle of 4 individual mares during period 1. Mare A from the control group shows an acyclic period between weeks 31 and 47. Mares B, C and D treated with Improvac® illustrate different types of reaction to the vaccine. Mare B showed the shortest period of suppression, followed by cyclic activity throughout the winter period, mare C showed a gradual return to follicular activity before achieving ovulation and mare D remained suppressed throughout period 1.

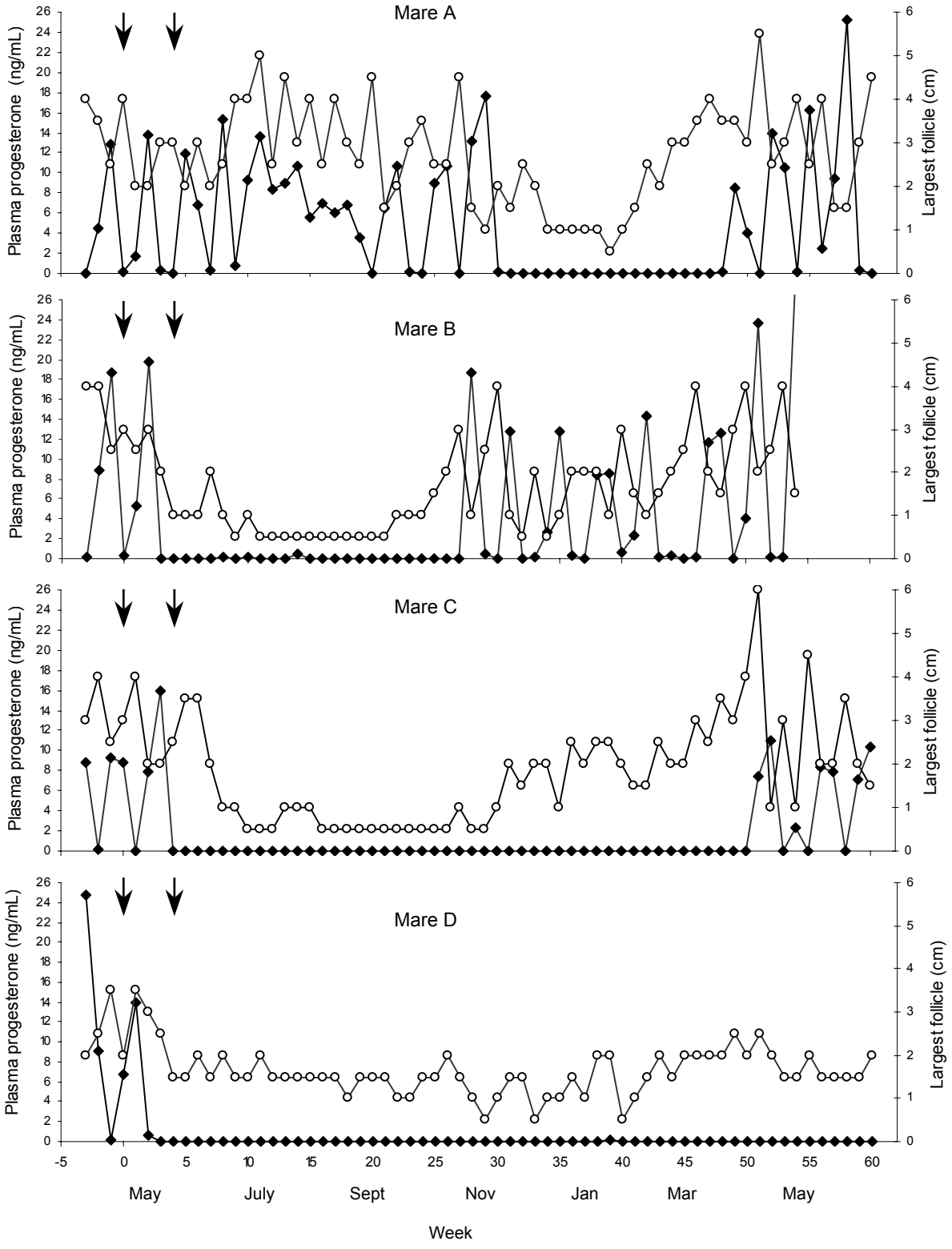


Fig. 3. Individual plasma progesterone concentration (◆) and diameter of largest follicle (○) of a control mare (A) and 3 vaccinated mares (B,C,D) before and after immunization (↓) during period 1.

In 4 vaccinated mares, estrous signs were absent during ovarian suppression and the first occurrence of large follicles was accompanied by estrous behavior. The other 5 mares expressed estrous signs that were not obviously related to follicle growth. Three of these mares showed sporadic estrous behavior, one was in estrus for several weeks before resuming follicular activity, while the last mare displayed estrus continuously from week 5 after vaccination until plasma progesterone concentration increased (week 84). All control mares demonstrated regular periods of estrous behavior throughout the study, except during seasonal anestrus.

3.4. Antibody titers

Antibody titers (Fig. 4) began to rise immediately after the first vaccination in all 9 mares and reached peak values 2 to 3 weeks after the booster injection. Maximum titers showed large individual variations and ranged between 35 and 70% binding (1:40 dilution). Thereafter, they slowly decreased to values between 15 to 40% binding at week 100 after the first vaccination. Values at resumption of ovulatory activity ranged from 24 to 35 % binding.

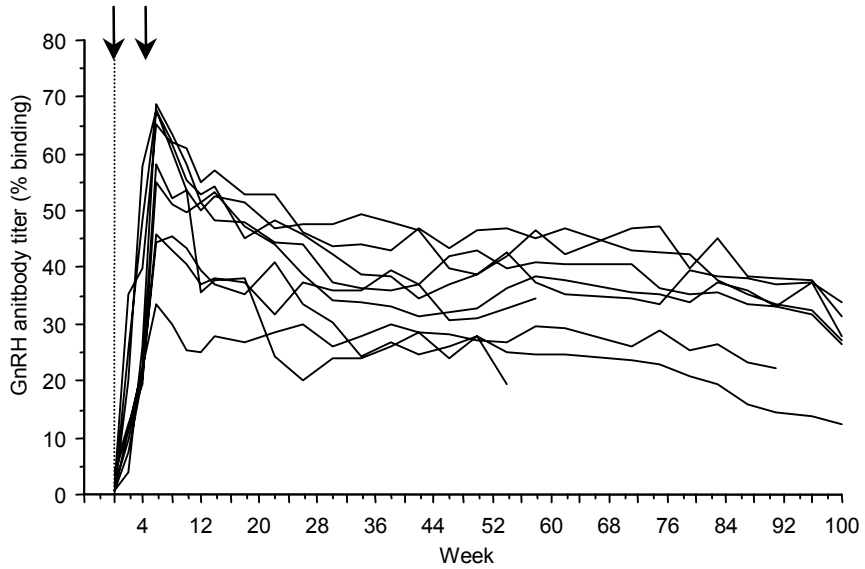


Fig. 4. GnRH antibody titers in 9 mares after GnRH immunization (↓) at weeks 0 and 4 (% binding at a 1:40 plasma dilution).

3.5. Adverse effects

Eight of 9 mares, which received 2 ml of Improvac®, showed reactions of varying severity, including swelling and pain at the injection site, stiffness of the neck, pyrexia and apathy (Table 2). The degree and duration of reactions varied individually and were more serious after the booster injection. A mean increase in body temperature was only evident on the day after each vaccination (mean 38.8 and 38.7°C). However, individual peaks up to 39.8°C were recorded. Swelling and pain was most evident on the second day after vaccination. All reactions had subsided by 4 and 5 days after primary and booster vaccination, respectively.

Table 2

Incidence of adverse effects of immunizing mares against GnRH using Improvac®

Effect	Number of mares	
	Primary immunization	Booster immunization
Number of mares immunized	9	9
Pyrexia	8 (88.9%)	7 (77.8%)
Pain at site of injection	4 (44.4%)	7 (77.8%)
Swelling at site of injection	3 (33.3%)	6 (66.7%)
Stiffness of the neck	0	7 (77.7%)
Apathy	0	2 (22.2%)

4. Discussion

The results of this study demonstrate that administration of the GnRH vaccine Improvac® to mares elicits clear inhibitory changes in endocrine, ovarian, uterine and behavioral parameters.

The effect of immunization against GnRH on ovarian activity was dramatic and rapid, and allowed clear differentiation between treated and control mares. Follicular development decreased such that by 4 weeks after the first vaccination, the number of medium and large follicles differed significantly between the treatment and control group. The variation in the amount of time (2-7 weeks) from vaccination to cessation of ovarian activity was probably due to immunization at different stages of the cycle. In mares with large follicles (estrus), cessation time was longer than in mares vaccinated during the luteal phase (diestrus). A similar study [27] carried out with a smaller number of mares also showed a rapid response to vaccination with follicular atresia and significant reduction in ovarian length 3 to 4 weeks after the first injection. In our study the ovarian response was always bilateral and the marked decrease in number and size of follicles resulted in ovaries that were significantly smaller than those in the control mares for 30 consecutive weeks. Resumption of ovulatory activity was achieved in 5 of 9 mares and varied between 27 and 100 weeks after the first vaccination. In these mares the period of time that elapsed between the first signs of follicular activity and the appearance of a progesterone-secreting CL was 1-5 weeks. These results demonstrate that resumption of follicular and ovulatory activity after immunization against GnRH is highly variable in spite of all mares being immunized at the same time with the same dose of the vaccine and despite showing a similar decline of anti-GnRH titers. A highly individual duration of ovarian suppression in vaccinated mares was also reported by Dalin et al. [28], where one mare showed a large time lapse between resumption of follicular activity and first ovulation and another was completely suppressed for approximately 14 months. The suppression of ovarian activity is brought about by the binding of endogenous GnRH to circulating antibodies, preventing GnRH from binding to receptors in the anterior pituitary. Studies on mares have shown that secretion (and possibly production) of LH is highly dependent on GnRH, while FSH secretion is at least partially independent of GnRH stimulation [25,26]. Therefore, the growth of small antral follicles that we observed in all vaccinated mares and that has been previously

described in mares [26,28], heifers [17] and ewes [31], may be relatively independent of GnRH availability. Because of the more comprehensive suppression of LH then of FSH achieved by vaccination against GnRH [25], further development, maturation and ovulation may be prevented.

Resumption of follicular and ovulatory activity in vaccinated mares may be comparable to the transitional period seen in mares between seasonal anestrus and the breeding season [32] where large (≥3 cm) anovulatory follicles grow and regress before an ovulatory follicle develops with sufficient estradiol production to increase LH secretion and induce ovulation.

A significant finding of the current study was that 3 mares showed no signs of luteal activity despite regular development of large follicles and that one mare showed neither luteal activity nor follicle development for 2 years after vaccination. Without having conducted further tests we are unable to say whether immunization resulted in reduced sensitivity of the pituitary gland to GnRH or decreased pituitary production and / or secretion of LH and FSH. Furthermore, we cannot entirely exclude the possibility of some degree of permanent impairment of hypothalamic or pituitary function, as observed after the immunization of ewes against GnRH early in life [31].

Levels of plasma progesterone and estradiol-17β concentration also showed marked differences between the treatment and control groups. Plasma progesterone concentration is considered the best indicator for the resumption of estrous cycles in mares [27,28]. Cyclic activity in control mares was demonstrated by regular progesterone increases, which correlated with recent ovulations. In all vaccinated mares the indirect effect of GnRH inactivation on LH secretion was best evidenced by the low progesterone concentrations (<1 ng/ml), indicating the absence of ovulations, during at least 5 months. The effect of vaccination on estradiol-17β concentrations was less dramatic. In control mares, levels fluctuated throughout the whole study and higher concentrations were observed when the number of large follicles was increased. In vaccinated mares, plasma estradiol-17β concentrations generally fluctuated at a lower level than in control mares until the end of the breeding season. From January to March mean estradiol-17β concentrations in vaccinated mares were significantly higher than in control mares due to abnormally elevated levels seen in 3 mares. As the number and size of the follicles during this time was not higher than at any other time during period

1, it is speculated that the origin of the estradiol-17β may be increased production by granulosa cells of small and antral follicles. It is also possible that the estradiol was of adrenal origin since sexual steroids are also produced by the adrenal cortex in response to ACTH [5].

The manifestation of estrous behavior in anovulatory mares as seen in this study is a phenomenon that has been described after vaccination against GnRH [28], after treatment with a GnRH agonist [14] and following ovariectomy [5]. In the absence of significant progesterone concentrations, only small amounts of estradiol are needed for induction of estrous signs. That estrous behavior in ovariectomized mares was successfully suppressed by dexamethasone treatment [5] further underlines the possible involvement of sexual steroids originating from the adrenal cortex.

All mares showed an immediate and distinct antibody response after the first vaccination with peak levels obtained 2 to 3 weeks after the booster injection. The titer decline was similar in all mares, with a rapid fall during the first few weeks followed by a steady decline until 100 weeks after the first vaccination. Antibody titers were not clearly related to the duration or the degree of ovarian suppression and titers at reversal varied considerably. Other studies in mares [26,28], heifers [17] and ewes [33] also report a large variation in antibody response, duration of suppression of reproductive activity and antibody titer at reversal. It may be concluded that thresholds at which antibody titers retain effectiveness are variable between individuals, and may well be related to genetic factors and the individuals' specific immune response [33].

The adverse effects to vaccination with Improvac® were quite severe, similar to those described by Dalin et al. in mares [28] and Dowsett at al. in stallions [23] using different vaccine formulations. Improvac®, originally developed to control boar taint in pigs has been shown to be well tolerated in pigs [34,35]. The high incidence of adverse effects using Improvac® in horses may either be caused by the adjuvant or the carrier protein present in the vaccine. A new vaccine (Equity™, Pfizer Animal Health, Australia), with a modified adjuvant and carrier protein has been designed specifically for use in the horse and is well tolerated (authors: unpublished observations).

In conclusion, this study demonstrates that reproductive cyclicity in adult mares can be reliably suppressed by active immunization against GnRH. However, the duration of the inhibitory effect is highly variable, not clearly related to GnRH antibody titers and

resumption of ovulatory activity may require more than 100 weeks. Moreover, ovarian suppression does not always prevent the occurrence of sexual behavior. Because of the failure of some mares in this study to return to reproductive cyclicity, it would be of practical significance to determine the long-term effects of repeated vaccination on the fertility of mares.

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Unterdrückung der Fortpflanzungsaktivität durch aktive Immunisierung gegen GnRH beim adulten weiblichen Schaf

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Zusammenfassung

Das Ziel dieser Arbeit war es, die Wirksamkeit einer anti-GnRH Vakzine auf die Zyklusaktivität beim adulten Schaf während der Zuchtsaison abzuklären. Für die Untersuchungen standen 22 zyklische Weisse Alpenschafe im Alter zwischen 2 und 4 Jahren zur Verfügung, die zufällig einer Versuchs- und Kontrollgruppe mit je 11 Tieren zugeordnet wurden. Die Schafe der Versuchsgruppe erhielten 2-mal im Abstand von 4 Wochen 2 ml (400µg GnRH-Protein-Konjugat) Improvac™ (Pfizer Animal Health, Australien) und die Kontrolltiere erhielten die entsprechende Menge physiologischer Kochsalzlösung subkutan am Hals verabreicht. Der Progesterongehalt im Blut wurde wöchentlich während 3 Wochen vor bis 32 Wochen nach der 1. Impfung und der Antikörpertiter monatlich nach der 1. Impfung bestimmt. Bei allen geimpften Schafen trat innerhalb von 2–8 Wochen nach der 1. Impfung eine Azyklie auf. Die Unterdrückung der Progesteronsekretion dauerte mindestens 12 Wochen (2 Schafe) mit individuellen Abweichungen von 14 (1 Schaf), 25 (1 Schaf) und mehr als 25 Wochen (7 Schafe). Bei 4 Tieren war die Impfwirkung reversibel, während die restlichen 7 Schafe bis zum Ende des Versuchs azyklisch blieben. Die Antikörpertiter erreichten 4 Wochen nach der 2. Impfung Maximalwerte und fielen anschliessend kontinuierlich bis Versuchende (8 Monate nach der 1. Impfung) auf Werte zwischen 10.9 und 40.8 % Bindung ab. Aufgrund unserer Ergebnisse kann gefolgert werden, dass beim adulten Schaf nach zwei Impfungen mit Improvac™ im Abstand von 4 Wochen die Zyklusaktivität während mindestens 12 Wochen gehemmt wird. Die Unterdrückung der Ovarfunktion ist jedoch individuell verschieden und kann mehr als 31 Wochen anhalten.

Schlüsselwörter: Schaf, GnRH Vakzine, Zyklus, Progesteron

Suppression of reproductive cyclicity by active immunization against GnRH in the adult ewe

The objective of the present study was to evaluate the effect of an anti-GnRH vaccine on cycling activity in the adult ewe during the breeding season. For the experiments 22 cycling White Alpine sheep, aged between 2 and 4 years, were randomly divided into a treatment and control group of 11 animals, each. Sheep of the treatment group were immunized twice at an interval of 4 weeks with 2 ml (400µg GnRH-protein conjugate) of Improvac™ (Pfizer Animal Health, Australia) subcutanously in the neck. Sheep of the control group received the same amount of saline solution. Blood progesterone concentrations were measured weekly from 3 weeks before to 32 weeks after first immunization and anti-GnRH titers were determined monthly. All vaccinated ewes ceased cycling within 2–8 weeks after first immunization. Plasma progesterone was suppressed for a minimum of 12 weeks (2 ewes) with individual variation of 14 (1 ewe), 25 (1 ewe) and more than 25 weeks (7 ewes). Four animals resumed cyclicity while 7 animals remained suppressed until the end of the study. Antibody titers peaked one month after the booster injection and thereafter continuously dropped until the end of the study (8 months after first immunization) to values between 10.9 and 40.8 % binding. From our results it can be concluded that two vaccinations with Improvac™ 4 weeks apart suppress cycling activity in adult ewes for at least 12 weeks. The inhibitory effect on ovarian activity, however, varies individually and may last more than 31 weeks.

Keywords: ewe, GnRH vaccine, estrous cycle, progesterone

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Einleitung

Die Steuerung des Sexualzyklus gewinnt beim kleinen Wiederkäuer zunehmend an Bedeutung. Neben der Zyklusinduktion ausserhalb der physiologischen Fortpflanzungsperiode und der Synchronisation ist auch die Unterdrückung der Brunst während der Alpung ein häufig geäussertes Anliegen der Schafhalter. In einem früheren Versuch (Janett et al., 2004) konnten wir zeigen, dass nach einmaliger Verabreichung hoher Gestagendosen (50 mg Chlormadinonazetat oder 140 mg Medroxyprogesteronazetat) der Sexualzyklus während mindestens 4 (Chlormadinonazetat) beziehungsweise 6 Wochen (Medroxyprogesteronazetat) unterdrückt werden konnte. Aufgrund der relativ kurzen Wirkungsdauer eignet sich diese Methode jedoch nicht für eine Unterdrückung der Brunst während einer gesamten Alpungsdauer von 3–4 Monaten. Eine Alternative zur hormonellen Brunstunterdrückung stellt die aktive Immunisierung gegen GnRH dar, die grundsätzlich bei allen Säugetieren (D'Occhio, 1993) möglich ist und bei Rind (Robertson et al., 1979; 1982; Jeffcoate et al., 1982), Schwein (Dunshea et al., 2001; Jaros et al., 2005), Pferd (Turkstra et al., 2005, Imboden et al., 2006), Hund (Ladd et al., 1994), Katze (Levy et al., 2004), Wildtieren (Miller et al. 2000; 2004), Ziegenbock (Godfrey et al., 1996) und Schaf (Jeffcoate et al., 1982; Brown et al., 1994; 1995; Janett et al., 2003; Earl et al., 2006) mit Erfolg angewendet wurde. Eigene Untersuchungen (Janett et al.; 2003) beim männlichen Lamm mit der mittlerweile in der Schweiz registrierten Schweinevaccine Improvac™ (Pfizer Animal Health, Australia) haben gezeigt, dass die Hodenfunktion nach einer 2-maligen Impfung im Abstand von 3 Wochen während mindestens 3 Monaten gehemmt werden kann. Da entsprechende Untersuchungen beim weiblichen Schaf fehlen, bestand das Ziel der vorliegenden Studie darin, die Auswirkungen einer Impfung mit Improvac™ auf die Zyklusaktivität sowie den Verlauf von Progesteron und des GnRH Antikörpertiters im Blut genauer abzuklären.

Tiere, Material und Methoden

Tiere

Für die Untersuchungen standen 22 adulte pluripare, klinisch gesunde, zyklische Schafe der Rasse Weisses Alpenschaf (WAS) im Alter von 2–4 Jahren zur Verfügung. Während des Versuchs, der von September 2004 bis Mai 2005 dauerte, wurden die Tiere zusammen mit einem vasektomierten Widder auf der Weide (Herbst und Frühling) oder im Stall (Winter) gehalten. Die Winterfütterung bestand aus Heu oder Grassilage und Wasser stand ad libitum zur Verfügung.

Versuchsanordnung

Vor Versuchbeginn wurde bei allen Schafen mittels vier aufeinanderfolgenden wöchentlichen Plasmaprogesteronbestimmungen die Zyklusaktivität nachgewiesen und je 11 Tiere zufällig einer Versuchs- (GnRH-Impfung, n=11) und einer Kontrollgruppe (physiologische NaCl-Lösung, n=11) zugeordnet. Anschliessend wurden die Tiere der Versuchsgruppe 2-mal im Abstand von 4 Wochen mit 2 ml (400µg GnRH-Protein-Konjugat) Improvac™ (Pfizer Animal Health, Australien) subkutan seitlich am Hals geimpft. Die Kontrolltiere erhielten jeweils die gleiche Menge physiologischer NaCl-Lösung.

Blutentnahme, Progesteron- und Antikörper-Bestimmung

Bei allen Schafen wurde wöchentlich in der Zeit von 3 Wochen vor bis 32 Wochen nach der 1. Impfung mittels heparinisierter Röhrchen (S-Monovetten™, Sarstedt, Sevelen) aus der V. jugularis Blut entnommen. Die Proben wurden unmittelbar nach der Gewinnung zentrifugiert (4000 x g, 10 Min.) und Plasma bis zur Analyse bei -18°C gelagert.
Die Progesteronbestimmung erfolgte mit einem Radioimmunoassay (Kit Prog-CTK-4 P001906, DiaSorin, Saluggia, Italien) unter Verwendung eines für Progesteron sehr spezifischen Antikörpers. Die Nachweisgrenze lag bei 0.05 ng Progesteron pro ml Plasma und der Intra- sowie der Inter-assay Variationskoeffizient betrugen 5.8% beziehungsweise 9.0%.
Die Antikörpertiter gegen GnRH wurden in monatlich gewonnenen Plasmaproben mit einem standardisierten ELISA (Röhn et al., 2006) unter Verwendung eines am N-Terminal konjugierten Peptids (RNase-Cys-GnRH-Konjugat) als beschichtetes Antigen (10µg/ml) und einem polyklonalen Kaninchen anti-Schaf-IgG Antikörper (Millipore, Zug) als sekundärer Antikörper bestimmt. Die Messergebnisse werden als Prozentsatz der Probe mit der höchsten Antikörperbindung bei einer Plasmaverdünnung von 1:540 angegeben.

Statistik

Die statistische Auswertung der Daten erfolgte mit dem Programm StatView 5.0 (SAS Institut, Dübendorf). Der Einfluss der Gruppeneinteilung, des Zeitpunktes der Blutentnahme und der Behandlung (Interaktion von Gruppe und Zeitpunkt) auf die Progesteronkonzentration wurde mit einer multivariaten Varianzanalyse (ANOVA) geprüft. Die Überprüfung von Gruppenunterschieden an einzelnen Zeitpunkten erfolgte mittels ungepaartem t-Test. Die Signifikanzschwelle wurde auf 0.05 festgelegt.
Als Beginn der Impfwirkung wurde der Zeitpunkt bezeichnet, ab dem in mindestens drei aufeinanderfolgenden Wochen die Progesteronkonzentration < 1 ng/ml Plasma war. Die Dauer der Impfwirkung wurde als Peri-

ode mit anhaltend tiefen Progesteronwerten (< 1 ng/ml) definiert. Der Impfeffekt wurde als reversibel bezeichnet, wenn als Hinweis für eine stattgefundenen Ovulation, erneut Progesteronwerte > 1 ng/ml Plasma gemessen wurden.

Ergebnisse

Progesteronverlauf vor und nach der Impfung

Der durchschnittliche Progesteronverlauf aller Schafe mit und ohne Improvac™ ist in Abbildung 1 dargestellt. Vor der Impfung schwankten die Progesteronkonzentrationen bei beiden Gruppen zwischen 2.0 und 4.3 (Improvac™) beziehungsweise 2.0 und 4.8 (Kontrolle) ng/ml Plasma. Während dieser Zeit bestanden keine signifikanten Unterschiede zwischen den beiden Gruppen (Tab.1).
Hingegen konnte nach der Behandlung ein signifikanter

Tabelle 1: Einfluss von Gruppe und Zeitpunkt der Blutentnahme sowie Interaktion von Gruppe und Zeitpunkt auf die Progesteronkonzentration im Blut vor der Behandlung.

Parameter	Gruppe *P*	Zeitpunkt *P*	Interaktion *P*
Progesteron	0.2667	0.0076*	0.7197

*Signifikant (P < 0.05)

(P < 0.05) Einfluss der Gruppe, des Zeitpunktes der Blutentnahme sowie der Behandlung (Interaktion von Gruppe und Zeitpunkt) festgestellt werden (Tab. 2).
Bei allen geimpften Tieren fiel das Progesteron 2 Wochen nach der 1. Immunisierung kontinuierlich auf Werte unterhalb 0.5 ng/ml Plasma ab und blieb, mit Ausnahme von Woche 7, bis zur Woche 19 auf diesem tiefen Niveau. Anschliessend stiegen die Progesteronwerte während der

Tabelle 2: Einfluss von Gruppe und Zeitpunkt der Blutentnahme sowie Interaktion von Gruppe und Zeitpunkt auf die Progesteronkonzentration im Blut nach der Behandlung.

Parameter	Gruppe *P*	Zeitpunkt *P*	Interaktion *P*
Progesteron	< 0.0001*	< 0.0001*	< 0.0001*

*Signifikant (P < 0.05)

Wochen 20–26 vorübergehend leicht an bevor sie ab Woche 27 bis Versuchende erneut auf Werte < 0.5 ng/ml Plasma sanken. Die durchschnittlichen Progesteronkonzentrationen der Kontrolltiere schwankten in den Wochen 1 bis 20 zwischen 1.6 und 6.6 ng/ml Plasma. Ab Woche 21 war ein deutlicher Progesteronabfall mit anhaltend tiefen Werten < 0.5 ng/ml Plasma in den Wochen 27 bis 32 zu beobachten. Signifikante (P < 0.05) Gruppenunterschiede waren in den Wochen 3–6, 8–20, sowie 25 und 27 vorhanden.

Individueller Progesteronverlauf, Wirkung und Reversibilität der Impfung

In Abbildung 2 sind die individuellen Progesteronverläufe bei allen 11 geimpften Schafen dargestellt. Es ist deutlich zu erkennen, dass die Progesteronwerte in den ersten 8 Wochen bei verschiedenen Schafen zwischen 0.13 und 10.00 ng /ml Plasma schwankten. Von Woche 8 bis 19 bewegten sich die Werte stets unterhalb 1 ng/ml Plasma. Ein erneuter Anstieg von Progesteron in den folgenden Wochen war bei 4 Schafen zu beobachten, während die restlichen 7 Schafe bis Versuchende azyklisch blieben. Die individuelle Wirkungsdauer und der Zeitpunkt des erneuten Zyklusbeginns sind in Tabelle 3 zusammengefasst. Daraus geht hervor, dass die Impfwirkung 2–8 Wochen nach der 1. Immunisierung einsetzte und mindestens 12 Wochen dauerte. Die individuelle Unterdrückung

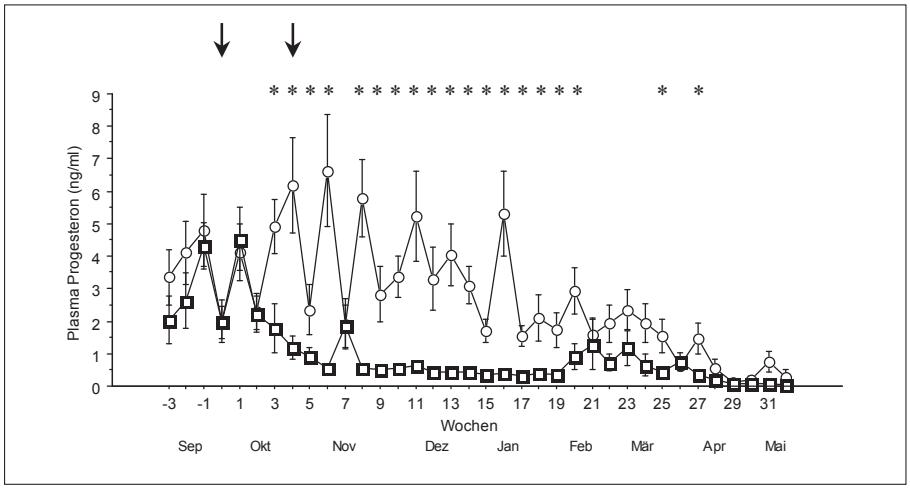


Abbildung 1: Durchschnittliche (*m* ± *SEM*) Progesteronkonzentrationen im peripheren Blut bei je 11 Schafen mit (□) und ohne (○) Improvac™. Pfeile stellen die Applikation von Improvac™ bzw. phys. NaCl-Lösung dar. *Signifikanter Gruppenunterschied (P < 0.05, ungepaarter *t*-Test).

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der Ovaraktivität betrug 12 (2 Schafe), 14 (1 Schaf), 25 (1 Schaf) und mehr als 25 Wochen (7 Schafe).

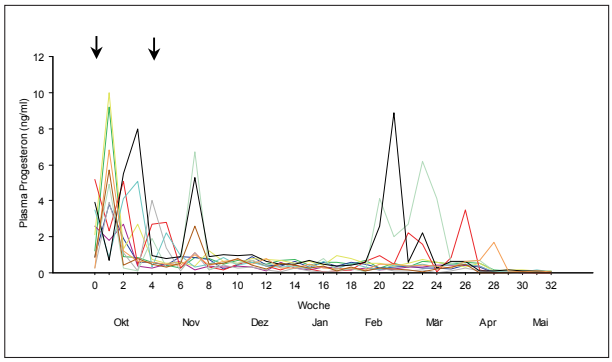


Abbildung 2: Individueller Progesteronverlauf bei 11 mit Improvac™ geimpften (↓) Schafen.

Antikörpertiter

Ein Monat nach der 1. Impfung stiegen die Antikörpertiter (Abb. 3) individuell auf Konzentrationen zwischen 41.5 (Schaf 260) und 89.7% Bindung (Schaf 258) an, erreichten dann 4 Wochen nach der 2. Impfung Maximalwerte zwischen 72.9 (Schaf 267) und 100% (Schaf 258) Bindung und fielen anschliessend bis Versuchende kontinuierlich ab. Am Ende des Versuchs, 32 Wochen nach der 1. Impfung, lagen die Antikörpertiter zwischen 10.9 und 40.8 % Bindung.

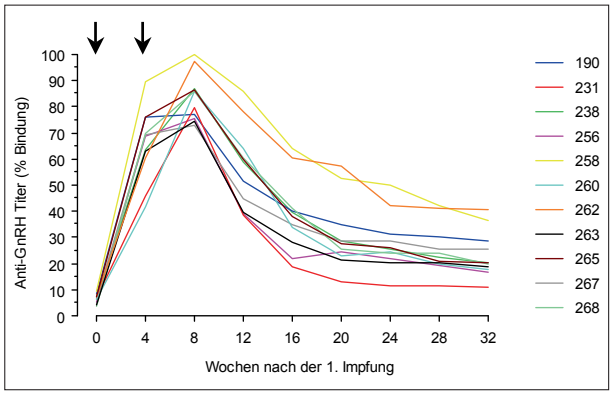


Abbildung 3: Individueller anti-GnRH Titer (% Bindung bei einer Verdünnung von 1:540) bei 11 mit Improvac™ geimpften (↓) Schafen.

Diskussion

Die Ergebnisse der vorliegenden Untersuchung zeigen, dass beim adulten Schaf eine 2-malige Vakzinierung mit Improvac™ im Abstand von 4 Wochen die Zyklusaktivität während mindestens 12 Wochen zu hemmen vermag. Die Impfwirkung setzte spätestens 4 Wochen nach der 2. Impfung ein und war bei 4 Tieren reversibel. Bei den übrigen 7 Schafen blieb der Zyklus während mindestens 25 Wochen bis zum Ende des Versuchs (8 Monate nach der 1. Impfung) gehemmt.

Aufgrund des Titerverlaufs konnten wir bei allen Schafen feststellen, dass im Gegensatz zum Schwein (Dunshea et al., 2001), bereits die 1. Impfung eine deutliche Immunreaktion mit Bildung von spezifischen Antikörpern (42–90% Bindung) auslöste. Ein rascher Titeranstieg bereits nach der 1. Impfung mit Improvac™ haben wir in früheren Untersuchungen (Imboden et al., 2006) auch bei der Stute beobachtet. Dies weist darauf hin, dass Improvac™ im Vergleich zu anderen GnRH Vakzinen bei Pferd (Turkstra et al., 2005) und Schaf (Earl et al., 2006) eine stärkere Immunreaktion hervorrufen kann.

Der Verlauf der individuellen Antikörpertiter zeigte, dass bei einem deutlich erhöhten Titer (> 70% Bindung) die Ovaraktivität zuverlässig gehemmt werden konnte. Tiefere Titer hingegen, liessen keine eindeutige Aussage über die Hemmwirkung zu. So zeigte Schaf 262 bei einem Titer von 41% Bindung in der Woche 28 einen erneuten

Tabelle 3: Beginn und Dauer der Impfwirkung nach 2-maliger Immunisierung im Abstand von 4 Wochen mit Improvac™ bei 11 adulten Schafen.

Schaf	Beginn der Impfwirkung (Woche)	Progesteronanstieg nach Impfwirkung (Woche)	Dauer der Impfwirkung (Wochen)
263	8	20	12
268	8	19	12
231	8	22	14
262	3	28	25
265	8	nicht beobachtet	> 25
267	8	nicht beobachtet	> 25
260	6	nicht beobachtet	> 27
258	4	nicht beobachtet	> 29
190	3	nicht beobachtet	> 30
256	3	nicht beobachtet	> 30
238	2	nicht beobachtet	> 31

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Progesteronanstieg, während dieser bei Schaf 258 mit einem ähnlich hohen Titer (42% Bindung in Woche 28) ausblieb.

Bei Schafen mit tiefem Antikörpertiter muss als mögliche Erklärung der Azyklie die saisonale Fortpflanzung bei gewissen Rassen berücksichtigt werden. So war ein jahreszeitlicher Einfluss auf die Kontrolltiere in den letzten 3 Monaten unseres Versuches (März–Mai), aufgrund der tiefen Progesteronwerte, deutlich zu erkennen. Dies ist ein weiterer Hinweis, dass das Weisse Alpenschaf entgegen der allgemeinen Annahme nicht asaisonal ist.

Bezüglich Reversibilität der Impfung muss erwähnt werden, dass nach Ende des Versuchs die azyklischen Tiere für weitere Untersuchungen nicht mehr zur Verfügung standen. Es kann jedoch davon ausgegangen werden, dass bei abnehmender Antikörperkonzentration die Zyklusaktivität wieder einsetzt (Keeling und Crighton, 1984). In diesem Zusammenhang ist von besonderem Interesse inwieweit, bei geimpften azyklischen Tieren, eine fertile Brunst induziert werden kann. Beim Einsatz einer GnRH Impfung bei präpubertären Schafen ist zu beachten, dass es zu einer bleibenden Hemmung der Fortpflanzungsfunktion und zu Sterilität kommen kann (Brown et al., 1994; 1995; Clarke et al., 1998; Janett et al., 2003). Aufgrund dieser Erkenntnisse sollte die Impfung gegen GnRH bei Jungtieren, die zur späteren Zucht vorgesehen sind, nicht angewendet werden.

Schlussfolgerung

Unsere Ergebnisse haben gezeigt, dass beim adulten Schaf eine 2-malige Impfung mit Improvac™ im Abstand von 4 Wochen zu einer zuverlässigen Hemmung der Fortpflanzung während mindestens 12 Wochen führt, die Impfwirkung jedoch individuell verschieden ist und länger als 31 Wochen anhalten kann.

Dank

An dieser Stelle möchten wir Urban Lanker und seiner Frau Gaby für die Tiere und ihre Hilfsbereitschaft ganz herzlich danken.

Blocage de l'activité reproductrice chez les moutons adultes par une immunisation active contre la GnRH

Le but de ce travail était d'étudier l'efficacité d'un vaccin anti GnRH sur le cycle de moutons adultes durant la période d'élevage. Pour cette étude, 22 brebis Blanc des Alpes, au cycle normal, âgées de 2 à 4 ans étaient à disposition; elles ont été réparties au hasard en un groupe d'essai et un groupe de contrôle de chacun 11 animaux. Les animaux du groupe d'essai ont reçu 2 fois à 4 semaines d'intervalle 2ml (400µg de conjuguât protéinique de GnRH) d'Improvac™ (Pfizer Animal Health Australie) et les animaux de contrôle une quantité correspondante de solution saline physiologique appliquée par voie sous-cutanée à l'encolure. Le taux sanguin de progestérone a été mesuré hebdomadairement dès 3 semaines avant à 32 semaines après la 1ère vaccination et le titre d'anticorps, mensuellement, après la 1ère vaccination. Chez tous les animaux vaccinés, une acyclie est apparue 2 à 8 semaines après la 1ère injection. Le blocage de la sécrétion de progestérone a duré au minimum 12 semaines (2 animaux) avec des variations de 14 (1 animal) 25 (1 animal) et plus de 25 semaines (7 animaux). Chez 4 brebis, l'effet de la vaccination a été réversible alors que les 7 autres sont restées acycliques jusqu'à la fin du test. Les taux d'anticorps ont atteint des valeurs maximales 4 semaines après la 2ème injection et ont décru ensuite continuellement jusqu'à la fin de l'essai (8 mois après la primo vaccination). Sur la base de ces résultats, on

Soppressione dell'attività riproduttiva tramite attiva immunizzazione contro il GnRH nelle pecore adulte

Scopo di questo studio è di chiarire l'efficacia di un vaccino anti-GnRH sull'attività del ciclo riproduttivo nelle pecore adulte, durante la stagione dell'accoppiamento. Per l'esame sono state messe a disposizione 22 pecore alpine bianche durante il ciclo riproduttivo di età tra i 2 e i 4 anni che sono state attribuite ad un gruppo sperimentale e uno di controllo con ciascuno 11 animali. Alle pecore del gruppo sperimentale è stato somministrato, per 2 volte a distanza di 4 settimane, 2 ml (400µg di proteine coniugate GnRH) Improvac™ (Pfizer Animal Health, Australia) mentre agli animali del gruppo di controllo si è somministrata la stessa quantità di soluzione salina per via subcutanea alla gola. Il contenuto di progesterone nel sangue è stato controllato settimanalmente a partire da 3 settimane prima fino a 32 settimane dopo la 1° vaccinazione e il titolo di anticorpi mensilmente a partire dalla 1° vaccinazione. In tutte le pecore vaccinate è sopravvenuta un'aciclia entro 2–8 settimane dopo la 1° vaccinazione. La soppressione della secrezione di progesterone è durata al minimo 12 settimane (2 pecore) con differenze individuali di 14 (1 pecora), 25 (1 pecora) e più di 25 settimane (7 pecore). In 4 animali l'effetto della vaccinazione è stato reversibile, mentre le restanti 7 pecore sono rimaste senza ciclo fino alla fine dell'esperienza. Il titolo di anticorpi raggiunse 4 settimane dopo la 2° vaccinazione dei valori massimi e di-

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peut conclure que chez les brebis adultes le cycle est interrompu pendant au moins 12 semaines après une double vaccination à l'Improvac™ avec un intervalle de 4 semaines. Le blocage de la fonction ovarienne est toutefois individuel et peut se prolonger plus de 31 semaines.

minuì in modo continuo fino alla fine dell'esperienza (8 mesi dopo la 1° vaccinazione) mostrando dei valori tra 10.9 e 40.8%. Sulla base di questi risultati si può dedurre che, l'attività riproduttiva nelle pecore adulte dopo due vaccinazione con Improvac™ a distanza di 4 settimane è inibita durante al minimo 12 settimane. La soppressione della funzione ovarica può essere bloccata per più di 31 settimane ed è differente a seconda dell'animale.

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Suppression of testicular function and sexual behavior by vaccination against GnRH (Equity™) in the adult stallion

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Abstract

The aim of this study was to evaluate the effects of an anti-GnRH vaccine on testosterone concentration, antibody titer, scrotal width, semen quality and sexual behavior in the stallion. Adverse reactions to the vaccine were also determined. Eight clinically healthy sexually experienced stallions aged between 6 and 15 years from the National Stud in Avenches (Switzerland) were used. Five stallions were immunized 3 times at an interval of 4 and 8 weeks, respectively, with 200 μg of a GnRH-protein conj (Equity™, CSL Limited, Australia) intramuscularly in the neck and 3 control animals received an equivalent volume of solution. Plasma testosterone concentrations and GnRH antibody titers as well as semen quality and libido were deter weekly during 1 year (52 weeks). In addition, scrotal width was measured in all stallions before and 4, 8 as well as 12 months after first vaccination. Our results demonstrate that in 4 stallions plasma testosterone started to decrease after the second vaccination and remained suppressed for at least 6 months whereas one stallion showed no effect. Until the end of the experiment 2 stallions reached prevaccination testosterone values. Antibody titers varied individually in all 5 stallions and reached peak concentrations within 2 weeks after the third vaccination. Scrotal width was significantly ($P < 0.05$) lower in vaccinated than in control stallions 8 months after first vaccination. Semen quality started to decreased after the second vaccination and improved towards the end of the experiment. In 4 stallions libido was clearly reduced after the second immunization but normalized in 2 animals before the end of the study while 2 stallions continued to show poor libido. From our results we conclude that three immunizations with Equity™ are well tolerated in the stallion and may effectively suppress testosterone secretion and reduce semen quality as well as sexual behavior. The inhibiting activity of Equity™ on these parameters is individually different and may last for a minimum of 6 months.

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Harem stallion changes are not associated with diminished reproductive performance of females in semi-feral Konik polski horses (*Equus caballus)*

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Credit Author Statement

Joanna Jaworska: conceptualization, methodology, investigation, resources, writing – original draft, writing – review and editing, formal analysis, funding acquisition, project administration

Zbigniew Jaworski: conceptualization, resources, investigation, validation

Sue M. McDonnell: conceptualization, writing – review and editing, validation

Aleksandra Górecka-Bruzda: conceptualization, methodology, software, writing – original draft, writing – review and editing, formal analysis, supervision

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Harem stallion changes are not associated with diminished reproductive performance of females in semi-feral Konik polski horses (*Equus caballus*).

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Short title: Bruce effect and infanticide in feral horses

ABSTRACT

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Infanticide, related to a stallion's aggression toward a foal sired by another stallion, and feticide related to a new stallion's aggression and/or pheromonal influence (the Bruce effect) inducing loss of a fetus sired by another stallion, a female's counteraction to infanticide, have been proposed for domestic horses *(Equus caballus*) in human-managed conditions. The aim of the present study was, in conditions close to natural, to investigate the influence of the natural succession of a harem stallion on the mares' subsequent reproductive performance.

In a population of semi-feral Konik polski horses observed for 31 years (reproductive seasons) in 8 bands, harem stallion changed 10 times. These changes involved 26 out of 48 mares and 60 out of 609 observed mare-seasons (MS, a year in which a mare experienced a reproductive event). Binary distribution and log link function were assumed. The marginal model included the classification variable (SCH) and the continuous variables (age of the mare and calendar year of reproductive event (birth of a live foal, abortion, foals lost or barrenness) in a given MS was analyzed with generalized linear mixed model. The reproductive fitness of mares and their reproductive success (foal surviving $\geq$ 1 year), did not differ between MS with and without SCH. Older females were more likely to stay barren, with chances increasing by 21% with each successive year; and less likely to give birth to a foal (13% decrease of chance), and rear a foal to one year of age (12% decrease of chance). The age did not affect the probability of abortions.

Of the 26 MS when mares were pregnant when the stallion had changed, there were 25 healthy foals born. For the entire 31 years of monitoring, no aggression toward any foal was observed and all foals that were born in the harem of a new, succeeding stallion successfully reached adulthood.

Due to the lack of incidents of infanticide and the lack of evidence suggesting that the presence of a new harem stallion leads to the termination of pregnancies sired by another stallion, the Bruce effect was not confirmed as a biological strategy to reduce investment in pregnancy and potential infanticide in studied population of semi-feral horses.

KEYWORDS: Horse, Bruce effect, Infanticide, Reproductive performance, Pregnancy

1. INTRODUCTION

Equine reproductive biology and mechanisms underlying reproductive success are still far from being well understood. In conditions closed to natural, horses usually achieve high reproductive performance [1]. However, in human-controlled conditions, the mares may experience reduced pregnancy rate due to various factors [2, 3], among which are sub-optimal conditions for expression of normal behavior [4, 5]. Decreased libido in stallions [4], silent estrus, equivocal agonistic behavior during pregnancy or pseudo-receptive behavior in early pregnancy in mares [6, 7] may mislead breeders, especially when ultrasound examination is not a routine stud practice. Also, other behavioral mechanisms such as infanticide and feticide are proposed to be responsible for decreased pregnancy rates in mares [8-10].

Infanticide and feticide, two phenomena related to a male's aggression potentially enhancing his reproductive success, have been suggested for several mammalian species [11-25], including wild equids [16, 26]. Infanticide is defined as killing vulnerable infants by a male who has not sired them, especially after taking over a group of females [16, 20, 23]. The rationale for this

male behavior is that it brings direct benefits, such as the more rapid return of the female to fertility than would not otherwise occur or the prevention of misled parental care [20].

In feral horses, only single cases of stallions killing foals have been described [13, 16, 18]. Moreover, the observed cases of stallions' infanticide occurred mainly under unstable conditions such as high population density [16] or in populations in which stallions were released only occasionally to breed with mares during the reproductive season [13].

Another potential aggression-related mechanism – termination of early pregnancy in females exposed to an unfamiliar male or its chemical signals (‘Bruce effect') – is believed to be females' counterstrategy to male infanticide [19, 20]. The Bruce effect was first observed and described in mice [27-29]. Since the costs of pregnancy and offspring upbringing are high for females, terminating a pregnancy sired by a predecessor prevents energy waste in production of offspring that most likely will be lost due to possible infanticide of successor males. The Bruce effect has been studied and described thoroughly in rodents; however, observations have also been performed for other species [9, 14, 30-33]. In a feral horse population, Berger [34] observed forced copulations by stallions when taking over a band of mares that he concluded led to abortions of early pregnancies. However, such behavior has not been described by any other author. Recently, questionnaire-based surveys of horse owners were conducted for domestic mares [8-10]. The results suggest that mares inseminated away from home, either naturally or artificially, were at higher risk of abortion after returning home compared to mares pregnant from the home stallion, especially when they experienced contact with a male separated by a fence. In this case, as suggested by latter authors, the mare cannot confuse the home stallion about his paternity, which may occur when she is placed in the same enclosure as the home stallion, so she " ‘manipulates' the male's paternity assessment by promiscuous mating (which)

may explain a common increased incidence of foetal loss in domestic horses" [8]. In contrast, Asa et al. [35], who observed a group of pregnant mares with a stallion that had not sired their pregnancies, never noticed such cases. Clearly, except for the cited studies, there is still much to learn about the drivers of variation in female reproduction in free-roaming horses.

The aim of the present study was, in conditions close to natural, to investigate the negative influence of the succession of a new harem stallion on the mares' subsequent reproductive performance. We discussed the concept of potential infanticide and feticide in horses [8-10, 13, 16, 18] regarding the hypothetical reproductive benefits. We hypothesize that if both infanticide and feticide occur in horses, the reproductive fitness of mares measured in terms of the numbers of abortions, foals born, and foals that survived ≥ one year will differ before and after stallion takeovers of the mares or after mare's migration. With direct observation, we can document stallions' aggression toward foals and forced or allowed copulations with pregnant mares. To investigate our hypotheses, we reviewed the database from 31 years detailing the migration and reproduction of a herd of Konik polski horses managed under semi-feral conditions. Such a long timeframe of observations provides detailed information about reproductive events. Considering the longevity of horses up to 30 years or greater, these records spanned the lifetimes of many of individuals.

2. MATERIAL AND METHODS

2.1 Ethical note

The study involved the analysis of breeding records and regular observations of animals from a distance. No experimentation was performed given European directive 2010/63/EU and the

105 Polish laws related to ethics in animal experimentation. The animals were under the care of the
106 staff of the Experimental Station of Polish Academy of Science in Popielno, Poland.

107 ***2.2 Study site***

108 Between 1986 and 2017 semi-feral Konik polski horses were observed daily in their
109 sanctuary situated on a peninsula in northeastern Poland by one of the coauthors (ZJ). Konik
110 polski horses were introduced to the sanctuary in 1956 as probable descendants of extinct
111 Tarpans (*Equus caballus gmellini Ant. forma silvatica Vet*.) to experimentally prove their
112 adaptability to and survival in natural East European environmental conditions [1, 36, 37]. The
113 available area is 16.2 km^2 of forests, some grassland, and wetlands. The sanctuary is surrounded
114 on the east and west by lakes, which are natural borders. On the north and the south, the
115 sanctuary is fenced. With annual surplus offspring removal, the habitat meets the nutritional and
116 spatial needs of all horses throughout the year, limiting the influences of ecological pressures
117 such as feed shortage or a high stocking rate on social structure of herds.

118 ***2.3 Animals***

119 For the present study we defined a "harem" as a social group of an individual stallion and
120 his mares (male-females group) while as a “band” we define a stable group of mares that can be
121 harems of several stallions in case they change. Horses run freely and are unmanaged except for
122 the annual removal of young offspring due to restricted area available to horses. Some colts and
123 fillies are left for parents' replacement. When remaining in the herd, young horses naturally
124 disperse from the natal group, usually at the age of 25 months ± 11 months. Male offspring
125 generally remain in their natal harem band longer than young females. Sporadically, new horses
126 from other sanctuaries are introduced. For approximately ten generations, this system has

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ensured the persistence of the population with minimal inbreeding. Individual horses are recognized based on markings and freeze brands. The horses live in distinct, freely formed bands. The succession of harem males occurs as a result of fights between stallions, mostly between a young stallion and its sire. In the majority of cases, the defeated stallion dies in the following months. Young mares are accepted by another stallion, and their lifetimes often reach 25 years or more (up to 34 years old for the most long-lived mare).

Over the 31 years of this study, the number of separate harem bands in the sanctuary at any one time ranged from three to five. Each band consisted of 1-10 mares and one stallion. No multi-stallion or bachelor groups were formed during the observation period. Altogether 8 bands, 11 harems, 11 stallions and 50 mares were observed and recorded. All horses are under a genetic resources protection program according to the Convention on Biological Diversity (https://www.cbd.int/) and are entered in the Konik polski studbook.

All dates of birth and death of the horses in the sanctuary were recorded. Additionally, the dates of the introduction of new horses were noted. The parents were recorded for each newborn foal; additionally, the parentage was genetically confirmed in a laboratory certified by ISAG (International Society of Animal Genetics). There was no discrepancy in parentage assignment between the manager and genetic assessment. The records also included, for each group, all cases of harem stallion change resulting from a harem stallion death or fight, the assignment of mares to a given stallion and the migration of mares from the natal band (forced) and between bands as adults (voluntary). During monitoring, observed estrous signs, matings, changes in harem and band composition, and all cases of birth, and death were noted.

2.4 Breeding records

149 Stallion change (SCH) is defined as a situation in which the stallion was succeeded by
150 another one within the same group of mares or a mare had migrated (changed the harem
151 voluntarily or as result of natal dispersal).

152 For each mare, the mare-seasons (MS), i.e. reproductive units (calendar years) in which a
153 mare experienced a reproductive event (foaling, abortion, foal loss), was reviewed for the whole
154 life or until 2017 (for dead and alive individuals, respectively). The death cause of individual
155 foals was inspected for cases of infanticide (general necropsy for evidence of external and
156 internal signs of physical abuse). Since the stallions changed usually for a group of mares, one
157 SCH is reflected in a given MS of several mares.

158 To assess the effect of stallion change, mares' reproductive fitness per MS included the
159 following outcomes (yes/no):

160 · foal born live;

161 · barrenness (defined as no pregnancy in a given reproductive season)

162 · abortion (defined as pregnancy loss after the 40th day of pregnancy, only in cases in which we
163 could see or find the fetus);

164 · reproductive success (foal raised to 1 year of age);

165 To assign reproductive indices to the above classification, we used the method based on
166 conception-to-foaling duration (in days). To calculate the probable conception date, we assumed
167 that first postpartum estrus (foal heat) occurred within 8-14 days and that pregnancy lasted 325
168 days [38]. First mating season of the maiden fillies was treated as MS0 and was not taken for

169 calculations as its result could be observed the next year (season). Also, two mares with evident
170 clinical signs of reproductive dysfunctions (throughout the observation period they got pregnant
171 and gave birth only to two per 12 MS and six per 27 MS foals respectively) were excluded from
172 the studied population since we focused on behavioral infertility only.

173 ***2.5 Statistical analyses***

174 The effect of SCH on the probability of the occurrence of the reproductive event of mares
175 (foals born alive, reproductive success, abortion, and barrenness per MS) was evaluated with the
176 generalized linear mixed model (PROC GLIMMIX, SAS 9.4 Statistical Package). As the
177 dependent variables have a binary outcome, a binary distribution and log link function were
178 assumed. The marginal model included the classification variable (SCH) and the continuous
179 variables (age of the mare and calendar year of reproductive event). Logit scale parameter
180 estimates (coefficient, Co) and odds ratio (OR) were presented for foals born alive, reproductive
181 success, abortion, and barrenness. Effect of age was reported as the mean probability (LSM ±
182 SE) of foals born alive, reproductive success, abortion, and barrenness. Effect of the increasing
183 age of the mare (by one year) to deliver live foals, reproductive success, abortion and barrenness
184 was given in percent. The mare nested in band was considered a residual random effect with a
185 compound symmetry covariance structure. A pseudo-likelihood estimation method and the
186 empirical Sandwich estimates for the standard error of the fixed effects were used. Average
187 number of foals born per mare was presented as mean ±SD.

188 Data used for the calculations are presented in the Supplementary Material 1.

189 **3. RESULTS**

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3.1 Stallion changes

A total of 609 mare-seasons (MS) for 48 mares were recorded. In total, SCH in 10 harems were recorded. On average, a mare changed harem stallion 0.8 ± 1.0 times (range: 0 – 3 times), and regarding whether the mare was dead or alive at the moment of database formation (2017) 0.7 ± 1.0 and 1.0 ± 1.0 times, respectively. Twenty-three mares experienced no SCH, 25 experienced at least one SCH: 15 mares one SCH, 5 mares two SCH and 5 mares three SCH.

3.2 Reproductive performance of mares

Altogether, 443 foals were born during the 31 years. No effect of calendar year upon reproductive efficiency: foal born alive (Co=0.01, F=0.33, P>0.05), barrenness (Co=-0.02, F=1.31, P>0.05), abortions (Co=-0.01, F=0.12, P>0.05) and reproductive success (Co=0.02, F=0.40, P>0.05) was stated. The majority (419) of foals survived either until their removal from the sanctuary (when the foals were approximately 7-9 months old) or at least until one year old in cases they remained in the sanctuary. Twenty-four foals died before 1 year of age due to congenital defects and/or disease (14 foals), natal isoerythrolysis (five foals), in accidents (two foals), and three were stillborn. The average number of foals born and raised per mare amounted to 9.0 ± 6.7 and 8.6 ± 6.3, respectively. Notably, within the observed timeframe and according to the age at database formation, some mares did not raise any or raised just one foal (eight very young or very old mares) and some raised 18 foals (five mares), with the most outstanding mare raising 24 foals.

3.3 Stallion change and the reproductive performance of mares

During the breeding seasons in which the stallion changed, there were 26 MS when mares were pregnant and nine MS when mares were barren; in the cases of the other seven MS, we were not able to diagnose the pregnancy in investigated mares with 100% certainty. Out of these 26 pregnancies, there were 24 healthy foals born, one mare gave birth to a healthy foal that was killed in a car accident in its 1st week of life, and one mare aborted. This particular mare was 22 years old and had previous reproductive problems: she aborted the year before, and the aforementioned abortion (in approximately the 7th month of pregnancy) occurred three months after the band had been taken over by a new stallion.

The reproductive fitness of a mare in the season following SCH, as compared to socially stable seasons, did not differ for the probability of giving birth to live foal (SCH seasons: 0.86 ± 0.07 vs. stable seasons: 0.91 ± 0.02, F=0.81, P>0.05; OR=1.63), to abort (0.04 ± 0.03 vs. 0.03 ± 0.01, F=0.45, P>0.05; OR=0.58), to stay barren (0.10 ± 0.05 vs. 0.06 ± 0.02, F=2.15, P>0.05; OR=0.52) or to have a reproductive success in a given season (0.79 ± 0.07 vs. 0.85 ± 0.02, F=0.79, P>0.05; OR=1.45).

Among 443 healthy foals, 28 were born in the harem of a new, succeeding stallion. All of these foals successfully reached adulthood. No forced copulations or copulations allowed by pregnant mares were ever witnessed. Additionally, no cases of stallion aggression toward pregnant mares or foals sired by other stallions were observed.

3.4 Age effect

Older females were more likely to stay barren (Co=0.19, F=20.5, P<0.0001), with chances increasing by 21% with each successive year; and less likely to give birth to a foal (Co=-0.13, F=12.0, P<0.0001, 13% decrease of chance), and rear a foal to one year of age (Co=-0.13,

232 F=24.7, P<0.0001, 12% decrease of chance). The age did not affect the probability of abortions
233 (Co=0.04, F=1.31, P>0.05).

234 Detailed reports for the seven mares for which we were not able to classify the reproductive
235 status at the moment of SCH is presented in Supplementary Table 1. Of these mares, five gave
236 birth to a foal sired by a new stallion in the year following the stallion change.

237 **4. DISCUSSION**

238 Our analyses showed that the seasonal reproductive fitness of observed mares was similar
239 whether a mare experienced harem stallion change or not. An important factor challenging
240 mare's reproduction was an advanced age. Since age is widely recognized as adversely affecting
241 reproduction and has been extensively studied [39 – 44], in the present work we focus on
242 mechanisms of purported male-provoked subfertility in mares. The analysis of reproductive
243 records of Koniks population does not support the hypothesis of the Bruce effect in semi-feral
244 horses with two findings: infanticide as underlying cause of early pregnancy failure was never
245 observed, and the negative effect of a stallion change on reproductive performance of mares was
246 not detected.

247 ***Male infanticide***

248 It was proposed that the potential gains from infanticide are: using the neonate as a
249 resource (i.e. as food), elimination of the potential competitor for resources, enhanced
250 reproductive success of a partner and the "increased access for individuals of one sex for
251 reproductive investment by the other sex at the expense of same-sex competitors"[12]. However,

252 any of the suggested reasons, including the last one assumed as rationale for decreased
253 reproductive performance in domestic mare [8 – 10], was not observed in studied population.

254 Infanticide is believed to bring direct benefits to the male due to the faster return of the
255 female to fertility [20, 23]. In the case of horses, mares start cycling within 8 – 14 days
256 *postpartum* and remain fertile the entire breeding season [45]. Thus, killing the newborn foal
257 would not change the mare's fertility and might be pointless in terms of male's reproductive
258 benefit. In contrast to lions and other animal species, for which infanticide may serve some
259 biological functions and has been observed in nature [46 – 50], no rationale for such a
260 mechanism is evident neither in domestic horses under natural conditions nor in Przewalski
261 horses after infanticide [16].

262 Observations of Feh and Munkhtuya [16] showed that Przewalski mares whose foals
263 were sporadically killed did not allow breeding with the infanticidal stallions. Therefore, these
264 stallions did not gain any reproductive benefits from the infanticide, as has been postulated for
265 other species. Since in wild equids males' incidental infanticide is observed towards unrelated
266 but also related foals [16] a pathological social behavior rather than a reproductive strategy in
267 horses was proposed as explanation [16]. Moreover, it can be hypothesized that wild equids,
268 especially zebra, have a generally higher level of aggression, especially toward neonates [51] of
269 own and other species like antelopes [52]. Importantly, no infanticide was confirmed by our
270 study, as none of 443 foals was killed by a stallion over 30 years of observations; although the
271 harassment of adults by other stallions present in the sanctuary and the succession of stallions
272 occurred spontaneously, and the animals were free to choose sexual partners from available
273 groups within the sanctuary. No attacks of a new stallion on newborn foals sired by the previous
274 stallion or on any other foals were ever witnessed or confirmed with the necropsy of dead foals.

In the observed population, twenty-eight foals were born after takeovers, and all of them successfully reached adulthood. Additionally, we observed that when these foals were left in the sanctuary, the stallion expelled unrelated immature horses as in case of own offspring as reported by other studies [53]. Twenty-five years of daily observations of the University of Pennsylvania's semi-feral pony herd have shown that of 375 foals born into that herd, 66 neonates have been with harem stallions that were not their sire (McDonnell, personal communication). No infanticide or aggressive behavior of stallions to foals has been observed and detailed focal observations have similarly indicated no difference in parenting behavior of harem stallions toward sired and non-sired foals in their band (McDonnell, personal communication). In the studied population, the finding that no foals were lost due to male aggression compared to 24 foals lost for other reasons before 1 year of age, questions the hypothesis that mares have to employ any strategy to avoid foal loss due to possible infanticide [8-10]. Hitherto results on possible Bruce effect in domestic mares were based on questionnaires filled by owners of mares kept in stabling conditions [8-10, 13, 16, 18] with no direct observation of the animals' behavior. Comparing to our results including regular inspection and behavior observations, we think that the question of whether females' reproductive counterstrategy to infanticide, i.e., the Bruce effect, has any biological rationale in domestic mares remains open and requires further research.

Bruce effect

Previous studies [8-10] imply that mares, similar to mice, may terminate pregnancy in the presence of a male that did not sire the fetus, especially when a female has not the direct access to the dominant male. The pregnancy rate was compared between mares mated or inseminated with home or unfamiliar stallions. The results suggest that when a mare was bred with an unfamiliar stallion and then kept in an enclosure adjacent to a home stallion or gelding, the

probability of pregnancy termination was the highest. However, the distribution of factors between compared groups that undoubtedly influence the reproductive performance, such as age, detailed reproductive and veterinary history of mares, use of pregnancy diagnostic technique (palpation or ultrasound examination since the questionnaire reaches back to the 1980s, when the use of ultrasound was uncommon), possible differences in feeding, stage of pregnancy after returning home [8-10], in which the placentation is one of the critical points [54], or the method of insemination (natural or artificial, the latter often including hormonal treatment) [8, 10], were not given. In general, the pregnancy rate in domestically managed mares is approximately 60 – 69% per cycle [41, 55]. Factors influencing conception and embryo development, including the signal of maternal recognition of pregnancy, are still not elucidated in horses [41, 53, 56]. Thus, without a scrutinized follow-up of the mare's reproductive history, behavior observations, and physiological and health status examinations, it may be difficult to interpret the results. A detailed analysis of the collected data yielded a large amount of relevant information in our study (Supplementary Table 1). The problematic cases (barrenness) following stallion change involved mare's congenital subfertility, advanced age, refusal to be mated by a son, poor post-winter condition and the refusal to be mated by a new stallion. Such information sheds additional light on the interpretation of potential causes of barrenness.

Misleading parental care

It has been proposed that mares manipulate their pregnancy status to avoid potential infanticide when not able to mislead a dominant male about his paternity [8]. In our study, three mares with evident reproductive problems, including serological incompatibility with the stallion, did not leave the harem to join a new stallion with whom they could improve the reproductive outcome. Based on this observation we speculate that mares may not be able to

predict the outcomes of their pregnancies to counteract possible loss, and manipulate the pregnancy outcome.

We acknowledge that the only human intervention, the annual removal of almost all born offspring, may have disturbed the natural composition of groups present in the sanctuary. Thus, the distribution of males and females of different ages is not completely natural and may affect the outcomes of the present study. However, to our knowledge, there is actually no completely natural (feralised) domestic horse population that would not be managed somehow (by area restriction, surplus young removal or contraception) otherwise it leads either to overpopulation or unnatural reduction of fertility. The example of the catastrophic outcome of uncontrolled reproduction of horses in a reserve where unlimited reproduction on limited area resulted in deaths of over half of the animals, mostly from starvation [57] shows that with no predators' harassment, present populations of feral horses have to be managed somehow in order to maintain the horses' welfare. It is proposed that feed availability is a probable reason for the formation of separate harems [58]. Also, in polygynous ungulates, the sex ratio is expected to be biased toward females [53] due to higher dispersal mortality of expelled males travelling to novel places in unrestricted areas. Nevertheless, the sanctuary area is limited, and the welfare of the horses is a priority, as a high stocking rate of horses would influence their behavior and condition. In such a case, the collected data might not reflect the natural behavior of horses but rather pathological one caused by shortages of food and space [4, 16, 59 - 61].

We recognize that another issue which could potentially bias the results is the frequency of stallion changes. Available literatures as well as our own observations indicate that a harem stallion manages a group of mares as long as he is able to defend them from other stallions [62, 63]. This period may significantly differ from stallion to stallion because the factors such as feed

and water availability, presence of other stallions, stallion's age, its qualities or random accidents may influence the leadership [52, 62, 63]. To our knowledge there is no reference research on average/routine feral stallion change and we believe that observational period of more than 30 years evidences for the first time how frequent changes of harem stallions are. Every SCH in our study occurred by defeating the predecessor harem stallion by the successor, that is as it has been reported for other feral populations. Hence, we think that the number of SCH in our study may reflect natural situation in feral equine population.

CONCLUSIONS

Due to the lack of incidents of infanticide and the lack of evidence that the presence of a new stallion leads to the termination of pregnancies sires by another stallion, our results suggest that the Bruce effect is unlikely to be a biological strategy to reduce investment in pregnancy and prevent potential infanticide in horses. No benefits underlying such mechanism were evidenced in horses living in semi-feral groups. Thus, our findings do not support the concepts of both infanticide and feticide (the Bruce effect) in *Equus caballus*, as they did not occur and seemed to have no biological justification, as shown by our long-term studies involving semi-feral horses. However, the limited number of closely monitored animals in feral groups, as well as subject-related research and contradictory results encourage further investigation of these phenomena in *Equus caballus* species.

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Conflict of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Authors' contributions

JJ analyzed the data and wrote the manuscript. ZJ collected and analyzed the data. SMM contributed to the data analysis and manuscript preparation. AGB supervised the work and contributed to the data analysis and manuscript writing and preparation. All authors critically revised and approved the final version of the manuscript.

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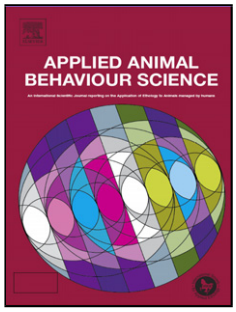
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HIGHLIGHTS

- Change of the harem stallion does not decrease reproductive performance in semi-feral mares
- Infanticide was not observed in semi-feral horses
- Bruce effect was not confirmed as a mares' reproductive strategy

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Accepted Manuscript



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ACCEPTED MANUSCRIPT

Decreased female fidelity alters male behavior in a feral horse population managed with immunocontraception

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Highlights:

- Stallions experiencing increased female turnover engage in more frequent contests
- These stallions engage in more escalated contests and spend more time vigilant
- Habitat visibility but not female turnover influenced male-female aggression
- Home range overlap also influenced male-male interactions
- Immunocontraception management indirectly affects stallion behavior

Abstract

In social species like the feral horse (*Equus caballus*), changes in individual

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behavior are likely to affect associated animals. On Shackleford Banks, North Carolina, USA, mares treated with the contraceptive agent porcine zona pellucida (PZP) demonstrate decreased fidelity to their band stallions. Here, we assess the effects of such decreased mare fidelity on male behavior and address potential interactions with habitat visibility, a component of the environment shown to significantly affect feral horse behavior. We compared the frequency and escalation of male-male contests, rates of aggressive and reproductive behaviors directed toward females, and the percentage of time spent vigilant among males experiencing varying levels of mare group changing behavior. We found that regardless of habitat visibility, males experiencing more female group changes engaged in contests at a higher rate ($P = 0.003$) and escalation ($P = 0.029$) and spent more time vigilant ($P = 0.014$) than males experiencing fewer group changes. However, while visibility had a positive effect on aggression directed by stallions toward mares ($P = 0.013$), female group changing behavior did not influence male-female aggressive or reproductive behaviors ($P > 0.1$), showing that decreases in mare fidelity altered male-male but not male-female interactions. These results have important implications for feral horse management; PZP-contracepted mares demonstrating prolonged decreases in stallion fidelity may have a disproportionate effect on male behavior. Moreover, our results shed light on the relative influences of female behavior and environmental factors like habitat visibility on male behavior. Such findings can ultimately improve our understanding of how the social and physical environments interact to shape male-male and male-female interactions.

Key words: aggression; *Equus caballus*; immunocontraception; social stability; stallion;

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vigilance

1. Introduction

Ungulate populations in the United States have expanded greatly in recent history (Eberhardt et al., 1982), demanding some form of management that balances efficient control of animal numbers and public opinion of the adopted strategy (National Research Council, 2013). While easily overlooked, maintenance of natural behaviors is an important consideration if management strategies are to sustain the health and wellbeing of wildlife populations. The feral horse (*Equus caballus*) provides an interesting case study for the impacts of management strategies on behavior and physiology at multiple levels. With few predators on the continent, feral horse populations have grown rapidly in the United States (Garrott et al., 1991; Turner et al., 1992; Turner and Morrison, 2001; DOI-OIG 2010). Subsequent overgrazing can be detrimental to native vegetation and wildlife communities and also conflicts with land use practices (Beever and Brussard, 2000; Levin et al., 2002). Viewed as both a culturally valuable species protected by federal law and a nuisance to humans and wildlife, feral horses present a unique challenge to wildlife managers (United States Congress, 1971). Recently, fertility control has become a popular option for managing feral horse populations. The immunocontraceptive agent porcine zona pellucida (PZP) has been used to manage several populations of feral horses and other social species including white-tailed deer (*Odocoileus virginianus*) and elk (*Cervus elaphus*) (Kirkpatrick et al., 1997; McShea et al., 1997; Heilmann et al., 1998; Ransom et al., 2010).

In feral horses, both physiological and behavioral effects of PZP on treated mares

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have been widely documented, with varying results among populations (Kirkpatrick et al., 1996; Powell, 1999; Turner and Kirkpatrick, 2002; Nuñez et al., 2009; Nuñez et al., 2010; Ransom et al., 2010; Madosky, 2011; Nuñez et al., 2014). However, the potential effects of immunocontraception management on non-target individuals have received less attention (Ransom et al., 2010). Such effects are especially important to consider in social species like the feral horse, as the behavior of one individual is likely to influence the behavior of its associates. Feral horses live in social groups (called bands) consisting of one or sometimes multiple stallions, one or more associated females, and their offspring (Feist and McCullough, 1976; Rubenstein, 1981). Bands are typically stable groups with females staying with the same male (band stallion) for most of their adult lives (Klingel, 1975).

However, this stability may be compromised when the behavior of individual group members changes. The feral horse population on Shackleford Banks, North Carolina, USA provides such an example. Between 2000 and 2009, the population was managed by treating a subset of females with PZP. This resulted in several changes in the behavior and physiology of treated mares (Nuñez et al., 2010; Nuñez et al., 2014) including increased group changing behavior (Nuñez et al., 2009). While PZP treatment was largely suspended in 2010, increased group changing behavior is still exhibited by previously treated mares experiencing prolonged subfertility (Nuñez et al., 2017). The resulting decrease in group stability may have implications for other group members, particularly the band stallions (Madosky, 2011).

During periods of social instability, males exhibit increases in aggression and

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testosterone across diverse taxa including primates, birds, and fish (Wikelski et al., 1999; Cavigelli and Pereira, 2000; Hirschenhauser and Oliveira, 2006; Arlet et al., 2009). On Shackleford, the social instability primarily created by decreases in previously treated mares' fidelity to band stallions is likely to similarly increase the frequency and escalation of stallion contests. Furthermore, changes in female behavior and movement patterns are likely to influence how males interact with these females. For example, male harassment of females is common in ungulate mating systems, with males more likely to initiate mating than females (Bro-Jørgensen, 2011). However, harassment intensity can vary greatly with the strength of social bonds between males and females (Linklater et al., 1999). Females demonstrating weaker bonds with the dominant male and/or female associates often experience increased male harassment (Linklater et al., 1999). On Shackleford Banks (and in the western United States), treated mares demonstrate prolonged reproductive cycling (Nuñez et al., 2010; Ransom et al., 2010) and weakened social bonds (Nuñez et al., 2009; Madosky, 2011). Not surprisingly, treated mares are often subject to increased stallion harassment when compared to untreated mares (Madosky, 2011), demonstrating the profound impacts female behavior can have on male behavior.

Still, to fully understand how males respond to changes in female behavior, male behaviors should be considered in the context of the animals' surrounding environment. The interactions between animals and various facets of their physical environment such as visibility and resource distribution profoundly influence mating systems by dictating how males exploit female movement and dispersal patterns (Emlen and Oring, 1977;

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Rubenstein, 1981). Historically, Shackleford has been defined by three distinct habitats: the East, which is flat and open with limited water sources (Marr, 1996); the Mideast, which is also flat and open with more evenly distributed water sources (Rubenstein, 1981); and the West, which contains two primary water sources and is dominated by high dunes and dense brush (Rubenstein, 1981). These regions vary in their resource availability and landscape features, both of which influence stallions' ability to defend territories (Rubenstein, 1981). For example, water and vegetation distribution determine the location and extent of horses' home ranges (Feist and McCullough, 1976; Miller, 1983; Manning and McLoughlin, 2017). Furthermore, the degree of visibility across a landscape can affect a stallion's ability to defend his band against intruders (Rubenstein, 1981). The history of territoriality in the eastern region of Shackleford demonstrates how high visibility and evenly distributed resources allow for the maintenance and defense of distinct boundaries (Rubenstein, 1981; Rubenstein, 1986). Therefore, shifts in resource availability or distribution, visibility, and the resulting degree of overlap between band home ranges are likely to impact the frequency of intergroup interactions and the degree of competition among stallions. Investigating the influences of both female behavior and ecological factors on male behavior can broaden our understanding of how these factors interact to shape male mating strategies and even the evolution of mating systems.

Here, we assessed the effects of decreased mare fidelity on male behavior in the feral horse population on Shackleford Banks, NC. We tested how the rate of female turnover experienced by males affected 1) the frequency and escalation of male-male contests and vigilance behavior and 2) the frequency of male aggression and reproductive

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behaviors directed toward mares. In addition, we tested the effects of ecological variables including visibility and home range extent on these behaviors. We hypothesized that males experiencing more group changes by females, defined as the number of times females enter or leave a stallion's band, would engage in contests with other males more frequently and at a higher intensity than those experiencing fewer group changes. Furthermore, we predicted that stallions experiencing increased female turnover would exhibit increased rates of aggression and reproductive behavior toward females. Finally, we hypothesized that increased habitat visibility and overlap among home ranges would heighten aggression between males and increase male aggression and reproductive interest directed toward females. Because females previously treated with PZP exhibit prolonged decreases in stallion fidelity, this study can improve our understanding of the potential for indirect effects of contraception management on non-target animals. In addition, our evaluation of the relative effects of female behavior and ecological factors on male behavior may provide insights into their influence on the expression (and evolution) of male mate-guarding and reproductive behaviors, critical components of male mating strategies.

2. Methods

2.1. Study Site and Subjects

The focal population of feral horses lives on Shackleford Banks, a barrier island off the coast of North Carolina, USA. The island is approximately 15 km long and ranges from 0.2 to 1.2 km wide. As a part of Cape Lookout National Seashore, Shackleford Banks is visited by approximately 100,000 tourists annually (National Park Service,

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2017). Therefore, the horses on the island are somewhat habituated to human activity (i.e., at distances of at least 15 m). The study was conducted during two breeding seasons, June through July, 2016 and May through August, 2017. In both years, we observed all band stallions and their females: 21 males and 70 females organized into 18 bands in 2016 and 20 males and 69 females organized into 19 bands in 2017. Most bands had one dominant stallion with the exception of two double-male bands in 2016 and one double-male band in 2017.

2.2. PZP Contraception

This feral horse population is federally protected under the Shackleford Banks Wild Horses Protection Act of 1997 and is managed jointly by the National Park Service and the Foundation for Shackleford Horses (United States Congress, 1997). To maintain the population at the predetermined size of approximately 120 animals, the National Park Service began utilizing immunocontraception in 2000. Mares received initial and booster doses of PZP, which were administered from February through April in designated years. Injections contained 100 µg of PZP plus an adjuvant, and initial doses contained Freund's Complete Adjuvant, Modified, *Mycobacterium butyricum* (Calbiochem #344 289). Booster doses contained Freund's Incomplete Adjuvant (Sigma #F5506). In 2010 and 2011, when the NPS and Foundation realized that some mares treated repeatedly experienced prolonged subfertility post-treatment, PZP treatment was suspended to allow the population to increase (Stuska, personal communication).

2.3. Behavioral Data

Behavioral data were collected primarily by one observer (M.M. Jones) and were

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supplemented with additional data from two other observers (M. Fatka and C.M.V. Nuñez). Both M.M. Jones and M. Fatka were trained by C.M.V. Nuñez. Study bands were located twice per week, and GPS location and group composition were noted. Individuals were identified with freeze brands, distinctive markings, coloration, and other identifying features. As witnessing group changing behavior by females was rare (n = 1), presence and absence of females in the band were noted to monitor this behavior. These data were used to calculate the number of group changes experienced by males (the number of times females entered or left a male's band).

Instances of rare behaviors were recorded *ad libitum* (Altmann, 1974). These included 1) male-male aggression (including biting, kicking, pushing, and kick threats), 2) male-female aggression (including both mate-guarding behaviors such as herding and chasing and the aggressive behaviors listed above), and 3) reproductive interest (including copulation, mounting, genital sniffing, and rump rubbing (Nuñez et al., 2009)). In addition, the amount of time that stallions spent vigilant was recorded during 15-minute focal samples (Altmann, 1974). The escalation of male contests was scored based on a scale of one to six adapted from Hynes (1998) with scores as follows:

1. Visual displays including parallel prance, pawing the ground, neck arch, and approach-retreat without physical contact.
2. Olfactory assessment including nose-to-nose and nose-to-genital contact and fecal pile sniff without physical contact.
3. Auditory assessment using aggressive male vocalizations called squeals.
4. Low intensity physical contact including pushing and kick threats.

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5. Moderate intensity physical contact including hind and front kicks that may result in injury.
6. High intensity physical contact including biting and rearing while kicking with front legs that are likely to result in injury.

Frequencies of observed behaviors were significantly correlated with the total time individuals were observed (male-male contests: $\rho = 0.330$; $P = 0.035$; male-female aggression: $\rho = 0.486$; $P = 0.001$; male-female reproductive behavior: $\rho = 0.663$; $P < 0.001$). To control for this bias, rates of male-male aggression, male-female aggression, male-female reproductive interest, and female group changes were calculated as the number of instances an individual was observed engaging in those behaviors divided by the total time each horse was surveyed during the season (expressed as observation days, defined as 12 hour periods (the approximate length of daylight during our study period)). Additionally, the escalation scores of all male-male contests that a single stallion engaged in were averaged to calculate a mean escalation score for that stallion. Finally, percentage of time spent vigilant, which was characterized by ears erect and directed forward, head and neck held high, and eyes alert (Figure 1) (Feist and McCullough, 1976), was measured as the minutes spent vigilant over all focal samples conducted for each male divided by the total number of focal minutes for each male.

During observations, detailed descriptions of the behaviors exhibited, identity of the participating individuals, and the date and time were recorded. All observations were conducted by eye or with the aid of binoculars (Nikon Monarch M511 10x42) when necessary at least 15-20 meters away from the animals (National Park Service, 2004) to

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ensure that they were not disturbed by our presence.

2.4. Assessing Visibility Across Shackleford Banks

We assessed visibility across the island as a potentially important environmental factor that can influence male behavior and inter-band conflict (Rubenstein, 1981). Visibility was quantified through viewshed analysis in ArcMap 10.4.1 (Alonso et al., 2012; Aben et al., 2017). Digital surface models were created using USGS LiDAR point cloud data to account for both bare earth elevation and vegetation structure (U.S. Geological Survey, 2015). GPS locations collected at every band sighting using Garmin eTrex 10 units were used as observer points, and an observer offset of 1.2 m was used as the average height of the horses (Stuska, personal communication). The Visibility tool in ArcMap was then used to predict the cells visible from each point, and the number of visible cells was averaged across all points for each stallion to produce an average visibility score.

2.5. Estimating Home Range Size and Overlap

To quantify band home range size and degree of overlap, as these factors can also influence stallion behavior (Rubenstein, 1981), we used the R package *adehabitatHR* (Calenge, 2006). Home ranges for each band were estimated with GPS locations collected at all band sightings in 2016 and 2017 using the 95% adaptive kernel method as described by Worton (1989). Home range area and each band's degree of overlap with other home ranges were then assessed in ArcMap. Home range overlap was calculated as the percentage of one stallion's home range area that was overlapped by other home ranges. Home ranges for each year were estimated separately, and bands with fewer than

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ten GPS positions were excluded from the analysis (Börger et al., 2006).

2.6. Statistical Analysis

To assess whether the increased group changing behavior by females previously treated with PZP reported by Nuñez et al. (2017) persisted during our study period, we used a permutation test with a significance level of 0.05 in the *geomorph* package in R (version 3.4.1) (Adams et al., 2017; R Development Core Team, 2017). The rate of group changing behavior by females was the response variable, and mare age and a binary variable representing whether or not a mare had been previously treated with PZP were the independent variables. For this permutation test, F-statistics with total and residual degrees of freedom and *P*-values are presented.

We used generalized linear mixed-effects models to examine how changes in female behavior influenced male behavior. Separate models were run for each of the following dependent variables: male-male contest rate, average male-male contest escalation, male-female aggression rate, male-female reproductive interest rate, and percentage of time males were vigilant. Each model included the following independent variables as fixed effects: the number of group changes a male experienced divided by the total time he was surveyed (female turnover rate), average visibility score, male age, average female age (within the band), and the average number of adult females present. The latter three variables were included to control for their potential impacts on male behavior (Berger, 1977; Vervaecke et al., 2007; Nuñez et al., 2017). Finally, all models included stallion identity as a random effect to control for pseudo-replication.

For all models, dependent variables were log or square root transformed as

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necessary to account for non-normality of model residuals. Best fit models were then selected via backward elimination with a cutoff level of $P = 0.1$ to eliminate variables from the models. Additionally, Spearman correlation tests were used to assess correlations among independent factors and to avoid collinearity within models. Final models did not include any highly correlated variables. The significance level of 0.05 was used to evaluate the effects of predictor variables on male behavior.

All band stallions that were observed for more than two hours were included in the analysis (n = 23 (18 stallions were observed in both 2016 and 2017, 3 additional stallions were observed only in 2016, and 2 were observed only in 2017)).

While male rank is also often a predictor of male aggression (Vervaecke et al., 2007), it was not included in these models because dominance data were not available for approximately one third of the stallions. However, age is typically associated with rank, with older males having higher ranks, and thus the inclusion of male age in the models likely controlled for at least some of the variation due to dominance status (Vervaecke et al., 2007). Further, for those stallions whose dominance could be determined, rank was not significantly correlated with any dependent variables in Spearman correlation tests, and thus it is unlikely that including rank in the models would have altered our conclusions (Table 1).

Finally, because home ranges could only be estimated for 17 stallions (there was sufficient data to estimate home ranges for the same 8 stallions in both 2016 and 2017, 1 additional stallion in 2016 only, and 8 additional stallions in 2017 only), including home range size and overlap in our models reduced our sample size. Therefore, we ran two

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subsets of models: those that included home range size and overlap and those that did not. For these generalized linear mixed-effects models, parameter estimates, standard errors (SE), t-values, and *P*-values are presented in Tables 2 and 3.

3. Results

3.1. PZP Treatment and Female Group Changing Behavior

When accounting for mare age ($F_{1,136} = 0.62$, $P = 0.432$), females that were previously treated with PZP made on average 2.4 group changes per day more than those that had never been treated ($F_{1,136} = 4.72$, $P = 0.026$).

3.2. Male-male Interactions

The rate of female group changes experienced (Table 2, Figure 2a) and male age (Table 2) both had positive effects on the number of male-male contests that stallions engaged in per day. Similarly, males experiencing more group changes by females per day (Table 2, Figure 2b) and older males (Table 2) engaged in more highly escalated contests.

3.3. Male-female Interactions

Males living in higher visibility areas initiated more aggressive behaviors toward females per day (Table 2). However, the number of female group changes experienced per day did not affect male-female aggression and was dropped from the final model ($P > 0.1$).

Male age was the only variable retained in the final model investigating factors affecting the rate of reproductive behaviors per day but did not have a significant effect on this behavior (Table 2). The number of female group changes that males experienced

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was dropped from the final model, suggesting it did not explain variation in the rate of male reproductive behavior ($P > 0.1$).

3.4. Vigilance Behavior

The percentage of time males spent vigilant during focal samples increased with a greater number of female group changes experienced per day (Table 2, Figure 2c). Additionally, males with younger females in their bands also spent more time vigilant (Table 2).

3.5. Analyses Including Home Ranges

Both the percentage of home range overlap and the rate of female group changes had positive effects on the rate of male-male contests (home range overlap: estimate = 1.430, t = 2.93, $P = 0.008$; group changes: estimate = 0.048, t = 2.43, $P = 0.024$). Additionally, contests between males with a high degree of home range overlap were more highly escalated than those between males with a lower percentage of overlap (estimate = 3.505, t = 2.93, $P = 0.008$). Finally, stallions experiencing more female group changes per day tended to exhibit higher rates of reproductive behavior (estimate = 0.048, t = 1.90, $P = 0.071$). The behavioral and ecological variables of interest (female group changes, home range size, home range overlap, and visibility) did not affect the rate of aggressive behaviors directed toward females or the percentage of time males spent vigilant (Table 3).

4. Discussion

Effects of PZP treatment on female behavior, including increased group changing behavior and extended reproductive behavior and cycling, have been observed among

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feral horses on Shackleford Banks and in three western populations (Nuñez et al., 2009; Ransom et al., 2010). However, the potential consequences of such changes for their male associates have not yet been fully explored. Here, we demonstrate that increased group changing behavior by mares is correlated with an increase in aggression among stallions and heightened male vigilance. The implications of these results for management strategies deserve careful consideration and further investigation. Specifically, treating females with immunocontraceptives or other agents that decrease social fidelity can have broader behavioral impacts on males in the population. Moreover, our results provide new insights into the relative influences of female behavior and the surrounding environment, specifically habitat visibility and home range overlap, on male behavior and the expression of reproductive and mate-guarding behaviors involved in mating strategies.

4.1. Effects of Female Behavior on Male Behavior

Stallions that experience increased rates of group changes by females engage in contests with other males more often and at more escalated levels than those experiencing fewer group changes (Figure 2a, Figure 2b). The feral horse social system is typically characterized as harem defense polygyny, in which females form stable groups defended by males (Emlen and Oring, 1977; Rubenstein, 1986). Typically, mares stay with the same stallion for most of their adult lives, and attempts by intruding males to gain access to a resident male's females are usually unsuccessful (Feist and McCullough, 1976; Rubenstein, 1981). In the Shackleford population, decreased female fidelity has compromised this group stability (Madosky, 2011); in response, it appears that males

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have responded by increasing their defense of females against rivals.

In addition, males that experience increased mare turnover spend a greater percentage of time vigilant than those experiencing fewer group changes (Figure 2c). Similar increases in vigilance behavior have been demonstrated across diverse taxa; during periods of high intrasexual competition, males spend more time monitoring conspecifics (Guillemain et al., 2003; Li et al., 2012). Such monitoring behavior allows males to guard female associates from rival males as well as detect and respond to wandering females (Guillemain et al., 2003; Li et al., 2012; Girard-Buttoz et al., 2014). In the Shackleford population, increased vigilance may allow stallions to monitor both rival males and group changing mares in the context of decreased social stability and high intrasexual competition.

Such changes in male behavior could have consequences for the overall health and wellbeing of these individuals. Male contests, particularly ones that are highly escalated, are costly, demanding significant energy expenditure and potentially leading to injury or even death in extreme cases (Feist and McCullough, 1976; Berger, 1986; Grogan and McDonnell, 2005). Devoting more time to vigilance behavior can detract from time available for other behaviors like foraging, limiting males' energy intake (Lima, 1998; Watson et al., 2007). This may be particularly consequential if this increase in vigilance behavior is also exhibited during the winter when horses spend more time grazing on lower quality forage (Stevens, 1990; Watson et al., 2007). Future work should explore the consequences of these changes in male-male competition and vigilance behavior on stallion health.

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4.2. Male-female Interactions

Contrary to our expectations, the number of group changes experienced by males did not affect the rate of aggressive or reproductive behaviors initiated toward females. Although previous work with the same population indicates that PZP-treated females receive more harassment and reproductive interest from males (Nuñez et al., 2009; Madosky, 2011), it does not appear that males engage in varying rates of male-female interactions based on the rate of female turnover they experience. It may be that stallion harassment and reproductive interest directed toward treated females are influenced more by differences in estrous cycling among treated versus untreated mares (Nuñez et al., 2010; Ransom et al., 2013) rather than the differences in group changing behavior that we examined here. As they rarely become pregnant, treated mares exhibit increased reproductive cycling, which can persist even after immunocontraceptive treatment is suspended (Ransom et al., 2010; Nuñez et al., 2017).

However, it is important to note that this study was conducted solely during the breeding season, when reproductive behaviors and male-female interactions are at a peak (Stevens, 1990). In horses and other ungulate species, behavioral effects of PZP on mares are often more evident during the non-breeding season when reproductive behavior is more variable (McShea et al., 1997; Heilmann et al., 1998; Nuñez et al., 2009). Similarly, variation in stallions' interactions with females may be more evident during the non-breeding season when behavioral differences between treated and untreated mares are more pronounced (Nuñez et al., 2010). For example, on Assateague Island, Powell (1999) found no difference in the harassment received by untreated versus treated mares during

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the breeding season, though that study lacked true controls (mares that had never been treated with PZP) for comparison. On the other hand, Madosky (2011) found that treated mares on Shackleford did receive more harassment from stallions than untreated mares during the breeding season. Unfortunately, the definition of "harassment," specifically whether or not it included reproductive behaviors, is unclear, making it difficult to directly compare these studies.

Studies of other species examining male behavior in populations managed via fertility control reveal similar inconsistencies (Ji et al., 2000; Poiani et al., 2002; Druce et al., 2013; Duncan et al., 2017). Ji et al. (2000) found that male brushtail possums (*Trichosurus Vulpecula*) were attracted to sterilized females during and after the mating season; however, Druce et al. (2013) found no evidence that bull African elephants (*Loxodonta africana*) associated more with family groups containing more estrous females, suggesting that PZP treatment did not alter male-female associations. This level of variation among species supports the need for careful monitoring not only of target animals', but also non-target animals', behavior in response to contraception management.

4.3. Effects of Visibility

Habitat openness (or visibility) often plays an important role in male behavior because higher visibility allows males to defend home range or territory boundaries and track band member activity more easily (Rubenstein 1981). Our data show that males residing in higher visibility areas are more aggressive toward band females (herding, biting, and kicking their females more often) than males in lower visibility areas (Table

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2). However, contrary to our predictions, we saw no effect of visibility on the frequency or intensity of male-male interactions (Table 2).

In high visibility areas, males can more easily see one another and each other's females, perhaps increasing the perceived threat of rival males. Males in these areas may herd their females more frequently to prevent them from wandering towards rivals. A higher frequency of herding behavior in high visibility areas may therefore allow for a lower frequency of male contests over roaming mares. In comparison, this pressure may be decreased in low visibility areas where stallions and their mares cannot readily see neighboring bands. In these areas, landscape features such as high dunes and dense brush likely make encounters between rival males relatively uncommon, limiting the frequency of male-male contests. Therefore, similar contest levels might be maintained in areas of low and high visibility albeit via different mechanisms, though future work should further explore this issue. Our use of viewshed analysis to assess the effects of visibility on feral horse behavior adds to a new but growing body of work that uses this GIS tool to explore questions in behavioral ecology (Alonso et al., 2012; Aben et al., 2017). Our results show that habitat visibility is an important environmental factor to consider in future studies. Visual cues are integral to the behavior of diverse taxa, and the reception and interpretation of these cues depend heavily upon the structure of the environment and the resulting visibility, as outlined by Aben et al. (2017).

4.4. Effects of Home Range Overlap

While both home range overlap and female group changes had positive effects on the frequency of male-male contests, only home range overlap influenced the escalation

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of those contests (Table 3). Because home range overlap affected both the frequency and escalation of these contests while female turnover only influenced contest frequency, home range overlap seems to be a stronger driver of whether and how males engage in fights with rivals. These results suggest that while female behavior is important, home range overlap and, potentially, habitat features like forage and water availability (which largely dictate the extent of home ranges), have a greater influence on male-male interactions (Feist and McCullough, 1976; Rubenstein, 1981; Miller, 1983; Manning and McLoughlin, 2017). In habitats necessitating a high degree of overlap between home ranges, inter-band interactions are likely to increase, with more heightened competition among band stallions over both females and resources (Michener and McLean, 1996; Haenel et al., 2003). However, the sample size from our analysis including home range variables was significantly reduced compared to our full analysis without those variables. Therefore, our results regarding the potential effects of home range size and overlap should be interpreted cautiously.

Nonetheless, these results provide preliminary but important insights into the factors driving the expression of behaviors involved in male mating strategies, specifically reproductive and mate-guarding behavior. In most wild populations, the effects of ecological patterns and female behavior on male behavior are nearly impossible to separate because ecological patterns typically dictate female behavior (Emlen and Oring, 1977; Kraus et al., 2003). However, on Shackleford, the increase in group changing behavior by previously treated females suggests that their behavior is now at least somewhat divorced from their physical environment; mares that would typically

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form stable groups are instead demonstrating behavior akin to a fission-fusion society (Rubenstein, 1986; Nuñez et al., 2009). Therefore, this system provides a rare opportunity to examine how female behavior and ecological factors may act separately or in interaction to drive male behavior. While visibility, home range size, and home range overlap do not encompass the full range of ecological variables that can influence animal behavior, they have been shown to be particularly important in determining stallion behavior and provide a platform for examining the effects of the surrounding environment (Rubenstein, 1981). Our data suggest that while both the social and physical environment influence male behavior, ecological factors may have a greater impact on the means by which males retain access to females (i.e. through herding of females and contests with rival males) than does female behavior itself. Future studies of similar populations (McShea et al., 1997; Ransom et al., 2010; Duncan et al., 2017), with larger sample sizes and long-term data have the potential to improve our understanding of how these factors influence important reproductive and mate-guarding behaviors that shape male mating strategies.

While we provide evidence that ecological factors like visibility and home range overlap play an important role in determining how males respond to changes in female behavior, much work remains to be done. Future studies should consider forage and water use and availability across seasons as these are dynamic factors that are likely to influence both male and female behavior (Feist and McCullough, 1976; Miller, 1983; Marr, 1996; Manning and McLoughlin, 2017). Furthermore, more intensive sampling of band locations to improve home range estimates, and use of more nuanced overlap

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calculations would allow for a clearer interpretation of how home range size and overlap influence males' intra- and inter-band interactions (Linklater et al., 2000; King, 2002; Fieberg and Kochanny, 2005; Börger et al., 2006). It is also important to note that while the extent of home ranges is strongly dictated by facets of the physical environment like food and water distribution (Feist and McCullough, 1976; Miller, 1983; Manning and McLoughlin, 2017), it can also be influenced by social factors such as dominance status (Höjesjö et al., 2007; Murray et al., 2007). How home range size and overlap represent an interaction between the social and physical environment should be considered when using these measures to assess the effects of the surrounding environment on behavior. Comparative studies in diverse environments with varying population densities may provide more generalizable conclusions about the influences of ecological factors on male behavior under different conditions.

4.5. Management Implications

While previous work has demonstrated the impacts of PZP on treated females in feral horses, white-tailed deer, and elk, this is one of only a few studies to explore the potential consequences of immunocontraception management for non-target individuals (Ji et al., 2000; Poiani et al., 2002; Gray and Cameron, 2010; Druce et al., 2013). If behavioral changes among treated females are likely to alter the behavior of untreated animals, we may need to reevaluate how we assess the behavioral impacts of immunocontraception. Our results show that increased mare group-changing behavior leads to increases in male-male competition and vigilance. Given the increased group changing among contracepted mares (Nuñez et al., 2009; Nuñez et al., 2017), it stands to

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reason that PZP-treated mares could have a disproportionate effect on these male behaviors. These results are not anomalous: indirect impacts of fertility control on male behavior have been demonstrated in three western horse populations in addition to brushtail possums (*Trichosurus vulpecula*) and eastern grey kangaroos (*Macropus giganteus*) (Ji et al., 2000; Poiani et al., 2002; Ransom et al., 2010). Compared to alternatives like gathers and removals, PZP is one of the best available options for controlling feral horse populations because it is generally highly effective, reversible, safe for pregnant animals, and relatively cost effective (Kirkpatrick et al., 1996; Kirkpatrick et al., 2009). However, when possible, care should be taken to develop management strategies that consider and attempt to minimize unintended consequences. Furthermore, as new methods of controlling these populations are developed (Ransom et al., 2014; Hall et al., 2016; Collins and Kasbohm, 2017), behavioral consequences for target animals and their associates should be monitored to ensure animal welfare and effective management.

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Figure captions

Fig. 1. Examples of (a) vigilant and (b) relaxed stances.

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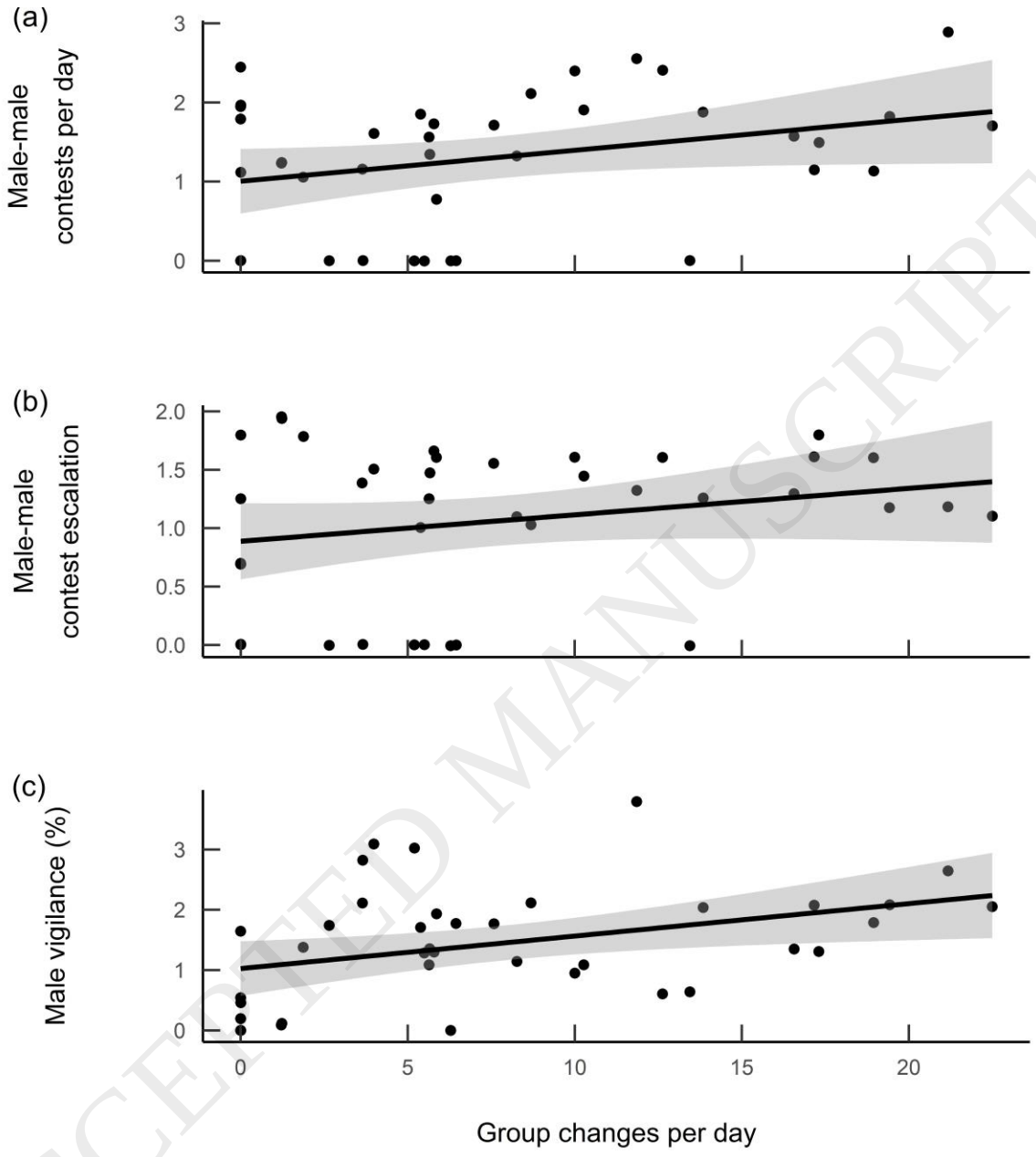
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Fig. 2. The effect of the number of female group changes that males experienced per day on various male behaviors: (a) the number of male-male contests per day, (b) the average escalation of contests, and (c) the percentage of time spent vigilant (all response variables are log transformed). All response variables increased as the number of group changes increased. Shaded bands represent 95% confidence intervals. Points are jittered to allow clear visualization of all males observed.

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Table

Table 1. Spearman rank correlations between response variables of interest and male rank.

Response variable	Correlation with male rank (ρ)	*P*
Male-male contests	-0.179	0.371
Average contest escalation	0.083	0.679
Male-female aggression	0.177	0.377
Reproductive behavior	0.180	0.370
Vigilance	0.184	0.367

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Table 2. Summary of the final mixed effects models describing the effects of explanatory variables on each male behavior of interest (fixed effects only). Significant main effects are in bold.

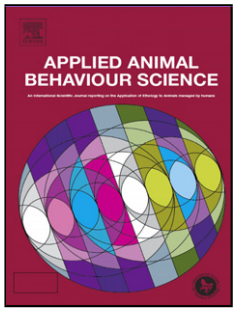
Response variable	Explanatory variable	Estimate	SE	t	*P*
Male-male contests	Group changes per day	0.058	0.02	3.13	**0.003**
	Male age	0.076	0.02	3.33	**0.002**
Average contest escalation	Group changes per day	0.035	0.01	2.28	**0.029**
	Male age	0.050	0.02	2.65	**0.012**
Male-female aggression	Visibility	0.804	0.30	2.66	**0.013**
Reproductive behavior	Male age	-0.053	0.04	-1.47	0.156
Vigilance	Group changes per day	0.048	0.02	2.62	**0.014**
	Average female age	-0.048	0.02	-2.15	**0.039**

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Table 3. Summary of the final mixed effects models describing the effects of explanatory variables on each male behavior of interest (fixed effects only) for analyses including only those males for which home ranges could be estimated (n = 17). Significant main effects are in bold.

Response variable	Explanatory variable	Estimate	SE	t	*P*
Male-male contests	Group changes per day	0.048	0.02	2.43	**0.024**
	Home range overlap	1.430	0.49	2.93	**0.008**
Average contest escalation	Home range overlap	3.505	1.20	2.93	**0.008**
Male-female aggression	Average female age	-0.545	0.22	-2.43	**0.023**
Reproductive behavior	Group changes	0.048	0.02	1.90	0.071
	# of adult females in band	0.184	0.09	1.94	0.065
Vigilance	Home range size	1.017	0.60	1.70	0.114

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Rising up to the challenge of their rivals: Mare infidelity intensifies stallion response to playback of aggressive conspecific vocalizations

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Rising up to the challenge of their rivals: Mare infidelity intensifies stallion response to playback of aggressive conspecific vocalizations

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Highlights

- Males were more vigilant after squeals (aggressive signals) than control playbacks
- Stallions were more likely to approach the speaker following squeals than controls
- Female turnover rate did not affect stallion responses to squeals
- Stallion responses were heightened during and after female group changes
- Altered social contexts in managed populations may affect male responses to rivals

Abstract

Management tools like immunocontraception can alter the behavior of target animals, but the extent to which they affect non-target individuals has received less attention. The feral horse (*Equus caballus*) population on Shackleford Banks, North Carolina is an ideal system with which

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these questions may be explored, as management of the population with the immunocontraceptive agent porcine zona pellucida (PZP) has resulted in an increased propensity for females to change social groups and thus, decreased social stability. During the study, on average, females made 1.4 group changes per day (range = 0—18.5 group changes per day): females previously treated with PZP made 1.8 group changes per day, while females that had never been treated made 1.2 group changes per day. Between May and August, 2017, we used playbacks of aggressive male vocalizations (squeals) and human voices (reciting "hello horse") to assess changes in stallion responses to male rivals versus socially irrelevant stimuli in the context of female turnover. Over the course of the study, males were observed for 9.4 hours on average (range = 2.4—20.5 hr). Males spent more time vigilant (estimate = 12.431, $P = 0.016$, $\bar{x}_{\text{squeal}} = 30$ s, $\bar{x}_{\text{control}} = 19$ s) and were more likely to approach the speaker following squeal playbacks than controls (estimate = 2.325, $P = 0.039$). Males' latency to return to normal behaviors varied depending on whether the playback was conducted in the weeks before, during, or after group changes occurred ($P = 0.025$, $\bar{x}_{\text{before}} = 26$ s, $\bar{x}_{\text{during}} = 39$ s, $\bar{x}_{\text{after}} = 53$ s). Male responses were not affected by the total number of female group changes a male experienced during the observation period ($P > 0.4$), suggesting the effects are more context-dependent and not long-lasting. These findings suggest mare turnover can impact stallion responsiveness to potential rivals. As previously contracepted mares change groups more often than untreated mares and stallions exhibit prolonged responses to aggressive vocalizations after experiencing a female group change, contraception-induced changes to mare behavior may lead to increased male aggression in response to intruding rivals, which could be associated with greater energy expenditure. Finally, our work demonstrates that playback experiments are a useful tool for studying feral horse behavior in the wild. As the need for population control of different species continues to expand, rigorous investigations of

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immunocontraception's effects on non-target animals are critical if agencies are to manage populations most effectively.

Keywords: *Equus caballus*, female turnover, immunocontraception, male aggression, playback, vocalization

1. Introduction

In the United States, feral horses (*Equus caballus*) are protected under federal law and are valued by much of the public for their historical and cultural associations (United States Congress, 1971). However, with few predators, feral horse populations have expanded rapidly (Garrott et al., 1991; Turner et al., 1992; Turner and Morrison, 2001; DOI-OIG, 2010), coming into conflict with human land use practices and certain native species (Beever and Brussard, 2000; Levin et al., 2002). As such, feral horses present a unique management concern. In lieu of traditional management practices like gathers and removals that tend to be costly, ineffective, and unpopular with the public, fertility control, and in particular the immunocontraceptive agent porcine zona pellucida (PZP), has become a popular option for managing feral horses and other ungulate populations (Kirkpatrick and Turner, 1991; Kirkpatrick et al., 1997; McShea et al., 1997; Heilmann et al., 1998; National Research Council, 2013).

In social species like the horse, maintenance of natural behaviors is an important consideration for managers trying to preserve the health and wellbeing of populations. Impacts of PZP on the behavior and physiology of treated mares have been explored in several populations (Kirkpatrick et al., 1996; Powell, 1999; Turner and Kirkpatrick, 2002; Nuñez et al., 2009; Nuñez et al., 2010; Ransom et al., 2010; Madosky, 2011; Nuñez et al., 2014), yet consequences of this management tool for non-target conspecifics have received considerably less attention (Ransom et al., 2010). Feral horses live in bands, social groups that remain together year-round and consist

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of one or sometimes multiple stallions, one or more associated females, and their offspring (Feist and McCullough, 1976; Rubenstein, 1981). Given this complex social structure, changes in the behavior of one individual are likely to affect the behavior of its associates. The potential effects of changes in the behavior of PZP-treated mares on their associates are important to consider if managers aim to preserve more naturally functioning feral horse populations.

PZP was used to manage the feral horse population on Shackleford Banks, North Carolina between 2000 and 2009. In this population, mares treated with PZP change bands up to ten times more often than untreated mares (Nuñez et al., 2009). Regardless of contraception status, mares with a greater proportion of years pregnant are less likely to change groups (Nuñez et al., 2009), suggesting that decreases in pregnancy (and possibly lactation) may be the mechanism(s) by which PZP treatment increases mare propensity to change groups (Nuñez et al., 2009). Despite suspension of PZP administration in 2010, mares previously treated with PZP experience prolonged subfertility and still exhibit this increased group changing behavior, with previously treated mares making 1.8 group changes per day compared to untreated females making 1.2 group changes per day (Nuñez et al., 2017; Jones and Nuñez, 2019). Before PZP treatment began on Shackleford, only 10.8% of mares in the population changed groups (Rubenstein, 1981). While the immunocontraception program was active, this number increased to approximately 69.5%, and in 2017, after the suspension of treatment, group changing behavior remained more frequent with 34.8% of females changing groups throughout the breeding season (Madosky, 2011; Jones and Nuñez, unpublished data). These changes to female behavior have decreased the typically high degree of social stability that characterizes feral horse bands (Klingel, 1975; Madosky, 2011). Historically, contests between intruding and resident stallions were won by the resident; furthermore, in fights to regain mares separated from the band, the females were usually recovered

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(Feist and McCullough, 1976; Rubenstein, 1981). However, mare retention is now destabilized (Nunez et al., 2017), increasing male-male competition in this population (Jones and Nuñez, 2019). Such changes in male competition are not unique to feral horses: similar decreases in social stability have been associated with increases in male aggression in other species including ring-tailed lemurs (*Lemur catta*) and gray-cheeked mangabeys (*Lophocebus albigena*) (Cavigelli and Pereira, 2000; Arlet et al., 2009).

In feral horses, vocalizations are commonly used in a number of social contexts including mating, aggression, and the formation of social bonds, with different types of vocalizations containing varying social information (Feist and McCullough, 1976; Proops et al., 2009; Yeon, 2012). Specifically, squeals are often used during contests between rival males and indicate dominance status, with more dominant stallions emitting longer and more forceful squeals than subordinates (Rubenstein and Hack, 1992). Most male-male contests are characterized by a typical sequence of behaviors, beginning with approaches, olfactory assessment, and acoustic signaling (Feist and McCullough, 1976; Miller, 1981; Hynes, 1998). If contests become escalated, these assessments are followed by physical contact ranging from pushing to kicking and biting (Feist and McCullough, 1976; Rubenstein and Hack, 1992). As squeal vocalizations are a clear indicator of male-male conflict, they may be used to experimentally assess how males respond to signals from intruding males, including whether or not they choose to engage rivals and how escalated the response becomes.

Here, we use experimental playbacks of squeals to determine how stallions with varying levels of experience with female group changing behavior respond to these aggressive signals. To our knowledge, ours is the first experiment to assess the effects of female turnover on male competition in a natural setting. We predicted that all stallions would respond more aggressively

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toward squeal playbacks than unrelated control stimuli (human voices), regardless of experience with female turnover. We also hypothesized that stallions experiencing more female group changes would exhibit heightened responses to squeal playbacks. We expected these males to approach the speaker more often and to exhibit decreased latency to respond, prolonged vigilance, and an increased latency to return to their normal behaviors when compared to stallions experiencing fewer group changes. This experiment provides a more complete understanding of the influence of female behavior on male competition. Furthermore, it provides insights into how playback experiments can be implemented as useful tools to assess feral horse behavior. Because mares previously treated with PZP demonstrate prolonged decreases in fidelity (Nuñez et al., 2017; Jones and Nuñez, 2019), investigating how increased female turnover affects male behavior will improve our understanding of the potential consequences of immunocontraception management for non-target animals.

2. Methods

2.1. Study site and subjects

The focal population of feral horses lives on Shackleford Banks, a barrier island off the coast of North Carolina, USA. The island is approximately 15 km long and ranges from 0.2 to 1.2 km wide. As a part of Cape Lookout National Seashore, Shackleford Banks is visited by approximately 100,000 tourists annually (National Park Service, 2017); therefore, the horses on the island are somewhat habituated to human activity (i.e., at distances of at least 15 m). The study was conducted during the breeding season from May through August, 2017. We observed all band stallions and their females: 20 males and 69 females organized into 19 bands. Most bands had one dominant stallion with the exception of one double-male band. Individuals were identified by their sex, color, facial markings, and, in some cases, freeze brands. We conducted all observations from at least 15-20 meters away (National Park Service, 2004) to ensure the horses were not disturbed

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by our presence. All observations were conducted by eye or with the aid of binoculars (Nikon Monarch M511 10x42) when necessary.

This study was approved by the Iowa State University Institutional Care and Use Committee (3-17-8456-E) and the National Park Service Institutional Care and Use Committee (1024-0265). The National Park Service granted permission to collect all observational data (CALO-2017-SCI-0006).

2.2. PZP contraception

This feral horse population is federally protected under the Shackleford Banks Wild Horses Protection Act of 1997 and is managed jointly by the National Park Service and the Foundation for Shackleford Horses (United States Congress, 1997). To maintain the population at the predetermined size of approximately 120 animals, the National Park Service began managing the population via immunocontraception in 2000. Designated mares received initial and booster doses of PZP, which were administered annually from February through April. Injections contained 100 micrograms of PZP plus an adjuvant, and initial doses contained Freund's Complete Adjuvant, Modified, *Mycobacterium butyricum* (Calbiochem #344 289). Booster doses contained Freund's Incomplete Adjuvant (Sigma #F5506). In 2010 and 2011, when the NPS noted that some mares treated repeatedly experienced prolonged subfertility post-treatment, PZP treatment was suspended to allow the population to increase (Stuska, personal communication).

2.3. Stimuli acquisition

Squeals made by stallions during male-male contests in a population of Misaki feral horses in Japan were recorded by L. Proops. Squeals tend to vary in duration with significant acoustic noise and medium to high amplitudes and tonality (Figure 1), differing in their acoustic properties from other vocalizations like whinnies and nickers (Yeon, 2012). Although stallions generally recognize familiar rivals using olfactory rather than auditory cues (Rubenstein and Hack, 1992),

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we used recordings of males from another population to avoid any question of potential confounds related to individual recognition by Shackleford stallions. Stallions are capable of discriminating between dominant and submissive males on the basis of acoustic cues in squeals (Rubenstein and Hack, 1992). To help control for the potential effects of rank on stallion behavior, we used vocalizations from band-holding (relatively dominant) Misaki males exclusively. Recordings were collected using a Sennheisser ME66/K6 directional microphone attached to a Marantz PMD661 audio recorder at a sampling frequency of 44.1kHz and bit depth of 32 at distances between 5 and 30 m from the subjects. Seven high-quality recordings were chosen and used as auditory stimuli. Selected squeals ranged in duration from 0.6 s to 2.0 s with an average duration of 1.0 s.

Control recordings were made at Iowa State University. Seven adult males ranging in age from 22 to 68 years (mean = 41 years) were recorded reciting the phrase "hello horse" using an AKG D790 microphone and a Focusrite Scarlett 18i8 preamp connected to a Dell Latitude E7470 PC. Recordings were captured using Audacity 2.1.3 (Audacity Team 2017) at a sampling frequency of 44.1kHz and bit depth of 24. All squeal and control recordings were normalized to 75 dB in Praat (64-bit edition) (Boersma and Weenink, 2017) and were standardized to play at the peak sound pressure of 98 dB at 1 m from the source (Proops et al., 2009).

2.4. Playback procedure

All playback trials were conducted by a single observer (M. M. Jones) between June and August 2017. Vocalizations were played to all band stallions throughout the study, with each stallion receiving one squeal and one control playback, randomized in order with at least one day between trials. In the case of the double-male band, we presented the same squeal and control sounds to both males only once and recorded each male's response separately.

Historically, Shackleford has been defined by three distinct habitats: the East, which is flat and open with limited water sources (Marr, 1996); the Mideast, which is also flat and open with

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more evenly distributed water sources (Rubenstein, 1981); and the West, which contains two primary water sources and is dominated by high dunes and dense brush (Rubenstein, 1981). The East, Mideast, and West were treated as blocks to account for the effect of region on stallion responses. Within each region, stallions were randomly assigned to different pairs of control and squeal exemplars.

Vocalizations were played on an Apple iPod Nano (2nd generation) connected to a small portable speaker (MIPRO MA-101C Personal PA System) hidden in vegetation approximately 20 m from the stallion. For each recorded exemplar, two minutes of silence were added before the vocalization to allow the observer time to move away from the speaker and out of sight of the stallion so as not to interfere with the subject's response. After the two minutes of silence, the vocalization played twice with ten seconds of silence between iterations (Proops et al., 2009). To capture the stallion's response, we conducted 15 minute focal samples (Altmann, 1974) starting immediately after the first vocalization played and recorded the following: whether stallions responded in any way to the stimulus, the latency to respond, whether stallions approached the speaker, length of vigilance behavior (characterized by ears erect and directed forward, head and neck held high, and eyes alert (Feist and McCullough, 1976)), and the latency to return to normal behaviors (measured as the time between the initiation of any response and the return to normal behaviors (e.g. grazing, standing)). A total of 40 trials were conducted among six horses in the East, eight in the Mideast, and six in the West receiving one squeal and one control vocalization. All playbacks were conducted when the stallion of interest was out of sight of other bands and when the weather was dry and winds were relatively calm.

2.5. Female group changing data

Study bands were located twice per week from late May to early August, and GPS location and group composition were noted. As witnessing group changing behavior by females was rare

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(n = 1), presence and absence of females in the band were noted to monitor this behavior. These data were used to calculate the number of group changes experienced by males (the number of times females entered or left a male's band). The number and rate of group changes experienced by all males is detailed in Table 1. For most males that experienced group changes (100% in 2016; 92% in 2017), at least one of the females that left their band returned for some period of time.

2.6. Assessing visibility on Shackleford Banks

Habitat visibility can affect a stallion's ability to protect his band from intruders because increased visibility allows males to defend territory boundaries and track band member activity more easily (Rubenstein 1981). Therefore, we included visibility as a potentially important environmental factor that could influence male responses to rival stallions. We quantitatively assessed visibility across the island by conducting a viewshed analysis in ArcMap 10.4.1 (Alonso et al., 2012; Aben et al., 2017). Digital surface models were created using USGS LiDAR point cloud data to take both bare earth elevation and vegetation structure into account (U.S. Geological Survey, 2015). GPS locations collected at every band sighting using Garmin eTrex 10 units were used as observer points, and an observer offset of 1.2 m (the average height of Shackleford horses) was added to each observation point to account for the height at which a horse would be able to see across the landscape at eye level (Stuska, personal communication). The Visibility tool in ArcMap was then used to predict the number of 10 m x 10 m cells visible from each point, and the number of visible cells was averaged across all points for each stallion and converted to km^2 to produce an average visibility score.

2.7. Statistical analysis

We used generalized linear mixed-effects models with the lmerTest package (Kuznetsova et al., 2017) in R (version 3.4.1) to examine how female group changing behavior influenced male responses to playbacks (R Development Core Team, 2017). Playback type (squeal or control),

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female turnover rate, and the timing of playback trials with regard to group changes were used as explanatory variables. Female turnover rate was calculated as the total number of group changes each male experienced divided by the total time that male was surveyed (expressed as observation days, which were defined as 12 hour periods (the approximate length of daylight during our study period)). Playback timing was determined by classifying each trial as occurring in the one to two weeks before the stallion experienced a female group change (weeks 1-2), during the week of and the week following a group change (weeks 3-4), or in the five weeks following a group change (weeks 5-10) (Figure 2). Interactions between playback type and female turnover rate as well as playback type and playback timing were included to assess whether experience with female group changes altered male responses to all stimuli or only relevant cues from potential rivals.

All response variables were analyzed independently due to small sample size. Binary response variables (whether stallions responded to the stimulus and whether they approached the speaker) were analyzed using models with binomial distributions, while models with normal distributions were used for continuous variables (response latency, length of vigilance behavior, and latency to normal).

The models also included male age, average female age (within bands), and the average number of adult females present as fixed effects as these factors are also likely to affect male behavior (Berger, 1977; Vervaecke et al., 2007; Nuñez et al., 2017). Visibility was included in the models to determine whether habitat openness affects how males respond to the stimuli. All models included stallion identity as a random effect to control for pseudo-replication. Best fit models were simplified using backwards elimination, removing terms with *P*-values > 0.1. In the final models, predictor variables were considered significant if $P \leq 0.05$. For these generalized linear mixed-effects models, parameter estimates, standard errors (SE), t- or z-values, and *P*-values are

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presented.

3. Results

Males were more likely to approach the speaker (Figure 3; estimate = 2.325, SE = 1.13, z = 2.06, P = 0.039) and spent more time vigilant (Figure 4; estimate = 12.431, SE = 4.56, t_{13} = 2.73, P = 0.016) following squeal playbacks than control trials. The probability of showing any response and the response latency did not differ based on the type of playback trial (all P-values > 0.2).

Main effects of the number of group changes males experienced per day and interactions between this variable and playback type were dropped from all final models (all P-values > 0.4), indicating that the rate of female turnover did not affect male responses to either stimulus.

However, there was a significant interaction between playback type and playback timing with regard to female group changes (before, during, or after a male experienced a group change) in the final model for latency to return to normal behaviors (Figure 5; P = 0.025). Tukey post-hoc pairwise tests revealed that males took significantly longer to return to normal following squeal trials than control trials after a group change (estimate = -53.380, SE = 16.77, t_{25} = -3.18, P = 0.040), and there was a strong trend showing the same pattern for trials conducted during a group change (estimate = -23.232, SE = 7.68, t_{19} = -3.03, P = 0.065). However, there was no difference in males' latency to return to normal between squeal and control trials conducted before a group change (estimate = 0.738, SE = 9.44, t_{20} = 0.08, P > 0.9).

With regard to the additional covariates, the average visibility score across all samples was 0.73 km^2 (range = 0.06—3.97 km^2); however, visibility had no effect on male responses. The number of adult females was the only factor retained in the final model predicting the probability of any response. While the effect was not significant, there was a trend by which males with more females in their bands were more likely to respond to all playbacks (estimate = 1.118, SE = 0.62, Z = 1.80, P = 0.072). Additionally, average female age and male age were retained in the final

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model predicting response latency. Males with younger females in their bands took longer to respond to playbacks than males with older females (estimate = -0.185, SE = 0.09, t_{15} = -2.15, P = 0.048). Finally, older males tended to take longer to respond than younger males (estimate = 0.035, SE = 0.01, t_9 = 2.13, P = 0.062).

4. Discussion

Here we show that males exhibit heightened responses to squeal playbacks compared to controls, and while the overall rate of female turnover does not affect those responses, responses to squeals are stronger during periods of group instability. These context-dependent responses suggest that female group changing events can influence how males respond to rivals. On Shackleford Banks, females previously treated with PZP change bands more often than untreated females, interfering with the population's social stability (Nuñez et al., 2009; Madosky, 2011). This playback experiment provides a more controlled assessment of how this altered social context affects stallion response to rivals than can observation alone. While the impacts of fertility control on the behavior of treated animals have been studied in a variety of species and populations (Hayes et al., 1996; McShea et al., 1997; Heilmann et al., 1998; Powell, 1999; Ramsey, 2007; Nuñez et al., 2009; Ransom et al., 2010; Madosky, 2011), its consequences for non-target individuals have received considerably less attention (Ji et al., 2000; Poiani et al., 2002; Gray and Cameron, 2010; Ransom et al., 2010; Druce et al., 2013). Here we provide evidence that immunocontraception management can have indirect consequences for non-target males.

Among the response variables measured, males were more likely to approach the speaker and spent more time vigilant following squeal playbacks than control stimuli (Figure 3, Figure 4). Rubenstein and Hack (1992) found similar responses in stallion vigilance and likelihood of approach in their playback experiment investigating agonistic encounters among stallions. However, contrary to our expectations, the likelihood of any type of response and response latency

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did not differ between squeal and control trials. The fact that playback type did not affect response likelihood may be attributed to a ceiling effect. Stallions responded in 80% of playback trials, suggesting that this measure may not be useful when examining response variation and that differences between playback types are more likely reflected in specific behaviors exhibited during responses. As horses are prey animals that respond to novel auditory stimuli as well as signals from conspecifics, it is important to consider which specific behaviors are relevant to the social context of interest when conducting playback experiments (Christensen et al., 2005). Vigilance and approaches are commonly demonstrated during male-male conflicts or when an intrusion by a rival is suspected, indicating that our playbacks served their intended purpose; males did engage, at least to some extent, in more typical contest behaviors following squeals than following control playbacks (Feist and McCullough, 1976; Miller, 1981; Hynes, 1998).

The rate of female group changes experienced by males did not explain variation in any of the measured response variables following squeal or control playbacks. Similarly, the likelihood of response, response latency, vigilance, and likelihood of approach did not differ depending on whether the trial was conducted before, during, or after a group change by a female. These findings contradict our prediction that males experiencing more group changes would exhibit more heightened responses to squeal playbacks than those experiencing fewer group changes. Low variation in male behavior may have been due to strong stabilizing selection on stallions' responses to potential threats in the environment. More responsive males are likely better able to monopolize and protect their females and offspring from rivals, thus increasing their own reproductive success, than are less responsive males (Rubenstein 1981, Asa 1999). It may be then that in feral horses, males respond strongly to potential threats, regardless of the social circumstances.

Alternatively, limitations of the experiment may have contributed to the insignificant

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findings. This study was conducted over the course of a single season with each male receiving only one squeal and one control playback. In addition, we could not control for the behavioral context at the time of testing; therefore, unknown external factors could have affected stallions' responses (Tyack, 2009). Furthermore, we presented only auditory stimuli in this experiment, while a typical encounter between rival males would also include visual and olfactory stimuli that play a significant role in determining responses (Feist and McCullough, 1976; Miller, 1981; Rubenstein and Hack, 1992). The limitation of the auditory stimuli used here likely restrained stallions' responses, potentially masking variation in the most aggressive behaviors that stallions would exhibit in response to visible intruders.

Moreover, our response measurements may not have been precise enough to capture subtle variations in stallions' responses to squeals. Transcribing responses as they occurred restricted our ability to note nuances such as laterality, subtle changes in ear positions, and nostril lifts, all of which have been noted as relevant responses to conspecifics in domestic horses (Austin and Rogers, 2014; Wathan et al., 2015; Smith et al., 2016; Wathan et al., 2016). In future playback studies, video recordings that allow for more nuanced measurements of responses may be an important tool to reveal variation missed in the measurements reported here (McComb et al., 2000; Proops et al., 2009; Austin and Rogers, 2014).

It is of interest that the short-term context of female group changes did influence males' latency to return to normal after playbacks. Males took longer to return to normal behaviors following squeals than controls but only when the playback was conducted during or after a group change by a female (Figure 5). This indicates that at least some responses are context-dependent, with males exhibiting prolonged responses to signals from perceived rivals compared to random stimuli in periods of social instability. Similar context-dependent changes in male behavior during

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periods of social instability have been observed in other systems. For instance, male ring-tailed lemurs were more aggressive toward rival males throughout the relatively unstable mating period (during female estrus) than they were during the more stable premating period (Cavigelli and Pereira, 2000). As in our study, this short-term period of social instability altered male-male interactions during that specific time-frame only. Because previously contracepted females change groups more often than other females (Nuñez et al., 2017; Jones and Nuñez, 2019), males associated with such females are likely to exhibit this increased responsiveness more often than other males. Admittedly, the sample size for trials conducted following a group change is low in our study (n = 5); as such these results should be interpreted cautiously. Additional playback trials would help to more clearly assess such patterns in stallions' latency to return to normal as well as other responses. Further, more nuanced exploration of these responses, perhaps in a more controlled setting, could reveal additional insights into the consequences of immunocontraception management for non-target animals.

More generally, our results support the use of auditory playbacks to experimentally investigate behavior in free-living populations (Reby et al., 2005; Fischer et al., 2013). Our data reinforce the importance of carefully considering the context of both stimuli and response measurements to ensure playback designs truly focus on behaviors relevant to the question of interest (Sayigh et al., 1999; McGregor, 2000). While playback experiments have been used extensively to assess domestic horse behavior (Lemasson et al., 2009; Proops et al., 2009; Lemasson et al., 2015; Smith et al., 2016; Wathan et al., 2016; Briefer et al., 2017), we posit that they are also a useful tool for feral horse behavior. They allow researchers to more precisely pinpoint causative relationships than do interpretations of correlative data and may be especially valuable when the behaviors of interest are rarely observed in the wild (Rubenstein and Hack,

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1992; McComb et al., unpublished data).

Declarations of interest

None.

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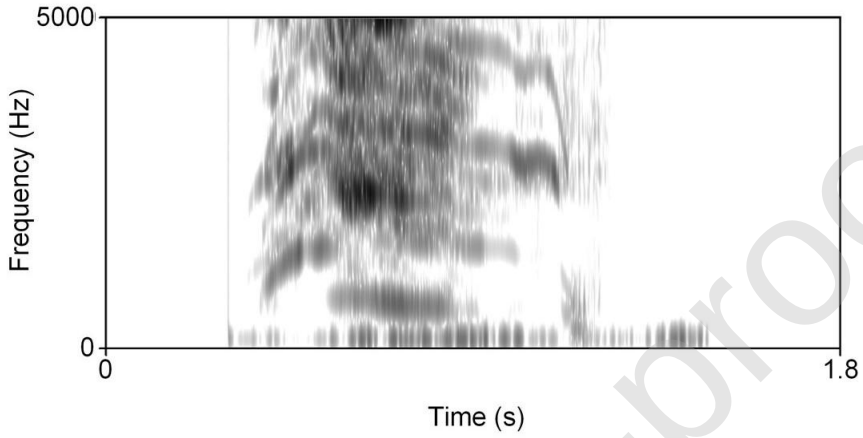
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Figure 1. Spectrogram of a squeal produced by a dominant Misaki stallion during an antagonistic encounter with another stallion.



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Figure 2. Timeline for playback trials evaluating stallion responses to control and squeal sounds on Shackleford Banks, North Carolina from May-August 2017.

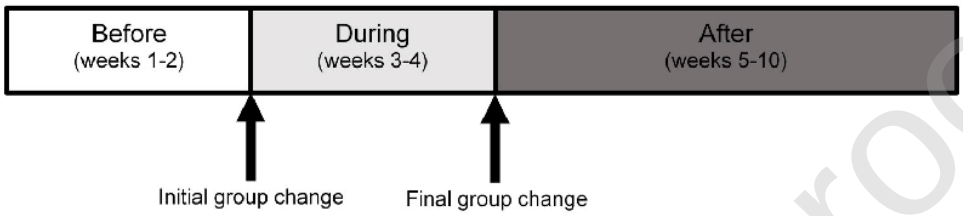


Figure 3. Stallion approaches toward the speaker for squeal and control playbacks on Shackleford Banks, North Carolina from May-August 2017. Black bars represent males that did approach the speaker; grey bars represent those that did not approach. Males were more likely to approach the speaker during squeal trials than controls.

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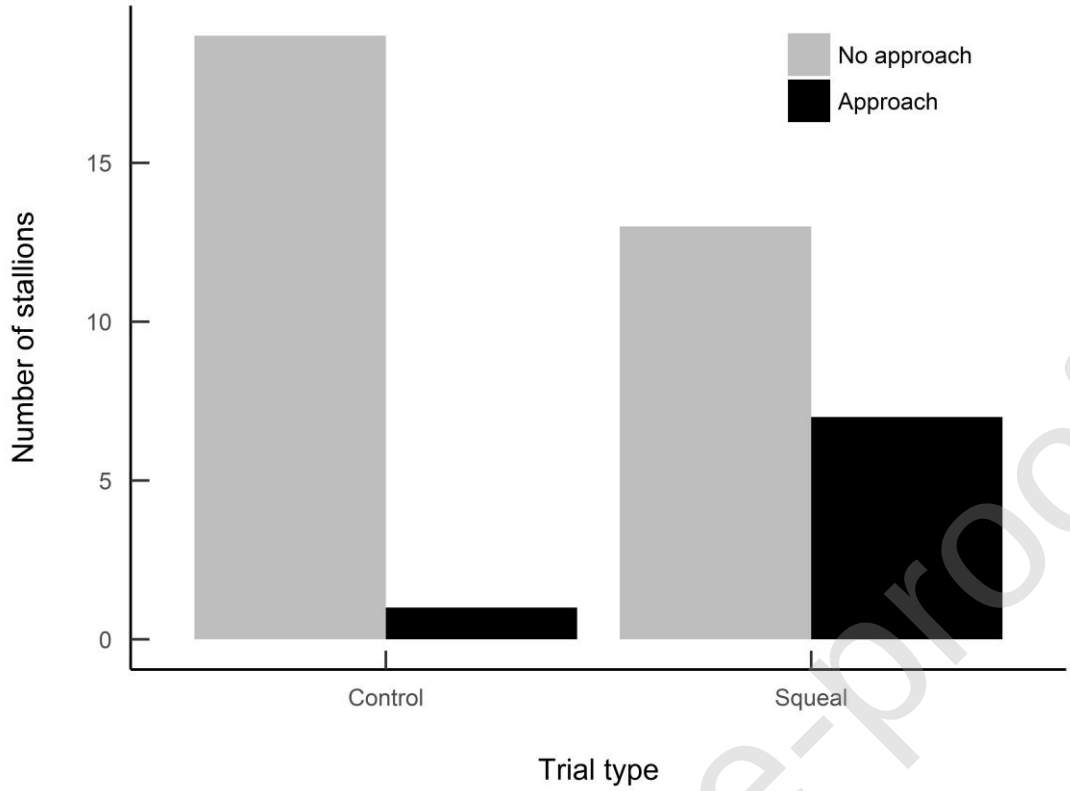


Figure 4. The amount of time (seconds) stallions spent vigilant following control and squeal playbacks on Shackleford Banks, North Carolina from May-August 2017. Males spent significantly more time vigilant following squeal stimuli than control stimuli. Segmented lines indicate means for each group.

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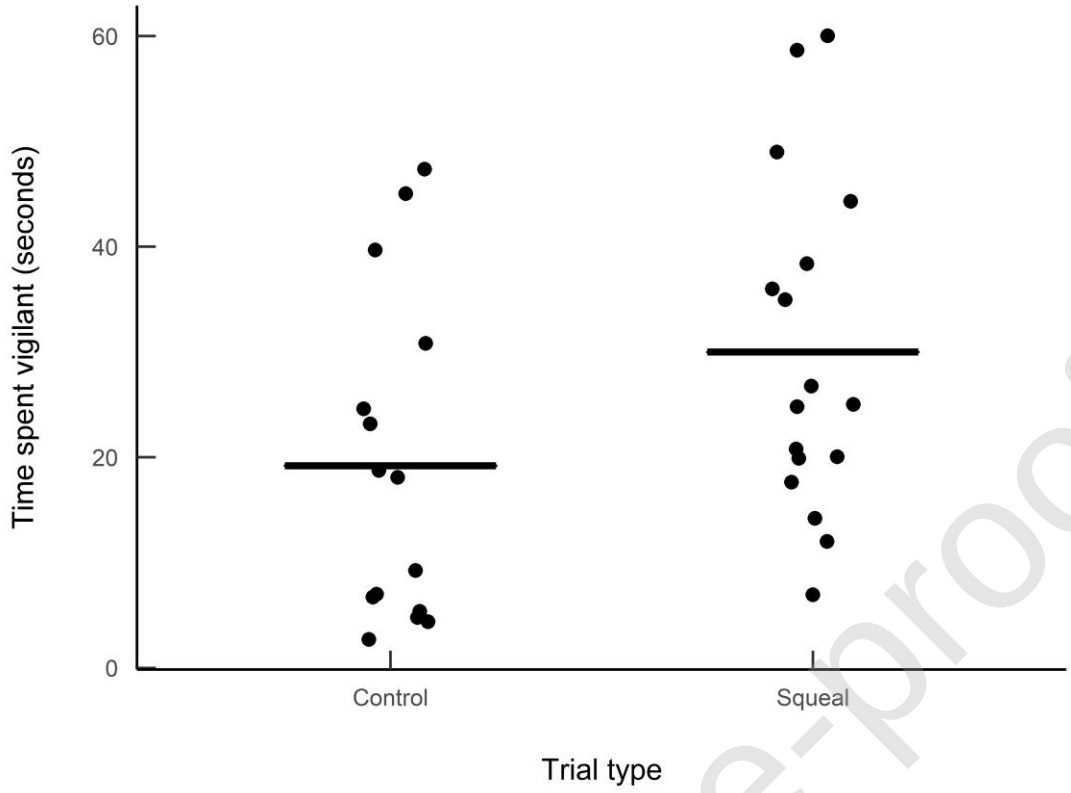


Figure 5. The length of time (seconds) between stallions' initial response and their return to normal behaviors following squeal (black) and control (grey) playback trials conducted before, during, or after a mare entered or left their band on Shackleford Banks, North Carolina from May-August 2017. Males took longer to return to normal after squeals compared to control trials during and after experiencing a group change. Segmented lines indicate means for each group.

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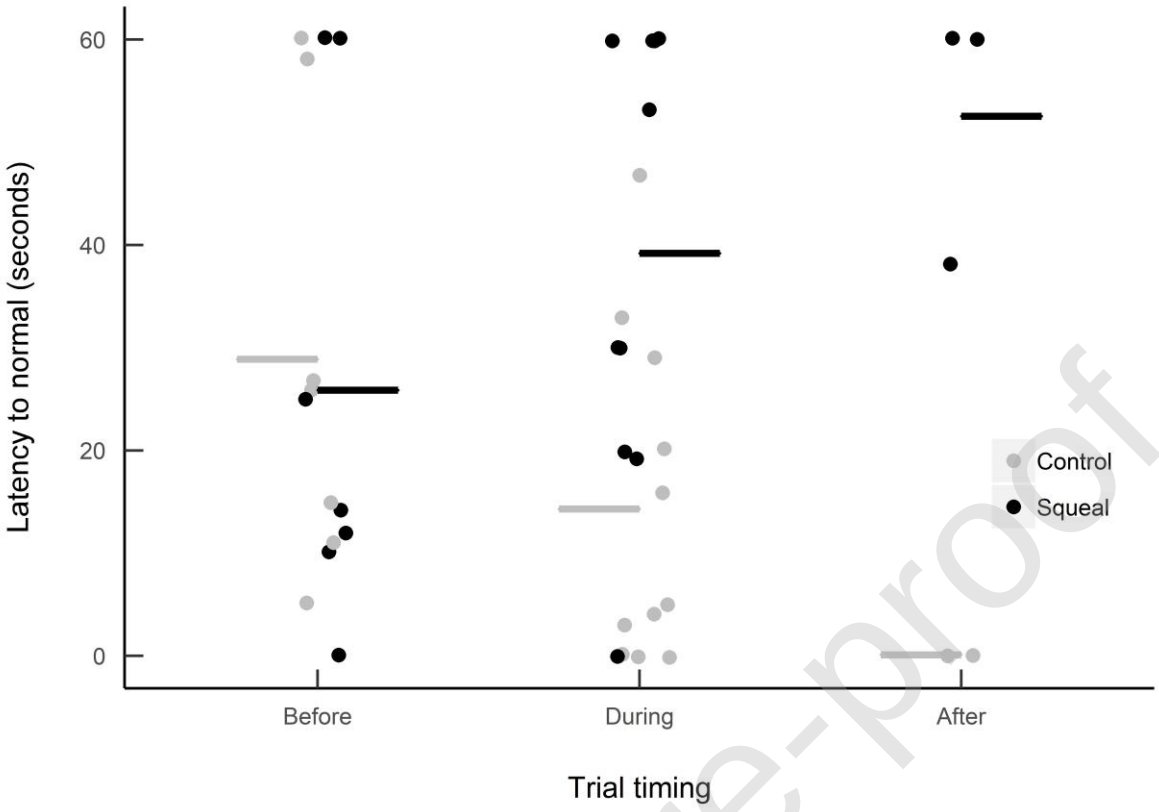


Table 1. The number and rate of female group changes experienced by all males observed in the playback experiment over the course of the 2017 breeding season on Shackleford Banks, North Carolina. Rates were calculated by dividing the number of group changes observed by the amount of time a given male was watched.

Stallion	**Number of female group changes experienced**	**Rate of group changes experienced (changes/day)**
Atari	5	17.3
Bilbo	16	17.2
Dale	1	1.2

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Dilbert	2	10.0
Dominic	5	13.8
Hernando	13	16.6
Homer	1	1.2
JFK	9	18.9
Judd	0	0.0
Kabuki	5	3.6
Katsu	2	1.9
Kenan	15	19.4
Largo	4	5.4
Noel	2	5.2
Scotch	9	8.3
Sebastian	9	10.3
Shakespeare	10	5.9
Simon	6	5.7
Toro	5	7.6
Waco	11	13.4

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Review

Ovarian dysfunction associated with zona pellucida–based immunocontraceptive vaccines

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ABSTRACT

Despite more than 40 years of research into zona pellucida (ZP)–based vaccines, relatively little is known about their mechanism of action. Early research demonstrated precipitation of ZP glycoproteins by antiovarian antiserum, rendering oocytes resistant to sperm binding *in vitro*. Subsequent work showed significantly decreased fertilization rates following passive immunization, sparking interest in anti-ZP immunocontraception for human and animal use. The primary mechanism of action of ZP vaccines is generally considered to be an antibody-mediated interference with sperm–oocyte binding and/or fertilization. However, this mechanism of action excludes the potential for ovarian dysfunction associated with anti-ZP treatment in some species. A review of relevant literature in pertinent model, domestic and wildlife species reveals a variety of previous and current hypotheses for ovarian effects following ZP-based immunization. Ovarian dysfunction has been suggested to be a species-specific response. In addition, cytotoxic T-lymphocytes and the use of Freund's adjuvants have been suggested to play a role. Finally, the type and extent of glycosylation of ZP antigens have been proposed to influence ovarian effects. The validity of these hypotheses is re-examined in the light of current knowledge. Further investigation of ovarian function in species believed to be resistant to the ovarian effects of anti-ZP vaccines is warranted. To this end, anti-Müllerian hormone may provide a novel tool for the assessment of ovarian function during ZP-based immunocontraception, particularly in wildlife species not amenable to frequent clinical examination.

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1. Introduction

The zona pellucida (ZP) is a complex glycoprotein matrix surrounding the mammalian oocyte and early conceptus. Comprised of either three or four glycoproteins, the ZP plays a pivotal role in the union of oocyte and spermatozoon during mammalian fertilization, arguably the most important joining event in biology. In addition, the ZP functions in the induction of the acrosome reaction, the prevention of polyspermy, and protection of the early embryo [1]. Furthermore, the ZP is intimately involved in communication between the oocyte and its surrounding granulosa cells in the developing follicle [2]. These critical functions of the ZP in reproduction and its tissue-specific nature have encouraged research into its role as an immunocontraceptive for over 40 years [3].

Porcine zona pellucida (pZP) with added adjuvant remains the most common native form of the vaccine due both to the homology between the ZP proteins of many mammalian species and its availability in relatively large quantities [4,5]. Approximately 80 zoo and wildlife species have been successfully contracepted using pZP [6]. Despite this widespread application, relatively little is reported describing the vaccine's mechanism of action. In research

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aimed at humans, initial enthusiasm for ZP-based immunocontraception waned sharply following reports of ovarian dysfunction in rabbits and nonhuman primates [7,8]. A number of hypotheses regarding the causes of ovarian dysfunction during ZP-based immunocontraception have since evolved.

This review revisits initial studies describing ovarian tissue, oocytes, or ZP as immunological agents, on which our current understanding of pZP's mechanism of action is based. In addition, several hypotheses advanced to explain ovarian dysfunction observed during ZP-based immunocontraception are re-evaluated based on relevant literature reporting on common laboratory and domestic animal species, as well as the feral horse, deer, and African elephant.

2. Early work on ZP antigens and antisera: a journey down the memory lane

Interest in immunological methods of fertility control dates back to the late 1890's with an initial focus aimed primarily at testes and spermatozoa as immunizing agents, reviewed by Tyler [9]. Reports of antisera to ovarian homogenates blocking fertilization processes in the sea urchin [10] and frog [11] encouraged interest in the mammalian oocyte and ovary as putative antifertility antigens. Initial studies demonstrated the existence of organ-specific antigens in the guinea pig ovary and testis [12,13]. Early immunofluorescence studies further localized common antigens to the ZP, atretic follicles, and the acrosome of spermatozoa [14].

Ownby et al. [15] injected golden hamster ovarian homogenates combined with Freund's complete adjuvant (FCA) into rabbits. Boosters, consisting of ovary homogenates with Freund's incomplete adjuvant, were followed by weekly serum sampling. The antisera produced included antibodies to at least one antigen unique to the ovary, demonstrated using agar-gel diffusion plates. Superovulated hamster eggs exposed to rabbit antiovary antisera formed a precipitate in the ZP that was visible under light- and phase-contrast microscopy. The precipitated ZP was found to be resistant to digestion by trypsin. Similar findings were reported by Sacco et al. [16].

Antiovarian antiserum, added to hamster ova before exposure to homologous spermatozoa *in vitro*, interfered with sperm–oocyte binding. None of 170 pretreated oocytes was penetrated by spermatozoa, in comparison to nearly half of 58 control oocytes [17]. The use of homogenized oocytes rather than the whole ovary as an immunogen produced similar results [18]. Despite ZP-based immunocontraception being in its infancy, researchers noted the potential advantages of this method of fertility control, including reversibility and the absence of somatic side effects because of the specificity of anti-ZP antibodies [17].

Jilek et al. [3], using mice passively preimmunized with rabbit anti-mouse ovary antisera, demonstrated via immunofluorescence the presence of anti-ZP antibodies bound to oocytes aspirated from antral follicles, as well as ovulated oocytes. This showed that anti-ZP antibodies were capable of reaching the ZP *in situ* within the follicle. In addition, passive immunization was found to decrease fertilization rates from over 91% to below 1%. The authors concluded that the effect on fertility *in vivo* "seems to be a block to sperm penetration through the ZP, on which antibodies were actually detected within the follicles".

Further study of aspirated oocytes and early embryos flushed from the uterus or uterine tubes of untreated hamsters showed that antihamster ovary antiserum precipitated the ZP of preovulatory oocytes as well as early embryonic stages *in vitro*. In addition, precipitation of the ZP following fertilization was thought to inhibit the attachment of transferred embryos to the endometrium [19], possibly as a result of interference with embryonic hatching [20]. In a similar study in mice, antioocyte and anti-ZP antisera had no effect on the development of early embryos to the blastocyst stage *in vitro*, although a small effect on zona shedding was noted [21]. A later study in the same species found that preincubation with anti-ZP antiserum had no effect on early embryonic development and zona hatching *in vitro*, nor implantation and further development of pretreated embryos transferred to pseudopregnant recipients, despite visible precipitation of the zonae [22]. Similarly, mice passively immunized with anti-ZP antiserum 2 days after mating showed no adverse effects on fertility or fecundity, and anti-ZP antisera had no effect on early embryonic development *in vitro* [23].

From these initial studies and those that followed, hypotheses regarding ZP vaccines' mechanism of action evolved as a primarily antibody-based interference with one or more of the following processes: sperm–oocyte binding, the acrosome reaction, sperm movement through the ZP, oocyte activation and/or the zona block; thus, at the level of the periovulatory oocyte. If so, estrous cyclicity and reproductive behaviors should remain unaffected following vaccination. This feature of pZP immunocontraception has been an important, although occasionally controversial, rationale supporting its application in species with complex social hierarchies, such as the African elephant and feral horse [24–28]. The detection, however, of ovarian dysfunction subsequent to treatment with ZP-based vaccines in some species has provided a challenge to researchers hoping to overcome this potentially undesirable outcome. During this process, a number of hypotheses regarding the cause of ovarian dysfunction have developed.

3. Hypotheses explaining ovarian dysfunction during ZP-based immunocontraception

3.1. Glycosylation causes oophoritis

Glycosylation refers to the pattern of binding of distinct carbohydrate moieties to amino acids, giving rise to the glycoprotein structure of the ZP. The diverse nature of ZP glycosylation across species may play a role in the species-specificity of sperm-zona binding [29]. Chemical deglycosylation of pZP3 was found to decrease its antigenicity and immunogenicity; precipitation of ZP *in vitro* by the relevant antisera was more superficial than precipitation produced using antisera to glycosylated pZP3 [30]. Consequently, early workers in the field considered the deglycosylation of ZP antigens to negate pZP's oophoritogenic effects. In rabbits, the degree of glycosylation of pZP was found to correlate directly with the degree of interference with

folliculogenesis, ovulation, and estrous cyclicity [31,32]. Researchers hoped that qualitative, rather than purely quantitative, differences in the immune response to deglycosylated versus glycosylated ZP antigens were responsible for the differences in oophoritogenic effect [32]. Subsequent trials in marmoset monkeys [33] and baboons [34] found that immunization with deglycosylated pZP3 did indeed result in lower levels of ovarian dysfunction compared with glycosylated pZP3; a result that correlated to poorer antibody responses. In addition, antisera to deglycosylated ZP antigens provided poorer contraceptive efficacy *in vitro* [33]. Although now abandoned as a possible means of preventing oophoritis, glycosylation remains a factor to consider when designing synthetic vaccines. For example, Hardy et al. [35] investigated the use of mammalian versus insect expression systems in the production of a recombinant murine ZP3 antigen. Recombinant ZP3 produced in a mammalian expression system caused a transient antifertility effect. However, recombinant ZP3 produced in an insect expression system had no antifertility effect despite evidence of an antibody response, possibly as a result of differences in glycosylation of the protein product. Furthermore, differences in glycosylation may contribute to the reduced immunogenicity associated with some synthetic vaccines, where multiple boosters have been required to maintain adequate antibody titers [36–38].

3.2. Contamination with non-ZP ovarian proteins causes ovarian dysfunction

An early study investigating pZP immunocontraception in cynomolgus monkeys suggested that contamination may have been responsible for the ovarian dysfunction detected, as a result of immune responses directed toward extra-ZP ovarian epitopes [8]. This hypothesis currently remains a commonly advanced argument [39].

Specific binding of anti-ZP antibodies within the developing ovarian follicle, primarily within the ZP, occasionally involving adjacent oolemma or granulosa cells, has been demonstrated in the rabbit [40], cat, dog, horse, and African elephant [41,42]. Similar findings have been demonstrated using immunofluorescence in both primates [43,44] and rabbits [45].

In addition, recombinant and synthetic peptide ZP antigens exclude the possibility of contamination by non-ZP ovarian proteins [46]. A review of the literature describing the use of these vaccines across a number of species includes multiple studies in which synthetic vaccines, including antigens delivered within a virus or as a DNA plasmid vaccine, are nevertheless associated with abnormalities on ovarian histology, including cellular inflammatory infiltration, and/or decreased follicle numbers [38,40,45,47–57]. Although these findings do not completely exclude the possibility of contamination of pZP vaccines as a cause of ovarian dysfunction, these reports suggest that oophoritis or interference with folliculogenesis may be an inherent feature of ZP-based vaccines.

In addition, attempts at purification of pZP to avert ovarian dysfunction have shown limited success in primates [34,58,59]. In the bitch, purified pZP failed to prevent ovarian pathology, although the observed pathology was milder and seen in association with lower antibody titers than that induced by a crude pZP preparation [60,61].

3.3. Freund's adjuvants are linked to ovarian dysfunction

Freund's complete adjuvant consists of a water-in-oil emulsion, incorporating nonmetabolizable oils (paraffin oil and mannide monooleate) and heat-killed *Mycobacterium tuberculosis* cells. Freund's incomplete adjuvant, generally preferred for booster vaccinations, consists of a similar water-in-oil emulsion without mycobacterial cells. Since its initial description nearly 80 years ago, initially incorporating only paraffin oil and mycobacteria (reviewed in [62]), FCA has enjoyed widespread application in immunological research due to its marked efficacy as an adjuvant. However, side effects associated with FCA, particularly granulomatous injection site reactions, have discouraged its commercial use [63].

Concerns regarding false positive results to tuberculosis testing following the use of FCA led to the development of Freund's modified complete adjuvant (FMCA), incorporating *M butyricum* instead of *M tuberculosis*. Although most ZP-based research involving Freund's adjuvants has employed FCA, recent research in horses and African elephants has made use of FMCA. In horses, pZP with FMCA was found to induce antibody titers consistently higher than, although statistically no different to pZP with FCA [64]. Although most researchers likely refer to FCA when discussing Freund's adjuvants, the following discussion encompasses both FCA and FMCA.

A third hypothesis within ZP-based immunocontraceptive research implicates the use of Freund's adjuvants in the pathogenesis of ovarian dysfunction. Early studies in primates detected disturbances of ovarian function in control groups administered Freund's adjuvant alone [59,65]. These findings were later contradicted by investigators who reported an absence of ovarian pathology in adjuvant control groups [33,66]. A similar lack of ovarian effects was found in rabbits administered Freund's adjuvant only, in comparison to saline-treated controls [31,32,67].

In two studies reporting a direct comparison between Freund's and alternative adjuvants in combination with pZP, only the inclusion of Freund's adjuvant was associated with ovarian pathology in two monkey species [66,68]. However, the alternative adjuvants induced antibody titers that were lower, and/or diminished faster than antibody titers induced by Freund's adjuvants.

Furthermore, ovarian dysfunction or oophoritis is clearly not limited to the use of Freund's adjuvants, having been reported in association with a number of non-Freund's adjuvants, including alum [8,61]; muramyl dipeptide (MDP) [34,49,69]; MDP with Morris adjuvant [36,70]; squalene with Arlacel-A (and SPLS; primary vaccination) [38,71]; MDP with squalene and Arlacel-A [52]; and CP20,961 [60,72].

3.4. Cytotoxic T-cells cause oophoritis and ovarian dysfunction

The adaptive immune system is mediated largely by T-helper and T-cytotoxic lymphocytes. T-helper ($CD4^+$)

cells recognize soluble and particulate antigens presented by professional antigen presenting cells, in association with major histocompatibility class (MHC) II molecules. In the classical endogenous pathway, intracellular-derived antigens presented in association with MHC class I molecules are recognized by cytotoxic T-lymphocytes ($CD8^+$; CTL). An alternative pathway has been suggested, whereby dendritic cells present extracellular antigens, which could include ZP antigens, via MHC I [73]. Although T-helper cells play a role in the production of antibody by B-lymphocytes, CTL produce a direct cytotoxic effect. Other cells capable of cytotoxicity include members of the innate immune system such as macrophages and natural killer cells.

Various authors have suggested that a CTL response may be involved in the development of oophoritis during pZP immunocontraception [1,74–77], often citing Rhim et al. [47], Lou et al. [78], and Lou et al. [79] despite no reference to CTL within the cited studies. Few studies have actively attempted to identify CTL responses during ZP-based immunocontraception. In mice, the infiltration of $CD4^+$ and $CD8^+$ T-lymphocytes was demonstrated in ovarian sections using immunohistochemistry, following infection by a murine cytomegalovirus expressing mZP3 [56]. However, the presentation of the antigen by a virus provides a direct link to the classical MHC I T-cell pathway, likely contributing to the $CD8^+$ response. Further work classifying the immune response to ZP vaccines, including the potential role of CTL in the pathogenesis of ovarian dysfunction, is warranted.

What does seem clear is the link between ovarian pathology, a helper ($CD4^+$) T-cell–mediated immune response and an antibody response. Rhim et al. [47] showed that the adoptive transfer of $CD4^+$ T-cells induced an oophoritis despite the absence of any detectable antibody response. This oophoritis was described as interstitial, excluding developing follicles, and did not appear to interfere with ovarian function in mice [50]. In a subsequent study, Lou et al. [51] reported that the presence of anti-ZP antibodies redirected the cell-mediated inflammatory response from the ovarian interstitium to the developing follicles, resulting in profound interference with ovarian function [51].

Furthermore, Lloyd et al. [57] found that immunoglobulin-deficient mice, incapable of mounting an antibody response but otherwise capable of normal adaptive immune responses, showed neither decreased fertility nor abnormalities on electron microscopy of ovarian sections following infection by a recombinant murine cytomegalovirus expressing murine ZP3 [57].

Immunocompetent mice infected with the same virus showed decreased fertility and fecundity, and evidence of abnormal ZP formation and vacuolization of oocytes on electron microscopy of ovarian sections. Although it could be argued that a recombinant ZP-expressing virus would be expected to show differences in immune response mechanisms to the conventional pZP vaccine, this study suggested a pivotal role for antibodies in the development of oophoritis during anti-ZP immunocontraception.

Taken together, these studies demonstrate the roles of both $CD4^+$ T-lymphocytes and antibodies, in unison, in ZP vaccine-induced interference with ovarian function in mice. Whether or not a similar dynamic plays a role in ovarian dysfunction in other species warrants further research.

3.5. Ovarian dysfunction is species-specific

Finally, ovarian dysfunction during ZP-based immunocontraception, characterized as a cellular oophoritis and/or interference with folliculogenesis has been described as a species-specific complication. Antibody responses to ZP-based vaccines show some variation between individuals within a species, and likely represent variation in the overall immune response to vaccination. Reasons for individual variation in immune response include factors influencing the physiological status of the individual during or after vaccine administration, including physiological stress, nutritional status, and the presence of concurrent systemic conditions. In addition, genetic differences in immune response to immunocontraceptive vaccines have been suggested to play a role [80].

Significant species differences in terms of the endurance of antibody titers have also been well documented. To illustrate this, Dall sheep maintained significant antibody titers for over 3 years following an initial pZP vaccination regime (primary plus booster) [81], whereas Muntjac deer required biannual boosters to maintain antibody levels [82].

A review of nine species groups (mice, rabbits, nonhuman primates, dogs, cats, sheep, deer, horses, and African elephants), arguably the most studied species to date in terms of ZP-based immunocontraception, revealed evidence of abnormal cyclicity in each one of these species following treatment, with the notable exception of the cat and African elephant (Table 1). Cats are refractory to pZP, showing neither cyclic disturbances nor any effect on fertility [92,93]. One study examining estrous cyclicity during pZP immunocontraception (2–3 years after the start of treatment) in African elephants included evidence of anestrus in a proportion of treated cows [106]. Although this effect was possibly ascribable to seasonal effects, the lack of controls complicates any definitive conclusions.

In horses, ovarian inactivity following pZP treatment, certainly in the short term, appears to have been undetected for over 20 years. An initial study of pZP in horses found no significant evidence of abnormal ovarian function following short-term treatment [101]. Similarly, Powell et al. [102] found no differences in estrous cycle characteristics between pZP-treated and untreated mares. Although researchers noted depressed excretory steroid levels and slower reversal of infertility following prolonged ($\geq$3 years) treatment [103,104], the mechanism of action of pZP immunocontraception in mares as an antibody-based interference with conception at the level of the oocyte remained until recently the generally accepted dogma in this species. Bechert et al. [105] compared two long-acting pZP vaccine formulations in mares. Treated mares demonstrated lower serum progesterone levels, smaller ovaries, and fewer follicles than control mares; 93% of treated mares ceased estrous cyclicity within 4 months of treatment. Similarly, six of seven mares treated with two doses of the conventional pZP vaccine demonstrated

Table 1
Overview of the literature reporting on ovarian dysfunction during zona pellucida–based immunocontraception.

Species	No evidence of ovarian dysfunction detected[a]	Evidence of ovarian dysfunction detected[a]	Inconclusive evidence of ovarian dysfunction detected	Fertility rates[b] reported for the relevant vaccine formulation, within the cited studies
Mice	[74,83–85]			9%–100%
		[48,50,53,55–57,86,87]		0%–100%
Rabbits	[7,54]			100%
		[7,31,32,40,45,54,88]		0%–30%
Nonhuman primates	[36,37,49,68,70,89–91]			25%–100%
		[8,33,34,36,49,52,58,59,68–71,89]		0%–50%
Cats	[92–94]			16%[c]–100%
Dogs	[38,60,72]			100%
		[60,61,72]		0%–34%
Sheep	[95]			100%
		[95]		0%
White-tailed deer	[96–99]			0%–95%
			[100]	11%
Horses	[39,101,102]			0%–57%
		[39,103–105]		0%–8%
African elephants			[106]	0%

[a] Evidence of ovarian dysfunction includes histological evidence of oophoritis or decreased follicle numbers, or behavioral or hormonal evidence of abnormal estrous or menstrual cyclicity.
[b] Expressed as a proportion of the control group, where control groups are available.
[c] Not statistically different to control group.

periods of anestrus posttreatment, characterized by baseline serum progesterone levels and a lack of follicular development [39]. In part, the discrepancy between the latter two studies and other, earlier studies might be explained by differences in antigen dose rates (100 μg [39] and 200 μg of a single administered formulation [105], compared with 65 μg [107]). In addition, most trials observed feral horses with associated constraints on clinical monitoring [27].

In white-tailed deer, ongoing estrous behaviors during pZP immunocontraception have been reported by a number of studies [96–98]. Yet, a study showing depressed progesterone levels following pZP treatment suggests that ovarian inactivity may nevertheless be a feature of pZP immunocontraception in this species [100]. The dose of pZP administered in the latter study (300–500 μg) was higher than that reported in other deer studies (65 μg; [96,97,108]). This, however, suggests that ovarian suppression is dose-dependent, rather than species-dependent. In a later study, fewer normal secondary follicles were detected in recently revaccinated does in comparison to controls and does vaccinated 2 years previously, although sample sizes were low [99]. A third study anticipated observing increased mating behavior in pZP-treated does but found no differences in behaviors, including estrus and dominance behaviors, between treated and untreated groups of fallow deer [109]. Given the similar difficulties in estrus detection between feral horses and deer, further study of the ovarian effects of pZP in deer is warranted.

In summary, ovarian suppression may be an inherent feature of effective ZP-based immunocontraception, associated with the generation of elevated antibody titers over a prolonged period of time and contributing to the vaccine's contraceptive effect, rather than a species-specific response. However, further research to confirm this hypothesis is indicated.

4. The link between antibody titers, contraceptive efficacy, and ovarian dysfunction

Although not an absolute rule, a recurring theme throughout the literature is the apparent link between the immunogenicity of a ZP vaccine with the antifertility capabilities of the vaccine and the presence of ovarian dysfunction. In the dog [60], sheep [95], and rabbit [7,32,67], ovarian pathology and contraceptive efficacy showed a direct correlation when comparing two or more alternative formulations. Similarly, in primates, significant antifertility efficacy is associated with evidence of ovarian dysfunction [70,71,89]; with the converse also demonstrated (poor antifertility effect with no evidence of ovarian effects) [90,91]. Finally, in the cat, the absence of contraceptive efficacy accompanies a consistent lack of ovarian effects [92–94].

These observations support the hypothesis that ovarian dysfunction is an inherent feature, or at least a component, of the mechanism of action of ZP-based vaccines [46,60]. To the best of the authors' knowledge, no vaccine formulation, whether native or synthetic, has yet achieved near complete contraceptive efficacy without some evidence of ovarian effects following further study. Attempts at vaccine design, aimed at the inclusion of B-cell epitopes while excluding putative oophoritogenic T-cell epitopes, have indeed shown decreased ovarian effects but, again, limited contraceptive success [70,79,83–85]. To further complicate matters, ovarian effects may inadvertently be missed. Periods of ovarian dysfunction may be transient and their detection consequently dependant on the timing of sampling interventions for ovarian histology in relation to vaccine administration [61]. In addition, the difficulty associated with the detection of cellular inflammatory infiltrates in ovarian tissue sections under light microscopy has been demonstrated. In an initial study, no

inflammatory infiltrate was detected in ovaries observed under light microscopy [86]. However, in a follow-up study using the same population and methodology, lymphocytic infiltration of the ovary was detected using immunohistochemistry [56]. The value of immunohistochemistry over conventional histopathology in detecting oophoritis was confirmed by Bagavant et al. [52].

Possible causes of interrupted folliculogenesis, leading to ovarian atrophy and anestrous, include immune-mediated follicular destruction and interference with normal follicle development [60]. Destruction of oocytes and follicles could occur as a result of antibody-mediated complement activation. Few studies have investigated the role of complement in ZP-based immunocontraception. In an early study, mice were treated with solubilized hamster ZP followed by superovulation and the flushing of oocytes from the oviducts. Antibody and complement binding to oocytes was assessed using fluorescein-conjugated rabbit anti-mouse IgG, and rabbit anti-mouse complement C3 followed by fluorescein-conjugated goat anti-rabbit IgG, respectively. In both tests, bright immunofluorescence was detected on oocytes from immunized animals, with no immunofluorescence detected on control oocytes [110]. In contrast, no complement binding was visualized in ovarian sections from immunocontracepted dogs; however, the small sample size and the lack of a positive control for the complement-binding assay were major limitations to the study [61].

Alternatively, or additionally, the immune response might alter ZP structure and function in developing follicles, affecting communication between the growing oocyte and its surrounding granulosa cells [88]. This scenario is mimicked in mice lacking the functional gene for connexin 37, one of a family of proteins involved in intercellular communication between oocytes and granulosa cells. Folliculogenesis is inhibited in connexion 37-deficient mice. In addition, ovaries show abnormal accumulations of luteinized tissue [111], possibly resembling that described in ZP-treated mice [87], rabbits [40,45,67], and primates [8,36]. This mechanism may be particularly plausible in the dog, where pZP treatment caused the formation of ovarian cysts associated with prolonged estrogen secretion [61].

4.1. Conclusions

The advantages of ZP-based immunocontraception include its efficacy as a contraceptive agent in many species, safety during pregnancy, reversibility (at least in the short term), and freedom from major side effects [112]. Apart from ovarian senescence, extensive macroscopic and microscopic postmortem examinations of the major organ systems in pZP-treated mares failed to reveal any pathology that could be linked solely to pZP [105]. The vaccine can be remotely delivered, important for use in feral species [113]. Furthermore, the protein nature of the vaccine precludes its entry into the food chain [29]. Permanent sterility, if indeed this could be induced by pZP vaccination with a degree of reliability, may be a desirable side effect in certain so-called pest species [46]. Importantly, pZP vaccination appears to show minimal adverse effects on animal welfare, particularly when compared to alternative means of population control such as culling [28]. No adverse behavioral or social effects were detected in a long-term study of pZP immunocontraception in elephants [114]. In the feral horse, pZP immunocontraception has been associated with both enhanced longevity and body condition [115]. Furthermore, the vaccine showed few significant effects on social behaviors and time budgets in horses [116,117]; one study reported an increased frequency of reproductive behaviors and a lengthening of the breeding season, which should be considered during the management of immunocontracepted herds [118]. Furthermore, studies to confirm the lack of effects on behavior and welfare are warranted.

In most species studied, annual and sometimes biennial boosters are required if the contraceptive effect is to be maintained. From a practical point of view, particularly in free-ranging species like the African elephant, this requires considerable time and resource investment. A long-acting pZP formulation which induces antibodies titers that are maintained for 2 years or longer after a single treatment would be a major advantage. A series of studies investigated the use of lactide-glycolide polymers to form pellets incorporating pZP. The release delay, depending on the ratios of lactide and glycolide in the pellets, was either 1, 3 or 12 months. Hand-injected pellets in combination with a conventional primary dose of pZP resulted in antibody titers that were sustained at contraceptive levels for 21 to 22 months. The contraceptive effect was evident for 2 years; a result which was promising [119]. An entirely different approach to achieve long-lasting antibody titers and thus contraception has been the use of a liposomal formulation consisting of lecithin and cholesterol and emulsified with FMCA or lyophilized and then reconstituted with FMCA [105]. Both formulations produced sustained antibody titers in mares (monitored for 22 weeks) and induced cyclic changes alluded to earlier. Moreover, the latter formulation maintained antibody titers for at least 7 years in African elephant cows [120]. The emulsified formulation has previously been tested in gray seals [121] and deer [122], both producing infertility that was maintained over 3 years or longer. Bechert et al. [105] proposed a number of possible mechanisms that may be responsible individually or, more likely, collectively for the sustained antibody titers. These were the sustained release of antigen from the injection site, increased production of long-lived plasma cells in the bone marrow, and mobilization of follicular dendritic cells within draining lymph nodes. Self-boosting by zona capsules in developing follicles was also mentioned but, in the absence of local adjuvant, seemed unlikely and would also apply to other pZP formulations. Although these results are extremely encouraging, the longer term effects on ovarian function have not been investigated satisfactorily. Reversibility and thus lack of long-term ovarian effects in species like the African elephant are an extremely important feature of this treatment.

The commercial availability of serum anti-Müllerian hormone (AMH) assays may provide access to a novel method of monitoring the ovarian effects of immunocontraception in females. This hormone, secreted by the

granulosa cells of, primarily, preantral, and early antral follicles, has been correlated to antral follicle counts and ovarian reserve in mice [123], cattle [124], and women [125]. Recently, AMH levels measured in mares during the course of 1 year of their immunocontraception with either pZP or GnRH vaccines were compared. Although the GnRH vaccine had little effect on AMH levels, pZP suppressed serum AMH in the short term (Joonè et. al., in preparation). Given the proposed link between low AMH levels and ovarian suppression in these mares, AMH may prove useful in species not amenable to direct clinical examinations of their reproductive organs. Likewise, pZP vaccination may provide an exciting tool for the study of AMH and its relationship to follicular dynamics and the ovarian reserve.

In conclusion, this review suggests a re-evaluation of dogmas that have emerged within the field of ZP-based immunocontraception, regarding these vaccines' ovarian effects. The suggestion that ovarian suppression could be an inherent feature of effective ZP-based immunocontraception, across all species, requires further investigation. Nevertheless, pZP remains a valuable, practical, and humane means of population management with application in a number of mammalian species.

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Ovarian function and pregnancy outcome in pony mares following immunocontraception with native and recombinant porcine zona pellucida vaccines

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Summary

Reasons for performing study: Few studies have investigated ovarian function in the mare undergoing porcine zona pellucida (pZP) immunocontraception despite reported ovarian dysfunction in other species.
Objectives: This study aimed to describe ovarian function and oestrous cyclicity in pony mares following treatment with either the conventional pZP vaccine or a novel recombinant form of the vaccine derived from porcine ZP3 and ZP4 (reZP). In addition, the contraceptive efficacy of pZP vs. reZP was assessed.
Study design: Blinded, randomised, prospective clinical trial.
Methods: Mares (n = 21) were randomised into 3 groups of 7: *Group I* received the pZP vaccine, with a booster 5 weeks later; *Group II* received the reZP vaccine, with a booster 5 weeks later; and *Group III* (controls) received 2 treatments, 5 weeks apart, of saline and adjuvant alone. Mares underwent weekly monitoring via transrectal palpation and ultrasound examination of the reproductive tract, with daily monitoring during oestrus. Data were collected over a 24 week period coinciding with the physiological breeding season; treatments commenced in Week 4. Serum samples were obtained for antibody titres and ovarian steroid level analyses at 7 day intervals. Cycling mares were bred via fresh semen artificial inseminations over a maximum of 2 consecutive oestrous cycles, commencing 5 weeks post booster vaccination.
Results: Control mares cycled throughout the trial. After treatment, 6 of 7 pZP mares (86%) and one reZP mare (14%) had an extended anoestrus that correlated with basal serum oestradiol and progesterone levels. All mares resumed cyclicity by 10 months post treatment. Pregnancies were diagnosed in all controls, 4 reZP- (57%) and none of the pZP-immunised mares.
Conclusions: The current study demonstrates the reversible suppression of ovarian function in pony mares following treatment with pZP. The effect of the reZP vaccine on pregnancy outcome requires further investigation.

Keywords: horse; ovarian function; anoestrus; progesterone; oestradiol; artificial insemination

Introduction

Investigation of porcine zona pellucida (pZP) as an immunocontraceptive in the mare began over 20 years ago [1]. In contrast to immunocontraceptive vaccines targeting gonadotrophin releasing hormone (GnRH), which causes reproductive quiescence [2], pZP has traditionally been associated with continued oestrous cyclicity [3]. The maintenance of reproductive behaviours has made the pZP vaccine the preferred immunological method of population control in species with complex social structures, such as the feral horse (*Equus caballus*) and the African elephant (*Loxodonta africana*) [4,5].

Research on pZP as a human antifertility vaccine waned following evidence of ovarian dysfunction in nonhuman primates [6]. Similar effects are reported in the rabbit, dog and sheep [7–9]. In contrast, little evidence for interference with ovarian function has been reported in the mare. In a previous study, 1 year of pZP treatment was found to have no effect on ovarian function [1]; however, longer periods of contraception, specifically ≥3 years, were associated with declining oestradiol levels and ovulation rates in feral horses of the USA [10,11]. A subsequent study in the same population reported no association between the incidence of ovulatory failure in mares and their duration of treatment [12]. Recently, investigators demonstrated ovarian inactivity in 13 of 14 mares within 4 months of treatment with single-dose pZP vaccine formulations [13].

A recombinant zona pellucida vaccine may provide potential advantages compared with native pZP vaccines that include production efficiency and the avoidance of contamination with non-ZP proteins [14]. Recently, recombinant vaccines based on the expression of porcine ZP3 and ZP4 in *Escherichia coli*, hereafter referred to as reZP, were developed [15].

The current study aimed to describe ovarian function and oestrous cyclicity in pony mares following treatment with either native pZP or reZP vaccines. In addition, the contraceptive efficacy of reZP in the mare was investigated.

Materials and methods

Mare management

Twenty-one Nooitgedacht pony mares, aged between 3 and 14 years and of variable parity, were studied from October 2013 to March 2014, coinciding with the physiological breeding season in the southern hemisphere [16]. Inclusion criteria were nonpregnant status, good physical and reproductive health and no previous immunocontraceptive exposure. Ponies were housed in outdoor grass paddocks, with free access to water and *Eragrostis tef* grass hay. Clinical examinations were performed weekly and mares were weighed using an electronic scale during Weeks 1, 8 and 24 (Table 1).

Vaccines

Native pZP vaccine was prepared according to standard methods [1,11][a]. Aliquots of 1 mg purified pZP in phosphate buffered saline (PBS) were

TABLE 1: Mare distribution according to parity, age (median (range)), and body weight (median (range)) for each study group

Mare information	*Group I*: pZP (n = 7)	*Group II*: reZP (n = 7)	*Group III*: controls (n = 7)	P value
Nulliparous	4	3	5	0.6
Foaled within last 3 years	3	2	1	0.5
Foaled >3 years ago	0	2	1	0.3
Age (years)	7 (4, 10)	8 (3, 13)	6 (3, 14)	0.8
Body weight (kg) at Week 1	416 (360, 503)	396 (329, 433)	436 (360, 473)	0.2
Body weight (kg) at Week 8	396 (352, 495)	405 (333, 433)	405 (361, 433)	0.6
Body weight (kg) at Week 24	435 (382, 515)	439 (359, 467)	445 (369, 472)	0.7

pZP, porcine zona pellucida vaccine; reZP, recombinant zona pellucida vaccine.

transferred to glass vials and lyophilised, sealed and stored at 4°C. Before vaccination, each vial was reconstituted with 5 ml sterile injection water with a final protein concentration of 200 μg/ml.

The reZP vaccines, TT-KK-ZP3 and bRNase-KK-ZP4, were supplied by Dr Satish Kumar Gupta (Reproductive Cell Biology Laboratory)[b]. Porcine ZP3 (amino acid (aa) residues 20–344) was expressed as a chimeric fusion protein encompassing a promiscuous T-cell epitope of tetanus toxoid (TT; aa residues 830–844) at its N-terminus and separated from ZP3 by a dilysine linker (TT-KK-ZP3) in *E. coli* [15]. Similarly, porcine ZP4 (aa residues 22–462) was expressed in *E. coli* as a chimeric fusion protein incorporating a promiscuous T-cell epitope of bovine RNase (bRNase; aa residues 94–104; bRNase-KK-ZP4) [15]. Recombinant proteins were purified from inclusion bodies followed by refolding, as described previously [17]. Recombinant TT-KK-ZP3 and bRNase-KK-ZP4 were dialysed separately in 20 mmol/l Tris pH 6.0 and the respective protein concentration estimated using a BCA Protein Estimation Kit[c] and adjusted to 500 μg/ml.

Study design

Mares were stratified by age and randomly assigned to one of 3 treatment groups; the primary investigator was blinded to treatment assignment. Treatments were administered into the gluteal muscles, commencing in Week 4, as follows.

Group I (n = 7) received a primary vaccination (V1) consisting of 100 μg (0.5 ml) pZP emulsified with 0.5 ml Freund's modified complete adjuvant (FMCA). Five weeks later, a booster (V2) consisting of 100 μg pZP emulsified with 0.5 ml Freund's incomplete adjuvant (FIA) was administered into the contralateral hindquarter.

Group II (n = 7) received 2 primary vaccinations (V1), one on each side of the hindquarters, consisting of 250 μg (0.5 ml) recombinant ZP3 and ZP4 proteins, respectively, each emulsified with 0.5 ml FMCA. Five weeks later, 2 boosters (V2) consisting of the same doses of recombinant ZP3 and ZP4 emulsified with 0.5 ml FIA, were similarly administered.

Group III (n = 7, control group) received an initial treatment (V1) consisting of 0.5 ml sterile saline emulsified with 0.5 ml FMCA. Five weeks later, a second treatment (V2) consisting of 0.5 ml sterile saline emulsified with 0.5 ml FIA was administered into the contralateral hindquarter.

Transrectal monitoring of the reproductive tract

Mares underwent examination by transrectal palpation and ultrasonography of the reproductive tract at 7 day intervals. In cycling mares, examinations coincided with Days 7 and 14 of consecutive oestrous cycles, with daily monitoring from Day 14 until ovulation (Day 0). Day 0 was defined by the ultrasonographic detection of a *corpus luteum*, correlated with the absence of a dominant follicle identified on the previous day. Ultrasound examinations were performed using a portable ultrasound machine (A6V)[d] and a 3–8 MHz linear array rectal probe.

Ovarian dimensions were estimated digitally and recorded in 3 perpendicular planes. Ovarian volumes were calculated using the prolate ellipsoid formula (length × height × width × 0.523) [18]. Identifiable structures on each ovary were recorded and follicles ranked according to approximate diameter (<15 , 15–20 and 20–25 mm). Follicles ≥25 mm in diameter were individually measured from the ultrasonographic image of the follicle at its maximum using the electronic calliper function of the ultrasound machine. The average of 2 perpendicular diameter measurements, one of which represented the widest diameter of the follicle, was recorded as the follicle diameter [19]. Anoestrus was defined as bilaterally small ovaries (both ≤25 cm^3), scant follicular development and the absence of any follicles ≥15 mm in diameter [20].

Artificial inseminations

All cycling mares were bred by artificial insemination over a maximum of 2 consecutive oestrous cycles using fresh semen collected from a single stallion of proven fertility, commencing ≥5 weeks post V2. Inseminations were performed according to standard practices once a mare's ovulation was adjudged imminent, i.e. a preovulatory follicle ≥35 mm together with maximal or decreasing endometrial oedema [21]. Semen doses consisted of ≥1 × 10^9 progressively motile spermatozoa, extended 1:1 in a prewarmed skim milk (MCT) medium[e]. Semen motility was evaluated subjectively under light microscopy. Semen concentration was quantified using a photometer calibrated for use with equine semen (Spermacue)[f]. Inseminations were repeated if a mare failed to ovulate within 72 h. Pregnancy diagnoses by transrectal ultrasound examination were performed 14 days post ovulation. If pregnant, mares were excluded from further breeding and sampling.

Blood samples for hormonal assays and antibody titre determination

Blood samples from all mares were collected by jugular venipuncture at 7 day intervals. In cycling mares, sampling coincided with Days 0, 7 and 14 of the mares' oestrous cycles. Samples were centrifuged and serum stored at -20°C until required.

Serum progesterone and oestradiol assays

Serum progesterone and oestradiol levels were determined by means of radioimmunoassay (Coat-A-Count progesterone and oestradiol kits)[g] [22]. Assay sensitivities for progesterone and oestradiol were 0.06 nmol/l and 29 pmol/l, respectively. For progesterone, intra- and interassay coefficients of variation were 6.1%, 3.5% and 4.7% and 10.3%, 4.3% and 5.2% for low, medium and high concentrations, respectively. For oestradiol, intra- and interassay coefficients of variation were 7.0%, 4.3% and 4.0%, and 8.1%, 6.8% and 4.2% for low, medium and high concentrations, respectively.

Antibody response

Anti-ZP antibody response was measured by enzyme immunoassay (EIA), using a modification of a method previously described [2]. Briefly, 96-well plates (MaxiSorp, cat. no. NUN430341)[h] were incubated at 2–8°C for 16 h with 1 μg purified pZP in 100 μl coating buffer (2.94% $NaHCO_3$, 1.59% Na_2CO_3, pH 9.6) per well. Plates were washed with PBS containing 0.05% Tween 20 and then blocked with 0.03% BSA in PBS for 16 h at 2–8°C. Plates were then incubated with serial dilutions (1:1000 to 1:8000 for test samples and 1:1000 to 1:64,000 for positive reference serum) of standard and test serum samples at 37°C for 1 h. The positive reference serum consisted of pooled sera from all 7 individuals in *Group I* at expected maximal antibody titre (4 weeks post V2). Blank wells were used as negative controls. After washing, antibodies were detected by incubating plates with recombinant protein G-horseradish peroxidase[i] at 37°C for 1 h. After further washing, plates were developed with trimethylene blue (SureBlue™; cat. no. 52-00-03)[j]. The reaction was stopped by adding 50 μl of 2 mol/l H_2SO_4 per well. Absorbance at 450 nm was measured using a microplate photometer (Multiskan™ FC)[k].

Antibody response was measured as the mean sample absorbance (minus blank) expressed as a proportion of the mean absorbance (minus blank) of the positive reference sample at the same dilution for each plate (1:2000; 1:4000; 1:8000). The overall proportion positive (PP) was calculated as the average value over the 3 dilutions.

Monitoring injection sites

All mares were monitored daily for visible lesions, including heat and swelling, and weekly by palpation of the approximate injection sites. Transcutaneous ultrasonography of the injection site area was performed when indicated by clinical findings. Monitoring continued following completion of the study as part of the routine care of experimental animals.

Reversibility

All mares underwent examinations at 3 and 6 months following the trial's completion to monitor reproductive activity. Teasing of mares continued after the study period as part of their routine management.

Data analysis

Data were assessed for normality through the plotting of histograms, calculation of descriptive statistics, and the Anderson–Darling test for normality, which was performed in commercially available software (MINITAB Statistical Software, Release 13.32)[l]. Categorical data were compared among treatment groups using Chi-square or Fisher exact tests in available freeware (Epi Info, version 6.04)[m]. The maximum oestradiol values and mean progesterone values before and after V2 were extracted for each mare and used for the statistical comparison among groups. Quantitative data satisfying the normality assumption were subsequently compared among groups using one-way ANOVA. Non-normal data were compared using Kruskal–Wallis tests followed by pairwise Mann–Whitney *U* tests with correction of P values for multiple *post hoc* tests. A linear mixed model was used to estimate the effect of treatment group and time on antibody responses measured as proportion of the positive control. 'Horse' was included as a random effect to account for the repeated sampling design. Mixed effects models were analysed in commercially available statistical software (IBM SPSS Statistics Version 22)[n]. Bonferroni adjustment was used to adjust for multiple *post hoc* testing and significance was set at $P \leq 0.05$.

Results

Transrectal monitoring of the reproductive tract

All mares demonstrated cyclic ovarian activity prior to V2, although one mare in *Group II* showed a period of anoestrus between normal oestrous periods prior to commencing treatment.

In *Group I* (pZP), one mare cycled regularly throughout the study period. Four mares demonstrated anoestrus within 5 weeks of V2 that persisted until the end of the study. One showed anoestrus from 12 weeks post V2 until study completion, while another cycled erratically, characterised by one brief period of oestrus between prolonged periods of anoestrus.

In *Group II* (reZP), one mare entered anoestrus within 5 weeks of V2, persisting until study completion. The remaining 6 mares cycled regularly throughout the study period.

In *Group III* (controls), 6 mares demonstrated regular cyclic activity throughout the study. One developed a persistent *corpus luteum* of unknown cause, which resolved spontaneously.

By Week 16 (7 weeks post V2, prior to any positive pregnancy diagnoses), left and right ovary follicle counts and maximum follicle diameters in *Group I* were significantly lower than in *Group III*. There were no significant differences in *Group II* between either *Group I* or *Group III* for these data points, suggesting an intermediate effect (Table 2).

Serum progesterone and oestradiol profiles

Mean progesterone profiles of *Groups I*, *II* and *III* mares prior to, and more than 5 weeks following, booster vaccination (V2) are shown in Figures 1 and 2, respectively. The mean progesterone concentrations before and after V2 for the 3 groups were: 20.4 vs. 6.4 nmol/l (*Group I*), 20.8 vs. 19.0 nmol/l (*Group II*) and 24.8 vs. 25.3 nmol/l (*Group III*). There were no significant differences in average progesterone concentrations between groups prior to V2 ($P = 0.6$); thereafter the change in average concentrations was significantly different among groups ($P = 0.05$). *Group I* had the largest average difference in progesterone values but *post hoc* pairwise comparisons did not indicate significant differences compared with *Groups II* and *III* ($P = 0.2$ and $P = 0.07$, respectively).

The mean for the maximum oestradiol concentrations measured before and after V2 for the 3 groups were: 42.0 vs. 6.8 pmol/l (*Group I*), 37.1 vs. 19.8 pmol/l (*Group II*) and 51.5 vs. 27.1 pmol/l (*Group III*). There were no significant differences in maximum oestradiol concentrations between groups prior to V2 ($P = 0.6$); thereafter the change in maximum concentrations in *Group I* was significantly different to that in *Group III* ($P = 0.01$), with no significant differences, in terms of the change in maximum oestradiol concentrations, between either between either *Groups I* and *II* ($P = 0.2$) or *Groups II* and *III* ($P = 0.8$).

Antibody response

Samples from *Group I* and *II* mares prior to the first vaccination and *Group III* mares at all 4 sampling times (pre-V1, four weeks post V1, four weeks post V2 and ten weeks post V2), effectively negative serum controls, showed a mean (± s.d.) optical density (OD) of 0.0841 (± 0.0218). This mean was statistically no different from the mean of all blank wells ($P = 0.2$; independent *t* test). All samples following immunisation with pZP (*Group I*) or reZP (*Group II*) rendered ODs that were greater than this mean plus 2 standard deviations.

Anti-ZP antibody response, expressed as proportion positive values as described in materials and methods, varied by treatment group ($P<0.001$) and time ($P<0.001$), with the time effect also varying by treatment ($P<0.001$). Proportion positive values for *Group I* were significantly higher than those of *Group II* ($P<0.001$), and those of *Group II* were significantly higher than those of *Group III* ($P = 0.006$; Fig 3) post V2.

Pregnancy outcome

In *Group I*, only 4 inseminations were performed owing to the paucity of oestrous cycles available. In *Group II*, one mare showed anoestrus throughout and could not be bred. A total of 11 and 9 inseminations were performed in *Groups II* and *III*, respectively. The proportion of pregnancies achieved in *Groups I*, *II* and *III* were 0%, 57% and 100%, respectively. Comparison of these proportions for *Groups I* and *III* was significant ($P<0.001$), with no significant difference detected between *Groups I* and *II*, nor between *Groups II* and *III* ($P = 0.07$ and 0.2, respectively).

Injection site reactions

No lameness or pyrexias were recorded. Swelling and/or palpable changes in muscular density at injection sites was detected in 20/21 mares post treatment. Overt, sterile abscessation occurred in 3 mares, all from *Group II*. Ultrasonography performed at the end of the study showed lesions affecting ≥one hindquarter in 17 of the 18 remaining mares. Lesions varied from mild changes in muscular architecture to poorly marginated areas of complex echogenic pattern ≤8 cm in width. A follow-up ultrasonographic examination 3 months later showed a distinct improvement in the appearance of lesions in 11 of these mares.

Reversibility

All mares that had demonstrated anoestrus following treatment had resumed oestrous cyclicity by 10 months post V2, based on follow-up oestrous monitoring or teasing records.

Discussion

The traditionally accepted mechanism of action of pZP in the equid involves, primarily, the interference of anti-ZP antibodies in sperm–zona binding, leading to contraception with continued oestrous cyclicity [1,23]. The current study, however, demonstrated suppression of ovarian function in 6 of 7 pony mares following pZP treatment, characterised by small,

TABLE 2: Results of transrectal monitoring of the reproductive tract for 21 pony mares prior to and following treatment with either pZP (*Group I*), reZP (*Group II*) or saline (*Group III*)

			Group I (n = 7)	*Group II* (n = 7)	*Group III* (n = 7)	P values‡
Pretreatment*	Week 1†	Left ovary volume (cm^3)	150.6 (52.3, 401.7)	78.5 (4.2, 153.8)	83.7 (28.2, 627.6)	0.5
		Right ovary volume (cm^3)	28.2 (6.3, 83.7)	6.3 (1.6, 94.1)	100.4 (9.4, 585.8)	0.1
		Left ovary follicle count	3 (0, 6)	3 (0, 6)	4 (2, 8)	0.4
		Right ovary follicle count	3 (1, 6)	3 (0, 3)	5 (1, 7)	0.3
		Maximum follicle diameter (mm)	30.3 (15.0, 56.1)	46.5 (0.0, 48.9)	45.1 (22.4, 55.8)	0.2
	Week 2†	Left ovary volume (cm^3)	58.6 (18.8, 150.6)	18.8 (4.2, 205.0)	50.2 (14.1, 131.8)	0.7
		Right ovary volume (cm^3)	28.2 (18.8, 78.5)	15.7 (4.2, 52.3)	18.8 (6.3, 418.4)	0.6
		Left ovary follicle count	4 (3, 7)	2 (2, 4)	3 (1, 9)	0.3
		Right ovary follicle count	4 (2, 6)	3 (2, 4)	3 (1, 6)	0.4
		Maximum follicle diameter (mm)	33.2 (20.0, 56.4)	28.1 (15.0, 48.9)	31.8 (12.0, 66.9)	0.8
	Week 3†	Left ovary volume (cm^3)	62.8 (9.4, 267.8)	18.8 (2.1, 205.1)	33.5 (6.3, 267.8)	0.6
		Right ovary volume (cm^3)	18.8 (6.3, 78.5)	15.7 (6.3, 78.5)	25.1 (18.8, 179.4)	0.3
		Left ovary follicle count	4 (0, 9)	2 (0, 4)	4 (1, 13)	0.9
		Right ovary follicle count	3 (1, 8)	3 (0, 6)	7 (3, 10)	0.09
		Maximum follicle diameter (mm)	42.1 (15.0, 49.2)	36.1 (15.0, 52.5)	34.5 (21.2, 49.1)	0.7
Post treatment*	Week 14†	Left ovary volume (cm^3)	23.5 (6.3, 78.5)	78.5 (18.8, 179.4)	78.5 (6.3, 94.1)	0.3
		Right ovary volume (cm^3)	12.6 (6.3, 78.5)	28.2 (18.8, 94.1)	18.8 (6.3, 50.3)	0.5
		Left ovary follicle count	1 (0, 6)	1 (0, 4)	3 (1, 6)	0.5
		Right ovary follicle count	1^{a} (0, 5)	$2^{a,b}$ (0, 6)	6^{b} (2, 9)	0.008
		Maximum follicle diameter (mm)	15.0 (0.0, 36.5)	18.0 (0.0, 47.6)	30.2 (15.0, 46.5)	0.8
	Week 15†	Left ovary volume (cm^3)	18.8 (4.2, 131.8)	23.5 (6.3, 150.6)	58.6 (8.4, 153.8)	0.5
		Right ovary volume (cm^3)	9.4 (6.3, 28.2)	18.8 (6.3, 205.0)	25.1 (9.4, 153.8)	0.1
		Left ovary follicle count	1^{a} (0, 2)	3^{b} (0, 8)	$2^{a,b}$ (0, 4)	0.02
		Right ovary follicle count	1^{a} (0, 4)	$2^{a,b}$ (0, 4)	5^{b} (2, 7)	0.006
		Maximum follicle diameter (mm)	10.0 (0.0, 48.3)	15.0 (10.0, 49.2)	25.0 (15.0, 42.3)	0.5
	Week 16†	Left ovary volume (cm^3)	23.5 (6.3, 50.2)	41.8 (18.8, 205.0)	131.8 (2.1, 205.0)	0.2
		Right ovary volume (cm^3)	12.6 (6.3, 31.4)	28.2 (6.3, 205.0)	25.1 (18.8, 153.8)	0.1
		Left ovary follicle count	0^{a} (0, 3)	$2^{a,b}$ (0, 4)	3^{b} (0, 7)	0.04
		Right ovary follicle count	0^{a} (0, 2)	$2^{a,b}$ (0, 5)	5^{b} (3, 8)	0.001
		Maximum follicle diameter (mm)	0.0^{a} (0.0, 22.0)	$20.0^{a,b}$ (0.0, 53.1)	40.3^{b} (16.0, 53.4)	0.006

*All data are reported as median (range). †If more than one data point for a mare existed during a particular week, the data point exhibiting the greatest follicle diameter was included. ‡For data points with superscripts, medians without superscripts in common are statistically different at P≤0.05 after adjustment for multiple *post hoc* testing.

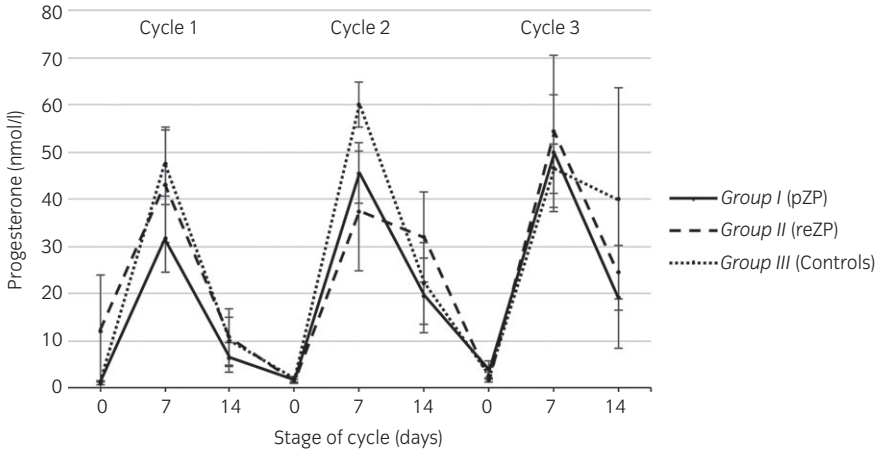


Fig 1: Graph showing mean weekly serum progesterone levels (s.e. bars) for each study group over 3 consecutive oestrous cycles prior to V2, where Days 0, 7 and 14 of each cycle have been synchronised in time.

inactive ovaries and basal ovarian hormone levels. The discrepancy between our findings and that of an earlier report of unaltered oestrous cyclicity during short-term treatment of mares with conventional pZP vaccine [1] may be due to the higher pZP dose administered in our study (100 μg vs. 65 μg pZP), selected to reflect the current dose administered to feral horses [24–26]. Our findings confirm recently reported ovarian quiescence in mares treated with long-acting pZP vaccines [13], suggesting that suppressed ovarian function is not unique to long-acting formulations.

Previous studies on the effects of pZP on behaviour and social structure in feral horse populations, at the same dose of pZP, suggested that treated

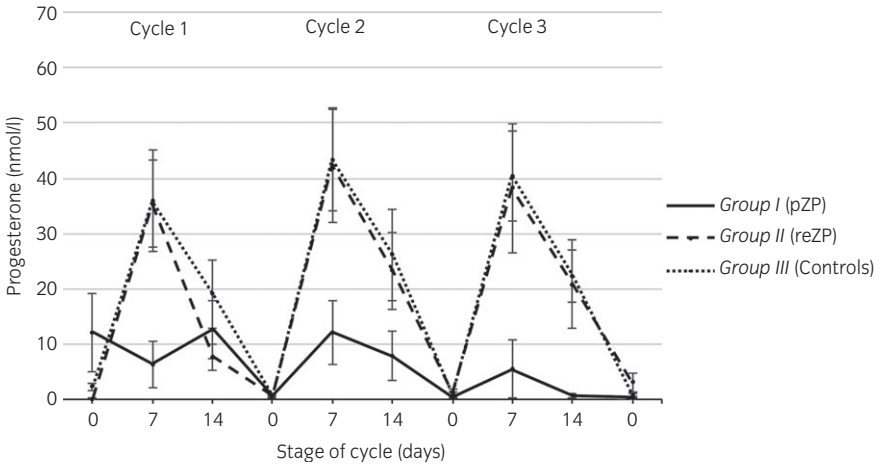


Fig 2: Graph showing mean weekly serum progesterone levels (s.e. bars) for cycling mares in each study group over 3 consecutive oestrous cycles ≥5 weeks post V2, where Days 0, 7 and 14 of each cycle have been synchronised in time. Weekly data are depicted for noncycling mares.

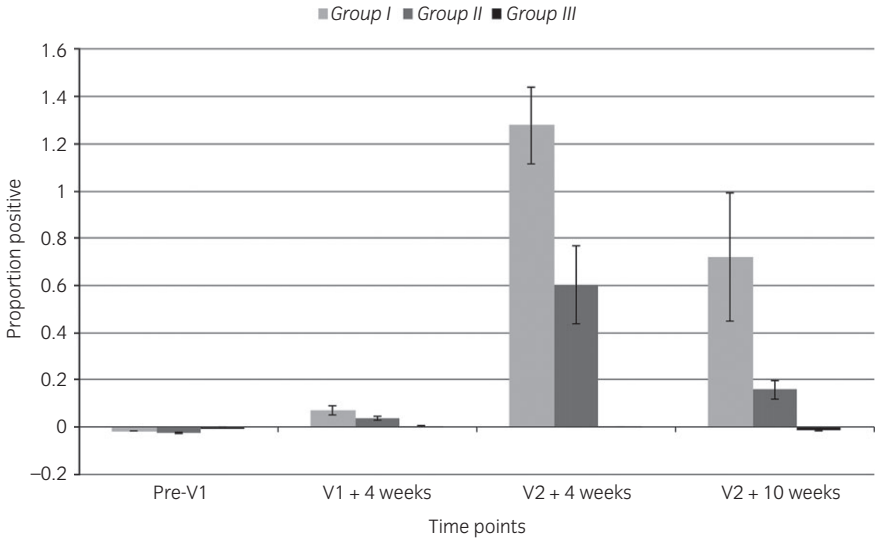


Fig 3: Mean anti-ZP antibody response expressed as a proportion of the positive control (with s.e.) for each treatment group at 4 successive time points.

mares show decreased harem fidelity and increased reproductive behaviours [27,28]. These findings are inconsistent with the current study, in which the majority of mares showed anoestrus following treatment, implying that there would be an opposite change (albeit transiently) in reproductive behaviours. The absence of these behaviours can be attributed to the significant decrease in follicular number and sizes, associated with decreased oestradiol concentrations.

Follicle counts and maximum follicle diameters for *Group II* showed no statistically significant differences from either *Group I* or *Group III* in Week 16, despite significant differences between the latter 2 groups. This partial effect parallels *Group II*'s intermediate antibody titres post V2. The pZP vaccine comprises all 3 native porcine zona glycoproteins (ZP2, ZP3 and ZP4), whereas reZP comprises only ZP3 and ZP4. The lower antibody titres post V2 in *Group II* compared with *Group I* may be due to the fact that the pZP vaccine will elicit an antibody response against ZP2 as well as ZP3 and ZP4, and the EIA read-outs using pZP antigen will reflect the summation of antibody titres against ZP2, ZP3 and ZP4. Ideally, antibody titres against purified ZP3 and ZP4 should be assessed to determine whether antibody titres are responsible for the intermediate ovarian response observed in *Group II*.

Further studies, involving either the administration of higher doses of the recombinant vaccine or using native pZP as the primary injection followed by reZP booster injections, are warranted. A third possibility would be to increase the number of booster vaccinations. Recently, it was shown that 2 boosters of recombinant dog ZP3, instead of one, showed better contraceptive efficacy in female mice [31].

An unexpected finding was the prevalence of injection site reactions, supporting Bechert *et al.* [13] who reported injection site reactions in 43% of treated mares. Our findings failed to support anecdotal reports of injection site reactions occurring less frequently when administered into the gluteal rather than the neck musculature [32,33]. The current findings, including the sterility of overt abscesses, also contradict previous reports linking abscessation to remote vaccine delivery, presumed to result from darts transferring dirt and bacteria into the subcutaneous tissues [34]. Reports of injection site reactions in feral populations, described as abscessation, varied from 0 to 11.5% and were associated with either Freund's complete adjuvant (FCA), (FMCA) or (FIA) [1,33,35–37]. Our use of domestic mares enabled closer inspection than can be achieved in a feral horse population. Although individuals from all groups showed lesions, *Group II* was particularly over-represented. This may be a result of either or both the double volume of FMCA and FIA in their vaccination protocol or the tetanus toxoid and bovine RNase linked to the ZP3 and ZP4 recombinant proteins, respectively.

The contraceptive efficacy of the pZP vaccine was confirmed in this study; however, the absence of oestrous cyclicity appears to be responsible for infertility to a larger extent than interference with sperm–zona binding. In addition to species differences in response, contamination with non-zona pellucida ovarian proteins has been proposed as a possible cause of ovarian malfunction in other species [29]. The latter cannot be completely ruled out for the pZP vaccine, although such contamination is impossible with the use of recombinant vaccines. Apart from oophoritis, a possible mechanism of ovarian suppression could be an interference with cellular communications between the developing oocyte and its surrounding granulosa cells, as a result of immune-mediated alterations to the zona pellucida. A family of proteins known as connexins is involved in oocyte–granulosa cell communication. Connexin gene-knockout mice were found to demonstrate suppressed ovarian activity with a lack of tertiary follicular development, reminiscent of the findings of the current study in mares [30].

All mares exhibiting anoestrus following treatment showed evidence of cyclic activity within 10 months of V2, which confirms the reported reversibility of pZP vaccines [38]. In the current study, follow-up examinations coincided partially with winter, thus the effect of seasonal anoestrus in biasing resumption of cyclicity remains undefined.

Conclusion

The current study demonstrates the reversible suppression of ovarian function in 6 of 7 (86%) pony mares following treatment with the native pZP vaccine. No significant contraceptive effect was produced by the (reZP) vaccine; however, further investigation of recombinant ZP vaccines, as an alternative contraceptive in the mare, is warranted.

Authors' declaration of interests

No competing interests to declare.

Ethical animal research

The study was approved by the University of Pretoria's Animal Ethics Committee (V051-13).

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Authorship

C.J. Joonè, H.J. Bertschinger and M.L. Schulman contributed to the study design, study execution, data analysis and interpretation, preparation and final approval of the manuscript. S.K. Gupta contributed to study design and preparation of the manuscript. A.P. Arukha and V. Minhas prepared the recombinant vaccines and were involved in final approval of the manuscript. E. Dieterman contributed to the acquisition of data. G.T. Fosgate is an epidemiologist who contributed to study design, data analysis and interpretation, and preparation of the manuscript.

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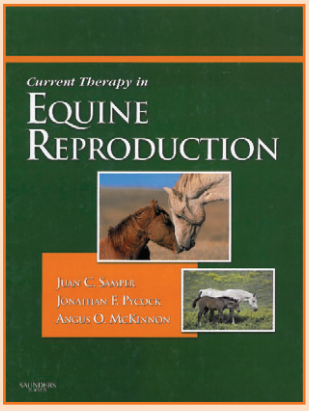
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Ovarian suppression following PZP vaccination in pony mares and donkey jennies

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Immunocontraceptive vaccines target one or more selected proteins essential to a critical step in fertility. Examples include anti-gonadotrophin release hormone (GnRH) and native porcine zona pellucida (PZP) vaccines. The successful application of PZP vaccines over several decades for population management in feral horse populations in the USA is well described. The currently hypothesised mechanism responsible for this vaccine's contraceptive effect is the prevention of sperm-zona binding, sperm penetration of the zona and thus fertilisation of the oocyte, which is prevented for as long as antibody titres are sufficiently high whilst treated females continue to cycle normally. Our group's recent research projects investigated PZP vaccination of pony mares in South Africa and donkey jennies in the Caribbean. These trials included intensive clinical and laboratory monitoring and clearly showed that the majority (85.7%) of both mares and jennies treated with the vaccine formulated with either Freund's complete modified (primary) or Freund's incomplete (booster) adjuvants developed small inactive ovaries. This was accompanied by baseline serum progesterone concentrations typical of anoestrus. All mares resumed normal cyclic activity after approximately 7-8 months during the following physiological breeding season. We additionally measured the concentrations of anti-Müllerian hormone (AMH) at strategic times during the mare study to better define the mechanism of ovarian shut-down. Notably, AMH, which is reportedly well correlated with small antral follicle counts in the mare, was not detectable during the intervals of ovarian suppression. Of further interest was an AMH rebound observed coincident with the recovery of ovarian cyclicity. As normal ovarian function was re-established in all mares, an inflammatory process (with or without cellular invasion) seems an unlikely explanation for this ovarian response. These results showed vaccination was followed by a period of ovarian suppression related to the loss or malfunction of small antral follicles and suggested that prevention of fertilisation was not the only potential mechanism responsible for infertility in PZP-immunocontracepted mares.

Accepted Manuscript



Serum anti-Müllerian hormone dynamics in mares following immunocontraception with anti-zona pellucida or -GnRH vaccines

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Serum anti-Müllerian hormone dynamics in mares following immunocontraception with anti-zona pellucida or -GnRH vaccines

Abstract

Circulating anti-Müllerian hormone concentration (AMH) is positively correlated to the number of small growing follicles in the mare and may reflect ovarian function. Dynamics of AMH during immunocontraception have not previously been investigated. This study aimed to compare serum AMH in mares following treatment with native porcine zona pellucida (pZP), recombinant pZP3 and pZP4 (reZP) or gonadotrophin releasing hormone (GnRH) vaccines, and saline-treated controls. Stored sera collected during two previous studies examining ovarian activity in mares during zona pellucida (ZP) or GnRH immunocontraception were analysed for serum AMH. Data were compared among treatment groups using mixed-effects linear regression and one-way ANOVA with post hoc testing. Correlations between AMH and previously reported clinical variables were estimated using Spearman's rho. Mares immunized against GnRH showed variable but detectable AMH throughout successive breeding and non-breeding seasons that were not significantly different to unvaccinated control mares. Mares treated with pZP demonstrated marked, reversible suppression of AMH. Mares immunized using reZP showed an intermediate effect. In the ZP study, AMH was positively correlated to serum progesterone concentrations, mean ovarian volumes and antral follicle counts, whereas no correlations between AMH and serum progesterone concentrations, mean ovarian volumes, or the presence of one or more follicles $\geq$ 20 mm in diameter were detected in the GnRH study. In conclusion, marked suppression of AMH during pZP immunocontraception, but not during GnRH immunocontraception, suggested enhanced suppression of ovarian follicular development

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and, or follicular function during pZP immunocontraception. Serum AMH concentrations may provide a novel tool for the assessment of ovarian function during ZP-based immunocontraception.

1. Introduction

The porcine zona pellucida (pZP) and gonadotrophin releasing hormone (GnRH) vaccines are effective forms of immunocontraception in the mare [1]. The pZP vaccine induces an immune response to endogenous zona pellucida (ZP) glycoproteins surrounding the mammalian oocyte. This has been the preferred vaccine for population control in species with complex social structures, such as the feral horse (*Equus caballus*), due to pZP's association with the maintenance of reproductive cyclicity [2]. In contrast, GnRH antibodies deplete effective GnRH within the hypothalamic-pituitary portal blood vessels, inhibiting GnRH-induced release of LH and FSH from the anterior pituitary and culminating in reproductive quiescence. This vaccine, therefore, shows promise as a means of preventing unwanted reproductive behaviours in the domestic horse [3].

The ovarian effects of pZP immunocontraception in the mare have been assessed clinically (transrectal palpation and ultrasonography), histologically (ovarian histology) and endocrinologically (measurements of ovarian steroids or their metabolites) [4-8]. Initial reports observed no ovarian effects following short-term pZP treatment [4]. Longer intervals (up to 7 years) of anti-pZP immunization resulted in decreased ovarian oestrogen production and ovulation rates [5, 9]. More recently, ovarian inactivity was detected in $\geq$ 86% of mares within four months of treatment with both the conventional pZP vaccine [8] and a single-dose formulation of pZP [7]. The latter two studies highlighted a need for further study of the mechanism of action of ZP-based vaccines in the mare.

The ovarian effects of GnRH-based immunocontraception in the mare have similarly been assessed both clinically and endocrinologically [10-14]. Immunisation against GnRH was significantly associated with decreased ovarian weight [10], length [12] and volume [10, 14]; decreased ovarian activity (the absence of corpora lutea and follicles $\geq$ 10 mm) [10-15]; suppressed or variable oestrus expression [10, 12, 13, 15, 16]; and suppressed progesterone [10, 12-15], oestradiol-17β [13, 15], LH [10, 11, 13] and FSH secretion [10].

Measurement of serum anti-Müllerian hormone concentration (AMH) may represent a novel method for the assessment of ovarian function during immunocontraception in mares and other species. This homodimeric glycoprotein hormone, a member of the TGFβ family of growth and differentiation factors, is expressed by granulosa cells of developing follicles in the post-natal ovary [17]. In the mare, follicles between 6 and 20 mm in diameter have the greatest influence on AMH [18]. Positive correlations between AMH, antral follicle count (AFC) and primordial follicle count are reported in mice [19], cattle [20] and women [21]. Similarly, AMH is positively correlated with AFC in mares over eight years of age and this correlation strengthens with increasing age [18].

This study aimed to investigate AMH in mares following their active immunisation against one of GnRH, native pZP or a combination of recombinant pZP3 and pZP4 (reZP; [22]) vaccines. Measured AMH was then compared to previously-published clinical variables, including mare age, serum progesterone concentrations, mean ovarian volumes and AFC or the presence of follicles $\geq$ 20 mm in diameter [8, 14, 23].

2. Materials and methods

2.1 Experimental design

Specimens were obtained from two previously-reported controlled studies in the mare that described the ovarian effects and reversibility of native pZP, recombinant porcine ZP3 and ZP4 [8] and GnRH vaccines [14, 23]. Briefly, native pZP (Trumpeter Farms and Veterinary Services, Winters, California, USA) was prepared according to standard methods [4]. The reZP vaccine (Dr. Satish Gupta; Reproductive Cell Biology Laboratory, National Institute of Immunology, New Delhi, India) consisted of pZP3 and pZP4 expressed by *Eschirichia coli* as chimeric fusion proteins linked to promiscuous T-cell epitopes of tetanus toxoid (TT-KK-ZP3) or bovine RNase (bRNase-KK-ZP4), respectively [22]. The GnRH vaccine is commercially available (Improvac; Pfizer Animal Health, Sandton, South Africa).

For the ZP vaccine study, mares were stratified according to age and randomly assigned to one of three study groups: pZP, reZP and saline-treated controls (n = 7 per group) [8]. All treatments commenced during the southern hemisphere summer, consisting of a primary vaccination incorporating Freund's modified complete adjuvant (V1) and a single booster with Freund's incomplete adjuvant (V2) 35 days later. Mares were monitored by transrectal palpation and ultrasound examinations of their internal reproductive tracts. Recorded variables included ovarian volumes, AFC (grouped according to size) and the presence of corpora lutea. Blood samples were collected weekly, centrifuged and sera stored at – 20°C until analysed. Follow-up gynaecological examinations and blood sampling occurred during the following winter and again at the start of the subsequent breeding season. Seven and four mares became pregnant in the control and reZP groups respectively.

For the GnRH study, mares were stratified according to age and randomly assigned to either GnRH vaccine (n = 9) or saline-treated control (n = 3) groups [14, 23]. Treatments similarly commenced during the southern hemisphere summer and consisted of a primary vaccination (V1) followed by a

booster (V2) 35 days later. Mares underwent three similar gynaecological examinations (to the ZP vaccine study) at V1, V2 and five weeks post-V2. Recorded variables included ovarian volumes and the presence of corpora lutea and follicles $\geq$ 20 mm in diameter. Blood samples were similarly acquired individually until resumption of cyclic ovarian activity, with sera being stored at – 20°C until analysed.

2.2 Data comparison between ZP and GnRH studies

Treatments for both ZP and GnRH studies commenced within a similar 6-week period of the year (12 November - 22 December). In order to compare data across studies while minimising temporal effects, data were normalised according to vaccination status within the following periods: period I correlated to pre-V1 to within one month post V1 (3 - 22 December), period II correlated to between V2 and within one month post-V2 (2 - 29 January), period III correlated to between one and two months post-V2 (12 February - 6 March), period IV correlated to the first winter post-treatment with a vaccination status of four to six months post-V2 (3 May - 26 June). Period V correlated to the approximate commencement of the following breeding season (29 Sep 2007, GnRH study; 6 October 2014, ZP study), or later, depending on the date of resumption of cyclicity (up to 3 October 2008, GnRH study) [12]. Anoestrus was defined as the period when mares had small ovaries (<45 cm^3) with follicles $\leq$20 mm in diameter and a progesterone concentration below 6 nmol/l. For the ZP study, anoestrus was further qualified by the absence of an ultrasonographically visible corpus luteum.

2.3 Anti-Müllerian hormone assay

Anti-Müllerian hormone concentrations were determined by means of a commercially available ELISA according to the manufacturer's instructions (AMH Gen II ELISA; Beckman Coulter, Brea, CA, USA). This assay has been previously validated for use in mares [24]. Briefly, standard dilutions, controls and samples were incubated in an anti-AMH antibody coated microtitration plate. Samples were sequentially incubated with biotin-labelled anti-AMH antibody, streptavidin-horseradish peroxidase and tetramethylbenzidine. An acidic stop solution halted further colour development. Absorbance was measured at 450 nm using a microplate photometer (Multiskan™ FCd, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and AMH concentrations determined by comparison to a calibration curve. The detection limit of the assay was 0.08 ng/ml. Intra- and inter-assay coefficients of variation were 3.7% and 4.4% respectively, for a low AMH concentration (3.82 ng/ml), and 3.4% and 4.0% respectively, for a high AMH concentration (16.45 ng/ml).

2.4 Statistical analysis

AMH concentrations below the assay detection limit of 0.08 ng/ml were assigned values of 0.07 ng/ml for statistical modelling. The normality assumption was assessed by calculating descriptive statistics, creating histograms, and performing the Anderson-Darling test for normality in commercial software (MINITAB Statistical Software, Release 13.32; Minitab Inc, State College, Pennsylvania, USA). Measured AMH values violated the normality assumption and were transformed using the natural logarithm prior to statistical analysis. The AMH values were compared among treatment groups using mixed-effects linear regression. Regression models included fixed effect terms for treatment group, sampling time (periods I to V), the interaction between treatment group and sampling time, and age categorized as <5, 5- 9, and >9 years of age to adjust for potential confounding. Horse identity was included as a random effect to account for the repeated measures sampling design. The complete model was followed by independent models for each treatment

group to evaluate changes in AMH over time using a linear mixed model incorporating a random effect for horse to account for the repeated sampling. One-way ANOVA was used to compare AMH among treatment groups within each sampling time. Post-hoc testing was adjusted for multiple comparisons using Bonferroni correction of P values. Correlations between AMH and previously reported variables, including mare age, serum progesterone concentrations, mean ovarian volumes, AFC (ZP study) or the presence of one or more follicles $\geq$20 mm in diameter (GnRH study), were estimated using Spearman's rho, across all time periods. Statistical testing was performed using commercially available software (IBM SPSS Statistics Version 23; International Business Machines Corp., Armonk, NY, USA) and results were interpreted at the 5% level of significance.

3. Results

According to the linear mixed model, measured AMH differed among treatment groups ($P < 0.001$) and there was a significant treatment by time interaction ($P < 0.001$). The pZP group was lower than all other groups ($P < 0.04$). The reZP group had lower AMH values than the GnRH-treated group ($P = 0.004$) but neither of the control groups ($P > 0.73$). The AMH values of the GnRH-treated group showed no difference to either control group ($P > 0.63$).

Data were further analysed within each time period (Table 1). Median AMH in the pZP group was below the detection limit of the assay, and significantly lower than that of control mares, at time periods II, III and IV. The reZP group demonstrated an intermediate effect between the pZP and control groups at time periods II, III and IV.

At time period V, AMH differed between the four groups ($P = 0.02$). Descriptively, the pZP and GnRH groups had similar concentrations that appeared different from the reZP and control groups, which

158 also appeared similar to one another. However, no pairwise differences were significant after
159 adjusting P values for the multiple post hoc testing.

160 Finally, data were analysed over time, within treatment groups (Table 1). Anti-Müllerian hormone
161 concentration in the control group changed significantly over time (P = 0.04). Although no pairwise
162 comparisons to time period I were significant, AMH tended to be lower at time period II (P = 0.07;
163 Figure 1a). In the pZP group, AMH showed a marked change over time (P < 0.001), characterised by
164 marked suppression of AMH at time periods II to IV. Furthermore, AMH increased significantly at
165 time period V in this group, with AMH exceeding 6 ng/ml in two mares (Figure 1b). In contrast, the
166 GnRH group showed a gradual increase in AMH over time (P = 0.001; Figure 1c). The reZP group
167 showed no significant change in AMH over time.

168 No correlation between AMH and age was detected in either vaccine study alone or in combination
169 (P > 0.2). In the ZP study (pZP and reZP), AMH was positively correlated to serum progesterone
170 concentrations (Spearman's rho = 0.244, P = 0.03; Figure 2) and mean ovarian volumes (Spearman's
171 rho = 0.391, P < 0.001; Figure 3). Moreover, AMH had a strong positive correlation to AFC
172 (Spearman's rho = 0.713, P < 0.001; Figure 4). In the GnRH study, AMH showed no correlation to
173 previously reported serum progesterone concentrations (P = 0.9), mean ovarian volumes (P = 0.2), or
174 the presence of one or more follicles $\geq$ 20 mm in diameter (P = 0.7).

175

176 **4. Discussion**

177 This is the first report describing variations in AMH in mares following their immunocontraception
178 with either pZP, reZP or GnRH vaccines. Marked differences in AMH of ZP-immunocontracepted
179 mares, particularly pZP, in comparison to GnRH vaccine-treated mares, were observed.

Post-treatment, AMH in pZP-treated mares was markedly suppressed and significantly lower than GnRH-treated and control mares. Clinically, six of seven pZP-treated mares demonstrated intermittent to persistent anoestrus during this time, characterised by bilaterally small ovaries, baseline serum progesterone concentrations and low AFC [8]. Decreased AMH during pZP-immunocontraception may be due to decreased AFC and, or downregulation of AMH expression by granulosa cells. The strong correlation between AMH and AFC in the ZP study supported the hypothesis that decreased AFC is a component of suppressed AMH.

Ovarian suppression following anti-pZP vaccination in the mare has been ascribed to immune-mediated destruction of developing follicles or inhibited folliculogenesis following antibody-mediated interference with oocyte-granulosa cell communication [7]. In the latter hypothesis, aberrant communication between the oocyte and its surrounding granulosa cells may lead to compromised competence of the developing follicle and diminished AMH expression. Expression of genes involved in steroidogenesis and follicular development, in relation to AMH, has been investigated in equine follicles. Mares with low AFC or circulating AMH were found to show decreased granulosa cell expression of AMH and AMH- and FSH-receptors [25]. A further study to examine molecular and hormonal changes within follicles during pZP immunocontraception is warranted.

In the mare, contraception as an outcome of anti-pZP vaccination has been shown to be reversible in the short term, associated with decreased antibody titres below a threshold level [4]. In feral populations however, prolonged treatment (up to 7 years) has been associated with prolonged intervals to reversal [5, 26]. Interestingly in the current study, in pZP-treated mares, AMH was markedly elevated in two mares at time period V, suggestive of a rebound effect of heightened folliculogenesis approximately concurrent with their reversal. Whether a similar resurgence in AMH occurred in the remaining five pZP-treated mares before or after the date of sampling is unknown. In

other species, particularly women, AMH has shown promise as an indicator of ovarian reserve, defined as the pool of primordial follicles [21, 27]. Assuming that a similar correlation between AMH and ovarian reserve exists in the mare, pZP immunocontraception may represent one instance where this correlation is rendered invalid.

The reZP-treated group showed an effect intermediate between the pZP-treated and control mares in terms of antibody titres, AFC and maximum follicle diameters [8]. Similarly, their AMH values were intermediate between pZP and control groups between one and 6 months post-V2. This finding encourages further research aimed at improved efficacy of reZP in mares, such as the administration of additional booster vaccinations [28] or alternative formulations such as the incorporation of liposomes [7].

Anoestrus during GnRH immunocontraception has previously been compared to winter anoestrus in the mare [10, 15]. Both are characterised by low bioavailability of GnRH associated with near-complete suppression of LH secretion. In contrast to LH, serum FSH levels are suppressed but detectable during both anti-GnRH vaccination [10] and winter anoestrus [29, 30], suggesting a non-GnRH dependent component of FSH secretion. Moreover, the ongoing presence of small antral follicles has been reported during anoestrus, whether induced via season [30] or anti-GnRH vaccination [11, 12]. In the current study, AMH values in GnRH-treated mares were similar to those in controls at each successive time period, likely due to continued AMH secretion by small antral follicles. However, AMH in GnRH-treated mares increased gradually over time, a pattern not detected in control mares. Increased AMH in anti-GnRH treated mares over time could be due to increased AFC or more efficient AMH production by granulosa cells. Increased AFC could result from either decreased atresia of growing follicles or increased recruitment of growing follicles from the ovarian reserve. Anti-Müllerian hormone was shown to play an important inhibitory role in primordial follicle recruitment, demonstrated using AMH *null* mice in which depletion of the

228 primordial follicle pool occurred at a faster rate than wild type mice [31]. Increased primordial
229 follicle recruitment as a hypothesis for increased circulating AMH concentrations in anti-GnRH-
230 treated mares is therefore unlikely. In goats [32] and women [33], transient decreases in AMH
231 following exogenous FSH administration was suggested in association with FSH-induced
232 development of large antral and preovulatory follicles, temporarily decreasing the number of smaller
233 follicles. A reversal of this process possibly occurs during anti-GnRH vaccination in the mare, where
234 anoestrous periods are characterised by the absence of follicular development to preovulatory
235 stages, resulting in decreased depletion of the population of preantral and small antral follicles.
236 Unfortunately, clinical data regarding AFC within the GnRH study included only the presence or
237 absence of follicles $\geq$ 20 mm in diameter. Since populations of follicles smaller than 20 mm in
238 diameter appear to correlate better to circulating AMH concentrations [18], limited conclusions can
239 be drawn. More detailed and frequent AFC assessments during GnRH immunocontraception are
240 required. Interestingly, a novel role for AMH as a stimulator of GnRH secretion was recently
241 demonstrated in mice [34]. Whether or not lowered GnRH levels during anti-GnRH vaccination
242 affects AMH over time due to a disrupted feedback mechanism between AMH and GnRH warrants
243 further research.

244 Mares show great individual variation in AMH [18, 35]. However, AMH values are repeatable within
245 mares, showing minimal changes throughout the oestrous cycle and gestation [35]. In the current
246 study, AMH in the control group were similarly variable and comparable to previously reported AMH
247 values in cycling and, or pregnant mares [18, 35]. Moreover, AMH in control mares, including four
248 mares pregnant at time periods IV and V, did not change significantly over time. The non-significant
249 decrease in AMH at time period II is of uncertain significance. Anti-Müllerian hormone
250 concentrations during the non-breeding season in the mare have not previously been reported. In
251 goats, a similarly seasonal breeder, AMH remains stable across seasons [32]. Although the findings

252 of the current study suggested minimal variation in AMH throughout the breeding and non-breeding
253 seasons in mares, both the unavailability of clinical data in controls from the GnRH study and
254 pregnancies in several mares from the ZP study at time periods IV and V resulted in a small sample
255 of relevant mares at these time periods, limiting valid conclusions. An important limitation of the
256 results presented here is the relatively small number of horses studied and the potential for low
257 power for the statistical tests to identify clinically important differences in AMH concentrations.

258

259 **5. Conclusion**

260 In mares, pZP immunocontraception caused a profound, temporary suppression of AMH. In contrast,
261 AMH values were maintained and rose gradually during GnRH immunocontraception. Maintenance
262 of AMH during seasonally-induced anoestrus, although suggested by current findings, requires
263 further study. The analysis of AMH provided a novel modality for the assessment of ovarian function
264 during ZP-based immunocontraception of domestic mares, potentially valuable in wildlife and feral
265 species where repeated gynaecological examinations are not feasible.

266

267 **Word count: 4493**

268

269 **Figure legends**

270 Fig. 1. Line graphs of serum anti-Müllerian hormone concentrations in a) saline-treated control
271 (n=10), b) native pZP-treated (n=7), and c) GnRH vaccine-treated (n=9) mares, over five consecutive
272 time periods. A bar graph depicting the percentage of mares showing oestrous cyclicity at each time

period, derived from data reported previously (for graph (a), n = 3 at time periods IV and V), is superimposed. Arrows depict approximate treatment times (V1 and V2). Asterisks indicate time periods at which AMHs are significantly different to time period I.

Fig. 2. Correlation between serum anti-Müllerian hormone concentration (AMH) and serum progesterone concentration in mares from the ZP study. Serum progesterone concentration was positively correlated (Spearman's rho = 0.244, P = 0.03) to AMH.

Fig. 3. Correlation between serum anti-Müllerian hormone concentration (AMH) and mean ovarian volume in mares from the ZP study. Mean ovarian volume was positively correlated (Spearman's rho = 0.391, P < 0.001) to AMH.

Fig. 4. Correlation between serum anti-Müllerian hormone concentration (AMH) and antral follicle count in mares from the ZP study. Antral follicle count was positively correlated (Spearman's rho = 0.713, P < 0.001) to AMH.

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293 **Table 1**

294 Mean (95% confidence interval)* serum anti-Müllerian hormone concentrations (AMH; ng/ml) in mares over five consecutive time periods during
295 anti-ZP or -GnRH vaccination or saline (control) treatment.

Time period	Treatment					P value†
	pZP (n=7)	reZP (n=7)	GnRH (n=9)	Control 1 (n=7)	Control 2 (n=3)	
I	0.80 (0.48, 1.35)	0.57 (0.26, 1.29)	0.70 (0.29, 1.67)	1.01 (0.56, 1.82)	0.82 (0.28, 2.45)	0.8
II	0.08[a] (0.05, 0.13)	0.29[b] (0.14, 0.61)	0.80[c] (0.46, 1.38)	0.33[b,c] (0.19, 0.56)	0.93[b,c] (0.17, 5.06)	<0.001
III	0.07[a,**]	0.29[b] (0.14, 0.63)	1.07[c] (0.74, 1.53)	1.26[c] (0.66, 2.41)	0.50[b,c] (0.12, 2.07)	<0.001
IV	0.10[a] (0.04, 0.21)	0.29[a,b] (0.10, 0.81)	1.54[c] (0.96, 2.47)	0.54[b,c] (0.19, 1.51)	0.48[a,c] (0.01, 29.8)	<0.001
V	1.86[a] (0.72, 4.84)	0.67[a] (0.22, 1.99)	1.81[a] (1.53, 2.14)	0.55[a] (0.22, 1.36)	NA	0.02
P value‡	<0.001	0.1	0.001	<0.001	0.763	

296 pZP, porcine zona pellucida vaccine; reZP, recombinant porcine zona pellucida vaccine; GnRH, anti-GnRH vaccine. NA = no animals.

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297 *Mean and confidence interval calculated on the natural logarithm transformed data and then back-transformed into the natural scale for
298 presentation.

299 †Based on 1-way ANOVA comparing AMH among groups within each sampling time. Medians without superscripts in common are significantly
300 different after post-hoc testing incorporating Bonferroni correction of P values.

301 ‡Based on mixed effects linear regression comparing AMH over time within each treatment group independently including a random effect for
302 horse to account for the repeated sampling.

303 **All values below the detection limit and not possible to estimate a confidence interval

304

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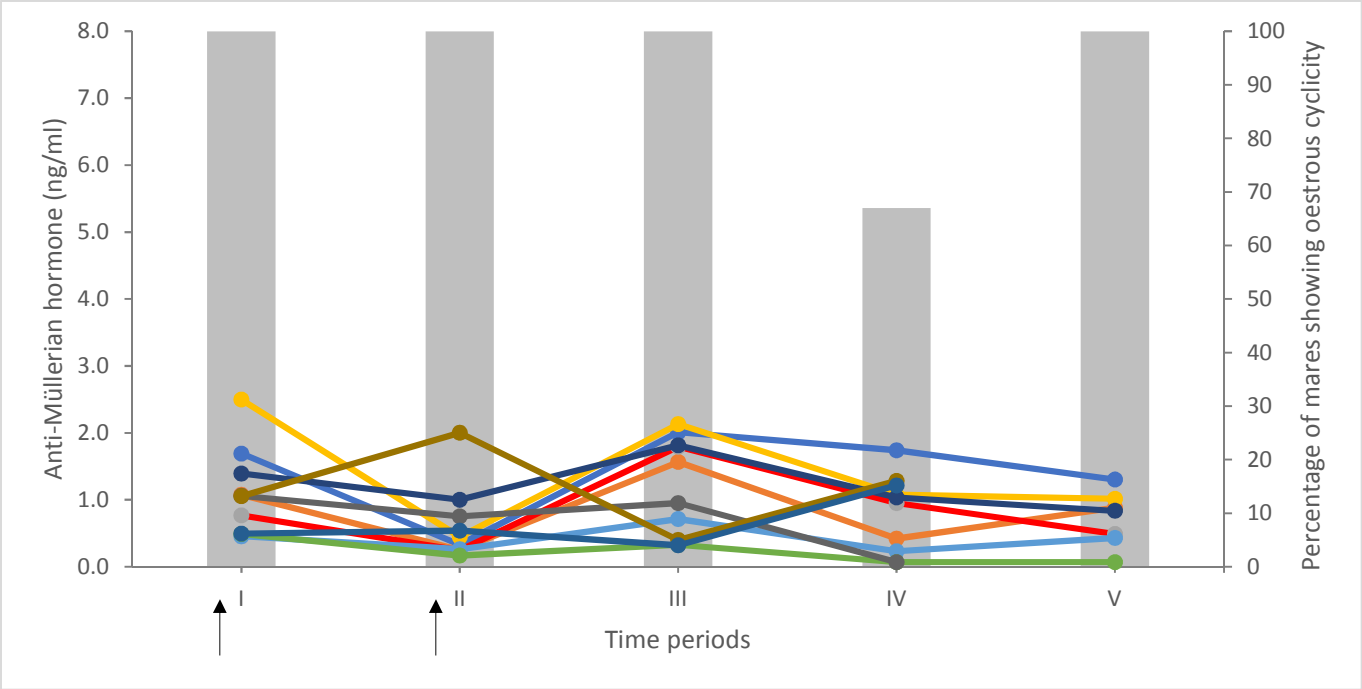
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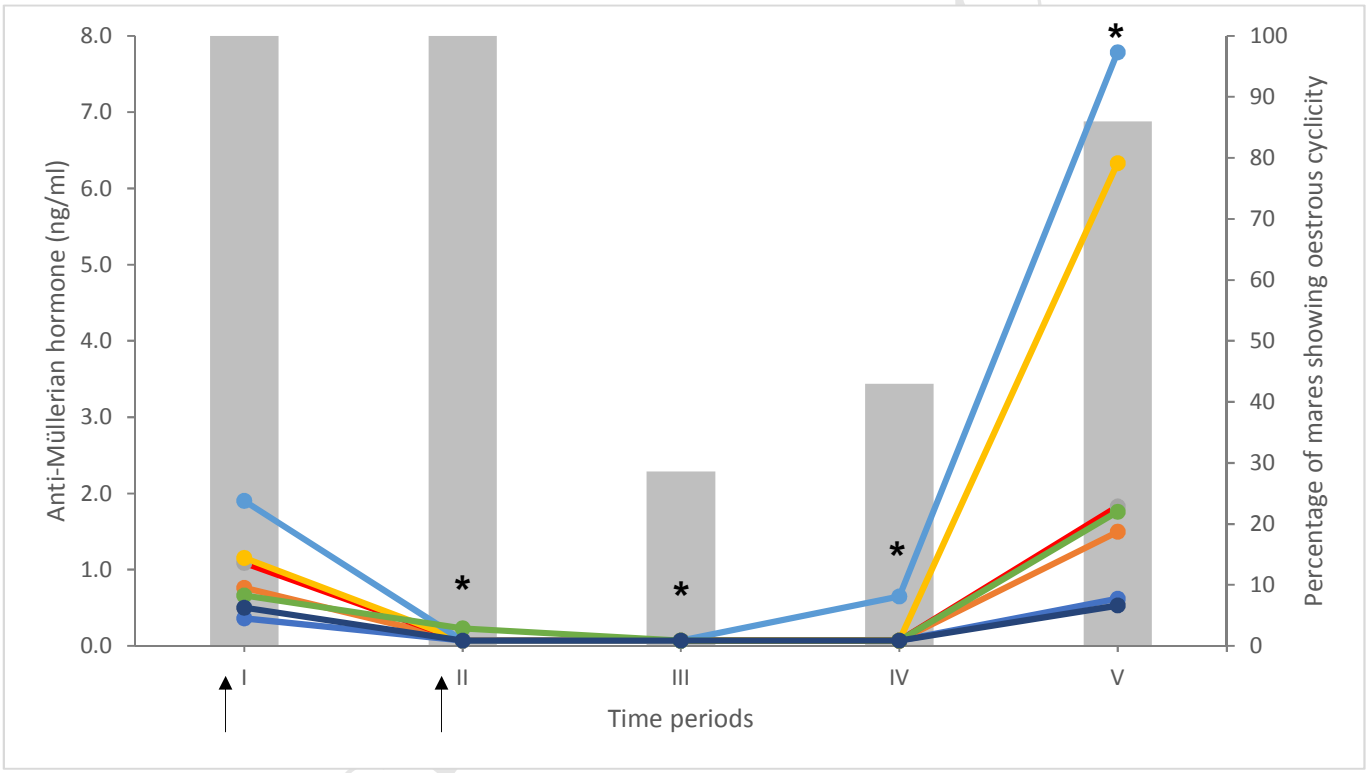
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a)



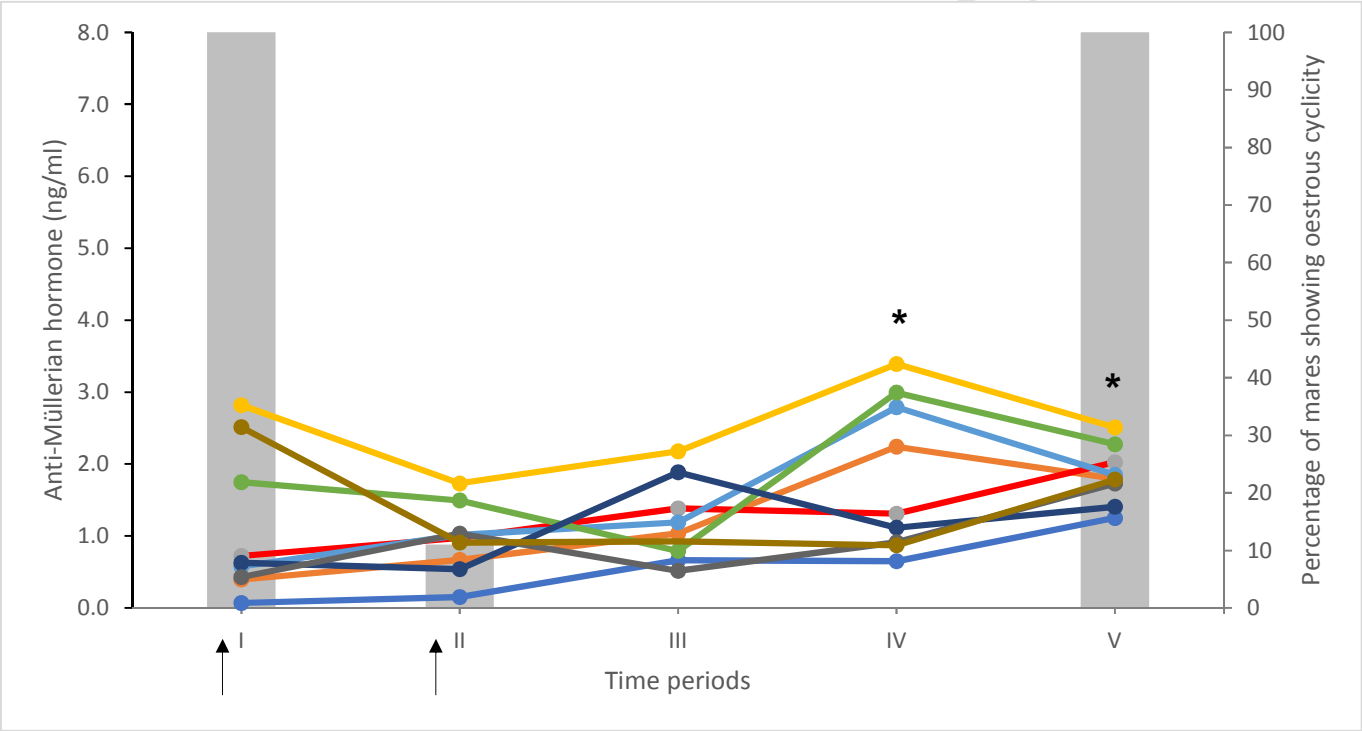
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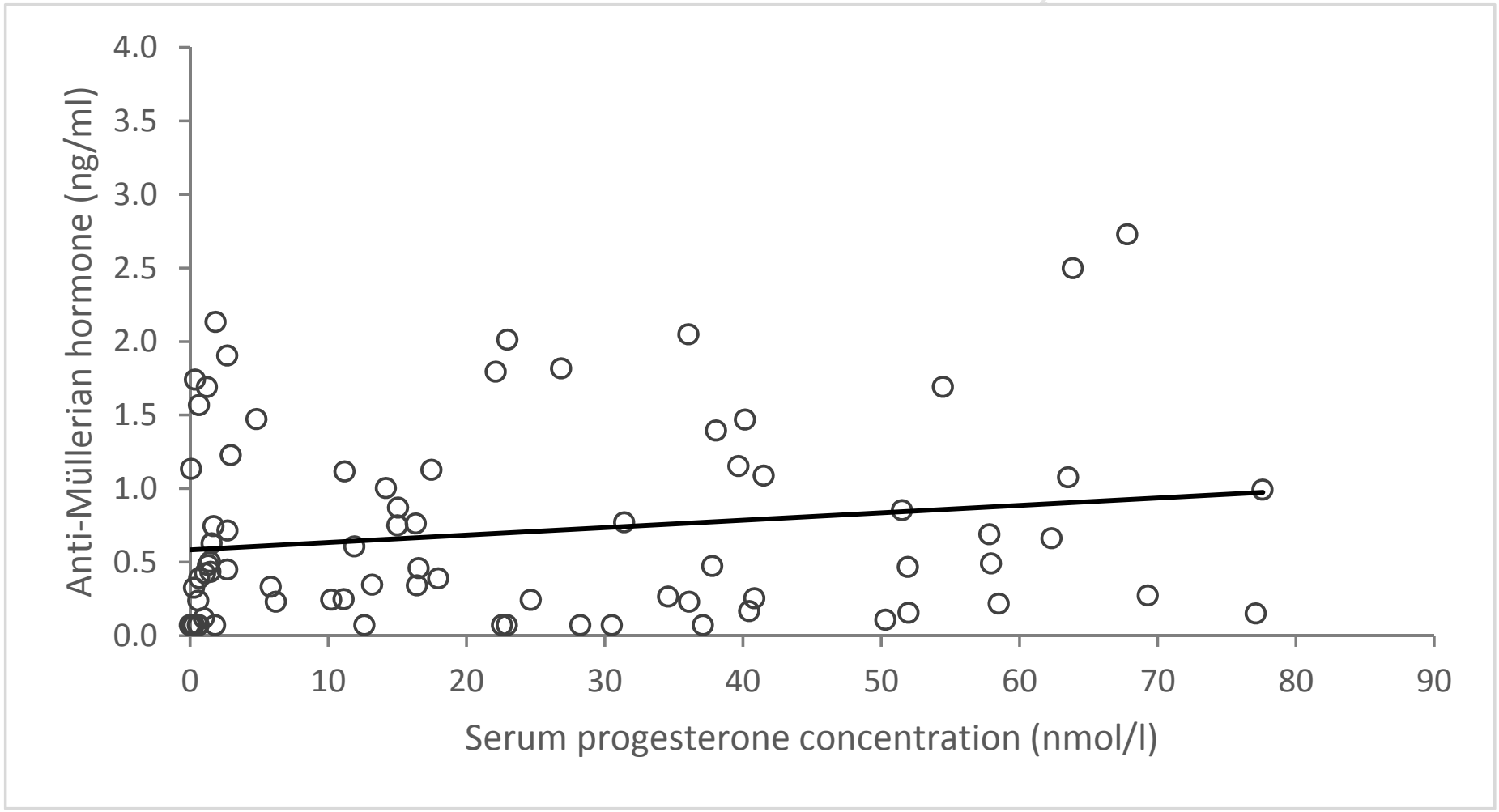


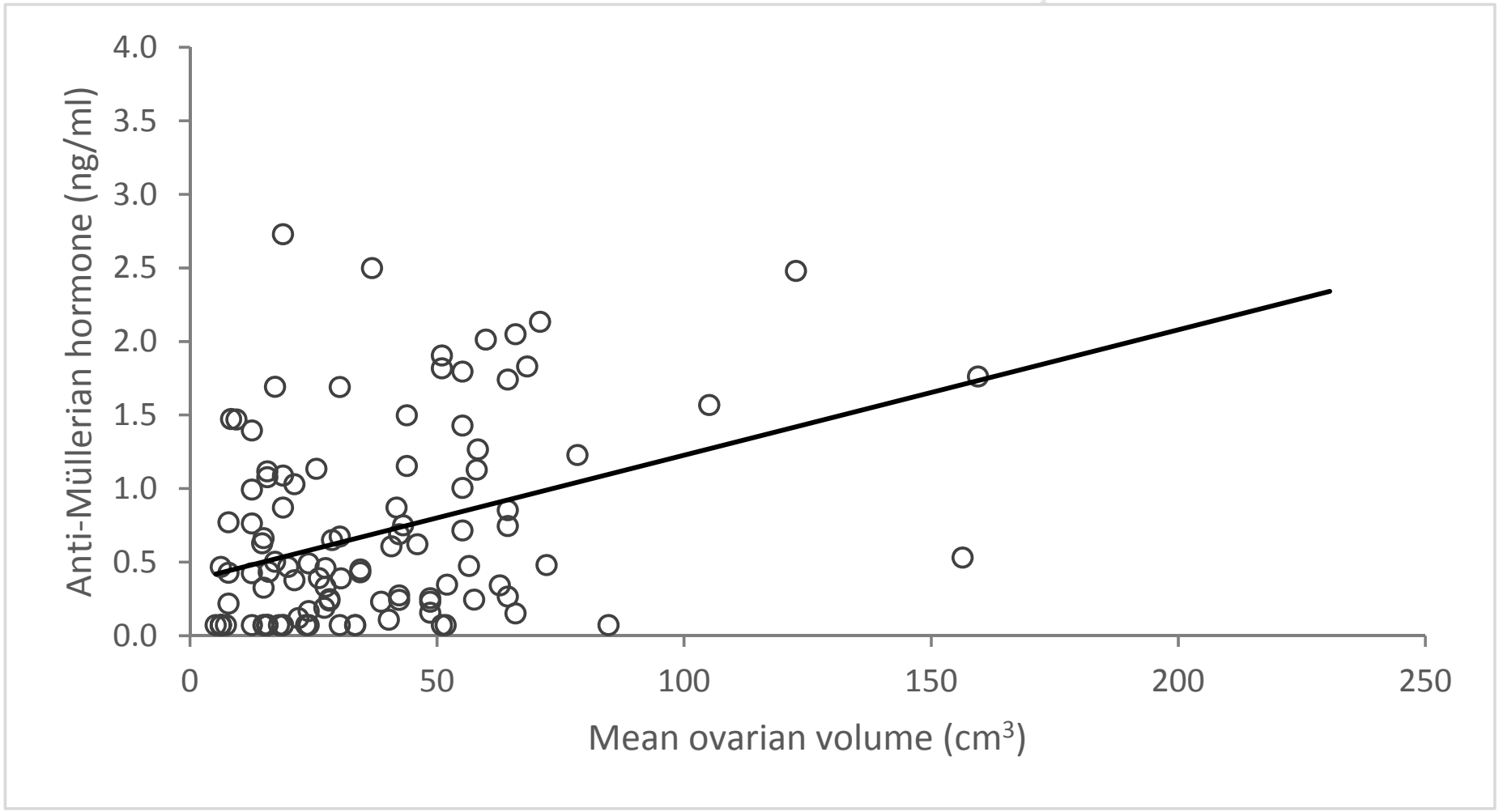
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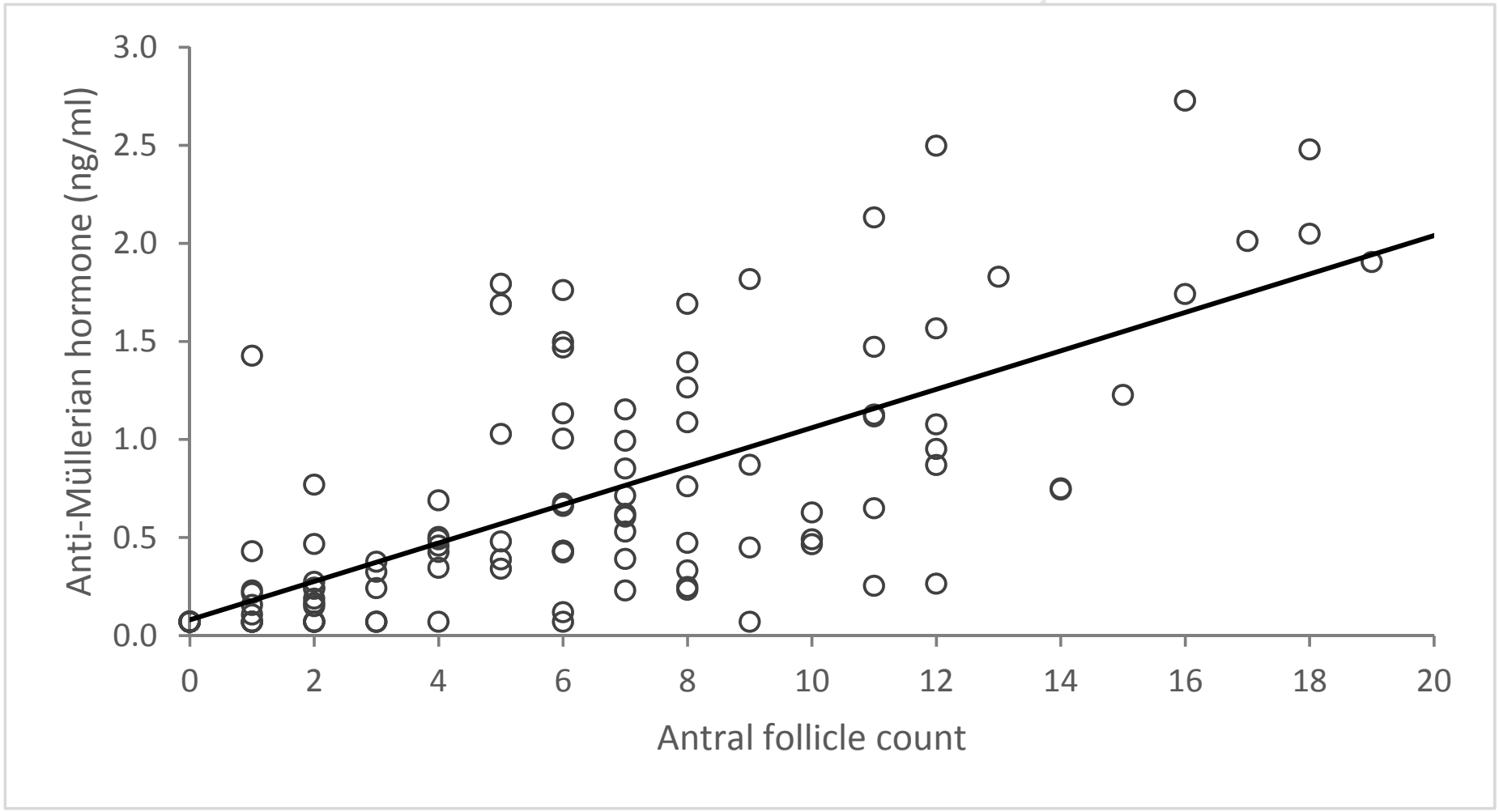
c)



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Anti-Müllerian hormone (ng/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
80
90
Serum progesterone concentration (nmol/l)


Anti-Müllerian hormone (ng/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
Mean ovarian volume (cm3)


Anti-Müllerian hormone (ng/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
16
18
20
Antral follicle count

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HIGHLIGHTS: Serum anti-Müllerian hormone dynamics in mares following immunocontraception with anti-zona pellucida or -GnRH vaccines

- Anti-Müllerian hormone (AMH) concentration has not previously been assessed during immunocontraception in the mare, or any other species, to the best of the authors' knowledge
- Measurement of circulating AMH provides novel data that contributes to our understanding of these contraceptive vaccines' mechanisms of action
- Mares immunized with native pZP vaccine showed profound suppression of AMH, followed by an apparent rebound effect in two of seven mares at the approximate time of reversal of the vaccine's contraceptive effect
- In contrast, mares immunized against GnRH showed little change in AMH over time
- Measurement of circulating AMH may provide an alternative method for monitoring of ovarian function during zona pellucida-based immunocontraception

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nicarbazin-treated bait for at least 3 consecutive years led to a visible and documented reduction of the number of pigeons. In turn, the number of public complaints and accidents had a documented reduction.

The talk will also discuss potential issues encountered by practitioners in delivering nicarbazin-treated bait, illustrate evidence-based solutions to overcome these issues and compare advantages and disadvantages of different methods to resolve human-pigeon conflicts. Combined with other pigeon population management methods, fertility control represents a modern, integrated solution to mitigate this man-made wildlife problem.

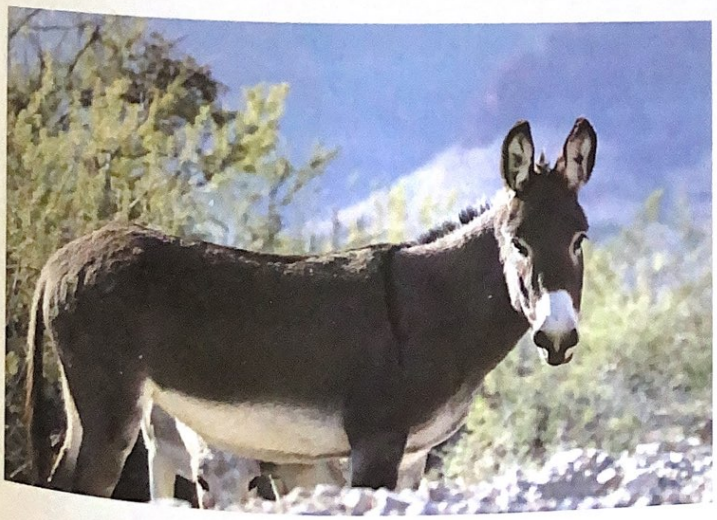
Field approaches to wild burro (*equus asinus*) identification and remote-delivery of ZonaStat-H in an American western landscape

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The use of immunocontraceptive vaccines is a well-established and viable approach for managing wild horse (*Equus caballus*) populations in western U.S. landscapes. Until recently, wild burro (*Equus asinus*) contraceptive research has not been a priority for the Bureau of Land Management (BLM) due to fewer populations of burros on public lands, slightly lower population growth rates, and historically higher adoption demand compared to wild horses. In 2017, the Humane Society of the United States (HSUS) partnered with the BLM to begin a four year pilot study to examine the efficacy and impacts of using the immunocontraceptive vaccine ZonaStat-H on wild burros in a western landscape. Since the project's inception, we have collected data associated with the treatment, observation, and management of the 109 wild female burros recruited from the Black Mountain Herd Management Area (HMA). The overarching goal of the project is to help build a scientific foundation for incorporating immunocontraception into the BLM's wild burro management programs. Although data collection for efficacy is ongoing, preliminary findings yield an opportunity to share the unique field experiences associated with identifying wild female burros and treating them with ZonaStat-H.

The Black Mountain HMA is the largest in Arizona and has field conditions applicable to other regions. Limited accessibility, diverse landscapes, windy and dry conditions, varying elevations, human activity, and extreme temperatures are factors that may impact efforts to successfully administer immunocontraceptive vaccines remotely to wild female burros. In addition, because wild horse identification and behavior has been the predominant focus of previous research, most available wild equid management resources exclude burro-specific attributes. Although similar to wild horses in many ways, wild burros exhibit different behaviors, social dynamics, and physical distinctions, making many horse-based identification strategies insufficient for field use. For a management program utilizing immunocontraceptive vaccines to be successful, the accurate identification of individual burros is essential. This presentation will focus on comparing different approaches to identifying burros and will summarize techniques used for effectively monitoring and administering ZonaStat-H to wild female burros in Arizona.



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Commentary

A review of contemporary contraceptives and sterilization techniques for feral horses

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Abstract: This commentary provides a brief review of the history of contraceptive research efforts for feral horses (*Equus ferus caballus*) as well as the contraceptives and sterilization techniques currently available for feral horses. Porcine zona pellucida (PZP) immunocontraceptives have received the most attention and use over the past 40 years, but other treatments such as the GnRH vaccine Gonacon™-Equine are also available. Optimization of these treatments as well as the development of other molecular approaches, intrauterine devices, and surgical techniques is ongoing.

Key words: contraceptive, *Equus ferus caballus*, feral horse, fertility control, sterilization

Since the late 1970s, researchers have sought a safe and effective equine contraceptive to help reduce population growth rates of feral horses (*Equus ferus caballus*) and burros (*E. asinus*). A good treatment that is safe, practical, effective, and long lasting has not yet been developed. Even after a treatment is developed that is effective for individual animals, there remain significant hurdles at the population level that must be overcome to reduce population growth rates (Garrott 2018).

Early fertility control efforts focused on both male and female contraception. However, as early as 1980, scientists with the National Research Council (NRC), part of the National Academies of Science, recommended that wild horse contraceptive efforts focus on reducing fertility in mares (NRC 1982). They recognized the polygynandrous nature of feral horses where multiple males may breed the same or multiple females. While it is true that wild horses live in harems that include a dominant stallion, much of the breeding may also be done by other stallions, and harem structures are fluid over time. It has been demonstrated that up to one third of foals may be sired by stallions that are not affiliated with a mare's band (Bowling and Touchberry 1990).

Steroid hormones to vaccines

Early contraceptive studies in stallions and mares used steroid hormones to alter fertility. While there was some success in mares, the treatments were cumbersome to administer, and there were concerns about the potential for persistence of the hormones in the food chain and the environment. About this same time, studies were done with surgical vasectomies applied to harem stallions. There was a small effect noted in 1 of the 2 groups studied, but it was short lived. The researchers concluded that, while it may be effective at the individual horse level, the efficacy of vasectomizing males for population growth suppression over time was doubtful.

As attention turned away from using steroid hormones to alter fertility, porcine zona pellucida (PZP) vaccines were developed in laboratory rodents and later applied to horses (Liu et al. 1989, Kirkpatrick et al. 1992). The PZP vaccine contains a glycoprotein antigen harvested from pig (*Sus scrofa domesticus*) ovaries. When mixed with a powerful adjuvant (in most cases it is emulsified with Freund's adjuvant), it stimulates the mare's immune system to make antibodies to ZP proteins. These antibodies block fertilization of the egg and over time bind to zona proteins in the ovary causing it to shrink and become nonfunctional. The most widely used PZP vaccine is Zonastat-H®. This liquid immunocontraceptive can be hand injected or darted into mares. When a booster shot is also administered about 30 days later and about 1–2 months prior to the breeding

season, it is highly effective at preventing conception for 1 year, with only about 10–20% of treated mares foaling each year. In smaller herds where the number of animals is at or very close to the appropriate management level (AML), Zonastat-H can successfully reduce or even eliminate the need to gather and remove animals over time.

The Bureau of Land Management (BLM) is currently using this approach in several herds (BLM 2017). The biggest limitation to this treatment is that it must be administered every year. Most animals on BLM ranges cannot be approached closely enough to allow darting, and repeated annual roundups to allow hand injecting the treatment get more difficult with each repetition, usually becoming impractical and ineffective after 2 or 3 iterations. Another limitation of this approach is how long it takes to achieve desired population numbers, called the appropriate management level (AML) by the BLM, when herds are even modestly above the desired numbers. The so-called Assateague prescription, where the vast majority of mares was darted every year (Kirkpatrick and Turner 2008), was eventually effective at achieving population targets on a small barrier island, but it took 13 years before a decline in numbers was achieved and several additional years before AML was achieved. That population of 156 horses started in 1993 only 30% over the desired AML of 120 horses, had 143 horses after 13 years, and today has about 90 horses. For comparison, many wild horse herds managed by the BLM in the west are currently at levels >100% greater than AML.

The quest for a longer-lasting PZP treatment began about the same time that Zonastat-H was being developed. An early study of the pelleted "PZP-22" treatment was most promising with only 6–18% of mares reported foaling in the first 2 years of the Clan Alpine study (Turner et al. 2007) and 30–40% foaling in years 3 and 4. Unfortunately, this level of success has never been repeated, with several captive and free-ranging trials reporting 25, 30, or even 70% foaling in the first 2 years following treatment. The reasons for this poor performance mostly remain a mystery. Recently, success of 15–40% foaling over a 3-year period was reported with reformulated PZP-22 after additional booster treatments (Rutberg et al. 2017), but whether or not this can be repeated remains to be seen. Currently, PZP-22 is the treatment used most frequently by the BLM; at the very least, it usually provides 1 year of good efficacy without requiring a 30-day booster shot, and when boostered 1–2 years later, efficacy should improve.

Spay Vac®, another formulation of PZP developed to offer longer-lasting efficacy, uses similar PZP antigens with a unique liposome technology expected to provide several years of efficacy. As with PZP-22, an initial study of the treatment was promising (Killian et al. 2008). However, subsequent trials aimed at demonstrating long-term efficacy in a captive pasture breeding setting could not duplicate the same results and in fact showed reasonable efficacy in 1 year (15% foaling) but poor efficacy over time with up to 70% of treated mares foaling (Roelle et al. 2017). Although this vaccine is not commercially available at this time, the proponents of the product have regrouped and hope to conduct testing of a new formulation of Spay Vac in the future (Bechert and Fraker 2018). Although this product shares the same limitations inherent to the PZP antigen as Zonastat-H and PZP-22, in some species including occasionally horses, it seems like it could be long-lasting or even permanent. The reliability of the newly formulated product will need to be established in captive breeding trials with horses before one might have the confidence needed for field applications.

Gonacon

At this time, there is only 1 other contraceptive product available for use in horses. Gonacon™-Equine is a vaccine that acts against gonadotropin releasing hormone (GnRH), a hormone critical to fertility. The vaccine was formulated and registered with the Environmental Protection Agency as a 1-shot treatment that was thought to provide good multiyear efficacy. Captive pen trials resulted in good efficacy (6% foaling) the first year (Killian et al. 2008), but again the efficacy of a single treatment in field trials conducted with feral horses at Theodore Roosevelt National Park never reached that level of effectiveness, with about 50% of animals foaling the first and second years (Baker et al. 2017). Fortunately, with a booster treatment as much as 3 years

following the initial shot, efficacy improved dramatically with 0% of mares foaling a year later and around 15% foaling in the following 2 years. Like PZP vaccines, Gonacon can be hand injected or darted. It has 2 advantages in that it does not require mixing in the field and is more stable when stored. The longer-term efficacy of Gonacon seems more promising than the PZP vaccines at this time; however, optimal booster schedules are still being investigated. The BLM has used Gonacon in a small pilot project on the range, and the early results are encouraging. Gonacon treatment that includes a booster may be among the best options currently available for the contraception of feral horses.

Intrauterine devices

After many years of focusing primarily on PZP and GnRH technologies, other contraceptive approaches are now being investigated for feral horses with support from the BLM (BLM 2017). In addition to injectable treatments, new intrauterine devices (IUDs) are being tested. Early studies of IUDs were promising, with reports of >80% retention and efficacy with no negative effects on mare health (Daels and Hughes 1995). However, once again, when additional captive breeding trials were done with the same design (as well as other more sophisticated designs promoted as effective with 100% retention), the results were disappointing with 60–100% of the devices falling out soon after stallions were allowed to breed the mares. A current BLM-supported study of a new IUD has had some success with a redesigned product that seems to offer good retention in the presence of stallions. Data are still being obtained at this time to demonstrate retention, efficacy, and safety over a period of years. The IUDs have the advantage of being almost 100% effective as long as they are retained. They appear to have no long-term negative effects on the mare's health or the uterus, and they could be 100% reversible if removed. They do have the distinct disadvantage that they can only be inserted into non-pregnant mares, and most feral horse mares of breeding age are pregnant for most of the year.

Surgical sterilization

Surgical sterilization, once considered taboo for feral horses because it is permanent, is also again being considered as a means of reducing population growth rates. For stallions, the same limitations of polygynandry that became apparent back in the 1980s are still present. It seems unlikely that castrating or vasectomizing only some portion of the more dominant males in a herd will significantly slow population growth over a period of several years, because enough stallions will still be present to eventually breed all the mares present. However, a saturation approach where some high percentage (e.g., 80–95%) of stallions are sterilized remains untested.

In their most recent review of the science behind feral horse contraception, the National Academy of Sciences recommended chemical vasectomy as a promising technique (NRC 2013). Despite acknowledging that it was not yet proven, they felt that it should not be difficult to adapt to feral horses. Unfortunately, this doesn't seem to be the case. When chemically vasectomized horses were reexamined after a recent study performed on the Sheldon Wildlife Refuge (Collins and Kasbohm 2016), it was revealed that the chemical vasectomies failed to block sperm transport in any of the dozens of animals treated (Scully et al. 2015). While it should be possible to develop and use a technique for chemical vasectomy, it seems to offer little advantage over the surgical procedure, which has been done with individual horses but remains unproven at the population level. The fact that a small number of fertile stallions can impregnate many mares suggests that any form of vasectomy is unlikely to be an effective means of long-term population growth suppression.

The consideration of surgical sterilization is not limited to stallions but also extends to spaying and tubal ligation procedures for mares. There is no established procedure for tubal ligation in mares. It just is not something that is called for among domestic horses and has not been developed or tested for feral horses. Spaying, while not as common as castration, is done in domestic horses and has been done with feral horses. The procedure in horses is much more difficult than it is in cattle and not as routinely practiced as spaying dogs and cats, for example. It is almost never done to pregnant domestic horses, so the safety and practicality of spaying feral horses on a large

scale, particularly while pregnant, remains mostly untested.

Feral mares were spayed on the Sheldon Wildlife Refuge with a report of fewer foals born to harems that included spayed mares and vasectomized stallions (Collins and Kasbohm 2016), but it remains to be seen if similar results could be obtained in other places when spaying is done on a large scale in the context of typical BLM roundups. Yes, individual mares can be spayed, but can practitioners do it safely on a large scale with pregnant mares, and will it help achieve the goal of controlling population growth rates? The BLM has attempted to answer some of these questions with applied research, but these questions remain unanswered largely due to litigation that prevented the projects from getting started.

What is the ideal contraceptive?

We are often asked, why hasn't anyone developed the ideal contraceptive for feral horses when the BLM has been supporting this research for >40 years? The answer is perhaps that an ideal contraceptive is an unreasonable expectation for any species. Safety for mares, unborn colts, and the environment are paramount, but is it really reasonable to expect a 1-shot, long-lasting, predictably reversible contraceptive that has no behavioral effects for horses when modern science has never even developed such a treatment for dogs, cats, or people? The emphasis for reproductive research in the domestic horse world has always been getting mares pregnant, not trying to prevent pregnancy. Until the last few years, there has been very limited funding for contraceptive research for horses with more funding obligated by the BLM to research projects in the last few years than the previous 40 years combined. From where we started in the 1970s, researchers have made significant discoveries and improvements in contraceptives for feral horses.

The desperate need for results in the field and the limited funding available have meant that several treatments were advanced to field trials or management use in the field after only 1 test under more limited conditions. It is not uncommon for treatments that work in controlled laboratory or clinical settings not to work as well when applied on a larger scale in the field. Unfortunately, we have seen this with feral horse contraception on several occasions. Were the early studies flawed by unblinded, biased outcome assessments? What about the blinded (Spay Vac) studies that also could not be replicated? Was it the biological variation in the PZP itself, changes in the adjuvants or preparation of the vaccines? All of these factors are the reason that research science prefers to look for replication of results by different investigators under expanded conditions before taking treatments to management application in the field. However, these are luxuries that feral horse contraceptive research didn't have.

The pressure and the push have been to take treatments to the field as soon as they offer some legitimate promise of success. The upside would have been faster results where they were needed most. The downside was several treatments didn't work as well as expected when applied on a large scale. These challenges were added to the practical limitations and expectations of trying to apply darting programs to the typically vast western rangelands that span not thousands of acres but hundreds of thousands of acres. Darting programs can work on a small scale where 100 or so named animals can be approached to within 30 or 40 yards. However, where 10 times as many horses might roam on land areas 30 times larger and the horses can't be recognized as individuals because they mostly look alike, this approach is not practicable. Most often these animals can't be approached to less than hundreds of yards. Under these more typical conditions, darting programs aren't likely to succeed. Despite all this, there have been small-scale successes on some BLM lands: the Pryor Mountains Wild Horse Range and the Little Book Cliffs, McCullough Peaks, and Spring Creek Basin Herd Management Areas come to mind.

Conclusion

The history of feral horse contraception research is one of desperate need, ambitious (perhaps sometimes unreasonable) goals, and the passionate pursuit of a solution by a handful of investigators challenged by limited resources as well as the biology of the endeavor. Unfortunately, today, despite the many advances that have been made, a contraceptive solution that is safe, practical, and effective for most

herds on typical western herds is not in hand. Right now, feral horse contraception research has better levels of funding than ever before, with major universities more engaged than ever and new, never previously conceived molecular techniques being investigated. Nevertheless, progress will likely be slow. For every idea that advances to the next level of investigation 2 or 3 others will fail. Despite the desperate need, if we can maintain current research funding levels, we are years or likely more than a decade away from a contraceptive solution to the challenge of significantly reducing feral horse population growth rates on western rangelands. The good news is that it is still conceivable, and there are still some very bright and passionate scientists working on solutions that will make a difference.

Acknowledgments

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Review Article

Immunocontraceptives: New Approaches to Fertility Control

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The rapidly increasing global population has bowed the attention of family planning and associated reproductive health programmes in the direction of providing a safe and reliable method which can be used to limit family size. The world population is estimated to exceed a phenomenal 10 billion by the year 2050 A.D., thus presenting a real jeopardy of overpopulation with severe implications for the future. Despite the availability of contraceptive methods, there are over one million elective abortions globally each year due to unintended pregnancies, having devastating impact on reproductive health of women worldwide. This highlights the need for the development of newer and improved contraceptive methods. A novel contraceptive approach that is gaining substantial attention is "immunocontraception" targeting gamete production, gamete outcome, or gamete function. Amongst these, use of sperm antigens (gamete function) seems to be an exciting and feasible approach. However, the variability of immune response and time lag to attain titer among vaccinated individuals after active immunization has highlighted the potential relevance of preformed antibodies in this league. This review is an attempt to analyze the current status and progress of immunocontraceptive approaches with respect to their establishment as a future fertility control agent.

1. Introduction

The world population has exceeded 6.43×10^9 and is increasing by 1×10^9 every 12 years [1]. Besides population explosion, unintended pregnancies and elective abortions are a major public health issue. This overgrowth will also worsen the impact of many social, ecological, economical, and medical trends [2]. With the serious global issue of overpopulation, especially in developing countries, it becomes a necessity to find an effective way to control the rapid growth of population.

Contraception is an accepted route for the control of population explosion in the world [2]. The need for contraception varies for different couples from postponing child bearing, spacing childbirth and limiting family size to absolute no child bearing which is highly personal decision based on their individual preferences, medical history, life style and other factors [3]. The characteristics of an ideal contraceptive include acceptability of user, no loss of natural feeling, no side effects, relatively inexpensive, effective, easy to use and reversible. Contraceptive choices are accessible in case of both men and women. Currently practiced contraceptive options available for men include condoms, withdrawal and vasectomy [4, 5]. In case of females, the contraceptive methods available worldwide include oral contraceptive (OC) pills, injectable contraceptives, contraceptive implants, intrauterine devices, contraceptive vaginal ring, barrier methods, surgical sterilization, spermicides and natural family planning methods.

A novel contraceptive approach that is gaining substantial attention is immunocontraception, which is the use of contraceptive vaccines (CVs) or preformed antibodies to prevent fertilization. As with the conventional vaccines, CVs utilise the body's defense system to wedge an essential step in the reproductive process [6]. A successful contraceptive vaccine must meet a number of criteria; it must be reliable, easy to administer, safe, affordable, widely acceptable, and capable of evoking homogeneous response and must bestow a high level of contraceptive efficacy. There are three major categories involved in the development of CVs including gamete production, namely, gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), and luteinizing hormone (LH); gamete outcome, namely, human chorionic gonadotropin (hCG), or gamete function, namely, zona

pellucida (ZP) and sperm antigens [7]. Of all, validation and practicability for the development of antisperm vaccines targeting prefertilization events seem to be much more encouraging and promising. In this article we have tried to review the current status of the immunocontraceptive approaches and discussed their relative merits as a future contraceptive.

2. Immunocontraception

The advent of "immunocontraception" represents the first truly novel approach to the development of family planning methods. It involves the administration of a vaccine that induces an adaptive immune response which causes an animal to become temporarily infertile [8]. Immunocontraception promises many advantages over the methods of contraception currently available for human use which include high target specificity, long term action but not permanent, relatively inexpensive, lack of endocrine or metabolic side effects, and easy to use and does not require insertion of an implant or device and does not require surgical intervention [9].

Currently, there are three major categories involved in the development of immunocontraception including gamete production, gamete outcome, or gamete function [7] (Figure 1).

2.1. Gamete Production. Gamete production includes the synthesis of reproductive cells from gonads, that is, sperm from testes and ovum from ovaries. The first hormone in the chain is GnRH. It regulates the release of two peptide hormones from anterior pituitary, namely, FSH and LH, collectively called gonadotropins. Immunoneutralization of any of these hormones can prevent the binding of respective hormone with their receptor which may result in failure of conception.

2.1.1. Gonadotropin Releasing Hormone (GnRH). The decapeptide GnRH stimulates the release of the gonadotropins and is present both in males and females; thereby a vaccine against GnRH is feasible in both sexes. The hypothalamic GnRH-I (also referred to as GnRH or luteinizing hormone releasing hormone (LHRH)) is clearly the main hormone in the hypothalamic-pituitary-gonadal axis, besides a possible direct role of this decapeptide in extrapituitary organs, such as testis, prostate, and placenta [10]. The gene coding for GnRH-I is located on chromosome 8p21-p11.2.

When GnRH vaccine was tested on human subjects, including breastfeeding women by The National Institute of Immunology, India, and the Population Control Council, USA, the vaccine essentially resulted in castration and halted testosterone production [11]. However, these results were accompanied with impotence and loss of body hair in case of men and affected menopause in women [12]. Hence, an analogue of GnRH was prepared by linking with different carriers in an attempt to make improved vaccine [7]. Different carriers, namely, diphtheria toxoid (DT), tetanus toxoid (TT), *Mycobacterium tuberculosis* hsp 70 [13], thioredoxin [14], keyhole limpet hemocyanin [15], and ovalbumin, were employed. It was observed that the conjugation with any of the carriers induced specific antibody response. However, even after multiple injections, low titer antibodies were generated. It is well known that conjugation of protein/peptide with appropriate adjuvant can augment the immune response. Therefore, different adjuvants namely, Quil A, aluminium hydroxide, or polylactide co glycolide acid (PLGA), were employed. Among all these adjuvants, PLGA was found to be the most effective adjuvant [16]. Further, encapsulation of GnRH-TT in PLGA microspheres induced an effective antibody response within 15 days of administration, thus negating the need of repeated injections [17]. Use of any of the combinations in different animals (rats, monkeys, and pig) was effective in reducing testosterone levels to castration levels thus inducing infertility. However, decrease in testosterone levels was accompanied with concomitant atrophy of prostate.

Therefore, vaccines targeting GnRH were developed primarily as immunocontraceptive and immunocastration agents in animals [18]. Few GnRH vaccines are already available for veterinary use such as Vaxstrate and Improvac whilst research is still under development to obtain potentially improved vaccines [19–21]. In humans, they can be used for the treatment of prostate cancer and various sex hormone-dependent disorders [22]. However, the inability of vaccine to induce azoospermia in all the cases, loss of body hair, atrophy of prostate, and impotency after chronic exposure hinders its application as a safe vaccine. Also, as the vaccine affects sex steroids, consumers are unlikely to find this approach acceptable. Therefore, this may be suggested that enough data needs to be accumulated before the vaccines against GnRH can be employed in humans without side effects.

2.1.2. Follicle Stimulating Hormone (FSH). Follicle stimulating hormone (FSH) plays an essential role in the initiation and maintenance of spermatogenesis in primates [23]. FSH has two subunits alpha (α) and beta (β). The α subunit of FSH, LH, thyroid stimulating hormone (TSH), and human chorionic gonadotropin (hCG) is identical. FSH-β is responsible for its binding with FSH receptor.

The use of FSH appears to be an attractive contraceptive candidate for application in males. Most of the research has been carried out using ovine FSH rather than human FSH. The rationale for this is associated with the fact that specific antibodies raised to ovarian FSH cross reacted and neutralized the activity of FSH of variety of species including humans. Also, the use of ovine FSH in place of human FSH lowers the chances of producing cross reactive antibodies with human LH and TSH (due to structural similarity with α-subunit) [24]. Immunization of male bonnet monkeys with ovine FSH resulted in testicular dysfunction, oligozoospermia, and subsequent infertility [25]. It was noteworthy that infertility was not associated with any change in testosterone levels thus adding an additional advantage of using FSH based vaccine. Following successful studies by ovine FSH, a phase 1 clinical trial was carried out using two different versions either ovine FSH $\alpha\beta$ heterodimer or isolated ovine β-chain. In both the cases alum was used as an adjuvant [26]. Immunization by any of these resulted in generation of FSH-specific antibodies in all immunised individuals. But, as the

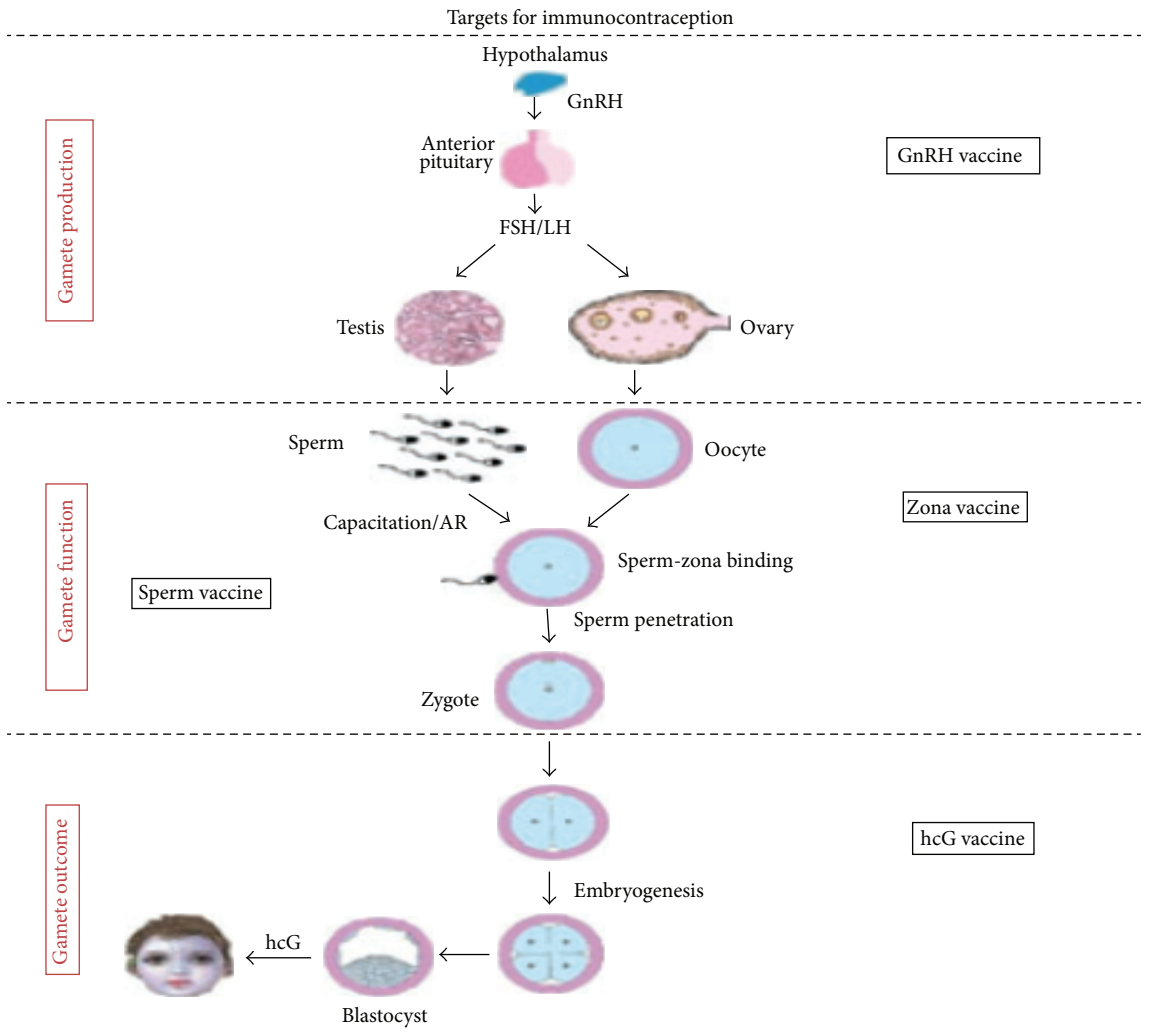


Figure 1: Schematic model indicating various targets that are being explored for the contraceptive vaccine development. These include targeting gamete production [gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH)], gamete function [zona pellucida (ZP) proteins of the oocytes and sperm antigens], and gamete outcome (human chorionic gonadotropin [hCG]) (adapted from [9]).

antibodies being generated were of fairly low titer, there was no reduction in the sperm count in the volunteers.

Further, an attempt was made by using FSH coding peptides instead of native protein form. Two peptides considered contained the sequences of β 33–53 and β 81–95 aa. Both the peptides were used in monomeric as well as dimeric form (tandem) and were subsequently used to immunize male rabbits, respectively. Immunization with β 33–53 tandem inhibited FSH bioactivity specifically and hence it was considered an attractive candidate to be used as the antigen of choice in a male contraceptive vaccine [27]. However, incidence of spermatogenesis in FSH-β knockout mice and considerable fertility in men homozygous for an inactivating mutation of FSH receptor [28] suggested that FSH suppression might not be a feasible alternative for the development of male contraceptive vaccine.

2.1.3. Luteinizing Hormone (LH). Luteinizing hormone (LH, also known as lutropin and sometimes lutrophin) is a heterodimeric glycoprotein hormone produced by gonadotroph cells in the anterior pituitary gland. An acute rise of LH triggers ovulation and development of the corpus luteum in females. It stimulates leydig cells to produce testosterone in males and also termed as interstitial cell-stimulating hormone (ICSH) in males. Similar to FSH, LH is also made up of one α and one β subunit which make the full, functional protein. Immunization of adult female sheep (ewe) with LH prohibited pregnancy in all the animals during two breeding seasons. The mode of action was assumed as prevention of ovulation presumably by inhibition of the preovulatory surge of LH [29]. Though immunization with LH has potential application as contraceptive agent, it is not widely accepted since it potentially affects sex steroids.

2.2. Gamete Outcome. The fusion of sperm and ovum is followed by gamete outcome, that is, the formation of fertilized egg which is maintained under the control of pregnancy specific enzyme, human chorionic gonadotropin (hCG). Soon after the conception, hCG is secreted during luteal phase and prepares endometrium to receive fertilized egg. Generation of antibodies against hCG can provide an immunocontraceptive agent which will target the establishment of fertilized egg.

Human chorionic gonadotropin is the only well-defined protein playing an essential role in the maintenance of pregnancy. The hormone is formed by the trophoblast and acts on the corpus luteum for stimulating progesterone production. If progesterone level drops, uterine endometrium will shed; thus preventing implantation [30]. As the substantial amounts of hCG are secreted only during pregnancy, its neutralization should have no other effects than on fertility. It also plays a critical role in implantation of the embryo onto the uterus.

Anti-hCG vaccine was the first and only birth control vaccine to go through Phase II efficacy trials successfully. The use of vaccine in sexually active women prevented them from becoming pregnant [31]. The vaccine was highly effective as long as the antibody titers remained >50 ng/mL. Action of the vaccine was fully reversible as women conceived readily when the antibody titers declined (<35 ng/mL). No side effects were observed during Phase I and Phase II trials as women showed normal ovulation and normal synthesis of their own sex hormones along with regular menstrual cycles. Also, no irregularity of bleeding in terms of spotting, amenorrhoea, or extra bleeding occurred [32].

Despite the encouraging results of phase I and phase II trials, major shortcoming of vaccine still existed which included the variation in generation of protective threshold titers. Only 60–80% women showed the presence of high titer protective antibodies. This percentage would have been satisfactory in case of infectious diseases but in case of a birth control vaccine, it has to be effective in >90–95% of recipients in order to be acceptable.

Therefore, in order to enhance the titers, recombinant approaches were tried using different carriers. Use of both diphtheria toxin (DT) and tetanus toxin (TT) evokes good antibody response but the repeated immunization with β-hCG-TT vaccine causes a carrier induced epitope suppression in some women. Various studies showed that conjugation of β-hCG to cocktail of peptides resulted in enhanced immune response as compared to single peptide [33].

Later, another study was carried out using heat labile enterotoxin subunit B (LTB) genetically linked with hCG-β chain. It was cloned and expressed in yeast *Pichia pastoris* and was purified using standard purification techniques [34]. Intramuscular immunization in Balb/c mice fortnightly resulted in generation of bioeffective anti-hCG antibodies. The titers when tested were several folds higher than 50 ng/mL/mouse. Similar results were obtained when this vaccine was used in different inbred mice of varying genetic background. The action of the vaccine was reversible and it received approval from the Indian National Review committee on Genetic Manipulation. The vaccine is being produced under GMP conditions for the preclinical toxicological studies. After passing these tests, next plan is to conduct clinical trials with the vaccine for preventing pregnancy, as well as for its possible therapeutic action on cancers expressing hCG or its subunits [35].

2.3. Gamete Function. The term gamete function is associated with the two main cells participating in gamete formation, that is, sperm and ovum. This approach targets the zona antigens of egg and surface antigens of sperm. The targets being nonhormonal provide much better safety upon other approaches. Exploiting ZP antigens as immunocontraceptive may interfere with the normal production of egg in female while sperm-based vaccine will be used in female reproductive tract to block fertilization by interfering with sperm movement or function.

2.3.1. Zona Pellucida (ZP). ZP is an extra cellular matrix, composed of glycoproteins, which surround the mammalian oocyte. This matrix serves multiple functions which play important role during fertilization. It is responsible for binding of spermatozoa to the oocyte due to presence of specific glycoproteins on its surface which acts as a receptor for spermatozoa. This interaction is followed by induction of acrosome reaction in the sperm bound to ZP which results in successful fusion between the two. ZP is composed of 3-4 glycoproteins named ZP1, ZP2, ZP3, and ZP4 [36].

The ZP glycoproteins have emerged as potential candidate for immunocontraception due to their essential role in the fertilization and tissue specificity (Figure 2). Immunization against ZP might result in generation of antibodies against themselves which go and bind to ZP. Thus, when a sperm encounters ovum in case of immunized animals, the binding is inhibited because ZP is already occupied by the antibodies. Immunization of female rabbits with porcine ZP proteins results in generation of antibodies against porcine ZP proteins which showed immunological cross reactivity with ZP from various other species including humans. Further, immunized female rabbits failed to conceive even after mating with fertile male rabbits [37]. This homology allowed the use of heterologous immunization in case of ZP [7].

Further studies revealed that the infertility induced in immunized female rabbits was irreversible which could not be restored even after the administration of exogenous gonadotropins. Histological examination of ovaries showed the destruction of oocytes in all the growing follicles along with the depletion of resting follicles [38]. This observation indicated that the infertility was a consequence of ovarian dystrophy rather than inhibition of sperm-oocyte interaction.

The irreversibility associated with immunization using ZP posed a major hurdle in the development of ZP based contraceptive. While irreversibility is not a major concern in case of wildlife management where long term infertility is often desirable. Therefore further development in this area resulted in production of various marketed products playing an imperative role in wildlife management.

The immunization of female bonnet monkeys was carried out using purified porcine ZP3. High antiporcine ZP3 antibody titers were formed and all the animals were rendered

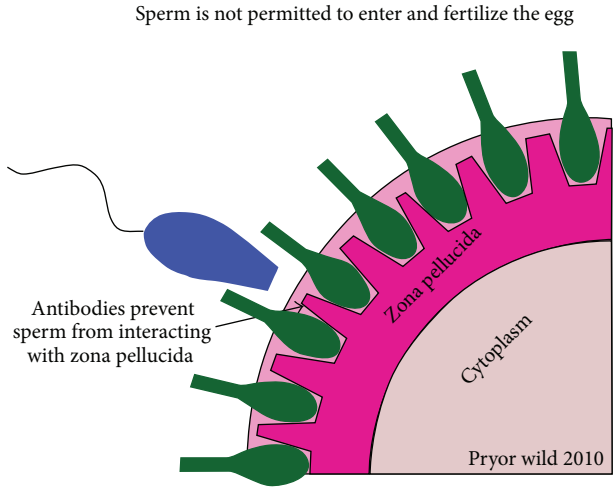


Figure 2: Immunocontraception using an immune response to zona pellucida (ZP). The immune response to the ZP antigen can inhibit sperm-egg binding or disrupt ovarian function (Source: http://www.newmalecontraception.org/risug/).

infertile [39]. Only 50% of the animals could regain fertility after the decline in antibody titers.

Ovarian histology of the animals that failed to regain fertility did not reveal any signs of inflammation or lymphocytic infiltration. The observed variations in the extent of ovarian dysfunction may be linked with differences in susceptibility among various species, purity of the ZP glycoproteins, and use of different adjuvants such as alum and synthetic muramyl dipeptide (MDP) [40–42]. It was suggested that these problems such as limited production of ZP glycoproteins from native source, batch to batch variation, and contamination with ovarian-associated proteins might be solved using recombinant form of ZP.

For this, the three glycoproteins, namely, ZP1, ZP2, and ZP3, were separately expressed in Chinese hamster ovarian (CHO) cells. A comparative analysis was carried out in two nonhuman primate species, namely, cynomolgus monkeys (*Macaca fascicularis*) and baboons (*Papio cynocephalus*) [43]. Animals immunized with any of the recombinant proteins remained infertile for some period of time but the animals immunized with recombinant ZP1 conceived later in contrast to control animals and animals immunized with remaining two proteins. Hence, another study was carried out in female baboons (*Papio anubis*) and immunization was done using *E. coli*-expressed recombinant bonnet monkey (*Macaca radiata*) ZP1 (bmZP1) conjugated to diphtheria toxoid (DT) [44]. After mating with males of proven fertility, immunized animals failed to conceive till the antibody titers were $> 2\times10^3$ antibody units. All the immunized animals became pregnant upon mating once the levels were less than the 2×10^3 antibody units. Significant curtailment of fertility was also observed by using recombinant possum ZP3 in grey kangaroos [45, 46]. Though the results were quite exciting, histological examination of ovaries of immunized animals revealed the presence of atretic follicles with degenerating oocytes.

These observations raised the hope of generation of ZP based CV for human use except for the histopathological damage. Thus, series of experiments were conducted to overcome these problems. It was found out that "oophoritogenic" T-cell epitopes existing in zona proteins might be responsible for ovarian dysfunction [47]. Hence, efforts were made to delineate B-cell epitopes which were devoid of "oophoritogenic" T-cell epitopes. As a result various synthetic peptides corresponding to ZP glycoproteins (ZP1, ZP2, and ZP3) were prepared and tested for immunocontraceptive potential *in vivo*. Female bonnet monkeys immunized with synthetic peptides remained infertile. Histopathological examination showed absence of any ovarian pathology in case of immunized animals. Similar results were obtained in case of mice immunized with ZP3 [48, 49].

Recently, murine ZP3 and sperm specific (YLP-12) epitope were expressed with Johnson grass mosaic virus coat protein to present antigens as virus like particles (VLPs) [50]. Immunization of animals resulted in significant infertility. The results were quite encouraging and suggestive of the possibility of using above mentioned approaches for immunocontraception. However, long term studies showed that immunization with zona antigens might induce immunological attack on many eggs in the ovary which might lead to premature ovarian failure [51]. Hence, enough data needs to be gathered before zona antigens are given any place in the market as immunocontraceptive measure [52].

2.3.2. Sperm Antigens. Development of CV based on sperm is a promising approach towards contraception [53]. The feasibility of employing sperm as a target is well documented. Sperm has both auto- and isoantigens and hence immunization of several species of animals and humans with sperm results in generation of antisperm antibodies (ASA), leading to infertility [54]. In a classic study carried out in 1932, fertile women (had at least one pregnancy) were injected with their husbands semen [55]. The women developed ASA which resulted in block of conception for up to 1 year of observation. More than 70% of the vasectomised men produce ASA after

vasectomy which interferes with regain of fertility even after successful surgical reanastomosis [56]. In addition, approximately 30% infertility may be attributed to the presence of ASA in either male or female partner. Disappearance of ASA from these males and females results in the recuperation of fertility. These findings provide evidence that sperm is an attractive candidate that can be exploited in case of both men and women.

Exploitation of whole spermatozoon/poorly characterized/crude sperm extracts is not a practical and feasible approach for the CV development as it has several antigens that are likely to be shared with various somatic cells due to molecular mimicry [6, 57]. Immunization with whole sperm can lead to immunopathological consequences with other tissues and organs. Thus, only sperm specific antigens can be regarded as a promising and attractive candidate for CV. The utility of an antigen is contingent upon its immunogenicity, expression on sperm surface (except from the acrosomal antigens that appear after the acrosome reaction) and involvement in fertility/fertilization. Fertilizing capacity of spermatozoa is dependent upon wide range of biological properties, namely, motility, ability to capacitate, undergoing acrosome reaction, penetrating ZP, and finally fusion with egg [58]. Antibodies generated against any of the antigens involved in above said phenomenon can result in suitable candidate.

Various approaches of genomics, proteomics, and vaccinology are being used to isolate sperm-specific antigens. In addition, several novel technologies such as subtractive hybridization, hybridoma technology, differential display technique, and gene knockout technology have also been employed. In addition to these approaches, sperm-bacteria interactions can also be targeted for the delineation of sperm antigens. Among various microorganisms that directly interact with spermatozoa leading to sperm immobilization/agglutination are well known causative pathogens of genitourinary infections such as *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Escherichia coli*, and *Staphylococcus aureus* [59]. These interactions use specific host cell-surface molecules known as receptors and such receptors provide a mechanism for intimate interaction with the ligand on bacteria. Harvey et al. [60] while studying the mechanism of interaction in case of *N. gonorrhoeae* with sperm showed that *N. gonorrhoeae* lipooligosaccharide (ligand) can bind to the asialoglycoprotein receptor (ASGP-R) on human sperm. Sulphoglycolipids have been confirmed as the only receptor molecule for the 70 kDa heat shock protein ligand on *M. hominis* [61]. Earlier studies have shown that sperm-*E. coli* adherence is mediated by mannose residues present on the sperm surface and mannose binding structures present on *E. coli* [62]. Addition of receptor antagonist resulted in inhibition of sperm-bacteria interactions. Therefore, it can be concluded that interaction of bacterium with spermatozoa is receptor mediated and involves interplay of multiple factors which cumulatively lead to sperm damage.

Amongst these microorganisms, *E. coli* is perhaps the most extensively studied microorganism in relation to infertility [63]. *E. coli* rapidly adheres to and agglutinates human sperm which results in significant decrease of motile sperm count. On the basis of this chemistry involved, the possibility arises that bacteria can also be exploited to identify these proteins on spermatozoa.

Various antigens that are being isolated from spermatozoa include sperm adhesion molecule 1 (SPAM 1), metalloprotease disintegrin cysteine (MDC), sperm protein (SP-10), fertilization antigen (FA-1), SP-17, NZ-1, NZ-2, lactate dehydrogenase (LDH-C_4), sperm agglutination antigen (SAGA-1), YLP-12 peptide, human equatorial segment protein (hESP), BS-17, rabbit sperm membrane protein-B (rSMP-B), sperm acrosomal membrane-associated protein (SAMP-32), and 80 kDa human sperm antigen (HSA). In addition few glycoproteins on the surface of spermatozoa were acquired from epididymal secretions during transit through epididymis, namely, dorsal head and equatorial (DE), epididymal protease inhibitor (Eppin), and sperm flagella protein (SFP-2). Izumo and AKAP are also being isolated.

Following antigens are being tried as CV. The antigens so obtained were tried in purified/semipurified forms for immunization. However, to achieve Food and Drug Administration (FDA) approval and to perform appropriate multicenter fecundity trials in a quality-controlled manner, recombinant or synthetic peptide molecules are required. Hence in addition to the use of native form, alternative forms of protein/antigens, namely, recombinant proteins and synthetic peptides, have also been tried. Among these two forms, synthetic form possesses advantage of being well-defined which can be synthesized and purified in large quantities at a relatively lower cost in contrast to recombinant proteins.

(1) The Sperm Adhesion Molecule 1 (SPAM1). The sperm adhesion molecule 1, a 64 kDa glycosyl-phosphatidylinositol (GPI)-linked protein, is expressed in testis, epididymis, sperm, and luminal fluid of the epididymis [64, 65]. SPAM1, a hyaluronidase enzyme, carries out three main functions during fertilization. These include penetration of cumulus [66], binding with ZP, and Ca^{2+} signalling in acrosomal exocytosis [67]. The protein is widely conserved among various mammals, hence it has been cloned from many species including fox [68], guinea pig [69], pig [70], rat [71], rabbit [72], monkey, and man [73].

In vitro incubation of sperm with antibodies generated against SPAM1 resulted in reduced sperm-ZP binding. Immunization of either male or female guinea pigs with purified SPAM1 from guinea pigs induced 100% infertility [74]. Infertility was associated with loss of normal sperm in epididymis and autoimmune disorders in case of immunized males [75]. In females, prevention of sperm-egg binding by antibodies resulted in infertility. However, the infertility induced by immunization was found to be reversible in both the cases as fertility was regained within 6–15 months.

Though the results were quite encouraging, but in order to produce an effective CV, it is necessary to generate protein in sufficient quantities. This shortcoming has been undertaken by the use of recombinant antigen. The cDNA encoding for the SPAM1 antigen has been cloned and sequenced. However immunization of both male and female rabbits carried out using recombinant SPAM 1 (rSPAM-1) did not result in

infertility in any of the immunized animal. These differences between native and recombinant form may be due to lack of sufficient quantities of anti-rSPAM1 antibodies which were required to block conception [72, 76].

(2) Metalloprotease/Disintegrin/Cysteine-Rich (MDC). The MDC proteins are a rapidly growing family of integral membrane proteins, expressed predominantly in mammalian testis. All the proteins have distinct conserved features such as a metalloproteinase-like domain, a disintegrin-like domain, a cysteine-rich domain, a prodomain, and a transmembrane domain (also known as ADAM family) [77]. Among these proteins, few are known to be expressed on male germ cells and/or mature sperm. Notable among them are fertilin α, fertilin β (collectively called fertilin), and cyritestin (tMDC I).

Fertilin, previously designated PH30, is a sperm-associated protein. It was originally isolated from detergent extracts of guinea pig caudal epididymal sperm. In guinea pig, it is composed of two related subunits, α and β, with molecular masses of 60 kDa (Fertilin α) and 44 kDa (Fertilin β), respectively, as observed on reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE). Role of fertilin α is associated with sperm-egg adhesion, but whether it has some part to play in membrane fusion or not is still not very clear [78]. Its role in adhesion is justified by the presence of cysteine-rich domain that appears to participate in cell adhesion. The use of recombinant form of fertilin α inhibits sperm-egg binding more effectively than does a shorter form with a truncated disintegrin-like domain. But the fact that the sperm lacking fertilin α are still capable of sperm-egg fusion has hindered the use of fertilin α [79].

Fertilin β was one of the first "cellular disintegrins" identified. It is one of the subunits of dimeric sperm antigen "fertilin" and the antibodies generated against fertilin cross reacts with fertilin α. The function of fertilin β is well known; it mediates sperm-egg binding and membrane fusion [80]. Inhibition in the presence of antidisintegrin loop antibodies pointed that fertilin β appears to utilize its disintegrin loop sequence to interact with the egg membrane. Although antibodies to fertilin β inhibit fertilization *in vitro* [81], but immunization with the same does not affect fertility *in vivo*, it is not suitable as a contraceptive agent [82, 83].

Cyritestin is the product of *Cyrn* gene and has an apparent molecular weight of 110 kDa but is subjected to processing during epididymal sperm transport, thus having an approximate weight of 55 kDa. It is involved in sperm-oocyte membrane adhesion and is similar to fertilin; it also uses its disintegrin loop for adhesion. Cyritestin gene knockout mice did not show any defect in sperm-oocyte membrane fusion, but they did show impairment in sperm-zona binding [84]. However, as human cyritestin gene is nonfunctional illustrating the risk of using rodents as models of human/primate fertility.

(3) Sperm Protein-10 (SP-10). Sperm-specific acrosomal protein, SP-10, was first identified in humans [85]. It was observed that SP-10 is a testis but not species-specific protein as antibody against SP-10 recognized sperm from baboon, macaque, and pig, but not from rabbit, bull, rat, guinea-pig, and cat [86]. The cDNA encoding SP-10 protein has been cloned and sequenced from a human cDNA expression library and was found to contain 1117 bp sequence encoding for 256 aa [87]. *In vitro* incubation of anti-SP-10 antibodies inhibited bovine fertilisation by reducing sperm-zona binding.

In vivo potential of SP-10 as an immunogen was tested using attenuated strain of *Salmonella* sp. expressing human SP-10. Immunization was carried out using oral route in mice and intramuscular route in monkeys. Presence of anti-SP-10 specific antibodies was seen in both cases [88]. However, antibody levels in the oviduct are of particular relevance in case of SP-10 since it is localised within the acrosomal compartment and the outer acrosomal membrane complex and is therefore only accessible to antibody after the acrosome reaction has been initiated.

(4) Fertilization Antigen-1 (FA-1). Fertilization antigen-1 (FA-1) is a 47 kDa glycoprotein which develops in testis during later stages of spermatogenesis and has autophosphorylating activity [89]. This tyrosine phosphorylation occurs during human sperm capacitation/acrosome reaction [90]. Thus, it was suggested that FA-1 has important role in capacitation/acrosome reaction which might also have effect on sperm-ZP binding. To check the specificity of FA-1 towards ZP, *in vitro* incubation of FA-1 antigen was performed with major components of ZP, that is, ZP1, ZP2, or ZP3. Results showed the specificity of FA-1 antigen towards ZP3, thus suggesting the presence of complementary sequence of FA-1 antigen on the murine oocyte.

Further, monoclonal antibodies raised against FA-1 antigen were used to ascertain the phenomenon involved in reduction of fertilization [91]. It was observed that antibodies to FA-1 block fertilization by affecting both capacitation/acrosome reactions as well as reducing sperm-zona binding. This blockage was not limited to humans as similar results were obtained in case of mice, cattle, and monkeys.

Active immunization of mice with rFA-1 resulted in an overall 64–70% reduction in fertility compared to that of controls [92]. The effect induced by antibodies was reversible and long lasting as animals delivered healthy babies when the titers reached control levels with no effect on litter size. Thus, involvement of rFA-1 antigen makes it attractive candidate for CV development. However, further studies in higher animal models are still awaited.

(5) Sperm Protein 17 (SP-17). Three sperm specific low molecular weight (~13 ± 2 kDa) proteins have been isolated from rabbit sperm and named rabbit sperm antigen-1 (RSA-1), RSA-2, and RSA-3/SP17 [93]. Monoclonal antibodies to RSA antigens showed cross reaction with human, baboon, and mice sperm. These antibodies also inhibited penetration of zona-free hamster oocyte by human spermatozoa [94]. Among these, SP-17 is the most important antigen which is exposed on the surface of mouse sperm after acrosome reaction. The localization of SP-17 was seen throughout the equatorial segment and this segment binds to ZP. *In vitro* studies revealed the specificity of SP-17 towards ZP3. When male and female mice were immunized with a chimeric peptide containing SP-17 and T-cell epitope, there was rise

in antibody titers. This rise in antibody titer confirmed the immunogenic nature of the peptide. Interestingly, the increase was pertinent in both sexes.

(6) NZ-1. The isolation of NZ-1 was carried out using polyclonal sera which was obtained from mice after immunization with human sperm antigens. The antigens so obtained had bands belonging to the molecular weight of 14–18 kDa on SDS-PAGE. Screening of the mouse λgt11 library using these antibodies recognized a cDNA clone coding for an antigen, designated as NZ-1. Neither the sequence of amino acid nor cDNA had matching with any known cDNA/amino acid sequence present in database. Recombinant form of NZ-1 antigen was used to immunize mice which resulted in reduced fertility rates [95].

(7) NZ-2. Another cDNA encoding for a sperm antigen, designated NZ-2, was also cloned and sequenced from human testis cDNA-lgt11 expression library by using polyclonal antibodies to human sperm surface antigens belonging to 14–18 kDa molecular regions. These sperm antigens are involved in binding to ZP of the human oocyte. The NZ-2 cDNA has 335-bp 58 and 139-bp 38 noncoding regions. The translated protein has a calculated molecular weight of 19 kDa. Extensive computer search in the Gen-Bank, National Biomedical Research Foundation (NBRF), and Swiss database indicates it to be a novel protein, having 99.5% nt sequence similarity, except for the first 40-bp, only with the human bacterial artificial chromosome (BAC) containing cloned human sperm DNA. The 20 kDa protein was recognized specifically by the antisperm IgG, not by the control IgG in the western blot procedure [92]. The recombinant human sperm NZ-2 antigen may find applications in the development of a contraceptive vaccine and diagnosis and treatment of infertility in humans.

(8) Lactate Dehydrogenase (LDH-C_4). The isozyme lactate dehydrogenase (LDH) plays important role in lactate metabolism and glycolysis of developing and mature spermatozoa. It has different subunits LDH-A, LDH-B, and LDH-C. Among these, LDH-C subunit is independent gene product which is expressed only in spermatogenic cells and is immunologically different from the other two subunits. Homotetrameric LDH-C_4 is perhaps the most extensively characterized sperm antigen which plays chief role in lactate metabolism and glycolysis. *In vitro* incubation of anti-LDH-C_4 antibodies resulted in sperm agglutination and cytotoxic effects on sperm cells. Active immunization using LDH-C_4 in variety of mammalian species including primates did affect fertility. Immunization of fertile baboons with synthetic form of LDH-C_4 conjugated to DT resulted in 75% reduction in fertility rates [96].

(9) Sperm Agglutination Antigen-1 (SAGA-1). Sperm agglutination antigen-1 was isolated from human spermatozoa using a monoclonal antibody generated against human sperm, designated as S19. Molecular weight of SAGA-1 ranges between 15 and 25 kDa. Immunolocalization by electron microscopy revealed the reactivity of S19 monoclonal antibody to the entire human sperm surface indicating the presence of SAGA-1 on whole sperm surface [97]. *In vitro* incubation of antibody generated against SAGA-1 resulted in agglutination/immobilization of sperm as well as inhibition of penetration of zona-free hamster ova [98].

(10) Human Equatorial Segment Protein (hESP). Human equatorial segment protein (hESP) is a sperm specific protein, first reported in the year 2003 [99]. Mouse ESP (mESP) has 81% homology with hESP. It is localized to the equatorial segment of acrosome and is associated with acrosome biogenesis. Interaction of hESP with sera of infertile male and female patients showed positive reaction. The use of antiserum raised against recombinant hESP inhibited the binding and fusion of human sperm in the hamster egg penetration assay [100]. These results suggested that ESP might be an interesting target for designing contraception vaccine.

When immunization of mice was carried out with polyepitope antigens, results showed that fertility of mice was significantly reduced. Further, an attempt was made to isolate the important part of mESP playing vital role in infertility. For achieving this, full mESP was divided in 3 fragments, namely, P1, P2, and P3. Inhibition of sperm-egg binding was seen only in the presence of anti-P1 or anti-P2 antibodies, while no inhibition was seen in case of anti-P3 antibodies. These results indicated that additional studies are needed to better characterize these peptides [100].

(11) YLP-12. A dodecamer sequence (YLPVGGLRRIGG), designated as YLP-12, was identified on human sperm using phage display technology [101]. This technology is an innovative tool which was first reported by George Smith in which the peptide sequences are presented on the surface of filamentous phage to examine their interaction with specific antibodies [102]. The peptide sequence of YLP-12 is primarily localized on acrosome and tail in case of humans and murine sperm. Extensive computer search in the database did not result in homology or identity with any known sequence indicating the novelty of the peptide. Immunoblotting analysis suggested that antibodies resulting from vaccination with YLP-12 recognized a specific protein band of 72 kDa in testis.

In vitro incubation of spermatozoa with YLP-12 Fab significantly reduced acrosome reaction (AR) [101]. Prevalence of antibodies against YLP-12 in sera of immunoinfertile patients highlighted its potential to be used as CV. Thus *in vivo* studies were carried out in mouse model using synthetic YLP-12 peptide conjugated with the binding domain of recombinant cholera toxin subunit B (rCTB). Immunization of female mice was done using two different routes, namely, intranasal and intramuscular. Both the routes resulted in overall reduction of 70.3% (intranasal) and 61.4% (intramuscular) in fertility, respectively. Though, further studies need to be carried out in higher animal models.

(12) Rabbit Sperm Membrane Protein-B (rSMP-B). One of the components with a molecular weight of 20.1 kDa was identified from the tail of rabbit sperm and designated as rSMP-B [103]. Immunolocalization of rSMP-B on spermatozoa and somatic cells was tested using polyclonal antibodies to rSMP-B. The results suggested that this antigen was

produced by germ cells during spermatogenesis. Monoclonal antibodies raised against rSMP-B were demonstrated for their ability to block the penetration and fertilization of zona-free hamster eggs by human sperm *in vitro*. Immunization of male rabbits with rSMP-B protein resulted in blockage of spermatogenesis resulting in azoospermia. Further it was found that immunization by rSMP-B 230 peptide resulted in 83.3% infertility which was observed in case of immunized female rats after mating.

(13) Sperm Acrosomal Membrane-Associated Protein (SAMP-32). A novel human sperm membrane antigen, SAMP32 (sperm acrosomal membrane-associated protein 32), was discovered from human sperm extracts [104]. When the experiments were carried out to reveal the expression of SAMP-32, results showed that the expression was testis-specific and subsequent stages of acrosome biogenesis. Before acrosome formation, SAMP32 expression did not occur. *In vitro* incubation of anti-SAMP antibodies significantly suppressed the binding and the fusion of capacitated human spermatozoa with zona-free hamster eggs in comparison to preimmune serum. The reason that serum from an ASA positive infertile man strongly reacted with the recombinant SAMP32 antigen suggested that it might be one of the antigens related to immune infertility which can be exploited as a target for CV [105].

(14) 80 kDa Human Sperm Antigen (80 kDa HSA). 80 kDa HSA was identified from human sperm extract by western blot analysis using serum of an immunoinfertile healthy woman. It was found to be a conserved glycoprotein localized only in testis and epididymis and not localized in other somatic tissues. The pI of the protein was found to be 4.5 [106]. Active immunization of both male and female rats was carried out using native protein and it induced infertility in both sexes. Use of synthetic form of 80 kDa HSA was also tried in order to augment the immunogenicity. Maximum inhibition in fertility was seen in case of male rabbits immunized with peptide-1 followed by peptide-NT and remaining peptides. Active immunization of male marmosets with synthetic peptide-1 induced antibody response in 7 out of 9 marmosets and 6 out of 7 became infertile. Semen samples of the animals from treated group showed complete loss of progressive motility. The effect was found to be reversible as antibody titer declined 8–10 weeks after last booster injection and the animals regained their fertility. Thereafter, no apparent effect on normal physiological processes was observed [107].

(15) BS-17. BS-17 is one of the antigens being identified using ASA from serum of infertile woman. When the human testis λgt11 cDNA expression library was probed with polyclonal anti-BS-17 antibodies, the positive clone yielded a cDNA fragment consisting of 758 bp. Polyclonal antibodies to the BS-17 antigen inhibited human sperm from penetrating and fertilizing zona-free hamster oocytes [108]. Due to 99.7% homology of BS-17 with calpastatin, it was suggested that this inhibition involved destabilization of the calpastatin-calpain complex by the anti-BS-17 antibodies. After this destabilization, calpain could trigger the premature sperm acrosome reaction before sperm would reach the ovum, thus resulting in deterioration of the sperm fertilizing capability. However, till now none of the *in vivo* experiments have been carried out.

(16) Contraceptive Vaccinogen. Complementary DNAs encoding few sperm antigens have been cloned and sequenced [92]. Subtractive cDNA hybridization technology was used to isolate novel human testis specific antigen which was designated as contraceptive vaccinogen [109]. The molecular mass of the deduced aa sequence of contraceptive vaccinogen protein was calculated to be 35.3 kDa. Contraceptive vaccinogen cDNA had no homology with existing sequences in database.

The recombinant contraceptive vaccinogen protein recognized ZP3 component of ZP of human oocytes rather than ZP1/ZP2. The specificity of this interaction was further confirmed when human sperm binding to ZP of human oocytes was significantly inhibited in the presence of antirecombinant contraceptive vaccinogen antibodies. In addition, immunobead binding technique (IBT), using live sperm, highlighted the predominant presence of recombinant contraceptive vaccinogen on surface, thus accessible to antibody binding. Though antibodies produced against recombinant contraceptive vaccinogen did neither agglutinate nor cause immobilization, they could block capacitation of human sperm as deduced by sperm penetration assay (SPA). Presence of contraceptive vaccinogen antigen on murine sperm raises the feasibility of exploitation of animal model for studying its immunocontraceptive potential and it may find application in CV development.

(17) Epididymal Protein-20 (EP-20). A 20 kDa glycoprotein designated as epididymal protein (EP-20) was isolated from rabbit cauda epididymal fluid. Immunolocalization of EP-20 protein was carried out using polyclonal antibodies to EP-20 which showed the presence of the protein in testis and epididymis. Interestingly, intense staining was also observed in case of human testes, whereas germ cells, interstitial cells, and nine other tissues remained unstained. This observation highlighted the conserved nature of the protein. Polyclonal antibodies raised against EP-20 not only immobilized and agglutinated human sperm but also blocked the penetration of zona-free hamster eggs by human sperm *in vitro* [110]. However, potential of EP-20 as an immunocontraceptive needs to be examined in *in vivo* model.

(18) Dorsal Head and Equatorial Segment (DE) Protein. Dorsal head and equatorial segment protein, also known as acidic epididymal glycoprotein (AEG), has a molecular weight of 37 kDa. It is associated with the dorsal region of the sperm head during epididymal maturation with binding characteristic of a receptor-type mechanism [111]. During acrosome reaction both *in vitro* and *in vivo*, the protein migrates from epididymal region to the equatorial segment of sperm [112]. The protein plays an important role in sperm-egg binding. Incubation of sperm with antibodies against the protein significantly inhibited the penetration of zona-free eggs *in vitro* [113]. This binding was significantly reduced when

sperm formerly exposed to anti-DE antibodies were used for artificial insemination *in vivo* [111]. Decreased fertilization rates were observed in male rats, after immunization with purified DE.

Further an attempt was made to evaluate the immunocontraceptive potential of recombinant DE in rats. Animals immunized with recombinant form showed statistically significant reduction in fertility in contrast to native DE. This observation supports the evidence of exploiting epididymal proteins as a target for CV development.

(19) Epididymal Protease Inhibitor (Eppin). An epididymal protein designated as epididymal protease inhibitor (Eppin) has a molecular weight of 26 kDa [114]. Immunizationof male monkeys was carried out using Eppin. After immunization, 78% of monkeys who developed high anti-Eppin antibody titers became infertile and 71% of those monkeys recovered fertility after immunization was stopped. Later, another study was carried out wherein active immunization with Eppin gave maximum reduction of 90% in animals [115]. These results highlighted the potential of Eppin as a very attractive candidate in contraception because none of the proteins isolated so far resulted in >75% of infertility. To enhance the sensitivity of eppin, recombinant form of Eppin was tried. However, immunization with recombinant protein resulted in only 70% of reduction in contrast to native protein. Therefore, further studies need to be employed using Eppin as a potential target for immunocontraception.

(20) Sperm Flagella Protein (SFP2). Several novel epididymis-specific proteins have been identified using the combinatorial approach [116]. SFP2 is localized on the surface of sperm and showed epididymis specificity. Antibodies to SFP2 peptide 1 recognized a doublet at 220–230 kDa only in epididymal extract. Antibody to peptide 1 recognized the cognate protein on mouse, rat, and human spermatozoa. Immunization of male mice with peptide1 resulted in production of specific antibodies.

In vitro incubation of immune sera from immunized male mice with sperm caused significant reduction in motility and viability but did not agglutinate sperm. Mating of immunized male mice was allowed with female mice. A reduced fertility rate of 20% was observed in female mice mated with immunized male when compared with control animals (100%). The antibody levels in the immunized males declined by 22 weeks after immunization, resulting in 100% reinstatement of fertility. Histological examination of reproductive organs of the immunized mice and control mice caput, corpus, and cauda region of epididymis was carried out. No gross histological difference was found between immunized group and control group. No sign of leucocyte infiltration was seen in any tissue thereby; data provide an experimental basis for the development of an effective contraceptive vaccine based on new epididymal target. Still, the research is at its infancy stage and has miles to go.

(21) Izumo. *Izumo* is named after a Japanese shrine dedicated to marriage. It is a typical type I membrane glycoprotein consisting of one immunoglobulin, like domain and putative N-glycoside link motif. It is expressed on both mouse and human sperm with a molecular weight of 56.4 kDa and 37.2 kDa, respectively. cDNA encoding for mouse and human Izumo protein has been sequenced. It is not detectable on ejaculated sperm but becomes recognizable once the sperm cell undergoes AR. Among all gene knockouts, Izumo has gained highest insight because gene knockout male mice for Izumo resulted in 100% block of conception. It was observed that even after mating of Izumo−/− male mice with proven females, none of the female delivered pups [117].

Thus to evaluate the immunocontraceptive potential of Izumo, three peptides of Izumo, namely, IZ1/IZ2/IZ3, were generated [118]. High titer specific antibodies were generated against all the peptides in three groups in contrast to control group (nonimmunized). Mating of immunized female mice with proven breeder male mice was allowed, and results showed block of conception leading to infertility. However, none of the peptides resulted in 100% contraceptive effect and maximum contraceptive effect was found to be only 53.4%. Regain of fertility was seen few months subsequent to the last booster. Thus, it may be stated that Izumo antigen holds potential for becoming CV. Also, as it is not exposed until the sperm cell undergoes AR; hence antibodies against the protein should be present at a particular space and time.

(22) A-kinase Anchoring Protein (AKAP). Phosphorylation of sperm proteins is regulated by a synchronized balance between kinase and phosphatase activities. It was demonstrated that two proteins, namely, A-kinase anchoring protein 3 (AKAP 3) and AKAP 4, are the most abundant proteins present in fibrous sheath of sperm. AKAP 3 is the basic organized structure of the sheath while AKAP 4 plays an imperative role in completing the assembly of fibrous sheath [119]. Relation of partial deletions of Akap3 and Akap4 gene sequences may therefore be linked to defective assembly and failure of compartmentalization of AKAP3 and AKAP4 proteins in the tail. These gene deletions may result in sperm immotility [119, 120]. However, the hypothesis needs to be verified using animal models.

(23) Testis Specific Antigen-1 (TSA-1). A 24 kDa testis specific antigen-1 (TSA-1) is expressed in murine sperm and human sperm. Northern blot procedure revealed the expression of TSA-1 on testis. TSA-1 cDNA did not have any sequence similarity with known nucleotide/amino acid sequence in the databases. Antibody recognized acrosomal, equatorial, mid piece, and tail regions of human sperm [121]. Antirecombinant TSA-1 antibodies inhibited the acrosome reaction and sperm egg binding in *in vitro* assays [122]. These findings indicated that the testis/sperm specific protein has role in human sperm function and may find clinical application in the contraceptive vaccine development.

Various sperm antigens have been delineated and characterized. Active immunization using most of these antigens resulted in generation of high titer specific antibodies; even then none of the antigens have resulted in 100% infertility. The reasons associated include variability in immune response at individual level, maintenance of high antibody titers, time lag to achieve reasonably good titers, that is, approximately

3 months, and uncertainty that for how long the required level of antibody will remain in circulation. All these concerns may be taken care by the use of preformed antibodies, that is, passive immunization approach. The success of application of passive immunization in case of various infectious diseases indicated the feasibility and viability of such an approach [123]. Furthermore, local application of preformed antibodies will not interfere with endocrine system of the individual and is independent of host's immune status, thus providing additional advantages over the use of active immunization.

2.4. Passive Immunization: An Effective Approach. Passive immunization includes two basic approaches, that is, use of polyclonal or monoclonal antibodies. In comparison to polyclonal antibody, use of monoclonal antibody is particularly appealing and feasible. Being specific, monoclonal will avoid any cross reaction with somatic cells and there will also be lesser chances of allergic reactions. Due to this reason, they have been heralded as "magic bullets." The term "magic bullet" was coined in the end of 19th century to describe a theoretical agent that could specifically target pathogens while leaving a healthy tissue intact [124]. As a further consequence of their high specificity, monoclonal antibodies are more potent; for example, use of monoclonal (Synagsis) against RSV infection in high risk infants is approximately 50 times effective than polyclonal preparation (RespiGam). Similar results were obtained in case of *Streptococcus* infection in neonates. Also, recent advances in hybridoma technology used for generation of monoclonal antibody have revitalized interest in passive immunization.

The guiding concept which led to the idea of using passive immunization for immunocontraception was based on the clinical reports of infertile men whose semen contains antibodies that immobilize sperm usually by agglutination. The data from previous studies indicated that sperm agglutinating/immobilizing antibodies must be present in the reproductive tract specifically vagina to provide significant immunoprotective effect from pregnancy. Earlier reports have examined the stability of antibodies in mucus secretions and found that antibodies whether polyclonal/monoclonal are stable in seminal fluid and cervical mucus at 37°C. The antibodies can provide protection for one to several days depending on their half-life [125] and the use of gels for the delivery of antibodies will result in sustained release.

Very few antigens have been explored to date via passive immunization approach. Polyclonal antibodies have been raised against three proteins, namely, 16 kDa sperm protein, HSA, and Eppin. Singh et al. [126] observed that polyclonal antibody raised against a 16 kDa human sperm protein when administered passively in female mice resulted in reduction of fertility. It was likely that the antifertility effect was due to agglutinating nature of the antibody which inhibited the motility of spermatozoa. In another study, passive administration of antibodies to 80 kDa HSA in male and female rats resulted in agglutination of the spermatozoa with the loss of motility and impaired fertility [127]. Though still detailed toxicological evaluation needs to be conducted using nonhuman primate model (bonnet monkey). Also, passive immunization in mice with antibodies to a 26 kDa epididymal protein showed 89% reduction in fertility [128].

Further, monoclonal antibodies have been raised against human sperm and rabbit sperm, namely, MA-24 and mouse anti-rabbit sperm (MARS), respectively. Naz et al. [129] found that systemic intraperitoneal injection of MA-24 ascites fluid in mouse models reduced the percentage of fertilized ova (from 69% in untreated to 29% in treated), thereby reducing the fertility *in vivo* [130]. For determining the *in vivo* contraceptive efficacy of MARS, the antibodies were incubated *in vitro* with a defined volume of semen, followed by insemination of females. All the mAbs were found contraceptively effective [131].

3. Conclusion

In conclusion, it may be suggested that better understanding of the mechanisms of immunocontraception will lead to large scale applications of these methods in future. The review highlights that use of passive immunization approach is more feasible when compared with the concerns associated with the active immunization since data from antibody therapies including clinical trials in infectious diseases indicate that it is an exciting, practical, viable, and durable proposition ready for experimentation. Further, among all exploitation of sperm antigens as an immunocontraceptive agent after evaluating its feasibility and effectiveness can result in potent immunocontraception. Moreover, the generation of monoclonal antibodies in this league will act as "cherry" on top of the cake.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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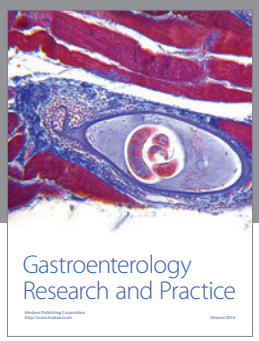
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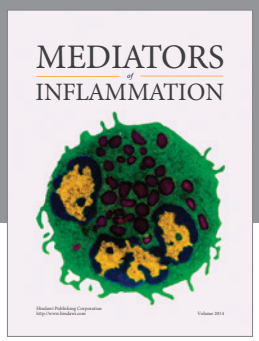
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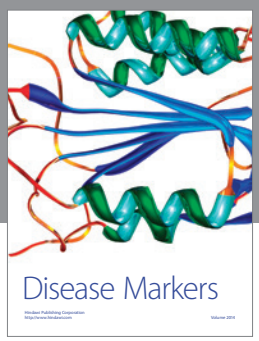
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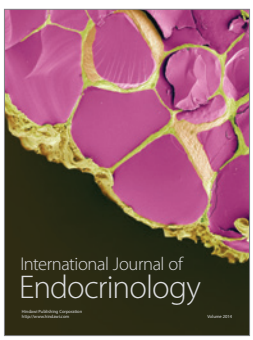






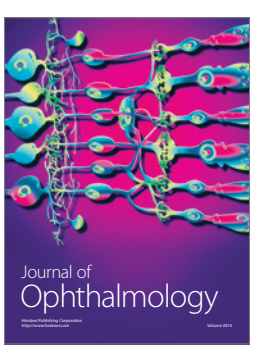


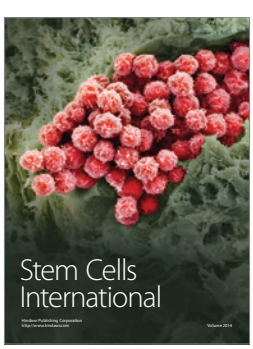


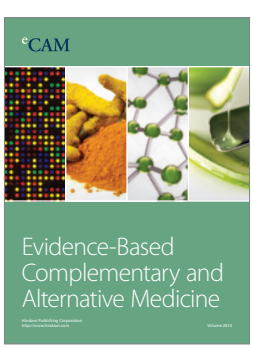






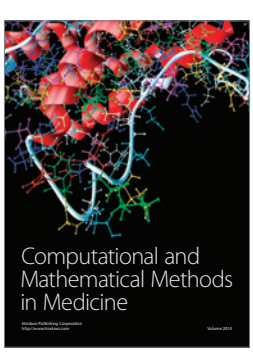


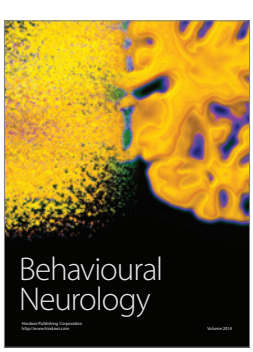




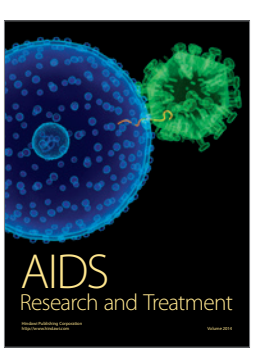


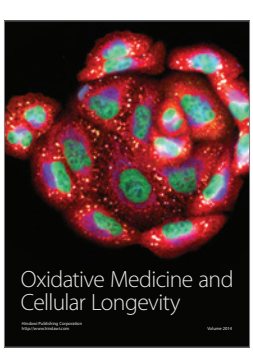












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Direct and Correlated Responses to Multitrait, Divergent Selection for Immunocompetence[1]

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ABSTRACT Leghorn lines had been selected for an immunocompetence index based on four traits: antibody production to *Mycoplasma gallisepticum* (MG) and *Pasteurella multocida* (PM) vaccines, reticuloendothelial clearance of colloidal carbon (CCA), and cell-mediated, wing web response to phytohemagglutinin (PHA). The purpose of this study was to produce replicated lines of chickens with divergent levels of multitrait immunocompetence by index selection. The objectives of analyses of Generations 5 to 7 of this study was to characterize these lines with respect to immune-response traits, correlations among these traits, and correlated responses in other important production traits.

Differences ($P < .05$) existed between the lines selected for high or low immune response and between the two replicates in mean breeding values and in individual immune-response traits. Averages of heritability estimates, weighted by number of offspring and pooled across three generations (two cycles of selection), estimated by using sire variance components and parent-offspring correlations were, respectively, .16 and .09 for the index, .31 and .08 for MG, .21 and –.02 for PM, .06 and .05 for CCA, and .08 and .12 for PHA. Realized heritabilities (response divided by effective selection differential) pooled across the two selection cycles, were .19 and .11 for the index, .06 and –.01 for MG, .44 and .32 for PM, 1.52 and –1.21 for CCA, and .48 and .15 for PHA, for Replicates 1 and 2, respectively. Phenotypic correlations among traits were generally small, and several estimates were negative. Estimates of genetic correlation varied widely. Juvenile and adult body weights, age of first egg, 32-wk egg weight, and rate of egg production were analyzed to evaluate effects of selection on these traits of direct economic importance. Very few differences were noted.

(*Key words*: index, selection, immune response, White Leghorn, chicken)

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INTRODUCTION

Improving disease resistance without actually challenging animals with a disease agent is desirable. Because an animal's response to the presence of a disease agent usually involves some form

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of immune response, selection for immunocompetence has been suggested as an indirect method of improving disease resistance (Gavora and Spencer, 1983; van der Zijpp, 1983; Warner *et al.*, 1987). Selection experiments using a variety of agents to elicit an immune response have been conducted (Bacon, 1992). Biozzi *et al.* (1979) reported selection experiments to several antigens in mice. The results of these experiments suggested that, in some instances, selection for increased antibody production to one antigen (for example SRBC) improved the responses to several other antigens. Antibody production against SRBC has also been used in selection experiments in chickens (Siegel and Gross, 1980; van der Zijpp *et al.*, 1983; Martin *et al.*, 1990; Pinard *et al.*, 1992). Pevzner *et al.* (1981a) reported on selection for antibody response to the synthetic amino acid polymer, GAT and also for antibodies against *Salmonella pullorum* (Pevzner *et al.*, 1981b). Antibody production against Newcastle disease virus and *Escherichia coli* (Pitcovski *et al.*, 1987) and against *E. coli* alone (Leitner *et al.*, 1992) have also been used in selection experiments. Karakoz *et al.* (1974) selected for cell-mediated immune response by using purified protein derivative of tuberculin.

Although these and other experiments have been conducted by selecting for immune response to some specific agent (or specific facet of immune response), selection for general immunocompetency, combining multiple facets of immune response, might be more desirable (Gavora and Spencer, 1978; van der Zijpp, 1983). Successful selection for different components of immune response simultaneously requires a knowledge of each individual trait and the interactions between the different facets of immune response and associations with disease resistance and other important biological traits. Biozzi *et al.* (1979) reported a negative correlation between antibody production and phagocytic ability in lines of mice divergently selected for SRBC response. Most of the other correlations found by Biozzi *et al.* (1979) between different measures of different facets of immune response were relatively small. Gross *et al.* (1980) found positive association between resistance to viral and protozoal diseases in lines selected for antibody production to SRBC but found negative correlation between anti-SRBC antibody production and resistance to bacterial diseases.

Mallard *et al.* (1992) characterized the immune-responsiveness of a random-bred population of Yorkshire pigs by using 14 indicator traits. Five traits [antibody production against hen egg white lysozyme (HEWL), total serum IgG, delayed-type hypersensitivity to a purified protein derivative of Bacillus (PPD), blastogenic response to concanavalin A (ConA), and monocyte killing of *Salmonella typhimurium*] were then chosen for multitrait selection. After one generation of selection based on a combined estimated breeding value (EBV), high and low lines differed significantly with respect to HEWL, PPD, and ConA, and also with respect to total EBV. Heritability estimates ranged from 0 to .25. The ConA and HEWL were positively correlated in the base population and no other significant correlations among these five traits were reported.

Experiments with selection for immune response have yielded varied results with respect to correlations with other important economic traits. In pigs, Meeker *et al.* (1987) found a negative correlation between growth traits and immune responses to both *Bordetella bronchiseptica* and pseudorabies virus vaccines but found no correlation between the two antigens and backfat thickness. Researchers working with dairy cattle (Detilleux *et al.*, 1991; Kehrli *et al.*, 1991; Weigel *et al.*, 1992) have used a somewhat reversed approach by selecting lines divergent in milk production and then comparing the lines' immune responsiveness. Several negative correlations with milk production have been found in these studies. In chickens, Siegel and Gross (1980) and Siegel *et al.* (1982) reported a negative correlation between body weight and antibody production in lines divergently selected for antibody production to SRBC. Van der Zijpp (1983) also reported negative correlation between antibody production to SRBC and body weight, and no other significant correlations with produc-

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tion traits were found. Although antagonistic correlations with important economic traits could diminish selection pressure and, therefore, slow improvements in immune response traits, simultaneous selection for negatively correlated traits is still possible (Falconer, 1989).

The overall purpose of this selection project was to develop replicated lines of chickens divergent in levels of multitrait immunocompetence and to characterize the genetic parameters of the traits involved. Cheng *et al.* (1991) reported parameters from the base population and the first two selected generations of chickens in this project. This report presents results from the fifth, sixth, and seventh generations of these lines. Genetic parameters of the selection index and the four individual traits that are used in that index, as well as associations between the immune response traits and production traits, are presented.

MATERIALS AND METHODS

Chickens

Divergent selection for multitrait immunocompetence has been conducted in a group of White Leghorn chickens derived from the Ottawa Control Strain 7 (Gowe and Fairfull, 1980). High-responding half-siblings from 10 sires and 36 dams were used to produce a high-immune-response line (1H) and corresponding low-responding half-siblings from the same 10 sires and 36 dams were used to produce a low-immune-response line (1L) in Replicate 1. Similarly, half-sib offspring from another 10 sires and 37 dams were used to produce a high-immune-response line (2H) and a low-immune-response line (2L) in Replicate 2. Matings for future generations were within lines, based upon breeding values (estimated by using the index described below), and sibling matings were avoided.

All progeny (approximately 500) were tested in each generation. In each generation, chickens with the highest ranking breeding values within each sire family within each high-immune-response line and chickens with the lowest ranking breeding values within each sire family within each low-immune-response line were chosen as breeders for the next generation. Ten sires and 40 dams were used as breeders in each of the four lines in each generation. Immunocompetence parameters of the base generation and first two selected generations have been reported previously (Cheng *et al.*, 1991).

All chickens in each generation were raised in floor pens until 20 wk of age and in individual cages after 20 wk of age. They were provided *ad libitum* access to feed meeting all NRC (1984) requirements and water at all times.

Immunocompetence Measures

The immunocompetence measures used in this project have been described in detail (Cheng *et al.*, 1991). Brief descriptions follow. Humoral immune response was measured as antibody production to two vaccines. Vaccines against *Mycoplasma gallisepticum* (MG bacterin)[3] (MG) and *Pasteurella multocida* (FC bacterin)[3] (PM) were injected at 6 wk of age. Serum samples, collected at 9 wk of age, were analyzed for specific antibodies by using commercial ELISA[4] kits according to the manufacturer's directions. Antibody production was calculated as a corrected ratio of the individual's sample and a positive control $(S - N)/(P - N)$, where S = sample; N = negative control; and P = positive control; per kit instructions. The positive and negative controls were chicken sera included in the commercial kits and were tested in duplicate on each plate of samples. The MG and PM responses are expressed as a ratio of sample to the positive serum. Sera collected before initial injection were also tested to evaluate any possible previous exposure and response to these antigens.

Cell-mediated immune response was measured by using wing web response to phytohemagglutinin (PHA-P)[5] (van der Zijpp, 1983; Lamont and Smyth, 1984) when the chickens were 10 to 12 wk of age. Wing web response (PHA), in millimeters, was

[3]Solvay Animal Health, Charles City, IA 50616.
[4]Idexx Corp., Portland, ME 04104.
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calculated as

$$PHA = (postINJ - preINJ) - (postPBS - prePBS), \quad [1]$$

where postINJ = thickness of test wing 24 h after injection of PHA; preINJ = thickness of test wing before injection; postPBS = thickness of control wing 24 h after injection of physiological saline (PBS); and prePBS = thickness of control wing before injection (van der Zijpp, 1983).

Reticuloendothelial clearance, a measure of phagocytic ability, was measured by using intravenous injection of colloidal carbon (Glick *et al.*, 1964; Lamont, 1986) as modified by Cheng *et al.* (1991). The CCA was calculated as in Equation 2

$$CCA = -\frac{\ln(5 \text{ min O.D.}) - \ln(1 \text{ min O.D.})}{5 - 1} \quad [2]$$

where 5 min O.D. was the optical density of the sample drawn 5 min after injection of carbon; 1 min O.D. was the optical density of the sample drawn 1 min after injection, and the denominator reflected the time between collection of samples (Lamont, 1986; Cheng *et al.*, 1991). The CCA values are reported as ln of absorbance values (at 675 nm) relative to the individual's normal serum.

Index

A selection index based on the four described traits was used to rank the chickens in each generation. Index weights were calculated by solving

$$[P] [b] = [G] [a] \quad [3]$$

for [b], a vector of unknown index weights, where [P] was the phenotypic variance-covariance matrix of the four traits (MG, PM, CCA, and PHA), [G] was the genotypic variance-covariance matrix of the four traits, and [a] was a vector of "economic" weights (Hazel, 1943). Some modifications were made to the index. Because no estimates of economic values of these traits were available, [a] was calculated as the inverse of the heritability of each trait in an effort to balance selection pressure on all four traits. With no estimates of relative economic worth for each trait, balanced gains in all three facets of immune response were deemed desirable. Cheng (1990) used parameter estimates from the base population and compared different weights for the four traits. He determined that the inverses of the traits' estimated heritabilities equalized gains better than did the inverses of the traits' variances or setting each trait's weight to unity. Also, for selection in the sixth and seventh generations, for breeders of the seventh and eighth generations, respectively, the weights for each measure of antibody production (MG and PM) were divided in half in an effort to increase selection emphasis for cellular immune response and reticuloendothelial clearance relative to humoral immune response. In addition, all genetic covariances in [G] were set to zero because no previous estimates of covariances for these traits in chickens were available in the literature, and estimates for similar traits in other species were relatively small (Biozzi *et al.*, 1979). Another important feature in this selection experiment was that the parameters used to calculate index weights were adjusted in each generation to reflect added information. Heritabilities, genetic variances, and phenotypic variances and covariances were estimated in each generation by using records from all previous generations and the present generation combined. Methods used to estimate these parameters are described below.

Production Records

Body Weights. Body weights were recorded on all individuals at 2, 6, 12, and 20 wk of age and on selected individuals (those chosen to be future breeders) at 32 and 51 wk of age in Generations 5 and 6. Body weights at 32 and 51 wk of age were not yet available for Generation 7 at the time of this study's analyses.

Egg Production. Egg production records were collected for hens in Generations 5 and 6 selected as breeders. Age at first egg (AFE), average egg weight at 32 wk of age (EGGWT), and egg production in periods 1, 2, 3, 4, and Total (P1, P2, P3, P4, and PT, respectively) were recorded. Each production period was 8 wk, and eggs were

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recorded for 4 d/wk, for a total of 32 d per period. Production was measured on a hen-housed basis from 20 wk of age. Egg production records were analyzed by number of eggs produced within each period and then converted to percentages for presentation.

Mortality. Mortality was recorded on all chickens to 20 wk of age. The number of accidental deaths was subtracted before analysis. After 20 wk of age, only those chickens selected as breeders 40 males and 160 females) were kept and mortality is, therefore, recorded only on these chickens.

Statistical Analyses

The General Linear Models (GLM) Procedure of SAS® (SAS Institute, 1985) was used to estimate least squares means of breeding values and the four individual traits of immune response in each generation. The model (Model [4]) used for these analyses was

$$Y_{ijkl} = \mu + R_i + D_j + R \times D_{ij} + s_{ijk} + e_{ijkl}, \quad [4]$$

where Y is the phenotypic trait (Index, MG, PM, CCA, PHA) of an individual; μ is the population mean; R is the fixed effect of the ith replicate (1, 2), D is the fixed effect of the jth selection direction (H, L), s is the random effect of the kth sire within the ij^{th} replicate and direction, and e is a random residual term. Differences between replicates and between selection directions, and the interaction, were tested for significance by using the mean square for sire as the error term.

Variance and covariance components were computed by using the restricted maximum likelihood (REML) method of the VARCOMP Procedure of SAS® (SAS Institute, 1985). Phenotypic variance and covariance components and sire variance components were used to calculate the index weights (by using Equation [3]) and were also used to estimate heritabilities in these lines. Heritabilities were estimated by using three methods. "Narrow sense" heritability ($\hat{h}^2_s$) was estimated by:

$$\hat{h}^2_s = \frac{4\,(\hat{\sigma}^2_s)}{\hat{\sigma}^2_s + \hat{\sigma}^2_e} \quad [5]$$

where $\hat{\sigma}^2_s$ = sire variance component and $\hat{\sigma}^2_e$ = error variance component (Falconer, 1989). The same model, Model 4, was used for this analysis.

Heritabilities were also estimated by using correlations between offspring values and their midparent values. The NESTED Procedure of SAS® (SAS Institute, 1985) was used to estimate within-line correlations. Estimates of correlations ($\hat{t}_{OP}$) were weighted by number of offspring for each parent. Heritability ($\hat{h}^2_P$) was calculated as

$$\hat{h}^2_P = \hat{t}_{OP}. \quad [6]$$

Realized heritabilities were also estimated for each replicate in the two selection cycles analyzed here. Because no control lines were measured, it was necessary to assume that response was symmetric in the high and low selected lines within each replicate (Hill, 1972). Response to selection (ΔR) was calculated as

$$\Delta R = (H_k - L_k) - (H_{k-1} - L_{k-1}) \quad [7]$$

where H = high line mean (within a replicate); L = low line mean (within a replicate); and k = generation.

Selection differentials (ΔS) were calculated as

$$\Delta S = (sH_k - sL_k) - (H_{k-1} - L_{k-1}) \quad [8]$$

where sH = mean (weighted by number of progeny) of effective breeders in the high line (within a replicate); sL = mean (weighted by number of progeny) of effective breeders in the low line (within a replicate); H = high line mean (within a replicate); L = low line mean (within a replicate); and k = generation. Selection differentials were calculated separately for sires and dams, and the mean of these differentials was used for the final analysis. Realized heritability ($\hat{h}^2_r$) was calculated as $\Delta R/\Delta S$ for each replicate in each selection cycle. The index weights from Generation 6 were used for chickens in all three generations to calculate realized heritabilities.

Phenotypic and genetic correlations between the index and the four traits were calculated from variance and covariance components. The variance components were estimated by using the REML method of the VARCOMP Procedure of SAS® (SAS Institute, 1985). Again, Model [4] was used. Covariance components ($\hat{\sigma}_{xy}$) of two traits, x and y, for example, were calculated by using Equation 9

$$\hat{\sigma}_{xy} = \frac{\hat{\sigma}^2_{x+y} - \hat{\sigma}^2_x - \hat{\sigma}^2_y}{2}, \quad [9]$$

where $\hat{\sigma}^2_{x+y}$ is the variance of the individual sums of the two traits, and $\hat{\sigma}^2_x$ and $\hat{\sigma}^2_y$ are the variances of x and y, respectively. Genetic covariances were calculated by using sire components of variance, multiplied by 4, in Equation [9]. Phenotypic correlations ($\hat{r}_{xy}$) were calculated by

$$\hat{r}_{xy} = \frac{\hat{\sigma}_{xy}}{\hat{\sigma}_x \hat{\sigma}_y}, \quad [10]$$

where $\hat{\sigma}_x$ and $\hat{\sigma}_y$ were phenotypic standard deviations of x and y, respectively. Genetic correlations were calculated by using sire standard deviations of x and y, multiplied by 4, in Equation [10].

Genetic correlations were also estimated by using parent-offspring correlations and were calculated as

$$\hat{r}_{xy} = \frac{\hat{r}_{o_x p_y} + \hat{r}_{o_y p_x}}{2\sqrt{\hat{r}_{o_x p_x} \hat{r}_{o_y p_y}}} \quad [11]$$

Some parent-offspring correlations were negative and therefore resulted in unestimable genetic correlations. Again, index weights used in Generation 6 were used for Generations 5, 6, and 7 for parent-offspring analyses.

The General Linear Models program of SAS® (SAS Institute, 1985) was used to estimate least squares means for body weights and egg production records. Again, Model [4] was used for this analysis. Standard errors were computed by using the mean square for sire as the error term, and these were used to calculate probabilities of significant differences between replicates, between high- and low-response-selected groups, and interactions.

Incidence of mortality within replicates and selection directions are reported (in percentages). Differences between replicates, between high- and low-selected groups, and the interaction were tested for significance by using 2 × 2 contingency tables (Snedecor and Cochran, 1989).

RESULTS

Least squares means for pools of lines by selection direction, Replicates 1 and 2, and their interactions are reported in Table 1. Comparing high lines and low lines, significant differences ($P < .05$) were present with respect to the overall index in Generation 7. Responses to MG differed by selection direction in Generations 5 and 7 but not in Generation 6. Differences were also seen between high-response lines and low-response lines in PM in Generation 7 and in PHA in Generation 5. Significant differences were present between replicates in antibody response to MG in Generations 5, 6, and 7, the overall index in Generations 5 and 6, in PHA in Generations 6 and 7, and in PM in Generation 6. Of the four instances of significant interactions, two (index in Generation 5 and CCA in Generation 6) were due to differences in direction and two (index in Generation 7 and MG in Generation 5) were due only to differences in magnitude.

Parameters used to calculate index weights were modified after each generation, with the assumption that additional records improved the accuracy of these estimates. As an example, the parameters used to calculate index weights in Generation 7 are reported below.

$$\mathbf{P} = \begin{bmatrix} & \text{MG} & \text{PM} & \text{CCA} & \text{PHA} \\ \text{MG} & 2.053 & .126 & -.027 & -.037 \\ \text{PM} & .126 & .420 & -.0001 & -.019 \\ \text{CCA} & -.027 & -.0001 & .053 & -.004 \\ \text{PHA} & -.037 & -.019 & -.004 & .230 \end{bmatrix},$$

$$\mathbf{G} = \begin{bmatrix} & \text{MG} & \text{PM} & \text{CCA} & \text{PHA} \\ \text{MG} & .737 & 0 & 0 & 0 \\ \text{PM} & 0 & .097 & 0 & 0 \\ \text{CCA} & 0 & 0 & .003 & 0 \\ \text{PHA} & 0 & 0 & 0 & .022 \end{bmatrix},$$

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$$\mathbf{a} = \begin{bmatrix} \text{MG} & 1.39 \\ \text{PM} & 2.17 \\ \text{CCA} & 16.13 \\ \text{PHA} & 10.64 \end{bmatrix}.$$

Index weights used for the fifth, sixth, and seventh generations, respectively, were: .379, .522, and .512 for MG; .335, .546, and .398 for PM; .335, 1.52, and 1.36 for CCA; and .409, 1.14, and 1.14 for PHA.

Heritability estimates of the four individual traits, MG, PM, CCA, and PHA and of the index were estimated by using three different methods. Table 2 presents Restricted Maximum Likelihood (REML) estimates of heritabilities from sire variance components of Generations 5, 6, and 7 and a mean (weighted by number of birds per generation) of the three generation means. Heritabilities ranged from 0 to .43 in individual generations and from .06 to .31 when averaged across generations. Estimates of heritabilities obtained by using parent-offspring correlations in the two cycles of selection and the weighted means of these estimates and from chickens pooled by selection direction are presented in Table 3. Heritability estimates ranged from –.13 to .16 in individual cycles of selection and from 0 to .12 when averaged across selection cycles. Estimates of realized heritabilities of the four traits and of the index are in Table 4. Again, estimates from each cycle of selection and a weighted mean are shown. These estimates ranged from –2.42 to 2.18 in individual cycles and from –1.21 to 1.52 when averaged across cycles.

Correlations, both genetic and phenotypic, between the four traits and between the individual traits and the index were estimated by using two different methods. Phenotypic and genetic correlations from REML estimates of variance and covariance are reported in Table 2. Phenotypic correlations among individual immune-response traits ranged from –.15 to .31 in individual generations and from –.06 to .12 when pooled across generations. Phenotypic correlations pooled across generations between the index and individual traits ranged from .21 between the index and CCA to .60 between MG and the index. Estimates of genetic correlations among immune response traits ranged from –.34 to .61. When pooled across generations, estimates were from –.34 to .58.

TABLE 1. Least squares means of four immune response traits and an index of those four traits, by selection direction and replicate, and levels of significance of Generations 5, 6, and 7 of lines selected for multitrait immune response

		Selection direction[2]			Replicate			Interaction
Trait[1]	Generation	H	L	*P*	1	2	*P*	*P*
Index	5	1.18	1.12	NS	1.09	1.21	.027	.0008
	6	2.96	2.77	NS	2.69	3.04	.020	NS
	7	3.86	3.38	.001	3.53	3.71	NS	.093
MG	5	1.31	.97	.005	1.02	1.26	.034	.011
	6	1.31	1.03	NS	.83	1.50	.017	NS
	7	1.74	1.22	.024	1.22	1.75	.022	NS
PM	5	.283	.305	NS	.258	.329	NS	NS
	6	.282	.205	NS	.078	.410	.0007	NS
	7	.826	.605	.022	.673	.758	NS	.008
CCA	5	.275	.258	NS	.274	.259	NS	NS
	6	.444	.445	NS	.463	.426	NS	.032
	7	.608	.562	NS	.587	.582	NS	NS
PHA	5	1.10	1.25	.035	1.17	1.18	NS	NS
	6	1.27	1.26	NS	1.32	1.21	.014	NS
	7	1.58	1.53	NS	1.61	1.51	.020	NS

[1]Traits are: Index = multitrait immune response value; MG = *Mycoplasma gallisepticum* antibody; PM = *Pasteurella multocida* antibody; CCA = carbon clearance response; PHA = wing web response.

[2]H = high immunoresponsiveness; L = low immunoresponsiveness.

TABLE 2. Heritabilities and genetic and phenotypic correlations,[1] calculated from sire variance and covariance components, among four immune response traits[2] and an index of the traits in three generations of lines of divergently selected chickens

		Trait				
Trait	Generation	Index	MG	PM	CCA	PHA
Index	5	**0**	. . .[3]	. . .	. . .	. . .
	6	**.24**	.95	.45	. . .	.29
	7	**.26**	1.17	.45	−.19	. . .
	$\bar{x}$[4]	**.16**	1.06	.45	−.19	.29
MG	5	.19	**.12**	. . .	. . .	. . .
	6	.85	**.43**	.17	.04	−.34
	7	.77	**.39**	.39	.25	. . .
	$\bar{x}$	.60	**.31**	.28	.15	−.34
PM	5	.15	.03	**.04**	. . .	. . .
	6	.25	.04	**.34**	.61	.33
	7	.55	.31	**.26**	.55	. . .
	$\bar{x}$	.31	.12	**.21**	.58	.33
CCA	5	.03	.02	.03	**0**	. . .
	6	.28	.02	−.15	**.13**	.46
	7	.32	−.06	.13	**.05**	. . .
	$\bar{x}$	.21	−.01	0	**.06**	.46
PHA	5	.10	−.02	−.01	0	**.10**
	6	.34	−.03	−.15	−.04	**.12**
	7	.39	−.07	−.01	−.04	**.01**
	$\bar{x}$	.27	−.04	−.06	−.03	**.08**

[1]Heritabilities are on diagonal (in bold print), genetic correlations are above diagonal, and phenotypic correlations are below diagonal.

[2]Traits are: Index = multitrait immune response value; MG = *Mycoplasma gallisepticum* antibody; PM = *Pasteurella multocida* antibody; CCA = carbon clearance response; PHA = wing web response.

[3]Values were not estimable when estimate of sire variance was nearly 0.

[4]$\bar{x}$ = mean of three generations, weighted by number in each generation.

TABLE 3. Heritabilities, estimated by using parent-offspring correlation, of four immune response traits[1] and an index of the traits in three generations of chickens divergently selected for multitrait immune response

		Trait				
Pool[2]	Generation	Index	MG	PM	CCA	PHA
H	5 to 6	.06	.03	.03	.16	.10
	6 to 7	.09	.15	−.13	−.05	.14
	$\bar{x}$[3]	.07	.09	−.05	.05	.12
L	5 to 6	.13	.04	.01	.04	.13
	6 to 7	.03	.06	.10	.03	.10
	$\bar{x}$	.08	.05	.04	.03	.11
Total	5 to 6	.10	.03	.03	.10	.12
	6 to 7	.07	.13	−.07	−.01	.12
	$\bar{x}$	.09	.08	−.02	.05	.12

[1]Traits are: Index = multitrait immune response value; MG = *Mycoplasma gallisepticum* antibody; PM = *Pasteurella multocida* antibody; CCA = carbon clearance response; PHA = wing web response.

[2]Pools are: H = High immune response lines combined; L = low immune response lines combined; Total = All lines combined.

[3]$\bar{x}$ = mean of two cycles, weighted by number per cycle.

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TABLE 4. Realized heritabilities of four immune response traits and an index of the traits in three generations of chickens divergently selected for multitrait immune response

Replicate	Generation	Trait[1]				
		Index	MG	PM	CCA	PHA
1	5 to 6	.23	.14	.27	2.18	.84
	6 to 7	.15	−.02	.61	.85	.11
	$\bar{x}$[2]	.19	.06	.44	1.52	.48
2	5 to 6	−.01	−.23	.06	−2.42	.10
	6 to 7	.24	.21	.59	0	.21
	$\bar{x}$	.11	−.01	.32	−1.21	.15

[1]Traits are: Index = multitrait immune response value; MG = *Mycoplasma gallisepticum* antibody; PM = *Pasteurella multocida* antibody; CCA = carbon clearance response; PHA = wing web response.

[2]$\bar{x}$ = mean of two cycles, weighted by number per cycle.

Parent-offspring correlations are reported separately for trait x in parents with trait y in offspring and trait y in parents with trait x in offspring (Table 5). These correlations ranged from −.11 to .06 between traits and from −.10 to .12 between the index and individual traits.

Estimates of genetic correlations, estimated from parent-offspring correlations (Table 6), ranged from −.66 to .21 for individual traits in a single cycle and from −.66 to .05 when the two cycles were pooled. Genetic correlations between the index and individual traits ranged from −.42 to .96 when the two cycles were pooled.

Some differences were noted between high-response lines and low-response lines and between Replicates 1 and 2 with respect to juvenile body weights (Table 7). In Generation 5, chicks in the low lines were heavier than chicks in the high lines at 2 wk of age. In Generation 6, the low lines were heavier at 6 wk and at 12 wk of

TABLE 5. Parent-offspring correlations among four immune response traits and an index of the traits in three generations of lines of divergently selected chickens

Progeny trait[1]	Generation	Parental trait[2]				
		PIndex	PMG	PPM	PCCA	PPHA
Index	5 to 6	. . .	.03	.06	−.02	.05
	6 to 7	. . .	.08	.01	−.04	.01
	$\bar{x}$[3]	. . .	.06	.04	−.03	.03
MG	5 to 6	.07	. . .	.06	−.07	.03
	6 to 7	.12	. . .	.04	0	−.05
	$\bar{x}$	.10	. . .	.05	−.04	−.01
PM	5 to 6	−.10	−.06	. . .	−.04	−.08
	6 to 7	−.01	.06	. . .	−.11	−.04
	$\bar{x}$	−.06	0	. . .	−.08	−.06
CCA	5 to 6	.05	.05	−.03	. . .	.02
	6 to 7	−.03	−.03	−.02	. . .	.01
	$\bar{x}$	.01	.01	−.03	. . .	.02
PHA	5 to 6	.12	0	.02	.03	. . .
	6 to 7	.02	−.05	.04	.01	. . .
	$\bar{x}$	.07	−.03	.03	.02	. . .

[1]Traits are: Index = multitrait immune response value; MG = *Mycoplasma gallisepticum* antibody; PM = *Pasteurella multocida* antibody; CCA = carbon clearance response; PHA = wing web response.

[2]Traits are: PIndex = multitrait immune response value of midparent; PMG = *Mycoplasma gallisepticum* antibody response of midparent; PPM = *Pasteurella multocida* antibody response of midparent; PCCA = carbon clearance response of midparent; PPHA = wing web response of midparent.

[3]$\bar{x}$ = mean of two cycles, weighted by number per cycle.

TABLE 6. Genetic correlations, calculated from parent-offspring correlations, among four immune-response traits and an index of the traits in three generations of lines of divergently selected chickens

		Trait			
Trait[1]	Generation	MG	PM	CCA	PHA
Index	5 to 6	.88	−.42	.15	.81
	6 to 7	1.04	. . .[2]	. . .	.12
	$\bar{x}$[3]	.96	−.42	.15	.47
MG	5 to 6		−.02	−.13	.21
	6 to 7		. . .	. . .	−.41
	$\bar{x}$		−.02	−.13	−.10
PM	5 to 6			−.66	−.54
	6 to 7			. . .	. . .
	$\bar{x}$			−.66	−.54
CCA	5 to 6				.05
	6 to 7				. . .
	$\bar{x}$				.05

[1]Traits are: Index = multitrait immune response value; MG = *Mycoplasma gallisepticum* antibody; PM = *Pasteurella multocida* antibody; CCA = carbon clearance response; PHA = wing web response.

[2]Correlations were not estimable when sire variance estimates were zero.

[3]$\bar{x}$ = mean of two cycles, weighted by number per cycle.

age. In Generation 6, chicks in Replicate 2 were significantly heavier than those in Replicate 1 at 2 wk of age but at 2 wk of age the ranking was reversed. In Generation 7, Replicate 1 chicks weighed more at 12 wk of age. All these differences, however, had disappeared by 20 wk of age, and no differences between lines were noted in adult body weights.

Egg production records from Generations 5 and 6 are described in Table 8. No differences ($P > .05$) were noted between

TABLE 7. Least squares means of body weights by selection direction and replicate, and levels of significance of Generations 5, 6, and 7 of lines selected for multitrait immune response

		Selection direction			Replicate			Interaction
BW	Generation	H	L	*P*	1	2	*P*	*P*
		(g)			(g)			
2 wk	5	116	122	.01	119	119	NS	NS
	6	129	132	NS	128	133	.02	NS
	7	125	130	NS	129	126	NS	NS
6 wk	5	410	419	NS	453	410	NS	.07
	6	425	443	.01	419	416	.0001	NS
	7	399	412	NS	412	399	NS	NS
12 wk	5	877	892	NS	896	873	NS	.08
	6	838	893	.08	842	890	NS	NS
	7	873	912	NS	917	868	.04	NS
20 wk	5	1,110	1,130	NS	1,123	1,117	NS	NS
	6	1,392	1,374	NS	1,373	1,394	NS	NS
	7	1,390	1,397	NS	1,420	1,367	NS	NS
32 wk	5	1,766	1,740	NS	1,763	1,742	NS	NS
	6	1,781	1,740	NS	1,761	1,759	NS	NS
	7	. . .[1]	. . .	. . .	. . .	. . .	. . .	. . .
52 wk	5	1,975	1,916	NS	1,977	1,933	NS	NS
	6	1,964	1,968	NS	1,958	1,954	NS	NS
	7	. . .	. . .	. . .	. . .	. . .	. . .	. . .

[1]Data were not available at the time of this analysis.

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TABLE 8. Least squares means of egg production measures, by selection direction and replicate, and levels of significance of Generations 5 and 6 of lines selected for multitrait immune response

		Selection direction			Replicate			Interaction
Trait[1]	Generation	H	L	*P*	1	2	*P*	*P*
AFE	5	167	169	NS	167	168	NS	NS
	6	170	172	NS	170	172	NS	NS
EGGWT	5	48.1	47.7	NS	47.8	48.1	NS	NS
	6	49.8	49.1	NS	49.7	49.2	NS	NS
P1	5	.381	.356	NS	.366	.372	NS	NS
	6	.344	.291	NS	.328	.306	NS	NS
P2	5	.725	.728	NS	.716	.741	NS	NS
	6	.844	.834	NS	.847	.831	NS	NS
P3	5	.734	.709	NS	.703	.741	NS	NS
	6	.734	.763	NS	.759	.738	NS	NS
P4	5	.653	.641	NS	.653	.638	NS	NS
	6	.622	.638	NS	.619	.638	NS	NS
PT	5	.509	.458	NS	.480	.486	NS	NS
	6	.634	.624	NS	.636	.622	NS	NS

[1]Traits are: AFE, age in days at first egg; EGGWT, average egg weight in grams at 32 wk of age; P1, egg production frequency from 20 to 28 wk; P2, egg production from 29 to 36 wk; P3, egg production from 37 to 44 wk; P4, egg production from 45 to 52 wk; PT, egg production from 20 to 52 wk.

replicates or between high- and low-response lines for the egg production traits measured, which included age at first egg, average egg weight at 32 wk of age, four 8-wk production periods starting at 20 wk of age, and total egg production in the 32-wk period from 20 to 52 wk of age.

Percentage mortality in each generation is shown in Table 9. Significant interactions were present because Line 1L had significantly ($P < .05$) lower juvenile mortality (0 to 20 wk) than did Line 1H in Generations 5 and 6 (data not shown). No significant differences were noted in adult mortality in Generations 5 or 6 or in Generation 7 up to 20 wk of age.

DISCUSSION

Divergent selection for multitrait immune response has produced phenotypic differences in immune response among the lines in this study. In Generation 7, lines selected for high immune-response had greater humoral immune response and greater index values than did lines selected for low immune-response. Because the selection has been on an index composed of all four traits, intensity of selection for each individual trait has been decreased. It may require several more generations before significant differences are realized among these lines for all of the immune-response traits. Estimates of

TABLE 9. Juvenile and adult mortality by selection direction and replicate, and levels of significance of Generations 5, 6, and 7 of lines selected for multitrait immune response

		Selection direction			Replicate			Interaction
Mortality	Generation	H	L	*P*	1	2	*P*	*P*
		— (%) —			— (%) —			
0 to 20 wk	5	9.3	7.3	NS	7.6	9.0	NS	<.02
	6	7.0	4.0	NS	5.3	5.7	NS	<.001
	7	8.0	6.0	NS	8.0	6.0	NS	NS
20 to 52 wk	5	11.0	10.0	NS	12.0	9.0	NS	NS
	6	6.0	8.0	NS	6.0	8.0	NS	NS

heritabilities, although low, predict that successful long-term selection is feasible.

Several of the estimates of heritability (ranging from 0 to .43 as estimated by using variance components or parent-offspring correlation) are similar to those found in other experiments involving traits of immune response. Biozzi *et al.* (1979) reported a heritability of .18 for antibody response to SRBC in mice. Eide *et al.* (1991) reported realized heritability of .19 for antibody response to diphtheria toxoid in goats. After one generation of selection, Mallard *et al.* (1992) characterized several immune response traits in swine and estimated heritabilities ranging from 0 to .25. In chickens, van der Zijpp *et al.* (1983) found realized heritability of .20 when selecting for increased antibody titer to SRBC and .52 when selecting for decreased titers. Martin *et al.* (1990) reported realized heritabilities of .23 and .25 in Generations 10 to 14 of selection for high and low antibody production to SRBC, respectively. Realized heritabilities of Leitner *et al.* (1992) for antibody response to *Escherichia coli* were similar, with estimates of .23 for high response and .32 for low response.

The estimates of realized heritabilities varied widely in the generations evaluated in the present study. These fluctuations may be influenced by many factors. Because the selection was conducted on the index, the actual selection differentials for individual traits were very small in some instances (data not shown). Additionally, because no control group was kept, responses in each direction of selection were assumed to be equal. This has not been confirmed and, based upon recent studies by Pinard *et al.* (1992), may possibly be unequal. Additionally, incidence of nonresponders, as has been noted in other immune response selection experiments (e.g., Pitcovski *et al.*, 1987), has been low (data not shown). This implies that the low-immune-response line has not been selected to a point below which no response can be measured.

The estimates of heritability may also have been biased because selection has occurred (Falconer, 1989). The selection pressure on each individual trait has not been overly intense, however, and because of the low selection pressure and small changes among lines, correction for bias was not included in this analysis.

Phenotypic correlations among the four traits of immunoresponsiveness were generally small (Tables 2 and 5), with the exception of the correlation between MG and PM. Positive, but often small, correlations between measures of antibody production have been found in other experiments conducted with pigs (Buschmann *et al.*, 1985) and with chickens (Cheng and Lamont, 1988; Heller *et al.*, 1992). Mallard *et al.* (1992) reported a very small positive and nonsignificant correlation of .01 in males and .05 in females. After one generation of selection, using EBVs that included response to HEWL but did not include response to TGAL, the high line exhibited a significantly greater response to TGAL than did the low line. Antibody response to SRBC, another unselected trait, was also greater in the high line than in the low line. Several of the other phenotypic correlations in this study, although small, were negative. Biozzi *et al.* (1979) also reported some negative correlations between measures of different facets of immune response, especially between phagocytic ability and antibody production, but found no significant correlation between either of these two traits and measures of cellular immune response. Mallard *et al.* (1992) reported a greater delayed-type hypersensitivity response to dinitrochlorobenzene (DNCB), an unselected trait, in the line of pigs selected for low multitrait immune responsiveness than in the corresponding line selected for high response.

Estimates of genetic correlation between immune response traits (Tables 2 and 6) from the present study were generally negative. These may slow further progress in this selection experiment. Improved accuracy, obtained by testing more generations, would seem to be necessary before these estimates should be included in index calculations.

More information concerning the true economic values of these immune-response traits is needed as well. Hazel and Lush (1942) point out that the first important part of using a selection index is to have the proper economic weights.

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Although this index was designed with the expectation that the weights (the relative amount of value given to responses to each of the four measures of immunocompetence) used would balance selection pressure evenly across the four traits, the correlations between the index and individual traits calculated from Generations 5 to 7 suggest that this may not have been effective. The correlations, both genetic and phenotypic, between the index and MG are consistently larger than those between the index and any of the other three traits (Tables 2 and 5). Gains in selection have also been more striking in MG, although this gain is probably also due to a higher heritability. In Generations 6 and 7, the economic weights (as in Equation 1) for MG and PM were divided in half to increase pressure on CCA and PHA. Index values still were more strongly correlated with MG (Tables 2 and 5) than with CCA or PHA but improvements were made in correlations between the index and CCA and the index and PHA in Generations 6 and 7.

The minimal differences among lines in measures of production traits may be a result of a lack of correlation between these traits and the immune-response traits included in this study or of low selection intensity. Negative correlations have been reported between body weight and antibody production to SRBC in other selection experiments (Siegel *et al.*, 1982; van der Zijpp, 1983). In another study, van der Zijpp (1984) found a small, positive correlation between antibody production to Newcastle's disease vaccine and body weight gain.

In Generations 5 and 6 in this experiment, Line 1L had lower mortality from 0 to 20 wk than did Lines 1H or 2L, causing a significant interaction. In recent studies (Nelson and Lamont, unpublished data) low-immune-response lines show greater resistance to Marek's disease than do high-immune-response lines, suggesting that specific resistance differences may contribute to observed differences in general mortality.

In summary, the results of this long-term, multitrait selection for immune response in poultry demonstrate that, with simultaneous selection, one can successfully alter humoral immune response without compromising cell-mediated immunity, reticuloendothelial clearance, and growth and egg production traits. Further tests, including direct challenge with specific pathogens to determine effects of altered immunophysiology on resistance traits, will elucidate the effects of multitrait immune-response selection on disease resistance.

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SHORT COMMUNICATIONS

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Observations on the Use of Gonacon™ in Captive Female elk (*Cervus Elaphus*)

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ABSTRACT: Overabundant populations of elk (*Cervus elaphus*) are a significant concern in some areas of the western United States because of potential ecologic damage and spread of brucellosis to domestic livestock. *Brucella abortus* is transmitted among elk through direct contact with aborted fetuses, placentas and associated fluids, or postpartum discharge of infected animals. Because transmission of brucellosis is dependent on pregnancy, contraception of cows could be used for both disease and population management. The objective of this study was to evaluate the contraceptive efficacy of a gonadotropin-releasing hormone vaccine (GonaCon™) in female elk. In September 2004, cows were given a single immunization of either 1,000 μg ($n=12$) or 2,000 μg ($n=10$) of GonaCon™ and compared with a group of adjuvant-treated controls ($n=15$). In November 2004, 2005, and 2006, cows were grouped with bulls for the breeding season. Blood samples were taken in February 2005 and March 2006 and 2007 for pregnancy testing, progesterone assays, and antibody titers. For cows given 1,000 μg GonaCon™ the percentages that were infertile for 2005, 2006, and 2007 were 86%, 90%, and 100%, respectively, compared with 90%, 100%, and 100% for cows given 2,000 μg GonaCon™. Rates of infertility for control cows were 23%, 28%, and 0% ($P<0.0001$). The results indicated that either dose of GonaCon™ prevented pregnancy of elk cows for at least 3 yr. We concluded that GonaCon™ use for population management of elk warrants consideration as part of a strategy to control brucellosis.

Key words: Elk, *Cervus elaphus*, GnRH vaccine, GonaCon™, immunocontraception.

INTRODUCTION

In previous studies, we have demonstrated that the gonadotropin-releasing hormone (GnRH) vaccine, GonaCon™, is highly effective for preventing pregnancy in white-tailed deer (*Odocoileus virginianus*; Miller et al., 2000, 2007), feral horses (*Equus caballus*; Killian et al., 2004, 2006a), domestic and feral swine (*Sus scrofa*; Miller et al., 2003; Killian et al., 2006c), bison (*Bison bison*; Miller and Drew, 2004), as well as several other species (Levy et al., 2004; Miller and Killian, 2004; Nash et al., 2004). In female deer and horses, a single intramuscular injection of GonaCon™ was effective for up to 5 yr (Killian et al., 2006b; Miller et al. 2007). GonaCon™ stimulates the immune system to produce antibodies against GnRH, a small peptide protein produced by the hypothalamus. When sufficient antibody is present, the antibody complexes with, and inactivates, GnRH and prevents it from stimulating reproductive hormone and gamete production. The result is an infertile animal that remains in this state until the concentration of antibody declines to an ineffective level. In safety and toxicity studies with white-tailed deer, GonaCon™ had no adverse effects based on blood chemistry, postmortem, and histopathology evaluations (Killian et al., 2006a).

Contraceptive vaccines can be used as a tool to manage overabundant wildlife populations where lethal means of management are not feasible (Fagerstone et al., 2002), such as in national parks or urban or suburban areas. The contraceptive management strategy works best when populations have been reduced to the desired level before the use of contraceptive vaccines.

Overabundant wildlife populations also

increase the potential for disease transmission. Brucellosis is a zoonotic disease transmitted among elk, bison, and domestic livestock by direct contact with aborted fetuses, placentas, and associated fluids of infected animals. The disease is present in free-ranging elk and bison in the Greater Yellowstone Area (GYA; Cheville et al., 1998). Because the ranges of elk and bison may overlap with grazing cattle, there is considerable concern the disease will be spread to livestock. Although elk cows, under natural conditions, typically seek isolation during calving, winter feeding of elk and livestock has increased congregation and commingling, resulting in increased opportunities for disease transmission.

Miller and Drew (2004) and Killian et al. (2006c) introduced the concept that brucellosis transmission could be limited by preventing pregnancy in conjunction with a *Brucella* vaccination campaign in bison and feral swine. Reversible contraception could prevent pregnancy, and thus abortion, of *Brucella*-infected elk until they cleared the bacteria. We undertook the present study to evaluate the contraceptive efficacy of two doses of GonaCon™, given as a single injection to captive female elk.

The study was conducted at the Sybille Wildlife Research Unit of the Wyoming Game and Fish Department (Wheatland, Wyoming, USA; 41°45′47″N, 105°22′36″W) from 2004–2007. In February 2002, female elk calves were captured in corral traps at the National Elk Refuge (Jackson, Wyoming, USA) and transported to Sybille. There elk were housed in 0.4-ha corrals and fed alfalfa hay supplemented with a pelleted ration. Water and a trace mineral block were provided ad libitum. Chronic wasting disease has existed in the Sybille facility for more than 15 yr and captive cervids often contract the disease, probably from a contaminated environment (Miller et al., 2004) Chronic wasting disease has not been found where the calves were captured (Kreeger, unpubl. data).

The GonaCon™ vaccine consisted of GnRH peptide conjugated to Keyhole Limpet Hemocyanin (KLH; Miller et al., 2000) made into an emulsion with Adju-Vac™ adjuvant (Pocatello Supply Depot, Pocatello, Idaho, USA). In September 2004, 12 cows received a 1-ml injection of 1,000 μg of GonaCon™, 10 cows received a 1-ml injection of 2,000 μg of GonaCon™, 13 control cows received a 1-ml injection of the adjuvant-buffer emulsion, and two control cows were untreated. Treatment groups were housed in individual pens. Intramuscular injections were delivered remotely by darts equipped with biodegradable barbs (Pneu-Dart, Inc., Williamsport, Pennsylvania, USA).

At the beginning of November 2004, 2005, and 2006, cows were grouped with bulls for breeding. Bulls were exchanged among the cow groups every other week for 3 mo to maximize conception. Blood samples were collected in February or March of 2005, 2006, and 2007 from the jugular vein while cows were restrained in a chute. After clotting, the serum was harvested by centrifugation and kept stored frozen at −20 C until assay. Serum was used to determine antibody titers to GnRH and progesterone concentration using methods described elsewhere (Miller et al., 2000). Assays for pregnancy-specific protein B were performed by BioTracking (Moscow, Idaho, USA).

Significant differences among treatments and controls for percentages of infertility and mortality were determined by chi-square. Differences between treatments for antibody titers and serum progesterone concentrations were determined by Student's *t*-test.

Antibody titers for cows treated with 1,000 μg of GonaCon™ averaged 1.04×10^5 at 5 mo after the immunization. Average titers were maintained for the 1,000 μg group at 7.8 and 8.5×10^4 in 2006 and 2007, respectively (Fig. 1). Cows receiving the 2,000-μg dose of GonaCon™ had an average titer of 1.29×10^5 in 2005, which compared with average titers of 1.48 and 1.44×10^5 ($P=0.016$) in

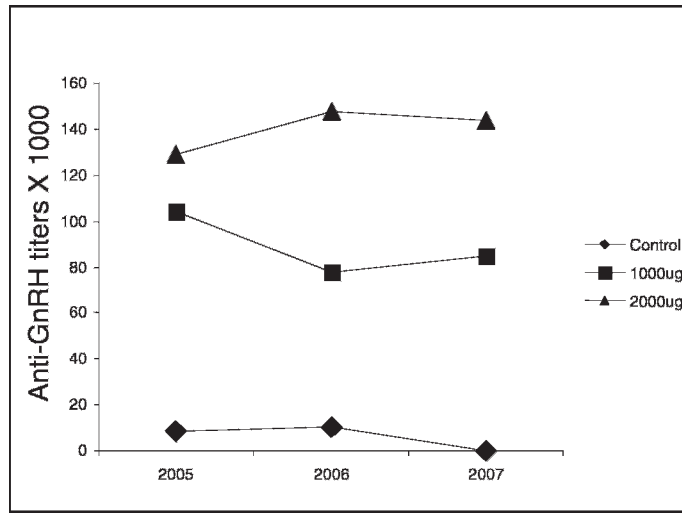


FIGURE 1. Average anti-GnRH titers for serum samples taken from control elk cows and from 1,000-μg, and 2,000-μg treated elk cows in February or March of 2005, 2006, and 2007, following a single vaccination with GonaCon™ or a control vehicle in September of 2004.

2006 and 2007 (Fig 1). These data suggested that there may be an advantage to using the 2,000-μg dose of GonaCon™ over the 1,000-μg dose to provide significantly greater titers.

Compared with the control animals, both doses of the GonaCon™ vaccine were highly effective in preventing pregnancy in elk cows throughout the entire study ($P<0.0001$). Annual infertility rates were lowest in the control animals where 27% (4/15), 25% (2/8), and 0% (0/6) were infertile in 2005, 2006, and 2007, respectively (Fig. 2). In contrast, 92% (11/12), 90% (9/10), and 100% (8/8) were infertile in the group receiving 1,000 μg of Gona-

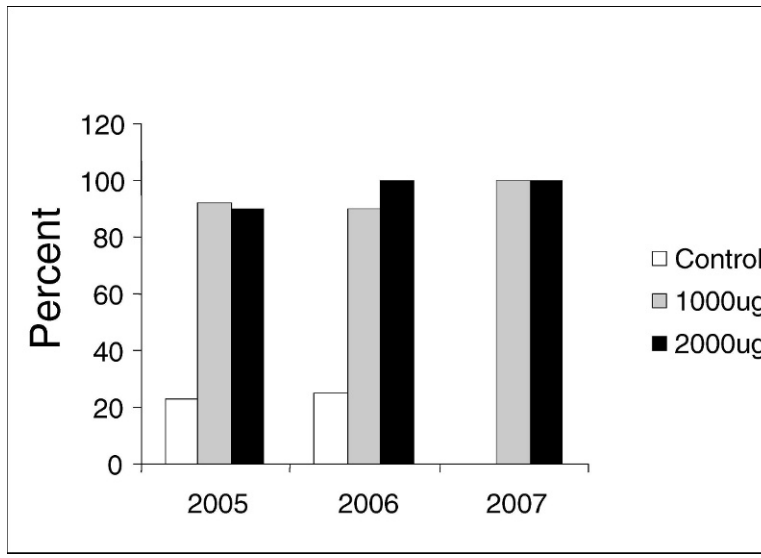


FIGURE 2. Average rates of infertility for control elk cows and for 1,000-μg and 2,000-μg treated elk cows for breeding seasons in 2005, 2006, and 2007, following a single vaccination with GonaCon™ in September of 2004.

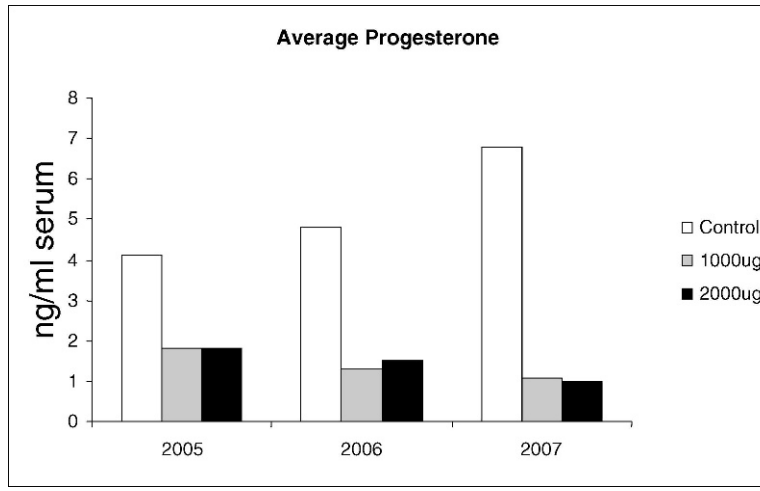


FIGURE 3. Average serum progesterone concentrations for control elk cows and for 1,000-μg and 2,000-μg treated elk cows for breeding seasons in 2005, 2006, and 2007, following a single vaccination with GonaCon™ in September of 2004.

Con™, and 90% (9/10), 100% (8/8), and 100% (6/6) were infertile in the group receiving the 2,000-μg dose of GonaCon™ (Fig. 2).

Although antibody titers were greater in cows treated with the 2,000-μg dose of GonaCon™, there were no differences in rates of infertility between the 1,000 μg and 2,000 μg GonaCon™ groups ($P = 0.697$) over the three breeding seasons studied. This outcome could change in future years if there is a greater decline in titers for the 1,000 μg group compared with the 2,000 μg group. Taken together, these results suggested that a single injection of either dose of GonaCon™ could be used for elk herds where population management by nonlethal means is desired.

Although some progesterone is secreted during the luteal phase of the estrous cycle, maximal serum concentrations of progesterone are produced by the corpus luteum during pregnancy. Control animals, most of which were pregnant when the blood samples were taken in February or March, had greater average concentrations of serum progesterone ($P<0.0001$) than cows treated with GonaCon™ (Fig. 3).

Because GonaCon™ was a highly effective immunocontraceptive vaccine for three breeding seasons, we suggest that it may prove useful in controlling brucellosis in infected elk herds' winter feed grounds in the GYA-concentrated elk, where the likelihood of disease transmission is increased. This is supported by the increased prevalence of *Brucella* antibodies in feedground elk, which is an order of magnitude higher than rates in elk not wintering on feed grounds (Kreeger, 2002). However, elk concentrated on feed grounds lend themselves to a large-scale vaccination program (Kreeger and Olsen, 2002). A challenge in managing elk in the GYA is that most herd sizes are higher than desired management levels, and hunting has been unable to reduce elk numbers. Thus, a single-dose, highly effective immunocontraceptive, like GonaCon™, would serve to reduce herd numbers while simultaneously preventing pregnancy and thus abortion, which would decrease transmission of brucellosis.

An unfortunate aspect of this study is that a large number of cows died. Of the cows that began the study in the fall of 2004, only 20 of the animals were alive when blood samples were taken in April of 2007. The cause of death in every instance

was CWD, diagnosed by immunohistochemistry. Exposure to the CWD prion was most likely environmental (Miller et al., 2004) because the facility has experienced continuous losses to CWD for more than 20 yr (Kreeger, unpubl. data).

Results of this study indicated that either dose of GonaCon™ prevented pregnancy of elk cows for at least 3 yr. We concluded that GonaCon™ use for population management of elk warrants consideration as part of a strategy to control brucellosis.

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Long-Term Efficacy of Three Contraceptive Approaches for Population Control of Wild Horses

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ABSTRACT: Controlling fertility of feral horses through the use of long-acting contraceptives or sterilization approaches has been championed as a reasonable and humane solution for addressing overpopulation problems in several western states. However, methods to accomplish long-term contraceptive efficacy of horses following a single treatment have been lacking. In fall 2002 and spring 2003, we initiated a study to compare the long-term efficacy of a single-shot contraceptive vaccine directed at gonadotropin releasing hormone (GonaCon™) to that of a single-shot vaccine directed at the zona pellucida (SpayVac) and to the use of intrauterine contraceptive devices (IUD). Both vaccines were administered with AdjuVac™, an adjuvant developed at the National Wildlife Research Center. The objectives of the study were to determine: 1) 3-year efficacy for preventing pregnancy, 2) whether the contraceptive effects are reversible, and 3) whether there are contraindications. The Nevada Department of Agriculture provided the feral mares, which were maintained at the Nevada State Penitentiary, Carson City, facility. Mares were dewormed and given health vaccinations annually. Eight untreated control mares were compared to 12 mares treated with SpayVac, 16 mares treated with GonaCon™, and 15 mares treated with copper-containing IUDs. Only 25% (2/8) of the control mares were not pregnant or infertile in the first year. All mares in the SpayVac group were infertile, and 94% (15/16) were infertile in the GonaCon™ group during the first breeding season. In Year 2, 80% (10/12) of the SpayVac-treated mares and 60% (9/15) of the GonaCon™-treated mares were infertile. In Year 3, 80% of the SpayVac mares and 53% (8/15) of the GonaCon™-treated mares were infertile. For IUD-treated mares, 80% (12/15) were infertile after Year 1, but only 29% (4/14) and 14% (2/14) were infertile after Years 2 and 3, respectively. For IUD mares that were infertile, it was possible to visualize the IUD by ultrasonography, leading us to conclude that mares that became pregnant had lost their IUDs. For mares given SpayVac, uterine edema was commonly observed. In Years 2 and 3, antibody titers for SpayVac were progressively lower compared to titers observed in Year 1.

KEY WORDS: contraception, *Equus caballus*, feral horse, fertility control, GnRH vaccine, intrauterine device, SpayVac-PZP vaccine

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INTRODUCTION

Contraception of wildlife and feral species has been considered a desirable option for addressing concerns of over population for many years. Ideally, the contraceptive approach should be easily administered, be effective for multiple years, and have few if any contraindications in the target species. Immuncontraceptive vaccines are believed to fulfill many of the attributes desired for population control. Data reported previously for several species, including horses (*Equus caballus*), support the notion that immunocontraceptive vaccines are safe and effective in the short term (reviewed in Killian *et al*. 2004). One injectable immunocontraceptive vaccine that has been extensively researched for use in wildlife and feral species is the porcine zona pellucida (PZP) vaccine. This vaccine presumably works by stimulating antibody production against the zona pellucida, the non-cellular outer covering of the egg. Consequently, the normal interaction between sperm and egg is prevented by the presence of antibodies on the surface of the ovum. One of the shortcomings of most formulations of PZP vaccines previously reported, however, is that a single vaccination is effective for only 1-2 years at most (Fagerstone *et al*. 2002). Although less extensively evaluated than the PZP vaccine for wildlife contraception, the gonadotropin releasing hormone (GnRH) vaccine is also of interest as an immunocontraceptive approach, since it provides a means of non-surgical sterilization by compromising reproductive hormone and gamete production of both males and females.

Considering the needs of population control of western, free-ranging mustangs, annual vaccination by injection is not a practical means to maintain infertility. Annual capture is inefficient, costly, and involves added risks of injury to horses and handlers. Although oral forms of contraceptive vaccines may make it practical to annually vaccinate free-roaming species with minimal difficulty, they are not now available and their development will probably take many years. Given the status quo, it was our belief that if the PZP and GnRH vaccines were modified or shown to act longer, they would eliminate the need for annual vaccination and provide a reasonable short-tem solution for controlling fertility in wild mustang populations.

In fall 2002 and spring 2003, we initiated a study to compare the long-term efficacy of a single-shot contraceptive vaccine directed at gonadotropin releasing hormone (GonaCon™) with that of a single-shot vaccine

directed at the zona pellucida of the ovum (SpayVac). Both vaccines were administered with AdjuVac™, an adjuvant developed at the USDA National Wildlife Research Center (NWRC). In addition, we evaluated the use of an intrauterine contraceptive device (IUD), the 380 copper "T", since in humans it has been shown to be effective for maintaining infertility for up to 12 years (Killian *et al*. 2004).

The objectives of the study for these contraceptive approaches were to determine: 1) rates of contraception for up to 3 years, 2) whether the contraceptive effects were reversible, and 3) whether there were contraindications. We now report the results following 3 years of study.

MATERIALS AND METHODS

The details pertaining to the horses used in the study and the treatments they received were previously reported (Killian *et al*. 2004). They are briefly summarized below.

Animals

The Nevada Department of Agriculture provided 53 mares and 3 stallions for use in the project. The mares weighed 225-360 kg and ranged in age from 18 months to 12 years at treatment. The horses were previously gathered from state lands and brought to the Nevada State Penitentiary, Carson City facility, where the study was conducted. All horses were given annual health vaccinations and dewormed routinely.

To handle feral mares for jugular blood sampling and vaccinations, they were run into a hydraulic chute and haltered. Contraceptive vaccines were given intramuscularly in the left lateral neck. For pregnancy evaluations by ultrasound or palpation, or IUD placement, the mares were chemically restrained.

Treatments

The studies were approved by the Institutional Animal Care and Use Committee of Pennsylvania State University. Eight mares were assigned to be untreated controls, 12 mares received a single shot of 400µg SpayVac PZP vaccine, 15 mares received a single shot of 1800µg GonaCon™ vaccine, and 3 mares received a single shot of 2800µg GonaCon™ vaccine. Fifteen mares received copper-containing IUDs placed into the uterus, trans-cervically. Doses of the SpayVac PZP vaccine were kindly provided by the vaccine's developer, Dr. Robert Brown (Brown *et al*. 1997). The single-shot GonaCon™ vaccine was developed at and provided by the NWRC. Both SpayVac and GonaCon™ were used in combination with AdjuVac™ adjuvant (Miller *et al*. 2004). The human copper 380 "T" intrauterine devices were purchased from Family Planning Sales Ltd., Littlemore, Oxford, UK. Mares were penned with a fertile stallion for a breeding trial lasting from June to September of each year. If mares failed to breed or become pregnant during the breeding season, they were considered infertile.

Observations

Nevada mares were routinely observed for breeding activity by staff and prisoner caretakers, and they were checked once or twice annually by ultrasonography for pregnancy, IUD retention, and uterine inflammation. Blood samples were assayed for estradiol, progesterone, and antibody titers to the contraceptive vaccines at the NWRC (Miller *et al*. 2000, 2001). Contraindications evaluated included general health and body condition, and uterine edema, which may be associated with hormonal changes or presence of IUDs. One mare receiving the GonaCon™ vaccine died after the first breeding season of causes not related to the treatment.

RESULTS AND DISCUSSION

Contraceptive Efficacy

All mares in the SpayVac group were infertile and 94% (15 /16) were infertile in the GonaCon™ group during the first breeding season (Figure 1). In Year 2, 80% (10/12) of the SpayVac-treated mares and 60% (9/15) of the GonaCon™-treated mares were infertile. In Year 3, 80% (10/12) of the SpayVac mares and 53% (8/15) of the GonaCon™-treated mares were infertile. For IUD-treated mares, 80% (12/15) were infertile after Year 1, but only 25% and 14% were infertile and after Years 2 and 3.

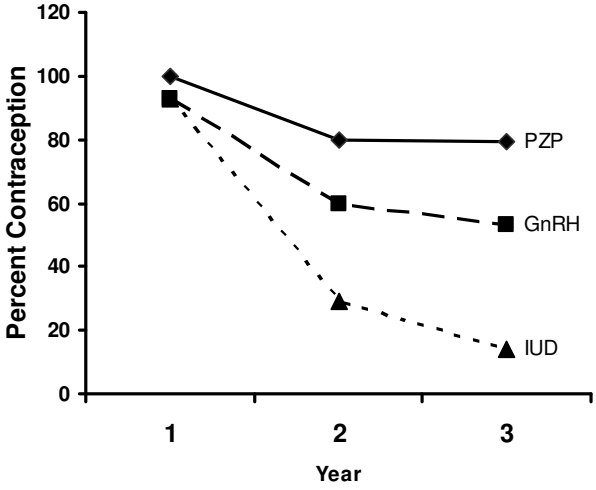


Figure 1. Percent mares contracepted in years following treatment with SpayVac (PZP), GonaCon™ (GnRH), or the 380 copper "T" human intrauterine device (IUD).

SpayVac

Mares receiving a single vaccination of SpayVac maintained a high level of contraception throughout the 3-year study. This rate of efficacy exceeds any previously reported rate for use of PZP vaccines in mustang mares or burros (Liu *et al*. 1989, Kirkpatrck *et al*. 1996, Turner *et al*. 2002). In previous studies with mares given a primary and booster vaccination, infertility was achieved in 80-90% of animals for 1 year and further sustained at this level by annual vaccination. Although Kirkpatrick, Turner, and colleagues have used annual vaccination with a PZP vaccine to effectively manage reproduction in some feral horse populations (Turner *et al*. 2002), annual recapture and vaccination of mares is not a practical approach for most field applications.

Various preparations of injectable PZP vaccines have

been used for contraception of a long list of species (Fagerstone *et al*. 2002), including deer (Turner *et al*. 1997, Miller *et al*. 2001, Fraker *et al*. 2002), elephants (Fayer-Hosken *et al*. 1999), dogs (Mahi-Brown *et al*. 1985), baboons (Dunbar 1989), and seals (Brown *et al*. 1997). However, the only formulation of single-shot PZP vaccine that appears to consistently produce a high degree of contraception lasting multiple years is the form known as SpayVac, developed by Dr. Robert Brown. Our findings confirm, in a controlled study, the multi-year efficacy for mares that has been reported for grey seals (*Halichoerus grypus*, Brown *et al*. 1997) and fallow deer (*Dama dama*, Fraker *et al*. 2002) using this formulation of PZP vaccine.

We observed that SpayVac-treated mares were more likely to have elevated serum concentrations of estradiol and show evidence of estrus at sampling than GonaCon™- or IUD-treated mares. These observations were correlated with a high incidence of uterine edema (>80%) in PZP-treated mares (Table 1). Uterine edema associated with estrus would normally be expected to be seen in ~25% of a population of randomly sampled mares during the breeding season. This prediction is based on the assumption that the normal mare estrous cycle is 22-24 days, and for approximately 6 days or 25% of that cycle she will be in estrus. From our observations, it is evident that uterine edema was present in a much higher percentage of PZP-treated mares. Although repeated estrous cycles during the breeding season have been reported for PZP-treated mares (Turner and Kirkpatrick 2002), it was generally concluded that the repeated estrous cycles were of normal length. However, we suggest that the high incidence of uterine edema and behavioral estrus observed for the Nevada mares during sampling in this study indicates abnormal estrous cycles. These abnormal estrous cycles are likely characterized by a prolonged follicular phase and a brief, if not absent, luteal phase. To better characterize the estrous cycle of mares treated with the PZP vaccine, it would be necessary to draw daily blood samples for hormone assay. Unfortunately, mustangs do not lend themselves to frequent handling regimens. Such studies would need to be done on domestic mares.

Table 1. Percentage of reproductive tracts with edema revealed by ultrasonography of mares for each of the treatments compared to the predicted number of mares expected to be in estrus.

	Year 1	Year 2	Year 3
Predicted %	25%	25%	25%
GonCon™ actual %	23% (3/13)	25% (2/8)	25% (2/8)
SpayVac actual %	82% (9/11)	91% (10/11)	100% (10/10)

GonaCon™

The single-shot GonaCon™ vaccine performed quite well during the first year, but the contraception rate decreased to 60% in Year 2 and 53% in Year 3. Although GonaCon™ did not perform as well as SpayVac, the multi-year results achieved with a single vaccination of GonaCon™ far exceed published results for contraception studies with wild horses using PZP vaccines (Turner *et al*. 2002, Kirkpatrick and Turner 2002). In addition, mares did not display evidence of frequent estrus or abnormally high incidence of uterine edema. It is noteworthy that the drop in contraception rate was greatest between Years 1 and 2, with only minimal decrease from Year 2 to Year 3 (Figure 1). A similar trend was seen with SpayVac, although to a lesser degree. This suggests that considering the immunological response, there are two sub-populations of mares: one population responded with antibody titers adequate for contraception that were maintained over several years, while in the other population contraception lasted no more than 1 year. Given the limited data we were able to collect from mustang mares, it is not possible to provide an explanation for the immunological differences between the multi-year and single-year responders.

Vaccine Antibody Titers

Contraceptive efficacy of both SpayVac and GonaCon™ was clearly related to antibody titer (Figures 2 and 3). Average titers for contracepted mares in each of the 3 years of study were considerably greater than those mares that became pregnant. The average titer for contracepted mares receiving SpayVac progressively declined during each year of study. However, the average titer in Year 3 for contracepted SpayVac mares was still nearly 8-fold greater than the average "breakthrough" titer for all SpayVac-treated mares that became pregnant. There was a 37% decline in titer between Years 1 and 2 and a 33% decline between Years 2 and 3. If we assume an average annual rate of decline in titer of 35%, this suggests that, on average, the majority of SpayVac-treated mares will remain contracepted for 4 additional years before the breakthrough titer is reached. This projection of a total of 7 years of contraception for SpayVac-treated mares is supported by the literature report of long-term efficacy of SpayVac use in grey seals (Brown *et al*. 1997).

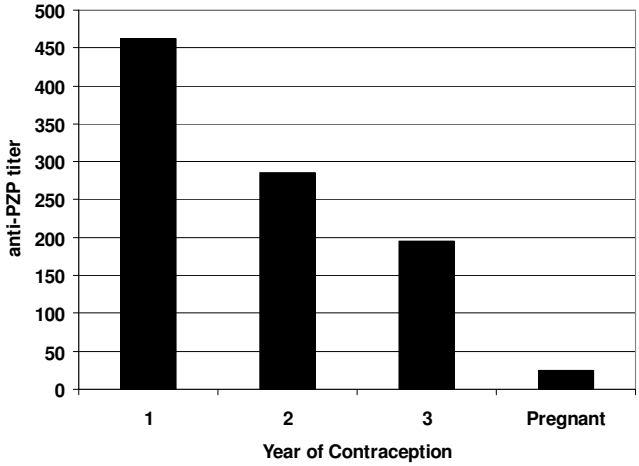


Figure 2. Average antibody titers for contracepted mares treated with SpayVac in years following treatment compared to the average titer of all pregnant mares.

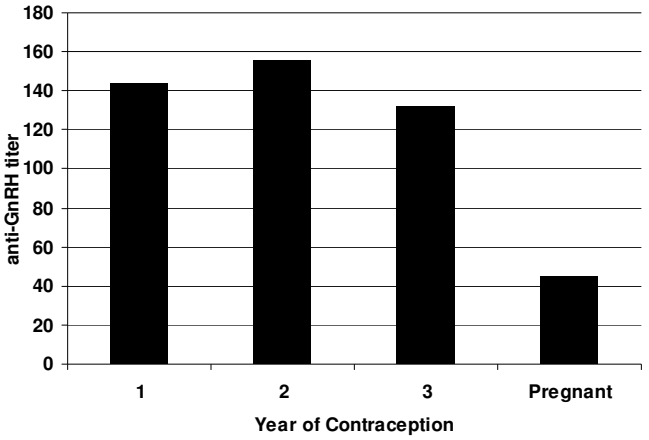


Figure 3. Average antibody titers for contracepted mares treated with GonaCon™ in years following treatment compared to the average titer of all pregnant mares.

Average titers for contracepted GonaCon™-treated mares did not show the same rate of decline in annual titer as seen with SpayVac. However, this observation is somewhat misleading, since in Year 2, 40% of the GonaCon™-treated mares became pregnant. The lower titers for these mares were averaged in the pregnant group, favoring a higher average titer for the mares in the GonaCon™-contracepted group. The fact that pregnant mares with lower titers were no longer included in the contracepted group explains the small artifactual rise in titer in Year 2. The 15% decline in GonaCon™ titer for the contracepted mares between Years 2 and 3 may more accurately reflect the annual rate of titer decline to expect for GonaCon™-treated mares following a single injection. The average titer for the 8 remaining GonaCon™-treated mares contracepted in Year 3 was 132×10^3, or nearly 3-fold greater than the 45×10^3 titer for the 5 mares that became pregnant. However, one of the mares that became pregnant had a titer of 128×10^3, while the average for the other 4 was only 24×10^3. This emphasizes the individual variability that can occur relative to titers and efficacy of a contraceptive vaccine.

IUDs

Although the 380 copper "T" IUD performed respectably during the first year of study with an 80% contraception rate (Figure 1), its performance as a contraceptive was poor during Years 2 and 3. Because we were able to visualize by ultrasonography the IUD in infertile mares, but in Years 2 and 3 we were not able to visualize the IUD in mares that were <50 days pregnant, we believe that the IUD was expelled from the reproductive tract. Daels and Hughes (1995) reported that 6 domestic mares implanted with a silastic ring IUD were infertile for 1 breeding season, and all mares returned to fertility after IUD removal the following breeding season. In unpublished studies we are conducting using the 380 copper "T" in domestic pony mares, 4 out of 5 mares have remained contracepted for 3-4 years. Given the difference in uterine size between the pony and mustang mare, it is likely that the human 380 cooper "T" is too small to be consistently retained in the uterus of the mustang mare. Retention of foreign objects in the uterus of the mare is clearly related to the size of the object. In studies using glass balls inserted into the uterus of mares to suppress estrous behavior, it was indicated that the size of the glass ball influenced its ability to be retained by the mare (Nie *et al.* 2001, Thomas 2002). Although the human 380 copper "T" did not perform for multiple years as we expected, based on studies with humans, further research to develop an IUD better suited to mustang mares could produce an effective means of long-term contraception.

Contraindications

We found no significant contraindications that would affect the well-being of mares given any of the treatments. All mares were in good body condition throughout the study. The presence of the copper-containing human IUD in the uterine lumen did not appear to cause a uterine inflammatory response, as reported for domestic mares equipped with silastic IUDs (Daels and Hughes 1995).

While a high incidence of uterine edema was observed in SpayVac-treated mares, which may have been due to irregularities in the estrous cycle, there was no indication that this naturally-occurring response to estrogen adversely affected the mares. There was no pattern of elevated estradiol or reduced progesterone in SpayVac-treated mares compared to GonaCon™- or IUD- treated mares. However, given the limited blood sampling we were able to do, it is not surprising. Frequent blood sampling is necessary to accurately characterize hormonal changes during the estrous cycle, a study better pursued with domestic mares.

Our results with the PZP vaccine agree with the report and general conclusion that mares treated with the PZP vaccine do not suffer from ill effects (Kirkpatrick *et al.* 1995, Turner and Kirkpatrick 2002). This conclusion is also supported by our study in white-tailed deer (*Odocoileus virginianus*) treated with PZP vaccine, which showed no significant adverse health effects (Miller *et al.* 2001). We also came to the same conclusion for white-tailed deer treated with GonaCon™, evaluated in a target safety study that is reported in a companion paper at this conference (Killian *et al.* 2006).

CONCLUSIONS

Three years of study on mustang mares treated with immunocontraceptive vaccines and an IUD suggest that they are safe and effective. Multiyear contraceptive efficacy was greatest for SpayVac, followed by GonaCon™ and the IUD. Given the number of mares becoming pregnant in Years 2 and 3, we conclude that the IUD and GonaCon™ approaches are reversible. Further study is needed to establish the rate of reversibility for the SpayVac vaccine, although the reversibility of other PZP immunocontraceptive vaccines has been reported (Kirkpatrick and Turner 2002).

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Four-year contraception rates of mares treated with single-injection porcine zona pellucida and GnRH vaccines and intrauterine devices

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Abstract. We evaluated the multiyear contraceptive efficacy of the gonadotrophin-releasing hormone (GnRH) vaccine GonaCon, the porcine zona pellucida (PZP) vaccine SpayVac and the human intrauterine device (IUD) 380 Copper 'T' in mustang mares provided by the State of Nevada. Eight untreated control mares were compared with 12 mares treated with SpayVac, 16 mares treated with GonaCon and 15 mares treated with the copper-containing IUD. Rates of contraception for Years 1, 2, 3 and 4 respectively for SpayVac were 100% (12 of 12), 83% (10 of 12), 83% (10 of 12) and 83% (10 of 12), rates for GonaCon were 94% (15 of 16), 60% (9 of 15), 60% (9 of 15) and 40% (6 of 15) and rates for IUD-treated mares were 80% (12 of 15), 29% (4 of 14),14% (2 of 14) and 0% (0 of 14). Antibody titres against PZP and GnRH declined over the four-year study. For mares given SpayVac, uterine oedema was commonly observed. IUDs were visible by ultrasonography in non-pregnant mustang mares, suggesting that pregnant mares did not retain their IUD. IUD retention may be a function of uterine size: pony mares with IUDs had high retention and contraception rates for 4–5 years. We conclude that long-term contraception of mustang mares with a single shot of either the SpayVac or GonaCon vaccine is possible.

Introduction

Overpopulation of wild horses is a significant concern in the western United States (Fisher 1983). In Nevada, where most of the wild horses are located, populations grow at a rate of 15–20% a year on State lands, while their range continues to shrink. Current management strategies of removal and adoption are expensive, logistically challenging, and minimally (if at all) effective in reducing and maintaining wild horse populations at a desired level. Conflicting interests associated with increased movement of people into wild horse ranges, sympathy to maintain wild horse populations because of their historic and cultural importance, competition among horses and indigenous plant and wildlife species, as well as ranching interests, are issues impacted by wild horse overpopulation. Controlling the fertility of free-ranging horses is considered a viable option for population control. However, this approach has many challenges for which solutions have been elusive. Ideally, methods for contraception of wild horses should be safe and potentially reversible, effective for several years, practical to administer and of reasonable cost and have minimal effect on reproductive or harem behaviour. Immunocontraceptive vaccines have garnered considerable attention in recent years as a means to address problems of overabundant wildlife and feral species (Fagerstone *et al*. 2002; Delves and Roitt 2005; Naz *et al*. 2005). Two immunocontraceptive vaccines that have been used in a variety of species and for which data exist on their safety and efficacy are porcine zona pellucida (PZP) vaccine (Kirkpatrick *et al*. 1992, 1995; Miller *et al*. 1999, 2001; Kirkpatrick and Turner 2002; Turner and Kirkpatrick 2002, 2003; Curtis *et al*. 2007) and gonadotrophin-releasing hormone (GnRH) vaccine (Miller *et al*. 2000, 2004; Killian *et al*. 2006*a*; Massei *et al*. 2008). Fertility control of mares using existing PZP vaccines has been shown to be safe and effective for up to 10 or more years. However, wild horses vaccinated with PZP preparations have required revaccination every year or two to maintain infertility (Turner *et al*. 2001, 2002). Limited data exist for the use of GnRH vaccines in mares to control fertility, ovarian function or behaviour (Dalin *et al*. 2002; Killian *et al*. 2004, 2006*b*; Imboden *et al*. 2006; Elhay *et al*. 2007). Information on multiyear efficacy and effects following a single injection of a GnRH vaccine in mares is lacking. Regardless of the contraceptive vaccine considered, most formulations that have been used do not appear to be effective for the long term without revaccination. Revaccination of mustangs involves considerable expense, manpower, and horse handling to maintain infertility. If a single-injection multiyear contraceptive were available it may be possible to achieve effective population reduction and reduce costs and risks associated with frequent horse handling.

We initiated a study in the autumn of 2002 and the spring of 2003 to compare the multiyear contraceptive efficacy of a single-shot contraceptive vaccine directed at GnRH with that of a single-shot vaccine directed at the zona pellucida of the ovum. We selected GonaCon to test as the GnRH vaccine on the basis of our positive experiences with it as a single-injection vaccine in deer and other species (Miller *et al*. 2000, 2004; Miller and Killian 2001; Killian *et al*. 2006*c*; Fagerstone *et al*. 2008). We selected SpayVac as the PZP vaccine to test in mares on the basis of our unpublished experiences with it as a single-injection vaccine in

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white-tailed deer and the reports of multiyear efficacy in harbor seals (Brown *et al*. 1997) and fallow deer (Fraker *et al.* 2002). Both vaccines were administered with AdjuVac, an adjuvant developed at the National Wildlife Research Center. In addition, on the basis of preliminary studies with pony mares, we evaluated the use of an intrauterine contraceptive device (IUD), the 380 copper 'T', which has been shown to be safe and efficacious in humans for multiple years (Fortney *et al*. 1999; Wu *et al.* 2000). Preliminary results of these studies with Nevada mustangs have been reported earlier (Killian *et al.* 2004, 2006*b*). This paper reports results for these contraceptive approaches after four years of study with Nevada mustangs and five years of observation on the use of IUDs in pony mares.

Materials and methods

Animals

Horses for the study were provided by the State of Nevada and were maintained at the Nevada State Penitentiary, located at Carson City. The studies were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University. In total, 51 mares and three stallions were used. The mares weighed 225–360 kg and their ages were estimated to be between 18 months and 12 years of age at the time the treatments were given. All mares except the 18-month-old filly were observed in the wild to foal normal healthy foals.

For jugular blood sampling and vaccinations, the mares were run into a hydraulic chute and haltered. Vaccines were given intramuscularly in the left lateral neck. Mares were chemically restrained for IUD placement and for pregnancy evaluations by ultrasound or palpation. Chemical restraint was achieved with an initial intravenous injection of a mixture of 1 mL Dormosedan (detomidine hydrochloride) 10 mg, 2 mL xylazine 200 mg, and 2 mL acepromazine 20 mg to produce sedation, followed in 5 min with a second intravenous injection of one bottle of Telazol (250 mg Tiletamine base, 250 mg Zolazepam base) resuspended in 3 mL xylazine 300 mg for anaesthesia. This regimen typically gave 15–45 min of anaesthesia.

Ten pony mares and two pony stallions belonging to the Department of Dairy and Animal Science of the Pennsylvania State University were used to develop methods and evaluate the use of IUDs as a means to block fertility. The mares ranged in age from 18 months to 12 years when the treatments were administered. Seven of the mare ponies were pastured with a stallion during the entire five-year study, except when treatments were being administered or when data were collected. They weighed 204–306 kg, Three of the mares were on the study for only one year and were pastured with a stallion for two months during the breeding season. They weighed 281–391 kg.

Ponies were haltered and restrained in a chute for examination and data collection. If sedation was necessary, mares were given intravenous 0.5–1.5 mL xylazine (50–150 mg) and 0.2–0.5 mL butorphanol, or 0.1–0.25 mL Dormosedan (10–25 mg) and 0.2–0.5 mL butorphanol (20–50 mg).

Treatments

For the Nevada study vaccinations were given in March of 2003. Eleven mares were given a single-shot GnRH vaccine containing 1800 μg of GonaCon and four mares were given a single-shot GnRH vaccine containing 2800 μg GonaCon, 12 mares received a single-shot PZP vaccine containing 400 μg SpayVac and 8 mares were assigned to be untreated controls. Copper-containing 380 'T' IUDs were placed in the uterus of 15 mares transcervically in October of 2002. The SpayVac PZP vaccine was provided Dr Robert Brown (Brown *et al*. 1997), who developed the vaccine. The GonaCon vaccine was provided by the National Wildlife Research Center, USDA-APHIS-WS. Both SpayVac and GonaCon were made into an emulsion with AdjuVac adjuvant (Miller *et al.* 2004) and injected as a 1-mL dose. To evaluate reproductive capacity, treated and control mares were randomly assigned to two breeding groups. 'Band Stallions' that had been observed to sire multiple generations in the wild were selected to be pen stallions. Most mares were maintained in the same pen throughout the study, but if behavioural issues resulted in mares fighting, the less dominant mare was moved to the other pen. Mares were penned with a fertile stallion for a breeding trial typically lasting from June through September of each year. If mares failed to breed, or failed to become pregnant or foal during the breeding season, they were considered infertile.

Ponies were used to evaluate three different types of IUDs for ease of placement, retention and efficacy. The 380 Copper T and the GyneFix IUDs were purchased from Family Planning Sales Limited, Littlemore, Oxford, UK. The ring IUD was fabricated from Sialistic tubing (2.5 mm o.d.), which was used to create a ring size of ~2.5 cm. Prior to closing the ring with Silastic cement, five or six small copper cable clamps were threaded over the tubing for inclusion in the ring.

In preliminary studies we attempted direct finger insertion of the IUDs into the uterus of the mare after dilation of the cervix with two fingers. Because this approach was somewhat cumbersome, time consuming and did not easily ensure placement of the IUD deep within the uterine lumen we attempted to use the IUD insertion devices that were supplied with the IUDs that were intended for humans. However, both of these insertion devices were unsatisfactory for the mare uterus. The insertion device for the copper 'T' was too short to traverse the mare vagina and cervix. Likewise, the insertion device for the GyneFix IUD, which is intended to attach the IUD to the uterine endometrium with a monofilament, was also too small for the mare reproductive tract. Consequently, we modified disposable large animal uterine swabs to accommodate the IUDs. The modified swabs enable the successful deposit of the IUDs transcervically into the uterine lumen of sedated ponies or anesthetised Nevada mares. The insertion device containing the IUD was placed between the two fingers used to dilate the cervix and guided into the uterine lumen where the IUD was discharged. With minimal practice the time needed to clean the perineum, palpate the cervix by hand, and then insert the device was 2–5 min, with actual insertion of the rod and placement of the device requiring ~30–60 s.

For the research trial, all IUDs were placed into the uterus transcervically following dilation of the cervix with one or two fingers. As detailed in the Results, several animals received more than one type of IUD during the course of the study. If a mare became pregnant after receiving an IUD she was either allowed to go to term or, in the case of two mares in which the pregnancy was 60 days or less, the pregnancy was terminated with prostaglandin F2α. Within 2–3 weeks of termination of pregnancy or

parturition, mares were treated with another IUD. As a result, the GyneFix IUD was evaluated in four mares, the copper ring in four mares and the 380 Copper 'T' in seven mares. Two untreated mares served as a control for each breeding season.

Observations

Blood samples were collected from treated Nevada mares once or twice a year. However, the only observations consistently made on the eight control mares were general health, body condition and foaling. In mid- to late-October of each year treated mares were examined by rectal ultrasonography for pregnancy, IUD retention and uterine inflammation. Pregnancy was established by ultrasonography by observance of an embryonic vesicle, a fetus or in the case of later gestation, rectal palpation of a fetus. These observations were later confirmed by birth of a foal. In a few cases where the behaviour of the mare prevented ultrasonography or rectal palpation, pregnancy was determined later by the birth of a foal. General health and body condition, and uterine oedema that may be associated with oestrous cycle changes or presence of an IUD were noted. Uterine oedema in healthy mares is an indication that she is in heat and that she is under the influence of oestrogen produced by ovarian follicles (Sample 1997). All blood samples were assayed for oestradiol, progesterone and antibody titres to the contraceptive vaccines (Miller *et al*. 2000, 2001). One mare receiving the GonaCon vaccine and one mare with an IUD died after the first breeding season of causes not related to the treatments.

From April through November of 2002–05 ponies were gathered and examined by ultrasonography every 4–6 weeks. During the 2004 breeding season, daily observations were also made from April through August on the breeding and harem behaviour of a group of ponies consisting of one stallion, two control mares, one mare with a GyneFix IUD, two mares with the ring IUD and four mares with the 'T' IUD. For 2006, ponies were pastured for the entire year and the only observations made were for general health status and foaling rates. In April of 2007, ultrasonography was performed to check for IUD placement, pregnancy and any contraindications.

Statistical evaluation

Data from hormone assays were subjected to one-way analysis of variance by treatment. Differences between means were detected by two-sided *t*-test. Mean values are reported plus or minus standard error. Regression analyses were used to evaluate changes in antibody titres during the study.

Results

Nevada mustangs

Foaling data for the eight control mares for Years 1–4 were 75% (6 of 8 mares), 75% (6 of 8 mares) 88% (7 of 8 mares) 100% (8 of 8 mares), respectively. Rates of contraception for SpayVac-treated mares for Years 1, 2, 3 and 4 were 100%, 83%, 83% and 83%, respectively (Fig. 1). Average anti-PZP titres for the autumn bleed for mares that were contracepted were sustained considerably above the titers of mares that became pregnant (Fig. 2). Although regression analysis indicated a significant decline of anti-PZP titres for the first three years of the study ($P < 0.05$), average titres tended to increase in the fourth year. Compared with titres in the third year, individual titres increased in 8 of the 10 contracepted mares, with the remaining 2 mares having titres similar to the titres they had in the third year. For all years, serum progesterone values ($ng\,mL^{-1}$) averaged 1.3 ± 0.40 for non-pregnant mares during the October bleed compared with 17.5, the average for two pregnant mares. We were unable to obtain serum oestradiol

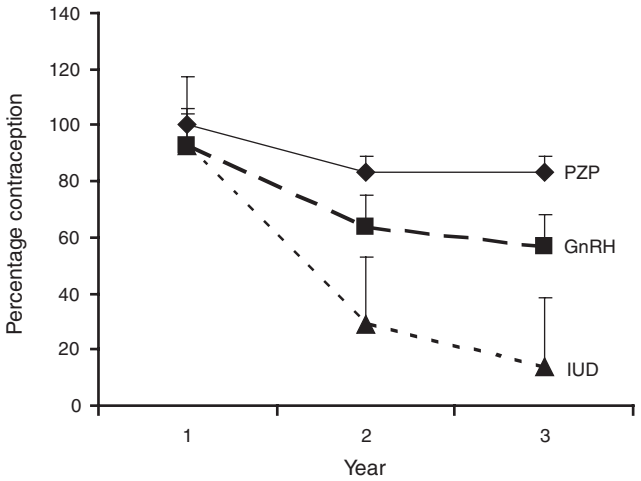


Fig. 1. Comparative rates of contraception for the three methods of contraception for each year of the four-year study. Rates of contraception for SpayVac-treated mares for Years 1, 2, 3 and 4 were 100% (12 of 12 mares), 83% (10 of 12), 83% (10 of 12) and 83% (10 of 12), respectively. Rates of contraception for GonaCon-treated mares were 93% (14 of 15 mares), 64% (9 of 14), 57% (8 of 14) and 43% (6 of 14) for Years 1–4, respectively. Contraception rates for intrauterine device (IUD)-treated mares were 80% (12 of 15 mares), 29% (4 of 14), 14% (2 of 14) and 0% (0 of 14), respectively in Years 1–4 of the study. In Year 2, one mare died in each of the GonaCon- and IUD-treatment groups of causes not related to the treatments.

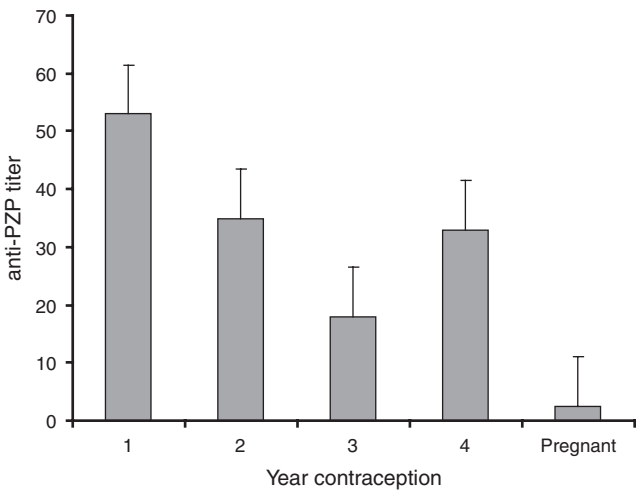


Fig. 2. Average anti-porcine zona pellucida (PZP) titres (±s.e.m.) for the autumn bleed for mares that were contracepted compared with titres of mares that became pregnant. The average titres for each of Years 1–4 were for the serum samples for the 10 mares that were infertile. The titre for the pregnant mares was the average for the 2 mares that became pregnant during the study.

values for the two SpayVac-treated mares that became pregnant, but the average serum oestradiol concentrations (ng mL^{-1}) for the cycling females at the autumn bleed was 25.9 ± 2.9.

Rates of contraception for GonaCon-treated mares were 93%, 64%, 57% and 43% for Years 1–4 (Fig. 1). For contracepted mares, regression analyses indicated that there was significant decline of average antibody titres over the last three years ($P < 0.01$). Nevertheless, anti-GnRH titres of non-fertile mares were significantly greater ($P < 0.01$) than average titres for mares that became pregnant during the study (Fig. 3). Serum progesterone concentrations (ng mL^{-1}) for non-pregnant GonaCon-treated mares at the autumn bleed averaged 0.3 ± 0.1, which was significantly lower ($P < 0.01$) than progesterone concentrations of pregnant mares treated with GonaCon (7.7 ± 1.5). Average serum oestradiol concentration (pg mL^{-1}) for the non-pregnant GonaCon-treated mares for all years was 32.6 ± 6.7, which was significantly less ($P < 0.001$) than concentrations detected in pregnant GonaCon-treated mares (1179 ± 225).

Contraception rates for IUD-treated mares were 80%, 29%, 14% and 0%, respectively in Years 1–4 of the study (Fig. 1). Average serum progesterone for all years at the autumn bleed was significantly lower ($P < 0.017$) for non-pregnant IUD-treated mares (4.3 ± 1.4 ng mL^{-1}) than for pregnant IUD-treated mares (11.3 ± 2.7 ng mL^{-1}). The average serum oestradiol concentration of 62.5 ± 34.2 ρg mL^{-1} for all years for non-pregnant IUD-treated mares was significantly less ($P < 0.001$) than the oestradiol serum concentrations of IUD-treated mares that became pregnant (1907 ± 505).

Comparing autumn serum progesterone values among treatments for non-pregnant mares, differences between GonaCon- and SpayVac-treated mares were not significant. However, progesterone values in non-pregnant GonaCon-treated mares and SpayVac-treated mares were significantly lower ($P < 0.02$) than values in non-pregnant IUD-treated mares (Fig. 4). In contrast, serum progesterone values at the autumn bleed were lower in pregnant GonaCon-treated mares than in pregnant SpayVac- or IUD-treated mares (Fig. 5). These differences were significant between IUD- and GonaCon-treated mares ($P < 0.025$), but because only two SpayVac-treated mares became pregnant, statistical comparisons could not be made with the other treatments. There were no significant differences in serum oestradiol concentrations in non-pregnant mares among treatments ($P = 0.13$) and differences between serum oestradiol concentrations of pregnant IUD-treated and pregnant GonaCon-treated mares were not significant ($P = 0.1$).

Observations made by ultrasonagraphy usually enabled visualisation of IUD location and the presence of uterine oedema or luminal fluid. In most instances IUDs were not observed in mares that were pregnant. When uterine oedema was observed it was recorded by treatment that the mare received (Table 1). These values were compared with the expected incidence of uterine oedema during oestrus based on a normal mare oestrous cycle. We assumed that within a normal 21-day oestrous cycle, 5–7 days of the cycle would be in oestrus, and the remaining 14–16 days the mare would be in dioestrus (Crowell-Davis 2007). Therefore, in a random sample of mares taken from a normal population, ~25–30% would be expected to be in oestrus



Fig. 3. Average anti-gonadotrophin-releasing hormone (GnRH) titres (±s.e.m.) for the autumn bleed for mares that were contracepted compared with titres of mares that became pregnant. The sample sizes for titres for Years 1, 2, 3 and 4 were 14, 9, 8 and 6 respectively. The average titre for the pregnant mares was for the 8 mares that became pregnant during the four-year study.

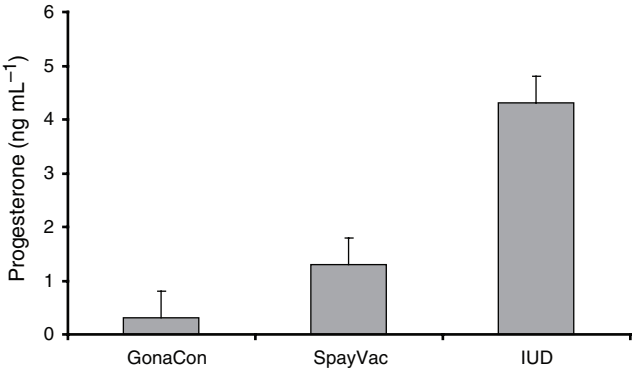


Fig. 4. Average progesterone values (ng mL^{-1}) (±s.e.m.) in non-pregnant GonaCon-treated mares ($n = 35$) and SpayVac-treated mares ($n = 46$) and non-pregnant intrauterine device (IUD)-treated mares ($n = 17$) for the autumn bleed for all years of the study. Values for GonaCon- and SpayVac-treated mares were significantly lower ($P < 0.02$) than values for IUD-treated mares.

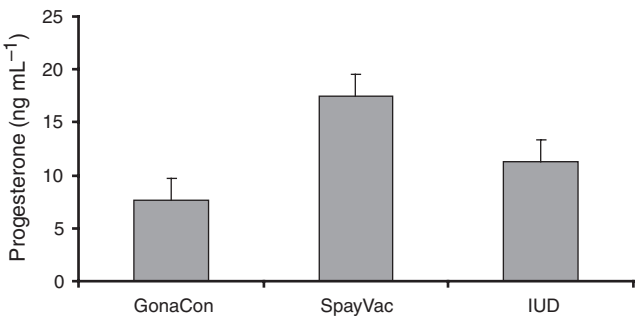


Fig. 5. Average serum progesterone (ng mL^{-1}) at the autumn bleed for pregnant GonaCon-treated-mares ($n = 8$), pregnant SpayVac-treated mares ($n = 2$) and pregnant intrauterine device (IUD)-treated mares ($n = 7$). Values were significantly different between IUD- and GonaCon-treated mares ($P < 0.025$); a comparison could not be run with the SpayVac-treated mares because of only two observations.

Table 1. Percentage of reproductive tracts with oedema revealed by ultrasonography of mares for each of the treatments compared with the predicted number of mares expected to be in oestrus

Numbers in parentheses indicate the number of mares showing uterine oedema out of the total number observed. Not all mares were observed at all time points. If a mare was found to be pregnant in a given year, she was dropped from the trial and not observed in subsequent years. Some mares on the trial could not be evaluated by ultrasonagraphy at a sampling date because their behaviour was unmanageable

Percentage	Year 1	Year 2	Year 3	Year 4
Predicted	25–30%	25–30%	25–30%	25–30%
IUD actual	20% (2 of 10)	0% (0 of 4)	0% (0 of 2)	–
GonCon actual	23% (3 of 13)	25% (2 of 8)	25% (2 of 8)	17% (1 of 6)
SpayVac actual	82% (9 of 11)	91% (10 of 11)	100% (10 of 10)	70% (7 of 10)

and to have oedematous uteri during the breeding season. Non-pregnant mares treated with GonaCon had rates of uterine oedema similar to the expected rate of 25–30% for normal non-pregnant cycling mares (Table 1). Likewise, IUD-treated mares in the first year had rates of uterine oedema similar to the expected rate, although in subsequent years too few non-pregnant mares remained in the IUD-treated group to obtain a reliable estimate. In contrast, SpayVac-treated mares had high rates of uterine oedema during all four years of the study (Table 1).

Ponies

A summary of the IUDs inserted into the pony mares is provided in Table 2. Only one of the four mares successfully retained the GyneFix IUD for a five-year period. Likewise, the copper-containing Sialistic ring was retained in only one mare for at least a year before she was sold. The copper 'T' device provided the greatest rates of contraception and retention, with several mares having the device in place for 3–5 years. We were able to observe only two of the mares (Table 2: Wanda, Remy) for one year before they were removed from the study.

Regardless of the type of IUD, the usual reason for failure was pregnancy. In most instances, we assumed that the pregnancy resulted from failed retention of the IUD, since we were unable to visualise the IUD by ultrasonography, or to identify it in the afterbirth when observed. In one case, we observed a 60-day pregnancy, but in a subsequent examination six weeks later the mare was not pregnant and the IUD was seen, suggesting that the pregnancy was aborted. In one case, a copper 'T' was removed because of pyometria. The recovery of that mare was unremarkable.

Two mares (Table 2: Libby, Maddie) were tested with all three IUD types. Both mares had the longest contraception rates with the copper 'T'. It is noteworthy that most mares given the copper 'T' IUD remained contracepted for multiple years. However, one mare (Libby) became pregnant within a year, regardless of the IUD type.

Observations made from April through August 2004 on the breeding behaviour of three non-pregnant mature mares equipped with a Copper 'T' IUD (Dewdrop, Maddie, Godiva) indicated that they had four or five oestrous cycles. The one mature mare equipped with a GyneFix IUD (Sprite) had six oestrous cycles and the one filly equipped with a 'T' IUD at 11 months of age had three oestrous cycles. These numbers were within the normal range of oestrous cycles for pony mares at the Penn State University facility.

Table 2. Summary of intrauterine device (IUD) types installed in pony mares, dates of installation, duration of contraception and reason for failure

n.a. = not applicable because the IUD is still intact

IUD type/ mare	Installation date	Date of last observation	Duration of contraception	Reason for failure
GyneFix				
Maddie	24.v.2002	25.vi.2002	30 days	Pregnant
Godiva	24.v.2002	23.vii.2002	60 days	Pregnant
Sprite	24.v.2002	28.iv.2007	5 years	n.a.
Libby (2)	23.ix.2005	23.iv.2006	7 months	Pregnant
Ring				
Maddie	2.vii.2002	23.viii.2002	10 days	Pregnant
Mandy	14.ix.2002	10.xii.2003	>1 year?	No data
Libby	25.v.2004	1.vii.2004	36 days	Pregnant
Libby	9.vii.2004	18.ix.2004	2 months	Pregnant
'T'				
Dewdrop	24.v.2002	1.xi.2006	5 years	Pyometria
Maddie	30.v.2003	28.iv.2007	4 years	n.a.
Godiva	14.vii.2003	28.iv.2007	4 years	n.a.
Libby	24.v.2002	15.v.2003	1 year	Pregnant
Connie	25.v.2004	28.iv.2007	3 years	n.a.
Wanda	4.ix.2002	30.v.2003	>1 year?	No data
Remy	4.ix.2002	30.v.2003	>1 year?	No data

Discussion

As with other species, population management of wild horses presents a specific set of challenges for a contraception method to meet in order for it to be of practical use. Assuming the contraception method does not pose serious problems to the health, behaviour or well being of the animal, two factors of considerable importance for contraceptive application in wild horses are long-term efficacy and whether the approach is easy to use. Wild horses need to be gathered from their range and managed under safe conditions for them to be hand injected with an immunocontraceptive vaccine. This approach has routinely been used for horses in the western United States. However, it is time consuming, expensive and, despite best efforts, not free of risk of injury to the horses or the human handlers. Although remotely darting individuals is possible (Kirkpatrick *et al*. 1990; Turner *et al*. 1996), it is not practical for vaccinating large numbers of horses in the western United States. For example, an internal cost–benefit analysis performed

by the Nevada Department of Agriculture (D. Thain, unpublished) for the Nevada Virginia Range horse-management area, consisting of over 145 000 ha of mountainous arid rangeland, concluded that equine bands did not aggregate in any one area to enable cost-effective darting. Because the Nevada Virginia Range is typical of many horse-management areas in the western United States, we believe that having a single-application contraceptive approach that is effective for multiple years would minimise or remove the need for additional gathers in these areas for revaccination in subsequent years. To date, a single-injection vaccine with multiple years of efficacy has not been available for horses despite considerable efforts of investigators working on the problem (Turner *et al*. 2001, 2002; Liu *et al.* 2005). The present study provides evidence that multiple years of contraceptive efficacy can be achieved with a single-shot immunocontraceptive vaccine in the mustang mare. SpayVac was shown to have the greatest contraception rate. For the four years of study, only two of the 12 SpayVac-treated animals had foals. This rate for a single vaccination far exceeds what has been reported by others for wild horses, although SpayVac has been shown to have long-term efficacy in other species (Brown *et al*. 1997; Fraker *et al.* 2002). Mare contraception with SpayVac was associated with antibody titre, since the two mares that became pregnant had titres much below the average titre of contracepted mares. Although the average titre for the non-pregnant SpayVac-treated mares declined in Years 2 and 3 relative to Year 1, the titres actually increased in the fourth year in most of the contracepted mares. This suggests that self boosting of the immune response may occur (Perry *et al.* 2006), perhaps as a result of the seasonality of mare reproduction. It is thought that self boosting occurs in the draining lymph node (Burton *et al*. 1994) as antigen is released from the follicular dendritic cell when the antibody flowing through the draining lymph node drops to a certain level. The released antigen then provides restimulation of antibody production. It is also possible that as the mare returns to breeding condition from a period of anoestrus, new zona pellucida proteins are produced with the initiation of follicle development which restimulate an immune response. If the titre at which mares became pregnant is assumed to be the set point for pregnancy to occur, we would predict that the mares currently contracepted with SpayVac will remain infertile for several more years. It is also possible that some of the mares may remain infertile indefinitely.

Compared with GonaCon and the IUD treatments, mares treated with SpayVac had a greater incidence of uterine oedema than would be predicted for normal cycling mares. Uterine oedema is associated with a predominance of oestradiol (Sample 1997), although there was no significant difference among treatments in oestradiol values for non-pregnant mares. However, expression of uterine oedema during the normal oestrous cycle is also influenced by the absence of serum progesterone (Crowell-Davis 2007). Relative to non-pregnant IUD-treated mares, which we found to have normal cycle lengths in the pony study, the serum progesterone values were significantly lower in SpayVac-treated mares than in IUD-treated mares. Although we were unable to make observations on the length of the oestrous cycle for the Nevada mares in this study, it has been reported that mares treated with PZP vaccine tended to have normal cycle length (Kirkpatrick *et al*. 1997; Powell 1999) although urinary oestrogen has been reported to be lower (Kirkpatrick *et al*. 1992), along with a short-term high incidence of persistent corpora lutea (Liu *et al*. 2005).

Serum oestradiol concentrations determined for SpayVac-treated mares suggest that some follicular development occurred. However, for SpayVac-treated mares showing some evidence of follicular development based on serum oestradiol, ovarian pathologies may be involved. Ovarian pathologies have been recorded for several species given PZP vaccines (Skinner *et al*. 1984; Mahi-Brown *et al*. 1988; Kirkpatrick *et al*. 1992; Lou *et al*. 1996; Stoops *et al*. 2006; Curtis *et al*. 2007), and are summarised as a disruption of folliculogenesis, depletion of primary oocytes and an infiltration of leucocytes. Given that serum progesterone was significantly lower in SpayVac-treated mares than in IUD-treated pony mares known to be having oestrous cycles of normal length, we suggest that SpayVac-treated mares may undergo some follicular development, but fail to ovulate and/or develop a normal corpus luteum.

Literature reports of circulating progesterone concentrations for untreated mares indicate considerable variation of values. Although concentrations of serum progesterone of $<1\ ng\ mL^{-1}$ are generally associated with oestrus, the concentrations progressively increase to high dioestrous values by Days 5–7 and are sustained there until Days 13–14. According to Ginther (1992, pp. 238–240), the range of means for 10 publications assaying serum progesterone during mare dioestrus was $4–22\ ng\ mL^{-1}$. Clearly, an average exceeding $4\ ng\ mL^{-1}$ for the IUD-treated mares suggests that most IUD-treated mares were in dioestrous when they were sampled. This is what would be expected when randomly sampling a population of mares. In contrast, an average of $1.3\ ng\ mL^{-1}$ for SpayVac-treated mares suggests that most of those mares were not in dioestrus. Whether they were in oestrus or transitioning into or out of dioestrus cannot be determined with only one sampling point for each mare. Nevertheless, we believe that the published values for average progesterone values during dioestrus, plus the reports of ovarian pathologies associated with PZP vaccines supports the notion that the lower progesterone values in SpayVac-treated mares may be due to a failure to form or maintain a normal corpus luteum. It is possible that antibodies to the zona pellucida prevent follicles from developing to normal ovulatory size and formation of the corpus luteum. Aside from these characteristics, there was no other evidence of contraindications associated with the SpayVac treatment.

Mares receiving a single vaccination of GonaCon showed a high degree of contraception during the first year, but this rate gradually declined to less than half after four years. This decline was associated with a gradual decline in antibody titre to GnRH over the same period. Unlike what was seen with SpayVac titres, there was no evidence for a self-boosting effect that occurs when the native protein is produced. The reason for this difference in response to the two immunogens is unknown, but it may relate to the fact that the PZP immunogen is a large glycoprotein compared with the GnRH decapeptide. Nevertheless, while the contraceptive efficacy of GonaCon was not as impressive as that of SpayVac, the GonCon results exceed rates of contraception reported by others for mares using other single-injection contraceptive vaccines (Turner *et al*. 2001, 2002).

The incidence of uterine oedema for GonaCon-treated mares was similar to what would be predicted in a population of normal cycling mares. This suggests that these mares may have some degree of oestrous cycle activity, although it is difficult to make firm conclusions. Theoretically, we would predict minimal steroid hormone production in GonaCon-treated mares if we assume that GnRH were inactivated by antibody to the vaccine. However, plasma oestradiol and progesterone concentrations in GonaCon-treated mares were similar to those in the other treatment groups. Because we have no direct observations on the reproductive behaviour of the GonaCon-treated mares, we cannot say whether these females expressed oestrus or showed evidence of an oestrous cycle. However, our unpublished data from white-tailed deer treated with GonaCon suggests that GonaCon does inhibit expression of oestrus at least in the first year or two following vaccination.

The presence of serum oestradiol in GonCon-treated mares is contrary to the notion that the GnRH is the sole regulator of follicle-stimulating hormone (FSH) secretion by the pituitary gland leading to stimulation of follicle development and oestrogen secretion. The GonaCon used in this study is prepared with the luteinising hormone-releasing hormone (LHRH) peptide (Levy *et al*. 2004) which has been shown to stimulate both luteinising hormone (LH) and FSH secretion. However, there is also evidence in several species for a follicle-stimulating hormone-releasing hormone (FSHRH) that specifically stimulates FSH secretion by the anterior pituitary gland (McCann *et al*. 1993, 1998; Yu *et al*. 1997; Padmanabhan and McNeilly 2001; McNeilly *et al*. 2003). If an FSHRH exists in the mare, it could explain the presence of serum oestradiol in infertile GonaCon-treated mares. Antibodies to LHRH in the serum would act to block some follicular development and the LH surge associated with ovulation, but some follicular development and oestrogen production would also occur in response to FSHRH and FSH secretion.

Contraception results for the Nevada mares treated with the IUD were encouraging in the first year of the study, but the performance was poor for the remainder of the study.

When IUDs were visualised, there was no evidence of uterine pathology as assessed by ultrasonography. It has been reported that mares implanted with a Sialistic ring IUDs were infertile for one year, but that device was associated with a uterine inflammatory response (Daels and Hughes 1995). In our study we did not see evidence of a uterine inflammatory response, and mares in the IUD-treatment group had the predicted number of occurrences of uterine oedema for the population size sampled. This, along with the serum progesterone and oestradiol data suggests that the IUD-treated mares were experiencing oestrous cycles.

Studies with ponies enabled more frequent observations of the IUDs and oestrous cycle events. From these observations we concluded that the 380 Copper ‘T’ IUD was superior to the other IUDs tested for long-term contraception. In addition, observations on oestrous cycle events for one breeding season led us to conclude that cycle length for mares with IUDs was within the normal range. The discrepancy in long-term rates of infertility between the mustang mares and pony mares equipped with a similar IUD is probably due to differences in uterine size. The retention of foreign objects in the mare uterus is related to the size of the object, relative to the size of the uterus. The ability of glass balls to be retained in the mare uterus has been shown to be related to the size of the glass ball (Nie *et al*. 2001; Thomas 2002). Although we have not found IUDs expelled by the uterus of an IUD-treated mare that became pregnant, we suspect the reason for the decline in efficacy of the Nevada mares was that the IUD was not retained in the uterus. This suggests that if larger ‘T’ IUDs were used, better rates of retention and contraceptive efficacy may be possible. On the basis of the pony studies, there is also evidence to suggest that shape of the IUD may also be a factor affecting retention, since neither the string GyneFix nor the ring IUDs were retained and performed as well as the ‘T’.

The mechanism preventing fertility in IUD-treated females has been argued to be either by interference with attachment of the early embryo to the uterus, or by induction of early abortion (Ortiz *et al*. 1996; Fortney *et al*. 1999). This mechanism may differ among species and no data have been published for the mechanism in the mare. Because the length of the oestrous cycle of pony mares in this study was within the normal range, we suggest that infertility in most instances was the result of the IUD interfering with events occurring between fertilisation and early embryo attachment. However, in one pony mare that was observed to be 50–60 days pregnant, in a subsequent examination she was not pregnant and the IUD was visualised. This indicated that abortion had occurred, but the IUD was retained. This observation raises the possibility that if pregnancy occurs followed by abortion, IUD expulsion may also occur. It is also possible that if pregnancy occurs but abortion does not, the IUD could be expelled with the placenta at parturition. This appears to have occurred with one Nevada mare that we observed to have the IUD when she was pregnant, and she went on to foal.

We believe that these studies provide evidence that long-term contraception of the mare is possible with the SpayVac PZP vaccine. Further improvements to the formulation of GonaCon that are now being tested in white-tailed deer suggest that rates of contraception similar to SpayVac are achievable with GonaCon. Development of larger IUDs that are better suited to the mustang mare may be possible. Regardless of the approach used, if a high rate of contraception is achievable for multiple years, population models suggest that contraception alone or used in conjunction with removal programs have the potential to stabilise and reduce population growth as well as reduce wild horse management costs (Garrott *et al*. 1992; Cameron *et al*. 2001; Bartholow 2004; Ballou *et al*. 2008; Kirkpatrick and Turner 2008). These observations lead us to conclude that population management of wild horses by single-application multiyear contraceptives will probably be possible in the near future for horse populations that can be gathered from their range for treatment and release.

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Immunocontraceptive Reproductive Control Utilizing Porcine Zona Pellucida (PZP) in Federal Wild Horse Populations

(Third Edition)

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Jay F. Kirkpatrick, Ph.D., Allen T. Rutberg, Ph.D., and Linda Coates-Markle

Compiler and Editor

~

Patricia M. Fazio, Ph.D.

June 1, 2010

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MEMORANDUM OF UNDERSTANDING
between the Humane Society of the United States and the Bureau of Land Management

After a workshop held in Santa Fe, NM, November 29 and 30, 2005 on wild horse fertility control, the Humane Society of the United States and the Bureau of Land Management have agreed to develop a Memorandum of Understanding to co-operate on: -

1) The further development and wider use of contraception in wild horse populations,
2) Resolve some of the uncertainties being faced in producing the vaccine and ensuring a continuing supply of a safe and effective vaccine,
3) Assist in public outreach on the issues, and
4) Maintain healthy and viable herds in the existing BLM wild horse Herd Management Areas.

"The BLM sees this as a way to reduce horse removals, to place fewer horses in short- and long-term holding facilities, and to achieve budgetary savings," said Don Glenn, Acting Group Manager of the BLM Wild Horse and Burro Program, Washington, DC.

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Source: HSUS-BLM Press Release on Fertility Control, e-mail from Tom Gorey WO/BLM/DOI, sent 11/30/2005 01:17 PM. Forwarded by Linda Coates-Markle /MTSO/MT/BLM/DOI on 12/05/2005 10:55 AM.
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Introduction

Two fundamental questions exist…

1) Does PZP harm wild horses?
2) Will its use eliminate entire herds?

The quick answers are that the Food and Drug Administration (FDA), The Humane Society of the United States (HSUS), and animal care committees all carefully review protocols for PZP use, and more than 20 years of data, carried out under these set of rules, clearly show that wild horses are neither injured by this drug, nor do aberrational behaviors occur as a consequence of its application. Too, oversight by The Humane Society of the United States assures that the vaccine is used only to slow reproduction and may not be used for the extermination of entire herds. PZP is designed to bring about short-term infertility and is reversible, if not used beyond five consecutive years. It reduces the need for gathers and preserves the original gene pool in each herd.

Expanding on these central points, the contributors and editor of material presented within this document have aspired to answer, with scientific objectivity, common questions and concerns raised by actual individuals and groups about porcine zona pellucida (PZP) and to provide citations and references that may be accessed through interlibrary loan, or other sources, for further study. Updates and additional questions and answers (Q&A's) will be provided periodically, as research progresses or protocols change.

PZP use in wild horse herds has been studied extensively for more than two decades, with papers published in peer-reviewed scientific journals by experienced reproductive physiologists, equine scientists, wildlife biologists, geneticists, and animal behaviorists, providing a portrayal of safety, high efficacy, and absence of long-term behavioral, physical, or physiological effects from the vaccine. Those involved in the creation of this Q&A have endeavored to produce a factual document of scientific merit, supported by field data, with statistically adequate sample sizes. Data was collected by trained, unbiased individuals, who adhere to established research methodology within his or her respective field.

Questions & Answers

THE PZP VACCINE

Q. What is porcine zona pellucida (PZP), and how does it work to block pregnancy?

A. A non-cellular membrane known as the zona pellucida (ZP) surrounds all mammalian eggs. The ZP consists of several glycoproteins (proteins with some carbohydrate attached), one of which, ZP3, is thought to be the primary component of the sperm receptor (the molecule that permits attachment of the sperm to the egg during the process of fertilization). The PZP vaccine is derived from pig eggs. When this vaccine is injected into the muscle of the target female animal, it stimulates her immune system to produce antibodies against the vaccine. These antibodies also attach to the sperm receptors on the ZP of her own eggs and distort their shape, thereby blocking fertilization. [see Paterson and Aitkin 1990; Miller et al. 2001]

Thus far, PZP has been a promising form of contraception in wild horses and other wildlife for the following reasons:

1. Pregnancy is prevented approximately 9o% of the time in treated animals;
2. The vaccine can be delivered remotely by small darts;
3. Contraceptive effects are reversible (up to five years in wild horses);
4. PZP is effective across many species;
5. No debilitating health side effects have been observed, even after long-term use;
6. No effects on social behaviors have been observed;
7. The vaccine cannot pass through the food chain;
8. It is safe to administer the vaccine to pregnant animals.

Q. How is it made, and who manufactures it?

A. The porcine zona pellucida (PZP) vaccine, used on BLM, U.S. Forest Service, and NPS wild horse mares, as well as within several wild horse sanctuaries, is produced by The Science and Conservation Center (SCC) in Billings, Montana. Each batch is subjected to a qualitative and quantitative quality-control program and shipped under the authorization of an Investigational New Animal Drug (INAD) exemption for wild horses (FDA

8857-G0002) issued to The Humane Society of the United States (HSUS) by the Center for Veterinary Medicine of the Food and Drug Administration. In collaboration with other investigators, The SCC continues to conduct research with the contraceptive vaccine, focusing on the ability to produce larger quantities, and increasing the efficacy of long-term contraception through a single inoculation (see Turner et al. 2002, 2008).

Q. How is the PZP vaccine obtained?

A. Once all necessary authorizations and approvals have been obtained for use of the vaccine, it may be ordered from:

Kimberly M. Frank
The Science and Conservation Center (SCC)
ZooMontana
2100 S. Shiloh Road
Billings, MT 59106
(406) 652-9719 (phone)
(406) 652-9281 (fax)
e-mail: sccpzp@hotmail.com

The vaccine is not commercially available and is provided at 60% of the cost of production, which currently runs about $24/dose. This is the price of the standard, one-year, 100 microgram dose. The 2-3 year vaccine uses considerably more than 100 micrograms, as well as more adjuvant, and includes the added cost of pelleting. The 2-3 year PZP vaccine costs about $200 per dose, plus the personnel costs of administration, which are minor, if horses are being gathered anyway. Compared with the $1,100 - $1,600 it takes to gather, remove, transport, hold, and adopt a horse (or care for it indefinitely), PZP is a bargain.

Q. Are any pigs killed, expressly to produce the vaccine?

A. Pig ovaries are obtained from a slaughterhouse in Iowa, as a by-product of hogs already destined for slaughter. Therefore, no fewer hogs will be killed if the PZP vaccine were no longer made. Major competitors for pig ovaries include Chinese restaurants, and pharmaceutical companies, that use ovarian endocrine components for research and production of products.

_Q_. Is this drug FDA approved and patented? If so, who is making all the profit from its use?

A. In FDA language, “approval” refers to approval for commercial distribution and marketing, and PZP is not a commercial product. No one is profiteering from PZP. The Humane Society of the United States holds the Investigational New Animal Drug exemptions (INAD), which are the oversight process by which FDA compiles data to examine vaccine safety and effectiveness. Basic and applied research that generated most of the knowledge about the vaccine was carried out with public funds (from the National Science Foundation, National Institutes of Health, U.S. Department of Agriculture, Bureau of Land Management, etc.). The research team considers products developed with public monies to be in the domain of the public, and therefore has no intention of commercialization. Therefore, it will always be called "experimental,” under FDA rules, despite the fact that PZP has been studied and field-tested extensively, for safety and efficacy, and is currently being used with more frequency on federal wild horse mares.

However, at the same time, use of public monies for research and development ***does not legally prohibit*** the commercialization of a product. Some researchers are, nonetheless, offended (due to a personal sense of ethics) and will not move forward with commercialization of a product developed with public funds. The rational is that the public has already paid for the product, and commercialization only allows private companies and individuals to profit from sale without having contributed to the process of research, development, and testing.

Because of this, PZP cannot be used on wildlife without the Investigational New Animal Drug exemption, or INAD. Once the INAD from FDA (one for horses, one for deer and zoo animals) was obtained, the INAD was turned over to The HSUS, leaving that organization to deal with the ethical issues. This means that each project – even at the management level – must have a research question attached to it, and The HSUS must approve the project. An added note is that the Investigational New Animal Drug exemption (INAD) issued to HSUS by the FDA requires a sound safety base before it is issued and would never have been issued were there a significant (or even an insignificant) health or safety concern. Therefore, neither HSUS nor The SCC make money from the vaccine. The SCC provides PZP vaccine at cost of production. Coupled with the paperwork required, The SCC actually

loses money. That is why The SCC is a non-profit. Currently, The SCC's annual budget is about $120,000, and PZP income results in less than half of that, meaning that a great deal of vaccine is donated.

The patent issue is a different question, not to be confused with the FDA process. Merck patented PZP in the 1970s, but the patent lapsed, and the technology is no longer patentable. Organon International, a large drug company based in the Netherlands, holds the patent for PZP use in humans, but that application may never take place, as scientists have not yet been able to make an effective synthetic form. Also, the variability in time for infertility reversal is significant and could potentially result in litigation.

In recent years, the FDA has transferred authority for wildlife contraceptives (exclusive of captive zoo animals) over to the Environmental Protection Agency (EPA). In 2009, an application is pending for approval of native PZP for use in wild horses by the EPA.

Q. What groups are on the PZP Contraceptive Research Team?

A. Today, the team consists of The Science and Conservation Center, Billings; Toledo University Medical College, Ohio; University of California-Davis; Tufts University, Medford, Massachusetts; The Humane Society of the United States, Gaithersburg, Maryland and Washington, DC; and the University of Iowa, Ames. Many other individuals contribute to the effort in one form or another. Governmental agencies that can be considered team members include the National Park Service, U.S. Forest Service, U. S. Department of Commerce, and the Bureau of Land Management.

The entire PZP contraceptive effort involves many people, several institutions, and numerous funding agencies. This team works together, bringing many disparate disciplines and talents in concert to solve the problems at hand.

Q. Who Funds PZP Contraceptive Research and Applications?

A. Funding for application of the vaccine to wildlife has been provided by many individual communities, agencies, and organizations, including but not limited to:

- The Humane Society of the United States

- Elinor Patterson Baker Trust
- Geraldine R. Dodge Foundation
- Bernice Barbour Foundation
- Leuthold Family Foundation
- Panaphil Foundation
- Delta-Sonics
- PNC, Inc.
- U.S. Navy
- National Park Service
- Bureau of Land Management
- Rachel Carson National Estuarine Reserve
- U.S. Department of Commerce
- National Institutes of Health
- Fire Island Community Association
- Burkett-Plack Foundation
- Bosack-Kruger Foundation
- Annenberg Foundation
- Cargill Foundation
- THAW Charitable trust
- 112 different zoos in North America, Europe, New Zealand and Australia
- South African National Parks Board
- U.S. Fish and Wildlife Service – African Elephant Conservation Fund
- Fripp Island (SC) Property Owners Association

- Morris County (NJ) Parks Commission
- Franklin County/Columbus (OH) Metro Parks
- and several anonymous donors.

This list is not all-inclusive but provides a picture of the breadth of support for this approach to wildlife management.

Q. Who controls vaccine use in wild horse populations?

A. No agency or organization can use the vaccine without the assent of HSUS, which monitors management plans and the INAD. In fact, the BLM approached the FDA and tried to circumvent HSUS control over vaccine use but was turned down by the FDA. Therefore, oversight and approval by HSUS still exists. No agency or organization will have control of the PZP vaccine in the foreseeable future. Every μg of vaccine that is produced can only be used in projects where HSUS has reviewed and approved a wild horse herd management plan.

All projects in which the vaccine crosses state lines must be on record with the FDA. As explained previously, the authority to carry out these projects is issued by two separate Investigational New Animal Drug documents (INADs) issued by the FDA to HSUS. As each new project is identified, HSUS reviews the need for the project in the context of scientific, ethical, and moral issues, and, if approved, issues permission to proceed. Notification of each project is accomplished by means of a form, filed with the FDA by The Science and Conservation Center, which specifies how much vaccine is being shipped and what species are to be treated. The INAD also requires that data from each project be gathered in a systematic way and filed, and be made available to the FDA when the need arises. These files are maintained at The Science and Conservation Center. Additionally, the legal managers of the horses (NPS or BLM) or the animal care committee of each zoo must also provide permission to treat animals. This regulatory process is similar for any wildlife species not classified as a food animal by the FDA or as a game animal by a state fish and wildlife agency.

Precisely how procedures will change – or remain the same – after EPA assumes regulatory authority, remains to be seen, but the HSUS will hold the approval; thus, we see no major changes at this time (6/09).

Q. Does an agency have to do an environmental assessment (EA) or an environmental impact statement (EIS) prior to using PZP on a wild horse herd?

A. Yes. Environmental Assessments are mandatory for any use by federal agencies. One difference between agencies is that the NPS does a single management EA, that is in force for years, while the BLM does one every five years for application in each herd management area.

Q. What wild horse populations, within the United States, are presently being managed with PZP?

A. The vaccine has been used successfully to manage the wild horse population of Assateague Island National Seashore (ASIS), in Maryland, under the sponsorship and authority of the National Park Service (NPS). The population has been treated for 22 years, without health problems, and the population has decreased by almost 40%, since management-level application began in 1995. Wild horses are also being treated on Cape Lookout National Seashore (Shackleford Banks), North Carolina, for the NPS; on Carrot Island, North Carolina; on the Rachel Carson National Estuarine Reserve, North Carolina; and on many areas of Nevada, for the Bureau of Land Management (BLM). Other treated herds include Return To Freedom (American Wild Horse Sanctuary), Lompac, California; Pryor Mountain Wild Horse Range (Montana/Wyoming); Little Book Cliffs National Wild Horse Range, Colorado; McCullough Peaks Horse Management Area, Wyoming; and Little Cumberland Island, Georgia; International Society for the Protection of Mustangs and Burros Wild Horse Sanctuary, Lantry, SD; Black Hills Wild Horse Sanctuary, Hot Springs, SD, Carson National Forest, NM, and the Navaho and Pima/Maricopa Indian reservations. In Nevada and Wyoming, at least 12 different wild horse herds are being treated "experimentally" to evaluate population effects. For Nevada references, see (1) Turner et al. 2001; (2) Turner et al. 1997; (3) Kirkpatrick et al. 1997; and (4) Kirkpatrick et al. 1997. In March, 2009, The Washington Office of the BLM Wild Horse Program i/ssued a memorandum that directs local horse managers to treat all mares returned to the range.

In the case of the four barrier island herds, the Pryor Mountain Wild Horse Range, Little Book Cliffs Wild Horse Range, and Return To Freedom Wild Horse Sanctuary, horses are treated remotely, with dart guns. In Nevada, they are treated in conjunction with gathers, as most of these HMA's are too large, and the horses too wild to dart.

In addition to controlling the horse population on Assateague Island, treatment has extended the lives and improved the health condition of older mares, by removing the stresses of pregnancy and lactation [see Kirkpatrick 1995; Kirkpatrick and Turner 2002, 2003; Kirkpatrick et al. 1990, 1991, 1992, 1995a, 1996a,b, 1997; Liu et al. 1989; Turner and Kirkpatrick 2002, 2008; Turner et al. 1996a]. Horses on Assateague are doing well. About 125 total animals roamed the area in 2009 (27% less than the starting number of 173 in 1995), and their body conditions have improved significantly since 1990 [Turner and Kirkpatrick 2002]. Mortality almost disappeared for the first eight years of management, and the horses are generating new age classes (large numbers between 20-25 years of age, and a growing population between 25-30).

Thus, at the management level, horses are being treated with PZP for the NPS, Rachel Carson National Estuarine Reserve, the BLM, and at least five private sanctuaries. In addition, new forms of the vaccine are being tested for the BLM in western horses, but not on a management level.

The following HMAs are sites that have been treated with long-acting PZP:

Onaqui Mountain, UT - 56 mares
Sand Springs, OR - 31 mares
Fox-Hog, NV - 28 mares
Green Mountain, WY - 38 mares
Monte Cristo, NV - 53 mares
Blue Wing, NV - 136 mares
Antelope Hills, WY - 28 mares
Black Rock East, NV - 19 mares
Black Rock West, NV - 19 mares
Warm Springs, NV - 27 mares
Antelope Complex, NV - 29 mares
Calico, NV - 92 mares
Groshuite, NV - 44 mares

Granite Range, NV - 79 mares
Nellis Air Force Base Bombing Range, NV (Nevada Wild Horse Range) - 358 mares
McCullough Peaks, WY - 34 mares
Sand Springs, CO - 75 mares
Cedar Mountain, NV - 85 mares

Additionally, another form of the long-acting PZP has been tested in captive mares at Canon City, CO; a reduced dose of PZP was tested successfully in captive mares at Canon City, CO; and a lyophilized form of the vaccine was tested successfully in domestic mares in Clark, WY and Billings, MT.

Q. What herds do you propose to treat with contraceptives in the near future? Why did you choose these particular herds? Who decides? What are your long-term goals?

A. While the PZP vaccine is currently being used on at least 20 horse management areas for the National Park Service or the Bureau of Land Management, its use is appropriate for all free-ranging wild horse herds. Application to particular herds is at the invitation of the managing agency. The long-term goal is to reduce or eliminate the need for gathers and removals.

DELIVERY, APPLICATION, AND BIOLOGICAL EFFECTS OF PZP

Q. How is the vaccine delivered?

A. The PZP vaccine must be injected into the muscle of the target animal. This can be done by hand if the animal is restrained, or by dart, for remote delivery. There are many commercial dart systems available, but the thick viscosity of the vaccine requires a large needle and a quick injection. Thus far, Pneu-Dart® systems (Williamsport, Pennsylvania) seem to work the best. The Pneu-Dart® 1.0 cc barbless darts can be fired from Pneu-Dart® capture guns or from several other commercial dart guns [Pax-Arms® or Dan-Inject®, for instance]. The darts are disposable, and after hitting the animal in the rump or hip (the only acceptable location for darting), they inject by means of a small powder charge, and then pop out. Because of

their bright colors, the darts are usually retrieved in the field. Darts that have not been discharged cannot be discharged by stepping on them or by any other kind of casual contact. Over a six-year period on Fire Island National Seashore, and with more than 1,000 dartings of deer, only two darts have not been recovered.

Normally, each animal is darted twice the first year, with the first injection being given up to a year before a booster, just preceding the breeding season (March for wild horses or September for deer). Thereafter, a single annual booster inoculation will maintain contraception. The second inoculation of the first year requires that…

a. you are able to recognize the individual animals; or

b. you do the first inoculation with a special "marker dart," which leaves a dye mark on the animal at the same time it injects the vaccine; or

c. selected mares are treated to allow for both genetic diversity within a specific herd and for the promotion of health and improved body condition of an individual animal (through temporary infertility).

An alternative strategy is to give only a single inoculation the first year, from which there will be little contraception, and then a single annual inoculation thereafter, from which there will be significant contraception (see McShea et al. 1997; Turner and Kirkpatrick 2002.)

New approaches using small non-toxic, biodegradable lactide-glycolide pellets, that result in several years of contraception after a single application, are being tested. [Turner et al. 2002, 2008].

Q. How would you describe the new pelleted form of PZP-22, and how does it work?

A. PZP is incorporated into lactide-glycolide pellets, small enough to be injected intramuscularly. After injection, the lactide-glycolide erodes (It is biodegradable and non-toxic) and releases the PZP at varying rates, depending on the ratio of lactide to glycolide. It is an injectable version of a contact cold pill. The pellets are made at the School of Pharmacy, University of Iowa. There have been spotty results in horses. It works in some herds and not in others, and it has worked well in deer, but not in elephants. Not all, but much of the data associated with the pellets has

come from helicopter counts, and it is difficult to match foals with mares from a helicopter. After the summer of 2010, more data will be available. Two herds in the Annenberg Foundation project have been treated with pellets. People are on the ground, who are familiar with the horses; so more solid data should be forthcoming.

***Q*. How does the liquid vaccine compare to the pelleted form?**

A. Nothing works as well as a primer dose of native PZP, followed by a booster 2 to 6 weeks later**,** and then annual boosters after that. Efficacy with the pelleted form, even where it has appeared to work, is not as good. The issue, of course, is having to treat mares every few years versus only once.

***Q*. Isn't darting mares painful and potentially harmful or even lethal? Will it result in mares being shot in critical anatomical areas – abdomen or chest, causing inhumane deaths?**

A. As long as only 1.0 cc Pneu-Darts are used, there is almost no risk of injury to the animal. These are very small, light darts. Over a 22-year period, no horse has ever been injured on Assateague Island, the Shackleford Banks, Carrot Island, the Pryor Mountains, or the Little Book Cliffs (translating to well over 1,000 dartings, over the course of 20 years).

On the Pryors, PZP remote-darting operations typically take place in late summer/early fall, and any wild mares receiving the vaccine are individually-identified and tracked regularly with data, non-intrusively gathered on behavior, estrus, fertility, reproduction, survival, and any health concerns. The field studies are conducted by seasonal and term USGS-Biological Resources Division and BLM biological technicians under the supervision of BRD research biologists and the BLM Wild Horse and Burro Specialist.

Several studies of injection site reactions have been carried out. In one study it was shown that of 38 total treated mares, 55% exhibit no reaction to darting, 6% have some level of swelling around the injection site, 22% have a small nodule (granuloma) about the size of a marble, and no animals had abscesses. Only one mare of 38 has ended up with a medium-level swelling, about one year after treatment. In a larger study, data were generated from the records of 329 different horses on Assateague Island, darted over a 19-year period. 1,094 different dartings resulted in a 0.2% abscess rate. The

abscesses healed within two weeks. In another study of captive zoo animals, 1,185 dartings of 25 different species resulted in 16 abscesses (1.3%) (Lyda et al. 2005). In the Pryor Mountains, 151 dartings resulted in 1 abscess (0.7%). At Return To Freedom Wild Horse Sanctuary, in California, 451 dartings of 85 total horses over six years resulted in 15 abscesses (3.0%). At the Black Hills Wild Horse Sanctuary, in 2009, 140 dartings resulted in one abscess (0.7%).

Abscesses typically appear as 25 mm swellings which open and drain within two weeks without complications. More common are granulomas, or subcutaneous nodules, about the size of marbles. These nodules are most notable post-injection but typically disappear over time. Ultimately these nodules are very difficult to discern amongst other natural scars within the coats of these wild mares. Furthermore, there is no indication that the presence of these nodules has compromised the quality of life for these horses. Field technicians have never recorded the mares showing any indication that these nodules are causing any level of discomfort during daily activities and/or interfering with reproductive activities. The granuloma is the counterpart of the smallpox vaccination scar found on humans, but beneath the skin instead of on its surface.

These injection site reactions should be given some context and compared with the same events in domestic livestock. The 1995 Beef Council Quality Audit reported that 11% of cattle inoculated with USDA-approved vaccines produced injection site reactions, including lesions and abscesses. In another study, the Kentucky Beef Council reported that fed cattle had abscess/lesion rates of 3.2-21.6% after vaccinations with USDA-approved vaccines, and in non-fed cattle, the abscess/lesion rate ran from 28.9 to 40.9%.

The most recent study of injection site reactions (Roelle and Ranson 2009) provides some interesting data. In any herd studies, often there are a few horses where abscesses appear more frequently than in other animals. Thus, for some reason, some animals are more prone to abscesses than others, and this often skews the abscess rate within a single herd. For example, in one herd, of a total of six abscesses that appeared after darting, four occurred in the same mare. The other data suggest that vaccinations given by dart produced more abscesses than those given by hand injection. This makes sense, if you consider that hand injections are preceeded by antiseptic swabbing of the skin surface, while field darting simply drives dirt and hair

follicles in to the wound, along with the vaccine. Thus, this all suggests that abscesses may not have any relationshop to the vaccine or adjuvant, but to the delivery method instead.

Q. How do immune system depression issues affect the formation of abscesses and lameness in horses?

We have insufficient data regarding how mares will react if they are in poor condition versus good condition, at least not enough for statistical analysis. Then, we just have visual indexes for body condition scores, from which you can simply infer immune competency; thus, we can offer only anecdotal information. Classic immunology says that animals in poor condition (or stressed for any other reason, as well) will not mount as strong an immune response as unstressed animals. Whether or not this effect will cause poorer efficacy, more abscesses, or other issues, is not known. In zoo animals, where we often administer PZP because of health problems, there seems to be no problem. Next, lameness and abscesses may not be at all associated with the vaccine *per se*. Rather, there is some limited data, by the Science and Conservation Center, and by Jason Ransom, U.S. Geological Survey, Ft. Collins, Colorado, that darted horses will produce more abscesses than hand-injected horses. When you hand inject, you wash the surface of the skin with ETOH. However, when you dart, you drive any surface dirt into the dart puncture. The same problem exists when darting wildlife with anesthetics, rather than vaccine. This issue is not any different from differing efficacies among individuals getting flu shots. In general, about all you can say is that animals with compromised immune systems do not do as well as those with competent systems, and there are many variables associated with what causes immune incompetency.

Q. Will PZP harm mares or foals, physiologically? Have any negative pharmacological side effects been observed? Are any benefits derived from its use?

A. Safety data has been accumulated over 20 years. It essentially says there are no short- or long-term health problems of any kind, and that the vaccine is reversible, unless the mare is treated for more than five consecutive years (in which case you probably didn't want her to reproduce again anyway). The data make clear that pregnancies in progress are not affected in any way by the vaccine, nor is the health or fertility of the foals compromised, once

they are born. Treating mares carrying female fetuses does not affect the fertility of the offspring.

In fact, as mentioned previously, mares on Assateague Island are living longer than ever, and their mortality has decreased, they are achieving new age classes never before seen on the island, and all of this happened because their body condition scores have increased steadily since 1989, when PZP application started. Historically, a mare never survived to 20 years of age, but now a significant percentage have passed twenty, and about 29 animals (20% of the herd) are between 25 and 30 years old. Removal of the stresses of pregnancy and lactation gives them an immense health advantage, both at the younger and older stages of life.

Foal mortality has dropped significantly. This is probably because their mothers, when they finally do become pregnant, after several years of contraception and then withdrawal of PZP treatments, are much healthier. All of this data (derived not from casual observation) is published.

The other victory for horses is that every mare prevented from being removed, by virtue of contraception, is a mare that will only be delaying her reproduction rather than being eliminated permanently from the range. This preserves herd genetics, while gathers and adoption do not. (Kirkpatrick and Turner 2002, 2008; Turner and Kirkpatrick 2002, 2003; Willis et al. 1994.)

Q. Does PZP application create late foaling in treated populations?

A. No. Available data from 20 years of application to wild horses contradicts this claim (Kirkpatrick and Turner 2003). From 1990 to present, Assateague Island has records for 178 horses whose month of birth is known (and in some cases, day of birth known). An examination of the published data, from 1984, of Ron Keiper (retired Distinguished Professor of Biology at Pennsylvania State University and currently Chairman of the Department of Biology at Valencia Community College, Orlando, Florida) in which he looked at eight years of birth dates for the same herd, which at that time was much smaller than we have today (considerably less than 100 horses versus 155) indicates that approximately 85% of the foals were born in April, May and June. Among the 178 horses with known birth dates, 95 were born to mothers who were never treated with PZP, with 70 born in April, May and June (73.6%), and 25 born outside this window. Another 83 foals were born to mares that had at some point been treated with PZP

before their pregnancies, and 65 were born in April, May or June (78.3%), with 18 outside this window. Thus, with a database of 178 horses over an eleven-year period, there is no evidence of late foals being born among treated mothers.

That corroborates published work (Kirkpatrick and Turner 1983), where it was demonstrated that Pryor Mountain wild horses did not extend their season of ovulation even when placed on high planes of nutrition. Mares do not extend their breeding season if they do not get pregnant.

One interesting issue is that the percent of untreated mares giving birth on Assateague Island in the April, May and June window has decreased from 85% down to 74% since 1984. This suggests that as herd size increases, variability in birth dates also increases, but this may simply be a function of larger numbers (in this case a 100% increase in herd size). There is also a moderate pattern among some mares (the N9BF line in particular) with regard to producing foals outside this window. This genetic line was consistently producing foals in March. If that observation is correct (This is only an untested observation.), then it corroborates Eric Palmer's theory that seasonal ovulatory patterns in mares are genetically controlled. In any case, these data, at least, demonstrate that contraception with PZP does not cause early or late births. Once again the Assateague Island (ASIS) horses and the 22-year treatment history produced a wealth of information (Kirkpatrick and Turner 2003).

In the Pryor Mountain Wild Horse Range, the normal foaling period has been well documented (EA #BLM MT010 FY05 -16, figure 10) to primarily take place in May and June, with limited foaling known to happen outside this window, from February to September. Thus, later foaling dates are not considered abnormal.

Q. For how many years is a mare generally treated with PZP?

A: This depends on the management plan of the agency, for a particular herd. Perhaps the most effective plan is the one used on Assateague Island, where all two-year-old mares are put on treatment, and then boosted at three and four years of age. After this, they are removed from treatment until they foal, which might occur anywhere from one to five years later. Mares that have already made their genetic contribution to the herd, in the context of the management plan, are treated until extinction.

***Q*. How do you determine which mares within a herd will be treated?**

A. The question of which mares to treat with vaccine is an important one, and the answer is embodied in the management plan. The approach used on Assateague Island has proven very effective and safe, i.e., two, three and four year olds are all treated and then withdrawn from treatment until they produce a foal. Older mares with good genetic representation in the herd are treated to extinction. Dr. E. Gus Cothran, an equine geneticist from Texas A&M University, College Station, monitors the plan on Cape Lookout. Immunocontraceptive control is more logical (genetically) than removing animals before they have had a chance to reproduce. Genetic representation is the key element within the management plan.

The most important consideration is to ensure that all genetics are represented, whether or not they reflect "wildness" or band stability, phenotype, or whatever social hierarchy exists. The bottom line is that wild horses are native North American wildlife, and humans should not be selecting for anything other than complete genetic representation and kinship.

***Q*. How effective is PZP? Won't some mares still become pregnant after treatment?**

A. PZP treatment in wild horses is about 95+% effective (Turner and Kirkpatrick 2002, 2008). The failure of some horses to respond to the vaccine results from an immune system that either doesn't "recognize" the vaccine's antigen, or from a compromised immune system. This is true for human vaccines as well (consider the less than 100% efficiacy with flu vaccines). Regardless, 95% efficacy is enough to manage wild horse populations effectively. In other species, efficacy varies in a species-specific manner (Frank et al. 2005).

***Q*. Why can't you block pregnancy with just one inoculation instead of the two shots you use now?**

A. The issue of the "one-shot" is complicated. Currently there are tests with SEVERAL forms of a one-shot vaccine. Despite that, this matter clouds the real issue of putting the vaccine to work NOW.

The initial “primer” dose of PZP causes the immune system to “recognize” and type the antigen, not so much for immediate long-term response, but to prepare the animal’s immune system for future exposures to the vaccine. Thus, a booster inoculation is required the first year, and an annual booster thereafter (Liu et al. 1989).

There is a second reason for the need for booster inoculations. Many human and veterinary vaccines use attenuated (weakened) or killed viruses as the vaccine, and these are powerful stimulators of the immune system. Often a single inoculation lasts for years. The PZP, however, is a relatively small protein that is not especially immunogenic. It is also very close in structure to the native PZP on the target animal’s own ova; thus, the target animal has difficulty in “recognizing” the PZP as foreign to the body. This, in turn, means that multiple inoculations must be given, and with a more immunogenic compound, known as an adjuvant (Lyda et al. 2005).

The subject of the one-shot also clouds the bigger topic of management because it only provides an advantage in the first year. After that, the horses are "one-shot" animals anyway. In 1994, almost every mare on Assateague Island was treated with a single shot. That shot was not meant to cause contraception but to set the herd up as a "one-shot" herd in preparation for management a year later. It was done this way because the National Park Service had to do an environmental assessment (EA) before they were allowed to manage with contraception. Then in 1995, the whole herd was managed with only a single shot per animal. The concept is fairly simple, and a "one-shot" vaccine only aids in the first year. Of course, a single inoculation that lasts multiple years would have more utility. (Turner et al. 2002, 2008)

The present advice given to the BLM is plain. Not a single mare that is gathered, for any purpose, and returned to the range should get back on the range without an inoculation. That makes them "one-shot" animals, and the expense of developing "one-shot" vaccines becomes moot. Once they have had that first shot, they can be re-treated anytime with a single shot.

A One-Inoculation Vaccine

Because of the need to inoculate animals twice the first year, and the difficulty of doing this with wild species, research is proceeding toward a "one-inoculation" vaccine. Such a vaccine would permit a single darting to

cause one or more years of contraception. The approach under study incorporates the PZP into a non-toxic, biodegradable material, which can be formed into small pellets. The pellets can be designed to release the vaccine at predetermined times after injection (at one and three months, currently), much the same way time-release cold pills work. Initial trials were encouraging, and continued trials are underway (see Eldridge et al., 1989; Turner et al., 2002, 2008). The downside to this approach is the immense amount of vaccine needed for the production of the pellets and the associated cost. While this approach may have some limited use, the difficulty in producing large amounts of vaccine make it unsustainable. What is needed is either a synthetic form of the PZP antigen or a pellet making process that reduces wastage by at least 65%.

Q. What do you mean by a one-year, two-year, or three-year vaccine?

Response to the PZP antigen is variable among individual mares. Some mares appear to be naturally poor responders to the vaccine and probably never develop sufficient antibody titer levels to confer infertility (hence the 95% efficacy of the vaccine in horses). Research with the Pryor Mountain wild horse herd indicates that immune response in mares may be correlated with age and fitness. One six-year-old mare, contracepted due to poor physical condition in September 2003, responded poorly to the vaccine, conceived in 2004, and foaled in 2005. Similarly, two 16-year-old mares last boostered in 2003, also produced a foal in 2005. Conversely, younger mares in good condition may have a stronger than expected antibody titer response resulting in a longer period of infertility. This appears to be the case with the first young mares treated with PZP on the Pryor Mountain Wild Horse Range (PMWHR).

Immunocontraception is not an exact science. The vaccines are designed to offer an effective time period based on the average response for many numbers of animals. As the treated number of animals increases, then the known response time for a specific formulation of PZP and adjuvant (carrying-agent) becomes better known. There are many variables to consider, the largest factors being animal condition and related immune response. Young PMWHR mares were healthy when injected (though a small sample size), and it would appear the resulting immune response (to a 90% effective one-year agent) has resulted in 2 years of efficacy. Older Pryor mares are relatively worn-out, and immune response appears limited. As such, the vaccine was not effective in a couple of mares. This is also true

with a younger mare that was treated, due her poor condition, but she still produced a foal.

Perhaps animal condition on Assateague Island (ASIS) is such that one year of efficacy is all that results with the same formulation of PZP and adjuvant that has been used on the Pryors. These are questions that still need to be dealt with, which is why the need exists for continued research. These questions are addressed in individual-based study herds within the Wild Horse & Burro Fertility Control Field Trial program and a project funded by the Annenberg Foundation. This is one of the reasons why the efforts on the Pryors have been critical to knowledge of this vaccine for use on western herds.

Q. How long does it take for native PZP to stop population growth in a herd?

A. This depends on the percentage of adult mares treated. On Assateague Island (ASIS), growth was stopped almost immediately. A large number of mares were given the PZP vaccine on ASIS during the first three years (about 87%), and growth stopped quickly. On the Pryor Mountain Wild Horse Range, fewer mares were treated (< 30%), but growth was slowed, as well. So, it depends mostly on the percentage of adult mares treated. However, fertility rates and mortality rates enter into this answer as well. It is precarious to address this any other way than to point out that results are site-specific and cannot be generalized.

Q. How long does it take for PZP to begin reducing a wild horse population?

A. On ASIS, the first sign of population reduction took about eight years, but herd reduction has been moving more quickly since then. The following eight years, herd numbers went down from 175 to 116, without any removal of horses, treating anywhere from 48 % to 79% annually. The lag time between initiation of treatment and decline in numbers is largely a function of increased longevity in treated horses, but, as pointed out above, there are other variable factors involved (fertility rates, mortality rates, sex ratios, and so forth). Fertility rates are average on ASIS (45-60%), and mortality is low (<5%).

***Q*. How can you tell if a wild mare is pregnant, so you don't treat her with PZP?**

A. In some cases, a fecal or urine sample is collected off the ground, or from yellow snow following urination. Reproductive steroid hormone metabolites are measured that tell us, with almost 100% accuracy, which mares are pregnant and which are not. A pregnancy can be diagnosed from 40 days post-conception until the day of parturition, and the animal does not have to be touched. That said, there is no danger to either the mother or the *in utero* foal, if the mare is treated with PZP during pregnancy (Kirkpatrick and Turner 2003, 2007).

***Q*. Is the drug residual in urine or feces or in the dead carcasses of treated mares, where PZP could get into the food chain or cause adverse effects to wildlife, or even contaminate water?**

A. Because PZP is primarily protein, it is readily destroyed in digestion, reduced to amino acids, and therefore cannot pass through the food chain intact and with biological activity (Oser 1965).

***Q*. What about compensatory reproduction in PZP-treated herds?**

Thus far, after 22 years of PZP treatment, there is no evidence for compensatory reproduction in a PZP-treated wild horse herd. This might be an issue if a herd is treated for long periods of time and then all treatment withdrawn, but that flies in the face of an effective management plan (Kirkpatrick and Turner 1991b).

BEHAVIORAL EFFECTS OF PZP

***Q*. Is wild horse behavior affected by PZP use? Are there any effects on motivation or drive, general contentedness, and the emotional stability of mares treated with PZP? Is band social stability affected negatively? Has any aberrational behavior been seen in PZP-treated mares, band stallions, or bands where mares have been treated with a contraceptive? What behavioral studies have been conducted on wild horses, both in eastern and western wild horse herds? Do you plan additional behavioral studies?**

A. After 22 years of treating the ASIS mares, there is still no evidence of altering behaviors. The baseline behaviors of eastern wild horses were the same as western horses. In order to understand this, a great deal of information must be read, which examines wild horse behavior from a variety of sites around the world. A huge body of literature exists on this subject, and a few of the more salient publications include: Berger 1977; Feist and McCullough 1976; Keiper 1976, 1986; Klingel 1975; McCort 1984; Rubnestein 1981; Rutberg 1990; Rutberg and Greenberg 1990; Salter and Hudson 1982. An independent investigator from the National Zoological Park has confirmed earlier results that show no behavioral changes (Powell 2000). Thirty-years of observing wild horses in North America and Australia and New Zealand have revealed no difference in fundamental behavioral structures. On the other hand, the affects of gathers on social behaviors are obvious (Ashley and Holcombe 2001; Hansen and Mosley 2000).

A good start on this subject can be obtained by reading Powell (1999), which reports on a study done by researchers from the National Zoological Park/Smithsonian. They found no behavioral effects, at that time, after almost eight (8) years of PZP treatment. The same results were reported in several of the Assateague Island papers, including (1) Kirkpatrick 1995, and (2) Kirkpatrick et al. 1995. Also, other studies (Fayrer-Hosken et al. 2000; Delsink et al. 2002) showed a lack of behavioral effects of this same vaccine on free-roaming African elephants, which have an even more complex social order than wild horses.

One critic of PZP claims, “The horses [on Assateague Island] seem more listless than western wild horses...” However, this could only be determined by time budget studies, and, in fact, no evidence for this has been found (referenced above). Casual observation of wild horses proves nothing. For example, casual observation has reported that Pryor horses travel less than ASIS horses, but that has no scientific significance. If one understands the biology of PZP, one would never suggest there is a "psychological" impact of the vaccine.

The research has already shown that band structures do not change and neither do hierarchies. The only major change in hierarchies that occurs is when mares get pregnant, and then they drop down the ladder even more. Most wild horse behavioral researchers still don't know how to measure hierarchy rank.

A more recent study (Nunez et al. 2009) on Cape Lookout National Seashore indicated that treated mares change bands more often outside of the breeding season. However, the control group for this study is a group of mares that become pregnant and have their foals removed from them. One could just as easily conclude that removing foals from untreated mares causes the mares to stay with the band for longer durations.

Q. What about behavioral changes, recently observed in mares, attributed to native PZP?

A. After 23 years of experience in the field, using native PZP, researchers observing wild horse mares feel that fundamental wild horse social behavior is not changed by the vaccine. However, other researchers are looking at issues such as "time budgets" to find any potential changes. It is the opinion of long-term PZP researchers, however, that these changes are neither of significance, nor are these studies controlled properly. For example, do time budgets differ for horses on good range as opposed to poor range? No one has looked at this, but if there are differences (and logic tells you there should be differences in time budgets between horses living in knee-deep alfalfa and those scratching out a living in a place like the Pryor Mountain Wild Horse Range), then we might expect the time budget of a chronically treated horse to change from untreated horses because of the better body condition scores of the former. These studies have not been controlled for mares with foals (usually untreated) and those without (usually treated), but the important point is that if the fundamental social structure and behavior do not change, it is of no consequence if time budgets change.

Q. Won't mares just keep coming back into estrus (heat) if they don't get pregnant? Won't prolonged estrus cycling make stallions "edgy" and aggressive, creating continuous "unrest?" In the chaos, won't foals be harmed or even killed?

A. At the heart of this issue is the subjective nature of casual observation. Science is based on data, not informal surveillance. Systematically collected data, reviewed by other scientists, accepted as legitimate, treated appropriately statistically, and published in a recognized journal is the only acceptable means for arriving at generalizable, accurate behavioral information. A good start for understanding the rudiments of behavioral research in social animals can be found in Craig (1986).

In 1983, it was shown (Kirkpatrick and Turner 1983; 1986 a,b) that wild horses do not have the same ovulatory patterns as domestic horses, and that wild horses have well-defined breeding seasons (usually from about late March until July, but this will vary somewhat from herd to herd). Wild mares do not extend their breeding season if they do not become pregnant. Keiper and Houpt (1984) also showed this for Assateague horses. This DOES happen, however, in deer (McShea et al. 1997) but not with wild horses. In 27 years of data collection on Assateague Island, only a single documented incident of a stallion killing another stallion, because of fighting, has been observed. This occurred in the middle of breeding season (late May).

The PZP vaccine does not prevent ovulation in horses and there is some limited data that it might interfere with ovulation in deer, at least after the initial treatments. Wild horses do not come into estrus every month, whether or not they have been treated with a contraceptive. They have a breeding season that barely makes it from April through July. Many wild mares have but a single estrous cycle and some have none in a given year, but they are highly seasonal and do not ovulate year-round, or even half the year.

The implication of the question is that by treating wild horse mares with PZP, they will continue to cycle throughout the year, and that this will cause stallion "unrest," aggression, and potential injury. By contrast, documented evidence shows that mares will NOT continue to cycle if they do not conceive, at least beyond the normal 3-4 month breeding season.

If critics were correct, and PZP did cause “unrest” and continuous cycling, which, in turn, led to worked-up stallions and foals dying as a result of this behavioral turmoil, then any area using PZP would have greater foal mortality. However, after 15 years of management-level treatment on ASIS, foal mortality has decreased. That is a data-driven fact.

Granted… wild horse behaviors are subtle, and individual horses will show a wide range of variability in behavioral patterns. It takes a great deal of observational experience to pick them up. We do know that hierarchies and band fidelity, are not affected by PZP application. We also know that aggressiveness and aberrational behaviors are not caused by PZP use. Perhaps, however, there may be a subtle change in daily routine. However, the larger question is, even if subtle alterations in behavior may occur, this

is still far better than the alternative of wild horses being rounded up, bands broken apart, and all of the other negatives that go with traditional management. These issues need to be put into the perspective of risk-benefit by PZP opponents.

There may be some minor time budget changes, but this was addressed several quesitons above. In any case they are not significant in the world of wild horse behavior.

***Q.* I "live" with wild horses and feel that some of your behavioral studies (measuring PZP effects) are wrong or incomplete.**

A. Living with wild horses and seeing them every day is one thing, but while of interest, this doesn't necessarily hold noteworthy meaning, unless a parameter for study is identified, a hypothesis established, and a means of testing that hypothesis is conducted. Additionally, this still does not indicate a significant behavioral or other casually observed pattern for an individual animal or for a band or herd unless the data is analyzed properly.

***Q.* I wish the BLM had used a bit of its research money or allocated new money to do a study regarding the effects of PZP on the social structure and health of wild horses in the west.**

A. The effects of PZP on social structure and herd health have been studied on herds within the Pryor Mountain Wild Horse Range and Little Book Cliffs Wild Horse Range, and no behavioral changes have been noted, nor have any behavioral changes been noted on wild horses at Return To Freedom (American Wild Horse Sanctuary) in California, where animals are observed daily.

***Q.* Isn't the use of PZP "against nature?" Why can't you just leave these animals alone?**

A. Except in a small number of wild horse herds (such as in the Montgomery Pass herd (Turner et al. 1992), Bordo Atravasado in New Mexico, and a few others), mountain lions cannot predictably predate a sufficient number of wild horses to keep herd levels at population numbers in balance with the carrying capacity of their ecosystem. The potential for both wolf and grizzly bear predation of wild horses is in question, as well,

especially if grizzlies and wolves are delisted from the Endangered Species Act, making grizzlies, wolves, and mountain lions all open to hunting pressures.

OPPOSITION TO PZP

Q. Why are some wild horse advocacy groups so vehemently opposed to PZP? It seems like the perfect solution.

A. Opposition to the contraception arises for several reasons. Wild horse advocates do not trust the BLM with the PZP vaccine, despite assurances that the agency cannot use it without approval from The Humane Society of the United States (HSUS). BLM has inflamed the issue by asking FDA for their own INAD, so they could bypass The HSUS. FDA told the BLM that no more "use" INADs would be issued; so, currently, the agency has no choice but to work with HSUS, unless they are willing to change horses in the middle of the race. The HSUS will permit the use of PZP to manage, even reduce, but not to eliminate wild horses.

This has now led the BLM to seek as many other forms of wild horse contraception as possible, from other groups, but it will take significant time to match the safety data studies that have been generated on PZP over many years.

All wild horse advocates want horses to have a better life, but if this entails a choice between having 130 healthy horses versus 200 living on the nutritional edge (in the event of a drought or a severe winter), some would choose the 130 healthy-horse option, out of (what they see as) concern for the well being of the horses. The irony is that those who seek to control wild horse populations through immunocontraceptive measures often spar with other horse advocates who (in the opinion of PZP supporters) object to what pro-PZP factions perceive as the humane treatment of wild horses. The anti-PZP community, who often question factual information and historic success, distrust the morals of those who strongly profess they care (about the well being of the herds). Pro-PZP individuals and groups believe, with conviction, that the primary motivation behind wild horse contraception is keeping healthy wild horses in the wild, on the land forever, in as natural a state as possible, with minimal interference from humans.

Many of these opponents dislike PZP because they fear it will reduce the herd to lower numbers than they want. That has been the major contention with most gathers. After 22 years of contraception on ASIS, and 15 at the management level, contraception has only been able to reduce that herd from 173 to 116. Zero population growth was achieved, and, to date, there has been no need for gathers, but reduction has been slow. So, there is little danger of massive reductions happening anywhere. Even the event of a catastrophic winter has less danger inherent than most advocates might think. The Pryor Mountain herd in Montana/Wyoming went from about 140 horses to 70 in a tragic winter die-off of 1977-1978, but the population had recovered within three years. The only thing that would have changed, had there been 200 animals instead of 140 is that more animals would have had less to eat, and therefore more would have died. The severity of the winter determined that 70 horses would survive, and not the starting population number.

Q. Aren't you trying to bring wild horses to extinction (using PZP)?

A. The concerns of some anti-PZP wild horse groups over BLM use of the vaccine are justifiable, and the BLM's proposal to use PZP to cause an Oregon herd to approach extinction, and other proposals that have surfaced to use PZP to manipulate herd composition, prove this. These apprehensions are legitimate and acknowledged by PZP researchers. However, Assateague Island data have proven the safety and efficacy of the vaccine, and it is obvious that the BLM is not going to be able to treat 30,000 wild horses to extinction with contraception, especially with continued involvement from The HSUS. Vigilance is important in the continued utilization of the PZP vaccine and in monitoring questionable research studies by state and federal agencies not sanctioned by The HSUS or the BLM, some involving the use of contraceptives that may potentially cause complete sterilization or deleterious effects in mares. Despite the utter rejection of scientific data as a legitimate form of persuasion by some anti-PZP individuals and groups, their hearts are in the right place. They are not the natural enemies of those promoting PZP as a humane management tool. Affable cooperation is needed to resolve issues of wild horse over-population, when and where it legitimately exists, once other means of population reduction have failed or are found to be undesirable.

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FOR ADDITIONAL INFORMATION

http://www.zoomontana.org/conservation_center/index.php
(The Science and Conservation Center, ZooMontana, Billings)

http://www.zoomontana.org/conservation_center/pzp_q_and_a.pdf
(online version of this document)



REVIEW ARTICLE

Contraceptive Vaccines for Wildlife: A Review

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Gonadotropin-releasing hormone, immunocontraception, porcine zona pellucida, wildlife

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Wildlife, free-ranging and captive, poses and causes serious population problems not unlike those encountered with human overpopulation. Traditional lethal control programs, however, are not always legal, wise, safe, or publicly acceptable; thus, alternative approaches are necessary. Immunocontraception of free-ranging wildlife has reached the management level, with success across a large variety of species. Thus far, the immunocontraceptive research and management applications emphasis have been centered on porcine zona pellucida and gonadotropin-releasing hormone vaccines. Contraceptive success has been achieved in more than 85 different wildlife species, at the level of both the individual animal and the population. At the population management level with free-ranging species, the primary focus has been on wild horses, urban deer, bison, and African elephants. The challenges in the development and application of vaccine-based wildlife contraceptives are diverse and include differences in efficacy across species, safety of vaccines during pregnancy, the development of novel delivery systems for wild and wary free-ranging animals, and the constraints of certain non-contraceptive effects, such as effects on behavior. Beyond the constraints imposed by the public and a host of regulatory concerns, there exists a real limitation for funding of well-designed programs that apply this type of fertility control.

Introduction

While elective contraception of domestic animals can be traced back hundreds of years, more recent contraceptive research has been almost universally associated with human applications. However, in the past 40 years a new application has arisen, involving certain species of both domestic animals[1] and wildlife.[2] In the past decade, increased public concern over unrestrained dog and cat reproduction has stimulated a great deal of research in this area, and issues such as the prevention of pregnancies in feedlot cattle have even brought the commercial agricultural community into the world of contraceptive research.[3] A few of these activities, however, provide methods that are directly applicable to non-domesticated animals. Not surprisingly, wildlife contraception involves a number of diverse challenges not seen with either human contraception or domestic species.

With regard to wildlife contraception, four commonly referenced case studies include wild horses (*Equus caballus*), urban and park cervids such as white-tailed deer (*Odocoileus virginianus*) and wapiti (*Cervus elphus*), African elephants (*Loxodonta africana*) in game parks, and more recently bison (*Bison bison*). There are other examples but these stand out with regard to free-ranging wildlife. A growing number of non-domestic species housed in zoos and wild animal parks could also be included. With rare exception, all are medium to large mammals.

Efforts at controlling wildlife overpopulation by means of contraception began as early as the 1950s, and most research involved natural and synthetic steroids, or a variety of non-steroidal compounds.[4–6] Virtually all of these early attempts, while

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pharmacologically successful, ended in failure, for a variety of reasons including toxicity, passage through the food chain, adverse effects on social behaviors, high cost of application, difficulty in delivering the compounds remotely, health risks in pregnant animals, and a host of regulatory issues. Of course, the constraints within these early approaches varied depending on the species. For example, remote delivery was not a requirement for captive exotic species, and behavioral changes were of less importance in urban deer, which were viewed as 'pests' in some quarters. Conversely, remote delivery is a virtual requirement for free-ranging wildlife, and behavioral changes would not be tolerated in social animals such as wild horses and African elephants. Thus, each species and setting brings with it specific needs and limitations. The cumulative effect of all these issues resulted in the realization that contraception with steroid hormones, the method of choice for humans and dogs, was not acceptable across the broader spectrum of wildlife. Steroids remain useful in some captive wildlife contraception efforts but have been abandoned for use in free-ranging wildlife.

It was only with the advent of practical immunocontraception that significant progress in free-ranging wildlife contraception occurred. Immunological approaches were considered for use in wildlife as early as 1987[7] and the earliest recorded attempts at wildlife immunocontraception included a gonadotropin-releasing hormone (GnRH) vaccine tested in wild horses on Cumberland Island National Seashore in 1986.[8] Shortly thereafter, a porcine zona pellucida (PZP) vaccine was tested in domestic and captive wild horses.[9] The results of the GnRH trial were not promising, while the PZP trial provided clear evidence that an immunocontraceptive could successfully inhibit fertility in equids. In relatively rapid succession, trials with PZP were successful in captive white-tailed deer[10–12] and a variety of zoo animals, including Przewalski's horse (*Equus przewalskii*) and Banteng (*Bos javanicus*)[13] Formosan sika deer (*Cervus nippon taiouanus*), axis deer (*Cervus axis*), Himalayan tahr (*Hemitragus jemlahicus*), Roosevelt elk (*Cervus elaphus roosevelti*), muntjac deer (*Muntiacus reevesi*), and sambar deer (*Cervus unicolor*)[14] and African elephants (*Loxodonta africana*) (J. F. Kirkpatrick, unpublished data).

Identifying an effective vaccine was only the initial step in developing a population management for wildlife. Very early it was recognized that the major challenges in wildlife contraception included (i) identifying successful contraceptive agents in captive animals, (ii) documenting safety associated with successful agents, (iii) developing a means of delivering these agents to free-ranging wildlife, and (iv) actually altering populations. With the success of PZP-induced fertility control in captive animals, which addressed the first of the four challenges, the next and more challenging step was the implementation of actual field trials with free-ranging animals. While the strategy remained the same, this represented a major change in tactics and one that is still underappreciated. Fortunately, one of the major advantages of immunocontraception became obvious during captive animal trials, which was the ability to deliver contraceptive doses in very small volumes, which could be delivered remotely. This is now recognized as a key requirement for practical wildlife contraception.

The first field trial of wildlife PZP immunocontraception occurred on Assateague Island National Seashore, a barrier island off the coast of Maryland 23 years ago. This trial proved successful in inhibiting fertility in wild horses.[15] Aside from demonstrating a high degree of contraceptive efficacy, this initial study proved beyond a doubt that the vaccine could be delivered remotely, without any handling of animals. It also demonstrated the safety of the vaccine in pregnant animals[15] and that fertility inhibition could be maintained with annual booster inoculations.[16]

The first field trial with white-tailed deer occurred at the Smithsonian Institute's Conservation and Research Center at Front Royal, VA.[17] Deer were captured, tagged, and given a primer dose of PZP and then released. Subsequent booster inoculations were given remotely via small 1.0 cc darts. As with earlier captive trials, the vaccine proved efficacious (85%) and remote delivery proved successful. In the case of both the wild horse and deer field trials, animals received a 65- to 100-μg primer dose emulsified with Freund's complete adjuvant (FCA) and a booster inoculation of 100-μg PZP emulsified with Freund's incomplete adjuvant (FIA). The first field trial with free-ranging African elephants occurred in 1996, in the Kruger Park, in South Africa. Animals were immobilized from a helicopter, given ultrasound examinations to determine pregnancy status, collared and hand injected with a 600-μg primer dose of PZP + a component of Ribi® adjuvant.[18] Subsequent booster inoculations occurred by remote

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treatment. Results were positive but efficacy was lower (75%) than with horses and deer. A change of adjuvants, to Freund's modified adjuvant (FMA), increased efficacy to 100%.[19,20] Since the completion of these trials, management level application of the PZP vaccine has been successful in altering entire populations, stopping population growth and even decreasing populations, of wild horses,[21] urban deer,[22] wapiti,[23] and African elephants.[19,20]

Safety

At this point, attention turned to safety issues. The questions at hand were (i) did PZP alter ovarian function, (ii) were the contraceptive effects reversible, (iii) were there adverse behavioral effects of PZP immunocontraception, and (iv) were there any short- or long-term adverse physical side effects?

A major advantage of zona proteins as immunogens is the absolute lack of cross-reactivity with other tissues and protein hormones.[24–26] Additionally, the site and mechanism of action (see Clark and Dell[27] for an eloquent discussion of the mechanism of action supporting zona vaccines) is as far 'downstream' for most of the reproductive processes as possible, such that the sequelae of reproductive events that are disrupted are inconsequential. This is essential for wildlife species but may be considered a disadvantage if the target animals are domestic species, such as dogs and cats, where extinguishing undesirable behaviors is as much a goal as contraception.

The safety of PZP immunocontraception was confirmed for horses, deer, and elephants through a series of follow-up investigations. Ovarian endocrine function was monitored in treated wild mares via urinary steroid metabolites,[28] and it was shown that there were no permanent or significant changes in ovarian endocrine parameters and estrous cyclicity even after long-term treatment.[29–31] This is consistent with the accepted theory of the mechanism of action of zona proteins.[32] Later studies in wild horses confirmed the safety of PZP use in pregnant mares[33,34] and reversibility of contraceptive action, at least through five consecutive years of treatment,[33] demonstrated increased body condition[35] and increased longevity in mares treated chronically,[36] and no significant changes in fundamental social organization or behaviors.[37] Two more recent studies found that PZP treatment resulted in less band fidelity among treated mares outside the breeding season[38] and minor changes in time budgets among treated mares.[39] However, the former study was not controlled for pregnant animals or mares that had foals removed annually. The latter study found that most behavioral changes were associated with changes in body condition or the presence of foals. Considering that untreated mares have more foals than treated mares, and that treated mares develop better body condition than untreated mares, small changes in time budgets would be expected. Ultrasound examinations of ovaries and uteri from PZP-treated elephants also revealed a lack of negative effects.[18,20]

While no long-term negative health effects were detected in PZP-treated wild horses, there was some concern over injection site reactions. Because of the homology of the zona glycoproteins across many mammalian taxon groups, PZP is a poor immunogen. Thus, a powerful adjuvant is necessary and between 1988 and 1998, FCA was the adjuvant of choice for initial inoculations, followed by booster inoculations of PZP + FIA. In reality, as long as inoculations were given in the rump or hip, injection site reactions were uncommon; however, there was concern over causing false tuberculosis-positive tests among some species of animals treated with FCA (there is no TB test for equids; thus, this was not a problem in these species). As a result, for initial inoculations, a new adjuvant was utilized with PZP in wild horses, and later among other species as well. FMA, which utilizes the fractionated cell walls from *M. butyricum*, a common soil bacterium with no known pathologies associated with it was tested and proved to be as efficacious as FCA.[40] Thus, this is the adjuvant of choice since 1998. Rare injection site reactions still occur after inoculation, even with FMA or FIA, but seldom exceed 1%.[40] One study[41] demonstrated a higher incidence of injection site reactions in horses treated remotely, with darts, than those treated by hand-injection. This suggests that injection site reactions may be as much a function of delivery as the actual vaccine. Delivery by dart must result in surface bacteria and debris being pushed into the injection site. In the absence of either short-term or long-term health issues, PZP contraception is now common in free-ranging and sanctuary wild horses, urban deer, zoo animals, and African elephants. Other safety issues have been reviewed in more detail elsewhere.[42]

Other immunogens have been used in horses but thus far none have been applied to free-ranging wild

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horses except in small-scale research trials. Several approaches targeted the stallion. GnRH vaccines have demonstrated that testicular function can be suppressed in the stallion.[43,44] However, the application of these vaccines to wild horses is contraindicated because of the suppression of testosterone-mediated reproductive behaviors, which are necessary for holding breeding bands together in the face of competition from other stallions. The entire social structure of wild horses is disrupted if the dominant stallion is chemically castrated. Further, studies have shown that control of dominant males, by means of vasectomy, in wild horses is not effective because this increases the reproductive success of bachelor stallions.[45] A second problem associated with targeting the male of the species, is that, if it is not a harem-based social structure, nearly every male in the population will need to be treated, and this is probably not logistically possible. Thirdly, the removal of functioning males from the breeding population may have far more serious genetic consequences that treating females[46,47] and certainly provides less management flexibility.

Three commercial GnRH vaccines have shown success in suppressing reproduction in mares. Both Equity® (Pfizer Animal Health, Sandton, South Africa) and Improvac® (CSL, West Ryde, NSW, Australia) suppressed cyclic activity in mares.[48,49] GonaCon®, a GnRH vaccine developed by USDA, primarily for use in deer, was tested in captive wild mares and also was successful in blocking cyclic activity and ovulation.[50] A fourth commercial GnRH vaccine, Repro-BLOC® (Amplicon Vaccine, Pullman, WA, USA) has been developed as an alternative to castration in cattle. This latter vaccine utilizes a series of GnRH genes, which were cloned into an ovalbumin carrier via an *Escherichia coli* expression vector. Aside from trials in cattle, Repro-BLOC® has been used to suppress estrus in captive Asian elephants and proposed as a contraceptive for this species;[51] however, five serial inoculations were required to achieve adequate anti-GnRH titers. Despite contraceptive success, and clear potential applications to several other species, the application of GnRH vaccines to wild horses brings to the fore issues that usually are not considered with human contraception. The complex evolutionary social organization of horses and other equids is driven to a large degree by reproductive steroids. In the case of GnRH vaccines, the result is, although temporary, a non-surgical castration. Public opinion, which directs so many aspects of contraception, human, or wildlife, requires in the case of wild horses an intact set of social behaviors. The same is true for elephants and most captive exotic species in zoos.

Other potential safety issues regarding GnRH vaccines remain. Safety for use in pregnant animals remains a species-specific issue. In those species that rely on pituitary Luteinizing hormone (LH) to maintain the corpus luteum of pregnancy throughout the entire gestation period, GnRH will cause abortion. This is not an issue in a species such as the horse, where placental hormones take over the task of supporting pregnancy, but use in bovids, for example, such as the bison, or during the first half of pregnancy in the goat family would interfere with pregnancies in progress. More troubling, however, are a variety of other issues associated with any vaccine that blocks GnRH. It has now been demonstrated that GnRH receptors exist in a variety of tissues throughout the mammalian body, including the cerebellum,[52] bladder,[53] and cerebrospinal fluid.[54] Now recognized as a form of neurotransmitter, GnRH has physiological effects throughout the central nervous system. GnRH activity in the hippocampus has been implicated in Alzheimers-like syndrome. GnRH activity can alter olfactory function, which is, of course, vital in the reproductive process for so many wildlife species. In the cerebral cortex, GnRH can cause depressed activity, and in the cerebellum GnRH has a correlative link to two genetically based disorders, including Gordon–Holmes Syndrome and Boucher–Neuhauser Syndrome. In the pituitary, it has also been demonstrated that GnRH affects more than just gonadotropic cells.[55] The action of GnRH in cardiac tissue, which is exceptionally rich in GnRH receptors, may also be an important safety issue. At least two studies have demonstrated a serious and negative effect on cardiac function and in men, blocking GnRH can place the patient at greater risk for coronary infarction.[56,57] Whether or not these particular safety issues have any clinical relevance to free-roaming or captive wildlife remains to be seen. However, it becomes intuitive that vaccines that exert their influence further 'upstream' in the reproductive process and which have interactions with non-reproductive tissues will be more problematic than those with target tissue specificity and that exert their effects further 'downstream' in the reproductive process.

In white-tailed deer, safety issues relating to PZP largely paralleled those of wild horses, but with

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some exceptions. Reversibility of contraceptive action occurred after 1–3 years in captive PZP-treated deer[11] and after 1–4 years in another study with captive deer.[12] An earlier study demonstrated that there were no changes in ovarian histology, but that the breeding season was extended by one to 2 months in PZP-treated deer.[17] In one study, this resulted in a pulse of late births the first year, but in subsequent years, the late births did not occur.[58] Treated deer were more active than untreated deer but did not spend more time feeding. Despite the increased activity, treated deer gained significantly more weight by the summer following treatment than untreated deer, presumably because of avoidance of lactation, but by the following fall, all weight differences disappeared.[59,60]

Potential changes in ovarian histology were a concern. Prior to application of PZP vaccines to wildlife, some temporal changes in ovarian function were shown to occur in PZP-treated non-human primates[61] and mice,[62] but long-term effects did not occur. Early studies with PZP-treated dogs revealed significant changes in ovarian histology.[63] Another study[64] demonstrated similar effects in PZP-treated rabbits, along with a depletion of primordial follicles. Among PZP-treated sheep, significant changes in the ovarian histology were found.[65] The dog study was probably compromised by a rather impure PZP, and the rabbit and sheep studies revealed species differences in response to the PZP. Collectively, this research raised an issue of concern as wildlife contraception moved forward and into the realm of management.

In the first trial with PZP and horses,[9] no changes in ovarian histology were found, and other investigators had demonstrated ovarian weights and histology were unchanged after PZP treatment of white-tailed deer.[17] In another study[66] ovarian eosinophilic oophoritis was found in six of eight treated deer, but this rate was not significantly different from controls. This same study revealed that this condition is common in untreated deer at the time of ovulation. Despite the absence of any significant changes in ovarian histology in wildlife studied thus far, it is vital that each species be evaluated for potential changes. Blood chemistry studies in treated white-tailed deer revealed only a few significant changes after PZP treatment, but none was associated with any physiological abnormalities.[66,67] Ongoing studies with Dall sheep (*Ovis dalli*)) and domestic goats (*Capra hircus*) reveal the same lack of blood chemistry changes in these two species.[42] Injection site abscesses were found in only 2 of 353 deer (0.5%)[68] and 100% of PZP-treated deer revealed granuloma formation at the injection site[66] but without any complications.

Efficacy

A major challenge in the development of human immunocontraceptives is the variability in immune responses by individuals, similar to that which is evident in so many prophylactic vaccines, such as influenza. It is unlikely that humans will use contraceptives with efficacy as low as even 95%. There are individual differences within a species in reactions to contraceptive immunogens. For example, examining the anti-PZP antibody titers from several groups of horses,[40] wapiti[69], fallow deer (*Cervus dama*),[70] and white-tailed deer[11] treated with PZP reveals a significant difference in response to the same immunogen, the same dose, and the same adjuvant. However, for wildlife populations, a variety of modeling[71] and results from field trials[21] have demonstrated that contraceptive vaccines with less than 100% effectiveness will suffice.

Clearly there are species differences as well. For wild horses treated with native PZP, efficacy is approximately 95% over 23 years.[21] In the case of white-tailed deer, there is more variability,[11,22] with an efficacy of approximately 75%. Among PZP-treated wapiti, efficacy ranged from a low of 84% to a high of 90%.[23] African elephants in one population have responded with a 100% efficacy over 10 years of management.[20] Among captive exotic species, efficacies ranged from lows of 70% in Sambar deer (*Cervus uniciolor*) to highs of 100% in addax (*Addax nasomaculatus*), gerenuk (*Litrocranius walleri*) markhor (*Capra falconeri*) bighorn sheep (*Ovis canadensis*), mountain goats (*Oreamnos americanus*), and several other ungulates.[72] Thus, a major challenge to the development of wildlife immunocontraceptives is the ability to inhibit fertility across diverse species. GonaCon-B® and SpayVac® have both been associated with efficacies in the ranges of 39–64% and 27–48%, respectively, in wild horses[73] while SpayVac® was associated with 100% efficacy in fallow deer over 3 years.[74] The GnRH vaccine Improvac®, when given to domestic mares in two inoculations over 35 days provided 100% efficacy with cycling and ovulation as the endpoints.[48]

This latter issue reflects both a strength and weakness of PZP immunoconception, and for that matter

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any immunocontraceptive targeting some reproductive tissue (ovum, sperm or pituitary cell) or protein hormone (GnRH). Unlike reproductive steroids, which have absolute homology across species, protein hormones reveal amino acid differences across species. In the case of PZP, there must be an immune response to an epitope of either or both the PZP-1 and the PZP-3 alpha glycoprotein for a successful antibody response and effective contraception. In the case of white-tailed deer, anti-porcine ZP antibodies recognized several epitopes of both the ZP-1 and ZP-3 glycoproteins[75,76] but in domestic cats, the same antibodies do not recognize any segment of the zona glycoproteins.[77,78] While a species-by-species analysis of cross-reactivity with PZP-induced antibodies has not occurred, it is clear that species-specific epitopes vary across taxonomic lines and will affect the contraceptive efficacy of the immunogen. Thus far, PZP-induced contraception has not failed in any of 64 members of Ungulata in which it has been attempted, whether members of Perissodactyla or Artiodactyla,[42] or bears[72,79] or pinnipeds.[72,80] This implies some evolutionary relationships among the epitopes of the zona sperm receptor. Although not directly related to contraceptive research, the application of zona proteins from, and directed toward, a variety of species across broad taxonomic lines will surely reveal interesting evolutionary pathways in the development of the sperm receptor.

Duration of contraceptive action

Vaccines generally, but not universally, employ killed or attenuated virulent organisms and the immune response by humans and domestic animals is very powerful, with lasting effects even after a single inoculation. In contrast, contraceptive vaccines generally target reproductive hormones or tissue receptors, and the ability to cause long-lasting elevations in antibodies, individual differences notwithstanding, is far more challenging, because of the relative homology of these protein molecules between species. It is intuitive that a single inoculation, long-lasting contraceptive vaccine is valuable and a worthy goal for free-ranging wildlife.

The current major shortcoming of the most widely used wildlife contraceptive, PZP, is that contraceptive duration is short-lived, at least during the first few years of treatment. An initial inoculation followed by a booster inoculation 2–6 weeks later generally provides a year of contraception. But then, an annual booster is required for a second year of contraception. At least for the first 2–3 years, this is a requirement for successful contraception. Following 3 years of treatment, the duration of high antibody titers is sufficient for successful contraception over several years and eliminates the need for annual booster inoculations. For example, in the wild mare, 3 years of treatment, from age 2 through 4, result in contraception that lasts for a mean of 3.7 years. However, the range is 1–8 years.[33]

This shortcoming of native PZP has stimulated a search for more lasting contraceptive vaccines. Several approaches to this challenge have occurred. The first approach has attempted to incorporate the PZP glycoprotein in biodegradable, non-toxic matrices, which can be injected and which then release the PZP molecule slowly in a sustained release or in pulsed releases. The matrix used thus far has incorporated various ratios of lactide-glycolide, either in the form of microcapsules[81] or in the form of small pellets that can be injected either by hand or by dart.[82–84] Results thus far have been encouraging, but not definitive, and a great deal of variability in both the manufacturing process and the results has resulted. Another difficulty with this approach is that it has not been possible to incorporate powerful oil-based adjuvants in the microcapsules and pellets.

A second approach has incorporated a novel 'packaging' of the PZP. In the case of a proprietary product, SpayVac®, native PZP is incorporated into a multilamellar liposome. This platform appears to provide a much longer duration of high antibody titers and contraception in seals,[80] fallow deer,[74] white-tailed deer[85,86] and horses.[71] Contraceptive duration appears to last for at least 3 years following a single treatment. The primary drawbacks to this approach appear to be the high viscosity of the preparation and the inability to deliver it remotely, and possible side effects on reproductive tissues,[50] at least in horses. The former is a technical problem and may be overcome, and the latter requires substantially more safety testing before this approach becomes practical. Nevertheless, an important point here is that native PZP vaccine must not be confused with more complex vaccines containing PZP.

A third approach that has shown promise is the GnRH vaccine GonaCon®. This vaccine targets the GnRH molecule and blocks ovulation and estrus by preventing endogenous GnRH from stimulating FSH and LH producing cells of the pituitary. In turn, the lack of gonadotrophs inhibits ovarian and testicular

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function and gamete production. As with PZP, there are structural differences in the GnRH of various species, with varying degrees of homology. This means that the vaccine will work in some species but not as well or at all in others. GonaCon® was developed primarily for application to white-tailed deer,[12,87–90] but also works in wapiti,[91] swine,[92] and horses.[50] In the case of GonaCon® the longer lasting duration of antibodies has been accomplished by the addition of aromatic amino acids to the structure, which enhances immunogenicity but without interfering with binding to pituitary receptors. Puzzling, however, trials with free-ranging deer resulted in significantly lower efficacy than those with captive animals.

Alternatives to longer acting vaccines have examined delivery systems. As early as 1993, the idea was put forward to engineer the genes for immunogenic proteins into bacterial or viral vectors.[93–95] Known collectively as genetically modified organisms (GMO), the theory was that a gene for an immunogenic molecule that causes fertility inhibition, such as PZP, or GnRH, might be engineered in a non-pathogenic virus, which could then be used to infect the target species and express the gene. There are multiple questions associated with this technology. Can the vector persist for a long enough time to express the immunogen? Could the vector express the immunogen in sufficiently large quantities to elicit adequate antibodies? Will the vector reach a sufficiently large portion of the population to alter density?[96] Research followed that examined a number of possibilities. Cloned genes for PH-20, LDH-C4, and ZP3, all of which encode for gamete proteins were proposed for incorporation into GMO vectors,[97,98] but none ever reached the field test stage. Significant issues of regulation were raised for a contraceptive method that, once released into the environment, could never be called back.[99] The issue of mutation rates in viruses, and the potential for the vector to infect non-target species, was also an issue. For example, a vector that could successfully infect and cause contraception in non-native foxes in Australia would probably also bring about the same result with domestic dogs. One study concluded that the effort to assess risks of contraceptive GMOs would be greater than the effort to develop the technology.[99] These and many other concerns have led to a cessation of this line of research for wildlife immunocontraceptives and have been summarized elsewhere.[100–102]

Table I Comparison of Zona Protein and Anti-GnRH Contraceptive Vaccines

Parameter	ZP	Anti-GnRH
Efficacy	80–100%[a]	39–100%[a]
Male	–	+
Female	+	+
Reversibility	+[b]	+[c]
Remote delivery	+	+
Safety in pregnant animals	+	Species Dependent[d]
Passage through food chain	–	–
Target tissue specificity	+	–
Debilitating side effects	–	??[e]
Behavioral effects	Minor	Major
Available as a synthetic	–	+

[a]Values are for horses and deer; beyond those, efficacy is variable depending on the species.
[b]Reversible through five consecutive years of treatment; not reversible after 7 years of consecutive treatment.
[c]Long-term data not available.
[d]Species in which the corpus luteum of pregnancy is supported by pituitary LH will abort if treated with an anti-GnRh agent.
[e]Long-term data not available.
GnRH, gonadotropin-releasing hormone; LH, Luteinizing hormone.

One exception to this trend is a research effort in New Zealand.[103,104] Here, the vector is a nematode parasite *Parastrongyloides trichosuri* and the target species is the invasive brushtail possum (*Trichosurus vulpecula*). The parasite is species-specific and mutation into some form that might infect other species is unlikely. If a gene for a contraceptive protein can be engineered into this parasite, and can be expressed effectively, it may open the door to similar approaches in other species. Of course, there is no guarantee that this particular parasite could not find its way to Australia and infect native possums.

One final approach to the problem of long-term immunocontraception of wildlife remains. Oral delivery of a contraceptive vaccine would overcome the need for laborious delivery systems currently necessary for sustained antibody titers in target animals.[105] Currently, this approach is not a practical reality, but the challenges have been clearly delineated. First, either the contraceptive vaccine itself has to be species-specific or the oral delivery system must be species-specific. Approaches under consideration include transgenic plants[102] and bacterial ghosts,[106] both carrying genetic information for a contraceptive immunogen. The research challenges

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are immense and the regulatory hurdles daunting, and it is unlikely that this approach will bear fruit for many years to come.

Conclusions

Immunocontraception for wildlife management has become a reality since its first application in 1988. Today, animals treated annually number in the thousands, and controlled alterations in population size are occurring in various settings at ever-increasing frequency. The most widely used wildlife contraceptive is native porcine zona protein, largely because of its ability to inhibit fertility in such a large breadth of species, its tissue-specific action, and because of its impressive safety record. Currently six different free-ranging wildlife species are being managed by means of PZP immunocontraception on 52 sites and 76 captive exotic species are being treated in 67 zoological gardens around the world. Several GnRH vaccines show promise, but widespread application will have to wait for more extensive safety testing. Table I summarizes and compares the major characteristics of PZP and anti-GnRH vaccines. The extraordinary success with actual field applications of wildlife immunocontraception over the past two decades has created a new paradigm. Only 23 years ago, the primary question was whether or not we could economically deliver, safely treat, and effectively regulate wildlife populations with vaccine-based contraception. Today, the new question is how we can achieve this goal easier, with less effort and cost and gain public approval at all levels. The remaining challenges now center on improving delivery systems, modifications of the vaccine components to enhance the duration of effectiveness, assuring comprehensive safety, developing a sustainable funding base for research, and achieving general acceptance.

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Immunocontraceptive Reproductive Control Utilizing Porcine Zona Pellucida (PZP) in Federal Wild Horse Populations

(Third Edition)

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Contributors

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Jay F. Kirkpatrick, Ph.D., Allen T. Rutberg, Ph.D., and Linda Coates-Markle

Compiler and Editor

~

Patricia M. Fazio, Ph.D.

June 1, 2010

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MEMORANDUM OF UNDERSTANDING
between the Humane Society of the United States and the Bureau of Land Management

After a workshop held in Santa Fe, NM, November 29 and 30, 2005 on wild horse fertility control, the Humane Society of the United States and the Bureau of Land Management have agreed to develop a Memorandum of Understanding to co-operate on: -

1) The further development and wider use of contraception in wild horse populations,
2) Resolve some of the uncertainties being faced in producing the vaccine and ensuring a continuing supply of a safe and effective vaccine,
3) Assist in public outreach on the issues, and
4) Maintain healthy and viable herds in the existing BLM wild horse Herd Management Areas.

"The BLM sees this as a way to reduce horse removals, to place fewer horses in short- and long-term holding facilities, and to achieve budgetary savings," said Don Glenn, Acting Group Manager of the BLM Wild Horse and Burro Program, Washington, DC.

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Source: HSUS-BLM Press Release on Fertility Control, e-mail from Tom Gorey WO/BLM/DOI, sent 11/30/2005 01:17 PM. Forwarded by Linda Coates-Markle /MTSO/MT/BLM/DOI on 12/05/2005 10:55 AM.
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Introduction

Two fundamental questions exist…

1) Does PZP harm wild horses?
2) Will its use eliminate entire herds?

The quick answers are that the Food and Drug Administration (FDA), The Humane Society of the United States (HSUS), and animal care committees all carefully review protocols for PZP use, and more than 20 years of data, carried out under these set of rules, clearly show that wild horses are neither injured by this drug, nor do aberrational behaviors occur as a consequence of its application. Too, oversight by The Humane Society of the United States assures that the vaccine is used only to slow reproduction and may not be used for the extermination of entire herds. PZP is designed to bring about short-term infertility and is reversible, if not used beyond five consecutive years. It reduces the need for gathers and preserves the original gene pool in each herd.

Expanding on these central points, the contributors and editor of material presented within this document have aspired to answer, with scientific objectivity, common questions and concerns raised by actual individuals and groups about porcine zona pellucida (PZP) and to provide citations and references that may be accessed through interlibrary loan, or other sources, for further study. Updates and additional questions and answers (Q&A's) will be provided periodically, as research progresses or protocols change.

PZP use in wild horse herds has been studied extensively for more than two decades, with papers published in peer-reviewed scientific journals by experienced reproductive physiologists, equine scientists, wildlife biologists, geneticists, and animal behaviorists, providing a portrayal of safety, high efficacy, and absence of long-term behavioral, physical, or physiological effects from the vaccine. Those involved in the creation of this Q&A have endeavored to produce a factual document of scientific merit, supported by field data, with statistically adequate sample sizes. Data was collected by trained, unbiased individuals, who adhere to established research methodology within his or her respective field.

Questions & Answers

THE PZP VACCINE

Q. What is porcine zona pellucida (PZP), and how does it work to block pregnancy?

A. A non-cellular membrane known as the zona pellucida (ZP) surrounds all mammalian eggs. The ZP consists of several glycoproteins (proteins with some carbohydrate attached), one of which, ZP3, is thought to be the primary component of the sperm receptor (the molecule that permits attachment of the sperm to the egg during the process of fertilization). The PZP vaccine is derived from pig eggs. When this vaccine is injected into the muscle of the target female animal, it stimulates her immune system to produce antibodies against the vaccine. These antibodies also attach to the sperm receptors on the ZP of her own eggs and distort their shape, thereby blocking fertilization. [see Paterson and Aitkin 1990; Miller et al. 2001]

Thus far, PZP has been a promising form of contraception in wild horses and other wildlife for the following reasons:

1. Pregnancy is prevented approximately 9o% of the time in treated animals;
2. The vaccine can be delivered remotely by small darts;
3. Contraceptive effects are reversible (up to five years in wild horses);
4. PZP is effective across many species;
5. No debilitating health side effects have been observed, even after long-term use;
6. No effects on social behaviors have been observed;
7. The vaccine cannot pass through the food chain;
8. It is safe to administer the vaccine to pregnant animals.

Q. How is it made, and who manufactures it?

A. The porcine zona pellucida (PZP) vaccine, used on BLM, U.S. Forest Service, and NPS wild horse mares, as well as within several wild horse sanctuaries, is produced by The Science and Conservation Center (SCC) in Billings, Montana. Each batch is subjected to a qualitative and quantitative quality-control program and shipped under the authorization of an Investigational New Animal Drug (INAD) exemption for wild horses (FDA

8857-G0002) issued to The Humane Society of the United States (HSUS) by the Center for Veterinary Medicine of the Food and Drug Administration. In collaboration with other investigators, The SCC continues to conduct research with the contraceptive vaccine, focusing on the ability to produce larger quantities, and increasing the efficacy of long-term contraception through a single inoculation (see Turner et al. 2002, 2008).

Q. How is the PZP vaccine obtained?

A. Once all necessary authorizations and approvals have been obtained for use of the vaccine, it may be ordered from:

Kimberly M. Frank
The Science and Conservation Center (SCC)
ZooMontana
2100 S. Shiloh Road
Billings, MT 59106
(406) 652-9719 (phone)
(406) 652-9281 (fax)
e-mail: sccpzp@hotmail.com

The vaccine is not commercially available and is provided at 60% of the cost of production, which currently runs about $24/dose. This is the price of the standard, one-year, 100 microgram dose. The 2-3 year vaccine uses considerably more than 100 micrograms, as well as more adjuvant, and includes the added cost of pelleting. The 2-3 year PZP vaccine costs about $200 per dose, plus the personnel costs of administration, which are minor, if horses are being gathered anyway. Compared with the $1,100 - $1,600 it takes to gather, remove, transport, hold, and adopt a horse (or care for it indefinitely), PZP is a bargain.

Q. Are any pigs killed, expressly to produce the vaccine?

A. Pig ovaries are obtained from a slaughterhouse in Iowa, as a by-product of hogs already destined for slaughter. Therefore, no fewer hogs will be killed if the PZP vaccine were no longer made. Major competitors for pig ovaries include Chinese restaurants, and pharmaceutical companies, that use ovarian endocrine components for research and production of products.

_Q_. Is this drug FDA approved and patented? If so, who is making all the profit from its use?

A. In FDA language, “approval” refers to approval for commercial distribution and marketing, and PZP is not a commercial product. No one is profiteering from PZP. The Humane Society of the United States holds the Investigational New Animal Drug exemptions (INAD), which are the oversight process by which FDA compiles data to examine vaccine safety and effectiveness. Basic and applied research that generated most of the knowledge about the vaccine was carried out with public funds (from the National Science Foundation, National Institutes of Health, U.S. Department of Agriculture, Bureau of Land Management, etc.). The research team considers products developed with public monies to be in the domain of the public, and therefore has no intention of commercialization. Therefore, it will always be called "experimental,” under FDA rules, despite the fact that PZP has been studied and field-tested extensively, for safety and efficacy, and is currently being used with more frequency on federal wild horse mares.

However, at the same time, use of public monies for research and development ***does not legally prohibit*** the commercialization of a product. Some researchers are, nonetheless, offended (due to a personal sense of ethics) and will not move forward with commercialization of a product developed with public funds. The rational is that the public has already paid for the product, and commercialization only allows private companies and individuals to profit from sale without having contributed to the process of research, development, and testing.

Because of this, PZP cannot be used on wildlife without the Investigational New Animal Drug exemption, or INAD. Once the INAD from FDA (one for horses, one for deer and zoo animals) was obtained, the INAD was turned over to The HSUS, leaving that organization to deal with the ethical issues. This means that each project – even at the management level – must have a research question attached to it, and The HSUS must approve the project. An added note is that the Investigational New Animal Drug exemption (INAD) issued to HSUS by the FDA requires a sound safety base before it is issued and would never have been issued were there a significant (or even an insignificant) health or safety concern. Therefore, neither HSUS nor The SCC make money from the vaccine. The SCC provides PZP vaccine at cost of production. Coupled with the paperwork required, The SCC actually

loses money. That is why The SCC is a non-profit. Currently, The SCC's annual budget is about $120,000, and PZP income results in less than half of that, meaning that a great deal of vaccine is donated.

The patent issue is a different question, not to be confused with the FDA process. Merck patented PZP in the 1970s, but the patent lapsed, and the technology is no longer patentable. Organon International, a large drug company based in the Netherlands, holds the patent for PZP use in humans, but that application may never take place, as scientists have not yet been able to make an effective synthetic form. Also, the variability in time for infertility reversal is significant and could potentially result in litigation.

In recent years, the FDA has transferred authority for wildlife contraceptives (exclusive of captive zoo animals) over to the Environmental Protection Agency (EPA). In 2009, an application is pending for approval of native PZP for use in wild horses by the EPA.

Q. What groups are on the PZP Contraceptive Research Team?

A. Today, the team consists of The Science and Conservation Center, Billings; Toledo University Medical College, Ohio; University of California-Davis; Tufts University, Medford, Massachusetts; The Humane Society of the United States, Gaithersburg, Maryland and Washington, DC; and the University of Iowa, Ames. Many other individuals contribute to the effort in one form or another. Governmental agencies that can be considered team members include the National Park Service, U.S. Forest Service, U. S. Department of Commerce, and the Bureau of Land Management.

The entire PZP contraceptive effort involves many people, several institutions, and numerous funding agencies. This team works together, bringing many disparate disciplines and talents in concert to solve the problems at hand.

Q. Who Funds PZP Contraceptive Research and Applications?

A. Funding for application of the vaccine to wildlife has been provided by many individual communities, agencies, and organizations, including but not limited to:

- The Humane Society of the United States

- Elinor Patterson Baker Trust
- Geraldine R. Dodge Foundation
- Bernice Barbour Foundation
- Leuthold Family Foundation
- Panaphil Foundation
- Delta-Sonics
- PNC, Inc.
- U.S. Navy
- National Park Service
- Bureau of Land Management
- Rachel Carson National Estuarine Reserve
- U.S. Department of Commerce
- National Institutes of Health
- Fire Island Community Association
- Burkett-Plack Foundation
- Bosack-Kruger Foundation
- Annenberg Foundation
- Cargill Foundation
- THAW Charitable trust
- 112 different zoos in North America, Europe, New Zealand and Australia
- South African National Parks Board
- U.S. Fish and Wildlife Service – African Elephant Conservation Fund
- Fripp Island (SC) Property Owners Association

- Morris County (NJ) Parks Commission
- Franklin County/Columbus (OH) Metro Parks
- and several anonymous donors.

This list is not all-inclusive but provides a picture of the breadth of support for this approach to wildlife management.

Q. Who controls vaccine use in wild horse populations?

A. No agency or organization can use the vaccine without the assent of HSUS, which monitors management plans and the INAD. In fact, the BLM approached the FDA and tried to circumvent HSUS control over vaccine use but was turned down by the FDA. Therefore, oversight and approval by HSUS still exists. No agency or organization will have control of the PZP vaccine in the foreseeable future. Every μg of vaccine that is produced can only be used in projects where HSUS has reviewed and approved a wild horse herd management plan.

All projects in which the vaccine crosses state lines must be on record with the FDA. As explained previously, the authority to carry out these projects is issued by two separate Investigational New Animal Drug documents (INADs) issued by the FDA to HSUS. As each new project is identified, HSUS reviews the need for the project in the context of scientific, ethical, and moral issues, and, if approved, issues permission to proceed. Notification of each project is accomplished by means of a form, filed with the FDA by The Science and Conservation Center, which specifies how much vaccine is being shipped and what species are to be treated. The INAD also requires that data from each project be gathered in a systematic way and filed, and be made available to the FDA when the need arises. These files are maintained at The Science and Conservation Center. Additionally, the legal managers of the horses (NPS or BLM) or the animal care committee of each zoo must also provide permission to treat animals. This regulatory process is similar for any wildlife species not classified as a food animal by the FDA or as a game animal by a state fish and wildlife agency.

Precisely how procedures will change – or remain the same – after EPA assumes regulatory authority, remains to be seen, but the HSUS will hold the approval; thus, we see no major changes at this time (6/09).

Q. Does an agency have to do an environmental assessment (EA) or an environmental impact statement (EIS) prior to using PZP on a wild horse herd?

A. Yes. Environmental Assessments are mandatory for any use by federal agencies. One difference between agencies is that the NPS does a single management EA, that is in force for years, while the BLM does one every five years for application in each herd management area.

Q. What wild horse populations, within the United States, are presently being managed with PZP?

A. The vaccine has been used successfully to manage the wild horse population of Assateague Island National Seashore (ASIS), in Maryland, under the sponsorship and authority of the National Park Service (NPS). The population has been treated for 22 years, without health problems, and the population has decreased by almost 40%, since management-level application began in 1995. Wild horses are also being treated on Cape Lookout National Seashore (Shackleford Banks), North Carolina, for the NPS; on Carrot Island, North Carolina; on the Rachel Carson National Estuarine Reserve, North Carolina; and on many areas of Nevada, for the Bureau of Land Management (BLM). Other treated herds include Return To Freedom (American Wild Horse Sanctuary), Lompac, California; Pryor Mountain Wild Horse Range (Montana/Wyoming); Little Book Cliffs National Wild Horse Range, Colorado; McCullough Peaks Horse Management Area, Wyoming; and Little Cumberland Island, Georgia; International Society for the Protection of Mustangs and Burros Wild Horse Sanctuary, Lantry, SD; Black Hills Wild Horse Sanctuary, Hot Springs, SD, Carson National Forest, NM, and the Navaho and Pima/Maricopa Indian reservations. In Nevada and Wyoming, at least 12 different wild horse herds are being treated "experimentally" to evaluate population effects. For Nevada references, see (1) Turner et al. 2001; (2) Turner et al. 1997; (3) Kirkpatrick et al. 1997; and (4) Kirkpatrick et al. 1997. In March, 2009, The Washington Office of the BLM Wild Horse Program i/ssued a memorandum that directs local horse managers to treat all mares returned to the range.

In the case of the four barrier island herds, the Pryor Mountain Wild Horse Range, Little Book Cliffs Wild Horse Range, and Return To Freedom Wild Horse Sanctuary, horses are treated remotely, with dart guns. In Nevada, they are treated in conjunction with gathers, as most of these HMA's are too large, and the horses too wild to dart.

In addition to controlling the horse population on Assateague Island, treatment has extended the lives and improved the health condition of older mares, by removing the stresses of pregnancy and lactation [see Kirkpatrick 1995; Kirkpatrick and Turner 2002, 2003; Kirkpatrick et al. 1990, 1991, 1992, 1995a, 1996a,b, 1997; Liu et al. 1989; Turner and Kirkpatrick 2002, 2008; Turner et al. 1996a]. Horses on Assateague are doing well. About 125 total animals roamed the area in 2009 (27% less than the starting number of 173 in 1995), and their body conditions have improved significantly since 1990 [Turner and Kirkpatrick 2002]. Mortality almost disappeared for the first eight years of management, and the horses are generating new age classes (large numbers between 20-25 years of age, and a growing population between 25-30).

Thus, at the management level, horses are being treated with PZP for the NPS, Rachel Carson National Estuarine Reserve, the BLM, and at least five private sanctuaries. In addition, new forms of the vaccine are being tested for the BLM in western horses, but not on a management level.

The following HMAs are sites that have been treated with long-acting PZP:

Onaqui Mountain, UT - 56 mares
Sand Springs, OR - 31 mares
Fox-Hog, NV - 28 mares
Green Mountain, WY - 38 mares
Monte Cristo, NV - 53 mares
Blue Wing, NV - 136 mares
Antelope Hills, WY - 28 mares
Black Rock East, NV - 19 mares
Black Rock West, NV - 19 mares
Warm Springs, NV - 27 mares
Antelope Complex, NV - 29 mares
Calico, NV - 92 mares
Groshuite, NV - 44 mares

Granite Range, NV - 79 mares
Nellis Air Force Base Bombing Range, NV (Nevada Wild Horse Range) - 358 mares
McCullough Peaks, WY - 34 mares
Sand Springs, CO - 75 mares
Cedar Mountain, NV - 85 mares

Additionally, another form of the long-acting PZP has been tested in captive mares at Canon City, CO; a reduced dose of PZP was tested successfully in captive mares at Canon City, CO; and a lyophilized form of the vaccine was tested successfully in domestic mares in Clark, WY and Billings, MT.

Q. What herds do you propose to treat with contraceptives in the near future? Why did you choose these particular herds? Who decides? What are your long-term goals?

A. While the PZP vaccine is currently being used on at least 20 horse management areas for the National Park Service or the Bureau of Land Management, its use is appropriate for all free-ranging wild horse herds. Application to particular herds is at the invitation of the managing agency. The long-term goal is to reduce or eliminate the need for gathers and removals.

DELIVERY, APPLICATION, AND BIOLOGICAL EFFECTS OF PZP

Q. How is the vaccine delivered?

A. The PZP vaccine must be injected into the muscle of the target animal. This can be done by hand if the animal is restrained, or by dart, for remote delivery. There are many commercial dart systems available, but the thick viscosity of the vaccine requires a large needle and a quick injection. Thus far, Pneu-Dart® systems (Williamsport, Pennsylvania) seem to work the best. The Pneu-Dart® 1.0 cc barbless darts can be fired from Pneu-Dart® capture guns or from several other commercial dart guns [Pax-Arms® or Dan-Inject®, for instance]. The darts are disposable, and after hitting the animal in the rump or hip (the only acceptable location for darting), they inject by means of a small powder charge, and then pop out. Because of

their bright colors, the darts are usually retrieved in the field. Darts that have not been discharged cannot be discharged by stepping on them or by any other kind of casual contact. Over a six-year period on Fire Island National Seashore, and with more than 1,000 dartings of deer, only two darts have not been recovered.

Normally, each animal is darted twice the first year, with the first injection being given up to a year before a booster, just preceding the breeding season (March for wild horses or September for deer). Thereafter, a single annual booster inoculation will maintain contraception. The second inoculation of the first year requires that…

a. you are able to recognize the individual animals; or

b. you do the first inoculation with a special "marker dart," which leaves a dye mark on the animal at the same time it injects the vaccine; or

c. selected mares are treated to allow for both genetic diversity within a specific herd and for the promotion of health and improved body condition of an individual animal (through temporary infertility).

An alternative strategy is to give only a single inoculation the first year, from which there will be little contraception, and then a single annual inoculation thereafter, from which there will be significant contraception (see McShea et al. 1997; Turner and Kirkpatrick 2002.)

New approaches using small non-toxic, biodegradable lactide-glycolide pellets, that result in several years of contraception after a single application, are being tested. [Turner et al. 2002, 2008].

Q. How would you describe the new pelleted form of PZP-22, and how does it work?

A. PZP is incorporated into lactide-glycolide pellets, small enough to be injected intramuscularly. After injection, the lactide-glycolide erodes (It is biodegradable and non-toxic) and releases the PZP at varying rates, depending on the ratio of lactide to glycolide. It is an injectable version of a contact cold pill. The pellets are made at the School of Pharmacy, University of Iowa. There have been spotty results in horses. It works in some herds and not in others, and it has worked well in deer, but not in elephants. Not all, but much of the data associated with the pellets has

come from helicopter counts, and it is difficult to match foals with mares from a helicopter. After the summer of 2010, more data will be available. Two herds in the Annenberg Foundation project have been treated with pellets. People are on the ground, who are familiar with the horses; so more solid data should be forthcoming.

***Q*. How does the liquid vaccine compare to the pelleted form?**

A. Nothing works as well as a primer dose of native PZP, followed by a booster 2 to 6 weeks later**,** and then annual boosters after that. Efficacy with the pelleted form, even where it has appeared to work, is not as good. The issue, of course, is having to treat mares every few years versus only once.

***Q*. Isn't darting mares painful and potentially harmful or even lethal? Will it result in mares being shot in critical anatomical areas – abdomen or chest, causing inhumane deaths?**

A. As long as only 1.0 cc Pneu-Darts are used, there is almost no risk of injury to the animal. These are very small, light darts. Over a 22-year period, no horse has ever been injured on Assateague Island, the Shackleford Banks, Carrot Island, the Pryor Mountains, or the Little Book Cliffs (translating to well over 1,000 dartings, over the course of 20 years).

On the Pryors, PZP remote-darting operations typically take place in late summer/early fall, and any wild mares receiving the vaccine are individually-identified and tracked regularly with data, non-intrusively gathered on behavior, estrus, fertility, reproduction, survival, and any health concerns. The field studies are conducted by seasonal and term USGS-Biological Resources Division and BLM biological technicians under the supervision of BRD research biologists and the BLM Wild Horse and Burro Specialist.

Several studies of injection site reactions have been carried out. In one study it was shown that of 38 total treated mares, 55% exhibit no reaction to darting, 6% have some level of swelling around the injection site, 22% have a small nodule (granuloma) about the size of a marble, and no animals had abscesses. Only one mare of 38 has ended up with a medium-level swelling, about one year after treatment. In a larger study, data were generated from the records of 329 different horses on Assateague Island, darted over a 19-year period. 1,094 different dartings resulted in a 0.2% abscess rate. The

abscesses healed within two weeks. In another study of captive zoo animals, 1,185 dartings of 25 different species resulted in 16 abscesses (1.3%) (Lyda et al. 2005). In the Pryor Mountains, 151 dartings resulted in 1 abscess (0.7%). At Return To Freedom Wild Horse Sanctuary, in California, 451 dartings of 85 total horses over six years resulted in 15 abscesses (3.0%). At the Black Hills Wild Horse Sanctuary, in 2009, 140 dartings resulted in one abscess (0.7%).

Abscesses typically appear as 25 mm swellings which open and drain within two weeks without complications. More common are granulomas, or subcutaneous nodules, about the size of marbles. These nodules are most notable post-injection but typically disappear over time. Ultimately these nodules are very difficult to discern amongst other natural scars within the coats of these wild mares. Furthermore, there is no indication that the presence of these nodules has compromised the quality of life for these horses. Field technicians have never recorded the mares showing any indication that these nodules are causing any level of discomfort during daily activities and/or interfering with reproductive activities. The granuloma is the counterpart of the smallpox vaccination scar found on humans, but beneath the skin instead of on its surface.

These injection site reactions should be given some context and compared with the same events in domestic livestock. The 1995 Beef Council Quality Audit reported that 11% of cattle inoculated with USDA-approved vaccines produced injection site reactions, including lesions and abscesses. In another study, the Kentucky Beef Council reported that fed cattle had abscess/lesion rates of 3.2-21.6% after vaccinations with USDA-approved vaccines, and in non-fed cattle, the abscess/lesion rate ran from 28.9 to 40.9%.

The most recent study of injection site reactions (Roelle and Ranson 2009) provides some interesting data. In any herd studies, often there are a few horses where abscesses appear more frequently than in other animals. Thus, for some reason, some animals are more prone to abscesses than others, and this often skews the abscess rate within a single herd. For example, in one herd, of a total of six abscesses that appeared after darting, four occurred in the same mare. The other data suggest that vaccinations given by dart produced more abscesses than those given by hand injection. This makes sense, if you consider that hand injections are preceeded by antiseptic swabbing of the skin surface, while field darting simply drives dirt and hair

follicles in to the wound, along with the vaccine. Thus, this all suggests that abscesses may not have any relationshop to the vaccine or adjuvant, but to the delivery method instead.

Q. How do immune system depression issues affect the formation of abscesses and lameness in horses?

We have insufficient data regarding how mares will react if they are in poor condition versus good condition, at least not enough for statistical analysis. Then, we just have visual indexes for body condition scores, from which you can simply infer immune competency; thus, we can offer only anecdotal information. Classic immunology says that animals in poor condition (or stressed for any other reason, as well) will not mount as strong an immune response as unstressed animals. Whether or not this effect will cause poorer efficacy, more abscesses, or other issues, is not known. In zoo animals, where we often administer PZP because of health problems, there seems to be no problem. Next, lameness and abscesses may not be at all associated with the vaccine *per se*. Rather, there is some limited data, by the Science and Conservation Center, and by Jason Ransom, U.S. Geological Survey, Ft. Collins, Colorado, that darted horses will produce more abscesses than hand-injected horses. When you hand inject, you wash the surface of the skin with ETOH. However, when you dart, you drive any surface dirt into the dart puncture. The same problem exists when darting wildlife with anesthetics, rather than vaccine. This issue is not any different from differing efficacies among individuals getting flu shots. In general, about all you can say is that animals with compromised immune systems do not do as well as those with competent systems, and there are many variables associated with what causes immune incompetency.

Q. Will PZP harm mares or foals, physiologically? Have any negative pharmacological side effects been observed? Are any benefits derived from its use?

A. Safety data has been accumulated over 20 years. It essentially says there are no short- or long-term health problems of any kind, and that the vaccine is reversible, unless the mare is treated for more than five consecutive years (in which case you probably didn't want her to reproduce again anyway). The data make clear that pregnancies in progress are not affected in any way by the vaccine, nor is the health or fertility of the foals compromised, once

they are born. Treating mares carrying female fetuses does not affect the fertility of the offspring.

In fact, as mentioned previously, mares on Assateague Island are living longer than ever, and their mortality has decreased, they are achieving new age classes never before seen on the island, and all of this happened because their body condition scores have increased steadily since 1989, when PZP application started. Historically, a mare never survived to 20 years of age, but now a significant percentage have passed twenty, and about 29 animals (20% of the herd) are between 25 and 30 years old. Removal of the stresses of pregnancy and lactation gives them an immense health advantage, both at the younger and older stages of life.

Foal mortality has dropped significantly. This is probably because their mothers, when they finally do become pregnant, after several years of contraception and then withdrawal of PZP treatments, are much healthier. All of this data (derived not from casual observation) is published.

The other victory for horses is that every mare prevented from being removed, by virtue of contraception, is a mare that will only be delaying her reproduction rather than being eliminated permanently from the range. This preserves herd genetics, while gathers and adoption do not. (Kirkpatrick and Turner 2002, 2008; Turner and Kirkpatrick 2002, 2003; Willis et al. 1994.)

Q. Does PZP application create late foaling in treated populations?

A. No. Available data from 20 years of application to wild horses contradicts this claim (Kirkpatrick and Turner 2003). From 1990 to present, Assateague Island has records for 178 horses whose month of birth is known (and in some cases, day of birth known). An examination of the published data, from 1984, of Ron Keiper (retired Distinguished Professor of Biology at Pennsylvania State University and currently Chairman of the Department of Biology at Valencia Community College, Orlando, Florida) in which he looked at eight years of birth dates for the same herd, which at that time was much smaller than we have today (considerably less than 100 horses versus 155) indicates that approximately 85% of the foals were born in April, May and June. Among the 178 horses with known birth dates, 95 were born to mothers who were never treated with PZP, with 70 born in April, May and June (73.6%), and 25 born outside this window. Another 83 foals were born to mares that had at some point been treated with PZP

before their pregnancies, and 65 were born in April, May or June (78.3%), with 18 outside this window. Thus, with a database of 178 horses over an eleven-year period, there is no evidence of late foals being born among treated mothers.

That corroborates published work (Kirkpatrick and Turner 1983), where it was demonstrated that Pryor Mountain wild horses did not extend their season of ovulation even when placed on high planes of nutrition. Mares do not extend their breeding season if they do not get pregnant.

One interesting issue is that the percent of untreated mares giving birth on Assateague Island in the April, May and June window has decreased from 85% down to 74% since 1984. This suggests that as herd size increases, variability in birth dates also increases, but this may simply be a function of larger numbers (in this case a 100% increase in herd size). There is also a moderate pattern among some mares (the N9BF line in particular) with regard to producing foals outside this window. This genetic line was consistently producing foals in March. If that observation is correct (This is only an untested observation.), then it corroborates Eric Palmer's theory that seasonal ovulatory patterns in mares are genetically controlled. In any case, these data, at least, demonstrate that contraception with PZP does not cause early or late births. Once again the Assateague Island (ASIS) horses and the 22-year treatment history produced a wealth of information (Kirkpatrick and Turner 2003).

In the Pryor Mountain Wild Horse Range, the normal foaling period has been well documented (EA #BLM MT010 FY05 -16, figure 10) to primarily take place in May and June, with limited foaling known to happen outside this window, from February to September. Thus, later foaling dates are not considered abnormal.

Q. For how many years is a mare generally treated with PZP?

A: This depends on the management plan of the agency, for a particular herd. Perhaps the most effective plan is the one used on Assateague Island, where all two-year-old mares are put on treatment, and then boosted at three and four years of age. After this, they are removed from treatment until they foal, which might occur anywhere from one to five years later. Mares that have already made their genetic contribution to the herd, in the context of the management plan, are treated until extinction.

***Q*. How do you determine which mares within a herd will be treated?**

A. The question of which mares to treat with vaccine is an important one, and the answer is embodied in the management plan. The approach used on Assateague Island has proven very effective and safe, i.e., two, three and four year olds are all treated and then withdrawn from treatment until they produce a foal. Older mares with good genetic representation in the herd are treated to extinction. Dr. E. Gus Cothran, an equine geneticist from Texas A&M University, College Station, monitors the plan on Cape Lookout. Immunocontraceptive control is more logical (genetically) than removing animals before they have had a chance to reproduce. Genetic representation is the key element within the management plan.

The most important consideration is to ensure that all genetics are represented, whether or not they reflect "wildness" or band stability, phenotype, or whatever social hierarchy exists. The bottom line is that wild horses are native North American wildlife, and humans should not be selecting for anything other than complete genetic representation and kinship.

***Q*. How effective is PZP? Won't some mares still become pregnant after treatment?**

A. PZP treatment in wild horses is about 95+% effective (Turner and Kirkpatrick 2002, 2008). The failure of some horses to respond to the vaccine results from an immune system that either doesn't "recognize" the vaccine's antigen, or from a compromised immune system. This is true for human vaccines as well (consider the less than 100% efficiacy with flu vaccines). Regardless, 95% efficacy is enough to manage wild horse populations effectively. In other species, efficacy varies in a species-specific manner (Frank et al. 2005).

***Q*. Why can't you block pregnancy with just one inoculation instead of the two shots you use now?**

A. The issue of the "one-shot" is complicated. Currently there are tests with SEVERAL forms of a one-shot vaccine. Despite that, this matter clouds the real issue of putting the vaccine to work NOW.

The initial “primer” dose of PZP causes the immune system to “recognize” and type the antigen, not so much for immediate long-term response, but to prepare the animal’s immune system for future exposures to the vaccine. Thus, a booster inoculation is required the first year, and an annual booster thereafter (Liu et al. 1989).

There is a second reason for the need for booster inoculations. Many human and veterinary vaccines use attenuated (weakened) or killed viruses as the vaccine, and these are powerful stimulators of the immune system. Often a single inoculation lasts for years. The PZP, however, is a relatively small protein that is not especially immunogenic. It is also very close in structure to the native PZP on the target animal’s own ova; thus, the target animal has difficulty in “recognizing” the PZP as foreign to the body. This, in turn, means that multiple inoculations must be given, and with a more immunogenic compound, known as an adjuvant (Lyda et al. 2005).

The subject of the one-shot also clouds the bigger topic of management because it only provides an advantage in the first year. After that, the horses are "one-shot" animals anyway. In 1994, almost every mare on Assateague Island was treated with a single shot. That shot was not meant to cause contraception but to set the herd up as a "one-shot" herd in preparation for management a year later. It was done this way because the National Park Service had to do an environmental assessment (EA) before they were allowed to manage with contraception. Then in 1995, the whole herd was managed with only a single shot per animal. The concept is fairly simple, and a "one-shot" vaccine only aids in the first year. Of course, a single inoculation that lasts multiple years would have more utility. (Turner et al. 2002, 2008)

The present advice given to the BLM is plain. Not a single mare that is gathered, for any purpose, and returned to the range should get back on the range without an inoculation. That makes them "one-shot" animals, and the expense of developing "one-shot" vaccines becomes moot. Once they have had that first shot, they can be re-treated anytime with a single shot.

A One-Inoculation Vaccine

Because of the need to inoculate animals twice the first year, and the difficulty of doing this with wild species, research is proceeding toward a "one-inoculation" vaccine. Such a vaccine would permit a single darting to

cause one or more years of contraception. The approach under study incorporates the PZP into a non-toxic, biodegradable material, which can be formed into small pellets. The pellets can be designed to release the vaccine at predetermined times after injection (at one and three months, currently), much the same way time-release cold pills work. Initial trials were encouraging, and continued trials are underway (see Eldridge et al., 1989; Turner et al., 2002, 2008). The downside to this approach is the immense amount of vaccine needed for the production of the pellets and the associated cost. While this approach may have some limited use, the difficulty in producing large amounts of vaccine make it unsustainable. What is needed is either a synthetic form of the PZP antigen or a pellet making process that reduces wastage by at least 65%.

Q. What do you mean by a one-year, two-year, or three-year vaccine?

Response to the PZP antigen is variable among individual mares. Some mares appear to be naturally poor responders to the vaccine and probably never develop sufficient antibody titer levels to confer infertility (hence the 95% efficacy of the vaccine in horses). Research with the Pryor Mountain wild horse herd indicates that immune response in mares may be correlated with age and fitness. One six-year-old mare, contracepted due to poor physical condition in September 2003, responded poorly to the vaccine, conceived in 2004, and foaled in 2005. Similarly, two 16-year-old mares last boostered in 2003, also produced a foal in 2005. Conversely, younger mares in good condition may have a stronger than expected antibody titer response resulting in a longer period of infertility. This appears to be the case with the first young mares treated with PZP on the Pryor Mountain Wild Horse Range (PMWHR).

Immunocontraception is not an exact science. The vaccines are designed to offer an effective time period based on the average response for many numbers of animals. As the treated number of animals increases, then the known response time for a specific formulation of PZP and adjuvant (carrying-agent) becomes better known. There are many variables to consider, the largest factors being animal condition and related immune response. Young PMWHR mares were healthy when injected (though a small sample size), and it would appear the resulting immune response (to a 90% effective one-year agent) has resulted in 2 years of efficacy. Older Pryor mares are relatively worn-out, and immune response appears limited. As such, the vaccine was not effective in a couple of mares. This is also true

with a younger mare that was treated, due her poor condition, but she still produced a foal.

Perhaps animal condition on Assateague Island (ASIS) is such that one year of efficacy is all that results with the same formulation of PZP and adjuvant that has been used on the Pryors. These are questions that still need to be dealt with, which is why the need exists for continued research. These questions are addressed in individual-based study herds within the Wild Horse & Burro Fertility Control Field Trial program and a project funded by the Annenberg Foundation. This is one of the reasons why the efforts on the Pryors have been critical to knowledge of this vaccine for use on western herds.

Q. How long does it take for native PZP to stop population growth in a herd?

A. This depends on the percentage of adult mares treated. On Assateague Island (ASIS), growth was stopped almost immediately. A large number of mares were given the PZP vaccine on ASIS during the first three years (about 87%), and growth stopped quickly. On the Pryor Mountain Wild Horse Range, fewer mares were treated (< 30%), but growth was slowed, as well. So, it depends mostly on the percentage of adult mares treated. However, fertility rates and mortality rates enter into this answer as well. It is precarious to address this any other way than to point out that results are site-specific and cannot be generalized.

Q. How long does it take for PZP to begin reducing a wild horse population?

A. On ASIS, the first sign of population reduction took about eight years, but herd reduction has been moving more quickly since then. The following eight years, herd numbers went down from 175 to 116, without any removal of horses, treating anywhere from 48 % to 79% annually. The lag time between initiation of treatment and decline in numbers is largely a function of increased longevity in treated horses, but, as pointed out above, there are other variable factors involved (fertility rates, mortality rates, sex ratios, and so forth). Fertility rates are average on ASIS (45-60%), and mortality is low (<5%).

***Q*. How can you tell if a wild mare is pregnant, so you don't treat her with PZP?**

A. In some cases, a fecal or urine sample is collected off the ground, or from yellow snow following urination. Reproductive steroid hormone metabolites are measured that tell us, with almost 100% accuracy, which mares are pregnant and which are not. A pregnancy can be diagnosed from 40 days post-conception until the day of parturition, and the animal does not have to be touched. That said, there is no danger to either the mother or the *in utero* foal, if the mare is treated with PZP during pregnancy (Kirkpatrick and Turner 2003, 2007).

***Q*. Is the drug residual in urine or feces or in the dead carcasses of treated mares, where PZP could get into the food chain or cause adverse effects to wildlife, or even contaminate water?**

A. Because PZP is primarily protein, it is readily destroyed in digestion, reduced to amino acids, and therefore cannot pass through the food chain intact and with biological activity (Oser 1965).

***Q*. What about compensatory reproduction in PZP-treated herds?**

Thus far, after 22 years of PZP treatment, there is no evidence for compensatory reproduction in a PZP-treated wild horse herd. This might be an issue if a herd is treated for long periods of time and then all treatment withdrawn, but that flies in the face of an effective management plan (Kirkpatrick and Turner 1991b).

BEHAVIORAL EFFECTS OF PZP

***Q*. Is wild horse behavior affected by PZP use? Are there any effects on motivation or drive, general contentedness, and the emotional stability of mares treated with PZP? Is band social stability affected negatively? Has any aberrational behavior been seen in PZP-treated mares, band stallions, or bands where mares have been treated with a contraceptive? What behavioral studies have been conducted on wild horses, both in eastern and western wild horse herds? Do you plan additional behavioral studies?**

A. After 22 years of treating the ASIS mares, there is still no evidence of altering behaviors. The baseline behaviors of eastern wild horses were the same as western horses. In order to understand this, a great deal of information must be read, which examines wild horse behavior from a variety of sites around the world. A huge body of literature exists on this subject, and a few of the more salient publications include: Berger 1977; Feist and McCullough 1976; Keiper 1976, 1986; Klingel 1975; McCort 1984; Rubnestein 1981; Rutberg 1990; Rutberg and Greenberg 1990; Salter and Hudson 1982. An independent investigator from the National Zoological Park has confirmed earlier results that show no behavioral changes (Powell 2000). Thirty-years of observing wild horses in North America and Australia and New Zealand have revealed no difference in fundamental behavioral structures. On the other hand, the affects of gathers on social behaviors are obvious (Ashley and Holcombe 2001; Hansen and Mosley 2000).

A good start on this subject can be obtained by reading Powell (1999), which reports on a study done by researchers from the National Zoological Park/Smithsonian. They found no behavioral effects, at that time, after almost eight (8) years of PZP treatment. The same results were reported in several of the Assateague Island papers, including (1) Kirkpatrick 1995, and (2) Kirkpatrick et al. 1995. Also, other studies (Fayrer-Hosken et al. 2000; Delsink et al. 2002) showed a lack of behavioral effects of this same vaccine on free-roaming African elephants, which have an even more complex social order than wild horses.

One critic of PZP claims, “The horses [on Assateague Island] seem more listless than western wild horses...” However, this could only be determined by time budget studies, and, in fact, no evidence for this has been found (referenced above). Casual observation of wild horses proves nothing. For example, casual observation has reported that Pryor horses travel less than ASIS horses, but that has no scientific significance. If one understands the biology of PZP, one would never suggest there is a "psychological" impact of the vaccine.

The research has already shown that band structures do not change and neither do hierarchies. The only major change in hierarchies that occurs is when mares get pregnant, and then they drop down the ladder even more. Most wild horse behavioral researchers still don't know how to measure hierarchy rank.

A more recent study (Nunez et al. 2009) on Cape Lookout National Seashore indicated that treated mares change bands more often outside of the breeding season. However, the control group for this study is a group of mares that become pregnant and have their foals removed from them. One could just as easily conclude that removing foals from untreated mares causes the mares to stay with the band for longer durations.

Q. What about behavioral changes, recently observed in mares, attributed to native PZP?

A. After 23 years of experience in the field, using native PZP, researchers observing wild horse mares feel that fundamental wild horse social behavior is not changed by the vaccine. However, other researchers are looking at issues such as "time budgets" to find any potential changes. It is the opinion of long-term PZP researchers, however, that these changes are neither of significance, nor are these studies controlled properly. For example, do time budgets differ for horses on good range as opposed to poor range? No one has looked at this, but if there are differences (and logic tells you there should be differences in time budgets between horses living in knee-deep alfalfa and those scratching out a living in a place like the Pryor Mountain Wild Horse Range), then we might expect the time budget of a chronically treated horse to change from untreated horses because of the better body condition scores of the former. These studies have not been controlled for mares with foals (usually untreated) and those without (usually treated), but the important point is that if the fundamental social structure and behavior do not change, it is of no consequence if time budgets change.

Q. Won't mares just keep coming back into estrus (heat) if they don't get pregnant? Won't prolonged estrus cycling make stallions "edgy" and aggressive, creating continuous "unrest?" In the chaos, won't foals be harmed or even killed?

A. At the heart of this issue is the subjective nature of casual observation. Science is based on data, not informal surveillance. Systematically collected data, reviewed by other scientists, accepted as legitimate, treated appropriately statistically, and published in a recognized journal is the only acceptable means for arriving at generalizable, accurate behavioral information. A good start for understanding the rudiments of behavioral research in social animals can be found in Craig (1986).

In 1983, it was shown (Kirkpatrick and Turner 1983; 1986 a,b) that wild horses do not have the same ovulatory patterns as domestic horses, and that wild horses have well-defined breeding seasons (usually from about late March until July, but this will vary somewhat from herd to herd). Wild mares do not extend their breeding season if they do not become pregnant. Keiper and Houpt (1984) also showed this for Assateague horses. This DOES happen, however, in deer (McShea et al. 1997) but not with wild horses. In 27 years of data collection on Assateague Island, only a single documented incident of a stallion killing another stallion, because of fighting, has been observed. This occurred in the middle of breeding season (late May).

The PZP vaccine does not prevent ovulation in horses and there is some limited data that it might interfere with ovulation in deer, at least after the initial treatments. Wild horses do not come into estrus every month, whether or not they have been treated with a contraceptive. They have a breeding season that barely makes it from April through July. Many wild mares have but a single estrous cycle and some have none in a given year, but they are highly seasonal and do not ovulate year-round, or even half the year.

The implication of the question is that by treating wild horse mares with PZP, they will continue to cycle throughout the year, and that this will cause stallion "unrest," aggression, and potential injury. By contrast, documented evidence shows that mares will NOT continue to cycle if they do not conceive, at least beyond the normal 3-4 month breeding season.

If critics were correct, and PZP did cause “unrest” and continuous cycling, which, in turn, led to worked-up stallions and foals dying as a result of this behavioral turmoil, then any area using PZP would have greater foal mortality. However, after 15 years of management-level treatment on ASIS, foal mortality has decreased. That is a data-driven fact.

Granted… wild horse behaviors are subtle, and individual horses will show a wide range of variability in behavioral patterns. It takes a great deal of observational experience to pick them up. We do know that hierarchies and band fidelity, are not affected by PZP application. We also know that aggressiveness and aberrational behaviors are not caused by PZP use. Perhaps, however, there may be a subtle change in daily routine. However, the larger question is, even if subtle alterations in behavior may occur, this

is still far better than the alternative of wild horses being rounded up, bands broken apart, and all of the other negatives that go with traditional management. These issues need to be put into the perspective of risk-benefit by PZP opponents.

There may be some minor time budget changes, but this was addressed several quesitons above. In any case they are not significant in the world of wild horse behavior.

***Q.* I "live" with wild horses and feel that some of your behavioral studies (measuring PZP effects) are wrong or incomplete.**

A. Living with wild horses and seeing them every day is one thing, but while of interest, this doesn't necessarily hold noteworthy meaning, unless a parameter for study is identified, a hypothesis established, and a means of testing that hypothesis is conducted. Additionally, this still does not indicate a significant behavioral or other casually observed pattern for an individual animal or for a band or herd unless the data is analyzed properly.

***Q.* I wish the BLM had used a bit of its research money or allocated new money to do a study regarding the effects of PZP on the social structure and health of wild horses in the west.**

A. The effects of PZP on social structure and herd health have been studied on herds within the Pryor Mountain Wild Horse Range and Little Book Cliffs Wild Horse Range, and no behavioral changes have been noted, nor have any behavioral changes been noted on wild horses at Return To Freedom (American Wild Horse Sanctuary) in California, where animals are observed daily.

***Q.* Isn't the use of PZP "against nature?" Why can't you just leave these animals alone?**

A. Except in a small number of wild horse herds (such as in the Montgomery Pass herd (Turner et al. 1992), Bordo Atravasado in New Mexico, and a few others), mountain lions cannot predictably predate a sufficient number of wild horses to keep herd levels at population numbers in balance with the carrying capacity of their ecosystem. The potential for both wolf and grizzly bear predation of wild horses is in question, as well,

especially if grizzlies and wolves are delisted from the Endangered Species Act, making grizzlies, wolves, and mountain lions all open to hunting pressures.

OPPOSITION TO PZP

Q. Why are some wild horse advocacy groups so vehemently opposed to PZP? It seems like the perfect solution.

A. Opposition to the contraception arises for several reasons. Wild horse advocates do not trust the BLM with the PZP vaccine, despite assurances that the agency cannot use it without approval from The Humane Society of the United States (HSUS). BLM has inflamed the issue by asking FDA for their own INAD, so they could bypass The HSUS. FDA told the BLM that no more "use" INADs would be issued; so, currently, the agency has no choice but to work with HSUS, unless they are willing to change horses in the middle of the race. The HSUS will permit the use of PZP to manage, even reduce, but not to eliminate wild horses.

This has now led the BLM to seek as many other forms of wild horse contraception as possible, from other groups, but it will take significant time to match the safety data studies that have been generated on PZP over many years.

All wild horse advocates want horses to have a better life, but if this entails a choice between having 130 healthy horses versus 200 living on the nutritional edge (in the event of a drought or a severe winter), some would choose the 130 healthy-horse option, out of (what they see as) concern for the well being of the horses. The irony is that those who seek to control wild horse populations through immunocontraceptive measures often spar with other horse advocates who (in the opinion of PZP supporters) object to what pro-PZP factions perceive as the humane treatment of wild horses. The anti-PZP community, who often question factual information and historic success, distrust the morals of those who strongly profess they care (about the well being of the herds). Pro-PZP individuals and groups believe, with conviction, that the primary motivation behind wild horse contraception is keeping healthy wild horses in the wild, on the land forever, in as natural a state as possible, with minimal interference from humans.

Many of these opponents dislike PZP because they fear it will reduce the herd to lower numbers than they want. That has been the major contention with most gathers. After 22 years of contraception on ASIS, and 15 at the management level, contraception has only been able to reduce that herd from 173 to 116. Zero population growth was achieved, and, to date, there has been no need for gathers, but reduction has been slow. So, there is little danger of massive reductions happening anywhere. Even the event of a catastrophic winter has less danger inherent than most advocates might think. The Pryor Mountain herd in Montana/Wyoming went from about 140 horses to 70 in a tragic winter die-off of 1977-1978, but the population had recovered within three years. The only thing that would have changed, had there been 200 animals instead of 140 is that more animals would have had less to eat, and therefore more would have died. The severity of the winter determined that 70 horses would survive, and not the starting population number.

Q. Aren't you trying to bring wild horses to extinction (using PZP)?

A. The concerns of some anti-PZP wild horse groups over BLM use of the vaccine are justifiable, and the BLM's proposal to use PZP to cause an Oregon herd to approach extinction, and other proposals that have surfaced to use PZP to manipulate herd composition, prove this. These apprehensions are legitimate and acknowledged by PZP researchers. However, Assateague Island data have proven the safety and efficacy of the vaccine, and it is obvious that the BLM is not going to be able to treat 30,000 wild horses to extinction with contraception, especially with continued involvement from The HSUS. Vigilance is important in the continued utilization of the PZP vaccine and in monitoring questionable research studies by state and federal agencies not sanctioned by The HSUS or the BLM, some involving the use of contraceptives that may potentially cause complete sterilization or deleterious effects in mares. Despite the utter rejection of scientific data as a legitimate form of persuasion by some anti-PZP individuals and groups, their hearts are in the right place. They are not the natural enemies of those promoting PZP as a humane management tool. Affable cooperation is needed to resolve issues of wild horse over-population, when and where it legitimately exists, once other means of population reduction have failed or are found to be undesirable.

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FOR ADDITIONAL INFORMATION

http://www.zoomontana.org/conservation_center/index.php
(The Science and Conservation Center, ZooMontana, Billings)

http://www.zoomontana.org/conservation_center/pzp_q_and_a.pdf
(online version of this document)



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Reversibility of action and safety during pregnancy of immunization against porcine zona pellucida in wild mares (*Equus caballus*)

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Contraceptive management of publicly valued wildlife species requires safeguards to ensure that these populations are preserved in a healthy state. In addition, reversibility of contraceptive effects and safety in pregnant animals are major concerns. A population of wild horses has been immunized against porcine zona pellucida (PZP) over a 12 year period on Assateague Island National Seashore, MD (ASIS). Mares initially received one or two 65 μg inoculations and once a year 65 μg booster inoculations, all delivered by dart. All young mares aged > 2 years were treated with PZP for 3 consecutive years regardless of whether they have bred successfully and they were then removed from treatment until they had foaled. All mares vaccinated for 1 or 2 consecutive years became fertile again and 69% of mares treated for 3 consecutive years returned to fertility. All five mares treated for 4 or 5 consecutive years have also returned to fertility, but over longer periods of time. Mares treated for 7 consecutive years have not returned to fertility, but several, while still infertile, have started ovulating again. There was no difference in survival rates between foals born to treated and untreated mares, and PZP treatment of pregnant mares did not affect subsequent fertility of their female offspring.

Introduction

Contraception is used increasingly to manage valued wildlife species; however, concern by the public for these animals requires safeguards to ensure that these populations will be preserved in a healthy state and with the potential to propagate successfully in the future. This is particularly true for species such as the North American wild horse, genetically valuable and rare captive exotic species in zoos, and animals with highly developed social behaviours, such as elephants.

In the USA, all wild and feral equids inhabiting public lands are protected under federal law, and public scrutiny of management by government agencies is intense. Where fertility control is used as a management technique, reversibility of contraceptive effects is crucial to the intelligent management of wild horses and is perhaps the single largest public issue. The only contraceptive agent used to treat wild horses routinely in the USA to date has been

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porcine zona pellucida (PZP). Most PZP studies to date have focused on contraceptive efficacy (Kirkpatrick *et al.*, 1990a, 1995a,b, 1996a,b; McShea *et al.*, 1997; Turner *et al.*, 1992, 1997) and short-term safety (Kirkpatrick *et al.*, 1991a, 1992, 1995a; Turner *et al.*, 1996), but certain management applications require a more complete understanding of long-term effects and safety in pregnant mares. In certain species, such as white-tailed deer, or captive exotic species in poor health, or older animals that have already made genetic contributions, reversibility of contraceptive effects is not an issue and often the goal is to prevent animals becoming pregnant in their declining years. However, in other cases, the management goal may be simply to delay reproduction until a later time to keep populations under control, or to allow for later breeding of genetically valuable animals to maintain genetic diversity in the population. In these latter cases, reversibility of contraceptive effects becomes a major issue.

The question of vaccine use in pregnant animals, the possible health effects on pregnancies in progress and the health and subsequent fertility of animals whose mothers were treated while they were *in utero* must also be considered. Again, in some species, for example white-tailed deer, in which seasonal breeding occurs and gestation is approximately 6 months, this is not a significant issue and these animals may be treated at any time during the other 6 months without danger of treatment during pregnancy. However, it is unlikely that large numbers of wild horses (gestation = 340 days) or elephants (gestation = 22 months) can be treated without including pregnant animals.

The published literature regarding PZP provides a diverse and often conflicting view of the reversibility of this vaccine. Reversibility has been documented after a single year of treatment in domestic horses (Liu *et al.*, 1989), wild horses (Kirkpatrick *et al.*, 1991a), white-tailed deer (Turner *et al.*, 1996) and African elephants (Fayrer-Hosken *et al.*, 2000). However, in other species, including dogs (Mahi-Brown *et al.*, 1985; Brandon *et al.*, 1998), rabbits (Wood *et al.*, 1981; Skinner *et al.*, 1984) and baboons (Dunbar *et al.*, 1989), PZP treatment indicates long-term impairment or complete cessation of ovarian function. Although no data have been presented to indicate deleterious effects on fetuses when the mother has been vaccinated during the pregnancy, this issue is nevertheless of concern with regard to the safety of the vaccine.

A herd of wild horses has inhabited Assateague Island National Seashore, MD (ASIS), for more than 300 years. The national seashore is charged with maintaining a herd of these horses as part of the island's historic and cultural resource, but the delicate ecology of the barrier island requires some form of population management. At the same time, there is concern about loss of genetic diversity through population control programmes, which remove horses and, therefore, genes, from the island forever. ASIS implemented immunocontraceptive research in 1988 and a fertility control management programme in 1994, based on the application of PZP to this population. In the present paper, the following are reported: (i) reversibility of contraception in treated mares over a 12 year period; (ii) survival of foals born to treated mares over the period 1988–1999; (iii) maintenance of pregnancy and live births among 26 mares treated while pregnant; and (iv) fertility of mares that were *in utero* at the time of PZP treatment of their mothers. On the basis of the accepted mechanism of the PZP contraceptive vaccine, the first hypothesis was that contraceptive effects would disappear in most animals, and the second hypothesis was that the vaccine would not interfere with pregnancy or the survival or fertility of the foals.

Materials and Methods

Study animals

The entire ASIS wild horse herd is observed for band composition and counted monthly. All individual animals are known by unique markings and band affiliations. Reproductive and

Table 1. Contraceptive reversibility in Assateague Island mares treated annually for 1–7 consecutive years with porcine zona pellucida (PZP) vaccine

Consecutive years of treatment	*n*	Interval between last treatment and pregnancy (years)[a]							
		1	2	3	4	5	6	7	8
1	16	14 (88)	14 (88)	16 (100)	–	–	–	–	–
2	5	5 (100)	–	–	–	–	–	–	–
3	32	5 (15)	12 (39)	21 (66)	22 (69)	–	–	–	–
4	3	0 (0)	0 (0)	1 (33)	2 (66)	0 (0)	0 (0)	0 (0)	3 (100)
5	2	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	–	–
7	5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a]Number (%) of mares returning to fertility. Number and percentage are cumulative for each group.

genealogy data for each individual animal have been maintained since 1974. The population has ranged in size from 43 horses in 1974 to 172 in 2000.

Vaccination

PZP glycoprotein was isolated and extracted by a modification of the method described by Dunbar *et al.* (1980) and stored at –44°C until used. The initial inoculation consisted of 65 µg PZP emulsified in Freund's complete adjuvant (FCA) and all subsequent inoculations were given with Freund's incomplete adjuvant (FIA) as described by Kirkpatrick *et al.* (1990a). All inoculations were administered i.m. remotely, using a 1.0 cc Pneu-Dart® dart (Pneu-Dart, Inc., Williamsport, PA). A single 65 µg PZP booster inoculation was given annually to those mares in which contraception was desired for > 1 year (Kirkpatrick *et al.*, 1991a). Initial inoculations were always administered in March, whereas booster inoculations were administered in either March or August–September. Contraception was considered successful if no foal was born during the 12 months after treatment. Depending on their role in the original study or their place in the management plan, mares were treated with the PZP vaccine for 1, 2, 3, 4, 5 or 7 consecutive years.

Remote pregnancy testing

Remote pregnancy testing was performed on 97 mares between 1988 and 2000, by measuring urinary or faecal oestrone conjugates (E1C) concentrations, or both, as described by Lasley and Kirkpatrick (1991) and Kirkpatrick *et al.* (1990b, 1991b), and immunoreactive pregnanediol-3-glucuronide-like progesterone metabolite (iPdG) concentrations, as described by Kirkpatrick *et al.* (1990c). Urine or faeces were collected directly from the ground following an observed excretion from an individual mare, placed on ice packs for approximately 12 h and frozen until assayed. Collection of these faecal samples was conducted from late August to December, or during the first third of pregnancy in horses.

Foal survival

Foals were defined as surviving if they lived for 12 months after birth.

Results

Of 53 mares treated with PZP for 1, 2 or 3 consecutive years, the reversibility of contraceptive action and a return to fertility (pregnancy) was 100, 100 and 68.8%, respectively, over a 4 year

period after the last PZP treatment (Table 1). Only a limited number of mares ($n = 10$) were treated for > 3 consecutive years, but 100% of the mares treated for 4 or 5 consecutive years returned to fertility, whereas none of the mares treated for 7 consecutive years has yet returned to fertility over a 7 year interval since the last PZP treatment.

Of 246 births between 1976 and 2000 among mares not treated with the PZP vaccine, 206 foals (83.7%) survived to 1 year of age. In a study of a subset of these 246 births, 76 of 86 (88.4%) foals born to mares never exposed to the PZP vaccine survived to 1 year (Keiper and Houpt, 1984). Of 80 foals born to mares that were treated with PZP during the course of their pregnancy between 1988 and 2000, 67 (83.8%) survived to 1 year. Of these 80 pregnancies among the PZP-treated mares, 26 were first diagnosed pregnant between month 8 and month 11 before parturition and all 26 mares produced live foals.

Fourteen filly foals that were born to mares exposed to the PZP vaccine during the course of their pregnancies have now lived to ⩾ 6 years of age. Of these 14 foals, eight were permitted to breed without PZP contraception upon reaching sexual maturity at 3 years old, and all eight have produced live foals (11 in total) that have survived to at least 1 year of age.

Discussion

Safety must be a major component of any wildlife contraceptive programme because of its potential application to genetically valuable and often publicly valued species. Reversibility of contraceptive action is one of the primary concerns, at least for certain species such as wild horses, free-ranging elephants and captive exotic species. The mechanisms of action of the PZP vaccine are not entirely understood, but there are several reliable models to explain the basic principles underlying its ability to inhibit fertility (Shivers and Liu, 1982; Florman and Wassarman, 1985). Administration of the vaccine to mammals results in a classic humoral immune response and B-cell-mediated production of antibodies against the glycoprotein components of the native PZP, namely ZP3, ZP1 and ZP2 (Skinner *et al.*, 1989). These IgG class antibodies will bind to the zona pellucida of mammalian ova and presumably block fertilization by causing steric hindrance of the zona sperm receptor (Florman and Wassarman, 1985). This model is consistent with the contraceptive effects of the PZP vaccine in wild mares, at least after 1–3 consecutive years of treatment. Although it was not possible to collect blood from the ASIS mares for measurement of antibody titres, similar studies with domestic mares (Liu *et al.*, 1989), deer (Turner *et al.*, 1996; McShea *et al.*, 1997; Miller *et al.*, 2000) and elk (Garrott *et al.*, 1998) support the model. As anti-PZP antibody titres increase, contraceptive effectiveness increases and as titres decrease, fertility recurs. This would explain the rather high degree of fertility recovery and the relatively short period of time necessary for return to fertility of the mares treated for 1–3 consecutive years.

The increased time for return to fertility in animals treated for 4 or 5 consecutive years, and the complete lack of reversibility in the five mares treated for 7 consecutive years, may be the result of sustained high antibody titres from longer periods of treatment. Alternatively, there may be additional mechanisms at work.

Immune responses can generally be partitioned into two general types, one of which is the B-cell-mediated humoral response, which, in the case of PZP, results in a fertilization block without accompanying oophoritis (Afzalpurkar and Gupta, 1997). The second response is a T-cell-mediated response, in which a variety of defensive phagocytic cells descend upon the target tissue and, in the case of PZP, cause moderate to severe oophoritis (Tung *et al.*, 1996). In most mammals, a particular immune response is dominated by one of these major forms of immunity, but not usually to the complete exclusion of the other. Oophoritis may become a serious long-term impediment to ovarian function and, therefore, fertility, usually as a result of

autoimmune responses and autoantibodies directed against the zona pellucida, or it may be a temporary condition with full recovery at some point (Lou *et al.*, 1996). Thus, the important question with respect to the mares treated for 7 consecutive years is whether the prolonged contraceptive effects are merely a function of sustained high antibody titres or are perhaps a result of a cellular response that has caused physical damage to the ovary.

Previous studies of the long-term effects of the PZP vaccine (Kirkpatrick *et al.*, 1992, 1995a) have indicated decreased oestrogen secretion, but fertility has always recovered except in animals treated for 7 consecutive years. This finding indicates both a humoral response, which interferes with fertilization and perhaps also a cellular response, which causes the mild reduction in oestrogen production. It is noteworthy that immediately after 7 consecutive years of PZP treatment none of the five mares was ovulating. However, a more recent investigation (D. Powell, personal communication) measuring urinary progesterone metabolites has shown evidence of high luteal phase progesterone concentration. Complete elucidation of the mechanisms at work in the mares treated for 7 years remains to be discovered.

Finally, the equal survival among foals whose mothers had and had not been treated during pregnancy strengthens the model for a simple fertilization block as the predominant immunological mechanism in horses. Furthermore, the normal fertility displayed by mares that were *in utero* at the time of their mothers' vaccinations provides further evidence of the safety of this vaccine in pregnant animals and indicates that the antibodies either do not cross the placenta or, alternatively, that their passage represents passive immunization, which would not be expected to last for the 3 years taken for the mares *in utero* at the time of vaccination to reach sexual maturity.

In conclusion, it is clear that, in horses, the contraceptive effects of PZP treatment administered annually for up to 5 consecutive years are reversible, although the time for reversal is highly variable and may extend to 6 or 7 years. The treatment of pregnant animals does not cause harm to the pregnancy, the survival of the foals or the subsequent fertility of the foals. This finding, coupled with the lack of behavioural side effects (Powell, 1999) indicates that the PZP vaccine represents an extremely safe approach to fertility control in wild horses.

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Long-term effects of porcine zonae pellucidae immunocontraception on ovarian function in feral horses (*Equus caballus*)

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Summary. Ten feral mares free-roaming in Maryland, USA, were inoculated with porcine zonae pellucidae (PZP) protein before the breeding season for three consecutive years (1988–90). Ovarian function was monitored for 51 days during the peak of the breeding season after the third annual PZP inoculation, in seven of these mares and in four untreated control mares, by means of urinary oestrone conjugates and non-specific progesterone metabolites. None of the ten inoculated mares became pregnant in 1990, compared with 55% of 20 control mares, which included two of the four monitored for ovarian function. Three of the untreated mares demonstrated apparent normal ovarian activity, characterized by preovulatory oestrogen peaks, concurrent progesterone nadirs at ovulation, breeding activity, and luteal-phase progesterone increases after ovulation. Two of the seven monitored PZP-treated mares demonstrated ovulatory cycles that did not result in conception. One was pregnant as a result of conception in 1989 and demonstrated a normal, late-gestation, endocrine profile. The remaining four PZP-treated mares revealed no evidence of ovulation, and urinary oestrogen concentrations were significantly depressed. The experiments indicated that (i) a third consecutive annual PZP booster inoculation is >90% effective in preventing pregnancies in mares and (ii) three consecutive years of PZP treatment may interfere with normal ovarian function as shown by markedly depressed oestrogen secretion.

Keywords: zona pellucida; contraception; ovary; immunocontraception; oestrogen; progestin; horse

Introduction

Immunization with porcine zonae pellucidae (PZP) is a promising approach to immunocontraception. This glycoprotein layer is composed of several protein fractions of which a fraction of M_r 55 000, referred to as ZP3, has been identified in the species studied to be the specific zona receptor for sperm recognition and attachment (Sacco *et al.*, 1984).

Immunization with PZP has resulted in significant zonae antibody titres and fertility inhibition in a range of laboratory species (Tsunoda & Chang, 1976; Gwatkin *et al.*, 1977) and non-human primates (Gulyas *et al.*, 1983; Sacco *et al.*, 1983a, 1987; Aitken *et al.*, 1984; Bamezai *et al.*, 1986; Dunbar *et al.*, 1989). Sacco (1977), Hasegawa *et al.* (1985) and Trounson *et al.* (1980) have demonstrated that human and porcine zonae share common antigens. In several of these studies, the administration of PZP or purified ZP3 was accompanied by disruption of ovarian function and

cyclic endocrine patterns, but, in most cases, normal ovarian function returned over an interval ranging from several cycles to 300 days.

A concern regarding the potential use of PZP, ZP3 or any antigenic epitope of ZP3 in humans or exotic species is the possible permanent loss of fertility or the disruption of normal reproductive endocrine events. Studies in three species have indicated adverse side-effects on ovarian function. Mahi-Brown *et al.* (1985) found that immunization of bitches led to long-term alterations in oestrous cycles and to abnormal steroid profiles. Immunization of rabbits with PZP also led to disruption of hormone profiles (Wood *et al.*, 1981; Skinner *et al.*, 1983). Ten baboons immunized with ZP3 showed reduced oestrogen concentrations in plasma and numbers of antral follicles; two of these animals failed to ovulate, and five became amenorrheic by 8 months after immunization (Dunbar *et al.*, 1989).

Liu *et al.* (1989) demonstrated that PZP immunization could render mares infertile, but that fertility returned during the following breeding season. Kirkpatrick *et al.* (1990a) also used PZP to inhibit fertility in free-roaming feral horses and demonstrated that fertility returned during the next breeding season. In a continuation of that study, Kirkpatrick *et al.* (1991) demonstrated that a single annual booster inoculation of PZP was sufficient to extend the contraceptive efficacy of PZP through a second breeding season.

Despite the data accumulated from studies of non-human primates and horses, little is known about the effects of continuous PZP treatment on the mammalian ovary over several years. The present study evaluated the effectiveness of monitoring ovarian function in uncaptured feral mares by means of urinary steroid metabolites and determined the effects of PZP immunization for three consecutive years upon fertility, ovarian endocrine function, and sexual behaviour in feral mares.

Materials and Methods

Animals

Ten free-roaming feral mares inhabiting Assateague Island National Seashore, MD, USA, were evaluated for the contraceptive efficacy of a third consecutive annual PZP inoculation; ovarian function was assessed in seven of these mares. Twenty untreated mares served as controls and ovarian function was studied in four of them. Ages and fertility records were known for six of the seven PZP-immunized mares and for three of the four control mares. Ages ranged from 3 to 16 years. Each mare possessed unique markings and was photographed for later identification.

PZP immunization

The PZP antigen was prepared from porcine ovaries as described by Liu *et al.* (1989) and stored frozen until used in the field. In 1988 (year 1 of this study), the ten experimental mares were immunized with two or three inoculations of PZP. The schedule of inoculations and the resulting contraceptive effects have been described (Kirkpatrick *et al.*, 1990a).

In February, 1989 (year 2 of this study) the ten PZP-treated mares were given a single PZP booster inoculation and the contraceptive efficacy was assessed (Kirkpatrick *et al.*, 1991). The ten mares were given a further single booster inoculation in March 1990 (year 3 of this study) identical to the booster treatment in year 2. Between 4 May and 24 June 1990, urine samples in soil or sand were collected daily or every other day, without capture, from seven of these ten treated mares and four untreated control mares. The selection of the seven mares from among the ten was made on the basis of accessibility for urine collections, and the timing of the collections was based on peak breeding activity among the Assateague population (Keiper & Houpt, 1984). The age distributions of the 20 mares selected as controls for the assessment of contraceptive efficacy was similar to that of the ten treated mares. Pregnancy was determined during October 1990, on the basis of concentrations of urinary oestrone conjugates as described by Kirkpatrick *et al.* (1988). Differences in rates of pregnancy between treated and control mares were tested for significance by means of binomial probability distribution (Freedman *et al.*, 1978).

Behavioural assessments

Sexual behaviour of treated and control mares was assessed in terms of behavioural oestrous patterns including (i) raised tail when stallions attempted mounting, (ii) eversion of the clitoris, (iii) absence of kicking or biting by the mare during attempted or successful mountings and (iv) copulation, as described by Waring (1983). The period for which

each mare was observed each day ranged from 1 to 3 h depending upon the duration of the interval between sighting and urination.

Assessment of ovarian function

Unextracted urine samples, collected from the soil, were assayed for oestrone conjugates and non-specific progesterone metabolites by microtitre enzymeimmunoassays. The oestrone conjugate assay has been described (Daels *et al.*, 1991) and validated for the Equidae (Evans *et al.*, 1984; Czekala *et al.*, 1990; Monfort *et al.*, 1991) and reflects plasma oestrogen concentrations (Daels *et al.*, 1991); the inter- and intra-assay coefficients of variation were 11·10 ($n = 7$) and 3·82% ($n = 7$), respectively. The lower limit of sensitivity for the assay was 6·25 pg and concentrations of oestrone conjugates in serially diluted urine samples extracted from soil have exhibited parallelism to the standard curve (Kirkpatrick *et al.*, 1988). Recovery of ^{3}H-labelled steroid metabolites from the soil has been demonstrated to be >95% (Kirkpatrick *et al.*, 1988).

The enzymeimmunoassay for non-specific progesterone metabolites has been described and validated in the Equidae by Kirkpatrick *et al.* (1990b) and employs monoclonal antibody 1284-1 raised against 20δ-hydroxyprogesterone (provided by R. Chatterton, Northwestern University). The inter- and intra-assay coefficients of variation were 12·38 ($n = 8$) and 10·42% ($n = 8$), respectively. The lower limit of sensitivity was 0·075 ng. To account for differences in urine concentrations, all urine samples were assayed for creatinine by the microcolorimetric method of Taussky (1954), and steroid metabolite concentrations were converted to ng/mg creatinine. Differences in mean hormone concentrations were tested for significance by means of Student's t test.

Results

None of the ten mares treated with PZP for the third consecutive year became pregnant by October 1990, but 11 of 20 control mares (55%) were pregnant in October 1990. The difference in pregnancy rates was significant ($P < 0·001$). Two of the four control mares for which breeding-season serial urine samples were collected were pregnant in October 1990.

The four control mares monitored for ovarian function demonstrated evidence of pregnancy and parturition (M13, 15 years), oestrous cycles without conception (Speckles, age unknown but < 10 years, and M4, 18 years) and a conceptive cycle (M17GL, 3 years). Pregnancy and parturition (Fig. 1a) was characterized by concentrations of urinary oestrone conjugates which fell rapidly ~7 days before parturition and urinary non-specific progesterone metabolite concentrations which fell abruptly on the day before parturition. There were no endocrine indications of *post-partum* ovulation during the 39 days of urine collection following parturition. A marked increase in oestrone conjugates occurred 6 days after parturition, but, on the basis of non-specific progesterone metabolite concentrations, did not culminate in ovulation; this increase in oestrone conjugates was accompanied by repeated behavioural oestrus in the presence of the band stallion. A second control mare demonstrated an ovulatory oestrous cycle (Fig. 1b) based on urinary oestrone conjugates and non-specific progesterone metabolites, with a clear peak in preovulatory oestrone conjugates (375 ng/mg creatinine) coinciding with a non-specific progesterone metabolite nadir (20 ng/mg creatinine) and a subsequent luteal-phase increase in non-specific progesterone metabolites (330 ng/mg creatinine). Urinary non-specific progesterone metabolites reached basal concentrations (15 ng/mg creatinine) by Day 36 of collection, without behavioural or endocrine evidence for a second oestrous cycle. At the same time as the preovulatory increase in oestrone conjugates, this mare demonstrated behavioural oestrus in the presence of the band stallion. A third control mare demonstrated a conceptive ovulatory oestrus (Fig. 1c) characterized by an exceptionally high peak in preovulatory oestrone conjugates (1·1 μg/mg creatinine) coinciding with a nadir in non-specific progesterone metabolite (27 ng/mg creatinine) and a subsequent and sustained rise in this urinary metabolite (1·7 μg/mg creatinine). Concentrations of non-specific progesterone metabolites remained above 1·0 μg/mg creatinine throughout the remainder of the collection period. Urinary oestrone conjugates fell to basal concentrations (15–55 ng/mg creatinine) for 35 days after the ovulatory nadir in non-specific progesterone metabolites and then rose abruptly to 1·45 μg/mg creatinine, which is a characteristic signal of pregnancy in mares (Evans *et al.*, 1984). During the first 5 days of urine collection, which coincided with the preovulatory oestrone conjugate increases,

M17GL displayed behaviour associated with oestrus and was witnessed being successfully mounted on Days 3–5 of collection. By October, M17GL had a urinary oestrone conjugate concentration of 31·3 ng/mg creatinine, indicating that the pregnancy had terminated, a common event in feral 3-year-old mares (Lucas *et al.*, 1990). The fourth control mare produced a foal on 6 April, 1 month before the initiation of urine collection. Urinary oestrone conjugates and non-specific progesterone metabolite concentrations revealed a portion of an ovulatory cycle during the first 20 days of the collection period and a second ovulatory cycle during the 31 remaining days of the collection period.

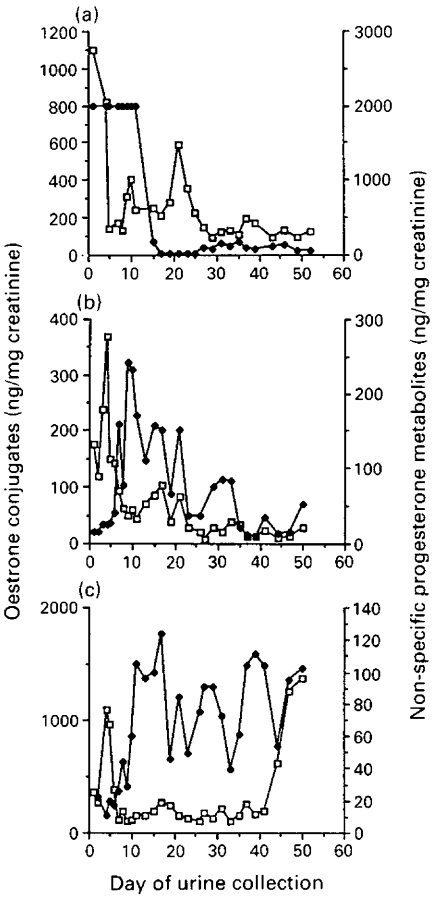


Fig. 1. Profiles of urinary oestrone conjugates (□) and progesterone metabolites (◆) in three control mares: (a) M13 (a foal was born on Day 12), (b) an oestrous cycle without conception in Speckles and (c) an oestrous cycle with conception in M17GL. Day 0 was 4 May.

Four of the seven PZP-treated mares (T5AE, 10 years; M17GJ, 5 years; M2E, 10 years; M13F's Bay, age unknown) displayed no endocrine evidence of ovulation during the 51-day collection period. Concentrations of urinary non-specific progesterone metabolites revealed no luteal-phase increases. Similarly, urinary oestrone conjugate concentrations and patterns revealed no signs of an oestrous cycle and concentrations during the 51-day collection period were extremely variable and significantly lower ($P < 0{\cdot}05$) than those of the control mares Speckles and M4, which demonstrated ovulatory cycles. Neither T5AE nor M17GJ displayed any signs of behavioural oestrus during the study. M2E displayed three incidences of behavioural oestrus on Days 16, 20 and 35 of

collection, in the absence of any significant increases in oestrone conjugates, but the band stallion showed no interest in this mare during these displays. The mare M13F's Bay had a significant increase in oestrone conjugates between Days 25 and 35 of collection, which was accompanied by eversion of the clitoris and four successful mounts by the band stallion during this time. Urinary endocrine profiles are shown for two of these non-cyclic mares in Fig. 2a, b.

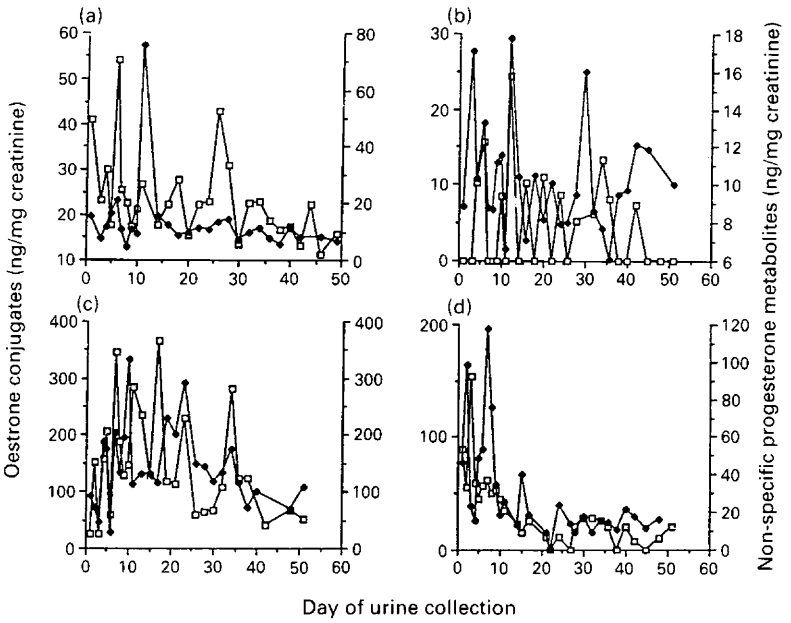


Fig. 2. Profiles of urinary oestrone conjugates (□) and progesterone metabolites (◆) in four mares treated with porcine zonae pellucidae: non-ovulatory patterns of urinary steroids in (a) T5AE and (b) M2E; ovulatory cycles without conception in (c) M6I and (d) T2BE.

The PZP-treated mares M6I (6 years old) and T2BE (10 years old) displayed urinary non-specific progesterone concentrations and patterns characteristic of ovulatory oestrous cycles (Fig. 2c, d, respectively), but mare M6I did not have the characteristic preovulatory peak in oestrone conjugates.

During two of the increases in oestrone conjugates, on the 10th and 34th days of collection, M6I displayed tail raising and evoked attention from the band stallion, but no mounting was observed. Urine collection with T2BE began in the latter stages of the luteal phase, concentrations of non-specific progesterone metabolites falling from 199 ng/mg creatinine to non-detectable concentrations 15 days later. T5AE displayed intermittent behavioural oestrus throughout most of the collection period despite very low oestrone concentrations of conjugates and on one occasion, on Day 27 of collection, was mounted successfully by the band stallion; this mounting coincided with a rapid rise in concentrations of oestrone conjugates from non-detectable to 45 ng/mg creatinine.

The seventh PZP-treated mare (M17G, 8 years old) was pregnant at the time of urine collection and produced a foal on 1 August 1990. This was the only foal produced among the mares treated with a booster inoculation in the second year of this study. The date of parturition indicates an unusually late conception (~25 August) for Assateague mares and suggests that her antibody titres had fallen to a concentration which allowed conception. Urinary endocrine data for oestrone conjugates and non-specific progesterone metabolites were consistent with data on plasma hormones for mares in late pregnancy (Ginther, 1979).

Discussion

This study demonstrates that a single annual PZP booster inoculation given after 2 years of treatment will extend the observed infertility in such treated mares for a third year. Only two of the seven monitored PZP-treated mares demonstrated ovulatory cycles and it is probable that treatment with PZP for 3 years altered ovarian function. The absence of a luteal phase in four of these mares, coupled with significantly lower and intermittent increases in oestrone conjugates may indicate incomplete folliculogenesis, with reduced secretion of oestrogen and failure to ovulate. These alterations in ovarian function are consistent with data from PZP-treated rabbits (Skinner *et al.*, 1983, 1984), dogs (Mahi-Brown *et al.*, 1985) and baboons (Dunbar *et al.*, 1989).

The urinary endocrine data and the correlated behavioural observations and events, such as parturition and breeding behaviour for the four control mares, reflect functional ovaries. These data provide an initial view of ovarian function in feral mares which is consistent with data derived from domestic horses based on plasma steroid concentrations (Ginther, 1979) and urinary steroid metabolites (Daels *et al.*, 1991).

Three previous studies with horses point to long-term treatment, as opposed to a single treatment, as the factor which resulted in the alteration of ovarian function in PZP-treated mares. Liu *et al.* (1989) treated mares with PZP for only one breeding season and demonstrated the occurrence of ovulation after 7 months of infertility. This was indicated by normal patterns of luteal-phase plasma progesterone and normal fertility rates for up to 3 years after the single inoculation. These results are consistent with the proposed mechanisms of contraceptive action of antibodies to PZP in mares, which include a fertilization block brought about by steric hindrance of the sperm receptor (Liu *et al.*, 1989) and an acrosome reaction in horse spermatozoa induced by zona pellucida (Arns *et al.*, 1990). Kirkpatrick *et al.* (1990a, 1991) demonstrated the return of normal fertility rates among feral mares 1–2 years after a single immunization with PZP and, although ovarian endocrine function was not monitored in these two studies, the normal fertility rates indicate that ovulation occurred and that preovulatory oestrogen concentrations were sufficient to elicit breeding behaviour. Similarly, short-term active immunization of non-human primates led to infertility, but with undetectable or only temporary disruption of ovarian function (Gulyas *et al.*, 1983; Sacco *et al.*, 1983a, b, 1987; Bamezai *et al.*, 1986).

Zonae antibodies have been shown to bind to the developing follicular zona pellucida as well as to the zona of ovulated ova (Henderson *et al.*, 1988). This may indicate that zonae antibodies disrupt some developmentally critical cellular communication between differentiating follicular cells and the growing oocyte (Skinner *et al.*, 1990), which ultimately leads to the destruction of a portion or all of the oocyte pool (Skinner *et al.*, 1984).

At least three factors may play a significant role in long-term alterations of ovarian function. The duration of contraceptive efficacy is directly related to the amount of PZP antigen administered (Henderson *et al.*, 1988) and to the resulting antibody titres (Millar *et al.*, 1989), and ovarian dysfunction may likewise be related to PZP dose.

The purity of the PZP antigen may be a second factor in the possible alteration of ovarian function. Most of the early studies with rabbits, dogs and some non-human primates which demonstrated ovarian dysfunction used crude preparations of PZP, which included the entire complement of zonae protein. These various preparations also include associated carbohydrate moieties which are thought to be important antigenic components (Paterson & Aitken, 1990). More recent studies have used preparations of higher purity and even genetically engineered epitopes of the ZP3 protein. Dunbar *et al.* (1989) used both ZP1 and ZP3, which share a common antigenic epitope, but serious ovarian dysfunction still occurred in baboons after 49 weeks and nine cycles. The horses in this study received crude PZP antigen which may have exacerbated long-term effects despite the small dose of 65 μg.

The duration of treatment may contribute to long-term effects. Small doses of high purity may produce long-term effects through cumulative effects upon follicular pools. No other mammals,

apart from the horses in this study, have been treated with PZP for 3 years. Thus, our current understanding of long-term effects is poor. Based on the work of Liu *et al.* (1989) with captive mares, and the initial field tests with feral mares (Kirkpatrick *et al.*, 1990a, 1991), all of which used the same antigen and doses and demonstrated a return to normal fertility, the data from three consecutive years of treatment suggest that the duration of treatment may be the primary factor for this observed ovarian dysfunction in mares.

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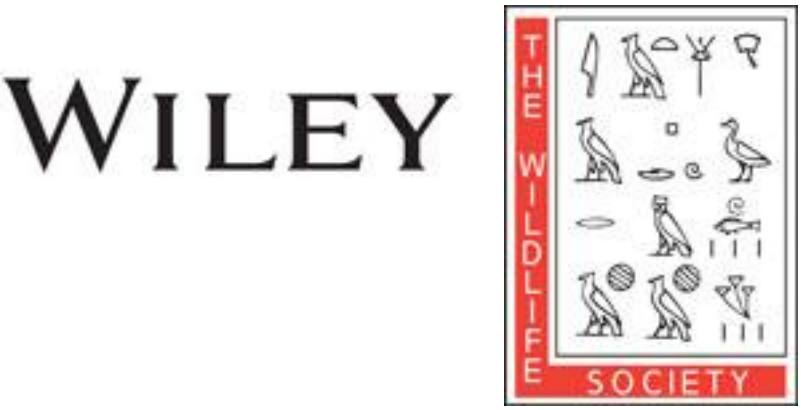
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COMPENSATORY REPRODUCTION IN FERAL HORSES

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Abstract: We estimated fetal loss rate in 2 separate herds of feral horses (*Equus caballus*) with significantly different foaling rates that inhabited the same barrier island. We estimated pregnancy rates for 40 feral mares from Assateague Island National Seashore (NS), where the foaling rate was 32.5% and for 48 mares from Chincoteague National Wildlife Refuge (NWR) where the foaling rate was 62.5%. Fetal loss rates were estimated by comparing fall pregnancy rates with foaling rates during the following spring and summer. The estimated fetal loss for mares from Assateague Island NS and Chincoteague NWR was 7.1 and 6.2%, respectively. Ten of the 40 mares from Assateague Island NS (25%) were lactating and only 2 produced foals, whereas 2 of the 48 mares from Chincoteague NWR (4.1%) were lactating and neither produced foals. These data indicate that (1) the differential foaling rates are not the result of fetal loss after 90-days postconception, (2) lactational anestrus is a contributing factor to differential foaling rates, and (3) the annual removal of foals from the Chincoteague NWR herd results in compensatory reproductive mechanisms and higher foaling rates.

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Compensatory reproduction might be an important adaptive response to various natural, population-limiting pressures such as predation, food availability, and disease. Human intervention into wildlife populations by means of hunting or removal of animals can also affect population growth. Two populations of feral horses inhabiting Assateague Island, a barrier island off the Maryland/Virginia coast, provide an excellent model for the study of compensatory reproduction. The island's feral horses are maintained in 2 separate herds, one on the Maryland portion of the island and the other on the Virginia portion. Peak breeding activity for both herds is May and June, with peak foaling activity during April and May (Keiper and Houpt 1984). Weaning occurs at age 1 year for mares foaling 2 consecutive years but often occurs at ages 2 or even 3 years for mares that do not foal on consecutive years.

The Maryland herd consists of approximately 150 horses living on Assateague Island National Seashore, and, in keeping with National Park Service policies of nonintervention, management is minimal. The Virginia herd consists of approximately 200 horses inhabiting Chincoteague NWR; these horses are intensively managed through the annual removal of approximately 80% of the foals, a practice dating back >30 years. In an 8-year study of reproduction among the Assateague Island horses conducted between 1975 and 1983, Keiper and Houpt (1984) reported an annual foaling rate of 74.4% among sexually mature mares on the Chincoteague NWR. In contrast, the foaling rate for the unmanaged Assateague Island NS horses was only 57.1%, with an age-specific range of 40–70%. Since 1986, the Assateague Island NS foaling rate has dropped below 50% (J. F. Kirkpatrick, unpubl. data).

The purpose of our study was to examine fall pregnancy rates in the 2 herds and to compare these with respective foaling rates during the following spring and summer, thereby determining the extent of fetal loss between the 2 herds. We hypothesized that the physiological stresses of concurrent pregnancy and lactation among the Assateague Island NS horses cause a greater incidence of fetal loss than among the horses of Chincoteague NWR, where the physiological stress of lactation is removed annually.

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METHODS

Forty mares whose home ranges were exclusively confined to Assateague Island NS and 48 mares whose home ranges were exclusively confined to Chincoteague NWR were included in our study. All mares were sexually mature and ≥3 years old. The ages of 29 of the Assateague Island NS mares were known, but the ages of the Chincoteague NWR mares were not known exactly. Ten of the 40 Assateague mares (25%) and 2 of the Chincoteague mares (4.1%) had foals at their sides and were lactating.

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Table 1. Pregnancy and foaling rates (%) for lactating and nonlactating feral mares in 2 separate herds.

	Assateague Island National Seashore		Chincoteague National Wildlife Refuge	
	n	%	*n*	%
Total mares tested	40		48	
Diagnosed pregnant	14	35.0	32	66.6
Live foals	13	32.5	32	66.6
Lactating mares	10	25.0	2	4.1
Diagnosed pregnant	2	5.0	0	
Live foals	2	5.0	0	

During October 1989 urine and/or fecal samples were collected from each mare as described by Kirkpatrick et al. (1988, 1990*b*). Urine was aspirated directly from the ground immediately following elimination by a mare, or it was hand-centrifuged from sand, placed on ice at the time of collection, and stored frozen at the end of each day. Fecal samples were placed in plastic bags, kept on ice during the day and frozen each evening. For the 40 Assateague mares, we collected urine samples from 18 mares, fecal samples from 17 mares, and both urine and fecal samples from 5 mares. For the Chincoteague mares, urine samples were collected from 23 animals and fecal samples from 25. All horses had distinctive markings which we recorded on drawings or with photographs for later identification.

Unextracted urine samples were assayed for (1) creatinine (Cr), (2) estrone conjugates (E_1C) by enzyme immunoassay as previously described by Shideler et al. (1990) and modified for use in the horse (Kirkpatrick et al. 1990*b,c*), and (3) nonspecific immunoreactive progesterone metabolites (iPdG) as described by Kirkpatrick et al. (1990*a*). The reliability for the E_1C assays and the 100% accuracy for predicting pregnancy, which has been tested in domestic horses and confirmed by rectal palpation (Evans et al. 1984, Kasman et al. 1988) and has been demonstrated in feral horses (Kirkpatrick et al. 1990*a,b,c,* 1991), have been reported elsewhere. Urinary steroid metabolite concentrations were indexed to urinary Cr to account for differences in urine concentrations, and they were reported as ng/mg or μg/mg Cr. We considered concentrations of E_1C in excess of 1.0 μg/mg Cr (Evans et al. 1984, Kasman et al. 1988, Kirkpatrick et al. 1988) and iPdG in excess of 100 ng/mg Cr (Kirkpatrick et al. 1991) as indicative of pregnancy.

We used methods described by Kirkpatrick et al. (1991) to measure fecal E_1C and iPdG concentrations. Fecal total estrogens were assayed by radioimmunoassay as described by Bamberg et al. (1984) and Kirkpatrick et al. (1990*c*). Fecal steroid metabolites and free estrogens were reported as ng/g feces. Pregnancy was predicted on the basis of >1.0 ng/g E_1C, >150 ng/g iPdG, and 5.0 ng/g total estrogens (Kirkpatrick et al. 1990*c,* 1991). During the summer and fall of 1990 we located all pregnancy-tested mares and recorded the presence or absence of foals. Ownership of foals was based on witnessed lactation. We used a binomial probability distribution (Freedman et al. 1978) to test for differences in pregnancy, lactation, and foaling between the 2 herds.

RESULTS

A significantly ($P < 0.001$) greater proportion of Chincoteague mares tested positive for pregnancy, and a significantly ($P < 0.001$) greater number of foals were produced by Chincoteague mares than by Assateague mares (Table 1). There was a 7.1% and a 6.2% fetal loss rate among the 40 Assateague and 48 Chincoteague mares, respectively, with no significant difference between the 2 herds. Annual foal removal resulted in a significant ($P < 0.01$) difference in lactating mares between the 2 herds, with only 2 lactating Chincoteague mares and 10 lactating Assateague mares. Collectively, the 12 lactating mares produced only 2 foals.

DISCUSSION

Early neonatal death is almost impossible to detect on Assateague Island habitat because of dense vegetation and the pregnant mares' behavior of isolating themselves at parturition. Thus, we could not distinguish between fetal loss and neonatal loss. Nevertheless, the nearly doubly great pregnancy rate for Chincoteague horses indicates that the differential foaling rates between the 2 herds is determined by October pregnancy rates, and not by fetal loss after approximately 90–150 days postconception.

Among domestic mares, Ginther (1979) reported an 80% seasonal pregnancy rate and a 70% live foal crop. Van Niekerk (1965) reported a 12.5% pregnancy loss among domestic mares, with greater pregnancy loss during the first half of pregnancy. The suggested critical period for pregnancy loss was Days 25–31. Estimates of abortion rates in the domestic mare, after Day 45 of pregnancy, range from 7 to 12% (Platt

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1973, Irwin 1975, Sullivan et al. 1975, Chevalier and Palmer 1982), and major causes of this loss are thought to include foal heat conceptions in lactating mares (Platt 1973) and periglandular fibrosis of the uterine mucosa (Kenney 1978). In our study fetal loss after Day 90 postconception in both herds of feral mares was not different from that reported for domestic mares after Day 45 postconception.

Among island feral horses, age has also been shown to be a major factor in fetal loss rates. Lucas et al. (1990) demonstrated that fetal loss among mares of Sable Island, Canada, between Days 120 and term was 9.7, 40.3, 25.0, and 26.3% annually over 4 years. The fetal loss rates were age-dependent, with a 16.7% loss for 3 year olds and a 5.6% loss for 4 year olds. In our study, only mares ≥3 years old were included, thus it is unlikely that age had a significant effect upon the 2-fold difference in pregnancy and foaling rates. Furthermore, an environmental basis for the differences seems unlikely, because the 2 herds inhabit the same island. Photoperiod, climate, and food supplies are equivalent, and both herds originate from the same genetic forebearers.

The most probable cause for the differences in pregnancy and foaling rates is lactational anestrus, a condition in mares during which ovulation does not occur (Day 1939, Burkhardt 1948, Roberts 1971, Lieux 1973). In domestic mares, this condition persists in a large percentage of lactating mares until the foal is weaned, usually at 4–6 months. This period of an ovulatory lactational anestrus has been characterized by very small or absent ovarian follicles (Day 1939). In controlled experiments with domestic mares, removing the foal at parturition hastened the return of estrus in 3 of 4 mares, whereas all 4 mares that nursed their foals normally were anestrus (Ginther et al. 1972). On Assateague Island, weaning seldom occurs sooner than 1 year, is fairly common at 2 years, and in some instances has been observed at 3 or 4 years. In contrast, about 80% of the foals of Chincoteague NWR mares are removed annually in July.

Our results differ significantly from those of Wolfe et al. (1989), who examined pregnancy rates among 553 feral mares >2 years old, in Nevada, Oregon, and Wyoming. The pregnancy rates for horses in these states were 57.0, 61.0, and 81.4%, respectively, with no differences between lactating and nonlactating mares. Some of the differences between the results of our study and those of Wolfe et al. (1989) could be the result of different pregnancy testing procedures and the accuracies of these tests. Wolfe et al. (1989) reported accuracy rates of 81.8% for plasma progesterone concentrations, 63.0% for plasma estrogen, and 53.7% for plasma pregnant mares' serum gonadotropin (PMSG). The use of only plasma progesterone measurements for pregnancy detection in horses is potentially inaccurate (Hyland 1990), and the same is true for urinary iPdG measurements alone, largely because of the high incidence of persistent corpora lutea in mares. Urinary E_1C concentrations, however, reflect the presence of a viable embryo and drop precipitously within hours of the death of the fetus (Kasman et al. 1988). Consequently, accuracy of pregnancy detection in mares approaches 100% with urinary E_1C concentrations alone (Evans et al. 1984, Kasman et al. 1988), 100% for urinary E_1C and iPdG concentrations together (Kirkpatrick et al. 1990*a*), and 95% for total fecal estrogens (Bamberg et al. 1984, Kirkpatrick et al. 1990*c*).

MANAGEMENT IMPLICATIONS

Control policies for feral horses have historically centered around the removal of horses, particularly young horses. Because our results suggest that feral horses increase conception rates in response to the removal of foals before weaning, a policy of removing young, unweaned horses can lead to greater fecundity among their mothers. Conversely, the removal of weaned horses will be unlikely to have any significant effect upon fecundity of the mothers. Future management policies for the control of feral horse populations should consider compensatory reproductive responses of the mares. Regardless of whether actual removal or contraception is employed as the control method, efforts should focus on mares >3 years old, and those without foals. Conversely, the removal of foals or yearlings should be avoided because of the probability of increasing fecundity among their mares.

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Absence of Effects From Immunocontraception on Seasonal Birth Patterns and Foal Survival Among Barrier Island Wild Horses

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Despite a large body of safety data, concern exists that porcine zonae pellucidae (PZP) immunocontraception—used to manage wild horse populations—may cause out-of-season births with resulting foal mortality. Our study at Assateague, Maryland indicated the effects of immunocontraception on season of birth and foal survival between 1990 and 2002 on wild horses from Assateague Island. Among 91 mares never treated, 69 (75.8%) of foals were born in April, May, and June (in season). Among 77 treated mares, 50 (64.9%) were born in season. Of 29 mares foaling within 1 year after treatment (contraceptive failures), 20 (68.9%) were born in season. Of 48 mares treated for greater than 2 years then withdrawn from treatment, 30 (62.5%) of 48 foals were born in season. There were no significant differences ($p < .05$) between either treatment group or untreated mares. Survival did not differ significantly among foals born in or out of season or among foals born to treated or untreated mares. Data indicate a lack of effect of PZP contraception on season of birth or foal survival on barrier island habitats.

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The use of porcine zonae pellucidae (PZP) contraceptive vaccine has been widely applied to wild horses in numerous research studies (Kirkpatrick, Turner, Liu, & Fayrer-Hosken, 1996) and also has been applied to several wild horse herds as a management tool (Turner & Kirkpatrick, 2002). Issues of efficacy, reversibility, long-term health (Kirkpatrick & Turner, 2002; Turner & Kirkpatrick, 2002), and behavior (Powell, 1999) have been addressed through past research efforts. Two remaining issues that have not been addressed are the effect of PZP on out-of-season births among treated mares and the effects of PZP on the survival of foals born to treated mares.

The concern with the possibilities of out-of-season births arises from the possibility that anti-PZP antibody titers may, over time, fall below contraceptive levels at times of the year when a pregnancy and the resulting birth would be injurious to the foal. Although no body of data suggests that foals born to treated mares are less healthy or survive at lower rates than foals born to untreated mares, this is a safety issue not addressed since the original study of PZP contraception of wild horses in 1988 (Kirkpatrick, Liu, & Turner, 1990). This retrospective study was conducted to determine if (a) PZP treatment led to significant out-of-season births, (b) PZP treatment led to a significant decrease in foal survival, and (c) foal births out of season led to decreased survival.

METHOD

The identities of all 172 wild horses inhabiting Maryland's Assateague Island National Seashore (ASIS) are known by individual markings and band affiliations. Birth dates, by month, exist for 168 foals born on ASIS from 1986 through 2002. Birth dates are acquired by visual sighting of foals during monthly surveys by National Park Service personnel. Foal survival is defined as surviving to age 1 or older.

To insure that very early neonatal losses do not go undetected, mares were tested for pregnancy each year by means of urinary and/or fecal steroid metabolite analysis (Kirkpatrick, Kasman, Lasley, & Turner, 1988; Kirkpatrick, Lasley, et al., 1990; Kirkpatrick, Shideler, Lasley, & Turner, 1991). Specifically, the excreta is analyzed for both estrone conjugates (E1C) or immunoreactive pregnanediol-glucuronide-like progesterone metabolites (iPdG). Both metabolites are significantly higher in pregnant animals. However, although progesterone levels (and therefore iPdG) will remain high for months after a fetal death, E1C concentrations fall within 12 hr of fetal loss (Hyland & Langsford, 1990). Thus, the pregnancy detection methods are virtually 100% accurate. In this way, very early neonatal losses do not go undetected.

The normal season of foaling is defined as April, May, and June (called *in season* hereafter), based on previous studies with wild horses (Hall, 1972; Keiper &

Houpt, 1984; Palmer, 1978; Satoh & Hoshi, 1932). Comparisons were made in the number and percentage of foals born in season and those born outside this seasonal window between mares (a) never treated with PZP, (b) treated with PZP for 1 year before the birth (i.e., contraceptive failures), and (c) those treated with PZP for at least 2 years before the birth of a foal. The group treated with PZP for 1 year before the birth (contraceptive failures) included 9 mares who became pregnant after only a single initial inoculation was given to 41 mares in 1994 as opposed to the standard two-inoculation protocol (Kirkpatrick, Liu, & Turner, 1990). Foal survival also was calculated as a function of whether the foal was born in season or outside this window and whether or when the mother had been treated with PZP prior to the birth. Differences in season of birth, foal survival as a function of birth date, and foal survival as a function of PZP treatment were tested for significance by Fisher's exact test for contingency tables.

RESULTS

Among foals born to mares who never had been treated, 69 of 91 (75.8%) were born in season; among 77 treated mares, 50 of 77 (64.9%) were born in season. The difference was not significant ($p = .122$; standard error [*SE*] of difference = .085). Among foals born to mares treated with PZP for 1 year before the birth of a foal, 20 of 29 (68.9%) were born in season. The difference was not significant from foals born to untreated mares ($p = .463$; *SE* of difference = .090). Among foals born to mares who had been withdrawn from treatment or who had experienced a contraceptive failure after at least 2 years of treatment, 30 of 48 (62.5%) were born in season. The difference was not significant from foals born to untreated mares ($p = .111$; *SE* of the difference = .090). Table 1 shows foal birth by month.

TABLE 1
Births by Months for PZP-Treated and Untreated Wild Mares

Mare Status	*Month of Birth*											
	J	*F*	*M*	*A*	*May*	*Je*	*Jy*	*Au*	*S*	*O*	*N*	*D*
UT[a]	2	0	3	23	23	23	10	4	2	0	0	1
T1[b]	1	1	1	12	1	7	1	3	2	0	0	0
T > 2[c]	1	4	1	12	14	4	4	3	1	1	1	2
Total	4	5	5	47	38	34	15	10	5	1	1	3

Note. J = January; F = February; M = March; A = April; JE = June; Jy = July; Au = August; S = September; O = October; N = November; D = December; UT = untreated; T1 = mares treated the year before the birth of a foal; T > 2 = mares withdrawn from treatment more than 2 years before the birth of a foal.

[a]69 of 91 (75.8%) of foals born in season to UT mares. [b]20 of 29 (68.9%) of foals born in season to T1 mares ($p = .463$). [c]30 of 48 (62.5%) of foals born in season to T > 2 mares ($p = .111$).

Foal survival did not differ significantly ($p = .471$; *SE* of the difference = .100) between foals born in season and foals born outside this seasonal window. Among foals born in season, 104 of 119 (92.9%) survived to age 1 or older; of those born outside this window, 42 of 49 (85.7%) survived. Among foals born to mares never treated with PZP, 77 of 91 (84.6%) survived and did not differ significantly ($p = .825$; *SE* of difference = .109) from foals born to mares treated at some time before their birth, of whom 67 of 77 (87.0%) survived.

DISCUSSION

These data indicate there was no significant effect of PZP treatment on out-of-season births, with only 13.3% separating the two treatment groups and untreated mares. This is in contrast to white-tailed deer in which significant extensions of the breeding season and, by implication, ovulation occurred after treatment of does with PZP (McShea et al., 1997), and almost all treated animals extended their breeding season by as much as 2 months. The failure of a larger effect in horses probably is a function of the reproductive biology of this species.

Photoperiod Influence on Ovulation

Ovulation in the modern horse is seasonal and influenced largely by photoperiod, with peak reproductive activity and foaling correlating with increased photoperiod (Kenney, Ganjam, & Bergmen, 1975). In the domestic mare, ovulation reflects this seasonal pattern, but significant percentages of mares will ovulate well into the fall and winter (Kenney et al., 1975). Photoperiods of 16 to 24 hr induced the onset of breeding in domestic mares, and photoperiods of less than 9 hr caused a cessation in ovulation and breeding (Kooistra & Ginther, 1975).

Patterns of Foaling

Among other horses, the patterns of foaling differ. On Assateague Island, Keiper and Houpt (1984) reported that 88% of foals were born in season. Feist and McCullough (1975) reported that no foals were born in the Pryor Mountains, MT outside season, and Tyler (1972) reported that 96% of births among New Forest ponies occurred in this same period. On Sable Island, Nova Scotia, Canada, foals were born year round, but 77% were born in season (Welsh, 1975). Similar patterns have been reported for wild horses of Wyoming's Red Desert (Boyd, 1979) and the Great Basin (Berger, 1986).

Foaling and Ovulatory Activity

Foaling patterns however, do not necessarily reflect ovulatory activity. Among domestic mares, the stallion does not always have access to the mare, and the timing of reproduction most often will reflect management of the stallion rather than environmental, nutritional, or genetic influences.

Palmer (1978) found that Welsh ponies had no ovarian activity during the winter months, whereas 66% of saddle breeds did. Satoh and Hoshi (1932) found that semiwild Korean ponies ceased ovulating by October. Kirkpatrick and Turner (1982) demonstrated that ovulation, determined endocrinologically, among Pryor Mountain wild mares was confined to April to July—even when the plane of nutrition was increased—whereas estrous behavior was observed occasionally well into October. Ginther (1979) speculated that wild horses, as they adapt to a free-ranging life and most often a harsh environment, develop a breeding pattern that begins and ends more abruptly and during a period when survival of the foal would be most favorable. This, in turn, implies a genetic basis for the control of ovulation.

Liu, Bernoco, and Feldman (1989) demonstrated that antibody titers to PZP treatment fall over time and that fertility will be restored when they reach minimal levels (below 64% of positive reference standards). If ovulatory activity is confined more or less to a highly seasonal pattern, successful reproduction out of season would not be significant. In any case, the more restricted breeding/ovulatory season in wild horses is the most plausible explanation for the lack of effect seen in PZP-treated wild horses.

Two Phenomena

This study draws attention to two additional phenomena that must be taken into consideration when examining the effects of PZP contraception in wild horses. First, there is a considerable spread in the percentage of foals born in season among the eight different populations referenced previously. This in turn requires, to assess any possible effects, that careful examination of foaling patterns be conducted prior to PZP treatment. If 23% of foals normally are born out of season among Sable Island horses, and only 4% are born out of season among New Forest ponies, these figures must be incorporated into any assessment of PZP treatment effects on a site-specific basis. Put another way, individual herds may be predisposed more or less to out-of-season births among PZP-treated animals, depending on their normal foaling patterns.

The second interesting aspect of this study is that out-of-season births in untreated mares have been increasing on ASIS since 1984, from 12% to 26% in 2001. The causes for this phenomenon are unknown but merely may reflect increased

variation coincident with increased population size. There were 80 horses on ASIS in 1984. Currently, there are 173.

A Surprising Discovery

The discovery that month of birth has no effect on survival on Assateague Island is surprising considering the harsh winter ocean-side weather patterns and storms. This suggests that horses living in milder climates such as the Shackleford Banks of Cape Lookout, NC, where horses also are being managed with PZP, will not suffer from out-of-season births whether they are associated with PZP treatment. In harsher climates, however, such as mountainous country where significant snow accumulates, the survival data derived here may not be valid or predictive.

The lack of any effect on survival as a function of PZP treatment in this retrospective study was expected. In the original study of PZP contraception in wild horses (Kirkpatrick, Liu, et al., 1990), 60% of treated animals were pregnant at the time of PZP treatment; survival of the foals was no different from that of foals born to untreated mares. This is consistent with the mechanism of the PZP vaccine: fertilization block. There is no known physiological link between an immunological block to fertilization and postfertilization gestational physiology.

SUMMARY

The use of PZP immunocontraception among selected wild horse populations can be carried out without fear of causing out-of-season births. Any population control measures applied to wild horses on public lands will come under severe scrutiny. The possibility of causing the death of foals, even indirectly, will be unacceptable to the public. The data in this retrospective study indicate that PZP immunocontraception is not implicated in out-of-season births and that this characteristic is driven by the unique reproductive physiology of equids. The difference in the percentage of out-of-season births between different wild horse ranges, and even the changing figures for the same herds over time point to the need to understand the reproductive biology of each individual herd before population models are constructed or contraceptive management plans are developed.

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The Effects of Porcine Zona Pellucida Immunocontraception on Health and Behavior of Feral Horses (*Equus caballus*)

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Abstract

The horse population on Shackleford Banks, a barrier island off the coast of North Carolina, has been controlled using a program of porcine zona pellucida immunocontraception since 2000. Prior studies found no negative physiological effects of the immunization, but suggested some adverse effects on behavior such as increased harem switching and extended reproductive season. Although no mares were immunized in the year of study (2013), we examined the effects of past contraception and foaling on health and behavior of the mares in an attempt to better understand its impacts on both the individual and the population. Current lactation was found to be the best predictor of health and behavior: lactating mares were in worse physical condition, spent more time grazing, and switched harems fewer times. Mares that had never foaled also were in better physical condition than mares that had foaled in the past, but did not differ in behavioral measures. There were indications that the PZP treatment has had effects other than preventing pregnancy: an increased number of years since immunization was correlated with better body condition and fewer harem switches, independent of lactation. Contraception treatment intensity or timing did not have any significant adverse effects on health and behavior on its own. However, three or more consecutive years of treatment or administration of the first dose before sexual maturity may have triggered infertility in some mares. Inducing sterility, while relieving the mares from the energetic costs of lactation and reducing the stress from harem switching, may have unintended consequences on population dynamics by increasing longevity and eliminating the mares' ability to contribute genetically.

Chapter 1
Introduction

Shackleford Banks, a small barrier island located off the coast of North Carolina, is home to a herd of approximately 100 feral horses (*Equus caballus*). The horses are of unknown origin, but have lived on the island for multiple centuries. In 1986, the cattle, sheep, and goats inhabiting the island were removed in an attempt to restore the vegetation to its native state. Released from the competition of the other herbivores, the horse population grew exponentially, reaching 185 in 1996. The Shackleford Banks Wild Horses Protection Act was passed in 1997 to ensure proper management of the herd to maintain its size between 100 and 110 horses (United States Congress, 1997). To that end, the National Park Service instituted a program of porcine zona pellucida (PZP) immunocontraception in 2000. Each spring, some of the females of reproductive age are immunized with the contraceptive agent, preventing them from conceiving in the upcoming summer breeding season. Other than the contraception, the management of the Shackleford Banks herd is "hands-off," with no human intervention such as feeding or veterinary care.

Despite its many advantages as a method of population control, prior studies both on Shackleford Banks and elsewhere have brought up potential concerns about its effects. This study examined the effects of the past 13 years of contraception treatment on the mares of Shackleford Banks in an effort to more fully understand its implications for the health and behavior of both the individual mares and the population as a whole.

1.1 Horse Behavior

1.1.1 Sociality

The horses of Shackleford Banks live in harems, the social system typical of horses and other equids. A harem usually consists of one or two stallions (sexually mature males), one or more mares (sexually mature females), and their immature offspring. Bachelor stallions are also found on the island, either alone or in groups. In the 1980s, the Shackleford herd consisted of temporary assemblages of horses of both genders, territorial harems that defended a permanent territory and water source, as well as non-territorial harems (Rubenstein, 1981). Since then, all Shackleford harems have transitioned to being non-territorial, inhabiting overlapping home ranges (Nuñez, Adelman, & Rubenstein, 2010; personal observation). Living in such groups has advantages such as increased vigilance and subsequent safety during grazing, as well as decreased harassment from unfamiliar stallions and aggression from other mares (Linklater et al., 1999; Rubenstein, 1986; Rubenstein, 1994). However, increased contact with other individuals can cause increased parasite infection and decreased health in the subsequent year (Rubenstein, 1986; Rubenstein & Hohmann, 1989).

1.1.2 Reproduction

The breeding season of feral horses runs between late winter and early fall. During the mare's period of estrus, the frequency of her interactions with the stallion increases; she often approaches him and assumes a receptive "sawhorse" posture. The precopulatory sequence includes vocalizations, sniffing, nuzzling, flehmen response (baring of upper teeth), and mounting without erection (McDonnell, 2000).

After successful conception, there is an 11-12 month gestation period. This timing ensures that foals are born in late spring or summer, when food is most abundant and the climate hospitable for survival (Rubenstein, 1994). During pregnancy, a female is forced to invest her energy in the production of fetal, uterine, placental, and mammary tissue; subsequent lactation is the most energetically costly part of foal-bearing (Gittleman & Thompson, 1988). Because of this investment in production and lactation, nursing mares

often are found to be in worse physical condition than non-nursing mares (Ransom, Cade, & Hobbs, 2010; Rubenstein, 1986; Turner & Kirkpatrick, 2002).

1.2 Horse Health Measures

There are multiple ways to measure health that are relevant to the study of feral horses. The first of this is the overall physical condition, as quantified by a body condition score indicating the amount of fat in the rump. For horses in the wild that are not able to be handled, this score is determined by examining the horse's rump, from behind, looking at the curve between the spine and the curve of the hip. On a commonly-used five-point scale, a body condition score of 5 indicates the best physical condition and 1 the poorest, as the amount of fat decreases and the rump takes on a more concave shape (Nuñez et al., 2010; Rubenstein, 1986).

Another, less direct, way of measuring overall health of a horse is to examine the amount of time it spends grazing. Horses spend a substantial portion of their time feeding on large quantities of low-quality grasses, so foraging success is limited by the time spent grazing (Rubenstein, 1994). Horses in lower body condition are likely to spend more time grazing, because they are in need of more energy to compensate for their poorer health (Ransom et al., 2010).

Endoparasite infection can also be used as a more specific indicator of health, measuring the health of the immune system. An increased number of endoparasites in the intestinal tract, as measured by the number of parasite eggs excreted in feces, signify a weaker immune system that is unable to fight off the infection, and thus a less healthy horse. The primary parasites found in horses are nematodes of the family Strongylidae, commonly known as "strongyles," which are divided into large and small strongyles (cyathostomes), based on their size (Lichtenfels, 1975). These parasites can cause anemia, diarrhea, and intestinal damage, and inflammatory enteropathy, respectively (Love, Murphy, & Mellor, 1999; Stoltenow & Purdy, 2003). Other nematodes that are commonly found in horses include large roundworms (*Parascaris equorum*) that damage the intestines, threadworms (*Strongyloides westeri*) that cause diarrhea, and pinworms (*Oxyuris equi*) that cause irritation of the anus (Stoltenow & Purdy, 2003). One study on Shackleford Banks by Rubenstein and Hohmann (1989) found the eggs of those

parasites, among others, in the feces of the horses and found that egg load was negatively correlated with body condition in the following year.

1.3 Porcine Zona Pellucida Immunocontraception

1.3.1 Vaccine Development

The porcine zona pellucida (PZP) vaccine is a form of *immunocontraception*: contraception that uses the animal's own immune system to render her infertile. The zona pellucida of a pig embryo (the outer glycoprotein membrane) is used to make a vaccine. When this vaccine is injected into the female animal, it acts as an antigen to which the immune system produces antibodies; these antibodies then cross-react with her own eggs, binding to the zona pellucida and preventing sperm attachment and subsequent fertilization (Sacco, 1977).

An early study on feral horses found that of ten PZP-immunized mares released back into their natural habitat, only one became pregnant within eight months. Anti-PZP antibodies were found to remain high in the immunized mares, demonstrating the importance of the antibody titer in the contraception's efficacy (Liu, Bernoco, & Feldman, 1989). Further studies on the feral horse herd inhabiting Assateague Island confirmed the vaccine's potential to control populations of feral horses, with a 90-100% success rate at preventing pregnancy in the year of immunization (Kirkpatrick et al., 1997; Kirkpatrick, Liu, & Turner, 1990).

The PZP vaccine has also been used for population control of deer and exotic zoo animals (Kirkpatrick et al., 1996; Kirkpatrick et al., 1997).

1.3.2 Implementation on Shackleford Banks

The PZP contraception program on Shackleford Banks was initiated by the National Park Service in 2000. Each year, the decision of which mares to treat is made based on various factors, such as the representation of her genetic line in the population and her foaling history (S. Stuska, personal communication). Between 2000 and 2009, an average of 63% of mares were inoculated each spring to prevent conception in the upcoming breeding season (Nuñez et al., 2010). However, the mares were not treated in

2010, 2011, or 2012; in those years, the population was deemed to be small enough and the trend in foaling and mortality indicated that the treatment was not needed. The exception to this was the mare Anastasia, who was treated in 2012 to prevent her from foaling because her low body condition and prior failed pregnancies indicated she was not healthy enough to reproduce. The contraception was resumed in the spring of 2014, the year after this study (S. Stuska, personal communication).

1.3.3 Practicality

PZP immunocontraception is an advantageous form of population control for several reasons. First, preventing pregnancy controls the population's size without having to physically remove foals from the herd, which is not always accepted by the public (Nuñez, 2009). Because PZP causes infertility through activation of the immune system, it does not alter hormones, which could have adverse effects; the mares continue to cycle normally throughout the year, and just fail to become pregnant after insemination. The ease of administration is also an advantage because the vaccine can be administered using a long-distance dart gun that eliminates the need for potentially stressful human interaction.

The PZP vaccine has been found to have no effect on somatic tissues because the antibodies only bind to egg tissue (Barber & Fayrer-Hosken, 2000). It also has no effect on intact pregnancies (foals born to an immunized mare are healthy a year after birth) and is reversible once the antibody titers naturally decrease over time (Kirkpatrick et al., 1990). Studies on herds other than Shackleford Banks have found no differences in behavior – activity budget, aggression, harem stability, spatial relationships with the stallion – between treated and non-treated females in a given year (Kirkpatrick et al., 1990; Powell, 1999).

1.3.4 Concerns

Recent research has discovered potentially significant effects of the contraception on behavior and health, especially in the Shackleford herd. Multiple studies have found that treated mares switch harems more times than non-treated mares, both in the reproductive and non-reproductive seasons (Madosky et al., 2010; Nuñez et al., 2009). Increased harem switching, in turn, is correlated with higher levels of fecal cortisol, an

indicator of physiological stress (Nuñez et al., 2014). Additionally, females that have been given PZP contraception were found to have an extended reproductive period past the normal breeding season, which could result in foals being born at times not optimal for their survival (Nuñez et al., 2010). In other herds in the western United States, treated females received more reproductive interest from stallions than the mares that had not been treated, though all other behaviors were not affected (Ransom et al., 2010).

There also may be detrimental effects of continued treatment on the mares' ovarian function. Assateague Island mares that had three consecutive years of treatment had depressed estrogen secretion, suggesting impaired ovarian function, but they all returned to fertility within three years of treatment. Mares that had more than three consecutive years of treatment took even longer to return to fertility after they were released from treatment, up to six or seven years (Kirkpatrick et al., 1992). It is possible, then, that even further treatment with PZP may permanently damage mares' ovaries.

1.4 The Current Study

The PZP contraception program was implemented in the Shackleford Banks horse herd to reduce its size in a way that would eliminate the need for foal removal. However, potential negative effects of the contraception on mare health and behavior must be explored in order to fully evaluate this program. Changes in the immunized mares' behavior, such as those found in prior studies, may have more far reaching effects that should not be ignored.

During the year of this study (2013), no mares on Shackleford Banks were given PZP contraception, so there were no true "treated" and "untreated" categories, and the direct effects of contraception could not be distinguished as in previous studies. Instead, we measured various indicators of health and behavior and first analyzed the relationships between them in an attempt to understand the interactions between the social and physical domains. We then examined each mare's contraception history since the program's induction in 2000, including factors such as intensity and timing of treatment and foaling history. Relating this history to the mares' health and behavior in the year of study will help us further understand the long-term effects of the treatment on the mares, as well as the implications for the entire population.

Chapter 2
General Methods

2.1 Study Site

This study was conducted on Shackleford Banks, a barrier island about 15 km long (east-west) and varying between 0.5 and 3 km in width (north-south). It is located approximately 3 km off of the coast of North Carolina and is a part of the Cape Lookout National Seashore, managed by the National Park Service. The island is comprised of a variety of habitats, including marsh, forest, swale, dunes, and beach. A maritime forest lines the majority of the north coast, separating the bayside beach from the dunes and swales covering the rest of the island; marshes are spread out along the north side. The island can be divided into three regions, distinguishable by landscape and each containing at least one permanent water source. The western end of the island contains more tall dunes, which flatten out into sparser dunes (and more flat swales) in the mid-east. The far-east is home to large areas of marshland, including a "submerge" marsh area that floods during high tide.

2.2 Study Subjects

During the majority of the study, the number of horses on the island held steady at 106; for a brief period there were 105, after the death of one young male (Scorpio) on July 4 and before the birth of a female foal (Djambi) on July 20. Most horses formed harems of one stallion, multiple females, and their young offspring. Two of the harems

also had an established beta stallion, and one had a suspected beta (Shakespeare, a four year old stallion still in his mother's harem). Additionally, several bachelor stallions roamed with no females. The harems inhabited overlapping ranges and were not territorial, as was found in the past (Rubenstein, 1981). There were five harems in the west, five in the mid-east, and seven in the far-east, ranging in size from three to 11 horses; throughout the study, about half of the females changed harems at least once. Of the 61 mares in the population, data was collected on only 45 of them because of ease of location and data collection.

2.3 PZP Contraception

In 2000, the National Park Service instituted a porcine zona pellucida (PZP) contraception program to control the growing horse population on the island. Eight mares were designated as controls based on age, prior breeding success, and genetic representation in the population. Each year, between late February and April, an average of 63% of the eligible mares were inoculated (range 37-88%); mares were given their first dose when they were approximately two years old. Each vaccine contained 100 μg of PZP mixed with an adjuvant (Nuñez et al., 2010). The National Park Service made the decision about which mares to inoculate each year; the researchers of this study had no involvement in the decisions. Whether or not a mare was inoculated was based on the representation of her genetic line; if a mare's line was not well-represented in the herd, then she was not administered the contraception so that her genes would have a chance to be passed on in the population (S. Stuska, personal communication). In 2009, the controls were administered the vaccine primarily because of their increased productivity. In the years after 2009, the Park Service did not inoculate mares because the herd was at a manageable size. Only one mare (Anastasia) was given contraception in 2012 and 2013 to prevent her from conceiving because she was in poor physical condition and pregnancy could have posed a danger to her survival. Contraception history of the mares used in this study can be found in Appendix I, along with foaling history.

2.4 Data Collection

Data was collected between June 13, 2013 and August 7, 2013.

2.4.1 Behavioral Sampling

When we located a harem, we identified its members using several factors: sex, coat color, mane direction (falling to the left or right of the head), facial markings, and brand (number, location on rump, brightness). Scan sampling methods were used to record the behavior of each member every three minutes for a one hour period (Altmann, 1974). The different behaviors that were differentiated can be found in Table 2.1. Additionally, during the hour period, we recorded the following qualitative observations:

- Incidences of reproductive interest (including genital sniffing, nose-to-nose behavior, rump rubbing, mounting, and copulation)
- A stallion herding some or all of his mares

We also noted interactions between mares that demonstrated dominance, including displacements from food or water sources and direct physical conflict/fights, both during the scan sampling periods and at other points during general observation.

2.4.2 Fecal Sampling and Testing

Samples of the mares' feces were collected immediately following defecation, before the stallions could mark them with urine. Portions from multiple fecal balls were collected to ensure a representative sampling of the pile. Samples were placed in plastic bags, labeled with the mare's name and the date and time of collection, and stored in

Table 2.1. Behaviors observed and recorded during scan sampling.

Behavior	Description
Grazing	Horse ingesting food with the head down, including grazing while walking
Standing	Horse standing still, but not alert
Walking	Horse walking while not grazing
Vigilant	Horse's head up and alert, i.e. looking at a nearby horse
Resting	Horse sitting or laying down
Grooming	Horse biting or scratching itself or another horse
Urinating/defecating	(when occurring exactly at time of data point collection)
Herding	Stallion herding mares, or mare being herded by stallion
Nursing	Mare nursing her foal
Fighting	Horse engaged in antagonistic interaction with other horses, i.e. kicking or chasing
Drinking	Horse drinking or digging for water
Reproduction	Horse engaging in mating behavior, i.e. genital sniffing, nose-to-nose, mounting
Out of sight	Horse not in sight of observer, i.e. behind a dune or in the forest

coolers with frozen ice packs until they could be taken back to the laboratory on the mainland.

Collecting the feces took preference to behavioral sampling, if being done simultaneously, and occasionally caused missed time points during scan sampling (which were then treated as "out of sight" points during analysis). If, after the hour-long behavioral sampling period, some of the mares had not yet defecated, we remained with the harem in an attempt to collect as many fecal samples as possible.

The eggs in each fecal sample were counted using a variation of the McMaster technique. Three grams of feces were measured using a hanging scale and a plastic bag and mixed with 12 ml of tap water in a 50 ml beaker using a tongue depressor. The mixture was poured through a tea strainer into another beaker to filter out the plant material, using the tongue depressor to press out as much of the liquid as possible. The resulting liquid was poured into a 15 ml disposable centrifuge tube, which was spun on a manual centrifuge for two minutes, and the supernatant was discarded. Ten ml of saturated salt solution (made by dissolving 400 g of table salt in 1 L of tap water) was poured into the centrifuge tube on top of the spun-down pellet. The tube was then covered and shaken manually to loosen the sediment from the bottom. The suspension was poured from the centrifuge tube into a clean 50 ml beaker and an additional 35 ml of salt solution was added. The solution was allowed to settle and the eggs to float to the surface. Using a disposable pipette, a sample of the eggs was drawn from the top of the solution and transferred into one chamber of a McMaster slide. The other chamber was filled with a new sample from a different section of the fecal float. The slide was transferred to a microscope and all of the eggs in the slide's grids were counted. The number of eggs in both chambers cumulatively was multiplied by 50 to calculate the number of eggs per gram of feces.

Eggs of strongyles could not be identified by species, but instead were grouped into "large strongyles" and "small strongyles." Eggs of the species *Parascaris equorum*, *Strongyloides westeri*, *Oxyuris equi* (pinworm), and *Anoplocephala spp.* (tapeworm) were identified separately; other eggs were counted in an "other" category, and adult worms were noted but not included in the egg counts. The counts of the various egg types were added together to give a total number of eggs per gram in the fecal sample.

Photographs of some of the eggs observed in the mares, including the common large strongyles, can be seen in Appendix II.

2.4.3 Body Condition Scoring

We gave each mare a body condition score based on the amount of fat in her rump, determined by examining the curvature of the rump between the spine and the point of the hip, as seen from directly behind her. Scores were allocated on a scale from 1 to 5, with 1 being the poorest condition and 5 being the best. A mare with a body condition of 3 displayed a flat line between the spine and the hip. A score of 4 manifested in a smoothly rounded rump across the spine, and a 5 had an "apple" shape, with the rump bulging above either side of the spine. In a horse with a body condition lower than 3, the rump muscle took on a gradually more concave shape. Physical condition of the mares was assessed at multiple times throughout the data collection period, and an average body condition score was calculated. Photographs of horses with common body condition scores can be found in Appendix III.

Chapter 3
Mare Health and Behavior

3.1 Introduction

Data collection, as described in Chapter 2, included measuring various indicators of the mares' health and behavior. Health was measured in two main ways: body condition and parasite load. Physical condition, as quantified by a body condition score between 1 and 5, revealed the mare's average overall health; a healthier horse would have more fat deposits in her rump, giving her a higher body condition score. Parasite load – the number of parasite eggs per gram in her feces – measured a more specific factor of health: immune function. A healthier mare with a stronger immune system should be better able to resist parasite infection and have a lower egg count at the time of sampling.

Behavior, in the form of the amount of time spent grazing, was also used to assess the mares' health. A mare in worse health would theoretically need more energy and subsequently spend more time grazing as a percentage of her total time budget. Additional behavioral factors such as harem switches, harem size, harassment rate, stallion temperament, and mare dominance were also measured to form a quantitative and qualitative description of the mares' social environment.

In this chapter, we describe the creation and calculation of these various health and behavior indices. We then examine the relationships between the variables in an attempt to describe what shapes the health and behavior of the mares. We also look at the effects of lactation on the mares before turning to the effects of contraception in the next chapter.

3.2 Data Analysis

From the various data collected (see Chapter 2), the following indices and variables were calculated to describe the social environment and health of the mares.

3.2.1 Time Budgets

During each one-hour time budget collection, 21 events were possible (one every three minutes for 60 minutes). The total number of events observed was then:

Events = 21 – # "Out of sight" instances

For each type of behavior (see Section 2.4.1), the proportion of time spent doing that behavior was calculated as:

Proportion(behavior) = # Instances of that behavior / # Events

This resulted in a calculation of how much of the time during that hour-long observation the horse spent on each type of behavior. Time budgets were collected throughout the study period, and an average proportion of time spent on each behavior was calculated by averaging the proportions from each individual time budget. For example, each mare had a data point of *Average time spent grazing*, given as a proportion (0.0-1.0).

3.2.2 Parasite Egg Counts

Over the period of data collection, we attempted to collect at least three fecal samples for each mare. This goal was met for all but a few mares because of difficulty finding them or a lack of defecation during observation. The samples were collected at times spread throughout the nine week study period. We first needed to determine if there was a temporal pattern of parasite infection. If there was none, then the egg count data could be pooled into an average for the entire study period; otherwise, we would have to look at the parasite data from the time periods separately.

To do this, we first had to find a date in the study period that would divide it approximately in half in terms of number of fecal samples in each half for each mare. For five dates that appeared to split the data approximately in half, the number of fecal samples before and after that date was recorded for each mare. For some mares, the different dates would not change their sample distribution (i.e. would have two samples in one half and one sample in the other half). The mares for which the choice of date

would change the distribution (i.e. turn a 2:2 to a 1:3) were then examined, and the date was chosen that had the least number of "bad" splits (1:3 instead of 2:2, 0:2 instead of 1:1, etc.). The "middle" date was determined in this way to be July 13.

We then needed to statistically determine if the parasite egg counts were significantly different between the two halves of the study period to see if they could be considered together in further analyses. Some mares only had samples collected in one half or the other, so they were excluded from this test. For each of the 29 mares with samples both before and after July 13, we calculated the mean number of total eggs found in the fecal samples during each time period – a *first half mean* and a *second half mean*. Then the difference between the second half and first half means was calculated for each mare. The resulting distribution was analyzed using a t-test against 0. The mean difference in the number of eggs between the second half and the first half was 308.929 eggs per gram (more eggs in the second half) and the t-test gave a p-value of 0.0107. This showed that the egg counts from the two halves of the summer were significantly different so the samples could not just be lumped into an overall mean for the whole study period. Therefore, each mare had two separate data points: *first half mean eggs* and *second half mean eggs*. The value for *total mean eggs* was also recorded, with the acknowledgement that it was not as significant of a measurement as the separate half means. The counts were all log-transformed to improve normality.

3.2.3 Age

The age of each mare was calculated as:

$$Age = 2013 - Birth\ year$$

This did not account for exact birth day/month but rather grouped the mares into approximate ages based on the year in which they were born. Because the foaling season is limited to a few months during the summer, this gave an accurate approximation of age to within a few months.

3.2.4 Lactation in 2013

Each mare was assigned a value for the categorical variable *Lactating in 2013.* If she was nursing during the study period – either a foal born in 2013 or a yearling – she was

said to be lactating (referred to in data analysis and graphing as "L"). If she was not nursing a foal, she was non-lactating ("NL"). In the mares studied, 10 were lactating and 35 were non-lactating. Two of the non-lactating mares (Hermione and Wallace) gave birth later in the 2013 foaling season after the study period was complete, and one gave birth in 2012 and in 2014 (Sydney). Because this was not during the period of study, these three mares remained in the non-lactating category for analyses.

3.2.5 Stallion Associations

In order to evaluate the mares' associations with different stallions throughout the data collection period, a large table was made with the mares' names down the left side and stallions' names across the top. A list of all sightings of all harems throughout the data collection period had been made previously. For each mare, we went down the sightings list and noted (in order) which stallion she was seen with. At each sighting, a tally was made in the column of that stallion. If she was seen with stallion A at one sighting, and then stallion A was seen without her at a subsequent sighting, a tally was made in an "unknown" column; she was no longer with that stallion but we could not identify her location on that day. From this, the *Primary stallion* could be identified: the stallion with which the mare was seen with the greatest number of times. From this, *Percentage of time with primary stallion* was also calculated: the number of times seen with the primary stallion divided by the number of total times seen.

3.2.6 Harem Switches

When going through the list of horse sightings for stallion associations (as in Section 3.2.4), switches between harems were also noted. If a mare was seen with stallion A at one sighting, then with stallion B at the next sighting, that was tallied as one harem switch. If she returned to stallion A at a later time point, that was another switch. Additionally, if she was seen with stallion A at one sighting, and then stallion A was seen without her at a subsequent sighting, this was considered a switch despite the fact that her exact location was unknown at the time of that sighting. If she was then seen again with stallion A, this was considered two switches (leaving stallion A to an unknown other location, then coming back). From these tallies, we determined the total *Number of harem switches* for each mare. Because a significant number of mares (25 mares, 56%)

did not ever switch harems, the mares were divided into two categories, "switchers" and "non-switchers."

3.2.7 Harem Size and Variation

The list of harem sightings was again traversed, and for each sighting of the mare, the number of horses in her harem (the one she was with at that specific time point) was noted. From those values, *Average harem size* was calculated for each mare.

The *Variance in harem size* was also calculated for each mare as a measure of group stability. This was done using the individual harem size values pulled from the sightings list, as above. Then the *Standard deviation of harem size* was calculated (square root of the variance). The *Coefficient of variance of harem size* was also calculated as a way to normalize the variance.

Coefficient of variance = Standard deviation / Average harem size

3.2.8 Stallion Harassment

The data necessary to determine the rate of mares' "harassment" by the stallions was extracted from the qualitative observations made during each hour-long behavioral observation (see Section 2.4.1). The behaviors of note were herding (when the stallion physically caused a mare to change location) and sexual approaches/behaviors (genital sniffing, nose-to-nose behavior, rump rubbing, mounting, and copulation); these behaviors will hereafter be referred to together as "harassment." The record of qualitative observations was traversed, and each time a herding or sexual behavior was observed, a tally was made for all mares involved. Adding up all instances for a mare gave a total number of harassment instances during all observation times. In order to standardize these numbers into a rate of harassment, we added up the total time that each mare was observed (i.e. the number of time budgets multiplied by their length). The only time budgets considered were those done by the primary researcher, because the other researchers only collected time budgets, not qualitative data, during their behavioral observations. From this the *Harassment rate,* a measure of total stress behaviors per hour, was calculated:

Harassment rate = Total herds and sexual approaches /
Number of hours of observation

3.2.9 Stallion Temperament

The temperament of the stallion with which each mare was primarily associated was used to measure a social variable similar to her harassment rate. However, rather than just measuring the harassment behaviors directed at the mare in question, it determined the overall harassment in her social environment by determining if her primary stallion was "mean" or "docile." When the record of qualitative observations was traversed (as in Section 3.2.7), in addition to tallying herding and sexual behaviors for each mare, it was noted which stallion was initiating such behavior. For harems that had both an alpha and a beta stallion, their harassment behaviors were added together. This resulted in a total number of "harassments" for each stallion/harem, which also needed to be standardized into a rate; this was done in the same way as the *Harassment rate* for the mares (Section 3.2.7). In order to classify the stallions based on aggression, the median value of *Harassments per hour* for the stallions was found to be 0.5. Stallions with *Harassments per hour* below this median were considered "docile," and above the median, "mean." Then, using this *Stallion quality* index, each mare was assigned a variable *Quality of Primary Stallion*, either *mean* or *docile.*

3.2.10 Mare Dominance

Dominance interactions between mares were recorded whenever they were seen (both during the hour-long behavioral observations and at other times) in an attempt to get the most accurate picture of harem hierarchy (Section 2.4.1). If mare A displaced mare B from her grazing spot, mare A was considered the "winner" of that interaction and mare B the "loser." Similarly, mare A chasing, biting, or otherwise fighting mare B was the "winner" of the interaction. From these observations, the total number of "wins" and "losses" were added up for each mare. The number of wins divided by the total interactions (wins + losses) determined *Percent wins*. From this, an index of *Dominance rank* was created; this assigned a rank to only the mares involved in dominance interactions, as some were not observed in any such interactions during the entire study period. "Low" dominance mares were those with a *Percent wins* of less than 50%. Those with a value of exactly 50% were considered "Neutral," and above 50% were "High" dominance.

3.3 Statistical Analysis

All statistical analyses were performed in JMP Pro (version 10.0.0). Quantitative data was checked for normality by histograms. Most data was not normally distributed, so nonparametric statistics were applied. However, the parasite egg count data was able to be made more normal through log-transformation, so parametric statistics were applied. T-tests performed were all two-tailed tests, because the null hypotheses were that the two groups were different (no directionality). When variances in two groups were determined to be unequal, a Welch's test was used. Significance was determined when $p < 0.05$.

3.4 Results

3.4.1 Relationships between Health Variables

Among all 45 mares in the study population, a nearly significant trend of negative correlation was found between body condition score and percentage of time spent grazing ($F_{1,43} = 4.03$, $r = 0.06$, $p = 0.0511$, Figure 3.1). No correlations were found between body condition score and parasite egg counts (first or second half mean, or total mean), or between the parasite egg counts and the time spent grazing.

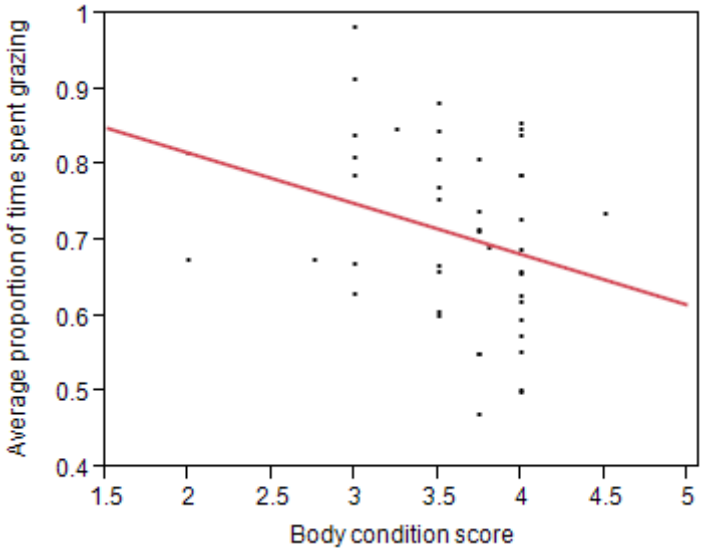


Figure 3.1. Average time spent grazing, as a proportion of the total time budget, by body condition score ($F_{1,43} = 4.03$, $r = 0.06$, $p = 0.0511$).

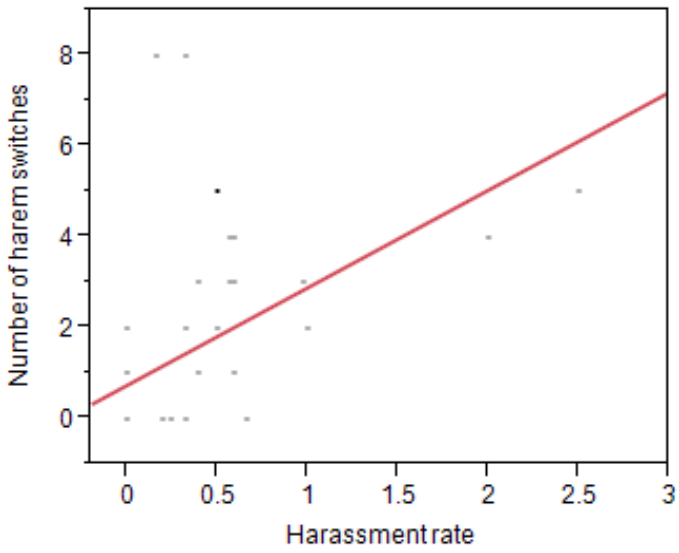


Figure 3.2. Number of harem switches during the study period by rate of harassment (number of reproductive behaviors and herding per hour of observation) ($F_{1,43} = 15.73$, $r = 0.25$, $p = 0.0003$).

3.4.2 Harem Switches

No correlation was found between the number of harem switches and body condition ($F_{1,43} = 0.24$, $p = 0.628$). There was no significant difference in body condition between those mares that switched harems at least once and those that did not switch at all ($t_{39} = 0.67$, $p = 0.5091$), nor a correlation between number of switches and body condition within the switchers. Number of harem switches was also not found to be correlated to parasite egg counts (first or second half mean, or total mean) or time spent grazing. However, a positive correlation was found between the number of harem switches and the rate of harassment ($F_{1,43} = 15.73$, $r = 0.25$, $p = 0.0003$, Figure 3.2).

3.4.3 Harassment Rate

Other than the relationship with harem switches (above), harassment rate was not found to be correlated with any of the other health and behavior indicators (body condition, parasite egg load, time spent grazing, dominance rank).

3.4.4 Average Harem Size and Variance

The average size of the harem with which the mare cohabitated was not correlated with body condition, parasite egg counts (either half mean or total mean), time spent

grazing, harassment rate, or harem switches. Variance in average harem size (*Coefficient of variance of harem size*) was also not correlated with those variables.

3.4.5 Dominance

Dominance rank (from the variable *Dominance rank*, which only included mares that were observed participating in interactions with other mares) was not correlated to parasite egg loads (either half mean, or total mean), time spent grazing, number of harem switches, or harassment rate. However, mean body condition was significantly higher for mares of high rank ($Z = -2.15$, $p = 0.032$, Wilcoxon test); mares of high dominance had an average body condition score of 3.85, and low dominance, 3.45).

3.4.6 Stallion Temperament

Stallion temperament, either "docile" or "mean" based on the number of aggressive actions towards his mares, was not correlated to any of the relevant variables (body condition score, parasite egg loads, time spent grazing, number of harem switches).

3.4.7 Age

Age was not significantly correlated to body condition, parasite egg loads, number of harem switches, or harassment rate. There was a trend for increased age to be correlated with decreased average time spent grazing ($F_{1,43} = 3.96$, $r = 0.062$, $p = 0.0531$).

3.4.8 Lactation Status

Lactating mares had a significantly lower average body condition than non-lactating mares ($t_{43} = 5.21$, $p < 0.0001$); average body condition was 2.98 for lactating mares and 3.75 for non-lactating mares (see Figure 3.3a). There were no significant differences in mean parasite egg load over the entire summer; notably, the ranges of average fecal egg counts were approximately equal for the two groups ($t_{37} = 1.17$, $p = 0.2485$, Figure 3.3b). Lactating mares spent more time grazing as a percentage of their average overall time budget, 79.64% in relation to the non-lactating mares' 68.59% ($t_{43} = -2.75$, $p = 0.0087$, Figure 3.3c). An analysis of variance model found that the main effect of lactation status on body condition was significant ($F = 16.21$, $p = 0.0002$), but the main

effect of time spent grazing was not significant ($F = 0.19$, $p = 0.6692$), nor was the interaction ($F = 0.005$, $p = 0.9442$); non-lactating mares had a higher average body condition, and the proportion of time spent grazing had no correlation to body condition when the variance due to lactation was removed (Figure 3.4).

Lactating mares switched harems fewer times than non-lactating mares: 0.40 and 1.74 times during the study period, respectively ($F_{1,39.8} = 8.21$, $p = 0.0066$, Welch's test, Figure 3.3d). Similarly, lactating mares also spent more time with their primary stallion ($F_{1,42,4} = 12.99$, $p = 0.0008$, Welch's test). There was a trend for lactating mares to have a lower harassment rate, but not significantly (Welch's test $F_{1,25.6} = 2.63$, p = 0.1174).

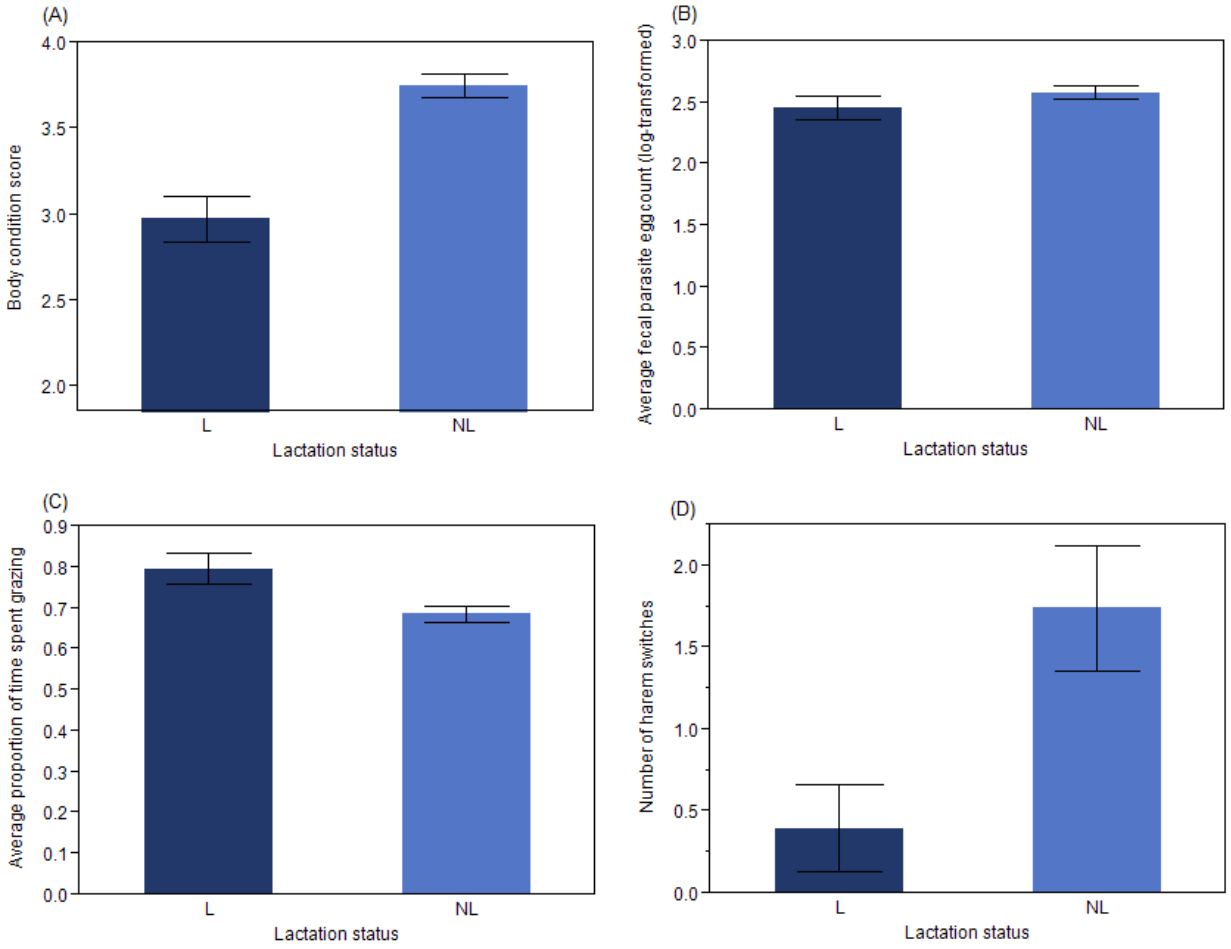


Figure 3.3. (A) Body condition score by lactation status (means ± S.E., $t_{43} = 5.21$, $p < 0.001$). (B) Fecal parasite egg count, averaged over the entire study period and log-transformed, by lactation status (means ± S.E, $t_{37} = 1.17$, $p = 0.2485$). (C) Average time spent grazing, as a proportion of the total time budget, by lactation status (means ± S.E, $t_{43} = -2.75$, $p = 0.0087$). (D) Number of harem switches during the study period by lactation status (means ± S.E, $F_{1,39.8} = 8.21$, $p = 0.0066$, Welch's test).

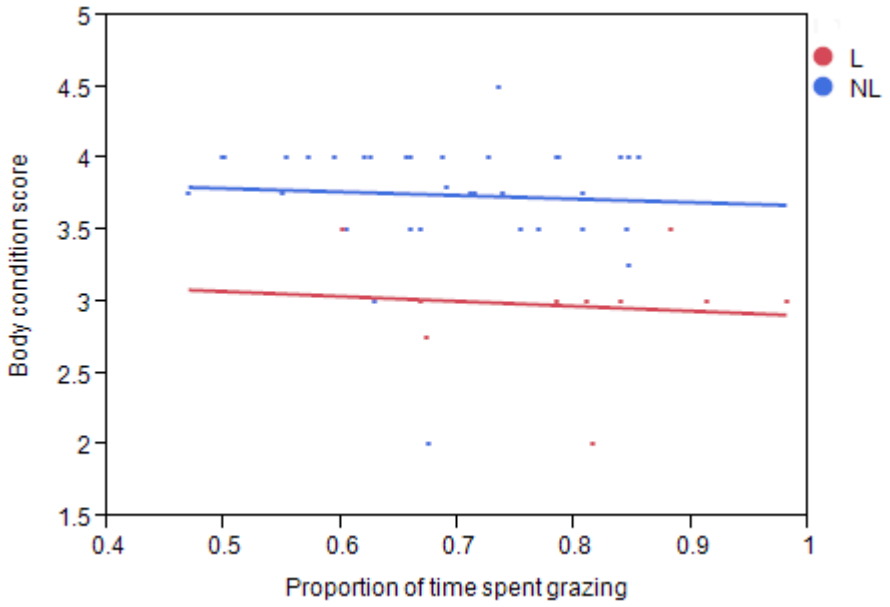


Figure 3.4. The separated main effects of current lactation ($p = 0.0002$) and proportion of time spent grazing (non-significant, $p = 0.6692$) on body condition score, with no significant interaction ($p = 0.9442$). L (red) = lactating; NL (blue) = non-lactating.

There was no significant difference in the distribution of dominance ranks between lactating and non-lactating mares ($\chi^2 = 0.321$, $p = 0.8516$). Lactating mares were on average 10.3 years old, significantly lower than the non-lactating mares average age of 15.37 years ($t_{43} = 2.46$, $p = 0.018$).

3.5 Conclusions

We proposed in this study to use parasite load, as quantified by the number of parasite eggs per gram of feces, to assess the mares' immune function and, indirectly, their health. However, the results from this primary analysis suggested that parasite egg load may not be a reliable or straightforward indicator. Egg counts were not significantly correlated with any other measure of health or behavior, including body condition score, which is a known measure of overall health. This, along with the fact that egg load did not differ between mares that are lactating or switching harems more (known physiological stressors), suggests that the parasite infection intensity is determined by a different mechanism. For this reason, we could not be sure what the egg levels were communicating, and could not use them to make any substantial conclusions about the immune health of the mares. Rubenstein and Hohmann (1989) found that parasite egg

counts were negatively correlated with body condition in the subsequent year; perhaps the same would be true if we measured body condition of these mares in the subsequent year. The body condition score will therefore be used in further analysis as the long-term average measure of health, disregarding the originally proposed finer scale measure of immune function through parasite eggs.

At first, we found a negative correlation between body condition score and time spent grazing, suggesting that less healthy mares were eating more, possibly to compensate for lower physical condition. However, this result was nullified when controlling for lactation. Lactating mares had a lower average body condition score. Analysis of variance modeling showed that there was no relationship between time spent grazing and body condition, as was initially believed, once this variance due to lactation was removed. The lactating mares, by spending more time grazing and also having lower body condition scores, drove the original correlation (Figure 3.1). This indicated that lactation, as a physical stressor that requires a large output of energy, required the mares to graze more to increase their energy input and simultaneously decreased their body condition.

Of the rest of the behavioral measures, only mare dominance rank was correlated with health as measured by body condition. Dominance was, however, not correlated with time spent grazing, suggesting that dominance and time spent grazing act on body condition in different ways.

The fact that the number of harem switches was not indicative of lower body condition was a surprising result. Prior studies on Shackleford Banks found that fecal cortisol levels were elevated during harem switching (Nuñez et al., 2014), but it seems that such physiological stress was not manifested immediately in body condition. The number of harem switches was not also correlated with time spent grazing; mares switching more were not taking time away from grazing, but rather from other activities such as sleeping.

Two behavioral measures, number of harem switches and rate of harassment, were positively correlated, but the direction of this correlation is ambiguous; a mare exposed to more instances of harassment (i.e. sexual advances and herding) from the stallion could switch groups more, or a mare switching more and not establishing stable social relationships could cause her to be harassed more by the stallion. Neither harassment nor harem switching were correlated with overall health, but they could still cause increased

physiological stress that was just not manifested immediately in body condition. Prior research on other herds has found that increased harassment is predictive of lower body condition (Linklater et al., 1999), but it is possible that just not enough instances of harassment were observed in this herd to make an accurate correlation. Additionally, the fact that harems switches did not differ based on stallion temperament suggest that it was the individual harassment, not the harassment occurring in the rest of the harem, that was related to harem switching. It is also possible that we were unable to sufficiently differentiate stallion temperament from the limited harassment data.

The fact that average harem size, harem size variation, or stallion temperament did not predict any changes in health or behavior indicated that they were not agents of stress significant enough to cause changes in health. This allowed us to assume that these factors did not need to be controlled for in further analyses, because they were not influencing our conclusions about health.

Age was similarly not correlated with body condition, suggesting that age is not influencing overall health. Increased age did predict decreased time spent grazing; this could be a function of lactation, because we found that non-lactating females were more likely to be older, and non-lactating females spend less time grazing. This could in turn be a function of increased contraception application in older females rendering them sterile, preventing them from lactating and therefore requiring less food intake, a topic which will be discussed further in the next chapter.

In addition to being correlated with lower body condition and more time spent grazing, current lactation was also a significant predictor of fewer harem switches. Remaining with a primary stallion would be favorable for a mare with a foal to ensure protection and reliable food and water sources. No difference was found in harassment rate between lactating and non-lactating mares. The fact that harem switches and harassment were previously found to be correlated, and switches but not harassment differed with lactation, could have two explanations: harem switches and lactation interact with harassment differently, or there were just not enough harassment instances observed to reach an accurate conclusion; we support the latter alternative.

From this preliminary investigation, we have concluded that body condition was a more reliable measure of health than parasite egg counts, which will hereafter be discarded as a health indicator. Lactation had significant effects on health and behavior:

non-lactating females had higher average body condition, spent less time grazing, and switched harems more. These differences will be taken into account in further analysis in the subsequent chapter.

Chapter 4
Contraception and Foaling History

4.1 Introduction

Each mare on Shackleford Banks has had a unique pattern of contraception and foaling. Mares born in the early 1990s were able to foal before the PZP program was begun in 2000. Some of those born after 2000 were able to foal before their first immunization, but some were not. The total number of times contraception was given varied, as well as if the doses were given in consecutive years or with breaks to foal in between. Such variation in contraception administration resulted in large variations in foaling frequency and timing. Each mare's contraception and foaling history since 2000 can be seen in further detail in Appendix I.

In this chapter, we use the contraception and foaling histories of each mare to explore the effects of PZP contraception on the mares' health and behavior, as measured by the variables described and deemed significant in Chapter 3 (body condition, time spent grazing, harassment rate, and harem switches). The different factors considered include: *recovery* from contraception, *timing* of contraception (first dose given before or after puberty, year of last dose), and *intensity* of contraception (number of doses, number of consecutive doses, and percentage of reproductive life treated).

4.2 Data Analysis

Multiple variables were created to describe the mares' history of contraception and

foaling. The abbreviation "BC," i.e. birth control, is used in place of "PZP contraception" in the names of variables for the sake of brevity.

4.2.1 Timing of Contraception

The year of last contraception was drawn from data provided by the National Park Service. This was used to calculate the number of years since the last dose of contraception:

$$\textit{Years since BC} = 2013 - \textit{Year of last BC}$$

This was an approximation, like age, ignoring slight differences in the timing of the last dose, instead providing a relative length of time since that dose.

Of significant interest was whether the mares were given their first dose of PZP before or after puberty and first foaling. The categorical variable *BC before puberty* was assigned to each mare: mares were given the value "no" if they foaled before their first dose, and "yes" if they were given the first dose before they were able to foal for the first time, either in the second or third year after birth.

Many of the mares were given their first dose of contraception before reaching sexual maturity (i.e. conceiving their first foal, at age two or three). A table was made distinguishing which mares had and had not foaled since the inception of contraception treatment, based on this timing (Table 4.1). A Chi-squared test was performed on this table to determine if differences in foaling based on timing differences were significant, or due to random chance.

4.2.2 Intensity of Contraception

The intensity of the contraception treatment for each mare was operationalized in three different ways. The first was the *Total number of years on BC*, a count of the number of separate years in which the mare was given PZP. The *Maximum number of consecutive years of BC* was also recorded as the maximum number of years in a row

Table 4.1. Foaling based on timing of first dose of PZP contraception.

	Foaled since PZP started?	
	Yes	No
PZP before puberty	7	14
PZP after puberty	13	7

that the mare was given PZP. The number of years that the mare had been of reproductive age between the start of the contraception program (2000) and the last possible year of reproduction (2012) was then determined; reproductive age was considered to start during the year in which they turned two years old. From this, the *Percentage of reproductive years on BC* was calculated:

% Reproductive years on BC = # Years on BC / # Reproductive years

4.2.3 Recovery from Contraception

A few different categorical variables (with "yes" or "no" values) were created to describe the mares' recovery from the contraception, i.e. whether or not the PZP had worn off, as evidenced by foaling. These variables included:

Ever foaled: If she had ever had a foal, whether before, during, or after contraception

Foal since end of BC: If she had foaled since the contraception was ceased, whether in 2009 or before, or after the study period (late 2013 or early 2014)

Foal since BC started: If she has foaled at any time since her first dose of PZP

4.2.4 Recovery Based on Intensity

A table was created to depict the mares' abilities to recover (i.e. foal again) after consecutive years of PZP immunization (see Appendix IV). For each mare, each stretch of years of consecutive doses was identified. Then the mare was written into the appropriate cell in the table based on the number of consecutive years and whether or not she foaled afterwards. A mare could be put into this table more than once if she had more than one period of multiple consecutive years of immunization; we were not looking at the maximum number of consecutive years of treatment for each mare, but rather each treatment period and the potential recovery. For the mares Tiger and Bridget, who each had two separate periods of consecutive years of treatment but did not foal in between, only the first period was considered and entered into the table; it was impossible to differentiate them as two separate data points without a foal in between to indicate recovery from the first period.

From this data, another table was created that indicated if the mares had been rendered sterile by their consecutive years of treatment (Table 4.2). A mare was considered "fertile" if she had foaled since the stretch of contraception. She was

Table 4.2. Foaling patterns based on consecutive years of PZP contraception treatment (for mares whose last dose was in 2006 or before)

Consecutive years of PZP	Foaled since that period	Not foaled since that period
1-2	4	1
3	5	3
4-5	2	9

considered "sterile" if she had not foaled, and her last dose was in or before 2006; it has been reported that mares with many consecutive years of PZP treatment can take up to six years to return to fertility (Kirkpatrick et al., 2002), so mares whose last dose was prior to 2006 could be assumed to be sterile. For each cell, rather than the names of all the mares with that fertility indication and number of consecutive years of PZP, the total number of mares with that outcome was entered. The mares with only one and five consecutive years were binned with those with two and four years, respectively, because of low group membership. A Chi-squared test was performed on this table to determine if differences in foaling ability after various numbers of years of contraception were significant, or due to random chance.

4.3 Statistical Analysis

All statistical analysis was performed in JMP Pro (version 10.0.0). All quantitative data sets were checked for normality by histograms. Most were not normally distributed, so nonparametric statistics were applied. When variances in two groups were determined to be unequal, a Welch's test was used. T-tests performed were all two-tailed tests, because the null hypotheses were that the two groups were different (no directionality). Significance was determined when $p < 0.05$. In tests relating to the contraception history (i.e. time since last dose, treated before puberty, etc.), the young mares that had never been given contraception were excluded from the analysis (Chacha, Hiphop, Desdemona, and Selena).

4.4 Results

4.4.1 Effects of foaling over the lifespan

Mares that had at least one foal during their lives (*Ever foaled* = Yes) had an average body condition score of 3.4, which was significantly lower than the average of 3.93 of

mares that had not foaled ($F_{1,33.6} = 25.0$, $p < 0.0001$, Welch's test). This effect remained when the mares that were currently lactating during the study were excluded from the analysis to eliminate lactation as a confounding factor ($F_{1,21.6} = 7.54$, $p = 0.0119$, Welch's test); the body condition scores were 3.61 and 3.93 for non-lactating mares that had and had not ever foaled, respectively (Figure 4.1). There were no differences in time spent grazing, number of harem switches, harassment rate, or dominance rank between mares that had and had not foaled.

There was no significant relationship between the total number of foals a mare had birthed and her body condition score ($F_{1,43} = 0.8861$, $p = 0.3518$). An analysis of covariance model was created to separate the effects of the number of foals and current lactation status on body condition. The main effect of lactation status during the study period on body condition was significant ($F = 29.2$, $p < 0.0001$), but the main effect of the number of foals was not significant ($F = 0.17$, $p = 0.6853$); the interaction was also not significant ($F = 3.78$, $p = 0.0588$). There was also no relation between the total number of foals and time spent grazing, dominance, number of harem switches, or harassment rate.

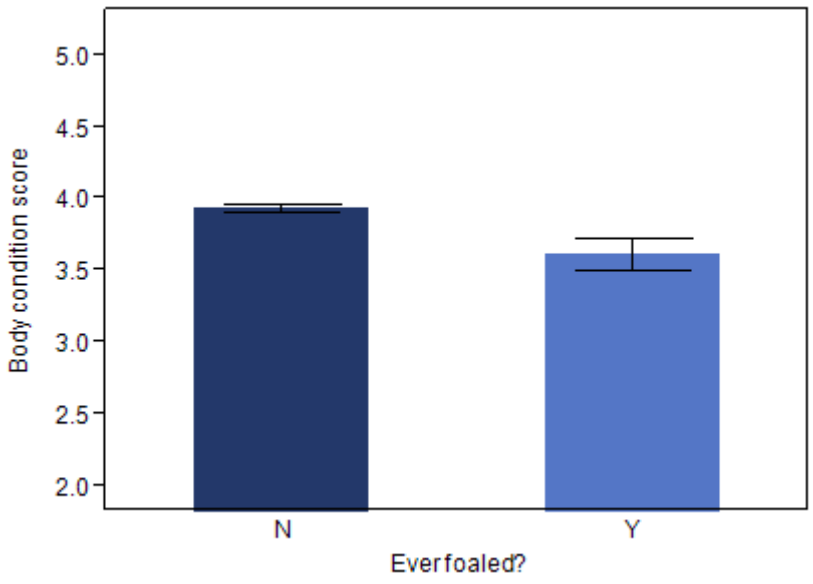


Figure 4.1. Mean body condition scores of mares that had never foaled (N) and mares that had foaled at least once (Y), while excluding those that are currently lactating (means ± S.E., $F_{1,33.6} = 25.0$, $p < 0.0001$, Welch's test).

4.4.2 Recovery from Contraception

The category of mares that had foaled since their last dose of PZP contraception (*Foal since end of BC* = Yes) aligned with those who were lactating during the study period (*Lactating in 2013* = Yes) with the exception of three individuals. Sydney, Hermione, and Wallace had recovered (*Foal since end of BC* = Yes) but were not lactating during the study period (*Lactating in 2013* = NL). Sydney foaled in 2012 and again in 2014; Hermione and Wallace foaled in 2013, but only after the study period.

Mares that had foaled since the end of contraception treatment had a significantly lower average body condition than mares that had not (t_{39} = -2.82, p = 0.0075); average body condition score was 3.25 for recovered mares and 3.74 for non-recovered mares. Mares that had foaled switched harems fewer times than those that had not ($F_{1,38.1}$ = 8.25, p = 0.0066, Welch's test). On average, mares that had foaled switched harems 0.40 times, and those that had not switched 1.81 times during the study period. There was no significant difference in time spent grazing (t_{39} = 1.77, p = 0.0844) or harassment rate ($t_{1,39}$ = -1.16, p = 0.2532) between the two categories of mares.

4.4.3 Timing of Initial Contraception Dose

A Chi-squared test on Table 4.1 (foaling based on if the mare's first dose was given before or after puberty) found that the difference in foaling pattern was significant (χ^2 = 4.11, df = 1, p = 0.04); mares that were given PZP before puberty were less likely to have foaled since the inception of the PZP regimen.

A two-way ANOVA test found that the main effect of having a foal since the inception of PZP contraception significantly impacted body condition score (F = 8.42, p = 0.0062), but the main effect of having PZP administered before puberty did not (F = 0.25, p = 0.6203); the interaction was also not significant (F = 0.65, p = 0.4249). Similarly, no effects of having the first dose before puberty were found on the amount of time spent grazing, number of harem switches, or harassment rate when the effects of current lactation were removed.

4.4.4 Time Since Last PZP Dose and Last Foal

The mares that foaled and did not foal since their last dose of contraception did not

differ in the number of years since that last dose (t_{39} = -1.16, p = 0.2551). There was a significant positive relationship between the number of years since the last immunization and the number of years since the last foal ($F_{1,25}$ = 10.36, p = 0.0035).

When looking at all of the mares studied, the number of years since the last PZP dose and the body condition score were significantly positively correlated; as years since treatment increased, so did body condition ($F_{1,39}$ = 13.80, p = 0.0006). An analysis of covariance model was created to remove the variance in body condition that we had already determined arose from foaling since the PZP was ceased (Section 4.4.2). The main effect of foaling since the end of treatment on body condition was significant (F = 6.17, p = 0.0177), as was the main effect of years since PZP treatment (F = 8.96, p = 0.0049), but the interaction of these two factors was not significant (F = 0.07, p = 0.7930). Mares that had not foaled since their last dose of contraception had a significantly higher body condition than those that had. And for both mares that had and had not foaled, as the years since last treatment increased, the average body condition increased. This model can be seen in Figure 4.2.

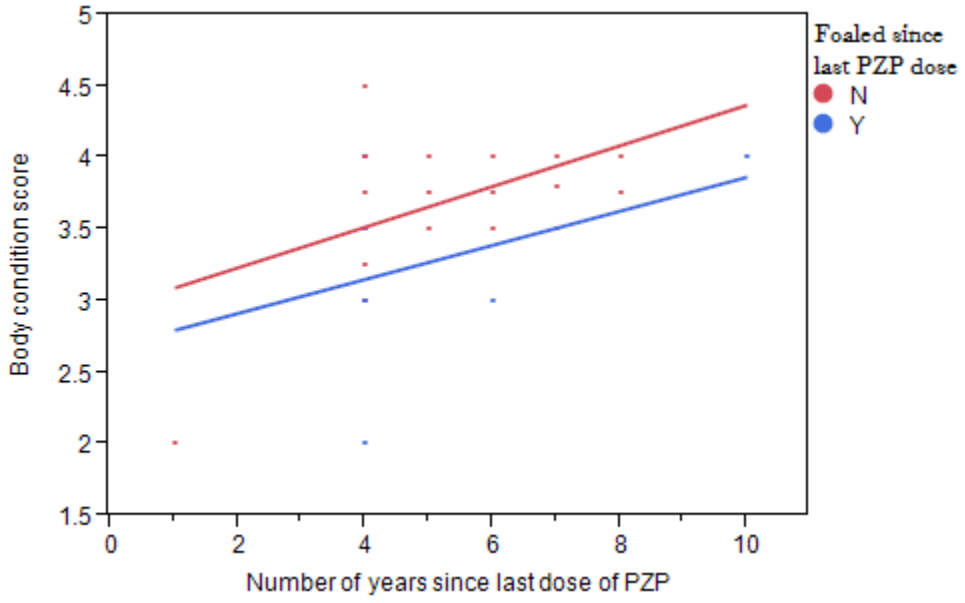


Figure 4.2. The separated main effects of the number of years since the last PZP dose (p = 0.0049) and foaling since that last dose (p = 0.0177) on body condition score, with no significant interaction (p = 0.7930). N (red) = not foaled since last dose, Y (blue) = foaled since last dose.

An analysis of covariance model (as above, to remove the effects of foaling since the last PZP dose) found no significant effects of number of years since the last foal on body condition ($F = 0.71$, $p = 0.4097$), or foaling since the last dose ($F = 1.52$, $p = 0.2305$), or any interaction between the main effects ($F = 2.02$, $p = 0.1691$).

When looking at all of the mares studied, the number of harem switches decreased as the time since the last PZP dose increased ($F_{1,39} = 4.08$, $p = 0.05$). There was no correlation between the number of switches and the time since the last foal ($F_{1,28} = 1.32$, $p = 0.2602$). This result was supported by an analysis of covariance model: the main effect of years since PZP on the number of harem switches was significant ($F = 4.92$, $p = 0.0368$), the main effect of years since a foal was not significant ($F = 3.22$, $p = 0.086$), nor was the interaction ($F = 0.18$, $p = 0.6782$).

This trend was more statistically significant when only considering the mares that had not foaled since their last treatment. The number of harem switches decreased as the time since the last PZP dose increased ($F_{1,29} = 8.36$, $p = 0.0072$, Figure 4.3). There was no correlation between the number of harem switches and the time since the last foal ($F_{1,18} = 0.0019$, $p = 0.9657$). In an analysis of covariance model, the main effect of years since PZP was significant ($F = 4.92$, $p = 0.0368$), but the main effect of years since a foal was not ($F = 3.22$, $p = 0.086$), nor the interaction effect ($F = 0.18$, $p = 0.6782$). An increased number of years since PZP, in non-recovered mares, predicted fewer switches even when the variance due to the number of years since the last foal was removed.

The same trend could not be examined in the mares that had foaled since their last dose of PZP because low sample size led to loss of degrees of freedom in models.

Analysis of variance models with the same main effects but harassment rate and grazing time as the response variables revealed no significant correlations or interactions.

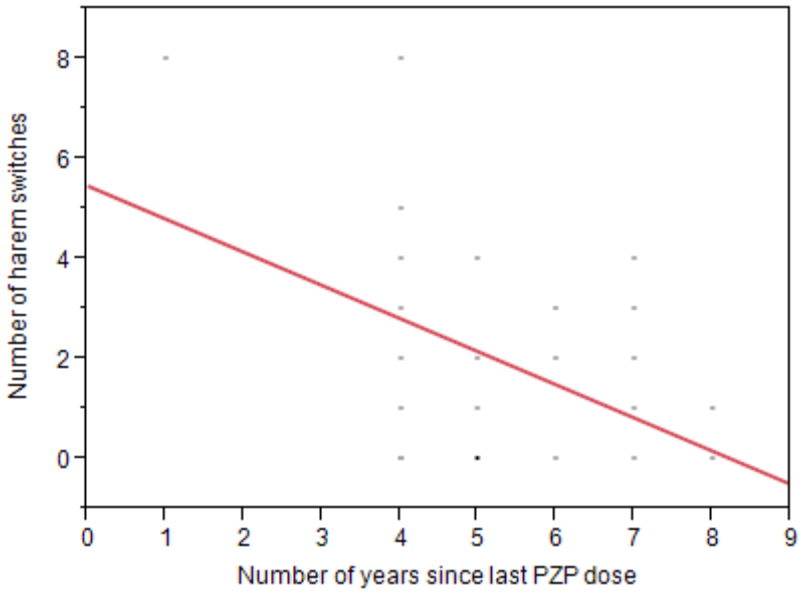


Figure 4.3. In only the mares that had not foaled since the last dose of PZP, the relationship between the number of years since that last dose and the number of harem switches ($F_{1,29} = 8.36$, $p = 0.0072$).

4.4.5 Intensity of Contraception

The Chi-squared test on Table 4.2 (foaling patterns based on consecutive years of PZP contraception treatment) indicated that the deviations from the expected values of mares who foaled and did not foal were not random ($\chi^2 = 6.634$, $df = 2$, $p = 0.04$). The mares with more consecutive years of PZP treatment (three or four years) had a significantly reduced chance of having foaled since that last treatment.

When looking only at the mares that were not lactating in this year (to eliminate the significant effects of lactation) there were no significant relationships between the measures of contraception intensity (number of consecutive years of treatment, total years of treatment, and percentage of reproductive life treated) and any of the measures of health and behavior (body condition score, proportion of time budget spent grazing, number of harem switches, or harassment rate); all *p*-values were greater than 0.05.

4.5 Conclusions

Based on the results from initial analysis in Chapter 3, parasite egg loads were not included as a measure of health, only body condition score. Time spent grazing, number of harem switches, and harassment rate were deemed to be the important indicators of

behavior and were used along with body condition score to determine the effects of past contraception and foaling.

It was previously determined that mares lactating at the time of the study were, on average, in worse physical condition than mares that were not lactating. Here, we found that mares had a lower average body condition score if they had ever had a foal, an effect that held true even when currently lactating mares were excluded from analysis. Additionally, there was no relationship between the total number of foals and body condition; mares that had one foal did not have a higher body condition than mares that had many foals, which would be expected if foaling had a cumulative effect. It appears from these analyses that the detrimental effect of foaling on physical condition is an "all or none" response: having at least one foal at any time over a mare's lifespan predicted that she was in worse physical condition at the time of study.

Having foaled at any point was not predictive of any of the other measures: time spent grazing, number of harem switches, or harassment rate. The fact that ever foaling predicted lower body condition but not increased grazing agrees with the former conclusion (Chapter 3) that current lactation, but not body condition itself, is what determines the amount of time spent grazing. Mares that had foaled in the past were in poorer body condition from that energetically-costly process of foal-bearing and lactation, but did not require extra intake of food if not currently nursing a foal in the year of study. The amount of time spent grazing therefore seems to be a measure of current, but not past, energetic needs; the increased cost of lactation leads nursing mares to graze more. These results suggest that harem switches and harassment rate are also indicators of current lactation status, but not past foaling.

Mares that had foaled since their last dose of PZP contraception were able to do so because the PZP antibody titers had dropped low enough to permit the sperm's binding to the egg. Mares that had recovered and foaled were found to have a lower average body condition than those who had not. This result is consistent with the above finding that foaling even once lowers body condition. However, the difference in average body conditions could be merely mirroring the difference caused by current lactation; the group of mares who had foaled since their last dose was the same as the group of mares who was currently lactating, plus three additional mares who foaled after the study period (Hermione, Sydney, and Wallace).

This possibility is supported by the fact that the difference in body condition between lactating and non-lactating mares was more significant ($p < 0.0001$) than the difference between recovered and non-recovered mares ($p = 0.0075$). Adding Hermione, Wallace, and Sydney to the lactating group (forming the "foaled since last dose" group) reduced the difference in body condition between the two groups, suggesting that the lowered body condition was primarily an effect of the current lactation, not recovery.

Mares that had foaled since their last dose of PZP switched harems significantly fewer times during the study period, but that was also probably because most of them currently had a foal and remained faithful to one stallion for protection of the foal. The mares that were pregnant during the study (Hermione, Sydney, and Wallace) also had good reason to remain with a single stallion because they were not in estrus and needed the protection and steady food source the stallion provided as they prepared to foal. Without a larger group of mares that had foaled since their last dose but were not currently lactating (larger than the three used in this analysis), no confident conclusions can be drawn about the differences between mares who had recovered from the contraception and mares who had not.

Two results from these analyses indicated that several of the mares were likely sterile as a result of repeated PZP contraception administration, a possibility that had been suggested previously (Kirkpatrick et al., 1992; Nuñez et al., 2010). Firstly, the mares that had and had not foaled since their last dose of PZP, i.e. recovered from the dose and become fertile again, did not differ significantly in the number of years since their last dose. We would expect that the longer it had been since a mare's last contraception dose, the more likely she would be to have recovered and subsequently foaled; if this were the case, the mares that had recovered would have had a higher average number of years since the last dose. Instead, there were mares that had not had PZP in many years, but also had not since recovered, suggesting that they had become sterile and could no longer foal. Another result implying sterility was that the length of time since the last foal and the length of time since the last dose of PZP were positively correlated. Again, the expected result would be a negative correlation: the longer since PZP, the less time since the last foal, because the mare would have more time to recover and foal. These two results showed statistically that there were mares in the population not foaling after their contraception treatment, and may have been made sterile by such treatment.

Kirkpatrick & Turner (2002) found that mares on Assateague Island with a higher number of consecutive years of treatment took longer to return to fertility. From this finding, we hypothesized that high intensity of treatment may be a cause of sterility in some Shackleford Banks mares. A Chi-squared test on the distribution of mares that had and had not foaled since their last dose, differentiated by the number of consecutive years of treatment, was significant enough to conclude that the differences in distribution were not by chance (Section 4.4.5, Table 4.2, $p = 0.04$). Only mares that had their final PZP dose in 2006 or earlier were considered in this analysis, because Kirkpatrick & Turner (2002) observed that mares could take up to six years to return to fertility; mares with the last dose in 2006 and not foaled since had surpassed this six year mark and were surmised to be sterile. From this test, we can conclude that the higher the number of years of consecutive treatment, the more likely it was that the mares had not returned to fertility. Of mares that had only one or two years of treatment, four out of five (90%) had since returned to fertility at the time of this study; of mares that had three years of treatment, only five out of eight (63%) foaled again. Most significantly, out of the 11 mares that had four or more years of PZP in a row, only two (18%) had recovered and foaled since the end of their treatment. Three or four years of PZP in a row appears to have significantly reduced chances of recovery and subsequent foaling, most likely because the higher number of consecutive doses compromised ovarian functioning to an irreparable extent.

It was noted upon preliminary inspection of the contraception histories that many mares were given their first PZP dose before they were able to foal for the first time. This was also considered a potential source of sterility, because those mares may have not yet reached sexual maturity before having their fertility compromised. Of those that had their first dose before puberty, seven of 21 had since foaled (33%), as compared to 13 of 20 of those who were allowed to foal before being administered their first dose (65%). This difference was found to be statistically significant by a Chi-squared test (Section 4.4.3, Table 4.1, $p = 0.04$): mares that were immunized before reaching sexual maturity were less likely to have recovered and foaled since the treatment ended. The effects of these two sources of sterility (intensity and timing) were not able to be separated because of relatively low sample size.

The contraception history itself (i.e. intensity or timing) was not found to be correlated to any of the measures of health and behavior when the variance from lactation was removed. However, the amount of time since the last dose of contraception was negatively correlated to the number of harem switches. In all of the mares, the number of harem switches was found to decrease as the number of years since the last dose of PZP increased. This effect was even stronger and resulted in a more statistically significant trend when only the mares that had not foaled since their last dose were considered, which removed the variance due to lactation. This could have two explanations. The contraception treatment could have caused an increase in harem switches during the year of its administration, an effect that wore off as the contraception wore off; Madosky et al. (2010) found that mares that had been administered contraception switched harems more times than mares that had not. Alternatively, an increased number of years since PZP in those mares that had not foaled could have been an indicator of sterility; sterile mares may switch harems fewer times because they are not reproductively active and do not solicit reproductive interest. These hypotheses will be more fully discussed in Chapter 5.

Also significantly, as the number of years since the last dose of PZP increased, the body condition score increased (Figure 4.2). This effect held true for both mares that had foaled since that last dose and those that had not, when they were assessed separately in an analysis of variance model. That is, regardless of if a mare had or had not foaled since her last dose of contraception, her body condition was higher the longer it had been since that last dose, with foaling mares at a significantly lower body condition at all time points. This suggests that the immunocontraception may have had some negative impact on the health of the mares, one that they are able to recover from over time, which cannot be explained by lactation status. Other than these two phenomena, lactation – both in the current year and over the lifespan – was found to be the most significant predictor of health and behavior, with contraception patterns only affecting whether or not the mares were able to foal.

Chapter 5
Discussion and Conclusions

5.1 Study Limitations

In this study, we were able to examine the relationships between various health and behavior indicators and the history of PZP immunocontraception and foaling of the mares of Shackleford Banks. However, various limitations prevented us from drawing certain conclusions. With no mares administered PZP in the spring of 2013, there were no immunized mares during our study period, the summer of 2013, to compare to non-immunized mares. Consequently, no conclusions about the direct effects of the contraception dose could be reached. This differed from previous studies on the Shackleford Banks mares, which were conducted during years of contraception administration, and from which the authors could make more specific conclusions about its effects (Madosky et al., 2010; Nuñez et al., 2009; Nuñez et al., 2010, Nuñez et al., 2014). Instead, rather than examining the direct effects of contraception in the current year, we took a more historical perspective and determined the effects of past years of treatment and the resulting foaling. We acknowledge that these are correlations and cannot determine causality with certainty.

Each mare had a unique history of contraception and foaling each year, making it impossible to statistically remove all variance to examine a single variable (i.e. mares who did not foal in the year of study differed not only in the number of years since PZP treatment, but the total number of years treated, the age, the total number of foals, etc.). When doing statistical analysis, we controlled for the most relevant variables to reach a

confident conclusion, but variation could still confound the results. This variation in histories also resulted in small group sizes for many combinations of variables, making certain tests impossible due to loss of degrees of freedom (i.e. looking at the differences in body condition due to varying intensity of treatment, in only mares that were immunized before puberty).

Additionally, we used foaling as the indicator of a mare's recovery from the contraception, which may not have been 100% accurate; a mare could have successfully conceived but not carried the foal to term, resulting in an incorrect judgment about fertility in that year. Pregnancy testing would have been a more accurate test of fertility, but this data was not available to us for the entire 2000-2013 period.

Limited data became a problem when determining mares' harassment rate and dominance rankings. Harassment events were rarely observed during the study period, so the calculated harassment rates may have been results of unequal sampling and thus not accurately reflect the actual daily harassment rates. Dominance rankings were determined based on mare-mare interactions, which were also relatively rare. Many of the mares were seen to participate in only one antagonistic interaction with another mare, if any, so conclusions drawn from such a small data set may be incorrect. For these reasons, the correlations between harassment rate or dominance ranking and other variables were not always as expected, and conclusions could not be made with much confidence.

5.2 Parasite Load as a Health Indicator

One of the original goals of this study was to examine the effects of PZP contraception on the health of the mares' immune systems, as quantified through the number of parasite eggs found in their feces; a mare with a healthier, stronger immune system is better able to fight off infection and should have a lower parasite load. Because PZP contraception works through activation of the immune system, we expected some effect of treatment on immune function. However, because there were no currently immunized females, there was no direct comparison and no conclusion about the effect of the contraception treatment on immune functioning to be made.

Even when looking at the relationships between the parasite egg loads and other health and behavior measures, surprisingly, no significant relationships were found.

Linklater et al. (1999) found that more parasite eggs predicted lower body condition and higher harassment rate; these results were not corroborated by our results. It was also surprising to find no difference in egg loads between lactating and non-lactating females since lactating females were under physiological stress which could have weakened their immune systems, along with lowering overall health. Rubenstein and Hohmann (1989) found that increased harem size predicted increased endoparasite load, another result that our results did not agree with.

This lack of correlation between parasite egg loads and any of the other measures could stem from one of two sources. It is possible that the parasite egg load is indicative of something other than overall health, resulting in it not being correlated with body condition score, which does measure overall health. The fact that the lactating and non-lactating mares had similar ranges and similar mean values of egg counts suggested that parasite load is more than just a simple measure of physiological stress or energy use. In fact, Rubenstein and Hohmann (1989) also found no differences in parasite loads between lactating and non-lactating mares on Shackleford Banks. It is also possible that the number of eggs produced in this year will be indicative of future body condition score, because the negative impacts of the parasites may not affect overall health immediately; this was also a finding by Rubenstein and Hohmann (1989).

Another alternative is that the sampling performed did not sufficiently capture the actual intensity of parasite infection in the mares. We collected three fecal samples from most of the mares throughout the study period, and only two for a few. This may not have been enough samples to obtain an accurate measure, because the average would have been easily skewed by outliers or unequal egg distribution within the fecal piles. The storage of the fecal samples until transfer to the laboratory for fecal flotation and egg counting may also have posed a problem. The samples were kept cool using frozen ice packs, but successive days of storage could have caused them to become too warm as the ice packs melted. Samples were stored for any amount of time between a few hours and two days before being taken to the lab for processing; this lack of consistency also could have affected the relative number of eggs found in the samples (i.e. by some eggs dying or hatching into worms because of the increased temperatures).

For whichever reason, the lack of patterns in the parasite egg count data led us to discard it as a measure of immune function or overall health. In future studies, sampling

the feces more frequently and having a more invariable form of fecal storage and preservation could result in more accurate and reliable parasite egg data from which conclusions about health and immune function could be drawn.

5.3 Effects of Lactation

Ransom et al. (2010) found that body condition was the strongest predictor of feeding and social behaviors in harems in the Midwest that were treated with PZP contraception. In this study, we instead found that lactation was the strongest predictor not only of feeding and social behaviors, but also of body condition itself. Lactating mares were not only in poorer physical condition – a finding found previously (Ransom et al., 2010; Rubenstein, 1986; Turner & Kirkpatrick, 2002) – but also grazed more in order to offset the high energetic cost of lactation. Lactating mares were found to spend an average of 11% more of their time grazing which, over a day of 20 hours of grazing, would equal 132 additional minutes of food intake, which they could then invest in milk production. Mares not currently nursing a foal, released from the demands of lactation, were found to have higher average body condition and spent less time grazing. No relationship between body condition and time spent grazing was found independent of the effects of lactation.

Significantly, this negative impact of lactation had no dose-response relationship: having more foals over the life span was not correlated with lower body condition, as might be expected. Rather, there was an "all or none" response that predicted mares that had ever had a foal, even just one, would have a lower average body condition score. This was corroborated by the fact that increased time since the last foal did not predict higher body condition, which would be expected if the lactation was causing a lower physical condition from which the mares could recover with time. This suggests that the negative impact of foaling is relatively long-lasting or permanent, which could be confirmed with longitudinal studies tracing the mares' body conditions over time.

Lactating mares also switched harems fewer times throughout the study period. In fact, only two of the ten currently nursing mares switched harems at all, and even they remained faithful to the primary stallion for all but a few days spent with a secondary stallion before returning to the primary stallion. Those two mares, Keller and Hezekiah, were not nursing new foals, but rather a yearling and a two-year-old, respectively. The

remaining four mares with yearlings (Carrot, Hedvig, Sadie, and Tahiti) did not switch harems, and neither did the four mares with new foals (Chacha, Daisy, Desdemona, and Hiphop). The mares who were later found out to have been pregnant during the time of this study (Hermione, Sydney, and Wallace) also did not ever switch harems. Remaining faithful to a single stallion is practical for both nursing and currently pregnant mares because it ensures protection and more stable food resources for both themselves and their foal. Nunez et al. (2009) found that reductions in mare fidelity, caused by increased switching harems, disturbs all of the mares in the harem and can promote aggression, which would not be favorable for a mare with a foal.

In Chapter 3, we found a positive correlation between harassment rate and number of harem switches. The direction of this correlation was unclear; a mare could have been switching harems to escape increased harassment from the stallion, or a mare switching into a group may have been experiencing increased harassment because she was unfamiliar or in estrus. Because of this correlation, we expected to find that lactating mares, who switched harems fewer times, experienced fewer instances of harassment during the study period.

This trend was observed but was not statistically significant ($p = 0.1174$). This could be a result of the small sampling, and therefore low reliability, of the observed harassment events (Section 5.1). If that were the case, more extensive observation that better captures the actual relative rates of harassment of the mares may reveal this trend to be significant. An alternative conclusion is that harassment and lactation interact with harem switching in different ways. A lactating mare may remain faithful to a stallion for protection, but may not necessarily have a lower harassment rate. She would receive more reproductive interest if she were in estrus again; the mares foaling during the study period would have gone into estrus a few weeks after foaling (S. Stuska, personal communication), and the mares with yearlings could also have been in estrus during the study period. The lactating mares then may be remaining with a primary stallion for the benefits of protection, despite harassment rate; the non-lactating mares may be the only ones subject to the positive correlation between harem switching and harassment. This would result in no definite relationship between lactation and harassment rates. These two hypotheses are not mutually exclusive.

Thus it can be concluded from our data that lactation is a significant predictor of both health and behavior. A lactating mare will be in lower body condition than a non-lactating mare; this negative impact of foaling is not cumulative, but a one-time hit that lowers average body condition for mares that have ever had a foal. To compensate for the increased energy output of lactating, nursing mares increase the amount of time they spend grazing. They also switch harems less frequently, if at all. These last two measures, grazing time and harem switches, were not found to be significantly different between mares that had ever foaled and those that had not; they measured current energy needs and social state, not historical. Body condition, on the other hand, is reflective of both current and past foaling, a long-term measure of overall physical health.

5.4 Effects of Contraception

5.4.1 General Effects

As can be gleaned from the contraception and foaling histories of each mare, the contraceptive's main effect was prevention of pregnancy. However, this was not always 100% effective, as some mares were able to foal in the year following immunization. Of the 59 foals born since 2000, 14 were born in a year following the mare's immunization with PZP. Some of these instances could be explained by either the contraception given or the foal born late in the respective seasons (the antibody would have been low enough to allow conception) but some were inexplicable. See Appendix I for full histories.

As explained previously, we were unable to make any direct conclusions about the effects of current contraception because of a lack of treated and non-treated groups during the year of study. But by releasing a mare from lactation, contraception leads to better physical condition, less time spent grazing, and more harem switches.

However, when removing the effects of lactation, the patterns of past years of contraception treatment did not seem to have any significant measurable effects on health and behavior. Mares that were given their first dose before reaching sexual maturity had no difference in physical condition or behavioral measures, nor were there any differences based on the intensity of treatment (number of total years of treatment, number of consecutive years, or percentage of reproductive life treated).

5.4.2 Effects on Body Condition

One significant finding independent of foaling or lactation was that a longer time since PZP treatment was correlated with higher body condition score. This was a trend that held true both for mares that had foaled since that last dose and mares that had not. Additionally, longer time since foaling was not predictive of higher body condition, suggesting that this finding was not mediated by merely lactation differences. However, there is a possibility that causality is reversed in this correlation. Mares that were in lower body condition may have been more likely to be given contraception in a given year to prevent them from carrying a foal, which could be hazardous to their already diminished health, which would result in fewer years since treatment being predictive of lower body condition score. A longitudinal study would be able to investigate this relationship further. But if this were found to be a true trend, higher body condition the longer it has been since PZP administration, it would suggest that the contraception was negatively impacting physical health in a way that could be recovered from in subsequent contraception-free years. This would shed light on a potentially detrimental physical effect of the contraception treatment that has not yet been explored.

5.4.3 Effects on Harem Switching

Lactating mares switched harems statistically fewer times; only two of them switched harems at all, and those were only short-lived switches. But there also appeared to be an effect of PZP on harem switching independent of lactation status. When looking at all mares, there was a significant negative correlation between the number of years since the last dose of PZP and the number of harem switches, when the variance due to the number of years since the last foal was removed. This relationship was even more evident in the mares that had not foaled since their last immunization. As more time since treatment passed without a foal, the mares switched harems fewer times.

This may be a result of the contraception treatment increasing the number of switches in the year in which it is administered, an effect that is able to wear off with time. Madosky et al. (2010) found in two separate years that mares administered PZP in that year switched harems more than the control mares that had never been given PZP. Because there were no control mares in the year of this study (except for the few who had just become of reproductive age) such a comparison could not be made to determine

if the contraception itself was increasing the number of switches above and beyond the lack of a foal. But it does appear that the number of years since the last treatment is predictive of the number of switches, pointing out a direct effect of PZP.

Prior to the contraception program's inception, only about 10.8% of mares changed harems in a given summer, which increased to 66% of mares in 2007, 73% in 2008, and 44% in 2013, the year of this study: the number of mares to switch harems has increased since they started being immunized with PZP (Madosky et al, 2010). The fact that the number was lower in 2013 than 2007 or 2008 could be indicative of the fact that there were no mares actually treated in this year, so the only ones manifesting the effects (more switches) were those whose antibody titers were still high enough to be physiologically effective.

If contraception treatment is in fact increasing harem switching, whether in the short- or long-term, such a disturbance to natural behavior could have significant impacts on the mares' health. Although we were unable to find a correlation between the number of harem switches and body condition or parasite load, that is not to say that switching does not create physiological stress that will result in poorer health in the future. Harem switches decrease harem stability, which disturbs mares in the harem, promotes aggression and stallion harassment, and prohibits the formation of dominance hierarchies or group cohesion (Nuñez et al., 2009). Our findings supported this positive correlation between harem switches and harassment. It was recently found that the mares on Shackleford in the middle of changing groups had higher fecal cortisol levels, a measure of physiological stress (Nuñez et al., 2014). Harem stability has also been correlated with yearly reproductive success (Rubenstein, 1986).

Increased harem switching and its resulting physiological stress caused by recent treatment thus could be one negative impact of the PZP contraception program. Further study during years in which mares are immunized could determine if contraception does increase the number of harem switches above and beyond the lack of a foal. This would lead to better understanding of this detrimental effect of PZP treatment.

5.4.4 Sterility: Causes

Negative effects of PZP treatment on ovarian function have been suggested in the past. Mares on Assateague Island had depressed estrogen secretion when treated for

three consecutive years (Kirkpatrick et al., 1992), and Shackleford mares that had ever been treated were found to have an extended reproductive period and to foal over a greater range of months, suggesting altered cycling (Nuñez et al., 2010). But despite these potential reproductive effects, sterility was not induced in the Assateague population; all mares returned to fertility after as much five consecutive doses of PZP (Kirkpatrick et al., 2002; Powell, 1999), and ovarian failures were not linked to contraception treatment (Powell & Monfort, 2001).

However, in this study of the Shackleford Banks mares, we did find evidence that sterility was likely being induced by the contraception treatment. If all mares were still fertile, mares not foaled since their last dose should have had that last dose more recently than mares that have foaled, since they would not have yet returned to fertility merely because of lack of time to recover; this trend was not found. Additionally, there was a positive correlation between the years since treatment and the year since a foal, which should have been an inverse relationship if all mares were fertile. These statistical indications can be corroborated by a visual assessment of the foaling and contraception history. Fourteen different mares whose last dose of PZP was in 2006 or earlier have not since returned to fertility. It is highly likely that these mares have been rendered infertile by the contraception treatment, because they have surpassed the expected time frame in which mares return to fertility after PZP immunization.

Two factors were found to be potential causes of this sterility. The first of those was the timing of the initial dose of PZP. Mares that were immunized for the first time before they reached puberty were statistically less likely to have foaled since the inception of treatment than mares that were able to reach puberty and foal before initial treatment. This is likely because they were not able to fully develop sexually before having their reproductive cycling altered by the immunocontraception, and this made them unable to reproduce in the future.

Increased intensity of contraception (more consecutive years of treatment) also appeared to decrease a mare's probability of foaling after PZP treatment, suggesting induced sterility. This was a statistically significant result. Of the five mares that had been given only one or two consecutive doses, with the last dose in 2006 or earlier (outside of the range of expected return to fertility), four had returned to fertility by the time of study. The chances of returning to fertility after three consecutive years of

treatment were decreased, with only five out of eight mares foaling again afterwards. A significant difference was seen after four years of consecutive immunization: of the 11 mares who had received four or more consecutive years of PZP ending in 2006 or before, only two had returned to fertility since the last dose. The other nine mares, having not foaled in six or more years, were assumed to be sterile because they were outside of the expected time frame of fertility returning. There were mares that may still return to fertility in the years after this study, those whose last dose was in 2007 or later. Examining these mares in the next few years may provide further insight and statistical evidence that an increased number of consecutive years of treatment increases the chances of inducing sterility.

While both consecutive years of treatment and early initial administration may independently increase the chance of resulting sterility, when combined they may be exceptionally hazardous to fertility. Of the 14 mares that had never foaled in their lifespan, 12 of them were both treated before puberty and given 3-4 years of consecutive treatment; the other two had only been given two doses, ending in 2009, and were still expected to foal at the time of this analysis. Although the effects of these factors were not able to be statistically separated because of low sample size, it remains a notable trend that the majority of mares that have never foaled were exposed to this combination of conditions, a likely cause of their sterility.

5.4.5 Sterility: Effects

Rendering a mare sterile through PZP immunocontraception treatment will subsequently prevent her from foaling. Since the detrimental effect of foaling on body condition is "all or none," as described above, the mare will have a higher body condition if she is sterilized before ever foaling. If the mare had foaled before treatment began and was subsequently rendered infertile by the PZP, she would not be expected to have a higher body condition. We did find that mares that had not foaled after treatment were in higher body condition, but this was most likely because many of them had never foaled at all; these two effects could not be statistically separated due to loss of degrees of freedom in modeling.

It is possible that the previously discussed negative correlation between harem switches and time since PZP treatment was also the result of induced sterility. The

mares that were last treated more recently were likely still fertile, but had not foaled yet because they were still under the contraceptive effects of the PZP. These mares would have increased reproductive cycling without successful conception; they may therefore switch groups more often in an attempt to conceive or to escape intense sexual interest and harassment by the stallions (Madosky et al., 2010). On the other hand, mares that had that last dose longer ago (without foaling since) were likely sterile and unable to foal at all, otherwise they would have foaled in the high number of years since treatment. If the intensity of contraception had effectively destroyed their ability to cycle normally (and therefore become pregnant) by depressing estrogen secretion, they would not solicit the same reproductive interest from the stallions or undergo normal estrus cycling, which could explain why they did not switch harems as often. Whether by male or female choice, the effect would be the same: effective contraception increases cycling and therefore switches, whereas sterility destroys cycling and motivation for switches.

This hypothesis and the previous proposal (Section 5.4.3, that contraception is increasing the number of harem switches in a way that can be overcome in time) are not necessarily mutually exclusive. The contraception could increase the number of switches, an effect that is maintained as long as the mares are not sterile. Sterility then could reduce the number of harems switches for the reasons explained above. Both would result in the observed trend of a longer time since PZP immunization predicting fewer harem switches. This differentiation cannot be confidently made without studies that include true control mares that were not administered PZP but also did not foal.

5.5 Population-Level Implications

We concluded that certain aspects of PZP contraception (specifically, a high number of consecutive years of treatment and administration of the first dose before sexual maturity) are likely causes of sterility in several of the Shackleford mares. Making a mare sterile appears to have positive impacts on her health. By preventing her from foaling, she will have a higher body condition on average, and will spend less of her time grazing because she does not require as much energy without the high cost of lactation. A sterile mare may also switch harems less, resulting in less physiological stress and less harassment.

However, this increase in average body condition and overall health may have unintended impacts on the dynamics of the population. Mares in better health will live longer on average, so PZP contraception may be artificially extending their lifespan. This was found in the Assateague Island herd, where mares treated for three or more years had the highest longevity, followed by mares treated for less than three years, and untreated mares had the lowest longevity (Kirkpatrick & Turner, 2007). In the population as a whole, even though the mares were not made sterile, mortality decreased, overall body condition increased, and a new higher age class emerged after inception of the PZP contraception program (Kirkpatrick and Turner, 2002).

We did not have historical information on the Shackleford mares to be able to make age class and longevity comparisons, but it is suspected that the PZP may be having the same effect on this herd. The contraception treatment and its probable induced sterility release mares from the energetic costs of lactation, resulting in better physical health that will cause them to live longer, resulting in a higher age class in the population. By reducing mortality, fewer mares will die each year. In order to maintain the goal population size of 100-110 individuals, more mares will have to be administered contraception each year to prevent too many foals from being born. In the year of this study, this did not appear to be a problem; only eight foals were born despite no PZP administration in the previous year, probably because many of the non-foaling mares were in fact sterile because of their contraception history. This may become an important consideration in the future. As new mares come to reproductive age, they may have to be immunized with PZP more frequently so that the number of foals born does not exceed the number of deaths in that year, and the population can remain at the intended size. This will raise the dilemma of how to increase their immunization frequency without inducing sterility.

Sterility induced by the immunocontraception program also has impacts on the genetic makeup of the population. Mares that are prevented from reproducing are not able to pass along their genes to future generations, thus reducing the different combinations of genes that are possible for the offspring of any given generation. This could result in an overall loss of genetic diversity in future generations as a few genetic lines are perpetuated while others are lost due to the sterile mares' inability to pass along their genes. Genetic diversity is what allows a population to adapt to changing

environments and reduces the chances of detrimental effects caused by inbreeding. By artificially limiting which mares are able to reproduce throughout their lifespan, it is possible that the PZP contraception program could be negatively impacting the health and long-term prospects of the population.

Representation of the mares' genetic lines within the population is currently factored into decisions about which mares on Shackleford Banks receive PZP in a given year: if a mare's genetic line is under-represented, she is less likely to be given contraception, so that the line can be perpetuated (S. Stuska, personal communication). However, if some mares are rendered sterile through intense PZP contraception, this eliminates the possibility of them ever foaling in the future. If their genetic lines do become under-represented, i.e. by death of many of their close relatives, there is no longer the choice to allow those mares to foal and contribute to the perpetuation of the lineage.

Our analysis has demonstrated the importance of considering not only a mare's foaling history and genetic line representation when making contraception decisions, but also her own contraception history and how to best prevent her from becoming sterile. Through examining current patterns of fertility on Shackleford Banks, we concluded that a mare is more likely to maintain her fertility if she is allowed to foal before her first dose of PZP and if she is only ever given three or fewer consecutive doses, with a break to foal in between. Each mare will vary in her physiological response to the PZP – she may become sterile after only one dose or have other health problems making her infertile – but these guidelines may help reduce the incidence of sterility in the future. If fewer mares are made sterile from PZP contraception, this will ensure more flexibility in which mares can be permitted to foal in a given year, leaving more mares able to contribute to the genetic diversity of the population.

5.6 Study Conclusions

Ideally, a feral horse population would not require any human intervention to endure. However, the rapid expansion of the herd on Shackleford Banks prompted the National Park Service to institute a program of porcine zona pellucida immunocontraception of the mares in 2000. By preventing pregnancy, the contraception is working to reduce the population so that horses will not need to be physically removed from the island, but it is important to consider the effects that PZP may have on the mares' health and behavior.

In this study, although we were unable to directly determine the effects of the contraception because it was not administered to any mares during the year of study, we were able to indirectly look at its effects through the histories of contraception treatment and foaling for each mare. Lactation was the most significant predictor of health (lower body condition) and behavior (more time grazing, fewer harem switches). By releasing mares from lactation, PZP contraception results in better physical condition, but also more harem switching, a physiological stressor. Independent of lactation, two main effects of the contraception treatment on the health and behavior of the mares were found. It appeared that the PZP treatment was lowering body condition and increasing the number of harem switches, both effects that diminished as time since the last dose passed. Increased group switching could subject mares to higher rates of harassment and resulting physiological stress that could later manifest in further poor body condition. Future studies that either directly compare treated and non-treated mares or more closely track changes in mare body condition and behavior over time could enhance understanding of these detrimental effects of the PZP immunization.

Administration of PZP before puberty and high intensity of treatment were found to be likely causes of sterility in many of the mares on Shackleford Banks. Though this may have the initial positive effect of freeing them from lactation and therefore increasing their physical health, this benefit should be weighed against the impacts on population dynamics when making decisions about the contraception regimen. Through careful execution of the PZP contraception program, the impacts of the human intervention on the natural behavior of the mares of Shackleford Banks can be minimized, and the population will continue to thrive.

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Appendix I: Contraception and Foaling Histories

PZP = treated with PZP contraception, F = foaled in that year (+ = yes; - = no).
"n/a" indicates the mare was not yet born.

Name	Birth year	2000		2001		2002		2003		2004	
		PZP	F	PZP	F	PZP	F	PZP	F	PZP	F
Anastasia	2001	n/a	n/a	-	-	-	-	+	-	+	-
Ariel	1998	-	-	+	-	+	-	+	-	-	-
Biff	1992	-	-	-	-	-	+	-	-	-	+
Bo	1996	-	-	-	+	-	+	+	-	-	-
Bridget	1998	-	-	+	-	+	-	+	-	-	-
Carrot	1994	-	-	-	-	-	-	-	+	-	+
Chacha	2008	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Daisy	1997	+	+	-	-	+	-	+	-	+	-
Darcy	1993	-	-	-	+	+	+	+	-	+	-
Darkface	1990	+	-	-	-	+	+	+	-	+	-
Desdemona	2009	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Djibouti	2002	n/a	n/a	n/a	n/a	-	-	-	-	+	-
Donoma	2006	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Dusty	1995	-	+	+	-	+	-	+	-	-	-
Hedvig	2007	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Helena	1994	+	-	-	-	-	-	+	-	+	+
Hermione	2005	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Hezekiah	1997	+	+	-	-	+	+	+	-	+	-
Hiphop	2008	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Hoorah	2005	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Jaquinkoke	1998	-	-	+	-	-	-	+	-	+	-
Julikmi	1993	-	+	-	-	-	-	-	+	-	-
Keller	2007	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Kelty	1994	-	-	-	+	-	-	-	+	-	-
Larissa	2001	n/a	n/a	-	-	-	-	+	-	+	-
MissIsabel	2003	n/a	n/a	n/a	n/a	n/a	n/a	-	-	-	-
Noir	1994	-	+	-	-	-	-	-	+	-	-
Paula	1995	-	+	-	+	-	+	+	-	+	-
Persia	2002	n/a	n/a	n/a	n/a	-	-	-	-	+	-
Sadie	1998	-	-	+	-	+	-	+	-	-	+
Sarah	1983	+	-	-	-	+	+	+	-	-	+
Sawathu	2006	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Selena	2011	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Serenac	1995	-	-	+	-	-	-	+	+	+	-
Shag	1997	+	-	-	-	+	-	+	-	-	-
Shiphrah	2003	n/a	n/a	n/a	n/a	n/a	n/a	-	-	-	-
Shira	2001	n/a	n/a	-	-	-	-	+	-	+	-
Slug	1990	-	-	-	-	+	-	+	-	+	+
Sydney	1995	-	-	-	-	+	-	+	-	-	-
Tahiti	2002	n/a	n/a	n/a	n/a	-	-	-	-	+	-
Tatya	2001	n/a	n/a	-	-	-	-	+	-	+	-
Texas	1990	+	-	-	+	+	+	+	-	+	-
Tiger	1998	-	-	+	-	+	-	+	-	-	-
Wallace	1999	-	-	-	-	-	-	-	+	-	-
Wire	1993	-	-	-	-	-	+	+	+	+	-

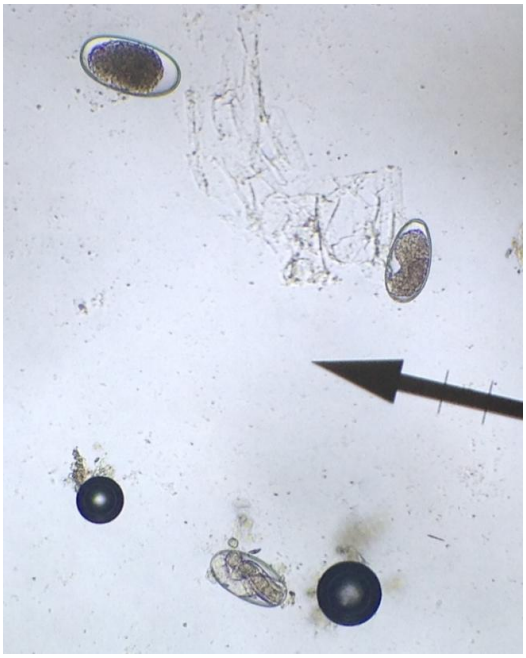
Name	2005		2006		2007		2008		2009		2010	
	PZP	F	PZP	F	PZP	F	PZP	F	PZP	F	PZP	F
Anastasia	+	-	+	-	-	-	-	+	+	-	-	-
Ariel	-	-	+	-	-	-	-	-	-	-	-	-
Biff	-	+	-	+	-	+	-	+	+	-	-	-
Bo	+	-	-	-	-	-	-	-	-	-	-	-
Bridget	+	-	+	-	-	-	-	-	-	-	-	-
Carrot	-	+	-	+	-	+	-	+	+	-	-	-
Chacha	n/a	n/a	n/a	n/a	n/a	n/a	-	-	-	-	-	-
Daisy	+	-	-	+	+	-	+	-	+	+	-	-
Darcy	+	-	-	-	-	-	-	-	-	-	-	-
Darkface	+	+	+	-	+	-	-	-	-	-	-	-
Desdemona	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	-	-	-	-
Djibouti	+	-	+	-	+	-	-	-	-	-	-	-
Donoma	n/a	n/a	-	-	-	-	+	-	+	-	-	-
Dusty	+	+	+	-	+	-	-	-	-	-	-	-
Hedvig	n/a	n/a	n/a	n/a	-	-	-	-	+	-	-	-
Helena	+	-	+	-	-	-	+	-	-	-	-	-
Hermione	-	-	-	-	+	-	+	-	+	-	-	-
Hezekiah	+	+	+	-	+	-	-	-	-	-	-	-
Hiphop	n/a	n/a	n/a	n/a	n/a	n/a	-	-	-	-	-	-
Hoorah	-	-	-	-	+	-	+	-	+	-	-	-
Jaquinkoke	+	-	+	-	-	-	-	-	-	-	-	-
Julikmi	-	-	-	-	-	+	-	+	+	+	-	-
Keller	n/a	n/a	n/a	n/a	-	-	-	-	+	-	-	-
Kelty	-	+	-	-	-	+	-	+	+	-	-	-
Larissa	+	-	+	-	-	-	-	-	-	-	-	-
MissIsabel	+	-	+	-	+	-	-	-	-	-	-	-
Noir	+	-	-	-	-	-	-	+	+	-	-	-
Paula	+	-	+	-	-	-	-	-	-	-	-	-
Persia	+	-	+	-	-	-	+	-	-	-	-	-
Sadie	+	-	+	+	+	+	+	-	+	-	-	+
Sarah	+	-	+	-	-	-	+	-	+	+	-	-
Sawathu	n/a	n/a	-	-	-	-	+	-	+	-	-	-
Selena	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Serenac	+	-	+	-	-	-	+	+	+	-	-	-
Shag	-	-	-	+	-	+	+	+	+	-	-	-
Shiphrah	+	-	+	-	+	-	+	-	-	-	-	-
Shira	+	-	+	-	-	-	-	-	-	-	-	-
Slug	+	-	+	+	+	-	+	-	-	-	-	-
Sydney	-	-	-	+	-	-	-	-	-	-	-	+
Tahiti	+	-	+	-	-	-	-	+	+	-	-	-
Tatya	+	-	+	-	-	-	-	-	-	-	-	-
Texas	+	-	+	-	-	-	-	-	-	-	-	-
Tiger	+	-	+	-	-	-	-	-	-	-	-	-
Wallace	-	+	-	-	-	+	-	+	+	-	-	-
Wire	+	-	+	-	-	-	-	-	-	-	-	-

Name	2011		2012		2013	
	PZP	F	PZP	F	PZP	F
Anastasia	-	+	+	-	-	-
Ariel	-	-	-	-	-	-
Biff	-	-	-	-	-	-
Bo	-	-	-	-	-	-
Bridget	-	-	-	-	-	-
Carrot	-	-	-	+	-	-
Chacha	-	-	-	-	-	+
Daisy	-	-	-	+	-	+
Darcy	-	-	-	-	-	-
Darkface	-	-	-	-	-	-
Desdemona	-	-	-	-	-	+
Djibouti	-	-	-	-	-	-
Donoma	-	-	-	-	-	-
Dusty	-	-	-	-	-	-
Hedvig	-	-	-	+	-	-
Helena	-	-	-	-	-	-
Hermione	-	-	-	-	-	+
Hezekiah	-	+	-	-	-	-
Hiphop	-	-	-	-	-	+
Hoorah	-	-	-	-	-	-
Jaquinkoke	-	-	-	-	-	-
Julikmi	-	-	-	-	-	-
Keller	-	-	-	+	-	-
Kelty	-	-	-	-	-	-
Larissa	-	-	-	-	-	-
MissIsabel	-	-	-	-	-	-
Noir	-	-	-	-	-	-
Paula	-	-	-	-	-	-
Persia	-	-	-	-	-	-
Sadie	-	+	-	+	-	+
Sarah	-	-	-	-	-	-
Sawathu	-	-	-	-	-	-
Selena	-	-	-	-	-	-
Serenac	-	-	-	-	-	-
Shag	-	-	-	-	-	-
Shiphrah	-	-	-	-	-	-
Shira	-	-	-	-	-	-
Slug	-	-	-	-	-	-
Sydney	-	-	-	+	-	-
Tahiti	-	-	-	+	-	-
Tatya	-	-	-	-	-	-
Texas	-	-	-	-	-	-
Tiger	-	-	-	-	-	-
Wallace	-	+	-	-	-	+
Wire	-	-	-	-	-	-

Appendix II: Parasite Eggs

Photographs of eggs found in feces through fecal flotation, as described in Chapter 2.

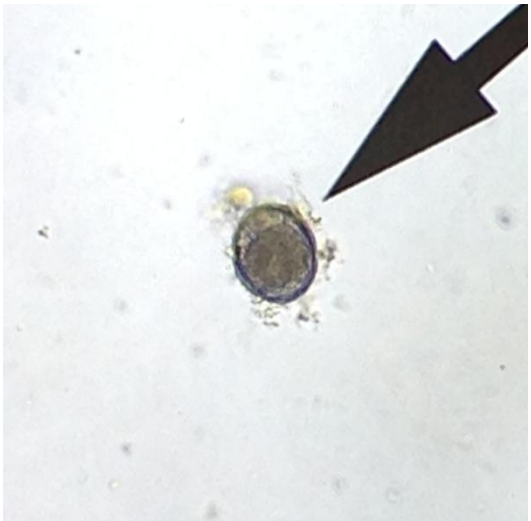
Three Large strongyle eggs in different stages of development; small strongyles were common but not photographed



Worm, most likely a hatched strongyle (Chacha, 6/30/13)



Possible *Strongyloides westeri* egg (Anastasia, 7/15/13)



Possible tapeworm egg (*Anoplocephala sp*) (Hedvig, 7/2/13)



Parascaris equorum eggs were also found, but not photographed.

Appendix III: Body Condition Scoring

As described in Chapter 2, each mare was given a body condition score from 1 to 5 based on the amount of fat in her rump. Commonly assigned scores are described below, with examples given.

Score	Description	Mare	Photograph
2	The rump takes on a concave shape from the spine to the point of the hip, which appears pointed above the rump.	Carrot	
3	There is a flat line of fat between the spine and the point of a hip, as if a book could be balanced on the surface of the rump.	Hiphop	

Score	Description	Mare	Photograph
3.5	In between a score of 3 and 4, the rump is longer a flat line but is not all the way rounded across the spine.	Keller	
4	The rump is rounded across the spine, like a dome.	Donoma	

Score	Description	Mare	Photograph
4.5	Above a score of 4, the rump begins to bulge above the spine on either side, shaped like an apple.	Shag	

Appendix IV: Recovery Based on Contraception Intensity

Numbers in parentheses designate the last year in the sequence of consecutive treatments. Mares that have more than one entry in the table are marked with *.

Consecutive years of treatment	Foaled after treatment period	Did not foal after treatment period
1	Carrot (2009) Hedvig (2009) Keller (2009) Wallace (2009)	Biff (2009) Bo (2005) Julikmi (2009) Kelty (2009) Noir (2009)
2	Sarah (2003)* Sarah (2008)* Hezekiah (2003)* Shag (2003)* Sydney (2003)	Donoma (2009) Sawathu (2009) Serenac (2009)* Shag (2009)*
3	Anastasia (2008)* Darkface (2004)* Dusty (2003)* Hermione (2009) Sadie (2003)* Slug (2004)* Tahiti (2006)	Ariel (2003) Bridget (2003) Dusty (2007)* Hoorah (2009) MissIsabel (2007) Persia (2006) Tiger (2003)
4	Anastasia (2006)* Daisy (2009) Hezekiah (2007)* Serenac (2006)*	Darcy (2005) Darkface (2007)* Djibouti (2007) Helena (2006) Jaquinkoke (2006) Larissa (2006) Paula (2006) Shiphrah (2008) Shira (2006) Slug (2008)* Tatya (2006) Wire (2006)
5	Sadie (2009)*	Texas (2006)

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Long-term fertility control in female cats with GonaCon™, a GnRH immunocontraceptive

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Abstract

The uncontrolled reproduction of free-roaming feral cats contributes to overpopulation and associated concerns regarding their welfare and impact on public health and the environment. Nonsurgical fertility control that could be administered to feral cats in the field would be a powerful tool for cat population control. The objective was to test the efficacy and duration of activity of a single-dose GnRH immunocontraceptive vaccine (GonaCon™) on the fertility of adult female laboratory cats. Vaccinated cats (n = 15) received a single injection of vaccine containing a GnRH-KLH conjugate (200 μg) emulsified in a mycobacterial and oil adjuvant on study Day 0. Sham-treated cats (n = 5) received a single injection containing all vaccine components except the GnRH-KLH conjugate. A breeding trial started on study Day 120. Vaccinated cats had a longer time to conception (median 39.7 mo) compared to sham-treated cats (4.4 mo; $P < 0.001$). A total of 93% of vaccinated cats remained infertile for the first year following vaccination, whereas 73, 53, and 40% were infertile for 2, 3, and 4 y, respectively. At study termination (5 y after a single GnRH vaccine was administered), four cats (27%) remained infertile. The GnRH antibody titers declined more rapidly in short-term responding cats with < 2 y of infertility (n = 4), compared to long-term responding cats that experienced fertility control for >2 y (n = 11) ($P < 0.05$). Non-painful but persistent late-onset granulomatous injection site masses appeared 2 y after initial vaccination in five cats. We concluded that GnRH immunocontraception is an ideal candidate for further development for feral cat control.

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Keywords: Immunocontraception; Contraception; GonaCon; GnRH; Anti-GnRH vaccination; Cat

1. Introduction

Unowned free-roaming (feral) cats number in the millions nationally and exist throughout the world [1]. Their uncontrolled reproduction contributes to cat overpopulation and associated concerns regarding their impact on public health and the environment [2]. In parts of the world where progressive animal control resources are not available, measures such as poisoning and shooting are still used to control cats [3]. Surgical sterilization followed by return to the colony is an increasingly popular method of controlling feral cat populations, but is expensive, labor-intensive, highly technical, and limited in scale [4,5].

Nonsurgical fertility control that could be administered to feral cats in the field would be a powerful tool

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for cat population control. Control of free-roaming species is often aimed at the level of the population, rather than at specific individual animals. Treatment must be efficacious in a high proportion of the population, but it is not essential that every treated animal be rendered infertile. Feral cats are wary of humans, and once trapped and released, are unlikely to return for repeated trapping episodes. A practical fertility control technique for feral cats would induce permanent or multiyear duration of action following a single treatment in the majority of animals treated.

Vaccines aimed at reproductive targets are one approach to nonlethal control of overabundant free-roaming species. Overcoming tolerance to self antigens and induction of durable immunity without the use of repeated booster vaccines are substantial challenges for the development of immunocontraceptive vaccines. A commonly used contraceptive antigen in wildlife is the zona pellucida. In previous studies, we demonstrated that vaccinating against the zona pellucida was ineffective in preventing pregnancy in cats [6,7]. Subsequently, we showed in a short-term study that a single dose of GnRH vaccine in a mycobacterial and oil emulsion (GonaCon™) effectively blocked testosterone production and spermatogenesis for at least 6 mo in a majority of vaccinated adult male cats [8]. This was apparently the first report to suggest that fertility control may be achievable in cats following a single vaccination against a reproductive self antigen.

We hypothesized that long-term fertility control in cats can be induced by immunological blockade of GnRH activity. The purpose of this study was to test the efficacy and duration of activity of single-dose GnRH immunocontraception on the fertility of adult female cats.

2. Materials and methods

2.1. Cats

Twenty-four 8- to 14-mo-old specific-pathogen-free female domestic shorthair cats were acquired from a commercial vendor (Liberty Research, Waverly, NY, USA). Cats were group-housed in one large enriched room with raised resting benches, maintained at ambient temperatures between 21 and 23 °C, and exposed to controlled lighting (explained later). Food and water were available *ad libitum*. The experimental design was approved by the University of Florida Institutional Animal Care and Use Committee, and facilities were accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All cats and their offspring underwent surgical sterilization and were adopted to private homes at the conclusion of the study.

Upon arrival, the 24 cats were housed for 30 d in a photoperiod regimen (8 h light: 16 h dark) that is inhibitory for estrous cyclicity. Forty-six days prior to treatment (Day −46), the photoperiod regimen was reversed to 16 h light: 8 h dark, which is permissive for estrous cyclicity within 15 d in most cats [9,10]. In cats, behavioral estrus occurs in conjunction with elevated concentrations of estradiol-17β secreted by developing follicles. Progesterone concentrations rise significantly within 4 d after ovulation, regardless of whether or not fertilization has occurred [9,10]. Serum progesterone and estradiol-17β concentrations were measured on Day −64, and then every other day from Day −46 to Day −26. The magnitude and duration (Day > 20 pg/mL) of elevated estradiol-17β concentrations in each cat were used to confirm normal photoperiod sensitivity and estrous cyclicity in cats selected for this study.

2.2. Vaccine construction

A GnRH vaccine was constructed using a synthetic GnRH peptide with the sequence [pEHWSYGLRPGGC-SH] produced by the Fmoc/tBU protection method (Global Peptide Services, Fort Collins, CO, USA). Immunogenicity was enhanced by coupling GnRH peptides to a protein carrier, keyhole limpet hemocyanin (KLH; Pierce Biotechnology, Rockford, IL, USA), in a 1:3 GnRH:KLH mass ratio. The first 10 amino acids represent the native GnRH molecule and "pE" signifies pyroglutamate. One glycine was added at the C-terminus as a spacer and a cysteine was added to ensure consistent alignment of the peptide to the maleimide-activated KLH. The aqueous-based GnRH-KLH conjugate (200 μg) was combined in a 1:1 ratio by volume with an emulsifying adjuvant, AdjuVac™, which was produced by modifying a USDA-licensed Johne's disease vaccine containing inactivated *Mycobacterium avium* in mineral oil (Mycopar®; Fort Dodge Animal Health, Fort Dodge, IA, USA). This GnRH vaccine (GonaCon™, USDA, Pacarello, ID, USA) is licensed as a "restricted-use pesticide" for prevention of estrous cyclicity in white-tail deer.

2.3. Treatment

Twenty cats were selected for use in the GnRH vaccination study based upon estrus induction following an increase in light to 16 h per day. These 20 cats were randomized based upon a similar peak estradiol-

17β concentrations in response to long-day photoperiod into a sham treatment group (n = 5) and a vaccination group (n = 15). The sham-treated group received placebo injections containing all components except the GnRH-KLH conjugate. The treatment group received vaccines containing 200 μg GnRH-KLH. Vaccines were administered intramuscularly under general anesthesia. Brief anesthesia was induced by administration of isoflurane (IsoFlo®; Abbott Laboratories, North Chicago, IL, USA) by face mask. The hair of the right cranial thigh was clipped, and the injection site was cleaned with 70% isopropyl alcohol. The vaccine (0.5 mL) was injected into the quadriceps muscle group. This site was selected to avoid critical structures such as the sciatic nerve in the event of a vaccine reaction. This site is also accessible when cats are resting in a crouched position, making it one of the likely injection targets in nonsedated cats in the field. The right pinna was tattooed with a treatment code for permanent identification.

Potential reactions to vaccination were evaluated by daily physical examination, including inspection of the injection site, and measurement of body temperature for 1 wk following treatment. The injection site was inspected at the time of each monthly blood sample collection throughout the study.

2.4. Breeding trial

The effects of treatment on fertility were evaluated in a breeding trial beginning 4 mo after treatment and continuing until each cat became pregnant or until 60 mo post-treatment, whichever came first. The daily photoperiod was alternated between the estrus-inhibiting short-day regimen (16 h dark:8 h light) and the estrus-inducing long-day regimen (8 h dark:16 h light). The cats were exposed to short-day photoperiod from Days 0 to 120, and from Days 330 to 360 (8% of post-treatment time). For the remainder of the study, the cats were exposed to long-day photoperiod. The switch from short-day photoperiod to long-day photoperiod was intended to induce estrus in any of the cats that were capable of an estrous response. A breeding male was housed continuously with the females during the long-day photoperiods beginning on Day 120. Four breeding males were alternated, to reduce the chance that inter-cat behavioral incompatibility would affect breeding success. Time-lapse videography was used to monitor breeding activity during lighted hours. The recordings were reviewed daily and the number of attempted and successful breedings was recorded.

Time to conception was defined as the interval between treatment Day 0 and fertilization date, which was estimated by subtracting the average length of cat gestation (63 d) from parturition date. Fertility was defined as full-term pregnancy. Litter size was defined as the number of total births (live and stillborn). Fecundity was defined as the number of live births. Pregnancy was scored as a treatment failure, and the cats were removed from the study after parturition. Short-term response to GnRH immunocontraception was defined as fertility control for two y or less, whereas a long-term response was defined as fertility control lasting more than 2 y after the single vaccination.

2.5. Blood collection

Blood (4 mL) was collected by jugular venipuncture monthly into serum separator tubes for determination of GnRH antibody titer and progesterone concentration. In addition, blood was collected every other day during an interval of 20 d following change from short-day photoperiod to long-day photoperiod beginning on Days 120 and 360 (for determination of serum concentrations of estradiol-17β and progesterone). Serum was separated by centrifugation and stored at −20 °C pending analysis.

2.6. Detection of GnRH antibodies

Serum was tested for GnRH antibodies using an enzyme-linked immunoabsorbant assay (ELISA). The 96-well plates (Thermo Electron Corporation, Waltham, MA, USA) were coated with BSA-GnRH and blocked with PBS buffer containing steelhead salmon serum (SEA BLOCK; Pierce Biotechnology, Rockford, IL, USA). Cat serum was added to wells in two-fold dilutions starting at 1:1,000. Two negative controls (buffer without cat serum and pre-vaccination cat serum) and one positive control (serum from a cat with a known high antibody titer) were included on each plate. The GnRH antibodies were detected with goat anti-cat IgG (Sigma Chemical Co., St. Louis, MO, USA) followed by rabbit anti-goat IgG conjugated with horseradish peroxidase (Sigma Chemical Co.), and color development with tetramethylbenzidine/phosphate-citrate buffer (Sigma Chemical Co.). Sulfuric acid (2M) was added to each well to stop the reaction. Absorbance was read at 450 nm with a Dynatech MR 5000 ELISA plate reader (Dynatech Laboratories Inc., Alexandria, VA, USA). The endpoint titers were defined as the dilution at which the absorbance was twice the absorbance of the pre-vaccination cat serum.

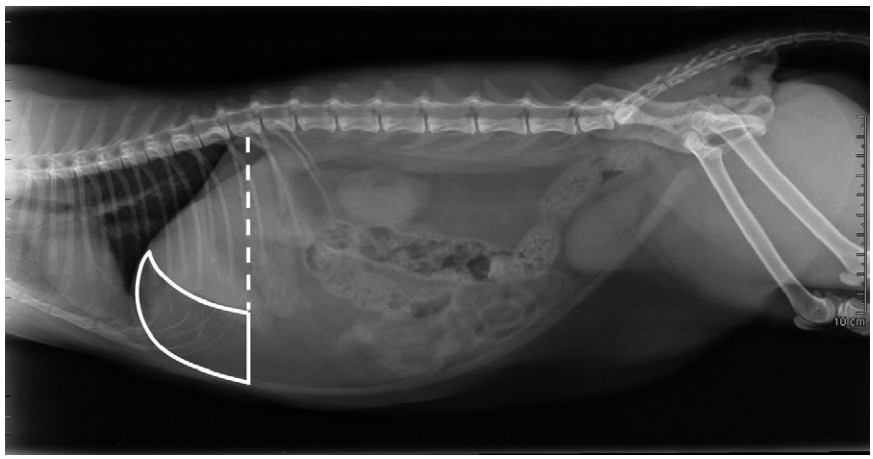


Fig. 1. The size of the falciform fat pad in female cats was assessed radiographically as an estimate of body fat content. The depth of the fat pad was measured at the level of the center of the T12 vertebral body (dashed line) from the ventral border of the liver to the ventral abdominal wall. The area of the fat pad was measured as the area confined by the ventral border of the liver, the caudal surface of the diaphragm, the ventral abdominal wall, and the line used for the depth of the fat pad measurement. The solid line shows the boundaries for measuring the area of the fat pad.

2.7. Determination of serum concentrations of estradiol-17β and progesterone

Serum samples were analyzed for total estradiol-17β and progesterone by radio-immunoassay (Coat-A-Count®; Diagnostic Products Corporation, Los Angeles, CA, USA) according to the manufacturer's instructions. In each run, controls included human serum with three concentrations of estradiol-17β (57, 127, and 934 pg/mL) and progesterone (1.78, 3.4, and 18.3 ng/mL).The manufacturer reports an assay sensitivity for estradiol-17β of 8 pg/mL with inter-assay CV (coefficient of variation) of 4 to 8% and intra-assay CV of 4 to 7%. Progesterone assay sensitivity was reported to be 0.02 ng/mL with inter-assay CV of 4 to 10% and intra-assay CV of 2 to 9%.

2.8. Body weight and composition

It has been well-documented that cats gain weight and percent body fat after ovariectomy [11,12], but it is unknown if immunocontraception has the same effect. To evaluate for this effect, an estimation of body fat was made by radiographic evaluation of the abdominal falciform fat pad on Day 420. Cats were sedated with medetomidine 40 mcg/kg IM (Domitor; Pfizer Animal Health, New York, NY, USA) and imaged in right lateral recumbency using computed radiography (Kodak, Rochester, New York, USA). The depth (mm) of the falciform fat pad was measured by dropping a perpendicular line from the center of the body of the 12th thoracic vertebra to the ventral body wall and measuring the distance between the caudoventral angle of the liver and the ventral body wall. The area (mm^2) of the fat pad was defined as the area outlined by the line used for the depth measurement, the ventral border of the liver, the caudal aspect of the diaphragm, and the ventral body wall (Fig. 1).

2.9. Statistical analysis

Differences in the median time to conception between the sham-treated group and the GnRH-vaccinated group were determined by the Kaplan-Meier survival analysis log rank test. Mean ± SEM were calculated for normally distributed data (monthly titers, body weight, fat pad measurements, litter size), and groups were compared with the Kruskal-Wallis one way ANOVA. Median and interquartile range (IQR) were calculated for non-normally distributed data (peak titer, titer at conception), and groups were compared with the Mann-Whitney rank sum test. Differences were considered significant when $P < 0.05$. All tests were performed using SigmaStat® statistical software, version 3.1 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Reactions to treatment

Body temperature in all of the cats remained normal after treatment, and there was no inflammation or tenderness at the injection sites. One vaccinated cat died

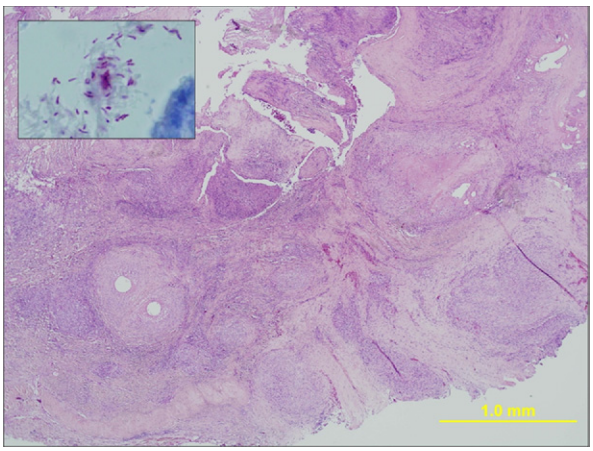


Fig. 2. Biopsy (stained with hematoxylin and eosin) of a subcutaneous injection-site nodule in a cat which appeared 24 mo following a single vaccination against GnRH. There was granulomatous inflammation, with acid fast bacilli evident within the lesion (inset). Five cats developed persistent late-onset, non-painful, injection-site nodules.

suddenly 45 d after treatment; necropsy revealed no gross or histological abnormalities to explain the death. This cat was replaced with a 2 y old cat of proven fertility from the colony and vaccinated with the GnRH vaccine according to the standard protocol.

Twenty-four months after immunization, it was discovered that one of the cats had a firm 4 cm × 5 cm mass at the vaccination site. No pain reaction was elicited on palpation of the mass. A biopsy revealed granulomatous inflammation with interlesional acid-fast bacteria (Fig. 2). Aerobic, anaerobic, and mycobacterial cultures were negative. Smaller single or multiple masses < 1 cm in diameter developed at the injection site of four other cats. The size of the masses in all five cats waxed and waned for the duration of the study, but the cats never appeared painful and the lesions never drained. Three of the cats with masses never became pregnant, and the other two became pregnant at 863 and 1212 d, respectively. No masses were observed in the sham-treated cats.

3.2. Breeding trial

All four breeding male cats sired litters during the study. The first male cat was introduced on Day 120 post-treatment at the beginning of a second long-day photoperiod. All sham-treated cats displayed behavioral signs of estrus and were receptive to breeding attempts by the male within 10 days of the change to long-day photoperiod. Time-lapse videography revealed that the male bred each of the sham-treated cats at least 15 times between Days 122 and 155. One sham-treated cat was observed breeding in two intervals during gestation (Days 7 to 11 and Days 25 to 26 of gestation). Four (27%) vaccinated cats that displayed estrous behavior and began breeding on Days 128, 418, 469, and 504 were classified as having a short-term response to GnRH vaccination.

Based on an average gestation period of 63 d, parturition date was used to estimate the date of conception. The GnRH-vaccinated group had a longer median time ($P < 0.001$) to conception following treatment (39.7 mo) compared to the sham-treated group (4.4 mo; Fig 3). All five sham-treated cats became pregnant 7–28 d (study Days 127–148) after the male cat was introduced. One short-term responder cat was bred in three successive periods of estrus between Days 129–164, before becoming pregnant on Day 164, whereas the other three short-term responder cats became pregnant during their first estrus following a prolonged vaccine-induced anestrous period (Days 418, 469, and 504, respectively).

Eleven (73%) cats were classified as long-term responders with fertility control lasting greater than two y following a single GnRH vaccination. A total of 93% of vaccinated cats remained infertile for the first year following vaccination, 73% for 2 y, 53% for 3 y, and 40% for 4 y (Fig. 3). At the time the study was terminated (5 y after a single GnRH vaccine was administered), four cats (27%) had never become pregnant.

There was a small but significant difference in fecundity (live births) in the GnRH-vaccinated group (2.6 ±

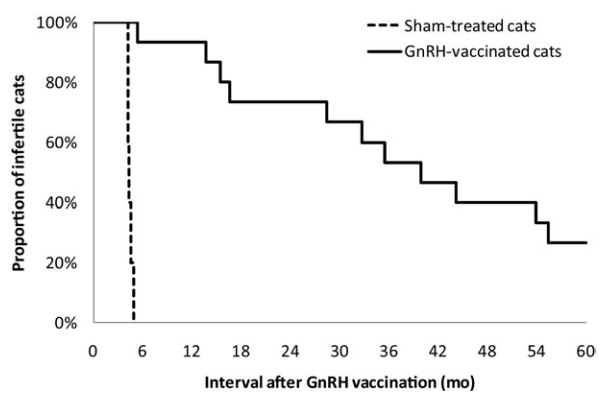


Fig. 3. Fertility control in cats. Vaccinated cats (n = 15) received a single injection of vaccine containing a GnRH-KLH conjugate (200 μg) in a mycobacterial and oil emulsion on study Day 0. Sham-treated cats (n = 5) received a single injection containing all vaccine components except the GnRH-KLH conjugate (n = 5). A breeding trial was commenced on study Day 120. Vaccinated cats had longer time to conception (median 39.7 mo) compared to sham-treated cats (4.4 mo; $P < 0.001$).

Table 1
Time to conception, litter size, and GnRH antibody titers in individual sham-treated cats (n = 5), short-term responding cats (n = 4), and long-term responding cats (n = 11). Four cats remained infertile for the entire duration of the 5 y study.

Cat	Conception (Day)	Peak GnRH titer	GnRH titer at conception	Live births	Still births	Injection-site reaction
Sham 1	127	0	0	3	0	No
Sham 2	128	1000	1000	3	1	No
Sham 3	131	0	0	4	0	No
Sham 4	137	0	0	5	1	No
Sham 5	148	4000	4000	4	0	No
Short-term 1	164	32,000	8000	3	0	No
Short-term 2	418	32,000	4000	2	0	No
Short-term 3	469	128,000	4000	3	0	No
Short-term 4	504	128,000	4000	1	0	No
Long-term 1	863	128,000	16,000	3	0	Yes
Long-term 2	995	128,000	64,000	2	1	No
Long-term 3	1078	128,000	64,000	3	1	No
Long-term 4	1212	256,000	128,000	4	0	Yes
Long-term 5	1343	256,000	128,000	4	0	No
Long-term 6	1640	128,000	0	3	1	No
Long-term 7	1684	256,000	0	1	3	No
Long-term 8	NA	256,000	NA	NA	NA	No
Long-term 9	NA	128,000	NA	NA	NA	Yes
Long-term 10	NA	256,000	NA	NA	NA	Yes
Long-term 11	NA	256,000	NA	NA	NA	Yes

0.3) compared to the sham-treated group (3.8 ± 0.4; P = 0.04; Table 1). There was no difference in the number of stillborn kittens between the groups (P = 0.9).

3.3. GnRH antibody titers

All vaccinated cats developed detectable GnRH antibodies (Table 1). The magnitude of GnRH antibody titers 1 mo after vaccination was similar in cats that responded to vaccination for < 2 y (short-term responding group) and those that responded for > 2 y (long-term responding group; Fig. 4). However, antibody titers decreased more quickly in the short-term responding group and remained lower at each monthly point beginning 5 mo post-vaccination (P < 0.05). Peak

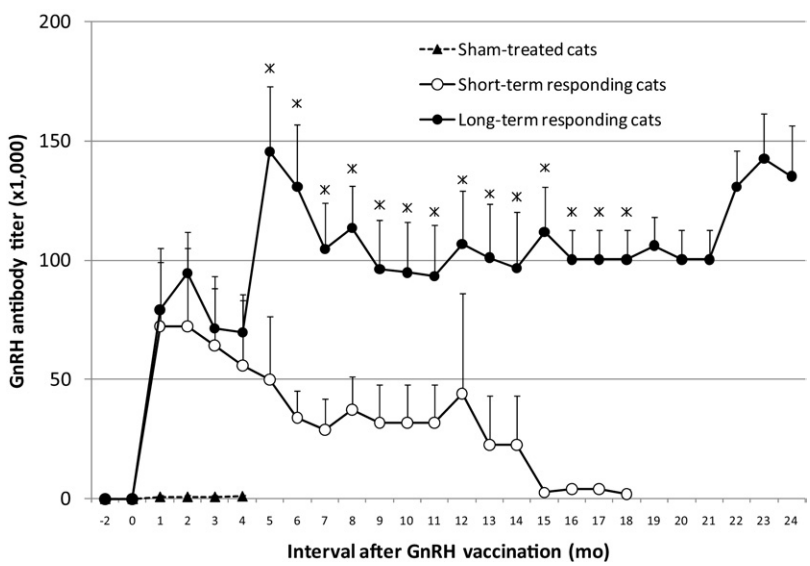


Fig. 4. GnRH antibody titers (mean ± SEM) in sham-treated cats (n = 5), short-term responding cats (n = 4), and long-term responding cats (n = 11) during the first 2 y following treatment with a single sham or GnRH vaccine. The GnRH antibody titers declined more rapidly in short-term responding cats with < 2 y of infertility compared to long-term responding cats that experienced fertility control for >2 y (*P < 0.05).

GnRH antibody titers were higher in the long-term responding group (median 256,000, IQR 128,000–256,000) than in the short-term responding group (median 80,000, IQR 32,000–128,000; P = 0.02; Table 1). Most cats that became pregnant had antibody titers of 16,000 or less at the time of conception, but four cats had antibody titers of 64,000–128,000. Antibody titers in the four cats that never became pregnant were 128,000–256,000 at the end of the study, 5 y after the single GnRH vaccination.

3.4. Hormone concentrations

Nineteen of the cats selected for the study responded to long-day photoperiod prior to treatment, with a surge in estradiol-17β > 20 pg/mL for at least four consecutive days and peak estradiol-17β > 40 pg/mL for at least 1 d. The remaining cat responded to long-day photoperiod with elevation of estradiol-17β from baseline, but exceeded 20 pg/mL on 1 d only. Six of 20 cats had an increase in serum progesterone > 2 ng/mL following the change to long-day photoperiod prior to treatment, indicating pseudopregnancy following estrus, even though the male cat had not yet been admitted to the colony. A cycle of 1 mo of short-photoperiod followed by return to long-day photoperiod on Day 360 failed to trigger behavioral estrus, breeding attempts, or increases in serum progesterone in any of the 14 vaccinated cats that remained in the study at that time.

The first elevation of progesterone > 2 ng/mL during monthly testing was associated with pregnancy in 14 of the cats (5 sham-treated cats and 9 vaccinated cats). In two other cats, both long-term responders, episodic non-fertile progesterone increases were detected during Days 1500 to 1590 and Days 1410 to 1650 before conception followed by term pregnancy occurred on Days 1640 and 1684, respectively. Progesterone elevation was also observed on Days 1230, 1650 to 1680, and 1830, respectively, in three of the cats that never became pregnant during the 5 y study period. Elevated progesterone was never detected in one of the cats that never became pregnant.

3.5. Body weight and composition

At the time of treatment on Day 0, there were no significant differences (P = 0.5) in body weight among the sham-treated (3.5 ± 0.2 kg), short-term responder (3.2 ± 0.2 kg), and long-term responder (3.3 ± 0.1 kg) groups. Similar to cats undergoing surgical sterilization, long-term responder cats had a higher percentage gain in body weight than sham-treated cats and short-term responder cats (P = 0.02; Table 2). Weight gain was also reflected in abdominal fat content. The mean falciform fat pad depth for the long-term responder group was greater than the mean for the sham-treated group (P = 0.02) and the short-term responder group (P = 0.03). The area of the falciform ligament was not significantly different among groups (P = 0.1).

Table 2
Body weight gain and abdominal fat (mean ± SEM) in cats as a function of response to GnRH vaccination. Body weight was measured at the time of treatment on Day 0 and again on Day 420 and the percentage change in weight calculated. Abdominal falciform fat pad depth and area were measured from lateral radiographs made on Day 420. Long-term responding cats (n = 11) gained more weight and had greater fat pad depth than sham-treated (n = 5) and short-term responding cats (n = 4).

Response group	Body weight gain (%)	Fat pad depth (mm)	Fat pad area (mm^2)
Sham (n = 5)	15 ± 7	19 ± 2	728 ± 145
Short-term responses (n = 4)	4 ± 10	19 ± 2	839 ± 172
Long-term responses (n = 11)	40 ± 6*	28 ± 3*	1193 ± 148

* P ≤ 0.02.

4. Discussion

The vaccine used in this study induced fertility control in 100% of cats following a single injection, but duration of contraception varied from 5 mo to > 5 y. Infertility was maintained for 3 y in approximately half of the cats and for more than 5 y in a quarter of the cats. Response to vaccination was accompanied by a cessation in estrous cyclicity and with weight gain, similar to cats undergoing surgical sterilization via ovariectomy.

This is apparently the first study to demonstrate multi-year fertility control following a single vaccination in cats. Although permanent sterilization is ideal, the relatively short life-span of many free-roaming feral cats suggests that a contraceptive that blocks fertility for several years in a high proportion of cats may be successful in reducing the population. Most models propose that more than 70% of the female cats in a population must be rendered infertile to trigger a negative population growth rate [13–15], but one model found that focusing a control program on juvenile cats and utilizing a contraceptive having 3 y duration of activity would stop population growth when just 60% of the female cats were treated [16].

Duration of infertility was associated with persistence of high GnRH antibody titers. Most cats with an antibody titer > 16,000 remained infertile. However, several cats, all long-term responders, successfully re-

produced in the presence of antibody titers as high as 256,000. We inferred that there is no absolute contraceptive antibody titer that is predictive of infertility and that other factors also influence the contraceptive effect of GnRH immunization in female cats. In a previous study in male cats, antibody titers of $> 32{,}000$ were associated with suppression of serum testosterone concentrations and spermatogenesis consistent with immunocastration [8]. Immunocontraception targeting GnRH appeared to be more effective in female cats than in male cats. All female cats in the current study experienced a delay in conception compared to sham-treated cats, whereas only 67% of male cats demonstrated infertility as determined by semen analysis in the previous study [8]. Higher efficacy in females has been observed in other species including feral swine [17], white-tailed deer [18], and domestic dogs (L. Miller, unpublished).

In addition to suppression of estrus, GnRH immunocontraception may have additional effects on reproduction. In this study, litter sizes in cats that recovered fertility were slightly smaller than litter sizes in sham-treated cats. Whether this is a transient phenomenon in the first pregnancy following recovery of fertility, is related to the older age of the vaccinated cats at the time of pregnancy, or is a persistent effect possibly related to the continued presence of GnRH antibodies is unknown, as cats were removed from the study following delivery of their first litter.

Granulomatous injection-site reactions containing acid fast bacteria, presumably from the adjuvant, were identified in five long-term responding cats. This evidence of an ongoing immune response may explain the ability of this depot vaccine to create durable immunity against a self antigen in a large proportion of cats in the absence of a booster vaccination protocol. Granulomatous and neoplastic masses at the injection site and systemic inflammatory responses have been previously reported in cats receiving zona pellucida antigens in the presence of mycobacteria and oil (Freund's complete or incomplete adjuvants) [6,19]. The production of durable immunity using non-replicating vaccines is a great challenge, particularly against a small self antigen such as GnRH [20]. Adjuvants and carrier proteins with T-cell epitopes that stimulate the immune system to react against the target antigen do so in the context of inducing a local inflammatory response. As a species, cats are at increased risk for development of life-threatening sarcomas at the site of inflammation resulting from vaccination and other injections [21]. In another study of GnRH immunocontraception in cats, all kittens vaccinated with a GnRH vaccine or placebo containing oil developed acute transient injection-site masses [22]. The initial reactions resolved, but examination of injection sites following booster vaccines up to 20 mo later was not reported. Consideration should be given to the development of vaccine formulations that reduce the risk of injection-site reactions and possible sarcoma formation in cats. However this concern should be balanced against the very real threats to the welfare of cats associated with their uncontrolled reproduction and with control programs that rely on long-term confinement or culling. Risk assessments associated with non-surgical fertility control should also include a comparison with the known morbidity and mortality associated with surgical sterilization, particularly in owned cats in which the benefit to the individual is a higher priority than population control.

GnRH has many of the characteristics of an ideal contraceptive target. Hypothalamic GnRH is a decapeptide "master hormone" that controls downstream pituitary and gonadal responses in both male and female mammals. In the absence of GnRH stimulation, the gonadal hormones estrogen, progesterone, and testosterone are suppressed and gamete maturation does not occur [8,22,23]. This results in infertility, elimination of repeated estrous cycles that can lead to uterine disease and mammary carcinoma, and reduction of undesirable nuisance behaviors such as fighting, roaming, spraying, and estrous calling. This is an advantage over contraceptive targets (such as zona pellucida) that block fertility, but not necessarily estrous cyclicity. The availability of a single product effective in both males and females following a single dose is also an advantage, because it eliminates the need to re-trap animals for repeat treatments, to maintain an inventory of multiple different products, to target trapping campaigns to one sex, or even to determine what sex a trapped cat is. The vaccine used in this study was approved in September 2009 for control of white-tail deer by the Environmental Protection Agency, which regulates the use of "pesticides," including nonlethal products for use in feral and "pest" species [24]. The vaccine has not been approved for use in any other species at this time, including feral cats.

Many feral cats are too unsocialized for safe handling. In contrast to surgical sterilization, the GnRH vaccine used in this study could possibly be administered without sedation to cats confined in a humane trap. This obviates the need for veterinarians to be directly involved with each cat targeted in a population control campaign, a distinct advantage in parts of the

world where veterinary services for companion animal species are not universally available. These findings suggest that GnRH immunocontraception is an ideal candidate for further development for the control of feral cats.

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Contraception in mares heteroimmunized with pig zonae pellucidae

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Summary. Ten fertile feral mares and 6 domestic horses (4 fertile mares, 1 infertile mare, 1 gelding) were immunized with heat-solubilized pig zonae pellucidae by 4 injections equivalent to 2000 or 5000 zonae each at 2–4-week intervals and a booster injection of 20 000 zonae 6–10 months after the last of the initial inoculations. The immune response was reflected by high antibody levels as measured by an enzyme-linked immunosorbent assay (ELISA) using immobilized pig zona antigen. In-vivo inhibition of fertility occurred in 12 (86%) of the 14 fertile mares studied and persisted for a minimum of 7 months. Repeated mating of the fertile domestic mares resulted in conception when anti-pig zona antibody concentrations had decreased from initial peak absorbance ratios (>1·0) to relatively lower levels (0·64 or less with one exception). An indirect immunofluorescence assay, revealed a considerably lower cross-reactive antibody titre with horse oocytes as compared to pig oocytes. Clinical, endocrinological and histological analyses of the ovaries and their function following regained fertility after immunization revealed no abnormalities. One mare remained infertile.

Keywords: horse; contraceptive vaccine; zona pellucida; antibodies; heteroimmunization.

Introduction

Attempts to reduce fertility in several mammalian species have been reported. Active immunization of female rabbits (Gwatkin *et al.*, 1980; Woods *et al.*, 1981; Skinner *et al.*, 1984), bitches (Shivers *et al.*, 1981; Mahi-Brown *et al.*, 1982) and monkeys (Sacco *et al.*, 1983) with pig zonae pellucidae has resulted in reduced fertility of the treated animals. Similarly, immunocontraception was achieved in mice (Gwatkin *et al.*, 1977) and rabbits (Gwatkin & Williams, 1978) by using hamster and cattle zonae. Other investigators have successfully inhibited fertilization *in vivo* by passive immunization, injecting rodents of several species with antisera against mouse (Shahani *et al.*, 1972; Jilek & Pavlok, 1975), rat and hamster (Tsunoda & Chang, 1976; Oikawa & Yanagimachi, 1975) ovaries.

Natural antibodies directed against the zona pellucida have been reported to occur in mares infertile for unexplained causes (Liu & Shivers, 1982). Further attempts to investigate the role of horse 'auto-antibodies' against the zona demonstrated immunological cross-reactivity of pig and horse zonae and in-vitro reduction of binding of boar spermatozoa to pig oocytes treated with horse zona-positive antiserum (Shivers & Liu, 1982).

According to several reports, however, the use of zona components as the antigen in active immunization results in altered cyclicity and ovarian malfunction. Such side effects have been described for the bitch by Mahi-Brown *et al.* (1982, 1985). A decrease in ovarian weight, marked reduction of follicular development, abnormal follicular differentiation and lack of response to induction of ovulation by human chorionic gonadotrophin were also reported for rabbits after

active immunization (Wood *et al.*, 1981; Skinner *et al.*, 1984). Sacco *et al.* (1983, 1987) have reported that fewer ovulated oocytes were recovered from monkeys after injections of purified pig zona antigen, but ovarian function was reduced only temporarily and returned to normal 7 months after the end of the treatment.

The present study was conducted to determine whether heteroimmunization of mares with crude pig zona antigen would temporarily inhibit fertilization *in vivo* and, if so, to assess the possible causes of the inhibition.

Materials and Methods

Selection of feral mares. Thirty (30) feral mares were captured at a wild horse sanctuary of $\sim 2000\ km^2$ in Northern California holding approximately 200 horses of different ages (~ 120 mares, 15 stallions and 65 geldings). After 6 months of confinement to a large area without stallions each mare was herded into a restraining chute and the reproductive tract was palpated *per rectum* to determine pregnancy status and potential abnormalities. Ten (10) presumably fertile mares were selected for this study on the basis of (1) a palpable normal reproductive tract, (2) a non-gravid uterus and (3) age (estimated to be 3–10 years old): 4 of the mares appeared to be maiden mares and the remaining 6 had foals by their sides. The individual mares were identified by their colour and markings and the group of mares treated was marked by removing their manes entirely with a pair of scissors. The 10 selected mares remained in a small corral for the period of the initial 4 immunizations, were subsequently released and recaptured 8 months later for assessment of pregnancy status and administration of booster injections.

Selection of domestic horses. These horses provided the opportunity for frequent serological analysis and continued examination of the reproductive tract for folliculogenesis, ovulation and pregnancy status. The group, housed at the Equine Research Laboratory's breeding facility of the University of California in Davis, consisted of 4 presumably fertile mares, 1 infertile mare with chromosome abnormality (63XO genotype) and 1 gelding. The 4 fertile mares were selected on the basis of (1) a palpable normal reproductive tract and speculum examination, (2) age, (3) history of pregnancy, and (4) normal cyclicity. The fertile mares were between 3 and 8 years old, had a history of never having been mated naturally or by artificial means and had normal physiological and behavioural oestrous cycles. The infertile mare and the gelding served as immunological controls.

Preparation of pig oocytes and zona antigen. Zonae pellucidae were isolated using procedures reported by Gwatkin *et al.* (1980) and Dunbar *et al.* (1980). The frozen–thawed ovaries were sliced in a ganged razor blade apparatus. The oocytes were separated by filtration through a series of screens of 210, 150 and 73 μm in the presence of phosphate-buffered saline (PBS, pH 7·2). The oocytes were counted and ground in a Potter–Elvehjem homogenizer. The zonae were trapped on a 48 μm screen, thoroughly rinsed with PBS and heat-solubilized at 70°C for 30 min in PBS for inoculation (doses of 2000 or 5000 zonae in 0·5 ml, 20 000 zonae in 2 ml), or in 0·1 M-glycine buffer (adjusted to pH 9·5 with NaOH) when used for the ELISA. The protein concentration of the heat-solubilized pig zona antigen was determined by the Automated Protein Assay (Technical Bulletin 1177: Bio-Rad Laboratories, Richmond, CA, U.S.A.) microassay procedure using bovine serum albumin as standard. Doses of 2000, 5000 and 20 000 zonae corresponded to 27·4, 68·5 and 274 μg protein, respectively.

Immunization adjuvants. The two initial inoculations used for all 16 horses consisted of a mixture of 3 parts zona antigen and one part amphogel (aluminium hydroxide gel) as adjuvant. As based on preliminary data obtained in an indirect immunofluorescence assay, this adjuvant seemed to be only moderately effective in most of the horses and was therefore substituted by Freund's adjuvant. Emulsions of equal parts of zona antigen and Freund's (complete for the initial, incomplete for the following injections) adjuvant were used for the remaining inoculations. However, serum analyses performed later by ELISA revealed more satisfactory immunization responses in 13 of the 16 horses after use of amphogel as adjuvant.

Immunization protocol. A series of 4 intramuscular injections of zona antigen was administered to each of the 16 horses at 2–4-week intervals. The doses for the 1st, 3rd and 4th injection consisted of 5000 zonae, the second injection dose consisted of 2000 zonae. At 6–9 months after the last injection, when antibody concentrations in the fertile domestic mares had decreased to levels that allowed conception at absorbance ratios of $<0{\cdot}64$ (one exception), the fertile domestic and feral mares received booster injections of 20 000 zonae each. Serum samples for antibody determination were obtained from all the horses before each inoculation and from the domestic horses monthly (with some variations) throughout the 3·5-year period of this study.

Mating of mares and pregnancy test. After the initial 4 inoculations, the feral mares were returned to their natural habitat with known fertile stallions: 8 months later they were recaptured and examined for pregnancy status by palpation *per rectum*. After immunization, the 4 fertile domestic mares were rectally palpated every other day through each oestrous cycle (every 18–21 days) of the mating season for observation of follicular development and

ovulation. (The natural mating season usually lasts from March through October and is followed by a 4-month anoestrous period.) Through each cycle and following the development of a preovulatory follicle of at least 40 mm in diameter, the mares were inseminated naturally or artificially with semen from known fertile stallions until ovulation. At 16–25 days after ovulation, ultrasonographic scanning was utilized for early pregnancy detection (embryo and heart beat). Additional pregnancy examinations by rectal palpation were performed at 45 and 60 days of gestation. The first pregnancies of the domestic mares that occurred during the study were terminated at 25 days by injection of 10 mg prostaglandin (Lutalyse: Upjohn, Kalamazoo, MI, U.S.A.). At 4 months after delivery of healthy foals in the second pregnancy, to Mares ST and GO, all 4 domestic mares were ovariectomized by colpotomy.

ELISA for determination of zona antibodies. The assay was performed essentially as described by Voller *et al.* (1980). Fox *et al.* (1981) and Drell *et al.* (1984) have measured antibody activity to pig zona antigen by the ELISA procedure. The biotin–avidin bridge method (S. Grimes, personal communication) was chosen for this study because of its low background reactivity.

Briefly, 50 µl of a 5 µg/ml zona antigen solution in 0·1 M-glycine buffer was placed in each well of a flat-bottom Microelisa Immulon 2 plate (Dynatech Laboratories, Alexandria, VA, U.S.A.) and incubated overnight at 4°C. The plate was washed once and incubated with 200 µl PBS–Tween for 30 min to block unspecific binding sites. After 2 more washes (PBS–Tween) the treatment of the plate consisted of subsequent 1-h incubations with 50 µl/well of PBS–Tween-diluted reagents used in the following order with 3 washes each in between: (1) test serum 1:1000, (2) biotinylated goat anti-horse IgG (Zymed Laboratories, San Francisco, CA, U.S.A.) 1:1000, (3) alkaline phosphatase avidin (Zymed) 1:2000. Finally, 50 µl substrate solution of 1 mg *p*-nitrophenyl phosphate/ml (5 mg tablets: Sigma Chemical Co., St Louis, MO, U.S.A.) in 10% diethanolamine buffer (pH 9·8) were added to each well, and the plate was scanned for absorbance at 405 nm (A_{405}) by an MR 580 Microelisa Auto Reader (Dynatech) when the absorbance of the positive reference serum had reached a level between 0·80 and 1·00 after incubation for 15–20 min.

Optimal concentrations of biotinylated goat anti-horse IgG and alkaline phosphatase avidin were selected after 'checkerboard' experiments in which, in an antigen–antibody (reference serum)-coated plate, doubling dilutions of one reagent are tested against doubling dilutions of the other reagent. The working dilution of the experimental sera (1:1000) was chosen from within the straight segment of the sigmoidal curve obtained after plotting various dilutions of the positive reference serum against their absorbances. Control reagents included PBS–Tween, 3 positive sera (weak, medium, strong) and 2 negative sera. The medium positive serum consisted of a pool of 8 different pre-tested serum samples and served as reference serum. The negative control pools were derived from 12 non-immunized random horses and from the pre-immunization samples of the 6 domestic horses in this study.

The experimental sera were tested in duplicate and their results are expressed as means ± s.e.m. of the ratios of the duplicate experimental absorbances divided by the mean absorbance of the reference serum tested on the same plate (EA_{405}/RA_{405}).

Indirect immunofluorescence assay with horse and pig oocytes. Frozen horse ovaries (obtained from an abattoir) were thawed and minced in ice-cold PBS and the mixture was filtered through gauze and a 73 µm screen. The oocytes were aspirated by micropipette from the cellular material trapped on the screen under a stereo microscope. The difficulty in obtaining horse ovaries and the poor recovery of oocytes from them (an average of 4 per ovary) did not permit any large scale usage as needed in the ELISA. Horse and pig (prepared as described earlier) oocytes (5 of each) were incubated for 30 min with 300 µl serum dilution in PBS on separate Boerner slides inside a humid chamber. They were washed 3 times by transfer to watchglasses holding PBS. After a 30-min incubation with 100 µl fluorescein isothiocyanate-conjugated rabbit anti-horse IgG (Miles-Yeda Ltd, Rehovot, Israel), diluted 1:10, the oocytes were washed and scored for surface zona fluorescence using an Olympus B2-2 microscope.

In-vitro assessment of sperm binding. Pig oocytes (n = 200) were incubated with 600 µl horse anti-pig zona serum dilution and washed 3 times by centrifugation. The oocyte pellet was resuspended in 30 µl PBS and transferred to a 5-ml sterile culture tube containing 0·4 ml boar sperm suspension at a concentration of 5×10^6/ml in basal medium (BM) with 0·5% bovine serum albumin (BSA) at pH 7·7. The fresh semen had been previously centrifuged on Percoll and the spermatozoa capacitated by incubation in BM with BSA and in an humidified incubator with 5% CO_2 at 39°C for at least 3 h (Berger & Horton, 1988). The oocytes were gently mixed with the spermatozoa and incubated overnight in the same incubator. The stop–fix technique of Saling *et al.* (1978) for recovering spermatozoa tightly bound to the zona was modified for treatment of the oocyte–sperm mixture. The mixture was carefully layered on discontinuous dextran (mol. wt 500 000: Sigma) gradients (1·8% over 2·25%) in BM using a 1-ml Fisher tube, and centrifuged in a Fisher microfuge at 100 *g* for 2 min. The pellet was resuspended in 25 µl 2·5% glutaraldehyde in PBS and transferred on a slide for microscopic examination. An average of 85 oocytes was counted per slide. The various degrees of sperm binding were classified as: (1) negative = no or less than 5 spermatozoa, (2) intermediate = 5–20 spermatozoa, (3) heavy = >20 spermatozoa bound per oocyte.

Enzyme immunoassay for progesterone. Luteal response was determined by progesterone analysis of plasma by an enzyme immunoassay (Munro & Stabenfeldt, 1984).

Preparation of ovarian tissue for histological examination. Ovarian tissue from the 4 fertile domestic mares was fixed in 10% formalin, embedded in paraffin wax, sectioned at 6 µm and stained with haematoxylin and eosin.

Results

Immune responses of feral mares

The anti-pig zona antibody responses of the 10 feral mares to the zona inoculations, as tested at 5 different time points, are summarized in Fig. 1. The antibody levels of all the mares were similar to one another at the first 4 test times as indicated by low standard errors. Pre-immune sera resulted in mean absorbance ratios of 0·04 and 0·05. The initial low response gradually increased with each inoculation and peaked after the 3rd, measuring a mean absorbance ratio of 1·03. Antibody responses of the feral mares to the 4th injection were not examined since the mares were released after the last inoculation. When 9 of the 10 treated feral mares were recaptured and tested 8 months later (Month 10·5) their results clearly fell into two groups: 7 mares still had remarkably high antibody levels (mean absorbance ratio = 0·82), and no antibodies could be detected in the 2 remaining mares (mean absorbance ratio = 0·06).

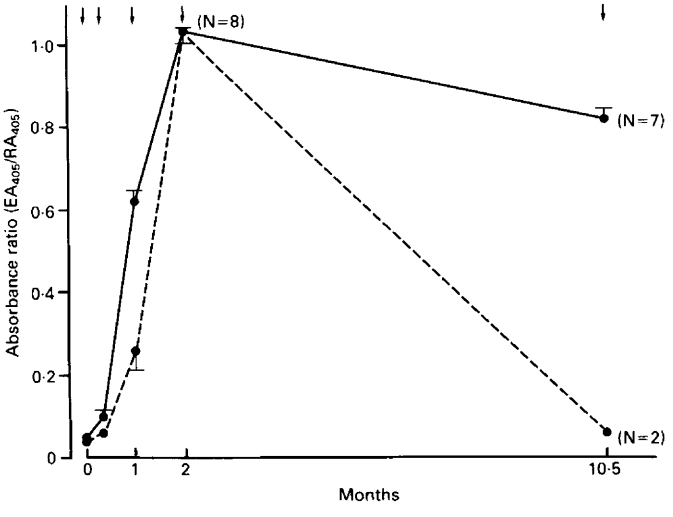


Fig. 1. Immune response to pig zona vaccinations (arrows) in 10 feral mares as measured by ELISA using pig zona antigen. Results of 7 mares (●——●), which had high antibody levels at Month 10·5 of the study, and results of 2 mares (●- - -●) with negative antibody levels at Month 10·5. One mare was not tested at Month 10·5. The mean absorbance ratios ± s.e.m. were calculated from the original duplicate absorbance ratios obtained per individual. Errors of $<0{\cdot}010$ are not shown.

Immune responses of domestic horses

The antibody responses of the 6 domestic horses to the zona inoculations were monitored over a total period of 22–41 months. The results, as found in the ELISA using pig zonae as antigen (Fig. 2), are presented individually for each horse because of treatment variability. The 4 initial inoculations gradually increased the antibody concentrations of all 6 horses to peak levels expressed by absorbance ratios between 1·00 and 1·20. When antibody levels of 3 (ST, AP, GO) of the 4 fertile mares had declined to absorbance ratios of ~0·50 at 7, 10 and 6 months (respectively) after the 4th inoculation, all 4 fertile mares received booster injections. As a result, their antibody levels peaked as before with the exception of Mare GO, which had developed an abscess at the injection site and

therefore received a second booster 4 months later. Antibody levels of the 4 fertile mares were monitored monthly for most of the period of 24–32 months following the booster injection. In 2 mares (ST, GO) antibody concentrations declined to lower levels (absorbance ratios 0·56, 0·42, respectively) 7 and 6 months after the booster. In Mare AP antibody levels remained above absorbance ratios of 0·82 for 23 months. Mare SH, however, maintained the high antibody level it had reached after the booster for 27 months (absorbance ratio at 27th month = 1·03). This originally healthy cycling mare developed bilateral blindness about 18 months after the initial vaccination. The mare's ovaries subsequently remained inactive, causing permanent acyclicity.

The infertile (XO) Mare SQ and the Gelding AL served as immunological control horses and were considered to be immunologically naive to zona components. Because they lacked antigenic exposure and subsequent antibody absorption from the circulation, higher and more persistent antibody levels were expected to result from immunization as compared to normal mares. However, the immune response actually reached by these control horses rather resembled some of the patterns observed in the healthy mares of this study and did not compare to the plateaued pattern of the Mare SH.

Antibody titre of horse versus *pig oocytes*

Because of the small numbers of available horse oocytes the indirect immunofluorescence assay was used instead of ELISA for antibody titre comparison of horse and pig oocytes. An antiserum of medium–strong reactivity against pig zona (Mare AP, after 3rd inoculation, absorbance ratio 0·94) was chosen and 2-fold dilutions were tested. The highest reactive serum dilutions were found to be 1:20 with horse oocytes and 1:800 with pig oocytes. The comparatively lower endpoint titre obtained with horse oocytes can be expected when cross-reacting antibodies are involved. The results confirm the cross-reactivity between pig and horse antigenic zona determinants as published earlier.

In-vivo block of fertilization

The 8-month period of exposure of feral mares to fertile stallions consisted of the last 2 months of the current physiological mating season, an intermediate 4-month anoestrous period and the first 2 months of the following year's mating season. Examination *per rectum* of the 9 feral mares that could be recaptured revealed one pregnancy of 21–23 days and no evidence of pregnancy in the remaining 8 mares. The pregnancy was not confirmed by ultrasonography because of the difficulty of applying this method to feral mares. Attempts to recapture the 10th mare failed, but observation from approximately 20 feet did not indicate any evidence of advanced pregnancy, and 8 months later it was seen without a foal by its side. When analysed for anti-zona antibodies, the pregnant mare's serum was negative. Among the 8 remaining non-pregnant mares, 7 demonstrated substantial antibody levels (mean absorbance ratio = 0·82) and one was found to be negative (Fig. 1).

Of the 4 fertile immunized domestic mares, 2 (AP, SH) did not conceive for an 8·5-month period after the 4th inoculation (Fig. 2). Of this 8·5-month period, 4·5 months consisted of active physiological mating period and the remaining 4 months of anoestrous and transitional phase periods. Conception occurred when antibody concentrations had decreased from peak absorbance ratios of 1·16 to 0·64 (AP) and from 1·14 to 0·91 (SH). At 25 days, the pregnancies were diagnosed by ultrasonography (embryo and heartbeat) and terminated. After subsequent booster injections, antibody concentrations regained high levels (absorbance ratios 1·11 and 1·12) and remained high for 27 months during which time continued insemination of Mare AP did not result in pregnancy. Because of persistent ovarian inactivity, possibly resulting from its blindness, Mare SH was not mated for the remaining part of the study.

How long the block of fertilization after the first immunization series lasted for the fertile domestic Mares ST and GO cannot be stated with certainty as the mares were in anoestrus with low

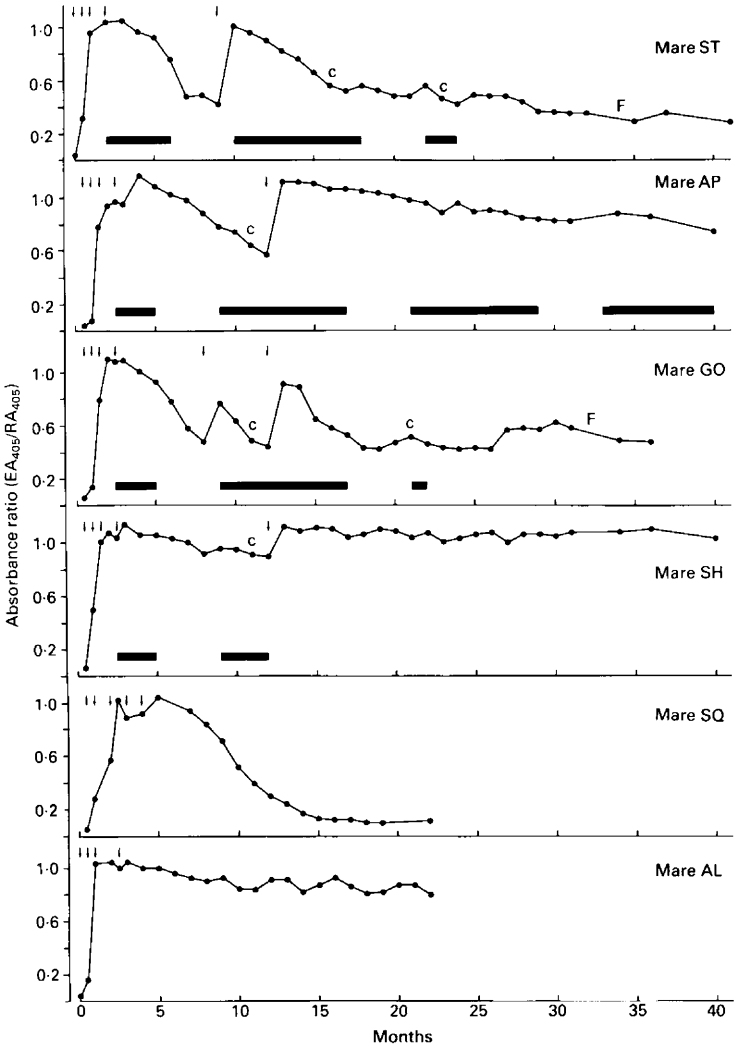


Fig. 2. Immune response to pig zona vaccinations shown individually in 6 domestic horses as measured by ELISA using pig zona antigen. Mares ST, AP, GO and SH were fertile, Mare SQ was infertile (63XO) and Horse AL was a gelding. Results are expressed as means of duplicate absorbance ratios: 57% of the standard errors (not shown) were <0·010, the mean of the remaining errors was 0·021 (range 0·010–0·052). ■, Period when inseminated; ↓, vaccination; C, conception; F, foaling.

antibody levels (0·42 and 0·49) at 5·5 and 7 months after inoculation and received booster injections before the start of the new mating season. In addition, the booster injection and subsequent immunological response of Mare GO was considered invalid because of the development of an abscess at the injection site. The injection was repeated 4 months later when a 25-day pregnancy was diagnosed (absorbance ratio 0·44) and terminated. Following the booster injection it required 7 months for Mare ST and at least 5 or at most 9 months (considering the anoestrous period) for Mare GO to regain fertility. At the time of conception the antibody levels were expressed as absorbance ratios of 0·46 and 0·51, respectively. The second pregnancies of Mares ST and GO were not interrupted and both delivered healthy foals after normal pregnancies.

Antibody-dependent in-vitro block of sperm binding

Repeated efforts to develop a binding assay for horse spermatozoa and oocytes failed because of inconsistent controls. This is consistent with the lack of reports of in-vitro fertilization or sperm binding techniques in the horse. The only alternative was to study the blocking abilities of the horse anti-zona sera using pig oocytes and boar spermatozoa. Sera collected at different periods from the same mare (AP) were selected on the basis of their different antibody levels and were tested simultaneously. The oocytes displayed normal sperm binding (95% heavy + 5% intermediate) after preincubation with pre-immune serum and similarly strong binding was found after treatment with a random non-immune serum and PBS (Fig. 3). Preincubation of the oocytes with the various antisera, however, reduced sperm binding in apparent relationship to the antibody levels of the sera. The weakest of the antisera used in this experiment (absorbance ratio = 0·57), collected in Month 12 of the study (Fig. 2), blocked sperm binding in 25% of the treated oocytes. Blocking of sperm binding gradually increased with higher antibody levels. The strongest antiserum (absorbance ratio = 1·16, Month 4) prevented sperm binding in 99% of the oocytes.

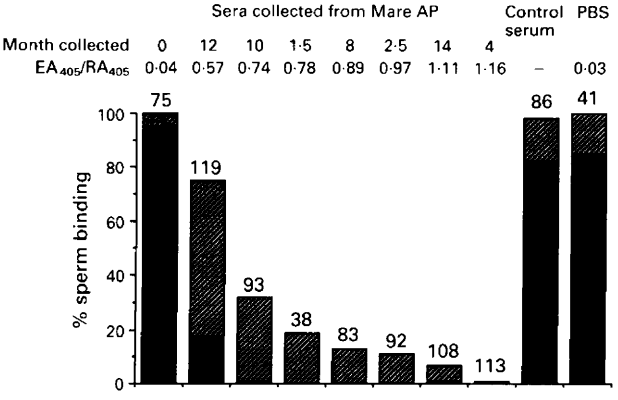


Fig. 3. In-vitro block of boar sperm binding (■, heavy; ▨, intermediate) to pig oocytes by horse anti-pig zona antibodies. Sera collected from Mare AP before (pre-immune) and at different times after immunization were selected for sperm binding experiments on the basis of their antibody level diversity. The results were placed in order of the antibody levels of the sera. The control serum originated from a non-immunized random mare. The total number of oocytes counted per test appears above each column.

Histological examination of the ovaries

The ovaries of 3 of the domestic mares appeared normal. Most of them showed severe hyalinization of the arteries in the capsules, a common finding in many ovaries of untreated mares. Graafian follicles, developing and atretic follicles, functional corpora lutea and recent ovulation sites were present in all the ovaries examined. No active inflammation was noted in the arteries. Both ovaries from Mare SH were inactive: no follicles or corpora lutea were noted in either ovary.

Progesterone profiles

Progesterone profiles obtained from the 4 domestic mares at the end of the study are shown in Fig. 4. Mare SH revealed no evidence of luteal function and remained acyclic, whereas Mares ST, GO and AP demonstrated evidence of ovulation, luteal function and normal cyclicity. Progesterone

concentrations of the 3 cycling mares peaked with a mean of 8·9 ng/ml, ranging from 5·6 to 13·1 ng/ml. Ovulation to ovulation (1 complete oestrous cycle) required a mean of 22·3 days, ranging from 21 to 25 days.

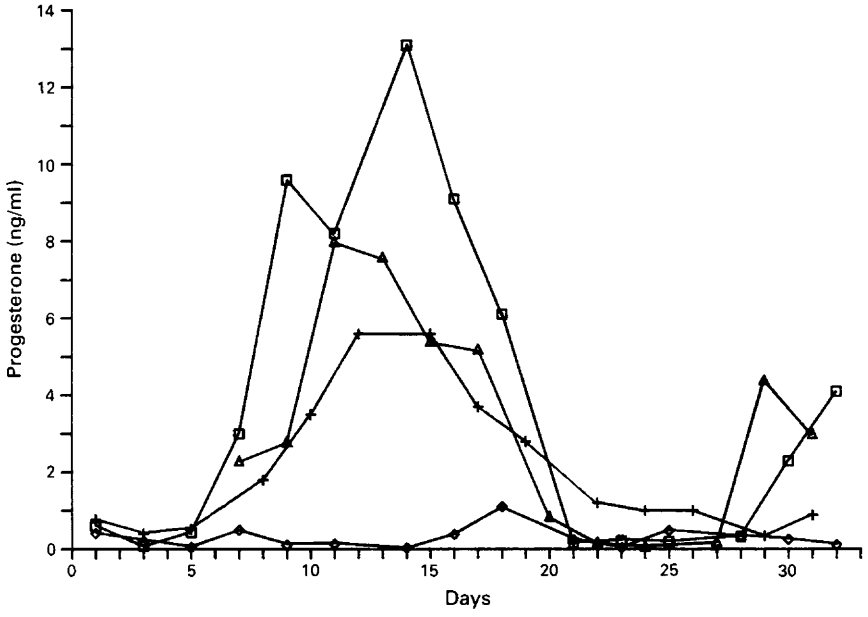


Fig. 4. Progesterone profiles of the 4 fertile domestic mares (△ = ST, + = AP, □ = GO, ◇ = SH) through one complete oestrous cycle (ovulation to ovulation, with the initial one occurring on Day 5) were obtained before ovariectomy.

Discussion

An immune response was provoked in horses by active heteroimmunization with heat-solubilized pig zonae. Other investigations confirm the effects of heteroimmunization when compared with alloimmunization (Gwatkin *et al.*, 1977; Wood *et al.*, 1981; Mahi-Brown *et al.*, 1982), whereby heteroimmunization appears to be the more immunogenic and therefore preferable method of eliciting anti-zona antibodies.

In this preliminary study, the contraceptive effect correlated with high anti-pig zona antibody concentrations, persisted for at least 8 months in most of the animals studied and the effect diminished as antibody levels declined. As in other investigations in which large quantities of zonae from the immunized species are unavailable, the horse anti-pig zona antibodies were studied by using pig and not horse antigen. Such data permit insight into the kind of immune response elicited, although correlation of the direct antibody titres with the more important cross-reactive antibody titres exists only to a certain extent within the same individual (Sacco *et al.*, 1983) and may vary between individuals, especially when the immune response is directed against different antigenic determinants of the zona. In an indirect immunofluorescent assay it was demonstrated that horse anti-pig zona antibodies cross-reacted with horse zona components at a considerably lower titre than with pig zona components. Such cross-reactivity (Shivers & Liu, 1982), as well as cross-reactivity among zona components of several mammalian species, has been reported elsewhere (Gwatkin *et al.*, 1978; Sacco *et al.*, 1981). While ELISA absorbance ratios of 0·64 or less appeared to indicate the level at which most mares became susceptible to pregnancy, one of the fertile

domestic mares conceived at an absorbance ratio of 0·91 and one feral mare had not conceived in the absence of antibody against pig zonae. It was not determined why conception occurred in Mare SH at such a high anti-pig zona antibody titre, but it is possible that the associated cross-reactivity with horse zona antigens may have had a lower titre than in the other mares. The failure of the feral mare to conceive could have been due to one or more of several reasons (e.g. uterine infections following copulation, persistence of a corpus luteum, or early embryonic loss at 40 days and over, resulting in persistence of PMSG secretion) unconnected with the immunization programme.

Binding assays with boar spermatozoa and pig oocytes pretreated with horse anti-pig zona sera demonstrated antibody-dependent interference with sperm binding. The sperm blocking ability of the horse anti-pig zona antibodies, in conjunction with the evidence of their cross-reactivity with horse zona, suggests that stallion sperm binding sites at the zona surface may be blocked by steric hindrance from cross-linked antibodies as described by Aitken *et al.* (1982). According to Ahuja & Tzartos (1981), the antibodies are interacting with structures in close proximity to the binding sites rather than directly with the less immunogenic sperm receptors. Thus, sperm attachment and subsequent penetration are prevented as long as sufficient levels of cross-reacting antibodies are present.

Unfortunately, the contraceptive effect of zona antibody injections was not evaluated during one continuous physiological mating season (March–October). Instead, the study was initiated in June and the effects on fertility were studied during the second half of one mating season and the first half of the following mating season. Because of the apparent block of in-vivo fertilization well into the second mating season and the persistence of high antibody levels, it is reasonable to assume that the contraceptive effect would have lasted through one continuous physiological mating season. Possible causes, other than immunological, for the temporary inhibition of fertility in the 4 fertile domestic mares after zona injections were investigated. Clinically, no abnormalities were found regarding follicular development, cyclicity, ovulation and duration of oestrus. After 2 mares had regained fertility they conceived normally and produced healthy foals following normal pregnancies. Endocrinological profiles and histological sections of the ovaries $\sim$3 years after the last immunization revealed no ovarian malfunction and/or abnormal effects on the oestrous cycle with the exception of Mare SH. It has not been determined whether the development of inactivity of the ovaries of Mare SH was due to the effects of zona injections or to adverse effects of bilateral blindness because the photoperiod is critical to the initiation of cyclicity in mares. Ovarian malfunction and follicular changes have been reported as resulting from approximate dose equivalents of 4000 pig zonae/kg in rabbits (Skinner *et al.*, 1984) and 4–6 monthly doses of 118 and 59 zonae/kg in dogs (Mahi-Brown *et al.*, 1985). In contrast, the horses of this study received only 4 doses of 4 or 11 zonae/kg in 3 months and a single dose of 44 zonae/kg 6–10 months later. The possible absence of ovarian side effects in this study may be attributed to the proportionately lower dose of zona antigen administered.

Preliminary data on the use of an aluminium hydroxide gel as immunization adjuvant suggested satisfactory immune responses in horses without apparent side effects. However, more information is needed before any conclusions can be drawn.

Control mares receiving sham injections were not used in this study. Instead, the conception rates of untreated feral mares at the sanctuary and untreated domestic mares at the Equine Research Laboratory's breeding facility were considered alternative controls. The normal breeding behaviour of a healthy mare in the presence of fertile stallions is to produce one foal per breeding season and year. Annual conception rates of the untreated feral mares maintained at the sanctuary are estimated at 60–70%. This is consistent with the findings reported in other feral herds throughout the northern hemisphere (Kirkpatrick & Turner, 1986). Untreated fertile domestic mares maintained at the same facility with the 4 fertile zona-immunized mares conceived at an estimated annual rate of 90% when mated to the same fertile stallions.

In the past, stallions in wild horse populations in the Northern hemisphere have been the target for temporary sterilization as a means of controlling overpopulation. These attempts have met with minimal success. Provided a mare is fertile and inhibition of fertilization can be accomplished for

one physiological mating season, a period of 2 years will yield the birth of only one foal. This represents approximately one half the normal number of foals produced annually for each mare immunized. This preliminary study suggests that the use of preparations of pig zonae pellucidae may serve as an attractive alternative for the control of wild horse populations for which management levels are desired.

Further studies are needed, however, to purify the antigen used for contraceptive purposes, minimize the number of doses required, and improve the method of its administration, before any realistic management procedures can be adopted for use in feral horse populations. Immunization of pregnant mares and its effect upon the fetus and the mare's long-term fertility also should be investigated.

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The effects of immunocontraception on harem fidelity in a feral horse (*Equus caballus*) population

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ABSTRACT

Feral horses on Shackleford Banks Island, North Carolina, are managed by the National Park Service in order to reduce their impact on the fragile barrier island ecosystem. Management techniques include removal of young horses and immunocontraception of many of the mares using Porcine Zona Pellucida immunocontraceptive. This immunocontraceptive reduces the number of horses that need to be physically removed from the island, but there is concern that the contraception may be influencing the social behavior of the contracepted mares. We investigated the effect of immunocontraception on harem stability by tracking the number of harem changes of each adult mare through the breeding season over two seasons. In both seasons the mares that had been treated with the immunocontraceptive changed harems significantly more than mares never treated (2007 $P = 0.037$ and 2008 $P = 0.016$) and visited significantly more harems (2007 $P = 0.021$ and 2008 $P = 0.011$). The number of years treated did not have a significant effect on the number of harem changes (2007 $P = 0.145$ and 2008 $P = 0.848$), nor did the number of years a mare had been off contraceptive once the contraceptive was discontinued (2007 $P = 0.443$ and 2008 $P = 0.826$). Additionally, there was no significant difference in harem changes between mares that were actively contracepted and mares that had been treated in the past but were not currently actively contracepted (2007 $P = 0.336$ and 2008 $P = 0.533$). These results indicate that the PZP immunocontraceptive has a significant effect on harem stability and that once a mare has been contracepted the behavioral effect of the contraceptive treatment may not be readily reversed.

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1. Introduction

Feral horse populations are often considered to be a nuisance due to their competition with livestock and their effect on native habitats (Rubenstein, 2001). Although commonly associated with grassland habitats, feral horses also occur on barrier islands, where they have been shown to have significant effects on native flora and fauna (Turner, 1969). De Stoppelaire et al. (2004) found that feral horse grazing had a significant negative impact on dune formation and contributes to dune erosion. Overgrazing of marsh areas may result in a loss of important nursery habitat for many marine species (Levin et al., 2002). Due to the impact of feral horse populations on the environment, populations are often actively managed to reduce population size through a variety of strategies, including selective removals, adoption programs, and fertility control (Rubenstein, 2001).

Feral horses generally associate in harems or bands consisting of up to three adult males, a group of adult females,

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and their offspring (Linklater et al., 2000; Rubenstein, 1981, 1986). Although these social groups are generally stable (Klingel, 1975; Linklater et al., 2000) several studies have documented harem changes by adult females (Blumenshine et al., 2002; Rubenstein, 1981; Stevens, 1990). Harem stability has been found to positively correlate with yearly (Rubenstein, 1986) and lifetime reproductive success in females (Kaseda et al., 1995), possibly because mares that are more stable are able to devote more time to grazing and thus are in better condition to support a foal (Rubenstein, 1986). Females that change harems less frequently are more likely to reproduce and produce foals that survive to independence than mares that change harems more frequently (Rubenstein and Nuñez, 2009). Linklater et al. (1999) proposed that stable relationships between females and a harem male or group of males serve to reduce intraspecific aggression and the resultant reproductive costs. Altering harem stability may therefore alter overall reproductive rate, effective population size, and patterns of genetic diversity critical to maintaining long term stable populations. Thus, successful management of feral horse populations requires an understanding of how management strategies may alter social structure.

Population management through contraceptive use may minimize disturbance to social structure by preventing reproduction without altering group size or composition (Garrott and Taylor, 1990). Porcine Zona Pellucida (PZP) immunocontraception has been proposed as a contraceptive agent because it can be administered remotely through the use of darts, is relatively inexpensive, and has been shown to be safe and effective in many animal species (Barber and Fayrer-Hosken, 2000; Martinez and Harris, 2000). Immunocontraception with PZP is also temporary, lasting approximately two years in horses when conventional dosing schedules are used (Powell and Monfort, 2001), although treatment for over seven years may not be reversible (Kirkpatrick and Turner, 2002).

However, the results of studies that have assessed PZP impact on social behavior in feral horses are inconsistent. In a study of Assateague Island horses, no impact on social behavior was noted (Kirkpatrick et al., 1995). Other studies have found no effect on aggression initiated or received, activity budgets of mares, sexual behavior, or proximity to stallions (Powell, 1999; Powell and Monfort, 2001). In contrast, Nuñez et al. (2009) documented an increase in harem changes, number of harems mares associated with, and reproductive behaviors in treated mares. This study was conducted over a span of five weeks, included a limited number of mares, and occurred during the non-breeding season (Nuñez et al., 2009). Given the potential impact of increased rate of harem change on individual fitness, additional study is clearly necessary to determine if there is a significant effect of PZP immunocontraception on harem changes.

In this study we investigated the effect of PZP immunocontraception on harem stability of the feral horse population on Shackleford Banks, North Carolina, USA, during the course of two breeding seasons. We examined the number of harem changes each mare on the island made and the number of harems each mare visited during the course of the 2007 and 2008 breeding seasons. Every mare on the island was included in the analysis and data were collected over a period of several months each year. Given the previous work conducted by Nuñez et al. (2009), we hypothesized that contracepted mares would change harems significantly more often than control mares over both seasons and that contracepted mares would visit more harems than control mares. We also analyzed the effect of foaling status on harem changes, hypothesizing that mares that foaled during the survey year would change less than non-foaling mares.

We also attempted to relate the historical record of immunocontraception with the current patterns of harem changes. We examined the relationship between number of years contracepted, number of consecutive years contracepted, or number of years off contraception and harem changes. We predicted that harem changes would increase as the number of years contracepted increased and the number of consecutive years contracepted increased. Conversely, we predicted that the number of harem changes would decrease as the number of years off contraception increased. Finally, we examined the number of harem changes made by females that were actively contracepted in comparison to those that had been contracepted in the past, but were not currently contracepted. We predicted that actively contracepted mares would change harems more than mares with lapsed contraception.

2. Materials and methods

2.1. Study area

Shackleford Banks is a small barrier island located approximately 3 km off the coast of North Carolina, USA, in Cape Lookout National Seashore. The island is approximately 17 km long and 1 km wide with a variety of habitats including beach, swale, dunes, salt and freshwater marshes and maritime forest (Rubenstein, 1981). Individual horse bands are generally distributed along the entire island but individual harems occupy home ranges within a portion of the island. These home ranges can be grouped into far-east, mid-east, and western distributions. Each area contains at least one permanent water source, although digging may be necessary to access the water. Subjects in all areas of the island were observed for this study.

2.2. Study subjects

There are currently approximately 120 horses on the island. Most horses on the island are found in harems including one to two males and a group of mature females and their immature offspring. Several harems have an alpha stallion and a beta stallion. Over the course of the study, harem sizes ranged from a single male and a single female to two males, multiple females, and multiple offspring. Both sexes disperse from their natal harems as found in other feral horse populations (Feh, 1990). Territoriality has been reported on the island in the past (Rubenstein, 1981), but does not appear to exist currently. This change may be the result of reduced water sources in the eastern end of the island where territories were found. Harems and soli-

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tary stallions tend to be found within the same general area of the island from season to season and year to year. There is some movement between areas, especially by dispersing juveniles, but most adult horses settle into one area of the island. There are exceptions – during a particularly dry summer several harems shifted areas or expanded their home range in order to access a better water source and there is more overlap between harems occupying the mid-east and far-east areas than between those in the mid-east and the west.

The minimum population of Shackleford horses is fixed by federal law at 100 horses (Prioli, 2007), and the National Park Service limits the population to approximately 130 individuals to reduce the potential for damage to the island ecosystem. The immunocontraception program was initiated in 2000 to reduce population size and control population growth (Nuñez et al., 2009). The contraceptive is administered by dart gun by the National Park Service between February and April every year. Each dose contains 100 μg of PZP mixed with 0.5 cm^3 of either Freund's Complete Adjuvant, Modified, *Mycobacterium butyricum* (Calbiochem, Gibbstown, NJ, USA, #344289) for initial doses, or Freund's Incomplete Adjuvant (Sigma, St. Louis, MO, USA, #F5506) for subsequent doses (S. Stuska, personal communication; Nuñez et al., 2009). Booster doses are administered at least 14 days after the initial dose. At the initiation of the program eight mares were chosen as controls, chosen from eight distinct genetic lineages present on the island. These controls have never been contracepted. One additional control was added in 2007 in order to increase the age variation in controls and is included in the data analyzed for 2007, but died before the breeding season in 2008.

We observed harem affiliations for every mare on the island. Only mares that had dispersed from their natal harems were included in the analysis: nine controls and 55 contracepted mares in 2007 and eight control mares and 57 contracepted mares in 2008. The contracepted mares consisted of any mare that had ever been treated with the contraceptive, regardless of whether they had been treated in the previous year. We included all mares ever contracepted as there is some evidence that the contraceptive effect may last longer than one year (Powell and Monfort, 2001) and it is important to determine if contraception at any point changes the mare's subsequent behavior.

2.3. Behavioral sampling

The study was conducted by multiple observers supervised and trained by the primary investigator (J M). All observations were collected during the breeding season. Observations were collected between May 22nd, 2007 and July 29th, 2007 and between May 22nd, 2008 and August 12th, 2008, totaling 2965 total sightings in 2007 and 2985 total sightings in 2008. A sighting was defined as an independent positive identification of a mare and her harem association. Each observer recorded initial sightings upon identifying individuals. Unless a mare changed harems more than 20 min after the initial sighting no additional sightings were recorded of the harem by that observer unless the harem was independently identified at a later point in the day. If two observers identified the same individuals within 20 min only one identification was included in the sightings count. Harem changes were recorded regardless of their timing during the observation session. Individual horses were identified by freeze brand, distinctive markings on the face and body, color, and sex.

Harems were located an average of five times a week during 2007 and approximately four times a week during 2008. Each mare was sighted an average of 46.3 times in 2007 (S.E. 1.70) and 45.9 times in 2008 (S.E. 1.64), or approximately every 1.2 days (2007) and every 1.6 days (2008). After examining the standardized Z score, no individuals were found to be outliers, and there was no correlation between the number of sightings and harem changes for either year (2007 $P = 1.82$ and 2008 $P = 0.502$). Each time a harem was located the harem members were all individually identified and the composition was recorded along with the GPS location of the harem. The number of harem changes for each mare on the island was quantified as a change from one harem to another over successive sightings. All sightings from all observers were compiled for each season and each move from one harem to another was counted. Absence from a harem without a sighting in a different harem was not counted as a harem change as in some rare cases mares were found without a harem stallion. The number of harems a mare visited was quantified as the number of harems a mare was affiliated with over a given season.

2.4. Statistical analysis

We analyzed the effect of contraception on the mean number of harem changes per individual per season using Mann–Whitney U tests. The Mann–Whitney U test was selected given the non-normality of the data and the large difference in sample size between the control (nine in 2007, eight in 2008) and contracepted (55 in both years) mares, which may cause problems when using t-tests (Zar, 1999). One-tailed tests were used as we predicted that contracepted mares would change harems significantly more than control mares given the previous work by Nuñez et al. (2009). Effect size provides a measure of the strength of the relationship between the variables and was calculated using G-power (Faul et al., 2007) for a one-tailed t-test, which provides a reasonably equivalent estimate for the Mann–Whitney U analysis (Zar, 1999). Data were analyzed independently for each year and statistical outliers (one per year) were eliminated from the analysis. In this study both statistical outliers were contracepted mares that changed harems repeatedly over the season. Eliminating the outlier in each year made the analysis more conservative.

We examined the effect of age on harem changes using nonparametric correlation (Spearman's rho) between age on June 1st of the survey year and number of harem changes during that survey season. We ran Mann–Whitney U tests to determine if the average age differed between control and contracepted females. We also ran Kruskal–Wallis tests to determine if mares that foaled during a given survey year changed harems less than mares that did not foal based on their contraceptive status. All mares that foaled during the survey year (not just during the data collection season)

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were included as mares that foaled even if the foal did not survive. The mares eliminated as statistical outliers for earlier analyses were also eliminated from the age and foaling analyses.

To investigate the impact of historical contraception patterns on current behavior, we analyzed the effect of total number of years contracepted, number of years contracepted consecutively with the survey year, total number of years ever consecutively contracepted, and number of years off contraception prior to the survey year on harem changes using Kruskal–Wallis tests. Our assessment of contracepted status in a given year was based on National Park Service records, and included only contraception attempts deemed successful (solid hit with the dart and immunocontraceptive solution ejected from the dart) were included. We analyzed the effect of active versus lapsed contraception on harem changes using Mann–Whitney *U* tests. Mares were considered to be actively contracepted if contraception had been administered in the past two years (Powell and Monfort, 2001). Again, data were analyzed independently for each year and statistical outliers were eliminated from the analysis. All analyses were conducted in SPSS 15 (SPSS Inc., 2006).

3. Results

3.1. Immunocontraception and harem changes

During 2007, there were nine controls and 55 contracepted mares; of the contracepted mares 25 received PZP in the previous year and 20 had not. Sixty-six percent of mares changed harems at least once over the season, with most changing harems multiple times. One individual mare changed harems 26 times over the course of the field season (the outlier in 2007 – a contracepted mare). Additionally, 70.9% of contracepted mares (39 of 55) changed harems at least once while 33.3% of control mares (three of nine) changed harems. Contracepted mares changed harems significantly more than control mares (Mann–Whitney $U = 157.00$, one-tailed $P = 0.0415$, effect size (d) = 0.61). The mean number of harem changes for contracepted mares was 3.62 ± 0.606 S.E., while the mean number of harem changes for uncontracepted mares was only 1.44 ± 0.766 S.E. (Fig. 1). Contracepted mares also visited more harems than control mares (Mann–Whitney $U = 145.5$, one-tailed $P = 0.021$, effect size (d) = 0.80). The mean number of harems visited for contracepted mares was 2.73 ± 0.213 S.E., while the mean number of harems visited for control mares was 1.67 ± 0.333 S.E.

During 2008, there were eight controls and 55 contracepted mares; 20 contracepted mares had received PZP in the previous year and 37 had not. Seventy-three percent of mares changed harem at least once, with a maximum of 27 harem changes (the outlier for 2008 – a different contracepted mare). In 2008, 77.2% of contracepted mares (44 of 57) changed harems at least once while only 50% of control mares (four of eight) changed harems. Again, contracepted mares changed harems significantly more frequently than control mares (Mann–Whitney $u = 124.5$, one-tailed $P = 0.0205$, effect size (d) = 0.92). In 2008, the mean number of harem changes for contracepted mares was 5.47 ± 0.758 S.E., while the mean number of harem changes for control mares was only 1.50 ± 0.732 S.E. (Fig. 1). Contracepted mares visited more harems that control mares (Mann–Whitney $U = 115$, one-tailed $P = 0.011$, effect size (d) = 1.10). The mean number of harems visited for contracepted mares was 3.05 ± 0.222 S.E., while the mean number of harems visited for control mares was 1.63 ± 0.263 S.E.

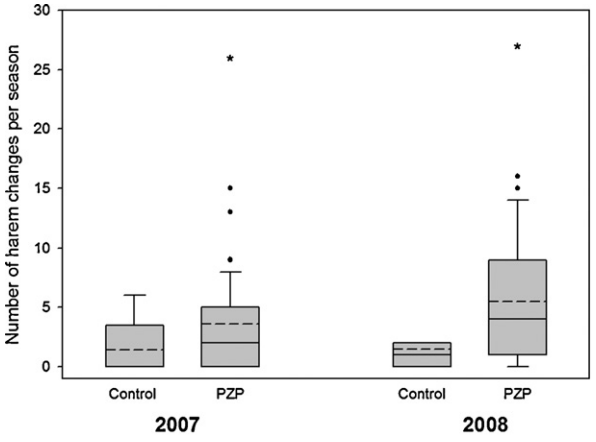


Fig. 1. Box plot showing the number of harem changes by contraceptive status for both 2007 and 2008 with median (solid line), mean (dashed line), and statistical outlier (asterisk). The statistical outlier is presented, but not included in analysis.

3.2. Age and harem changes

Age of the mare was not found to correlate with harem changes in either year (2007 rho = −0.073 (61), $P = 0.569$, 2008 rho = −0.023(62), $P = 0.854$). Additionally, average age was not significantly different between contracepted mares and control mares (2007 Mann–Whitney $U = 200.5$, $P = 0.403$, 2008 Mann–Whitney $U = 139$, $P = 0.084$).

3.3. Foaling and harem changes

In 2007, six control mares foaled and four non-control mares foaled; two non-controls had been off PZP for at least a year while two others had been contracepted the previous year. In 2008, seven control mares foaled and eight non-control mares foaled; again two mares contracepted the previous year foaled. In 2007, the 92% of treated mares did not foal while in 2008 90% of treated mares did not foal. There was a significant difference in the number of harem changes between mares that foaled and those that did not foal in 2008 (Mann–Whitney $U = 240.5$, $P = 0.042$) and a borderline significant difference in 2007 (Mann–Whitney $U = 165.0$, $P = 0.054$). In both cases mares with a foal changed harems less than mares without a foal. There was no significant difference in the number of harem changes between mares that were previously contracepted and foaled, mares that were controls and foaled, and mares that did not foal (Kruskal–Wallis 2007 $P = 0.120$, df = 2; 2008 $P = 0.112$, df = 2), but in both years mares that had previously been contracepted and foaled changed harems less

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than contracepted mares without foals and more that control mares with foals.

3.4. Prevalence of harem changing

The distribution of harem changes each season significantly differed from the Poisson distribution (2007: Chi-square = 269.11, df = 10, $P < 0.001$; 2008: Chi-square = 761.15, df = 10, $P < 0.001$) suggesting that harem changes are not random. In general, there were more mares at the extremes and fewer mares in the center of the distribution than expected in a Poisson distribution. Prior to the immunocontraception program only 10.8% of mares changed harems in the same study area (Rubenstein, 1981) as opposed to the 66% and 73% found over the two seasons of this study. This difference was significant for both years (2007: Chi-square = 316.293, df = 1, $P < 0.001$; 2008: Chi-square = 401.599, df = 1, $P < 0.001$). In 2007, 94% of harems experienced at least one mare changing into or out of the harem and in 2008 97% of harems were affected by the harem changes.

3.5. Historical PZP treatment and harem changes

The number of years treated with PZP did not have a significant effect on the number of harem changes (Kruskal–Wallis 2007 $P = 0.199$, df = 6; 2008 $P = 0.871$, df = 7), nor did the number of years a mare had been off contraceptive after it had been discontinued (Kruskal–Wallis 2007 $P = 0.310$, df = 4; 2008 $P = 0.823$, df = 5). The number of years a mare had been treated consecutively with the survey year did not have a significant effect (Kruskal–Wallis 2007 $P = 0.273$, df = 7; 2008 $P = 0.093$, df = 4), nor did the maximum number of years a mare had ever been consecutively treated (Kruskal–Wallis 2007 $P = 0.782$, df = 6; 2008 $P = 0.889$, df = 6). There was no significant difference in harem changes between mares that were actively contracepted and mares that had been contracepted in the past but were not currently actively contracepted (2007 Mann–Whitney $U = 275.0$, $P = 0.196$, 2008 Mann–Whitney $U = 365.0$, $P = 0.751$).

4. Discussion

It is important in any management program to reduce the impacts of the management on the natural behavior of the population, though the importance of preserving behavior is often overlooked (Clemmons and Buchholz, 1997). In a highly social species such as feral horses it is critical to ensure that management strategies do not negatively impact social behavior. The results of this study indicate that the PZP immunocontraceptive used to control population numbers on Shackleford Banks Island has a significant negative effect on harem stability and that this behavioral effect is more persistent than the physiological contraceptive effect documented in previous studies (Powell and Monfort, 2001). This study demonstrates that the negative effect of the PZP immunocontraception on harem stability during the non-breeding season (Nuñez et al., 2009) was also present in the breeding season and that age was not related to number of harem changes in a given season. The significant increase in the historic rate of harem changes (from approximately 10% to approximately 66 and 73%) indicates that the overall rate of harem changes has increased since the immunocontraceptive program was initiated. Additionally, the high percentage of harems affected by harem changes indicates that the change in stable social structure after PZP immunocontraception affects the entire population, not just the mares contracepted.

Although the control sample size in this study was necessarily small due to management concerns, the replication of these results over two breeding seasons increases our confidence in the significance of differences in harem changes between contracepted and control mares. Given that mares stayed in the same general area, they frequently revisited the same harem several times during the season. The average number of harems visited for all mares was 2.58 in 2007 and 2.88 in 2008. Contracepted mares visited more harems than control mares in both years. It is important to note that some mares changed harems several times over the course of a week or even a day. High temporal resolution is critical to insure that these rapid harem changes between small numbers of harems are documented.

4.1. Impacts of historical contraceptive treatment

The fact that the number of harem changes did not differ among mares contracepted for different numbers of years, or different numbers of consecutive years, suggests that any exposure to PZP contraception may alter female behavior in fundamental ways. Additional years of contraception did not result in a higher number of harem changes, indicating that contracepting for multiple years does not decrease social stability further. However, increasing the number of years between contraception did not mitigate the behavioral effect of the contraception as expected. Surprisingly, mares that were not currently contracepted but had been in the past did not change harems less than mares that were actively contracepted. These analyses indicate that contraception schedule does not seem to mitigate the behavioral effects of the contraception and that the behavioral effects of the PZP contraception may persist long after the contraceptive should no longer be physiologically effective.

4.2. Influence of foals on harem fidelity

As seen in the non-breeding season (Nuñez et al., 2009), mares that had foals changed harems less than mares without a foal, regardless of their contraceptive status. The presence of a foal may increase mare fidelity. Unfortunately, it is difficult to separate the effect of the contraceptive itself from the result of the contraceptive – a lack of foals. If the presence of a foal is a driver of harem fidelity all contraceptive strategies may reduce social stability. Although not significantly different, the pattern of harem changes suggests that previously contracepted mares with foals may change more than controls with foals, but less than contracepted mares without foals suggesting that the contraceptive may have an effect beyond the presence of a foal. The failure to find a significant difference in this com-

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parison may be due to low sample size. Further studies should be conducted with higher sample size to determine if contracepted mares with foals change harems at the same rate as control mares with foals.

It is possible that foaling returns mares to pre-contraceptive behavior – the sample size of this study was simply too low to conclusively answer this question. Relatively few mares that had been previously contracepted, but were not currently contracepted, foaled in either year – two out of 30 in 2007 and six out of 37 in 2008. Further studies should be conducted to determine if contracepted mares cease changing harems the season after they are reproductively successful or if they continue to change harems at a higher rate than mares that are never treated.

4.3. Potential motivations for harem infidelity

While it is clear that PZP immunocontraception causes a decrease in harem fidelity in this population regardless of season, it is unclear if this decrease is due to male or female choice. Males have been shown to discriminate between females based on female reproductive success in many taxa (Berglund et al., 1986; Berven, 1981; Johnson and Hubbell, 1984; Jones et al., 2001; Szykman et al., 2001; Verrell, 1985). This male choice may result in males defending reproductively successful mares (as evidenced by their foals) more than contracepted mares, allowing contracepted mares to change harems more often due to reduced male attentiveness. Previous studies found increased herding of mares with foals, but no difference in harem tending behaviors between currently contracepted mares and controls (Ransom et al., 2010). Alternatively, continuous cycling may lead to increased breeding attempts (Ransom et al., 2010) and male harassment of contracepted females. Contracepted females may elect to change harems more frequently to escape harassment or in an attempt to become pregnant. Future work aimed at distinguishing between male and female choice would be of particular interest.

4.4. Fitness impacts of decreased harem stability

Finally, the pervasive pattern of decreased harem stability may affect individual fitness of both males and females. In feral horses the breeding season corresponds to the foaling season (McCort, 1984) and thus a decrease in harem stability may lead to decreased grazing time while caloric demands on lactating mares are highest (Pagan, 1998; Pilliner, 1999) and decreased body condition even among mares that do not change harems. Ransom et al. (2010) found no difference in grazing time between treatment groups, but did not consider the overall effect of decreased harem stability on grazing. With decreased social stability, males may be required to spend more time guarding their harem to prevent harem changes. This increase in time spend guarding harems may lead to reduced time grazing and result in lowered body condition for the harem stallions. Future work will test if body condition of males or females has decreased since the implementation of the immunocontraception program.

4.5. Management implications

The contraception of the majority of adult females on the island may have substantially changed the social structure of the population, and it is unclear if contracepted mares will ever return to pre-contraception social stability. Immunocontraception using PZP offers many potential benefits, but managers should also consider the potential impacts PZP may have on social structure and stability. One of the key benefits of PZP immunocontraception is the temporary nature of the contraceptive affect. However, even if mares can be physiologically restored to reproductive condition; their continuing propensity to move may reduce their subsequent fitness and complicate management. The persistent behavioral effects are not reduced by manipulating the number of years treated with PZP, so reducing these behavioral effects may not be possible.

Given that PZP immunocontraceptive is administered to a wide variety of species including deer, African elephants, feral water buffalo, feral burros, elk and more than 95 species of zoo animals (ZooMontana, 2000), it is critical to further examine the behavioral effects the contraception may have on other species. Although immunocontraception does not have some of the potential physiological effects of steroidal contraceptives and while behavioral effects may vary with different social systems present in different species, this study indicates that social behavior can be changed by PZP immunocontraception. If, as is known in horses, social behavior affects reproductive success, population management may be complicated by lingering changes in social dynamics. Managers of other species treated with PZP should be cautious in assuming that PZP immunocontraception ameliorates the behavioral effects of other types of contraception.

5. Conclusions

This study, combined with previous work, demonstrates that there is a significant effect of PZP contraception on behavior in feral horses. Contracepted mares changed harems significantly more often than control mares. Further, a high percentage of mares change harems over the course of each season, indicating that this change is affecting the entire population. The duration of contraception does not have an effect on harem changes, nor does the duration of time off contraception reduce harem changes. Mare behavior is altered after contraception and this alteration appears to be long-lived. Given the pervasive behavioral effect of the PZP immunocontraception and the duration of this behavioral effect, it is especially important to investigate the potential costs of the reduction in harem stability to both sexes regardless of the direct effect of the contraceptive.

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Theoretical Article

Immunocontraception for population control: Will resistance evolve?

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Summary The prospect for successful biocontrol using immunocontraception is threatened if there is adaptation to the vaccine through natural selection of individuals that are genetically resistant to the contraceptive agent. To assess this possibility we examined the literature and found that little relevant data are available for any species on the appropriate trait, fertility variation among immunized individuals, or about appropriate population and genetic parameters influencing the likelihood of a selection response. Some data are available on variation in antibody response to immunocontraceptives, but the relationship between antibody response and fertility levels is poorly documented. The antibody response data indicate low heritability for this trait suggesting that fertility levels of contraceptive-resistant individuals will also have a low heritability. Slow evolution of contraception resistance might therefore be anticipated. The absence of information about relevant parameters makes the construction of quantitative models premature. We discuss factors in particular need of investigation if predictions about resistance evolution are to be made. These include: 1. the genetic basis of fertility retention, 2. the proportion of the population resistant to the contraceptive agent and how this is affected by gene flow from refuge populations, 3. the genetically-based fitness tradeoffs of resistant individuals that often accompany selection, 4. cross-generation effects that can thwart the effects of selection, and 5. the efficiency of delivery of the contraceptive agent. An understanding of the above for particular species, and the development of appropriate divergently acting multiple vaccines that can be used in temporal rotation or in mixtures, should facilitate the development of management options to minimize resistance evolution.

Key words: immunocontraception, population control, resistance evolution.

Introduction

Environmental degradation and economic losses due to pest populations of native and exotic mammals are widespread problems[1,2] and immunocontraception has been heralded as a potential solution to these problems.[3–5] Immunocontraceptive vaccines are designed to target the reproductive system of pest mammals by promoting antibody production against various proteins necessary for reproduction.[6] The potential end result is a publicly acceptable and humane control over wildlife numbers. Nevertheless, the application of this method to wildlife populations is still in its infancy and several concerns have been raised regarding detrimental consequences of immunocontraception.[7] One of the most controversial issues involves selection for individuals who fail to mount a significant contraceptive response to the immunogen and hence remain fertile in the presence of the vaccine. It is possible that the progeny of these individuals will inherit this resistance in which case alleles of genes responsible will increase in frequency. Thereby biocontrol via immunocontraception will become increasingly ineffective over time due to strong selective pressures in favour of non-response to the immunocontraceptive.[8]

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There are many examples of rapid evolutionary changes in populations faced with an environmental shift, such as the evolution of pesticide resistance in insects, resistance to anticoagulants in rodents, and resistance to heavy metals in plants and invertebrates.[9–11] Adaptation to environmental shifts, however, can also be unsuccessful. Many fossil studies suggest that species move away from stressful conditions rather than adapt to them.[12–14] There are also many documented examples of population extinction following stressful conditions.[15,16] Even in the case of pesticides and fungicides, resistance is not necessarily a foregone conclusion as there are agrichemicals that have been used for many years without resistance problems being encountered. Thus it is not clear that resistance adaptation to immunocontraceptives is inevitable.

To address the likelihood of resistance evolving a number of factors and population processes need to be considered. While extensive research has been conducted on developing immunocontraceptive vaccines, far less research has been directed towards assessing population variability in fertility response to vaccines, and towards identifying factors that might influence resistance evolution. Many such factors are intrinsic properties of the species or population, such as the degree to which contraception resistance is heritable. Other factors however, might be manipulated in ways that minimize resistance evolving, as has been done for pesticide resistance.[17]

Here we elaborate on the factors and processes that need to be understood to assess the likelihood of the evolution of

contraception resistance. We draw upon examples from other types of traits and from non-mammalian species and make suggestions for research directions to address concerns. Once relevant factors and processes are understood, strategies could be put in place that reduce the likelihood of this occurring.

Heritable variation and contraception resistance

Traits that vary are not always heritable. For any quantitative trait heritability is defined as the extent to which the phenotypic variance in a trait (V_P) is a consequence of genetically caused, as distinct from environmentally caused, variation (V_G as distinct from V_E). In the broadest sense it can be thought of as the proportion of the variance of a trait attributable to genetic causes. Note that in a 'contracepted population' fertility variation will not be all-or-none. Individuals will vary in the level of induced immunocontraception. Heritability is normally expressed as V_G/V_P, where $V_P = V_G + V_E$. The genetic variation component is normally further subdivided into three components, that due to additive effects (V_A), dominance effects (V_D) and genetic interaction (epistatic) effects (V_I). In predicting the response to selection, the additive effects are considered particularly important because they determine the similarity between parents and offspring; in other words the extent to which variation for a trait in one generation is passed to the next generation. Interaction effects can also have an impact on resemblance across generations. Because of the importance of V_A, the extent to which a trait is genetically determined is often expressed as V_A/V_P, defined as the narrow-sense heritability.[18] An important point is that if a high proportion of the population phenotypic variation is caused by environmental factors (V_E is large) then even intense selection will have little consequence in changing the phenotype of future generations. While levels of contraception resistance may vary markedly among individuals this does not mean heritability is high.

Immunocontraception research has largely ignored the heritability of fertility effects. However, there are heritability data on one component of the immune response, the antibody response, and there are some studies of associations between antibody response and resultant fertility. It is important to distinguish between heritabilities of these two measures because only effects on fertility are selected; non-response of antibodies will not be selected in the absence of associated fertility effects. Also, while antibody response to an immunocontraceptive treatment may be a primary mechanism that reduces fertility, it is unlikely to be the only mechanism since other processes such as cell-mediated immunity may be involved.

Heritabilities of antibody levels have been estimated from selection experiments. For example, selection of chickens for high and low antibody response to two different bacterial vaccines was carried out for seven generations.[19] All high response lines exhibited significant increases in antibody production to both vaccines when compared to the low lines. Unfortunately the omission of a control line means that we cannot be confident the increase was other than a response to general rearing conditions. Assuming this was not the case heritabilities for antibody response were low (ranging from –0.23–0.61). In a similar chicken experiment, selection over a period of 14 generations was the likely cause of high and low antibody titres attained and heritabilities ranged between 0.02 and 0.20.[20] In mice selected for high and low response to antigens for 15 generations, immune responses were 60 times higher in high lines compared to low lines.[21] Heritability was estimated at 0.18. Similar antibody heritabilities were estimated for pure-bred pigs immunized with two different vaccines.[22] Here low heritabilities of 0.18 ± 0.09 and 0.15 ± 0.07 were recorded for immune response to a modified-live pseudorabies vaccine and an inactivated bacterial bacterin, respectively, both measured 56 days after inoculation (however, it was 0.52 ± 0.15 for the bacterin after 119 days). Overall the data indicate that the level of an antibody response is heritable and will respond to selection, but heritabilities are not necessarily high relative to other traits.[23–25]

Immunization followed by a booster can elicit an immune response that results in reduced or zero fertility, provided antibody titres remain high.[26–28] However, in some cases a high immune response did not ensure against contraception resistance,[29–31] there was no apparent correlation between antibody titre and fertility[32] or associations between these traits were ambiguous because appropriate controls were either absent or inadequate.[29] These data indicate that high antibody titres do not ensure contraceptive efficacy. However such associations might be higher in future efforts to relate fertility to immune response levels if the efficacious epitopes are identified and quantified.

While the heritability for antibody response levels are low to intermediate, the heritability of fertility effects are likely to be even lower, since antibody levels are only one component of fertility effects and underlying components of traits have higher heritabilities than the traits themselves.[24] This might be particularly the case under field conditions where environmental variance is likely to be higher and contribute to each subcomponent of the fertility trait.[33] While most studies of heritable variation in non-response have taken place in defined laboratory situations, natural conditions will be more stressful and/or more variable, reducing the heritability of contraception resistance. Also, levels of heritable variation expressed in one environment are not necessarily the same as those in another environment.[34] Considering the above, in all likelihood contraception resistance will have a low heritability and therefore respond only slowly to selection.

Note that small sample size is an area for concern in the estimation of heritabilities for immunological responses. Heritabilities have usually been estimated using a few individuals and these estimates have little statistical power. They may be unrepresentative of a population, especially given the large amount of genetic polymorphism associated with immunological responses to contraception.[30,35] Estimates for outbred animals tend to be particularly variable (for example, natural variability in pregnancy rates of foxes after mating was between 35 and 90%).[36] Note also that heritabilities are not constant but change as selection proceeds because they depend on underlying gene frequencies that change under selection. Predictions of selection responses based on heritabilities therefore only apply for a few generations. Finally, note that heritabilities do not necessarily provide information on the extent to which a trait will shift under selection; the extent of change is also influenced by the mean of a trait.[25,37]

Genetic basis of contraception resistance and selection intensities

Heritable variation alone does not ensure the evolution of a particular phenotype. The type and level of genetic variation, and the intensity of selection are also important. If selection intensities are relatively high, a response to selection may not be successful and the population will go extinct even when there is some genetic variance in the population.[38] There are several cases in the pesticide resistance literature where resistance has not evolved or has not persisted despite the presence of genes with small effects on resistance variability and strong selection pressures.[39] Strong selection may kill all individuals unless they carry major resistance genes (although see Groeters and Tabashnik).[40] A similar situation is thought to be relevant for the evolution of resistance to other toxicants, such as heavy metals.[41] High selection intensities can favour the spread of genes having large effects on a trait, and genes that are often initially rare, particularly if the genes show some dominance. However, local populations may not be large enough to harbour such individuals and evolution does not occur. The frequency of genetic variants, the nature of the way the genes affect the phenotype and selection intensities are all important for predicting the occurrence and speed of evolutionary change.

The genetic and molecular basis of immunocontraception resistance has yet to be explored. However genetic variation in some components of the immune response, especially antibody response, has been investigated. Early research suggested that the genetic basis for antibody response is under the control of a dominant Mendelian locus.[42] Later data suggested both a dominant autosomal effect of a major gene and some polygenic influences.[43,44] The MHC complex, containing an array of tightly linked genes that are highly polymorphic, appears to be a strong candidate for a major autosomal dominant gene that affects antibody response.[45,46] However non-MHC linked genes, such as those involved with Class I antigen processing or genes that influence antibody isotope usage, may have substantial effects.[47] Therefore the genetic control of antibody non-response might be realistically modelled as two or more independent (unlinked) polymorphic genes, one with a major effect on fitness, the MHC cluster, and several with minor effects.

For contraception resistance on the other hand, where associations between antibody response and contraception resistance may not be strong, other genes again are likely to be involved. For example, genes that effect variation in cell-mediated immune responsiveness,[48] or expression of cytokines (reviewed in Reiner and Locksley).[49] A further level of complexity is introduced if the antigen is delivered using a genetically modified vector organism, in which case the target species may evolve resistance to the vector itself. The evolutionary genetics of host resistance to a virulent horizontally transmitted pathogen is well-researched and modelled[50,51] and may help our understanding of contraception resistance evolution in these situations. While single genes can be important in host-pathogen systems, this complexity makes the prediction of contraception resistance even more difficult. It may be appropriate to model contraception resistance using a small number of major genes and a large number of minor genes. Polygenic models that rely on an infinite number of loci each with small effect, or simple single gene models, are likely to be unrealistic. More information is needed if models relevant to natural populations are to be developed.

Proportion of the population that is contraceptive resistant

To predict the outcome of selection for contraception resistance it will be important to know both the initial frequency of resistant individuals and the ongoing impact of immigration from non-immunized sources, from 'refuges', that can dilute the frequency of resistant individuals. Such inward gene flow could prevent resistance evolution. The proportion of a population that retains any level of fertility after contraception treatment is likely to be highly variable.[52,53] For a given immunocontraceptive in a particular population it is this proportion that determines the initial selection response. If the initial frequency is very low resistance will build up slowly and populations may become extinct because they do not reach effective demographic population size. Data useful for predictive purposes need to be obtained from outbred animals in the field, as laboratory-reared or inbred populations will have less genetic variability affecting contraception resistance. Also, captive populations are likely to show high fertility levels, having been selected for breeding in captivity and buffered against natural stresses that decrease fertility.

Because average fertility levels of non-immunized individuals are below that of the most fertile individual, one must assume that the functional, or effective level of contraception resistance (remaining fertility) is higher than the published data indicate. For example, using data from fox,[52] the effective contraception-resistance fertility after immunization would be close to 53% (36%, of control fertility at 68%), and for the feral mares[53] the effective level of fertility would be between 8 and 51% (between 4.5 and 28.6% of about 55%). We suggest that the effective contraception resistance levels be used in any evolutionary modelling exercises. However, these levels might be overestimates since breeding failure in controls may be related to other factors (for example, lack of opportunity or poor conditions compared to treated individuals).

To predict the results of selection for contraception resistance, information on immigration from refuge populations is needed. Gene flow often limits adaptation, as has been recognized for a number of traits and organisms.[54–56] An influx of susceptible individuals into a population previously exposed to controlling treatment will dilute the frequency of resistance genes. The dilution effect is likely to be particularly significant in the case of polygenic resistance. When individuals with polygenic resistance mate to susceptible individuals, the gene combination needed for resistance will be lost in the ensuing generations because there is only a low probability of recovering offspring with polygenic resistance. This dilution effect has a much lower impact in the case of major genes. The dilution effect will depend on the species, population, and environment being considered. For example interpopulation movement level is likely to be very different between the red kangaroo[57] and the brushtail possum.[58] Clearly in captive populations gene flow will have no influence on the response rates to selection for contraception resistance.[55]

Cross generation effects, resistance tradeoffs and costs of contraception

Cross-generation effects

Cross-generation effects can have a negative impact on selection responses.[59] Cross-generation effects arise when environments experienced by the parent influence the phenotype/performance of the offspring, or when genes carried by the parent (usually the mother) influence offspring phenotype (even when the offspring has not inherited a causal gene). Often this results in a fitness cost in the F1 generation[60] that can influence selection responses. They can even lead to phenotypic changes in the direction opposite to that being selected.[61]

The potential for an immunocontraceptive vaccine to have cross-generation effects is suggested by one study on rats.[62] Both males and females were subject to vaccination against luteinizing hormone receptor (LHR), and mated with untreated individuals. For both treated sexes the number of progeny per coupling was reduced compared to control groups but was more apparent in the couplings of a treated male. A cross-generation fertility effect was evident for the F1 progeny from treated females where, in the absence of vaccine, the F1 progeny had reduced fertility. Such effects have the potential to reduce the selective intensity for contraception resistance and alter the rate of evolution of contraception resistance.

Genetic trade-offs

Much of life history theory is concerned with genetic tradeoffs and how they limit evolutionary change.[25] There is a lot of evidence that alleles favoured under one set of environmental conditions can be selected against under a different set of conditions[15,25] thus leading to tradeoffs between environments. Moreover, genetic change in one trait often has costs in terms of a different trait even when the environment does not change. There are good examples of tradeoffs associated with resistance to chemicals. For instance, while both warfarin resistance in rodents and pesticide resistance in insects lead to increased survival when these poisons are present in the environment, resistance is often selected against when the chemical is absent resulting in the loss of resistance in populations that are no longer exposed to the toxin.[60,63,64]

Subsequent fitness improvement that counteracts these deleterious effects may occur in the longer term however, when treatment persists.[9,10] This is particularly well studied in the case of sheep blowflies where resistance to the pesticide diazinon is associated with a fitness cost.[65] In this example there is no fitness cost detectable in the genetic background of a contemporary field population where the pesticide has been present for many years. In this population genes have been selected that modify the deleterious effects of the major resistance gene. If a major gene for contraception resistance was associated with fitness costs and reached high frequency in a treated pest population, modifier gene selection could nullify the cost, helping to maintain the gene in the population and thwart control efforts.

Little research has been conducted into defining or clarifying potential tradeoffs for immunocontraception resistance. This issue is pertinent given that a low level of responsiveness to some antigens could potentially influence responsiveness to others. These individuals may be more susceptible to particular categories of infectious or parasitic agents that retard selection for contraception resistance, particularly if the target population experiences intermittent applications of the vaccine. With intermittent selection, any change in contraception resistance gene frequencies could theoretically be reversed during periods when the immunocontraceptive agent is absent.

Tradeoffs will only limit adaptation if they have a genetic basis. To establish this in mammals individuals should be followed over two or more generations and sample sizes need to be large.[18] While for morphological traits there may be a good correspondence between simple phenotypic tradeoff associations and genetic correlations, this is not the case for other traits[25] making it difficult to test for genetic tradeoffs involving immunocontraception resistance.

Loss of genetic variability and immunological fitness

A possible outcome of long-term selection for contraception resistance is a general loss of genetic variability. In the laboratory, strong selection for a single trait can reduce levels of genetic variability in that trait as favoured alleles go to fixation. While in theory this would lead to a reduction in trait variance it often does not.[18] Intense selection can also lead to a general decrease in genetic variation if the number of breeding individuals in a population is small. However there is also evidence that sharp reductions in population size can increase levels of trait variability due to the 'unveiling' of nonadditive genetic variance.[66] A reduction in population size as a consequence of immunocontraception may therefore not invariably decrease phenotypic variability.

Another possible outcome of long-term selection for contraception resistance is a loss of immunocompetence.[7,8] As the fertility response to an antigen may be largely controlled by the MHC locus, individuals resistant to contraception could show reduced diversity in their immunological response. At this stage a few laboratory investigations have reported that selection for high antibody responders is associated with altered immunological fitness against other antigens/pathogens, but this is not a general finding.[21,67] If hypersensitivity to a specific infectious agent was a recognized tradeoff for a contraception resistance gene, a synergistic treatment program (treatment with both vaccine and infectious agent) could become part of a management control strategy, similar to what has been implemented for integrated insect pest management schemes.[68]

Under field conditions, the outcome of contraception-resistance selection on levels of genetic variation in target and other traits is difficult to predict. Effects are likely to be species-specific and dependent on the duration and intensity of selection. To determine the impact of such selection on levels of genetic variation and overall immuno-competence it will be necessary to undertake both field and laboratory-based experiments looking at longer term effects. Of course loss of genetic variability and immunological fitness will be less of a problem for invasive or exotic pest populations targeted for elimination.

Also note that within the lifetime of immunized individuals deleterious direct effects of immunization may occur that

lead to behavioural changes or low 'quality of life', effects that are over-and-above those that reduce fertility.[7] These changes may or may not be an undesirable outcome of the immunocontraception, however, they can effect social dynamics within the population and impact on selection for contraception resistance.

The component of the population targeted for contraception

The proportion of a population immunized will impact directly on the rate of increase of contraception resistance in a population, since the selection intensity will be reduced if not all breeding individuals are targeted. If the species to be controlled is contained within restricted habitats, it may be possible to inoculate the entire population. However in many cases this will be impossible. Several modelling and laboratory investigations have suggested that, to obtain the necessary reduction in population size, approximately 50–75% of individuals need to be targeted.[69–71] If only a small proportion of the breeding population is targeted effectively, selection intensity for increased levels of contraception resistance will be low.

The sex that is targeted can influence potential selection intensity. In most case studies female-specific contraceptive agents have been tested[27,31,72] although males have also been investigated.[62,73] When the immunogen targets the reproductive response of both sexes, there will be fewer progeny from contraception-resistant individuals. However selection for contraception resistance will be greater as all progeny will contain contraception-resistant genes from both parents, as opposed to only the treated sex.

The social and reproductive dynamics of the targeted population will influence selection intensities for resistance. For example, under a harem system where a dominant male mates many females, control programs may target the dominant male in the group. Alternatively, when reproduction occurs in distinct times of the year, it may be more effective to only target populations at these times, perhaps when climatic or nutritional stress is maximum. These approaches could more effectively reduce population size without increasing selection intensity for contraception resistance. Knowledge of a species' social and mating structure can help the design of a strategy to minimize resistance evolution.

Optimal contraception requires multiple and booster immunizations

Multiple applications of an immunogen are required to elicit the maximum level of infertility response.[28] This is generally the case for a range of laboratory and outbred species. Thus to achieve high levels of infertility individuals need to receive multiple applications, spread out over time. In addition, immunocontraceptive effects have a finite duration and booster vaccinations are required to maintain a high level of infertility.[74] This is particularly relevant for species with a long generation time and/or multiple reproductive seasons – additional factors that would impinge on the timeframe for the evolution of immunocontraception resistance. While the goal for much of the research is for a long-lasting single shot vaccination this does not yet appear to have been achieved. It is therefore likely that in natural populations it may be difficult, even impossible, to implement complete control by a single treatment or repeated regular delivery of the immunogen to many or most individuals. In large, or complex populations, a number of strong short-term selective episodes at intermittent times would be interspersed with periods of reduced selection for resistance. If there are genetic tradeoffs associated with contraception-resistance there may even be negative selection at these times, decreasing the chance of resistance evolving.

Multiple vaccines in rotation or mixtures

A multiple-vaccine approach has been advocated as a strategy to improve contraception and population control;[30] it might also minimize the chance of resistance evolving. This strategy has been successfully used to reduce the rate of insecticide resistance evolution in field situations.[17,75,76] By rotating pesticides, the number of applications of a particular chemical is reduced. This effectively reduces the selection pressure for resistance to each pesticide and also allows any fitness costs of resistance alleles to decrease resistance incidence between applications. Moreover, any influx of susceptible individuals into a target population in the intervening period can further reduce the incidence of resistance. The other strategy involves mixing pesticides. This may delay the evolution of resistance more effectively than pesticide rotation strategies,[77] however, if resistance occurs it is more likely to elicit a single cross-resistance response.[78] Pesticides with a contrasting mode of action are the best choice, whether rotating or mixing, since a common mechanism of resistance is more likely for related pesticides. The experimental and theoretical aspects of a multiple-treatment approach with respect to pesticides has been discussed by Tabashnik.[79]

Exposure to multiple antigenic determinants may therefore be effective, particularly where the mode of action of the immuno-contraceptives varies and the same genes are unlikely to confer cross-resistance. While in some systems a response to a particular antigen carries a similar high response to other antigens, this is not always the case, as has been demonstrated in mice.[80] A range of antigenic determinants within a vaccine might elicit diverse contraceptive-effective responses within individuals, and thus decrease the fertility level of the average individual, and the frequency of contraception-resistant individuals. In fact the growing understanding of the complexity of fertility controlling processes, and growing number of candidate target molecules, are leading to suggestions that multiple antigen approaches will be most effective in future immunocontraceptive efforts.[5,81,82] Multiple genetic bases for contraception resistance will be more likely under this approach and resistant individuals would be rarer. As well as improving control a multiple antigen approach would retard the evolution of contraception resistance.

Conclusion

It seems likely that the use of immunocontraception will result in selection pressure for contraception resistance. The development of contraception resistance will vary among target species and may only occur over such a long time frame that changes in control approaches or community

priorities remove the need for concern. However until levels of heritable variation in contraception resistance are assessed in each particular situation, and unless other pertinent factors that are likely to impinge on resistance evolution are evaluated, it is not clear if or how rapidly contraception resistance will develop in a population. The other pertinent factors include:

1. the genetic basis of the resistance
2. the frequency of resistant individuals, both initially and on-going as influenced by immigration from non-contracepted refuges
3. fitness costs associated with the resistance phenotype
4. cross-generation effects, and
5. the efficiency of delivery of the vaccine

If enough information is available on the contraception response and population biology of the target species it should be possible to implement strategies for reducing the rate of evolution of contraception resistance.

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Serum antibody immunoreactivity to equine zona protein after SpayVac vaccination

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ABSTRACT

Immunocontraception with porcine ZP (pZP) can be an effective means of fertility control in feral horses. Previous studies suggest that antibodies produced after pZP vaccination may both inhibit fertilization and cause follicular dysgenesis. Zonastat-H, PZP-22, and SpayVac are three pZP vaccines proposed for use in horses. Although all these vaccines contain the pZP antigen, variations in antigen preparation and vaccine formulation lead to differences in antigenic properties among them. Likewise, despite numerous efficacy and safety studies of Zonastat-H and PZP-22, the contraceptive mechanisms of SpayVac remain unclear. The preparation of pZP for SpayVac is thought to include more nonzona proteins, making it less pure than the other two vaccines. This may result in increased antigenicity of the vaccine. We therefore investigated the immunoreactivity of serum antibodies from SpayVac-vaccinated mares to equine zona protein. Western blot analyses revealed an immunoreactivity of these antibodies to protein isolated from mature equine oocytes, ZP, follicular tissues, and ovarian tissues. Immunohistochemical analyses were used to locate the binding of serum antibodies to the ZP of immature oocytes in ovarian stromal tissue. We also found serum antibodies from SpayVac-treated mares to be predominantly specific for zona protein 3. Collectively, our results suggest a model where serum antibodies produced in response to SpayVac vaccination are immunoreactive to equine zona protein *in vitro*. Our study lends insight into the contraceptive mechanisms underlying the infertility observed after SpayVac vaccination.

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1. Introduction

Feral horse (*Equus caballus*) populations residing on lands managed by the Bureau of Land Management and United States Forest Service are federally protected under the Wild Free-Roaming Horses and Burros Act (Public Law 92–195). Provided this protection, in combination with a lack of natural predators, many horse populations can grow 10% to 25% annually [1]. Because their habitats remain constant in size and limited in forage availability, feral horse populations often overwhelm available resources.

Researchers have investigated the use of immunocontraceptive vaccines as an approach to control feral horse population growth [2]. Immunocontraceptive vaccines stimulate an animal's own immune system to interfere with a critical reproductive event [2]. When an antigen is combined with an immunologic adjuvant and injected into an animal, antigen-specific antibodies are produced [3]. These antibodies are thought to bind not only to the antigen for which they are specific but also to structurally similar endogenous

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proteins in the target animal. Immunocontraceptive vaccines can therefore target specific hormones or proteins within the animal [4].

Zonastat-H (Humane Society of the United States, Washington, DC, USA), PZP-22 [5], and SpayVac (Immunovaccine Inc., Halifax, Nova Scotia, Canada) are immunocontraceptive vaccines that incorporate the porcine ZP (pZP) antigen. Vaccination with the pZP antigen is reported to stimulate an immune response that can be measured by serum antibody titers [6,7]. Infertility is thought to be achieved by the binding of these serum antibodies to ZP proteins on mature oocytes, thereby inhibiting sperm binding and penetration [8–10]. Follicular dysgenesis characterized by diminished ovulatory ability, absence of mature oocytes and follicles, decreased ovarian weights, and a decline in ovarian estrogen secretion has also been reported after pZP vaccination [11–13]. These studies suggest that serum antibodies produced in response to pZP vaccination may act both on ZP of ovulated oocytes in the oviduct and during folliculogenesis in the ovary.

Despite these valuable pathologic studies in multiple species, the molecular mechanisms by which antibodies produced in response to SpayVac vaccination result in infertility remain unclear. Although serum antibodies may not accurately predict the contraceptive efficacy of a vaccine, understanding whether these antibodies are capable of binding key proteins involved in folliculogenesis and fertilization is a central part of understanding how the vaccine may be acting to inhibit fertility. Our objectives were to (1) determine whether vaccination of feral horse mares with SpayVac results in the production of antibodies immunoreactive to equine zona protein and ovarian tissues and (2) identify the location of binding of these antibodies to equine oocytes and ovarian tissue.

2. Materials and methods

2.1. Animal use

No live animals were used exclusively for this study. All tissue and serum samples from nonvaccinated mares were collected as part of other ongoing research under protocols approved by the Institutional Animal Care and Use Committee at Colorado State University. Serum from SpayVac-vaccinated mares was obtained from samples previously collected under protocols approved by the Fort Collins Science Center's Institutional Animal Care and Use Committee as part of a study on SpayVac immunocontraception (Roelle, U.S. Geological Survey, Pauls Valley, OK, USA).

2.2. Samples

Equine ovaries and renal tissues were harvested from necropsy samples collected at the Animal Reproduction and Biotechnology Laboratory, Colorado State University (Fort Collins, CO, USA). Oocytes were collected by aspirating follicular fluid from follicles on the ovaries. Oocytes were stored in PBS at 4 °C. Tissue samples were either fixed in 4% paraformaldehyde and sent to the Colorado State University's Veterinary Diagnostics Laboratory to be embedded in paraffin or frozen and stored at −80 °C.

Serum from vaccinated mares of known titer levels was obtained from a study on SpayVac immunocontraception. Mares were vaccinated with 200-μg pZP (Immunovaccine Inc.). Blood samples were collected and processed 3 months after vaccination, and serum was stored at −80 °C. Serum antibody titers from these samples were determined using ELISA by Immunovaccine Inc. Titers were read as average optical density across three readings of the same sample and then reported as a percent of standard, where the standard was a serum sample containing a large number of antibodies specific to the vaccine. Percent of standard values, or titers, ranged from less than 50.00 to as high as 178.33, with 100.00 representing a sample that had an antibody profile similar to that of the standard. Mares with higher titer levels, specifically values greater than 100.00, were considered to have an increased number of vaccine-specific antibodies in their serum. Because antibodies against the vaccine are thought to be responsible for the contraceptive effect, higher titer levels correlate to increased vaccination efficacy. Serum samples from eight mares of high titer levels ranging from 97.83% to 178.33% of standard were therefore used interchangeably throughout the study. Serum from two nonvaccinated mares was obtained from other ongoing research at the Animal Reproduction and Biotechnology Laboratory. All serum samples were stored at −80 °C.

2.3. Protein isolation and quantification

Isolation of protein was performed using radioimmunoprecipitation assay (RIPA) lysis buffer (150-mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50-mM Tris, pH 8.0, 1-mM EDTA). Samples were suspended in RIPA, homogenized and sonicated twice, and subjected to centrifugation for 10 minutes at 10,000 revolutions per minute (Centrifuge Model #5417R; Eppendorf, Germany). The supernatant containing protein was recovered, and the DNA-containing pellet was discarded. All protein was isolated just before use and never frozen.

To isolate protein from equine oocytes, cumulus cells were mechanically stripped from four oocytes by a series of passages through a 10-μL embryo pipette (Bioniche, Pullman, WA, USA), while the oocytes were suspended in PBS. Oocytes were suspended in 40-μL RIPA lysis buffer and processed as detailed previously. The final supernatant volume was divided into two 20-μL samples.

To isolate protein from the ZP of equine oocytes, the cumulus cells were mechanically stripped from four equine oocytes and ultrasharp splitting blades (Bioniche) were used to make an incision in each ZP. Turbulence was created to free each ZP from the ooplasm, and the ZP of the four oocytes were suspended in 40-μL RIPA lysis buffer. Protein was isolated as previously described, and the final volume of the supernatant was divided into two 20-μL samples.

To isolate protein from ovarian and renal tissues, a small section of each tissue was excised from the sample, suspended in 300-μL RIPA lysis buffer, and processed as detailed previously. Protein content was quantified following the manufacturer's protocol for the Pierce BCA

Protein Assay Kit (Catalog #23227; Thermo Scientific, Rockford, IL, USA). An aliquot containing 40 μg of each protein sample was transferred to individual 1.7-mL conical tubes and brought up to 20 μL in volume with PBS.

2.4. Electrophoresis and Western blot analysis

Protein samples were incubated with 4-μL 6X Laemmli sample buffer (375-mM Tris-HCL, pH 6.8, 48% glycerol, 6% sodium dodecyl sulfate, 0.01% bromophenol blue, 20% dithiothreitol), loaded into 10% Mini-PROTEAN TGX precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA) in duplicate, separated by electrophoresis, and transferred to Protran nitrocellulose membranes (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Membranes were blocked in 5% blocking buffer (5-g nonfat dried milk in 100-mL 1X TBST—50-mM Tris-HCL, pH 7.4, 150-mM NaCl, 0.1% Tween 20) for 1 hour and then cut down the middle such that each half contained one set of the duplicate protein samples. One half of each membrane was incubated in a dilution (1:50 in 1% blocking buffer—1-g nonfat dried milk in 100-mL 1X TBST) of either serum from a SpayVac-vaccinated mare or serum from a nonvaccinated mare overnight. Both membranes were then incubated with horseradish peroxidase (HRP) –conjugated goat polyclonal secondary antibody to horse immunoglobulin G (IgG; 1:10,000 in 1% blocking buffer; ab102396; Abcam, San Francisco, CA, USA) for 1 hour. Membranes were exposed to SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) for 5 seconds and imaged immediately on a Molecular Imager ChemiDoc XRS+ System (Bio-Rad). Membranes were washed between each step with 1X TBST for 15 minutes.

A mouse anti–β actin monoclonal primary antibody (1:100 in 1% BSA; ab8226; Abcam) followed by an HRP-conjugated donkey antimouse IgG polyclonal secondary antibody (1:1000 in 1% BSA; ab97030; Abcam) was used as a loading control for protein isolated from follicular and ovarian tissues. A rabbit anti-ZP3 polyclonal primary antibody (1:100 in 1% blocking buffer; ap17235b; Abgent) followed by HRP-conjugated goat antirabbit IgG polyclonal secondary antibody (1:1000 in 1% blocking buffer; #170-6515; Bio-Rad) was used for protein isolated from oocytes and ZP as a loading control. Binding specificity was determined by preincubation of the Western blot membranes with either the rabbit anti-ZP3 polyclonal antibody (ap17235b; Abgent) or rabbit anti-ZP4 polyclonal antibody (ap12724b; Abgent) overnight before incubation with serum from a SpayVac-vaccinated mare overnight and use of the HRP-conjugated goat polyclonal secondary antibody to horse IgG (1:10,000 in 1% blocking buffer; ab102396; Abcam).

2.5. Immunohistochemistry

Three consecutive 5-μm sections were sliced from tissue embedded in paraffin blocks and placed on two glass slides. The slides were incubated at 55 °C for 30 minutes. Citrisolv (Thermo Scientific) along with an alcohol gradient was used to dewax and rehydrate the tissue slices.

After rehydration, one of the three slices of tissues was subjected to a hematoxylin and eosin staining protocol. The slide was incubated in hematoxylin for 1 minute, rinsed under running tap water for 5 minutes, incubated in eosin for 5 minutes, rinsed once more under running tap water, and dehydrated through an alcohol and citrisolv gradient. The slide was then left to air dry before being mounted with a coverslip using Cytoseal (Thermo Scientific).

The other slide, containing two tissue slices, was boiled in 10-mM sodium citrate (pH 6.0) for 20 minutes and blocked in 5% natural goat serum in 1% BSA (0.1-g BSA in 10-mL 1X PBS) for 2 hours. The two tissue slices were then incubated with a dilution (1:50 in 1% BSA) of serum from either a SpayVac-vaccinated mare or nonvaccinated mare overnight. The following day, both tissue slices were incubated with the HRP-conjugated goat polyclonal secondary antibody to horse IgG (1:1000 in 1% BSA; ab102396; Abcam) for 1 hour. Impact DAB Peroxidase Substrate Solution (Vector Laboratories, Burlingame, CA, USA) was then applied for 10 minutes. The slide contents were dehydrated through an alcohol and citrisolv gradient, allowed to dry, and mounted with a coverslip using cytoseal. The slide was washed between each step with 1X PBS for 10 minutes, and each incubation step took place in a moist chamber. All samples were imaged using light microscopy.

Binding specificity of serum antibodies from SpayVac-vaccinated mares was determined by presaturating a sample of serum from a SpayVac-vaccinated mare with ZP3 and ZP4 synthetic blocking peptides (bp17235b and bp12724b, respectively; Abgent). An aliquot of 30 μg of each peptide was added to 10 μL of serum in 500-μL 1% BSA before use as the primary antibody for immunohistochemical analysis. The HRP-conjugated goat antihorse IgG polyclonal antibody was used as the secondary antibody (1:1000 in 1% BSA; ab102396; Abcam).

3. Results

3.1. Immunoreactivity of serum antibodies from SpayVac-vaccinated mares to equine zona protein

Western blot analysis revealed the immunoreactivity of serum from SpayVac-vaccinated mares to equine zona protein. Antibodies in the serum of SpayVac-vaccinated mares bound to protein isolated from whole equine oocytes and equine ZP (Fig. 1A), as well as to protein isolated from equine ovarian and follicular tissues (Fig. 1C). These antibodies are not endogenously found in serum from nonvaccinated mares (Fig. 1B, D) and did not bind protein isolated from equine renal tissue (Fig. 1C). Western blot analyses of equine oocytes and ZP were repeated twice and analyses of equine tissues were repeated four times with different protein and serum samples to verify consistency.

3.2. Location of binding of serum antibodies from SpayVac-vaccinated mares to equine ovarian tissue

Immunohistochemical staining of equine ovarian tissue located the binding of serum antibodies from SpayVac-vaccinated mares to the ZP of immature oocytes of primary follicles (Fig. 2E). These serum antibodies did not bind oocytes in primordial follicles, where the ZP was not yet formed as part of folliculogenesis (Fig. 2B). They are also not found in serum from nonvaccinated mares (Fig. 2C, F).

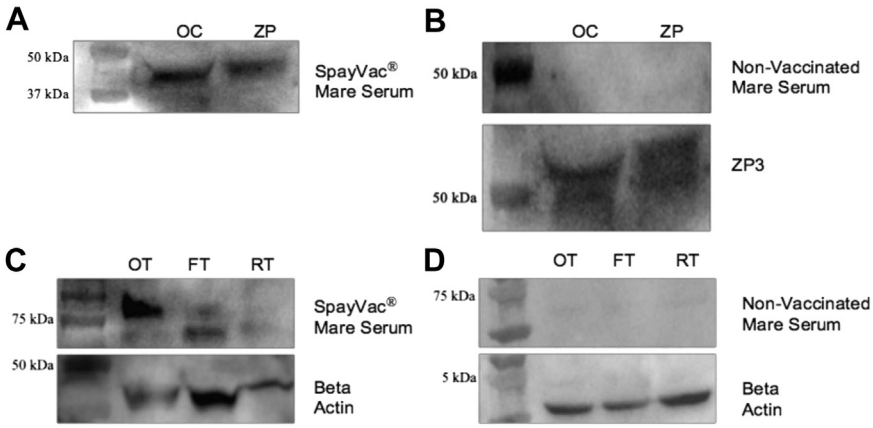


Fig. 1. Immunoreactivity of serum antibodies from SpayVac-vaccinated mares for equine zona protein. Western blot analysis of protein isolated from the equine ZP of two oocytes, two whole equine oocytes (OC), 40-μg equine ovarian tissue (OT), 40-μg equine follicular tissue (FT), and 40-μg equine renal tissue (RT) using serum from a SpayVac-vaccinated mare (A and C) or serum from a nonvaccinated mare (B and D). Protein quantities are expressed per lane. Anti-ZP3 (B) and anti–β actin (D) are used as loading controls. Renal tissue was used as a negative control. Molecular weight standards appear in the leftmost lane of each gel.

Immunohistochemical analyses were repeated twice with different serum and tissue samples.

3.3. *Specificity of serum antibodies from SpayVac-vaccinated mares for equine ZP3*

The addition of ZP3 and ZP4 blocking peptides to a sample of serum from a SpayVac-vaccinated mare successfully saturated antibodies in the serum and blocked their ability to bind zona proteins in the tissue (Fig. 3C). In the absence of blocking peptides, serum antibodies from the SpayVac-vaccinated mare bound to an immature oocyte in the ovarian tissue (Fig. 3B).

Preincubation of a Western blot membrane with anti-ZP3 polyclonal antibody before incubation with serum from a SpayVac-vaccinated mare blocked the ability of serum antibodies to bind to the protein sample (Fig. 4A). These serum antibodies maintained their ability to bind the protein samples after preincubation with anti-ZP4 polyclonal antibody (Fig. 4B).

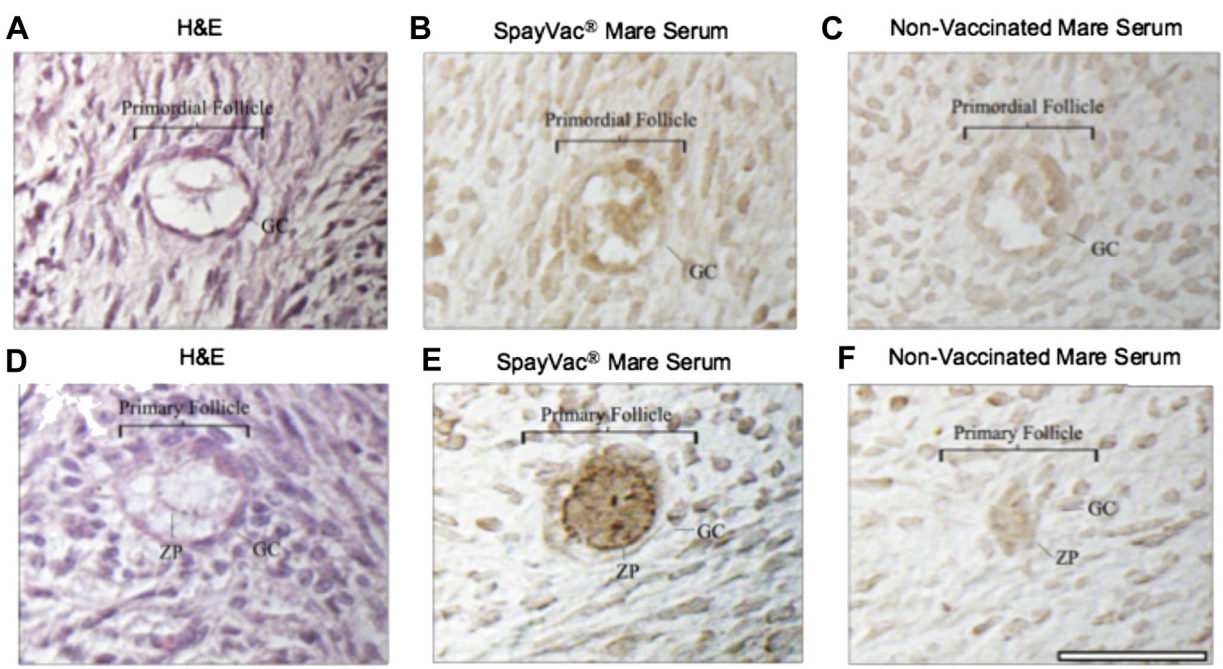


Fig. 2. Localization of serum antibodies from SpayVac-vaccinated mares to the ZP of immature oocytes. Immunohistochemical staining of equine ovarian tissue using serum from a SpayVac-vaccinated mare (B and E) or serum from a nonvaccinated mare (C and F). Serum antibodies bind the ZP of oocytes in primary follicles (E) but did not bind primordial follicles (B). Immunohistochemical staining using serum from nonvaccinated mares is not observed (C and F). H&E staining of ovarian tissue containing a primordial follicle (A) and primary follicle (D). Bar = 20 μm. GC, granulosa cells.

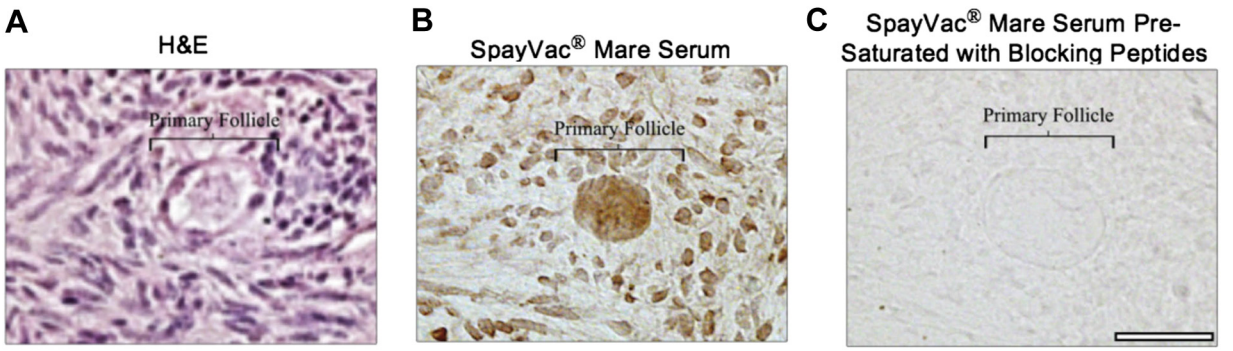


Fig. 3. Specificity of serum antibodies from SpayVac-vaccinated mares for zona protein. H&E staining of of ovarian tissue containing a primary follicle (A). Immunohistochemical staining of equine ovarian tissue using serum from a SpayVac-vaccinated mare (B) or serum from the same SpayVac-vaccinated mare after presaturation of the serum with ZP3 and ZP4 blocking peptides (C). Bar = 20 μm.

4. Discussion

4.1. Immunoreactivity of serum antibodies from SpayVac-vaccinated mares to equine zona protein

Through Western blot analyses of mature preovulatory equine oocytes and ZP, we revealed the presence of antibodies in serum from SpayVac-vaccinated mares that are immunoreactive to equine zona protein. The use of serum from nonvaccinated mares as a control verified that these antibodies are not endogenous to the mare. Western blot analyses also confirmed the immunoreactivity of serum antibodies from SpayVac-vaccinated mares to protein isolated from equine ovarian and follicular tissues.

4.2. Location of binding of serum antibodies from SpayVac-vaccinated mares to equine ovarian tissue

The immunoreactivity of serum antibodies from SpayVac-vaccinated mares to protein isolated from equine ovarian tissue is explained by the localization of antibodies to the ZP of immature oocytes in the tissue. We failed to locate the binding of these serum antibodies to follicular tissue. Because the synthesis of the ZP is an integrated process involving both the oocyte and granulosa cells [14,15], the immunoreactivity of serum antibodies from SpayVac-treated mares to equine follicular tissue is thought to be due to the presence of zona protein precursors in the granulosa cells. Although Western blot analysis may have detected these zona protein precursors because of its high level of sensitivity and stringent cell lysis protocol to release intracellular proteins, high background staining observed in immunohistochemical analyses may have masked any localization to these cells [16]. High background staining is common when the tissue is of the same species as the secondary antibody used [16]. Conversely, the high background staining observed may also be indicative of an immunoreactivity between serum antibodies from SpayVac-vaccinated mares to other proteins located in follicular tissue. Because it is likely that the preparation of SpayVac results in nonzona proteins, such as ovarian and follicular proteins [17], it is possible that serum antibodies from SpayVac-vaccinated mares are immunoreactive to nonzona proteins as well. More research however is required to investigate the immunoreactivity of serum from SpayVac-vaccinated mares to other ovarian and follicular proteins and how this immunoreactivity may relate to efficacy and reversibility of the vaccine.

4.3. Specificity of serum antibodies from SpayVac-vaccinated mares for equine ZP3

Our presaturation and preincubation experiments are indicative of both a specificity of serum antibodies from SpayVac-vaccinated mares for equine zona protein 3 and a lack of nonspecific binding. Because zona proteins are large glycoproteins with varying levels of posttranslational

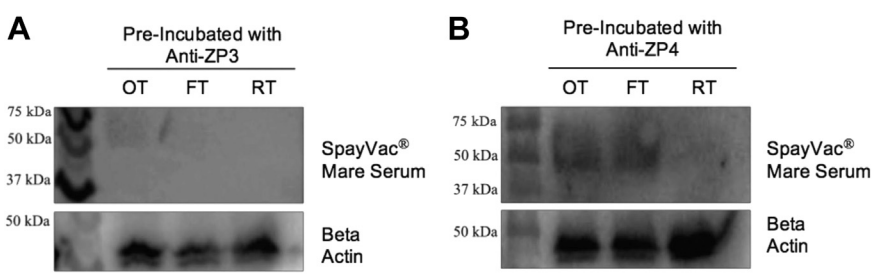


Fig. 4. Specificity of serum antibodies from SpayVac-vaccinated mares for equine zona protein 3. Western blot analysis of protein isolated from equine ovarian tissue (OT), equine follicular (FT), and equine renal (RT) tissues using serum from a SpayVac-vaccinated mare after preincubation with rabbit anti-ZP3 polyclonal antibody (A) or rabbit anti-ZP4 polyclonal antibody (B). Anti–β actin antibody was used as a loading control. Renal tissue was used as a negative control. Molecular weight standards appear in the leftmost lanes of each gel. Forty micrograms of protein was loaded per lane.

modifications, each protein can be observed in a range of molecular weights making the protein difficult to accurately identify by electrophoresis [18–20]. Our experiments lend clarity to the specificity of these serum antibodies. Likewise, this specificity for ZP3 is in agreement with the finding by Wassarman [18] that ZP3 is the major component of the porcine ZP, representing as much as 70% to 80% of total protein content. When the pZP antigen is isolated, the entire ZP is homogenized [21], resulting in a compilation of all zona protein families. It follows that 70% to 80% of the pZP antigen is likely accounted for by ZP3 and, when injected, the mare may produce substantially more antibodies against ZP3 than the other proteins. Furthermore, the pZP antigen is thought to work by eliciting the production of antibodies that bind to ZP proteins on mature oocytes, thereby interfering with sperm binding and penetration [8–10]. Because the zona protein families of ZP1, ZP2, and ZP4 are thought to only be involved in ZP structure, whereas ZP3 has a known role in sperm binding and penetration [20], this specificity for ZP3 coincides with the conclusions of prior studies.

4.4. Limitations of results

There are a few limitations to our study. First, our study focused exclusively on SpayVac as it is less documented in published literature and potentially induces greater duration of infertility in field application. Although all three vaccines incorporate the pZP antigen, they are not a homogenous set of compounds. For instance, it is likely that the preparation of SpayVac results in more nonzona proteins, such as ovarian and follicular proteins, than the other two vaccines [17]. This variation in purity may account for the immunoreactivity of serum antibodies from SpayVac-vaccinated mares to ovarian and follicular tissues seen in our experiments. Specifically, the high background staining observed during our immunohistochemical analysis of equine ovarian tissue may be due to the presence of antibodies immunoreactive to ovarian proteins produced as a result of SpayVac vaccination.

Second, serum samples from vaccinated mares were selectively chosen for their reported high antibody titer levels. Differences in antibody titer and quality depend on the animal immunized and contribute to variability in efficacy among individuals [22]. Accordingly, although serum samples were taken from mares immunized with the same dose of SpayVac, one mare was still able to conceive despite vaccination. Nonetheless, our experiments showed that all eight mares produced antibodies immunoreactive to equine zona protein, regardless of whether the antibodies successfully suppressed the mare's fertility. It is likely that although each mare had sufficient serum antibodies to successfully bind the protein in our experimental samples, overall availability of these antibodies *in vivo* may not have been enough to saturate all possible protein-binding sites and thus result in infertility.

4.5. Conclusions

Our results suggest a model where serum antibodies produced in response to SpayVac vaccination are immunoreactive to equine zona proteins *in vitro*. When zona proteins are isolated from equine tissues and made available to serum samples from SpayVac-vaccinated mares, antibodies in the serum are consistently able to recognize the protein. These serum antibodies appear immunoreactive not only to the ZP of mature preovulatory oocytes isolated from Graafian follicles but also to immature oocytes embedded in ovarian tissue. Although we illustrate a pathway by which serum antibodies from SpayVac-vaccinated mares could act if they are biologically available to these tissues under the correct conditions, it has yet to be investigated whether these antibodies are present in the follicular fluid of follicles or able to permeate ovarian tissue after SpayVac vaccination. We simply showed a model by which antibodies produced in response to SpayVac vaccination may bind zona proteins *in vivo*, thereby interfering with a critical reproductive component, and result in infertility.

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Delivery of GonaCon™ -Equine to Feral Horses (Equus caballus) Using Prototype Syringe Darts

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Remote delivery of injectable fertility control products is a crucial component for both feasibility and acceptability of contraceptive management practices. While there has been much work in remote delivery of the traditional porcine zona pellucida vaccine, there has been relatively little research with respect to the GnRH based GonaCon vaccine. Due to its high viscosity, it is difficult to find a dart and pressure combination that allows this vaccine to be delivered without loss of product or dart failure. In 2013 a beta design of a slow injection (Slo-inject™) remote delivery device (RDD), equipped with 3.81 cm 14 gage Tri-Port needles and a gel collar positioned 1.27 cm forward of the ferrule, was created and donated by Pneu-Dart Inc. The design promoted a reduced rate of injection while maintaining optimum performance to insure delivery of dart contents. Closed to the public, we tested the performance of this device to deliver GonaCon™-Equine to penned and free-ranging horses at Theodore Roosevelt National Park, North Dakota.

Shots were taken from distances ranging from 10-21 meters. Darts were fired from JM-Special (DanInject) and X-caliber (Pneu-dart) projectors using pressures of 3.0-4.5 bars and 3.5-4.0 bars respectively. Darts hit their intended target at 37-47 m/s which minimized bounce yet allowed for dart discharge. A total of 88 darts were loaded and fired; 74 were recovered and weighed and determined to have delivered a full dose, 6 were lost or otherwise un-weighed but were observed to stick in the animal, there was 1 miss, 2 darts bounced out, and 5 were determined to have delivered an insufficient payload of vaccine. Overall, we observed an 84.1% success rate for delivery of payload among darts fired and 100% overall success in eventual dosing (i.e., with follow-up shots) of all study animals with remote delivery systems.

We found that the contraceptive efficacy in mares that received dart delivered GonaCon™-Equine was comparable to that observed for hand-injected mares in the same population. In the first foaling season when a contraceptive effect was expected, 45.5% of treated mares (5 of 11 individuals) had foals, whereas 84% of control mares (21 of 25 individuals) had foals in 2015.

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ORIGINAL ARTICLE

The Single-Shot GnRH Immunocontraceptive Vaccine (GonaCon™) in White-Tailed Deer: Comparison of Several GnRH Preparations

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Keywords
contraception, gonadotropin-releasing hormone, infertility, *Odocoileus virginianus*, overabundance, wildlife

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Problem
An effective, single-injection, multi-year, GnRH contraceptive agent is needed to control reproduction in overabundant white-tailed deer populations.

Method of study
Two GnRH conjugates, GonaCon™ (GnRH–KLH) and GonaCon-B™ (GnRH–blue protein), were prepared in emulsion form as one-injection and two-injection immunocontraceptive vaccine formulations. In addition, the GnRH–KLH protein conjugate was lyophilized and suspended in AdjuVac™ adjuvant to produce a fifth vaccine formulation. Each formulation was administered to a group of five captive adult female white-tailed deer. Reproductive performance of treated female deer was monitored for 5 years to determine the comparative efficacy of the various treatments.

Results
The longevity of the contraceptive response (2–5 years) was strongly influenced by the design of the conjugate antigen, the adjuvant used, and the delivery form of the vaccine.

Conclusion
One-injection and two-injection formulations of GonaCon™ and GonaCon-B™ produced multi-year contraception in adult female white-tailed deer. GonaCon-B™ provided a longer lasting contraceptive effect.

Introduction

Non-lethal tools are needed for the control of overabundant wildlife populations in settings in which traditional management methods, such as regulated sport hunting, cannot be used because of legal prohibitions or safety concerns. The development and use of safe, effective, and humane reproductive inhibitors may provide a partial solution to the problem of overabundant populations of deer and other wildlife in situations in which hunting is not an option. Reproductive inhibitors must be tailored to the physiology of the target species, as no contraceptive agent is effective, practical, and appropriate for use in all species.[1]

GonaCon™ Immunocontraceptive Vaccine is a highly effective contraceptive agent that prevents pregnancy in white-tailed deer (*Odocoileus virginianus*),[2] elk (*Cervus elaphus*),[3] wild horses (*Equus caballus*),[4,5] domestic and feral swine (*Sus scrofa*),[6–8] bison

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(*Bison bison*),[9] domestic cats (*Felis catus*),[10] California ground squirrels (*Spermophilus beecheyi*),[11] and Norway rats (*Rattus norvegicus*).[12]

Immunocontraceptive vaccines based on porcine zona pellucida (PZP) and gonadotropin-releasing hormone (GnRH) control fertility by stimulating the production of antibodies that bioneutralize proteins or hormones essential for conception. PZP immunocontraceptive vaccines have been widely tested in white-tailed deer[2,13,14] and other wildlife species.[15,16] Female white-tailed deer vaccinated with PZP may cycle repeatedly and mate several times during the breeding season while still remaining infertile.[17] The multi-cycling problem associated with PZP vaccines prompted investigation into the potential use of GnRH as a contraceptive vaccine.

Gonadotropin-releasing hormone is produced and secreted by the hypothalamus and is responsible for the release of two pituitary hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and the subsequent release of hormones that control the functioning of the ovaries and testes. Antibodies to GnRH induce infertility by binding to circulating endogenous GnRH and preventing the GnRH from binding to pituitary receptors, thereby precluding the release of FSH and LH. The severe reduction or absence of FSH and LH in the bloodstream leads to atrophy of the gonads and concomitant infertility in both sexes. Immunoneutralization of endogenous GnRH through the introduction of a GnRH conjugate in a vaccine appears to be highly specific, and does not appear to affect other hypothalamic-releasing hormones.[18]

An ultimate objective of efforts to develop infertility agents such as immunocontraceptive vaccines is the application of these materials to overabundant populations of free-ranging wildlife to slow the growth of such populations. Much of the early research effort to use GnRH immunization as an infertility method was focused on the contraception of males.[19–21] Reduction of population growth via contraception would be most effectively accomplished by targeting females, however, so most of our studies have dealt with the use of GnRH vaccine to induce infertility in females.

In this paper we describe the use of a newly developed GnRH vaccine in captive, adult, female white-tailed deer. We also describe recent improvements to the vaccine including (i) development of a single-injection technology that provides a multi-year contraceptive effect, (ii) development of a new adjuvant to replace Freund's™ adjuvant, and (iii) replacement of keyhole limpet hemocyanin (KLH) as a protein carrier molecule with a more cost-effective mollusk protein ('blue protein') for conjugation with synthetic GnRH peptide. All of these improvements contributed to the development of a more effective and practical contraceptive vaccine for use in wildlife.

Materials and methods

Deer Research Center at Pennsylvania State University

White-tailed deer used in these studies were born, raised, and maintained at the Pennsylvania State University (PSU) Deer Research Center, where a captive deer herd has been maintained since 1972. The original source of animals for the PSU deer herd was the wild white-tailed deer population in central Pennsylvania. Supplements to the PSU captive deer herd occurred approximately every 5 years until 1996. Since then, the PSU Deer Research Center has maintained a closed herd. During our study, the PSU deer facility encompassed 22 acres of natural woodland/forest habitat, which were divided into nine separate paddock areas that ranged in size from 0.12 to 1.54 ha (0.3–3.8 acres). Vegetation on the open areas consisted of a mixture of clover and orchard grasses, but most of the land was covered with dense eastern deciduous forest that had little understory vegetation. Deer were kept in outdoor paddocks at a density of 25–37 animals per hectare (10–15 animals per acre). During the non-breeding portion of the year, treated female deer were isolated from males. To test vaccine contraceptive efficacy, however, each year, from the first week of November through the end of the following February, four bucks of proven sire ability in the control herd were confined with the aggregated females from all five vaccine treatment groups. The animal facility at the PSU Deer Research Center was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Our study was approved by the Institutional Animal Care and Use Committee of PSU.

Development of the One-shot GnRH Vaccine

The GonaCon™ Immunocontraceptive Vaccine formulations used in this study consisted of synthetic GnRH peptide conjugated to one of two mollusk hemocyanins: KLH (*Megathura crenulata*) or blue

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protein hemocyanin (*Concholepas concholepas*). Each 1.0-mL dose contained 850 or 1000 μg of the GnRH-mollusk hemocyanin conjugate. Four different treatments resulted from combinations of the two amounts of conjugate and the two protein carriers. A fifth treatment consisted of 1000 μg of the GnRH–KLH conjugate that was lyophilized and then suspended in AdjuVac™ adjuvant.

The Peptide (Hapten)

The Gonadotropin-releasing hormone used in this study was synthesized at Global Peptide Services (Fort Collins, CO, USA), with the structure [pE-HWSYGLRPGGC-SH]. The underlined amino acids represent the native GnRH molecule. The 10-amino-acid GnRH peptide hormone was made immunogenic by coupling it to a large carrier protein that contained numerous T-cell epitopes. Immune responses to the GnRH peptide vary with the coupling position of the GnRH molecule.[22–24] A glycine (G) was added at the C terminus as a spacer, followed by a cysteine (C) to ensure consistent alignment of the peptide to the maleimide-activated protein carrier KLH. This GnRH analog, called GnRH-glycys, has a conformation similar to that of native GnRH.[25]

Mollusk Hemocyanin Protein Carrier

GnRH is a small peptide hormone that is not naturally immunogenic. It becomes immunogenic when it is conjugated to a large carrier protein that contains numerous T-cell epitopes. Carrier proteins used in vaccine production include tetanus toxoid,[21] diphtheria toxoid,[26] bovine serum albumin (BSA), ovalbumin, and KLH.[22,25] BSA is not a good carrier for use in cattle or deer because of the phylogenetic proximity between cattle and deer. Our vaccine formulations incorporated the mollusk hemocyanin proteins KLH (Pierce Chemical, Rockford, IL, USA) and 'blue protein' (Biosonda, Santiago, Chile). These mollusk proteins are highly immunogenic in mammals because of their large sizes (molecular weights of 6–8 million), and because the mollusks are phylogenetically distant from the vertebrate target species.

Optimal Conjugate Design

Bachmann et al.[27] showed that the design of the immunogen is a critical factor in obtaining a strong B-cell response to an individual epitope. In a review of studies of immune responses to hepatitis B vaccines, Banatvala et al.[28] reported that the strength of the primary response is influenced by the antigen dose and structure, and that stronger primary responses result in longer lasting immune memory. To make the conjugate used in our vaccine highly immunogenic, we dissolved the hemocyanin proteins in a 7.5-pH, high-salt buffer that kept the large molecules intact so that coupling would occur on their surfaces. The coupling of as many as 300 GnRH peptide molecules on the surface of a mollusk protein mimicked the immune image of many pathogens, including viruses and bacteria that exhibit rigid, highly organized, highly repetitive protein epitopes. This mimicry of the repetitive nature of pathogen epitopes is an important aspect of the GnRH-hemocyanin conjugate design. Consistent alignment of the peptide facilitates the coupling of the immunogenic protein to the sulfhydryl group of the terminal amino acid and leaves the 10-amino-acid GnRH peptide exposed to the immune system.

Before the GnRH peptide can be coupled to the carrier protein, the carrier protein must be activated so that the available maleimide groups on the carrier protein can react with the terminal sulfhydryl-containing peptides on the GnRH peptide. KLH purchased from Pierce Chemical was pre-activated Imject® (Pierce Biotechnology Inc., Rockford, IL, USA) maleimide-activated mcKLH. Our conjugation process followed Pierce Chemical's recommended procedure with minor modifications. Our primary modification reduced the quantity of GnRH from the recommended GnRH:KLH ratio of 50:50 to 30:70, thus reducing the quantity of excess free GnRH remaining in solution after conjugation.

Blue protein was not maleimide activated when purchased from Biosonda. Maleimide activation was accomplished using sulfo-SMCC purchased from Pierce Chemical. To conjugate the GnRH peptide to the maleimide-activated mollusk protein, we followed the Pierce Chemical instructions except that we changed the ratio of blue protein to GnRH just as we did in the GnRH–KLH procedure.

Adjuvant Design – AdjuVac™

An important component of any vaccine is a non-specific immune stimulant called the adjuvant. A widely used adjuvant is Freund's Complete Adjuvant (FCA; Difco Laboratories, Detroit, MI, USA), which induces strong and long-lasting immunity to a broad

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range of antigens.[29] Concerns over FCA's severe injection-site reactions and its potential carcinogenicity, however, prompted NWRC scientists to develop a new adjuvant by modifying the USDA-licensed Johne's vaccine, Mycopar™ (Fort Dodge Animal Health, Fort Dodge, IA, USA). Mycopar is approved for use in food animals, thus mitigating the concern for use in deer that may be hunted for human consumption.

The new adjuvant, called AdjuVac, contains a small quantity of killed *Mycobacterium avium*, a common bacterium found in many species of wildlife and domestic animals throughout the world.[30–32] Because *M. avium* is widely distributed in natural environments,[33–35] many animals have been exposed to it, and for that reason they may respond to the bacterium in AdjuVac as a previously encountered bacterial antigen.

Preparation of the Vaccine Emulsion

The creation of a depot at the vaccine injection site may promote a long-lasting immune response through the slow release of antigen.[36,37] To produce a depot effect, we prepared our vaccine as an emulsion. This was accomplished through the dropwise addition of 0.5 mL of the GnRH-carrier protein conjugate in mollusk-stabilizing buffer (MSB) to 0.5 mL of AdjuVac adjuvant with vigorous vortexing. The emulsion was stiffened by passing it through a 22-gauge needle three times.

Animal Handling, Vaccination, and Sample Collection

Twenty female deer were divided into four treatment groups with five deer per group. Four preparations were tested (i) GnRH–KLH-AdjuVac (850 μg one-shot), (ii) GnRH–KLH-AdjuVac (1000 μg/1000 μg two-shot), (iii) GnRH–blue protein-AdjuVac (1000 μg one-shot), and (iv) GnRH–KLH-AdjuVac (1000-μg lyophilized suspension).

Prior to handling and vaccination, animals were sedated with Xylazine™ (100 mg/mL; Bayer, Leverkusen, North Rhine-Westphalia, Germany), which was administered intramuscularly (i.m.) at 2.2–4.4 mg/kg (depending upon the nervousness of the individual animal). Each deer was then given an i.m., hand-injected dose of 1.0 mL of one of the vaccine preparations. Each injection was delivered to the right hindquarter with a 3 mL syringe and a 2.54 cm (1-inch), 19-gauge, hypodermic needle. The sedative effect was then reversed with Tolazine™ (100 mg/mL; Lloyd Inc., Shenandoah, IA, USA) administered intravenously (i.v.) or i.m. at 4.0 mg/kg.

Blood samples were taken before vaccination in July and then again during September, October and February each year. Approximately 7–10 mL of blood was collected from the jugular vein with 10-mL vacutainer blood collection tubes and 2.54 cm (1-inch), 18-gauge needles (Becton Dickinson, Franklin Lakes, NJ, USA). Blood samples were centrifuged and serum was isolated and then stored at −70°C. During the first week of February each year, a blood sample was collected and centrifuged, and the serum was evaluated for progesterone and anti-GnRH antibody titers. At the same time in February, ultrasound examinations were performed to determine if the does were pregnant. Ultimate reproductive efficacy was assessed by evaluating annual fawn production.

Laboratory Testing

Progesterone

The Coat-A-Count Progesterone *In-vitro* Diagnostic Test Kit™ (Diagnostic Products, Los Angeles, CA, USA) was used in these studies according to the manufacturer's recommended procedure. For each test, we used 0.1 mL of serum.

Anti-GnRH antibody titers

The enzyme-linked immunosorbent assay (ELISA) was used to measure anti-GnRH antibody titers. Fifty microlitres of serum was used for each assay. A 96-well plate was prepared by adding 100 ng of BSA-GnRH antigen to each well and then blocking with SeaBlock™ from Pierce Chemical. As either KLH-GnRH or blue protein-GnRH was used in the vaccine, BSA-GnRH was added to the ELISA plate, causing only antibodies to GnRH to be detected.

Deer blood serum was serially diluted from 1:1000 to 1:128,000 in phosphate-buffered saline containing SeaBlock. Antibodies in the deer serum to GnRH on the plate were directed with the following linkages: deer anti-GnRH bound to GnRH on the plate, rabbit anti-deer IgG bound to the deer IgG, and goat anti-rabbit-peroxidase bound to the rabbit IgG. Chromogen tetramethylbenzidine was used to develop the color, and 2 M H_2SO_4 was used to stop the reaction. The color intensity of the sample was read at 450 nm with a Dynatech MR 5000 ELISA plate

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reader (Dynatech Laboratories, Alexandria, VA, USA).

Contraceptive efficacy: The mean reproductive success of the entire PSU captive deer herd was used as a control value that was compared with that of the GnRH-treated deer. Deer intentionally bred at the PSU facility during the period 1994–1998 produced an average of 1.7 fawns per year.[2] The deer in the current study were managed at the same facility and were maintained under conditions similar to those during the earlier period. In this study, if a vaccinated doe bore twins, she was physically removed from the treatment group (to reduce animal maintenance costs), but she was retained in our data analysis with the assumption that she would have remained fertile for the remainder of the study. Treated does that bore single fawns remained in the treatment groups.

Results

Group 1: GnRH–KLH-AdjuVac™ (850 μg one-shot)

All five does vaccinated in July 2000 with the formulation of GonaCon™ that contained KLH (850 μg single-shot) were infertile during the 2000–2001 breeding season (Table I). A year later, three of the five does (60%) remained infertile, whereas the remaining two treated does each bore a single fawn. One of the infertile does died in July 2002, leaving four deer remaining in the treatment group. One of the fertile does that produced a fawn in 2002 bore twins in 2003 and was then physically removed from the treatment group. In our analysis, however, we assumed that, in the absence of revaccination, she would have remained fertile throughout the rest of the study. Of the two remaining infertile does, only one produced a fawn (during 2005).

Table I Contraceptive Efficacy of Four GnRH Vaccine Formulations Given to Captive Adult Female White-Tailed Deer

	Year post-treatment				
Treatment group	1	2	3	4	5
GnRH–KLH one-shot	100	60	50[a]	50	25
GnRH–KLH two-shot	100	100	80	60	n/a[b]
GnRH–blue one-shot	100	100	80	80	80
GnRH–KLH lyophilized one-shot	40	0	n/a[b]	n/a[b]	n/a[b]

All vaccine formulations were emulsions except the lyophilized formulation, which was a suspension. Table values are the percentages of treated deer that were infertile each year. Five deer were injected with each vaccine formulation.
GnRH, gonadotropin-releasing hormone; KLH, keyhole limpet hemocyanin.
[a]One doe died during previous year, leaving $n = 4$.
[b]Treatment group was removed from study.

Group 2: GnRH–KLH-AdjuVac™ (1000 μg/1000 μg two-shot)

For 2 years, all five does in this treatment group were infertile. During the third year, four does remained infertile, and during the fourth year, three remained infertile (Table I).

Group 3: GnRH–blue protein-AdjuVac™ (1000 μg one-shot)

All five treated does remained infertile through the first 2 years of the study (Table I). During each of the third and fourth years, only one of five does became pregnant. Interestingly, one doe produced a single fawn in the third season, but did not reproduce during the following year. Another doe that had been infertile for the first three breeding seasons produced a single fawn during the fourth season. Although she was then given a booster vaccine dose (same formulation as the primary dose) that caused her to be infertile during the fifth year of the study, we assumed for the purposes of our analysis that she would have remained fertile in year five had she not been revaccinated.

Group 4: GnRH–KLH-AdjuVac™ (1000 μg lyophilized suspension)

The GnRH–KLH vaccine given as a lyophilized suspension did not provide a long-lasting immune response. Only two of the five deer in this treatment group developed a sufficiently strong antibody response to achieve infertility during the first year (Table I). Titers in these two does dropped below contraceptive level (antibody levels ≥1:64,000 generally are associated with infertility in adult white-tailed deer[2]) during the second year, when all five does became pregnant (Fig. 1, Table I). Hence, the trial was terminated.

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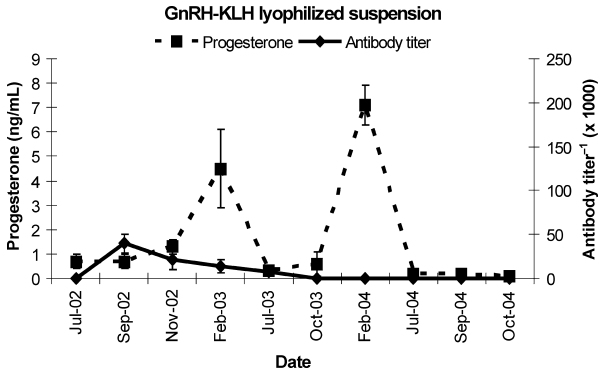


Fig. 1 Gonadotropin-releasing hormone–keyhole limpet hemocyanin lyophilized suspension, one-shot. A single injection (1000 μg) of this GonaCon™ vaccine formulation was given to five captive adult female white-tailed deer during July 2002. The GnRH–KLH conjugate was lyophilized and made into a suspension with the oil-based adjuvant AdjuVac™. A contraceptive effect was induced only in two deer by this vaccine, and it lasted for only 1 year, as reflected in the elevated blood serum progesterone concentrations observed in February 2003 and 2004, as well as in the low anti-GnRH antibody titers, especially after 2002. When vaccine efficacy reached zero during 2004, this trial was terminated.

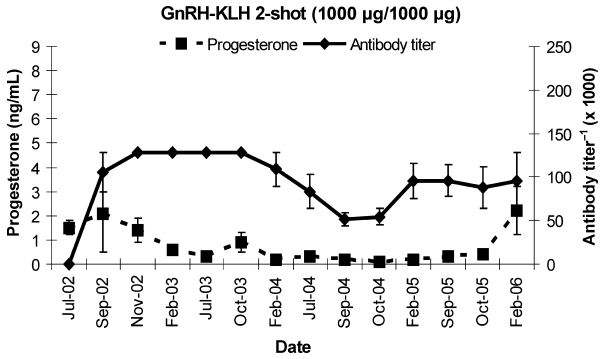


Fig. 3 Gonadotropin-releasing hormone–keyhole limpet hemocyanin emulsion, two-shot. A primary injection (1000 μg) of this GonaCon™ vaccine formulation was given to five captive adult female white-tailed deer during July 2002, and a booster injection (1000 μg) was given during September 2002. The GnRH–KLH conjugate was made into an emulsion with the oil-based adjuvant AdjuVac™. Elevated anti-GnRH antibody titers persisted until 2004, when an apparent self-boosting effect re-elevated the titers. The low blood serum progesterone concentrations observed in deer in this treatment group throughout the 3.5-year trial were associated with a lack of cycling and pregnancy.

Serum Progesterone Concentrations and Ultrasound Examinations

Except in the deer treated with the lyophilized suspension (group 4), serum progesterone concentrations were generally low in treated deer throughout the study (Figs 2–4). Progesterone levels assayed on

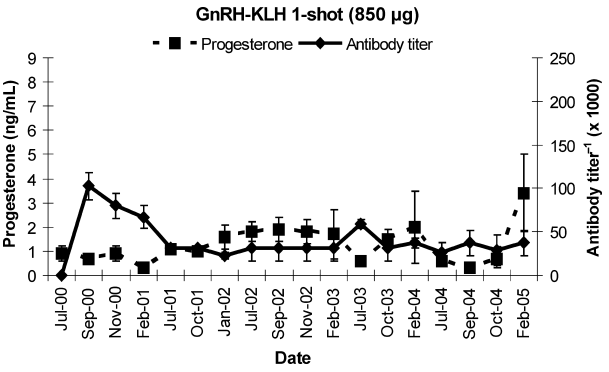


Fig. 2 Gonadotropin-releasing hormone–keyhole limpet hemocyanin emulsion, one-shot. A single injection (850 μg) of this GonaCon™ vaccine formulation was given to five captive adult female white-tailed deer during July 2000. The GnRH–KLH conjugate was made into an emulsion with the oil-based adjuvant AdjuVac™. An initially strong immune response, as indicated by high anti-GnRH antibody titers, resulted in the contraception of all five deer during the first year after vaccination. The immune response declined during the second year and then remained fairly stable throughout the remainder of the 4.5-year trial.

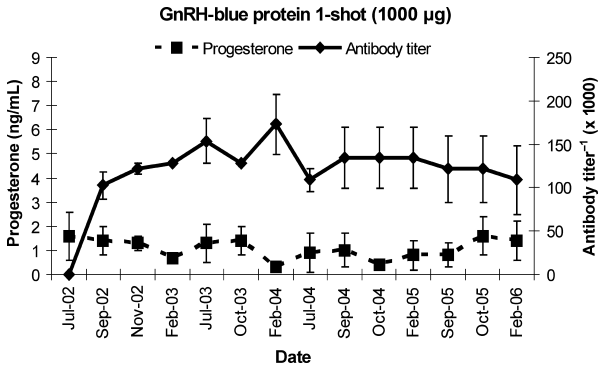


Fig. 4 Gonadotropin-releasing hormone–blue protein emulsion, one-shot. A single injection (1000 μg) of this GonaCon-B™ vaccine formulation was given to five captive adult female white-tailed deer during July 2002. The GnRH–blue protein conjugate was made into an emulsion with the oil-based adjuvant AdjuVac™. Elevated anti-GnRH antibody titers persisted throughout the 3.5-year trial, during which several apparent self-boosting effects occurred. The low blood serum progesterone concentrations observed in deer in this treatment group throughout the trial were associated with a lack of cycling and pregnancy.

serum drawn each February had a strong, positive correlation with spring fawning. Multiple studies have demonstrated that GnRH contracepted deer have serum progesterone concentrations of <1.5 ng/mL in the month of February while controls and deer that are no longer contracepted by GnRH have concentrations >3.0 ng/mL. In this study using

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progesterone concentrations of >3.0 ng/mL as a positive indicator of pregnancy, we found that the correlation between elevated progesterone and spring fawning was 0.90 ($r^2 = 0.95$, $P < 0.0001$). Ultrasound performed at the same time also had a high correlation with spring fawning ($r^2 = 0.95$, $P < 0.0001$).

Anti-GnRH Antibody Titers

An examination of the anti-GnRH antibody titers measured in deer in the various treatment groups (Figs 1–4) and the corresponding contraceptive effects (Table I) indicated that these factors were directly related. The 850 μg, single-shot KLH treatment produced initially high antibody titers and contraception in all five does during the first year after vaccination (Fig. 2). The decline in antibody levels during the second and subsequent years, however, corresponded with a decline in the contraceptive rate. In vaccine trial 2, the 1000 μg/1000 μg KLH primary and booster doses provided a much longer lasting antibody response (Fig. 3), which produced longer lasting contraceptive effects (Table I). The replacement of KLH by the blue protein carrier produced an immune response with a single injection that was stronger than that observed in response to the KLH two-shot treatment (Figs 3 and 4). This strong antibody response produced a 5-year contraceptive effect in four of five blue protein-treated does (Table I). On the other hand, the use of 1000 μg of GnRH–KLH conjugate, lyophilized and suspended in AdjuVac, provided only a weak, short-term immune response (Fig. 1). Only two of five does were infertile during the first year after vaccination, and, as antibody titers declined during the second year, all five does became pregnant (Fig. 1, Table I).

Discussion

GonaCon™ Immunocontraceptive Vaccine suppresses reproductive activity in female white-tailed deer, and may be effective in reducing undesirable behavior associated with the autumn breeding period or rut. PZP vaccines, on the other hand, may actually increase the incidence of such behavior by extending the duration of the breeding season.[17,38,39] By lengthening the rut, PZP vaccines are likely to increase rut-associated movements of deer, thereby increasing the occurrence of deer–vehicle collisions. On the other hand, two-shot formulations of GnRH-based vaccines such as GonaCon™ have limited potential for practical application as wildlife contraceptive agents, and the Freund's™ adjuvant used in early formulations of GnRH vaccines caused large injection-site granulomas.[40] The development of a non-Freund's, single-injection immunocontraceptive vaccine represents an important breakthrough in wildlife contraception.

Several factors were involved in the production of this single-injection, multi-year vaccine. It has been known for more than 20 years that to produce antibody to GnRH, the peptide must be coupled to a larger protein. Many different proteins and coupling techniques have been used (as discussed in the *Materials and methods* section), but in many cases, much of the resultant antibody production targeted the carrier protein and not the GnRH peptide. Therefore the vaccines required a primary and several booster doses to produce a 6-month-long response.[21,26,41]

In our conjugate, the mollusk protein remained intact because of the presence of a stabilizing buffer, and the GnRH peptide coupled to the surface of the carrier molecule. The immune response of the target animal was highly directed to the repeating GnRH peptides on the surface. This directed response provided a strong antibody response to a normally small, non-immunogenic peptide.

Factors Essential for a Multi-year Response to a Single Vaccine Injection

Emulsion form of delivery

The longevity of the contraceptive response appears to be related to the delivery of the antigen in an emulsion form. A properly prepared emulsion is stable at 4°C for months to years; if the emulsion is not prepared properly, it may begin to separate within weeks.[42] A poor emulsification would preclude the long-term effectiveness of the vaccine. The need to produce a stable emulsion is probably related to the need for a depot effect at the injection site to provide a slow release of the antigen. Although lyophilization of the GnRH-mollusk hemocyanin conjugate and addition of the lyophilized antigen to the AdjuVac™ adjuvant in a suspension form did not provide a long-term, single-injection contraceptive effect (Table I), lyophilized delivery may be effective in a two-shot form.

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Presence of Mycobacterium avium in AdjuVac

GonaCon™ vaccine is a mixture of a new, highly immunogenic antigen (GnRH-mollusk hemocyanin) with a previously experienced *M. avium* bacterium. In a GnRH vaccine study with black-tailed deer (*Odocoileus hemionus columbianus*), Perry et al.[43] injected a group of deer with GnRH vaccine in which *M. avium* had been replaced by DD-Dextran in the adjuvant. The first-year response to the DD-Dextran was 14% contraception ($n = 14$). The deer were revaccinated during the following year, resulting in a 90% contraception rate. In the same study, deer ($n = 8$) given a single injection of GonaCon™ vaccine that contained *M. avium* exhibited contraception rates of 79% during the first year after vaccination, and 75% during the second year.[43]

Self boosting: follicular dendritic cells provide continued presence of antigen

The antibody titers illustrated in Figs 2–4 indicate that self-boosting of the GnRH antibody is essential for the single-injection, multi-year contraceptive effect. Follicular dendritic cells (FDC) in the lymph node that drains the site of injection may provide the basis for the long-term immune response associated with some antigens. An immunoglobulin G (IgG) antibody needed for an immunocontraceptive response has a half-life of 23 days.[44] Although antigen-specific memory B-cells are produced with an initial immunization, the induction of immunocontraception that persists for months or years requires a repeated stimulation of these B-cells by the injected antigen.

The long-term immune response is thought to be dependent on the formation of immune-complexes (ICs). In the two-dose paradigm, specific antibodies produced by the primary dose are present at the time of injection of the second dose of antigen, resulting in the formation of ICs. The second dose of antigen selects for the higher affinity antibody-producing B-cells. The higher affinity antibodies are more likely to bind to the target antigen. Affinity maturation of the antibody occurs gradually as new memory B-cells are selected by exposure to small (picogram) quantities of retained antigen.

To be effective in producing long-lasting antibodies, antigen administered in a single dose must be retained in the body long enough to produce specific antibodies that will bind with the antigen to form ICs. These ICs could then bind FDCs, protecting the antigen from macrophage and liver degradation. ICs may begin to form within 7–14 days after a primary injection, or within minutes after a booster injection.[45]

The presence of a specific antibody is the significant difference between primary and secondary antibody responses, as the specific antibody from the primary response combines with the antigen to form immune complexes for transport into draining secondary lymphoid tissues. Many of these immune complexes are eliminated by phagocytic cells, but some are trapped on the surface of FDC for months to years.[27,46]

In the absence of specific antibody, soluble antigen freely diffuses through the draining lymph nodes and interacts with the appropriate immune cells.[45] Initial clearance of antigen is because of catabolism.[45] By the fourth day, there is enough specific antibody to begin a second phase of immune clearance, in which antigen complexes with specific antibody are cleared by macrophages in the afferent and efferent sinuses of the lymph nodes, the sinusoids of the liver, and the marginal sinuses of the spleen.[46,47] FDC trap immune complexes and retain picogram amounts of antigen on their surfaces for long periods.[45]

Antigen availability to the immune system is highly regulated throughout this process. Low levels of specific antigen induce antigen release from FDC, and high antibody concentration inhibits the release of antigen.[45] High concentrations of antibody interact with the antigen on FDC processes and crosslink them so that the antigen is not available to the surrounding cells.[45] In addition, it appears that the antigen released from FDC is significantly more immunogenic than free antigen, such that it may take 1000 pg of free antigen to elicit antibody production, whereas it takes only 10 pg of FDC-retained antigen to elicit the same response.[48] This greater immunogenicity may also be caused by the proximity of the antigen-containing FDC and the B-cells in the draining lymph nodes.

Conclusions

GonaCon™ Immunocontraceptive Vaccine is a highly effective, single-injection, multi-year contraceptive agent based on conjugated GnRH–KLH or GnRH–blue protein. Although both formulations of the vaccine caused infertility in female white-tailed deer, GonaCon-B™, which incorporates the GnRH–blue protein conjugate, was a more effective, longer lasting, and more economical contraceptive agent than the GnRH–KLH formulation. Both conjugate formulations of GonaCon™ vaccine used the adjuvant

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AdjuVac™ in an emulsion that was injected into the hind limb musculature of the target animal, and both were effective as a single-injection contraceptive agent in captive adult female white-tailed deer. Vaccine delivered as a lyophilized suspension is not as effective when compared to the emulsion form of delivery.

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TWENTY YEARS OF IMMUNOCONTRACEPTIVE RESEARCH: LESSONS LEARNED

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TWENTY YEARS OF IMMUNOCONTRACEPTIVE RESEARCH: LESSONS LEARNED

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Abstract: The National Wildlife Research Center (NWRC) began immunocontraception vaccine research by testing porcine zona pellucida (PZP) on white-tailed deer (*Odocoileus virginianus*). Early PZP research demonstrated that PZP induced infertility; however, increased length of the rut was observed in PZP-treated deer. An alternative vaccine using a keyhole limpet hemocyanin-gonadotropin-releasing hormone (KLH-GnRH) conjugate formulated with modified Freund's adjuvant was developed at NWRC. Suppression of GnRH has reduced reproduction in both sexes but is most effective in females. This vaccine was effective in preventing contraception in female deer for several years after a prime and boost. Due to adverse side effects of Freund's adjuvant, NWRC developed a new adjuvant called AdjuVac, a mineral oil/surfactant adjuvant with the addition of *Mycobacterium avium* as an immunostimulant. The price of KLH prompted a search for a more economical hemocyanin carrier protein for the GnRH peptide. Blue protein, derived from the mollusk *Concholepas concholepas*, proved to be a successful option. Formulation improvements resulted in a vaccine that can be effective as a single injection for multiple years, now called GonaCon. GonaCon is registered with the Environmental Protection Agency (EPA) for use in white-tailed deer in urban/suburban areas and for wild horses (*Equus caballus*) and burros (*Equus asinus*). Future GonaCon applications may include reducing reproduction to manage populations of other wildlife species, such as prairie dogs (*Cynomys ludovicianus*) in urban areas and suppressing reproduction to reduce the spread of venereal diseases such as brucellosis. Research is being conducted to develop a GnRH vaccine used in combination with the rabies vaccine to control population growth in free-roaming dogs, with the secondary effect of managing the spread of rabies. The EPA would regulate all these uses. Research is also ongoing on a GnRH vaccine to delay the onset of adrenocortical disease in pet ferrets (*Mustela putorius*), a use regulated by the United States Department of Agriculture.

Key words: AdjuVac, GonaCon, immunocontraceptive vaccine, KLH, *Mycobacterium avium*.

INTRODUCTION

In 1992, the United States Department of Agriculture National Wildlife Research Center (NWRC) began a project to develop reproductive control methods as an additional nonlethal tool to manage overabundant wildlife. The early focus of the project was on white-tailed deer (*Odocoileus virginianus*) because stakeholders identified overabundant deer populations in urban/suburban areas as a concern because of increases in deer-motor vehicle collisions, property damage, and habitat destruction. The project began by studying porcine zona pellucida (PZP) immunocontraception technology for management of white-tailed deer.[23,47,48] The project's focus later shifted to development of vaccines using gonadotropin-releasing hormone (GnRH) as the antigen for several reasons: first, the PZP vaccines are only effective in females and resulted in prolonged periods of estrus cyclicity because normal reproductive hormones were maintained; second, the project's focus broadened beyond white-tailed deer, and a broadly effective vaccine in most mammals was wanted that would affect both sexes; and third, other researchers were developing PZP vaccines, so duplicate research efforts needed to be minimized. Initial studies were conducted with various formulations of GnRH vaccines. Eventually, the vaccine GonaCon was developed at NWRC, which suppresses reproductive hormone production, estrus, and spermatogenesis in mammals, often after a single injection. For this review, GonaCon is a GnRH-based immunocontraceptive vaccine comprised of numerous GnRH peptide molecules coupled to a mollusk carrier protein (either keyhole limpet hemocyanin [KLH] or blue protein [*Concholepus concholepus*]) mixed with a mineral oil-based adjuvant containing *Mycobacterium avium* (AdjuVac) and made into an emulsion (Table 1). Generally, efficacy for longer than 1 yr is achieved from a single injection, and contraception may be extended by administering a boost vaccination.[48,50] GonaCon proved efficacious in deer, reducing

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Table 1. Formulations for GnRH vaccines tested by the National Wildlife Research Center.

Name of formulation[a]	Species tested	Mollusk protein[b]	Adjuvant used[c]	Amount of *M. avium*[d]	Citation
GnRH vaccine	White-tailed deer	KLH	FCA for prime, FIA for boost	NA	23, 29, 42, 48
GnRH vaccine	White-tailed deer fawns	KLH	FCA, FIA boost	NA	43
GnRH vaccine	Norway rats	KLH	FA prime; FIA boost	NA	44
GnRH vaccine	New Zealand white rabbits	KLH	FCA; FIA; AdjuVac	Standard	57
GonaCon	White-tailed deer	KLH and blue	AdjuVac	Standard	42
GonaCon	Black-tailed deer (*Odocoileus hemionus*)	KLH	AdjuVac	Standard, one-half standard, one-fourth standard	55
GonaCon	Elk	KLH	AdjuVac	Standard	22
GonaCon	Elk	Blue	AdjuVac	Standard	56
GonaCon	Bison	KLH	AdjuVac	Standard	50
GonaCon	Domestic swine	KLH	AdjuVac	Standard	51
GonaCon	Feral swine	KLH	AdjuVac	Standard	26, 27
GonaCon	Wild boar	KLH	AdjuVac	Standard	37
GonaCon	California ground squirrel	KLH	AdjuVac	Standard	53
GonaCon	Eastern gray squirrel	Blue	AdjuVac	Standard	54
GonaCon	Black-tailed prairie dog	Blue	AdjuVac	Standard	66
GonaCon	Domestic cats	KLH	AdjuVac	Standard	32, 34
GonaCon	Brushtail possum	KLH	AdjuVac	Standard	8
GonaCon	Tammar wallaby; Gray kangaroo	Blue	AdjuVac	Standard	60
GonaCon	Domestic ferret	KLH and blue	AdjuVac	Standard	40
GonaCon-Cervid (EPA registration)	White-tailed deer	KLH	AdjuVac	Standard	13, 14, 21
GonaCon-Equine (EPA registration)	Wild horses	KLH	AdjuVac	Standard	24, 25, 28
GonaCon-Equine (EPA registration)	Wild horses	Blue	AdjuVac	Standard	18
GonaCon	Dogs	KLH	AdjuVac	Standard	19
GonaCon	Dogs	KLH	AdjuVac	One-half standard	2
GonaCon-Canine (Proposed EPA registration)	Dogs	Blue	AdjuVac	One-fourth standard	63

[a] GonaCon is defined as a GnRH immunocontraceptive vaccine comprised of numerous GnRH protein molecules coupled to a mollusk carrier protein (either keyhole limpet hemocyanin [KLH] or blue protein) mixed with a mineral oil-based adjuvant (AdjuVac) and made into a stiff emulsion.

[b] The EPA registered formulations for GonaCon can contain either keyhole limpet hemocyanin (KLH), or blue protein (*Concholepas concholepas*).

[c] GonaCon is formulated with the adjuvant Adjuvac, a dilution of Mycopar, which contains *Mycobacterium avium*. FCA, Freund's Complete; FIA, Freund's Incomplete.

[d] The standard amount of *M. avium* (approximately 0.17 mg/ml) is the concentration in AdjuVac for the Environmental Protection Agency (EPA) registered products. NA, not applicable.

reproductive behaviors in females and reducing the length of the rut;[13,23] it was also found to induce infertility in many different species of mammals.[9] NWRC has subsequently improved the manufacturing process for GonaCon to include aseptic manufacturing in a clean room, as well as scaling up production to allow GonaCon to be made in larger batches. An adjuvant (AdjuVac) was developed by the NWRC to increase the effectiveness of both PZP and GnRH vaccines.

This manuscript will briefly describe much of the research conducted by the NWRC during the last 20 yr, the vaccines and adjuvant that have been developed, the changes that have been made to improve those products, the lessons NWRC has learned, and what still needs to be determined after 20 yr of research on immunocontraception in wildlife.

VACCINE DESIGN FOR DIFFERENT REPRODUCTIVE SYSTEM TARGETS

Immunocontraceptive vaccines use the animal's immune system to produce antibodies against gamete proteins, reproductive hormones, and other proteins essential for reproduction.[30] The antibodies interfere with the biologic activity of the reproductive proteins,[61] and depending on the antigen and formulation, the vaccines can be effective for 1–4 yr or longer with single or multiple injections.[47,62] Two antigens, PZP and GnRH, have been used to develop immunocontraceptive vaccines for wildlife. The NWRC has conducted research on both PZP and GnRH vaccines. The antigen used, including its mode of action on the reproductive system and the design of the vaccines, is vital in determining vaccine efficacy in different mammalian species.

PZP immunocontraceptive vaccines

The NWRC began its contraceptive research program in 1991 by studying PZP, a combination of three zona pellucida (ZP) proteins extracted from pig ovaries, because PZP had previously been shown to be effective as an antigen in an immunocontraceptive vaccine used for horses (*Equus caballus*).[35] In the authors' early work, it was effective in coyotes (*Canis latrans*) as well.[38] The PZP vaccine targets the ZP, a glycoprotein layer located on the outer surface of the egg. Antibodies to ZP result in infertility either by blocking sperm from penetrating the ZP layer or by interfering with egg maturation within the follicle.[6]

Extracted PZP proteins were initially provided to the NWRC by Dr. Bonnie Dunbar (Baylor College of Medicine, Houston, Texas, 77030, USA) and Dr. Irwin Liu (University of California-Davis, Davis, California 95616, USA) and were produced by the method of Dr. Dunbar.[7] For later NWRC studies, native PZP and SpayVac (PZP encapsulated in liposomes) were produced and provided by Dr. Robert Brown (ImmunoVaccine Technologies [IVT], Nova Scotia, B3H OA8 Canada).[3]

During early research with PZP prepared by the Dunbar method, 11 white-tailed deer does at Pennsylvania State University (University Park, Pennsylvania 16802, USA) received a prime vaccination in July to August and two boost vaccinations in September and October; in November, the does were paddocked with bucks. Extensive observations of breeding activity by a team of Penn State students were made comparing the breeding activity of female deer vaccinated with the vaccine. This PZP preparation was quite effective in preventing pregnancy when deer received both prime and boost doses, with 89% reduction in fawning over a 4-yr period and 76% over 6 yr.[47]

After demonstrating that PZP vaccines could be effective when given as a prime and a boost, NWRC researchers attempted development of a vaccine that could induce a long-lasting immune response after a single injection. A study was subsequently conducted comparing the effectiveness of six different single injection vaccine formulations,[41] containing different PZP preparations (Dunbar preparation, IVT preparation without liposomes, and SpayVac). The study demonstrated that the different production methods for PZP affected the duration of effect of the resulting vaccines. The PZP isolated by IVT produced a longer-lasting response as compared witj the Dunbar PZP isolation method. The IVT PZP with liposomes (SpayVac) and without liposomes were the most efficacious formulations, with 8 of the 10 does in each group contracepted for 5 to 7 yr after a single injection.[41] The normal fertility level for this herd was 1.7 fawns/doe per year.[42] The deer study also compared different adjuvants used with the SpayVac PZP: SpayVac prepared in AdjuVac; lypophilized SpayVac suspended in AdjuVac; and SpayVac mixed with alum as the adjuvant. The water-in-oil emulsion produced with AdjuVac provided a longer-lasting response than the alum or suspension.[41] NWRC researchers were encouraged that vaccines formulated

with AdjuVac provided the long-lasting efficacy with a single injection that would be required for most uses in wildlife.

Because the process of isolating PZP from pig ovaries obtained from pig slaughterhouse facilities is costly and time consuming, an alternative source of PZP was sought. In one study,[45] different recombinant rabbit ZP proteins produced in *Escherichia coli* were studied. The proteins RC55, RC75a, and RC75b all showed cross reactivity with native PZP. Deer vaccinated with these recombinant proteins were compared with deer vaccinated with native PZP. The native PZP was much more effective in contracepting deer than the rabbit recombinant proteins. In another study,[49] the NWRC used selected PZP-derived peptides (generated by Chiron Mimotope Systems, San Diego, 92121, California) identified from published sequence information (National Institutes of Health gene bank, www.ncbi.nlm.nih.gov/genbank) to identify which peptide or peptide groups were responsible for the contraceptive effect. The peptides were screened using serum from a PZP-treated doe, and a ZP1 mimitrope (pins 10–16) demonstrated the greatest response. When this ZP1 peptide was used to vaccinate a group of nine deer, some efficacy was observed, but there was also large variation in the immune response between deer. The deer with the highest antibody titer to this ZP1 peptide exhibited three estrous cycles and had no fawns; however, the average fawning rate in this group was 0.89 fawns/doe, which was indicative of a partial contraceptive effect. It was concluded that the native PZP, which apparently maintains the natural folding of the glycosylated protein, was more consistent in inducing infertility than this or any of the other peptides tested.[49]

By effectively blocking sperm from penetrating the egg without reducing circulating hormone levels, PZP vaccines caused repeated cycling during the rut season in deer at Penn State University,[23] extending the breeding season for the PZP-treated deer for up to seven estrus cycles ($\bar{x} = 2.4$ cycles), with a mean of 98 days and a few deer cycling up to 150 days.[47] This increased length of the breeding season has the potential for increasing car-deer collisions because of increased deer movement. It also causes increased energy expenditures by rutting deer, and if antibody titers drop below a critical threshold late in the breeding season, late season fawns can be produced that may not survive the northern winter climates. Moreover, other researchers were developing PZP vaccines,[30] so duplication of research efforts needed to be minimized. For these reasons, the NWRC discontinued testing of PZP in deer and focused research on developing an alternative contraceptive vaccine for use in ungulates.

GnRH immunocontraceptive vaccines

The NWRC researchers desired a vaccine that would eliminate most reproductive behaviors. GnRH is a key reproductive hormone that controls steroidogenesis and gametogenesis by stimulating the release of gonadotropins from the pituitary gland, triggering the cascade of reproductive hormones that lead to sperm production and ovulation. The NWRC began development of GnRH vaccines in the early 1990s, and the first GnRH vaccine was tested at Penn State University beginning in 1994[23,46] (Table 1). This GnRH vaccine was given to females ($n = 8$) and males ($n = 4$) as a prime injection followed by a boost 1 mo later. Extensive observations of breeding activity were made. Fawning rates in the study were 0.21 fawns/doe per year over 4 yr in the treated does compared with 1.8 fawns/doe per year in control does. The contraceptive activity was correlated with the antibody titer for the vaccine, with fertility returning as the antibody titers dropped. GnRH-treated does showed no breeding behavior, and bucks had no interest in them; the reduction in progesterone shown in the GnRH-treated does suggested that they were not cycling. GnRH immunized bucks had a significant reduction in testosterone and had no interest in sexual activity when paired with control females. Depending on the immunization schedule, antlers in GnRH-treated bucks either dropped early in the fall or remained in velvet.

After years of refinement, a new vaccine (GonaCon Immunocontraceptive Vaccine, NWRC, Fort Collins, Colorado, 80521, USA) was developed by the NWRC that has been shown to suppress reproduction in treated animals of both sexes, can be administered as a single injection,[52] is effective for at least a year in most mammalian species,[9] and is often effective for multiple years.[9] Like earlier GnRH vaccines, GonaCon stimulates the production of antibodies that neutralize GnRH, suppressing reproductive hormone production (i.e., follicle-stimulating hormone [FSH] and luteinizing hormone [LH] from the pituitary gland and estradiol, progesterone, and testosterone from the gonads). The design of GonaCon is the key to its long-term efficacy with a single injection; the

characteristics of the design that allow it to be effective without a boost injection will be discussed in further detail.

VARIATION IN VACCINE EFFICACY AMONG INDIVIDUAL ANIMALS

Users of a contraceptive vaccine need to be aware that, unlike surgical sterilization, which is 100% efficacious, the efficacy of immunocontraceptive vaccines is subject to individual variation, so it is difficult to predict the percent and duration of induced infertility. A certain percentage of vaccinated animals will always be nonresponders or have a reduced immune response to a vaccine.[59] This is true for both disease vaccines and PZP and GnRH immunocontraceptive vaccines. For human or companion animal vaccines, it is possible to give an injection and a follow-up boost that increases the effectiveness of the vaccine and may provide almost 100% efficacy. For wildlife or feral animals, a boost injection is less desirable because the animal must be relocated, it is difficult to individually mark animals, and it is difficult and time consuming to handle an animal more than once. A single injection, long-lasting contraceptive vaccine like GonaCon or SpayVac is a more attractive wildlife management tool because animals would need to be handled or darted less frequently. However, additional studies and/or population models[20,67] will be required to provide managers with recommendations for implementing contraceptive programs as management tools to reduce or maintain wildlife population levels.

Contraceptive vaccine users also need to know that, while GonaCon is effective in both males and females, it has been demonstrated to be effective for a longer period of time in females than in males. In a GnRH vaccine study at Penn State University, male deer ($n = 5$) given a single injection showed reduced testicular size and plasma testosterone for at least three breeding seasons.[29,46] Males given two immunizations tended to remain infertile for an additional 2–3 yr. In contrast, six of nine female deer were contracepted for 4 to 6 yr after a single injection.[41] Similar differences between longevity of vaccine effectiveness in males and females have been demonstrated with cats (*Felis catus*). When a single injection of GonaCon was given to 15 female cats, 93% of vaccinated cats remained infertile for the first year, with 73, 53, 40, and 27% infertile for 2, 3, 4, and 5 yr, respectively. The median duration of contraception was 39.7 mo based on suppression of hormone levels.[32] In a similar study with males given a single injection of GonaCon, 9 of 12 cats responded with high antibody titers and a drop in testosterone to a nondetectable range; however, three treated cats failed to produce a high GnRH titer and showed no suppression of testosterone.[34] The median duration of effect for the male cats that responded to the vaccine was 14 mo, with one treated cat having undetectable testosterone at 34 mo.

As indicated previously, GonaCon-treated white-tailed deer showed a reduced number of estrus cycles or did not cycle at all, an effect probably due to the lack of estrogen. This reduced breeding behavior associated with infertility was typically seen during the first few years following vaccination. Although the single injection of GonaCon induced infertility for multiple years, an occasional return to breeding behavior 4–5 yr after vaccination, prior to the return of fertility (Miller, unpubl. data),[42] was observed. This return of breeding behavior prior to the return of fertility has also been observed in wild horses (Gray, unpubl. data)[18] and in wild boar (*Sus scrofa*).[37] It is speculated that the reason for the return of breeding behavior prior to the return of fertility could be that GonaCon controls LH levels more completely than it controls levels of FSH. As the level of GnRH antibodies drops in immunized animals over several years, sufficient gonadotropins are released to allow estrogen to reach levels that can induce signs of estrus, but GnRH is still sufficiently inhibited to block the LH surge and ovulation. As the GnRH antibody titer drops further, more LH is released, ovulation resumes and the animal can become pregnant. A vaccine boost will enhance the longevity of the vaccine's efficacy in both males and females. Male white-tailed deer were given a boost of GonaCon that lengthened the time that they remained infertile by several years.[29] In a study with female bison (*Bison bison*) at the Northwest Trek Zoo in 2001 (Eatonville,Washington, 98328, USA), a prime dose was effective in contracepting three of five bison; however, a boost given 1 yr later rendered all bison infertile the second year, and they remained infertile for at least 3 yr.[58] Untreated bison in the herd calved normally.

The authors' research has also demonstrated the importance of timing when injecting the vaccine. The vaccine is most effective if given at least a month or more before the onset of reproductive activity. This allows time for an adequate titer level to develop so contraception can occur.[42]

UNDERSTANDING THE SINGLE INJECTION PRINCIPLE

There are very few vaccines on the market that are effective with a single injection, so the statement that GonaCon is effective in contracepting animals with a single injection is often received with skepticism, especially from knowledgeable immunologists. GonaCon is effective as a single injection due to three primary factors: 1) the GnRH antigen is coupled to a large foreign molecule in a systematic manner; 2) the vaccine antigen is made into a stable emulsion; and 3) *M. avium* is included in the adjuvant.

GnRH is a small peptide hormone that is a weak antigen due to its low molecular weight and its being a "self" hormone. However, due to its small size, GnRH can easily be made synthetically. In the GonaCon vaccine, GnRH is made immunogenic by ensuring that each synthetic GnRH peptide molecule is conjugated in a very systematic and predictable manner to a large, nonself, highly immunogenic hemocyanin protein harvested from marine mollusks;[51,52] thus producing a GnRH-mollusk protein conjugate. Many pathogens, including viruses and bacteria, exhibit rigid, highly organized, and highly repetitive surface protein epitopes. The GonaCon design provides a consistent alignment of up to 300 GnRH peptide molecules on the surface of the large mollusk protein, mimicking the repetitive nature of pathogen epitopes, an important aspect of the GnRH-hemocyanin conjugate design. The original mollusk protein used was KLH. However, the high price of KLH (now used in cancer therapy vaccines) prompted a search for a more economical hemocyanin carrier protein for the GnRH peptide. Blue protein, derived from the mollusk *Concholepas concholepas,* proved to be a successful option. The single injection blue protein formulation has been shown to provide a longer-lasting effect compared with the KLH preparation in white-tailed deer.[39,42]

This water-soluble GnRH/mollusk conjugate is then made into a water-in-oil emulsion with the adjuvant AdjuVac. The mineral oil-based emulsion provides a depot effect for the vaccine and protects the antigen from rapid destruction by the macrophages of the target animals' immune system.[39] This provides for a strong prolonged immune response because of the slow release rate of the GnRH conjugate antigen at the injection site. Remnants of vaccine have been observed at the injection site during necropsy up to 2 yr after vaccination (unpubl. obs.). A water-in-oil emulsion appears to be the only formulation that provides a long-term contraceptive response in both PZP and GnRH vaccines with a single injection.

In all of the successful immunocontraceptive vaccine studies, adjuvants have been used as part of the immunocontraceptive vaccine to enhance the immune response of the host animal to the PZP or GnRH antigen. Adjuvants for contraceptive vaccines are critical in providing efficacy, but unfortunately, the immunostimulatory properties of some of the most effective adjuvants can result in localized inflammation and tissue destruction.[1] In initial research on white-tailed deer, the PZP used by the NWRC was formulated as an emulsion with Freund's Complete Adjuvant for the first injection and Freund's Incomplete Adjuvant for subsequent boosts. During discussions with the Food and Drug Administration (FDA) regarding the potential for gaining regulatory approval of immunocontraceptives, the FDA stated their opposition to the use of any vaccine containing Freund's adjuvant because of its known inflammatory effects. The NWRC began work on a safer adjuvant in 1998, leading to development of a new oil-based adjuvant called AdjuVac, which for currently registered uses contains $<$200 μg of killed *M. avium* per 1-ml dose. Inflammatory reactions caused by AdjuVac are less severe than those caused by Freund's complete adjuvant.[2,57,] AdjuVac has been used as the adjuvant for both PZP and GnRH vaccines by the NWRC since 1998.

In addition to the stable emulsion, the long-lasting contraceptive effect of GonaCon is dependent on the presence of *M. avium* in the adjuvant. In early NWRC studies with PZP immunocontraceptive vaccines in deer using Freund's adjuvant, it was necessary to boost with incomplete Freund's at least once, and sometimes several times, to maintain high antibody titers. When it was switched to the new adjuvant AdjuVac, which contains killed *M. avium*, it was found that in most animals (e.g., deer,[42] horses,[24] and prairie dogs [*Cynomys ludovicianus*][66]) a boost was not needed to achieve a long-lasting immune response. *Mycobacterium avium* has been shown to be ubiquitous in nature,[10] having been recovered from almost every environmental compartment that has been investigated, including fresh water, brackish water, biofilms, aerosols, soils, food plants, plant products, and fish. Furthermore, isolation from a hospital water system over a period of 18 mo demonstrated that *M. avium* is commonly found in drinking-water because of its high resistance to ozone and chlorine-based

disinfectants.[31] The authors hypothesize that due to the ubiquitous nature of *M. avium,* it is a key factor in the success of GonaCon.[39,42,55] Most animals will have previously experienced and produced an immune response to *M. avium*; therefore, the immune system is already primed, leading to a more immediate response to the vaccine than would normally be expected after only a single dose.

Current research from the authors' laboratory has shown that a single injection of Gonacon can provide a multiyear contraceptive effect and has also shown that prolonged infertility with GonaCon is directly related to high serum concentration of GnRH antibodies.[42] Burton et al.[4] writes, "To be effective in producing long-lasting antibodies, antigen administered in a single dose must be retained in the body long enough to produce specific antibodies that will bind with the antigen to form immune complexes (ICs)." Fukanoki et al.[11,12] demonstrated in chickens that the slow release of antigen from oil-based emulsions was positively correlated to immune response. A slow antigen release allows ICs to form, creating a greater immune response. These ICs can then bind to follicular dendritic cells (FDCs) that protect the antigen from macrophage and liver degradation. ICs may begin to form within 7–14 days after a primary injection or within minutes after a booster. The FDCs can provide the continued presence of antigen for months to years.[36] Antigen release from the FDCs relates to the level of the antigen-specific antibody. When specific antibody levels drop, the FDCs will release the specific antigen, boosting the circulating antibody.[41] It is believed that the carefully prepared emulsion used in GonaCon serves as a depot, allowing retention and slow release of the target antigen (i.e., GnRH).

USE OF GONACON FOR REPRODUCTIVE, BEHAVIOR, AND DISEASE MANAGEMENT

GonaCon was initially developed as a management tool for urban/suburban populations of the ever-expanding white-tail deer populations that can be used when other management tools, such as hunting, are impractical or not legal. However, it soon became apparent that a GnRH-based contraceptive could apply to other species as well. Because GnRH is common to all mammals, the GonaCon immunocontraceptive vaccine has induced contraception in many overabundant mammalian species, including white-tailed deer,[47] elk (*Cervus elaphus*),[22] wild horses,[24,25,18] bison,[50] California ground squirrels (*Spermophilus beecheyi*),[53] prairie dogs,[66], gray squirrels (*Sciurus carolinensis*),[54,65] captive Norway rats (*Rattus norvegicus*),[44] domestic and feral swine and wild boar,[26,27,51,37] brushtail possums (*Trichosurus vulpecula*),[8] and tammar wallabies (*Macropus eugenii*).[59] In the United States, all of these uses in wildlife will be regulated by the Environmental Protection Agency.

GonaCon is also being tested as a technique to assist in control of feral or wild dogs and in management of rabies. Rabies in domestic dogs (*Canis familiaris*) has been eliminated in most of the developed world through extensive vaccination, and now rabies only persists in wildlife in these areas.[64] However, in the developing world and in poverty-stricken areas, domestic dogs remain the principle vectors of rabies, with dog bites contributing to more than 95% of human rabies cases.[5] Effective rabies control relies on a combination of large-scale vaccination as well as effective dog population control strategies. Most population control programs have moved away from culling of animals toward surgical sterilization, which is expensive, labor intensive, and does not always reach enough of the dog population to be effective. GonaCon is being investigated as a tool to provide contraception of dogs through a single injection that can be administered in conjunction with a rabies vaccine during annual vaccination campaigns. Simultaneous injection of GonaCon with a canine rabies vaccine in dogs did not affect the development of rabies antibodies.[2] Further research is being conducted to determine the effectiveness of combined contraceptive and rabies vaccine programs as a rabies control tool in field situations.

GonaCon has also been tested in zoo animals, livestock, and companion animals for situations in which animals are not intended to be bred. It has induced contraception in domestic cats,[32,33] and domestic swine.[26,27] GonaCon has also been proposed as a technique for eliminating unwanted behaviors associated with reproduction in companion or work animals. However, because the current GonaCon formulation is designed to last for several years, which may not be desirable in certain situations, the formulation would need to be redesigned to last only a few months to a year as needed. Regardless, uses of GonaCon in domestic or zoo animals would be regulated by the FDA's Center for Veterinary Medicine; these uses will not be pursued by the NWRC but could be pursued by collaborators.

Contraception with GonaCon offers potential as a disease management tool for certain diseases,

most notably, venereally transmitted diseases or diseases transmitted at parturition, such as brucellosis. Swine brucellosis is transmitted through the venereal route as well as through contact with aborted fetuses and placental membranes and fluids. Bovine brucellosis is transmitted among cattle (*Bos taurus*), bison, and elk, primarily through contact with infected aborted fetuses and placentas and to calves from infected milk.[58] GonaCon has been and is currently being tested to prevent parturition in bison. Studies in bison showed that a single dose of the vaccine resulted in infertility in all vaccinates for multiple years.[50] If ongoing studies demonstrate GonaCon's utility in decreasing shedding of *Brucella abortus*, the vaccine could provide a potential nonlethal management tool to prevent transmission of the disease in an infected population.

GonaCon has been tested as a tool for the treatment and prevention of adrenal cortical disease (ACD) in domestic ferrets.[40] ACD is a common problem in neutered middle-aged and older ferrets, affecting the majority of animals by 7 yr of age, often causing alopecia, adrenal hyperplasia, and tumors. ACD is caused by the excessive continuous production of LH due to the lack of negative feedback from gonadal hormones in neutered ferrets, resulting in overproduction of sex steroids by the adrenal glands. Injection with GonaCon reduces the production of LH, and thus the adrenal sex hormones, thereby reducing ACD and its clinical signs. In an ongoing study, 88 neutered young male and female ferrets were vaccinated with GonaCon.[40] Only 25% of treated ferrets developed clinical signs of ACD by 8 yr of age compared with 84% of control ferrets, indicating that GonaCon may offer a therapeutic tool for the prevention of ACD in ferrets.

SAFETY AND TOXICITY OF GONACON

The NWRC has conducted several studies that have shown that GonaCon can reduce fertility in deer for multiple years without any obvious side effects other than injection site reactions visible upon necropsy. GonaCon is currently registered with the Environmental Protection Agency (EPA) for use in white-tailed deer and in wild horses and burros (GonaCon-Equine). A safety study was designed to evaluate the potential for negative health effects if deer accidentally received multiple injections of GonaCon.[21] In the study, seven does were given a single injection of GonaCon (EPA-registered formulation) and another six does were given three injections at 2-wk intervals. Both groups were compared with a saline control group ($n = 6$). Blood samples were drawn periodically, and the deer were observed for general health. At 20 wk, the deer were euthanized, necropsies were conducted, and the blood was tested using hematology and chemistry health profiles. Aside from some granulomata formation seen at necropsy in the muscle at the injection site, typically evidenced by a tan-yellow appearance and a bulge at the cut surface, there were no toxic or health effects associated either with GonaCon given in a single injection or in multiple injections.

Another potential concern addressed by the NWRC was the health of animals when injected with GonaCon while pregnant. The mechanisms required for the maintenance of pregnancy differ between species; however, in all mammalian species progesterone, from the corpus luteum and or placenta, is required at some stage. In many years of using GonaCon in white-tailed deer, no indications of abortions after treatment were noted; however, to test this, GonaCon was injected into six deer that tested positive for pregnancy by ultrasound in the first week of February. All deer gave birth to healthy fawns but were then infertile for 2 to 3 yr.[43] Other animals have also been injected with GonaCon while pregnant, including elk, bison, and wild horses;[56,50,28] all delivered normal offspring the next year and then were infertile in the following years. Thus, in species tested to date, GonaCon is safe for use in pregnant animals.

Research was also conducted to determine the effect of using GonaCon on white-tailed deer fawns, because it is sometimes difficult to tell a fawn from a doe in the late summer through winter, when darting with an immunocontraceptive vaccine could occur.[43] To determine the effects on fawns, six male and six female fawns were given two injections of 450 μg of GonaCon 1 mo apart at 3 and 4 mo of age, in September and October. All fawns developed initial elevated titers to GnRH; however, the GnRH antibody titers in 11/12 fawns dropped by the following fall, and those fawns came into breeding condition. Only one female retained a high antibody titer and remained infertile throughout the 3-yr study.[43]

Another of the concerns with using an immunocontraceptive vaccine is the occurrence of injection site reactions. Successful stimulation of the immune system requires some level of injection site reaction because the injection site represents the area where the animals' immune system sees and attacks the invading foreign

material, creating an immune response. As discussed, vaccines that are able to remain at the injection site, without being destroyed, can provide a long-lasting immune response. Injection site reactions vary among vaccine formulations, species, and animals. For example, vaccines containing mineral oil-based adjuvants produce more frequent injection site reactions because of the vaccine emulsion remaining at the injection site. If the vaccine is not cleared from the injection site area, the body walls this area off, forming a granuloma. In some cases, more severe reactions occur that result in open sores or draining abscesses.

There are large differences among species in susceptibility to injection site reactions. Some species, including cats,[32] wallabies,[60] and grey kangaroos (*Macropus giganteus*),[60] appear to be less prone to injection site reactions than others after treatment with GonaCon. After a single GonaCon injection, Levy reported no injection site reactions in female cats during the first 2 yr of a study; at 2 yr, the appearance of a nonpainful but persistent late-onset injection site mass was reported in some animals.[32] Lyn Hinds (pers. comm.) also noticed no initial injection site reactions in grey kangaroos or wallabies but observed in some wallabies late-onset nonpainful injection masses (up to 2 cm wide) that would come and go over a period of months in the GonaCon injection site.

In contrast, dogs ($n = 3$) treated with GonaCon (registered formulation; Table 1) experienced severe injection site reactions.[19] This may be due to general differences between species, with dogs being more susceptible to these types of reactions. It is suspect that the amount of *M. avium* in the vaccine is, at least in part, responsible for the injection site reactions in dogs. In a subsequent trial, dogs ($n = 12$) were treated with GonaCon that contained half the amount of *M. avium* than was previously tested. Dogs still experienced injection site reactions, but they were less severe (S. Bender, pers. comm.). A third trial was conducted in Mexico, where dogs were treated using only one-fourth of the *M. avium*.[63] No severe injection site reactions occurred, and there were no signs of pain or limping in the dogs because of vaccination. At necropsy, there was evidence of gross and microscopic lesions in muscle tissue, but reactions were much reduced compared with previous trials. Additional trials are ongoing with GonaCon-Canine, a formulation with the reduced *M. avium* concentration.

In the authors' studies, injection site reactions have occurred more frequently in field trials than in pen trials with white-tailed deer.[13,14] During the 19 yr of vaccinating deer housed in Penn State University pens, there were no visible injection site reactions in any of the deer, and necropsies following the studies did not show any remarkable injection site reaction. These Penn State deer were fed a high-quality diet and were medicated three times a year to ensure they were parasite free. Because of the complete lack of injection site reactions, it was a surprising that some of the deer in the field studies[13,14] developed injection site reactions that were visible upon necropsy. The deer were in poor health because of overpopulation of the deer herd, and at necropsy we found the deer were infested with many parasites. It is possible that poor general health and/or high parasite load may be correlated with an increase in injection site reactions, but further research is necessary to explain the mechanism behind the differences seen.[15]

Similarly, contraceptive efficacy of GonaCon was consistently higher in captive than in free-ranging white-tailed deer and feral horses.[13,14,18] It was hypothesized that parasites in the free-ranging study animals may have reduced their hosts' immune responses to vaccination with GonaCon. Captive animals in these trials received optimal nutrition and regular veterinary care, including routine deworming, whereas free-ranging wild deer and horses received no veterinary intervention. Similar variation in vaccine efficacy has been observed in humans, where several vaccines used in infants failed to confer the expected protection in heavily helminth-parasitized populations but were highly efficacious in populations in which parasitism was uncommon.[15] Abundant experimental evidence from studies in humans and animal models indicates that parasitic helminths can suppress or divert host immune responses and reduce vaccine efficacy. Therefore, inferences on effectiveness of a contraceptive vaccine based on pen studies may be overly optimistic.

DEVELOPING A PHARMACEUTICAL-GRADE VACCINE

To move from a benchtop experimental vaccine to one that could be registered by the EPA and potentially licensed to a manufacturer, the NWRC needed to make changes in some of the GonaCon vaccine inert ingredients and the manufacturing process. Changes included using higher-quality mineral oil and surfactant, moving the

manufacturing process from a chemistry benchtop to a clean room, and developing an aseptic manufacturing technique to deliver a sterile vaccine product.

For GonaCon to be mass-produced for sale, the need for a more efficient and economical production system is needed. Several steps have been made to produce larger GonaCon batches, reduce the cost of reagents, and reduce the time involved in the manufacturing process. Production of GonaCon requires the proper conjugation of GnRH peptides to a mollusk carrier protein and the preparation of a stable water-in-oil emulsion of the GnRH conjugate with the AdjuVac adjuvant. The authors' were able to shorten the time required to make the GnRH conjugate and the amount of reagents used. This allowed the production of a quality emulsion by using a commercial emulsifier machine (Microfluidics, Newtown, Massachusetts, 02464, USA). This helps to reduce variability between batches and allows for the vaccine to be preloaded in syringes, thus eliminating the need to mix vaccines in the field. Quality control steps have been added to the manufacturing process for GonaCon, including a determination of the amount of the active ingredient, the GnRH peptide, in the GonaCon vaccine.[16] Determination of the stability of the active ingredient is required by the EPA for the GonaCon registration, so the chemistry section of the NWRC developed an analytical method to determine the amount of the active ingredient in GonaCon; this method was used to perform a vaccine stability study. In addition to the chemistry analytical method, a biologic assay in rabbits was used to determine efficacy of the GonaCon vaccine. These studies have allowed for a 6-mo shelf life on the outgoing GonaCon vaccine when it is stored and shipped at 4°C.

SUMMARY AND FUTURE DIRECTIONS FOR NWRC REPRODUCTIVE CONTROL RESEARCH

During the 20 yr that the NWRC has been conducting research on immunocontraceptives, the NWRC has had successes and failures and has learned some important general lessons. Both PZP and GnRH antigens can be effective antigens for immunocontraceptive vaccines in many species. Each of these products has advantages and disadvantages, and its use needs to be based on evaluation of the wildlife problem to be solved. The effectiveness of the vaccines is dependent on their formulation and on the proper presentation of the target antigen. The vaccines require a powerful adjuvant and a water-in-oil emulsion to be most effective. The presence of a ubiquitous *Mycobacterium* (killed) in the adjuvant enhances vaccine efficacy.

When used in combination with AdjuVac, SpayVac (a PZP vaccine), and GonaCon (a GnRH vaccine), formulations can provide single injection, multiple year effectiveness. A boost vaccination can extend the effectiveness of the vaccines, sometimes making the animal permanently infertile, but the effectiveness of vaccines varies between species and among individuals. A certain proportion of animals will be nonresponders and will not generate a high antibody titer. Vaccines are most effective if injected at least a month or more before the onset of reproductive activity to allow time for antibody development. Field efficacy of a vaccine may be lessened by high parasite load and poor body condition. Localized, nonpainful injection site reactions caused by the long-term presence of the vaccine can occur but may be part of the process of developing a long-lasting antibody response after a single injection. Animals treated with either PZP or GonaCon vaccines can be consumed because the protein antigens are broken down into amino acids in the gastrointestinal tract.

In addition to these general lessons, the NWRC has gained considerable insight into PZP vaccines. For example, the native PZP was shown to be much more antigenic than peptides or recombinant proteins. Also, PZP vaccines are not effective in all mammalian species.[17] PZP vaccines may cause undesirable behavioral effects such as multiestrus.

During its development and testing, the NWRC has observed that GonaCon is effective in most mammalian species, with contraceptive effects lasting longer in females than in males. GonaCon can be safely given to pregnant animals without interfering with the pregnancy. GonaCon can eliminate almost all reproductive behaviors, including estrus cycles and theoretically some undesirable behaviors in companion animals. It may also provide a potential management tool for venereally transmitted diseases or diseases transmitted at parturition. GonaCon can effectively prevent ACD in domestic ferrets by reducing LH levels. However, approval for use of GonaCon in companion animals or for disease prevention will be through regulatory agencies other than the EPA and will not be pursued by the Animal Plant Health Infection Service.

Future research at the NWRC will continue to look for new applications for the use of GonaCon and will continue research into understanding the immunology behind the variations in effectiveness from species to species. It is hoped that information on the timeframe in which to give a boost to improve the length of the contraceptive response can be developed. The NWRC also hopes to pursue studies on its effective use as a management tool and to develop private sources of manufacturing for GonaCon in efforts to commercialize the product and make it more widely available.

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The One-Migrant-per-Generation Rule in Conservation and Management

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Abstract: *In the face of continuing habitat fragmentation and isolation, the optimal level of connectivity between populations has become a central issue in conservation biology. A common rule of thumb holds that one migrant per generation into a subpopulation is sufficient to minimize the loss of polymorphism and heterozygosity within subpopulations while allowing for divergence in allele frequencies among subpopulations. The one-migrant-per-generation rule is based on numerous simplifying assumptions that may not hold in natural populations. We examine the conceptual and theoretical basis of the rule and consider both genetic and nongenetic factors that influence the desired level of connectivity among subpopulations. We conclude that one migrant per generation is a desirable minimum, but it may be inadequate for many natural populations. We suggest that a minimum of 1 and a maximum of 10 migrants per generation would be an appropriate general rule of thumb for genetic purposes, bearing in mind that factors other than genetics may further influence the ideal level of connectivity.*

La regla de Un-Migrante-Por-Generación en Conservación y Manejo

Resumen: *Debido a la continua fragmentación y aislamiento de habitats, el nivel óptimo de conectividad entre poblaciones se ha convertido en un tema central en la biología de la conservación. Una regla común sostiene que un amigrante por generación en una subpoblación es suficiente para minimizar la pérdida de polimorfismo y heterocigosidad en las subpoblaciones y al mismo tiempo permite la divergencia de frecuencias alélicas entre subpoblaciones. La regla de un-migrante-por-generación se basa en numerosas suposiciones simplifican que tal vez no sucedan en poblaciones naturales. En este trabajo examinamos las bases conceptuales y teóricas de la regla y consideramos factores genéticos y no genéticos que influyen en el nivel deseado de conectividad entre subpoblaciones. Concluimos que un migrante por generación es un mínimo deseable, pero puede ser inadecuado para muchas poblaciones naturales. Surgerimos que un mínimo de uno y un máximo de diez migrantes por generación seria una regla general apropiada para fines genéticos, sin olvidar que factores no genéticos pueden influir en el nivel ideal de conectividad.*

> . . . evolution depends on a certain balance among its factors. There must be gene mutation, but an excessive rate gives an array of freaks, not evolution; there must be selection, but too severe a process destroys the field of variability, and thus the basis for further advance; prevalence of local inbreeding within a species has extremely important evolutionary consequences, but too close inbreeding leads merely to extinction. A certain amount of crossbreeding is favorable but not too much. In this dependence on balance the species is like a living organism. At all levels of organization life depends on the maintenance of a certain balance among its factors.
>
> —S. Wright (1932:365)

Introduction

By the 1930s Sewall Wright had clearly established—based on experiments and historical breeding records—

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that inbreeding depression in small, isolated populations typically leads to adaptive decline and extinction (Provine 1986). In Wright's view small amounts of gene flow between partially isolated demes are necessary for long-term persistence. Prompted by Wright's work, the question of how many immigrants ("migrants" in population genetics literature) are needed to prevent population differentiation became the subject of a vast body of theoretical analysis (for review see Slatkin [1985, 1987]). From this analysis emerged a genetic rule of thumb that one migrant individual per local population per generation (OMPG) is sufficient to obscure any disruptive effects of drift (Spieth 1974; see also Kimura and Ohta 1971; Lewontin 1974).

Biologists concerned with population insularization caused by habitat fragmentation began advocating the application of this principle for conservation purposes. Several of the most cited papers advocating, in some sense, the movement of one individual per generation between isolated populations for conservation purposes include Franklin (1980), Frankel and Soulé (1981), and Allendorf (1983). More recently, the OMPG concept has been widely applied by scientists and managers charged with initiating connectivity between potentially isolated populations. For example, Gogan (1990) makes the statement for mammals in general that "Immigration to a subpopulation of only one individual once every five years reduces concerns about inbreeding depression to a negligible level." Triggs et al. (1989) suggest for the critically endangered kakapo (*Strigops habroptilus*) that "In future generations, exchanges of one young kakapo per generation between populations should be made to help maintain genetic variation and reduce inbreeding."

Mace and Lande (1991) have used the OMPG rule as a criterion for fragmentation in defining threatened species categories of the World Conservation Union. In the United States nearly every recovery plan that considers genetic issues and insularization applies the OMPG rule. For example, the black-footed ferret (*Mustela nigripes*) recovery plan states that "If the effective population size is to be maintained among many smaller populations, then exchange of genetic material at the rate of one individual/generation between local populations to ensure random and adaptive divergence among populations is necessary" (U.S. Fish and Wildlife Service 1988:18).

The simplicity of the OMPG—and its widespread adoption—belies confusion over the derivation of the rule, its real generality, and the specifics of implementing it (Varvio et al. 1986). Indeed, immediately after stating that subpopulations would not become fixed for different alleles if there were "an interchange of only one individual every other generation," Wright noted that violations of simplifying assumptions may mean that ". . . an interchange of the order of thousands of individuals per generation between neighboring subgroups of a widely distributed species might well be insufficient to prevent a considerable random drifting apart in their genetic compositions" (Wright 1931:128). Thus, from the outset Wright acknowledged the complicating role of the social, ecological, and genetic characteristics of immigrants; these real-world complexities are often neglected in contemporary reference to the OMPG rule.

Human-caused habitat modification is reducing many continuous populations to isolated or semi-isolated fragments (Soulé et al. 1988; Mills 1995). In light of these changes, concerns over the appropriate level of gene flow in fragments are well grounded, both because of the increased potential for demic extinction in isolated populations (Senner 1980; Soulé 1987; Lynch & Gabriel 1990; Mills & Smouse 1994) and because of potential consequences for adaptation to future disturbances (Soulé 1980; Frankel & Soulé 1981). The issue of actually prescribing gene flow in a management context is complicated, however, and it has received little empirical or theoretical consideration.

We describe the OMPG rule, present its theoretical underpinnings, and discuss its application and limitations from a genetics perspective. Finally, we make recommendations that apply both to highly manipulated systems, where managers physically move individuals, as well as to systems in which the movement rate is affected by the size and shape of available habitat or movement corridors.

The One-Migrant-per-Generation Rule

The one-migrant-per-generation rule originated in Wright's analysis of the interplay between genetic drift and gene flow in determining the expected patterns of genetic divergence among a series of subpopulations. Consider a large number of subpopulations in which genetic drift produces genetic divergence among the subpopulations at a rate inversely proportional to the local effective population size (N_e), which is assumed to equal the census size, N. Assume that in each generation a proportion m of the individuals in each subpopulation are migrants from outside that subpopulation and that the allele frequency in migrant individuals is the average allele frequency in all subpopulations. Such migration will limit the amount of divergence that will occur among subpopulations.

This model leads to the surprisingly simple result that at equilibrium the amount of divergence among subpopulations for reasonably small values of m is approximately

$$F_{ST} \approx \frac{1}{4mN + 1}, \quad (1)$$

where F_{ST} is the proportion of total gene diversity (heterozygosity) due to divergence among subpopulations. Note that because m is the proportion of migrants and N is the population size, mN is the actual number of migrants entering a subpopulation each generation.

The amount of divergence among subpopulations as measured by F_{ST} is independent of the total amount of gene diversity in a population (H_T). The F_{ST} can also be thought of as the reduction in heterozygosity within subpopulations (H_S) caused by random genetic drift and divergence among subpopulations. That is,

$$F_{ST} = 1 - \frac{H_S}{H_T}, \quad (2)$$

and F_{ST} is usually called the fixation index. In the case of more than two alleles, each allele may have a different fixation index. Nei (1973) introduced an analogous value (G_{ST}, the coefficient of genetic differentiation) that can be used to describe genetic differentiation for many loci with more than two alleles (Chakraborty & Leimar 1987).

This analysis has two notable results. First, the amount of divergence is dependent only on the number of migrants (mN) and is in a sense independent of population size. For example, we expect the same amount of divergence among subpopulations of size 1000 with 2.5% migration as we do among subpopulations of size 50 with 50% migration; in both cases there are 25 migrants per generation.

The dependence of divergence on the number of migrants, rather than the population size (N) or rate of migration (m), may at first seem counter-intuitive. Divergence, however, results from the opposing effects of migration and genetic drift. The larger the subpopulations, the slower they are diverging through drift; thus, proportionally fewer migrants are needed to counteract the effects of drift. Small subpopulations diverge rapidly though drift, and thus proportionally more migrants are needed to counteract drift.

The second notable aspect of this result is that remarkably small amounts of migration can have powerful genetic consequences on divergence among subpopulations at equilibrium; for example, OMPG is expected to yield an equilibrium F_{ST} of 0.2 (Fig. 1). If we examine the actual distribution of allele frequencies among subpopulations, it is clear that OMPG will also make it unlikely that polymorphism will be lost within subpopulations (Fig. 2; Wright 1931; Fig. 6). With at least one migrant per generation, subpopulations are unlikely to reach equilibrium gene frequencies where one allele or the other is lost (frequency of zero) or "fixed" (frequency of 1.0).

Nevertheless, substantial divergence in allele frequencies across populations is expected with one migrant per generation (Wright 1931, 1969; Allendorf & Phelps 1981). Thus, OMPG provides a desirable balance between drift and gene flow by preventing the loss of alleles and minimizing loss of heterozygosity within subpopulations but allowing genetic divergence to exist among subpopulations. Low amounts of exchange will maintain qualitative similarity among subpopulations and also allow substantial quantitative divergence in allele frequencies.

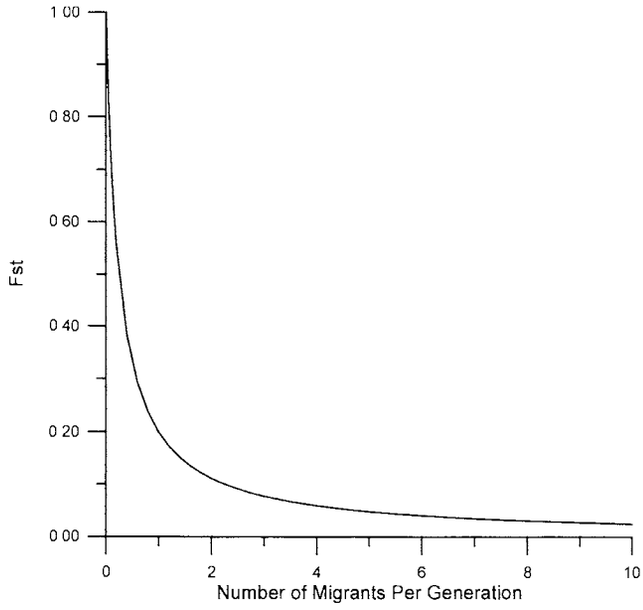


Figure 1. Relationship between number of migrants per generation (mN) *and the fixation index* (F_{ST}).

We want to emphasize that OMPG is not a discrete quantitative threshold and does not imply panmixia (random mating) among individuals of different populations. Rather, the basis of OMPG originates from a qualitative evaluation of tradeoffs between loss of genetic diversity within populations versus homogenization among populations.

Assumptions of the Rule

The OMPG rule results from a simple model of population structure based upon a host of unrealistic assumptions. We consider the primary assumptions that affect the applications of the results of this basic model to conservation.

(1) *Island model of migration*: A migrant is equally likely to come from any subpopulation so that there is no geographical pattern to gene flow. The assumption is also usually made that there are an infinite number of subpopulations of equal size.

(2) *Selective neutrality and no mutation*: There are no selective differences among genotypes, and no new mutations enter the population. That is, gene-frequency dynamics are determined entirely by the interaction between genetic drift and gene flow.

(3) *Ideal populations*: The subpopulations have the characteristics of an ideal genetic population, so that the census number of individuals equals the effective population size. For general purposes, the ideal population consists of a constant number of N diploid individuals ($N/2$ females and $N/2$ males) in which all parents have

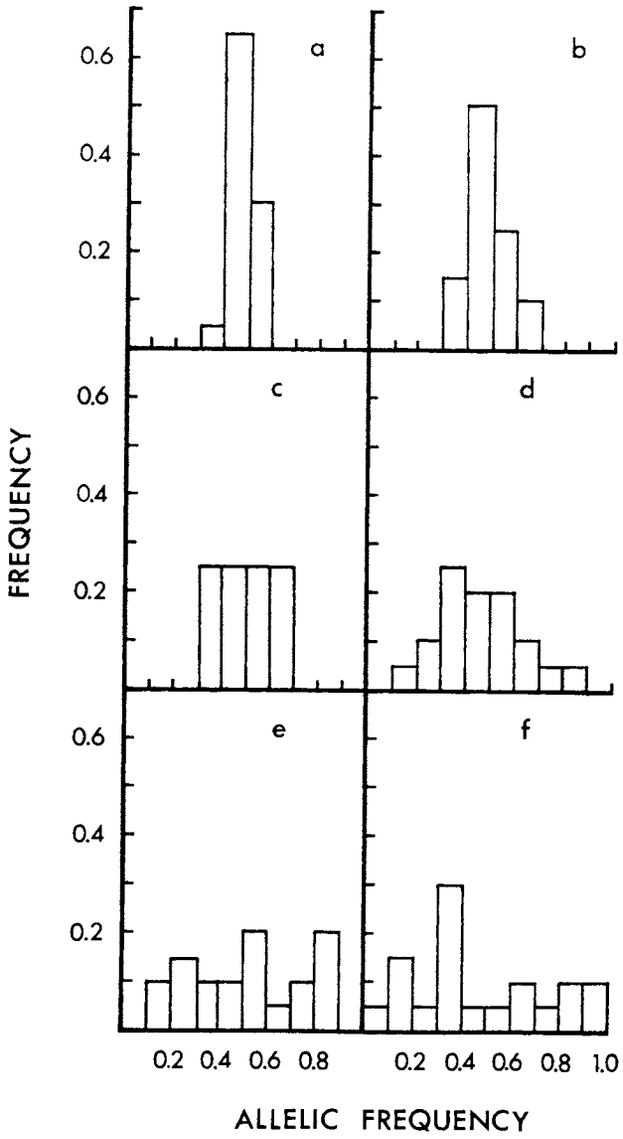


Figure 2. Distribution of allele frequencies in 20 subpopulations (N = Ne = *200 each) with different amounts of migration, based upon computer simulations of the island model for two alleles at a single locus (Allendorf & Phelps 1981). For panels a-e, number of migrants per generation decreases and* F_{ST} *increases, as follows:* mN = *25,* F_{ST} = *0.0080 (a);* mN = *10,* F_{ST} = *0.0175 (b);* mN = *5,* F_{ST} = *0.0509 (c);* mN = *2,* F_{ST} = *0.1104 (d);* mN = *1,* F_{ST} = *0.2513 (e); and* mN = *0.5,* F_{ST} = *0.3221 (f).*

equal probability of contributing an offspring to the next generation.

(4) *Demographic equality*: Immigrants have the same demographic attributes, in terms of survival and reproduction probabilities, as resident individuals.

(5) *Equilibrium*: The subpopulations persist long enough to reach steady-state or equilibrium conditions with respect to gene frequencies.

Limitations of the Rule

What are the consequences of violating the simplistic assumptions of the OMPG rule? We consider how population size may affect the predicted effects of OMPG, then evaluate each of its assumptions. In each case we determine whether each complicating factor is expected to increase or decrease the effectiveness of OMPG or whether "it depends."

Census Population Size

As noted in the derivation of the OMPG rule, one of its interesting and attractive features is that it is independent of population size, as long as gene dynamics are driven entirely by drift and gene flow (mutation and selection play no role). There are, however, at least two caveats to this generalization. First, mN migrants into a small population will represent a larger proportion of the population than if they had entered a larger population; any disruptive social or mating system effects of the immigration will therefore be magnified in smaller populations (Frankel & Soulé 1981; Frankel 1983).

Second, the usual formulation for the loss of genetic variation under drift-migration equilibrium (Eq. 1) is an approximation of the exact equation and holds true only when m (migration rate) is "small" (Wright 1969:291). For a fixed mN, such as one migrant, m becomes large with small population size. For example, when $N = 2$, $m = 0.5$, a large value that leads to less loss in variation (smaller F_{ST}), with the exact equation than expected with the approximation (Fig. 3). We note, however, that the overestimation of F_{ST} with the approximate equation is insignificant with subpopulation sizes large enough to be likely to persist in the face of demographic stochasticity for even relatively short periods of time. For example, for $N = 20$, the exact F_{ST} is 94% of the approximation.

Both of these issues imply that when populations become quite small less migration may be appropriate, both because of possible social disruption and because very small populations are not losing variation as fast as predicted by the F_{ST} approximation. Other factors, such as Allee effects and demographic or environmental stochasticity, lead to the opposite conclusion, with immigrants initiating an important "rescue effect" (Brown & Kodric-Brown 1977).

Island Model: Pattern of Migration

The immigrants in Wright's infinite island model are randomly chosen from the pool of an infinite number of subpopulations. If individuals in the real world come from nearby populations, their gene frequencies would differ little from the target population, so more immigrants would be necessary to prevent loss of variation

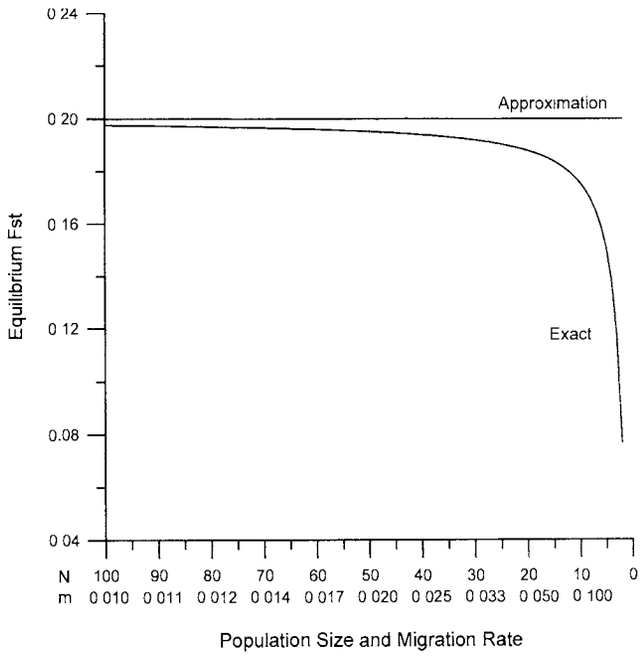


Figure 3. Expected F_{ST} *at equilibrium for one migrant per generation (*mN = *1) using the exact equation (from Eq. 12.2 in Wright 1969) and the commonly used approximation (Eq. 1). Unlike the approximation, which assumes small* m, *the exact equation changes as* m *changes.*

(Wright 1931; Levin 1988). Crow and Aoki (1984) conclude, however, that unless the number of populations is very large, this neighborhood effect is relatively small; in a finite population model, migrants from neighboring groups are about half as effective in their genetic effects. Kin-structured migration—in which individuals disperse in the company of relatives—will also have the effect of requiring more immigrants for the same effect on genetic drift (Levin 1988).

Directional movement patterns are an important and widespread exception to the assumption of the island migration model. In many situations migrants from a relatively large subpopulation may be moved into a smaller subpopulation that is isolated; this is referred to as a continent-island pattern of migration (Hedrick 1983:278). For example, grizzly bears (*Ursus arctos*) from the Yellowstone National Park region are isolated from other populations. The recovery plan for grizzly bears includes regularly moving bears from more northern populations in Montana that are contiguous with grizzly bear populations in Canada into the Yellowstone region (U.S. Fish and Wildlife Service 1993).

The genetic effects of this type of directional movement pattern are compatible with the OMPG rule. The isolated population can be considered one subpopulation of the island model in which migrants from the larger population represent the migrant pool of the island model. Thus, the predictions of the OMPG rule will be valid as long as the donor population is large so that genetic drift is minimal. Weishampel (1990) cautions that if the donor population has lower genetic variation than the receiving population, migration can actually decrease genetic variation in the population of interest.

Another characteristic of the pattern of migration is the interval between immigrants. In its simplest form the island model assumes that probability of migration per generation is constant. Will 1 migrant per generation have the same effect as 10 migrants every 10 generations? Lacy (1987:153) concludes that sporadic immigration should be just as effective as a steady supplementation, provided that the same long-term average rate is maintained. On the other hand, Levin (1988:645) concludes that "a variable migration rate homogenizes neutral allele frequencies less than a uniform migration rate with the same mean." This effect is quite small, however, based upon theory (Nagylaki 1979). Experimental work offers no insight into this question. In many cases the driving criteria for the timing of immigrants will probably not be genetic calculations but logistical concerns (transplanting one animal per generation will often be impractical) and behavioral and social considerations whereby groups of immigrants are more likely to experience successful reproduction.

In short, real-world deviations from the simple pattern of migration used to derive the OMPG rule mean that somewhat more than one migrant per generation may be necessary to achieve the desired genetic goals; the necessary increase will depend on particular circumstances, but it is probably small.

Island Model: Number of Subpopulations

The expected value of F_{ST} at equilibrium can be corrected as shown below to take into account a finite number (n) of subpopulations (Slatkin 1995):

$$F_{ST} \approx \frac{1}{4mNa + 1}, \tag{3}$$

where

$$a = \left(\frac{n}{n-1}\right)^2. \tag{4}$$

Total gene diversity (H_T) can be partitioned into within- and between-subpopulation components. Isolation causes a decrease in within-subpopulation variation (H_S), whereas fixation for different alleles maintains total gene diversity (H_T). As long as the subpopulations are connected by gene flow, an allele will eventually be lost from all subpopulations (and therefore the entire population) by genetic drift if there are a finite number of subpopulations. If the subpopulations are isolated, however, then different alleles will drift to fixation in differ-

ent populations, so more alleles will be maintained in the entire population.

There may be merit (Chesser 1983) in maintaining isolated populations so that more alleles can be retained in the entire population. Nevertheless, these benefits must be weighed against increased probability of extinction due to within-population inbreeding depression (Varvio et al. 1986; Mills & Smouse 1994). In short, the tradeoff between within- and among-population variation may dictate more or less migration than the OMPG rule does (Figs. 1 & 2).

Natural Selection and Mutation

The theoretical model upon which the OMPG rule is based assumes selective neutrality—that is, there are no differences in fitness among genotypes. Nevertheless, the OMPG rule itself does take into account natural selection. The OMPG rule was chosen because it is sufficient to avoid the loss of alleles in subpopulations caused by genetic drift but will allow the allele frequencies within subpopulations to respond to local selective pressures.

Even weak natural selection can have a major effect on the amount of genetic divergence among populations (Allendorf 1983). The effects of natural selection will be greater in larger subpopulations because of the reduced effects of drift in larger populations. In general, natural selection will not have a major effect on the distribution of allele frequencies if the selection coefficient (s) is less than the migration rate (m) (Wright 1940). Thus, natural selection will have less effect on allele frequency divergence among subpopulations with smaller subpopulations for the same amount of migration (mN) (Allendorf 1983).

The effect of selection upon the expected amount of divergence with a given amount of gene flow depends upon the pattern of selection. Similar selection in different subpopulations will tend to reduce divergence, whereas different selective conditions will increase divergence among subpopulations (Allendorf 1983). It is likely that both of these patterns of selection will occur at different loci within the same population. Thus, the OMPG rule seems appropriate when we consider the potential effects of natural selection.

Mutation is expected to have little effect on the application of the OMPG rule. The expected amount of divergence at equilibrium for G_{ST} (the multiple-allele form of F_{ST}) is independent of the mutation rate and the number of alleles, as long as the migration rate is much greater than the mutation rate (Hartl & Clark 1989). This condition will be met for all loci in which migration rates are on the order of one per generation ($mN = 1$) or greater.

Ideal Populations and Demographic Equality

The ratio of effective population size to census population size is expected to affect the consequences of varying migration rates. Wright (1931:128, 1969:292) noted that the smaller N_e is relative to the census N, the larger the number of migrants necessary to minimize differentiation. This is because as N_e decreases below N the population loses variation faster via drift, and a given migrant contributes less to the breeding population, than when N_e equals N. Therefore, populations with highly skewed N_e/N ratios, as is often the case in wild vertebrates (Frankham 1995), may require more than one migrant per generation. How many more are necessary is uncertain; presumably it will depend on why N_e is less than N (for example, because of skewed sex ratio or variance in family size) and on the reproductive characteristics of the immigrants.

Demographic equality also assumes that immigrants are equivalent to residents. To the extent that an immigrant is more or less likely to survive and breed relative to individuals in the target population, it may count as more or less than one migrant. It seems unlikely that immigrants will be demographically equivalent to resident individuals in most situations. In the case in which immigration is imposed by moving captured individuals, there are a variety of reasons to expect immigrants to be less likely to survive and reproduce than residents.

There are also a few situations in which immigrants may be more successful than residents. For example, individuals with rare genotypes have a reproductive advantage in plant species with self-incompatibility systems (Charlesworth 1985). Thus, immigrants from a genetically different subpopulation may have a reproductive advantage in this case.

Because deviations from ideal population structure will tend to compromise the effectiveness of migration relative to expectations, including social structure and taking into account the relative reproductive success of immigrants will mean that more than one migrant per generation may be necessary.

Equilibrium

The OMPG rule is based upon the assumption of equilibrium between the effects of migration and genetic drift. Equilibrium will be reached more quickly with smaller population sizes and higher migration rates (Varvio et al. 1986). Thus, greater migration may be initially desirable with larger subpopulations in order to reach equilibrium more quickly. Equilibrium between gene flow and drift is approached fairly quickly with small population sizes (Table 1; see also Allendorf & Phelps 1981; Table 2).

Before the population reaches equilibrium, the restorative effect of immigration is initially greater for small populations than for large because they have drifted to lower levels of variation (Lacy 1987). This implies that increases above OMPG may well be beneficial when migration is initiated for the first time.

Table 1. Observed and expected genetic divergence among five subpopulations of Leadbeater's opossum connected by gene flow in a simulation study (Lindenmayer & Lacy 1995).*

mN	H_s	*Expected* F_{ST}	*Observed* F_{ST} *20 yr*	*50 yr*	*100 yr*
0	0.28	1.000	0.170	0.309	0.509
0.7	0.60	0.186	0.105	0.128	0.130
1.5	0.70	0.096	0.074	0.068	0.079
7.4	0.80	0.021	0.021	0.022	0.024

**The* mN *values were calculated with the annual migration rate (*m*) used in the simulations, a generation interval of 3.71 years and an* N *of 40 based on carrying capacity (see text for discussion of using* N *versus* N_e*).* H_s *values represent observed mean heterozygosity within subpopulations at 100 years. Expected* F_{ST} *at equilibrium was calculated using Eq. 3 and the actual* mN *values used in the simulations. Observed* F_{ST} *values were calculated using Eq. 2.*

Implicit in the equilibrium assumption is that populations all persist long enough to reach equilibrium. McCauley (1993) reviews the consequences of relaxing this assumption: incorporating extinction and recolonization dynamics can either increase or decrease genetic differentiation, depending on how high population turnover rates are, where the migrants originate, how many migrants move together, and whether the migrants settle in unoccupied or occupied patches. Extinction and recolonization dynamics tend to accentuate population differentiation most when migrant individuals move into established populations more than colonizing new patches. Therefore, if conspecific attraction (Reed & Dobson 1993) tends to increase the likelihood of a dispenser settling in an already occupied area rather than an unoccupied area in a metapopulation, then those migrants will be less effective at minimizing differentiation in the metapopulation; if they settle more in empty patches, then extinction and recolonization dynamics predict that fewer migrants are needed to prevent differentiation (McCauley 1993).

In general, then, violation of the equilibrium assumption means that somewhat more than one migrant per generation may be beneficial. Extinction and recolonization dynamics could either increase or decrease the desirable number.

Empirical and Simulation Results

What insights can be gained from field studies, laboratory experiments, and comprehensive computer studies of natural populations that violate many of the above assumptions at the same time? Because it is not possible to test the predictions of the OMPG rule within a reasonable time frame for most species of interest, empirical tests will initially come from model genetic species with short generation times.

Little experimental work considers the issue of how many migrants are optimal in natural—or even captive—populations. The most direct experimental work is that of Spielman and Frankham (1992). They found strong and significant increases in reproductive fitness in 10 replicate inbred lines of *Drosophila melanogaster* that received only a single immigrant per generation. On average, OMPG restored nearly 50% of that fitness lost under isolation. Newman (1996) has found similar results with experimental populations of the plant *Brassica campestris*. One migrant per generation among 10 subpopulations had a significantly positive effect for five of six fitness components measured. In contrast to these results, Backus et al. (1995) found no increase in fitness associated with a single immigrant (although higher levels of gene flow did increase fitness) among five subpopulations of the housefly (*Musca domestica*).

Studies that use simulations or analytical approaches incorporating actual life-history information for long-lived species also provide insight into how effective OMPG is expected to be in the face of real-world deviations from the simplifying assumptions. For example, Hedrick (1995) used a variety of theoretical population genetic models to test the validity of the OMPG rule in the case of the Florida panther (*Felis concolor coryi*). The report of a workshop for genetic restoration of the Florida panther recommended an initial influx of 20% of the population, followed by gene flow of one animal per generation. Hedrick's models included various combinations of gene flow and genetic drift. He also examined several violations of the assumptions of the basic model: natural selection, directional gene flow, and nonequilibrium initial conditions. Despite these complicating factors, he concluded that this recommendation was sufficient to eliminate lowered fitness due to inbreeding depression and to retain adaptive alleles in the Florida population.

Allendorf et al. (1991) used simulations with the computer program GAPPS (Harris et al. 1986; Harris & Allendorf 1989) to examine the effects of immigration on effective population size and the rate of loss of genetic variation in an isolated population of grizzly bears in the Rocky Mountains. These simulations used a directional (continent-island) pattern of gene flow in a discrete-time, stochastic computer program that followed each individual bear from birth to death. The estimated effective population size of the isolated population was approximately 30% of the census population size. Their results are in general agreement with those expected with the basic model: the introduction of a few bears per generation greatly reduced the rate of loss of variation in the isolated population. The introduction of males had a greater effect than the introduction of females in the model, but this effect may be outweighed by the reduced likelihood of males being incorporated in the breeding population into which they are introduced be-

cause of the behavioral differences between males and females (Allendorf et al. 1991; U.S. Fish and Wildlife Service 1993).

Dobson et al. (1991) used GAPPS to simulate the viability of isolated populations of black rhinos (*Diceros bicornis*) in Kenya. They used an island-continent pattern of migration and demographic data obtained from black rhino sanctuaries. They also included the effects of inbreeding depression in some of their simulations. They found that one immigrant every 10 years had a major effect on the population size and persistence of isolated populations.

The most realistic test of the OMPG rule using simulations that we are aware of was done by Lindenmayer and Lacy (1995) with Leadbeater's possum (*Gymnobelideus leadbeateri*). They used the computer program VORTEX (Lacy 1993) to model the viability of metapopulations with subpopulations occupying a small number of habitat patches of varying sizes. They used values for life-history parameters drawn from extensive field studies, allowed local subpopulations to become extinct, and included the effects of inbreeding depression. Dramatic effects of migration were observed in their largest metapopulation, which consisted of five subpopulations of 40 individuals each (Table 1).

The results for Leadbeater's possum also can be used to quantitatively test the predictions in a system that violates many of the assumptions upon which the OMPG is based. The observed amount of genetic divergence among the five subpopulations estimated from the data of Lindenmayer and Lacy (1995) is similar to that predicted by the simple equilibrium model (Table 1). As expected, near-equilibrium values were obtained more rapidly with greater amounts of gene flow. Even with $mN = 1.5$, however, the observed F_{ST} value was nearly 75% of that expected at equilibrium after approximately five generations.

Recommendations for Implementing Gene Flow

When connectivity is implemented for genetic purposes, we are interested in finding the middle ground between loss of alleles in local populations (fixation, or loss in heterozygosity) and uniformity of allele frequencies across local populations. One migrant per generation is an appropriate lower limit to the amount of gene flow that is desirable. Nevertheless, sometimes more than an average of one migrant per generation will be necessary because of deviations in real populations from the ideal conditions assumed in developing the OMPG rule.

We hesitate to give "cookbook" prescriptions, but an increase in migration above the mean of one migrant per generation may be desirable under the following conditions: (1) inbreeding depression is thought to be a problem in the local population; (2) migrants are closely related to each other or to the local population; (3) social, behavioral, or logistical factors prevent single individuals from immigrating, so that movement is in pulses of several animals every several generations; (4) N_e is much less than total population size; (5) migrants are likely to be at a disadvantage in terms of survival and breeding success; (6) the receiving population has been isolated for many generations; and (7) demographic or environmental variation indicates a high danger of extinction without aggressive supplementation.

If one migrant per generation is not enough in many circumstances, then the next question is how much gene flow may be too much to maintain the balance we are seeking. This question is difficult to answer without extensive genetic and demographic information on the population under consideration. Concerns over local adaptation and outbreeding depression dictate that, under some conditions of phenotypic or genotypic dissimilarity, immigration into an isolated population should be minimized (for cogent discussions see Templeton [1986] and Leberg [1990]). Frankel and Soulé (1981) proposed an upper limit of 5 migrants per generation because of concerns with disruptive effects on social structure in small populations. We suggest that up to 10 migrants per generation is not likely to tip the balance too far by causing uniformity of allele frequencies across subpopulations (Fig. 2).

The characteristics of the migrants is as important as the number of migrants. Social, behavioral, and demographic characteristics that affect the likelihood of survival and reproduction of migrants may overwhelm genetic considerations in assessing the "ideal" candidates to recruit as migrants (Kleiman 1989; Stanley-Price 1989; Short et al. 1992). Likewise, considerations of mating system and social structure will affect the genetic impact of an immigrant. Greater numbers of randomly chosen migrants may be necessary to achieve the same genetic effect as fewer migrants chosen to maximize contribution to the effective population size.

Finally, we stress that criteria other than genetic variation will often play pivotal roles in determining the appropriate level of connectivity between semi-isolated fragments. For example, small populations subject to blinking out due to demographic or environmental stochasticity can experience a "rescue effect" (Brown & Kodric-Brown 1977), with immigrants increasing numbers away from the boundary of zero. In this case more migrants increase population persistence, regardless of genetic considerations. On the other hand, contagious diseases have been raised as a factor decreasing the benefits of connectivity (Wilson et al. 1994; Hess 1994).

Conclusions

The OMPG rule has provided much-needed guidance in management and has illuminated the need for genetic

connectivity among isolated populations. Like many theoretical applications in conservation, its utility is strongest when its complexity is appreciated (Doak & Mills 1994). We have seen several compelling real-world factors that suggest more than one migrant per generation may be necessary to achieve genetic goals, and some situations (albeit fewer) in which less connectivity is desirable. One key to practical decision making is to define clearly the desired genetic outcome, as well as other objectives and constraints of the supplementation program (Lacy 1987).

One migrant per generation is an acceptable minimum, but it may be inadequate for many natural populations. Different levels and schedules of supplementation are appropriate under different environmental conditions and with different species. Nevertheless, we suggest that a minimum of 1 and a maximum of 10 migrants per generation would be an appropriate general rule of thumb for genetic purposes.

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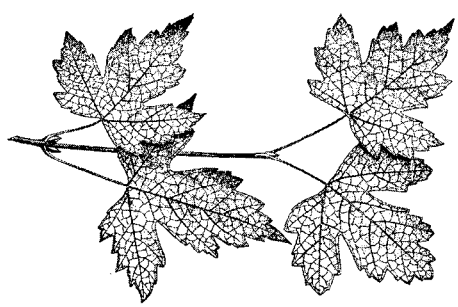
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Potential consequences and problems with wildlife contraceptives

Victor F. Nettles

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Abstract

This presentation reviews multiple wildlife health considerations associated with contraception of wildlife, at the level of both the individual animal and wildlife population. Review of the literature demonstrates that many contraceptives studied in the past have had potential adverse effects characterized as follows: harmful effects on pregnant animals, inhibition of parturition or dystocia, changes in ovarian structure or function, changes in sex ratio, changes in lactation or mammary glands, impact on fertility of young, changes in testicular structure or function, changes in secondary sex characteristics, changes in bodyweight, changes in behaviour, changes in annual breeding season, other physiologic and pathologic changes, abscesses or inflammatory reactions, toxicity, interference with diagnostic tests and ecological alterations. Concern is expressed that the use of immunocontraception could create genetic changes in the target population that would influence disease resistance. The use of infectious agents as vectors to deliver immunocontraceptives was not considered wise animal health management because the product will be a new reproductive disease that could be difficult to contain within the target population. Criteria that need to be fulfilled for the safe use of contraceptives in wildlife are offered.

Keywords: contraception, health.

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Serum anti-Müllerian Hormone Following Zona Pellucida Immunocontraceptive Vaccination of Mares

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Ovarian suppression was recently suggested as an inherent feature of effective zona pellucida (ZP) based immunocontraception across various species including horses. Measuring anti-Müllerian hormone (AMH) in immunocontracepted mares may provide a novel and minimally-invasive tool for the assessment of ovarian function (Joone. et. al. Theriogenology. 2018; 106(2): 214-220). This study aimed to assess the utility of measuring serum AMH concentrations by comparison with clinical parameters for monitoring ovarian activity following ZP-based immunocontraception in a population of horse mares. Mares (n=31) were subdivided into age-categorised (<3y, 3-6y, >6y) treatment groups for active immunisation with either: native porcine ZP proteins (pZP; University of Pretoria, South Africa) (n=7; 2 treatments), novel recombinant ZP proteins (reZP; CSIR, South Africa) (n=8; 3 treatments) or combined pZP and reZP proteins (n=8; 2 treatments). Antigens were prepared in a combination adjuvant formulation (6% polymeric adjuvant (Montanide™ Pet Gel A, Seppic, France) and 500 μg polyinosinic-polycytidylic acid - TLR3-agonist (Poly (I:C) HMW VacciGrade™, Invivogen, USA) all with a 1ml total injection volume. A control group (n=8; 2 treatments) received the same combination adjuvant formulation without antigen. Treatments and measurements were initiated in December (D=0) (southern hemisphere summer) with measurements and, or blood sampling for AMH and serum progesterone concentration (SPC) repeated in January (D=35), February (D=70), March (D=105) and May (D=175). Mares were examined by trans-rectal palpation and ultrasonography of the reproductive tract to establish individual ovarian activity status. Recorded variables included; ovarian volume, uterine and cervical tone, uterine oedema, presence and number of follicles ≥15mm diameter and presence of corpora lutea retrospectively confirmed by SPC assay. Ovarian activity established at each time-point on a composite outcome measurement categorised mares as displaying cyclic ovarian activity if ovarian activity was detected by; trans-rectal palpation, ultrasonographic examination or SPC ≥ 1ng/ml. The various measures of ovarian cyclic activity agreed in 172 of 195 cases. Ovarian suppression was established if all 3 measures of detection were negative. Serum AMH concentrations were determined by ELISA (AMH Gen II ELISA; Beckman Coulter, Brea, USA). Data were analysed using general and mixed linear models. Both time, treatment and time treatment interaction had significant effects on functional ovarian status ($P<0.001$, $P<0.001$, $P<0.001$, respectively) becoming evident from D105, most markedly in the reZP group (7/8 inactive) and furthermore a significant treatment effect on serum AMH concentrations was observed on D175 ($P<0.05$). Notably, mare age positively influenced serum AMH with lower concentrations observed in mares ≤3y. Both pZP and reZP immunisation were associated with ovarian suppression in treated mares. Treatment effects associated with serum AMH concentrations, however, only became apparent 5 weeks later. Serum AMH concentrations proved useful for inferring suppression of ovarian function following completion of the ZP immunocontraception protocol.

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Ovarian function following immunocontraceptive vaccination of mares using native porcine and recombinant zona pellucida vaccines formulated with a non-Freund's adjuvant and anti-GnRH vaccines

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Highlights

- Ovarian suppression is evident in the short term following immunocontraception of mares using reZP proteins formulated with non-Freund's adjuvant.
- Serum AMH concentrations are correlated with ovarian volume, serum progesterone and mare age when employing infrequent sampling techniques.
- Serum AMH is higher in older than younger, albeit sexually mature, mares.

Abstract

the following vaccine formulations, native porcine zona pellucida (pZP), recombinant zona pellucida proteins ZP3 and ZP4 (reZP), pZP and reZP combined or a commercially available anti-GnRH vaccine. The ZP antigens were prepared in an adjuvant formulation consisting of 6% polymeric adjuvant (Montanide™ PetGel A, Seppic, France) and 500 μg polyinosinic-polycytidylic acid - TLR3-agonist (Poly(I:C) HMW VacciGrade™, Invivogen, USA). A vehicle-only control group was administered the adjuvant formulation without antigen. Ovarian activity was monitored using clinical observations (transrectal palpation and ultrasonography of the reproductive tract) in addition to blood sampling for serum progesterone and anti-Müllerian hormone (AMH) concentrations while employing a low sampling frequency. Treatments and measurements were initiated in December (southern hemisphere summer) and subsequent data collection was performed in January, February, March and May. Both reZP and anti-GnRH vaccination were associated with clinically evident ovarian suppression in the short term. Ovarian activity in mares administered a reZP or anti-GnRH vaccine was significantly different to adjuvant control and pZP treated mares. Serum AMH concentrations were different between pZP and anti-GnRH treated mares 3.5 months after the final vaccination. Serum AMH concentrations were significantly correlated with mare age, serum progesterone and ovarian volume.

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Keywords

Horse; Immunocontraception; Ovary; Anestrus; anti-Müllerian hormone

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Antibody Response and Safety of a Novel Recombinant Zona Pellucida Vaccine Formulation in Mares

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Commercial and regulatory limitations associated with porcine zona pellucida (pZP) vaccines may be overcome by developing effective recombinant ZP-based vaccines. Similarly, reported undesirable side effects associated with traditionally-utilised Freund's adjuvants in ZP-vaccines warrants investigation of alternative formulations. This study aimed to assess serum anti-ZP antibody response and safety following administration of novel ZP-based formulations to horse mares. Mares (n=31) were subdivided into age-categorised (<3y, 3-6y, >6y) treatment groups for active immunisation with either: porcine ZP proteins (pZP; University of Pretoria, South Africa) (n=7; 2 treatments), novel recombinant ZP proteins (reZP; CSIR, South Africa) (n=8; 3 treatments) or combined pZP and reZP proteins (n=8; 2 treatments). Antigens were prepared in a combination adjuvant formulation (6% polymeric adjuvant (Montanide™ Pet Gel A, Seppic, France) and 500 μg Polyinosinic-polycytidylic acid - TLR3-agonist (Poly (I:C) HMW VacciGrade™, Invivogen, USA) with a total injection volume of 1ml. A control group (n=8; 2 treatments) received the same combination adjuvant formulation without antigen. Treatments and measurements were initiated in December (D=0) (southern hemisphere summer) with measurements or jugular blood sampling repeated in January (D=35), February (D=70), March (D=105) and May (D=175). Injection site reactions were assessed by inspection and palpation of gluteal intramuscular injection sites for 7 days post-treatment using a 3-point scale and rectal temperature was measured. Anti-ZP antibody response was measured by enzyme immunoassay. Linear models examined data for treatment and time effects. Both treatment and time had a significant effect on anti-ZP antibody titres ($P<0.001$, $P<0.001$ respectively). Treatment group differences were assessed at the time of assumed maximal antibody titres (D=105). All treatment groups' antibody concentrations differed significantly to the control group ($P<0.001$). No differences were measured between Groups 1 (pZP) and 2 (reZP) ($P>0.05$) but Groups 1 and 3 (pZP and reZP) differed significantly, with lower concentrations in Group 3 ($P<0.001$) and differences remained at the end of monitoring (D=175). Temporal changes in antibody titres in all ZP treatment groups followed patterns seen previously in various species treated with pZP and Freund's adjuvants. Correlation analysis for anti-ZP antibody titres with age and BCS were non-significant ($P=0.92$, $P=0.16$, respectively). Injection site reactions, categorised as 0 (none), 1 (slight), or 2 (visible and pain) occurred in 55%, 46%, 47%, and 22% and elevated temperatures (≥39°C) in 20%, 26%, 9%, and 20% of examinations in Groups 1, 2, 3, and 4, respectively. Significant effects of treatment and time on injection site reactions were evident ($P<0.01$), occurring more frequently with subsequent treatments. The majority of reactions were mild (0-1: 97.5%), resolving within 7 days. A similar pattern was observed with elevated rectal temperatures. This represents the first application of a reZP vaccine evoking a similar antibody titre response to native pZP vaccine in mares. Furthermore, incorporation of a novel non-Freund's adjuvant provided an alternative effective formulation for ZP-based immunocontraception.

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Immunocontraception in Wild Horses (*Equus caballus*) Extends Reproductive Cycling Beyond the Normal Breeding Season

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Abstract

Background: Although the physiological effects of immunocontraceptive treatment with porcine zona pellucida (PZP) have been well studied, little is known about PZP's effects on the scheduling of reproductive cycling. Recent behavioral research has suggested that recipients of PZP extend the receptive breeding period into what is normally the non-breeding season.

Methodology/Principal Findings: To determine if this is the case, we compiled foaling data from wild horses (*Equus caballus*) living on Shackleford Banks, North Carolina for 4 years pre- and 8 years post-contraception management with PZP (pre-contraception, n = 65 births from 45 mares; post-contraception, n = 97 births from 46 mares). Gestation lasts approximately 11–12 months in wild horses, placing conception at approximately 11.5 months prior to birth. Since the contraception program began in January 2000, foaling has occurred over a significantly broader range than it had before the contraception program. Foaling in PZP recipients (n = 45 births from 27 mares) has consistently occurred over a broader range than has foaling in non-recipients (n = 52 births from 19 mares). In addition, current recipients of PZP foaled later in the year than did prior recipient and non-recipient mares. Females receiving more consecutive PZP applications gave birth later in the season than did females receiving fewer applications. Finally, the efficacy of PZP declined with increasing consecutive applications before reaching 100% after five consecutive applications.

Conclusions/Significance: For a gregarious species such as the horse, the extension of reproductive cycling into the fall months has important social consequences, including decreased group stability and the extension of male reproductive behavior. In addition, reproductive cycling into the fall months could have long-term effects on foal survivorship. Managers should consider these factors before enacting immunocontraceptive programs in new populations. We suggest minor alterations to management strategies to help alleviate such unintended effects in new populations.

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Introduction

The extirpation of predator species has resulted in the expansion of free-ranging ungulate populations in North America [1], necessitating their regulation. Immunocontraceptive management has become increasingly popular as culling programs are seldom well-received by the general public. In females, the most common form of immunocontraception, porcine zona pellucida (PZP), stimulates the production of antibodies that bind sperm receptors on the egg's surface, thereby preventing sperm attachment and fertilization [2].

In recent decades, the wild horse (*Equus caballus*) has become a model for evaluating the effects of PZP, thanks chiefly to the seminal nine-year study of contracepted mares on Assateague Island National Seashore [3]. Subsequent research on this population has shown that PZP has little to no effect on recipient physiology or behavior [4,5,6]. For example, in Assateague horses, PZP has no effect on the duration of individual estrous cycles [6], and researchers have reported only minor ovulation failure and depressed urinary oestrogen concentrations with repeated applications [4]. However, behavioral research on the horses of Shackleford Banks, North Carolina, and other wild ungulate species suggests that PZP affects the reproductive physiology of recipient animals [7,8,9]. In each of these studies, females treated with PZP extended reproductive behaviors into the non-breeding season. While these results are consistent with an extension of ovulatory cycling into the post-breeding season when most females are normally anovulatory [10,11], this possibility has not yet been tested.

Mares are seasonally polyestrous and extended estrous periods have been documented [11]. Tropical species, for example, are less strictly seasonal, and in some cases, reproduce throughout the year [12,13]. In addition, variability in the cycling schedules and receptivity of individual mares [14], and the performance of estrous behavior and copulatory activities during the non-breeding season [15] have been documented in temperate species. Such

variation in ovulatory scheduling and receptivity suggest that the seasonality of reproductive behavior in *Equus caballus* females is characterized by a substantial degree of plasticity. As the physiological state of contracepted animals has been significantly altered, the possibility of prolonged extended cycling is even more feasible.

In wild horse societies, the harem is the core social group, consisting of usually one, but sometimes two or three harem male(s), one to several female(s), and their offspring [16,17,18,19,20,21]. Harem males will sometimes fight to acquire mares from other groups, but stallions almost always retain their mares [18,19,20,22]. In temperate environments, food availability is lower during the fall and winter months and free-ranging horses will alter their activity to maximize food intake and reduce energetic costs [23,24]. Mares are typically anovulatory at this time and sexual behavior in males is largely absent [11,14].

On Shackleford Banks, increased reproductive behavior in the post-breeding season by mares has resulted in increased male attentiveness [9]. Such behavior (by males) has been shown to restrict the movement of females, thus reducing their grazing efficiency [21,25]. The occurrence of this behavior during a time of year when animals typically increase group spread to acquire adequate forage [19,24], represents a change in behavior fundamental to the animals' survival [26,27]. Offspring conceived during the post-breeding season are likely subject to decreased resource availability as lower quality forage can affect mares' ability to produce sufficient milk [27]. Finally, regardless of the timing of titer decline post-treatment [9,28,58], when anti-PZP antibody titers decrease during the fall months [28,29], extended reproductive cycling among recipient mares will increase their chances of conception, thereby lowering the vaccine's overall efficacy. Determining whether PZP recipients are likely to extend reproductive cycling is therefore of great importance if managers are to limit animal numbers while still maintaining functional, healthy populations.

Here we use the birth dates of foals to estimate dates of conception for PZP recipient and non-recipient mares on Shackleford Banks, North Carolina. Gestation length in wild horses is 11–12 months [30]. Therefore, dates of conception can be reliably estimated as approximately 11.5 months prior to birth. Breeding normally occurs from March through August, with most births occurring in April and May [11]. Given the extension of reproductive behavior in PZP- treated mares [9], we hypothesize that PZP recipients will extend cycling into the non-breeding season more often than will non-recipients. Therefore, when PZP recipients conceive and give birth, they will do so later on average and over a wider range of months than will non-recipients.

Methods

Study area

This study was conducted on Shackleford Banks, a barrier island located approximately 3 km off the coast of North Carolina, USA. The island was 15 km in length, and varied between 0.5 and 3 km in width. The horse population on Shackleford Banks has been co-managed by the National Park Service and the Foundation for Shackleford Horses since 1996.

Study subjects

The reproductive units of Shackleford horses are typical of feral equids. They are coherent harem groups of one, or sometimes two or three stallion(s), one to several mare(s) and their offspring [18]. Predominantly, the harem groups are not territorial and animals move within overlapping home ranges, although this has not always been the case [18].

Management

PZP Contraception. In January 2000, the National Park Service began the application of PZP for the purposes of immunocontraception. The National Park Service administers PZP in the spring (late February through April) each year. Mares receive their initial treatment at 1.5–2 years of age. Each injection includes 100 micrograms of PZP with an adjuvant (combined at the darting site). Initial doses include Freund's Complete Adjuvant, Modified, Mycobacterium butyricum (Calbiochem #344289). All succeeding doses include Freund's Incomplete Adjuvant (Sigma #F5506). In a given year, an average of 63% of all reproductive mares are inoculated with the vaccine (range = 37–88%). The authors of the present study are not and have never been in charge of making management decisions regarding this herd.

Gathers and Removals. The National Park Service began their management of the Shackleford Banks population prior to the use of PZP, performing five gathers between November 1996 and January 2000. During these gathers the majority of the population was rounded up and individuals testing positive for equine infectious anemia were removed and either euthanized or quarantined. As such forms of management could conceivably influence reproductive cycling, we analyzed foaling dates before and after gathers (but before contraception management) using a linear mixed effects model (see Statistical Analyses).

From January 2000 – January 2008, 38 foals (conceived due to contraception failure or administration scheduling) were removed from the island for the purpose of population control. The majority of removals (92%) were conducted in the January following the foals' birth.

Foaling Data

We recorded foaling data before contraception management (1995–1997) during a study of mother-infant behavior [31]. We obtained foaling data for 2000 and post-contraception years (2001–2008) from the National Park Service at Cape Lookout National Seashore, North Carolina. Although PZP was first administered in 2000, foals born in this year were conceived before contraception management. As such, we considered 2000 a pre-contraception year.

We identified individual horses by color, sex, age, physical condition, and other distinguishing markings including freeze brands. We monitored pregnant mares 2–3 times per week to ensure accurate estimation of foaling date. Births are not commonly witnessed, as mares will generally give birth away from their harem group [31]. This does not significantly affect the determination of birth dates because typically, mares return to their groups within hours (depending on the length of the foaling process and the mares' and foals' condition afterward). In the event that we did not locate mares for more than one week, the birth dates of new foals were estimated by comparing the condition of the foals' coat, mane and tail hair, and locomotor ability to that of foals for which the exact age was known. Using these methods, we were able to estimate birthdays within a range of 1–2 days to 1 week. Focal animals in this study were observed from a minimum of 15 m away at all times. The observers did not obstruct or manipulate the animals' natural behavior in any way. As such, approval for this study by a review board or ethics committee was unnecessary.

We designated births as coming from current recipient, prior recipient, or non-recipient mares. Current recipients received PZP

treatment the year they conceived (the year previous to foaling). Prior recipients received PZP treatment at some point earlier in their lifetime, but not the year of conception. Non-recipients had never received PZP at the time of conception.

Physical Condition

Physical condition is an important factor to consider when monitoring reproductive behaviors. Animals in better condition will have more resources to allocate to mating behavior and physiology than will animals in poorer condition. We assessed mare condition via rump scoring. We determined rump scores examining the curvature of the line between the tailbone and the point of the hip. Scores were based on a scale from 1 to 5; a score of 1 being the poorest [32].

Weather Data

We collected all climatology data from the Morehead City WNW Station at 34° 44′N; 76° 44′W, approximately 8 km from the study site. We obtained all data from the National Climate Data Center (2009) [33].

Statistical Analyses

We analyzed data in R version 2.11.1 (R Core Group, Vienna Austria) and JMP, version 7 (SAS, Cary NC, USA). We used Fligner-Killeen tests to determine if variation in foaling dates differed before and after contraception management and between PZP recipients and non-recipients. This test is more conservative than Fisher's or Bartlett's tests and is less sensitive to outliers and non-normal data [34]. Results from these tests show Bonferroni corrected *P*-values.

We used linear mixed effects models to analyze monthly weather patterns. These statistical models included year as a random effect and the following fixed effects: linear through fourth order polynomial functions of month (to account for nonlinear fluctuations in temperature and rainfall), management regime (before or after contraception management), and two-way interactions with management regime. We selected best fit models using Akaike's Information Criterion adjusted for small sample size (AICc). Because temperature data were temporally auto-correlated, we included an autoregressive moving average correlation structure [35]. For each year of the study, we calculated the mean of the monthly residual values from both the rainfall and temperature models from July through November. We included these terms in the mixed effects models described below to determine if weather affected foaling date.

To test for differences in foaling date with PZP treatment, while controlling for pseudo-replication and unequal variances, we used a linear mixed effects model. The model included mare ID as a random effect and a function allowing different variances across groups (pre-contraception management, post-contraception management non-recipient, post-contraception management current recipient, and post-contraception management prior recipient) [35]. We included mare age as a covariate, as this has been suggested to influence female reproductive behaviors and physiology [36,37]. Additionally, a one-way ANOVA revealed no differences in mare age among treatment groups ($F_{2,84} = 2.03$, $P = 0.14$), suggesting that age did not confound differences among groups. From the above mixed effects model, differences in foaling dates among current, prior, and non-recipients were calculated using an F-test for linear combinations [35].

As gathers could have affected foaling date, we used a linear mixed effects model to test for differences between foaling dates before and after gathers (but before contraception management). After controlling for weather and mare age, mean foaling date did not differ before and after gathers ($P = 0.63$). Additionally, the range of birth dates did not differ significantly between these groups (SD before gathers = 1.04, SD after gathers = 0.77, $P = 0.36$). Finally, AICc from our analysis of foaling dates (see Results) suggested that combining data before and after gathers yielded a more parsimonious model than did separating those categories (AICc separating pre- and post-gathers = 551.02, AICc combining pre- and post-gathers = 550.60). Consequently these groups were combined as pre-contraception management animals for all analyses.

To determine whether the number of PZP applications (total or consecutive) or the number of years between pregnancies (total or consecutive) influenced foaling date, we ran separate linear mixed effects models using these as predictor variables. Each model used only data from PZP recipients and included mare ID as a random effect. To determine if results from this analysis could be influenced by year, we ran a linear mixed effects model on post-management, non-recipients predicting foaling date by year.

We analyzed PZP efficacy using a generalized linear mixed effects model with a binomial error distribution and mare ID as a random effect [38]. This analyzed the probability of conceiving using age at first PZP application and the number of consecutive PZP applications as fixed effects. All mixed effects models initially included two-way interactions, which were removed if their *P*-values were >0.10.

Results

Foaling

After contraception management, foaling occurred over a broader range of months than before contraception management (see Fig. 1, SD pre-contraception = 0.91 months, SD post-contraception = 2.04 months; Fligner-Killeen Test for Homogeneity of Variances: Median Chi Square = 28.10, $P<0.0001$; pre-contraception: n = 65 births from 45 mares over 4 years; post-contraception: n = 97 births from 46 mares, over 8 years). In the eight years following contraception management, mares receiving PZP foaled over a broader range of months than did non-recipient mares (see Fig. 1, SD recipients = 2.40 months, SD non-recipients = 1.54 months; Fligner-Killeen Test for Homogeneity of Variances: Median Chi Square = 11.76, $P = 0.001$; recipients: n = 45 births from 27 mares; non-recipients: n = 52 births from 19 mares).

On average, current PZP recipients gave birth 3.36 months later than did pre-contraception mares, according to our linear mixed effects model, which controlled for heterogeneity of variances, weather influences, and mare age (estimate = 3.36, SE = 0.51, t = 6.64, $P<0.001$; overall model: Likelihood ratio (compared to null model): 40.79, generalized $r^2 = 0.48$, $P<0.001$). Mares that had received PZP earlier in their lifetime, but not during the year of conception (prior recipients), gave birth 0.90 months later than pre-contraception mares on average (estimate = 0.90, SE = 0.40, t = 2.27, $P = 0.03$). Non-recipient mares that never received PZP themselves, but gave birth after the general population was managed with PZP, gave birth 1.01 months later than pre-contraception mares on average (estimate = 1.01, SE = 0.25, t = 4.04, $P = 0.001$). This translates into current PZP recipients giving birth 2.34 months later than non-recipient animals and 2.46 months later than prior recipients (F-tests for linear combinations: $F>16$, $P<0.001$ for each comparison). Furthermore, birth dates were about 0.38 months later for each degree centigrade above average in the latter half of the breeding season and 0.05 months later for each centimeter of rain above average (temperature residuals July-Nov: estimate = 0.38, SE = 0.13, t = 2.84, $P = 0.001$; rainfall residuals July-Nov: esti-

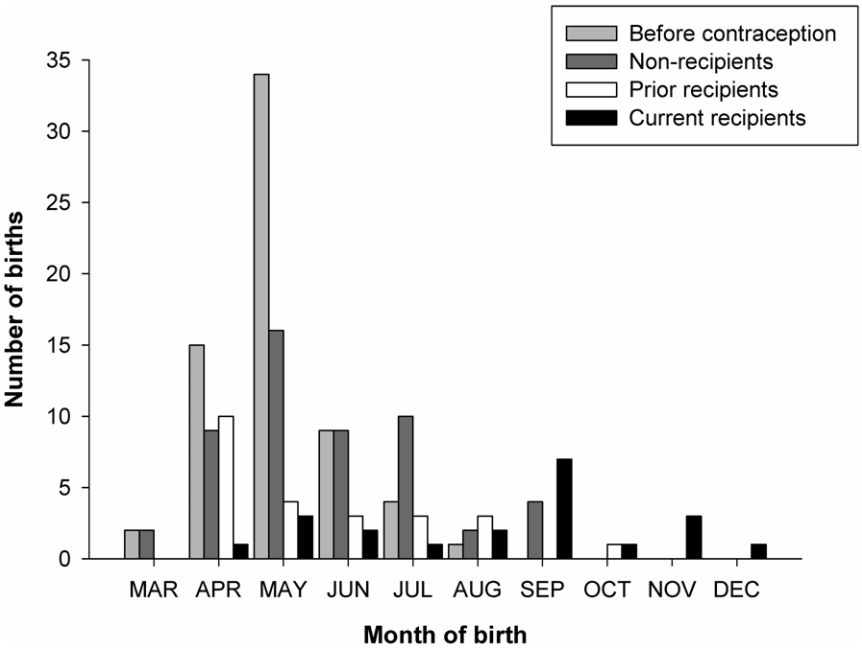


Figure 1. The distribution of births for mares on Shackleford Banks, NC, pre-contraception and post-contraception management. Mares gave birth over a wider range of months after the onset of contraception; this effect was more pronounced in PZP recipients than non-recipients.
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mate = 0.05, SE = 0.03, t = 1.83, $P = 0.07$). In general, mares gave birth 0.05 months earlier for every year of age (estimate = −0.05, SE = 0.02, t = −2.03, $P = 0.05$).

Among mares treated with PZP, those receiving a higher total number of applications foaled later in the season than did those receiving fewer applications, with each additional application associated with a 0.5 month delay in foaling date (Linear Mixed Effects Model: estimate = 0.55, SE = 0.21, t = 2.61, $r^2 = 0.65$, $P = 0.01$, see Fig. 2A). In addition, each consecutive PZP application was associated with a 0.8 month delay in foaling date, on average (Linear Mixed Effects Model: estimate = 0.83, SE = 0.23, t = 3.64, $r^2 = 0.65$, $P<0.0008$, see Fig. 2B). While the consecutive and total number of PZP applications were highly correlated, comparing the AICc between the prior two models suggested that the number of consecutive PZP treatments more accurately predicted month of birth than did the total number of PZP treatments (AICc total = 202.96, AICc consecutive = 196.83). The number of years that mares did not conceive (total or consecutive), however, did not correlate with foaling date (Linear Mixed Effects Model: total years: estimate = 0.31, SE = 0.26, t = 1.19, $r^2 = 0.57$, $P = 0.24$; consecutive years: estimate = 0.26, SE = 0.28, t = 0.92, $r^2 = 0.44$, $P = 0.36$). It is unlikely that these patterns resulted from a general trend across years, as birth dates among non-recipient animals did not change with calendar year (Linear Mixed Effects Model: $r^2 = 0.17$, estimate = 0.05, SE = 0.09, t = 0.57, $P = 0.57$).

Mare Condition

A generalized linear model, which controlled for mare age, showed that among post-contraception animals, recipient mares (both current and prior) were in better physical condition, with rump scores approximately 0.4 points higher than those of non-recipients (overall model (compared to null model): Chi Square = 7.74, $P = 0.05$; PZP treatment: estimate = 0.38, SE = 0.15, Chi Square = 6.13, $P = 0.01$; age: estimate = −0.005, SE = 0.06, Chi Square = 0.007, $P = 0.93$; PZP treatment × age: estimate = −0.02, SE = 0.06, Chi Square = 0.13, $P = 0.72$).

Weather

The best fit model of monthly mean temperature showed that overall temperatures were approximately 0.6°Cwarmer after the onset of contraception management (see Fig. 3A; overall model: Likelihood ratio (compared to null model) = 358.40, $P<0.0001$, generalized $r^2 = 0.88$; month: estimate = −8.49, SE = −.97, t = −8.78, $P<0.0001$; $month^2$: estimate = 3.88, SE = 0.29, t = 13.43, $P<0.0001$; $month^3$: estimate = −0.45, SE = 0.03, t = −13.70, $P<0.0001$; $month^4$: estimate = 0.015, SE = 0.001, t = 12.20, $P<0.0001$; contraception management (before): estimate = 0.58, SE = 0.27, t = −2.13, $P = 0.06$). The best fit model of monthly precipitation showed no overall differences between the periods before and after contraception management, but there were significant interactions between month of the year and the periods before and after contraception (see Fig. 3B; overall model: Likelihood ratio (compared to null model) = 38.43, $P<0.0001$, generalized $r^2 = 0.20$, $P<0.0001$; month: estimate = −11.19, SE = 4.96, t = −2.25, $P = 0.03$; $month^2$: estimate = 3.66, SE = 1.46, t = 2.51, $P = 0.01$; $month^3$: estimate = −0.38, SE = 0.17, t = −2.27, $P = 0.02$; $month^4$: estimate = −0.01, SE = 0.006, t = 1.88, $P = 0.06$; contraception management (before): estimate = 6.99, SE = 4.22, t = 1.66, $P = 0.12$; month × contraception management (before): estimate = −2.97, SE = 1.43, t = −2.07, $P = 0.04$; $month^2$ × contraception management (before): estimate = 0.22, SE = 0.11, t = −2.03, $P = 0.04$).

PZP Efficacy

We defined PZP efficacy during the year of administration as the number of vaccinated mares that did not became pregnant divided by the total number receiving the vaccine. Across the first four consecutive PZP applications, this efficacy declined from 97% to 87%, returning to 100% after five or more consecutive applications (see Fig. 4). A generalized mixed effects model shows that this pattern

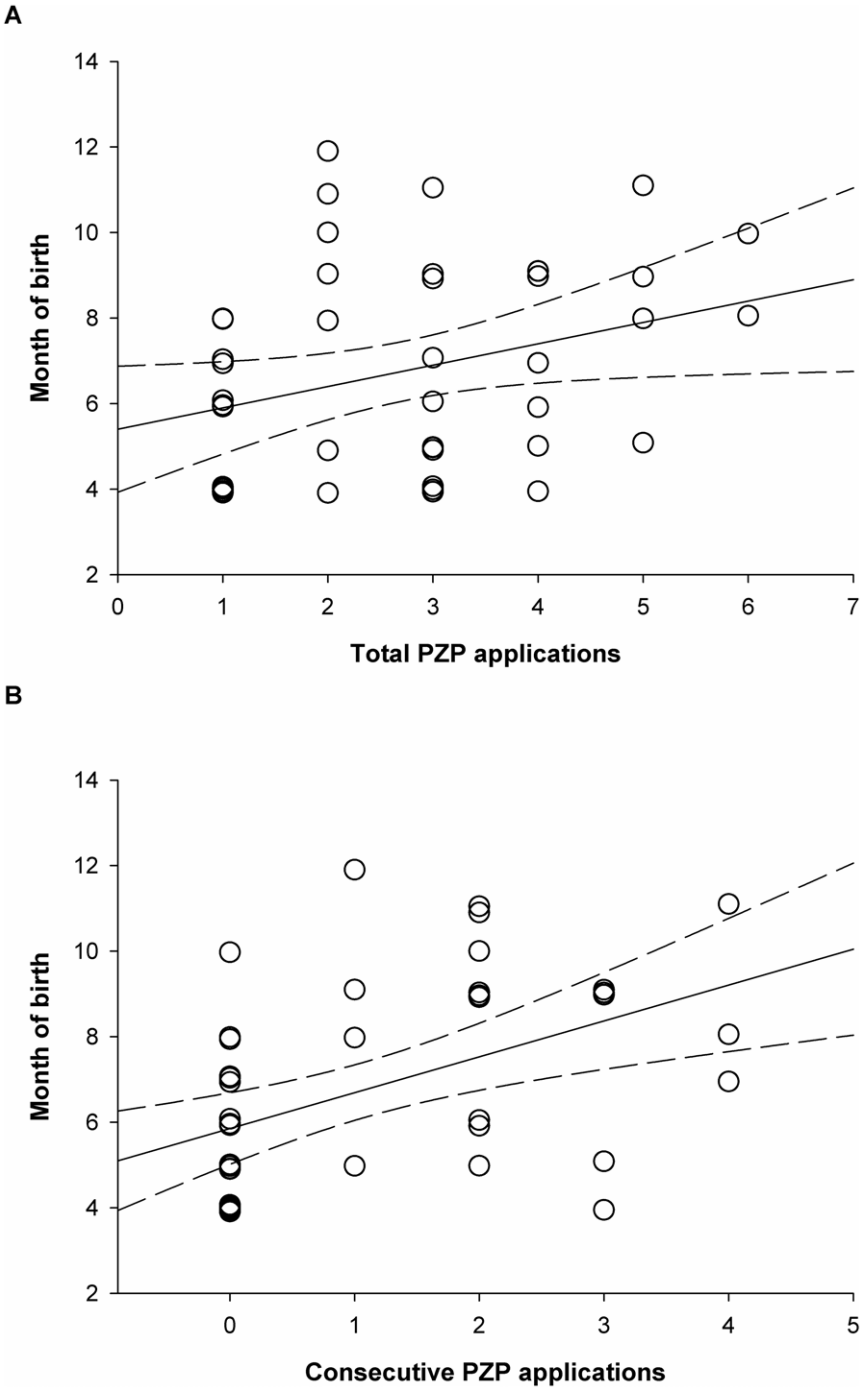


Figure 2. Birth month and number of A) total PZP applications, and B) consecutive PZP applications. In the events of ties, month of birth has been jittered by 0.2 years to allow clear visualization of every individual. Mares receiving more applications of PZP foaled later in the year on average than did mares receiving fewer applications. Although the number of total and consecutive applications is highly correlated, AICc suggests that the number of consecutive applications explains more of the variation in the data.
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is significant, even when controlling for mare age (overall model with binomial error distribution: Log Likelihood = −61.79, $P = 0.01$, generalized $r^2 = 0.17$; consecutive PZP applications: estimate = 2.98, SE = 1.20, z = 2.49, $P = 0.01$; $(\text{consecutive PZP applications})^2$: estimate = −0.51, SE = 0.22, z = −2.33, $P = 0.02$; age at first PZP application: estimate = 0.10, SE = 0.05, z = 1.84, $P = 0.07$). Prior research has shown that five to seven years of consecutive PZP treatment can be associated with ovulation failure [4]. The present dataset is consistent with this result, as no mare receiving the vaccine for five or more consecutive years became pregnant.

Discussion

Here we show that PZP recipients exhibited a change in their reproductive schedule: recipient mares gave birth over a broader time period than did non-recipients, with current recipients giving

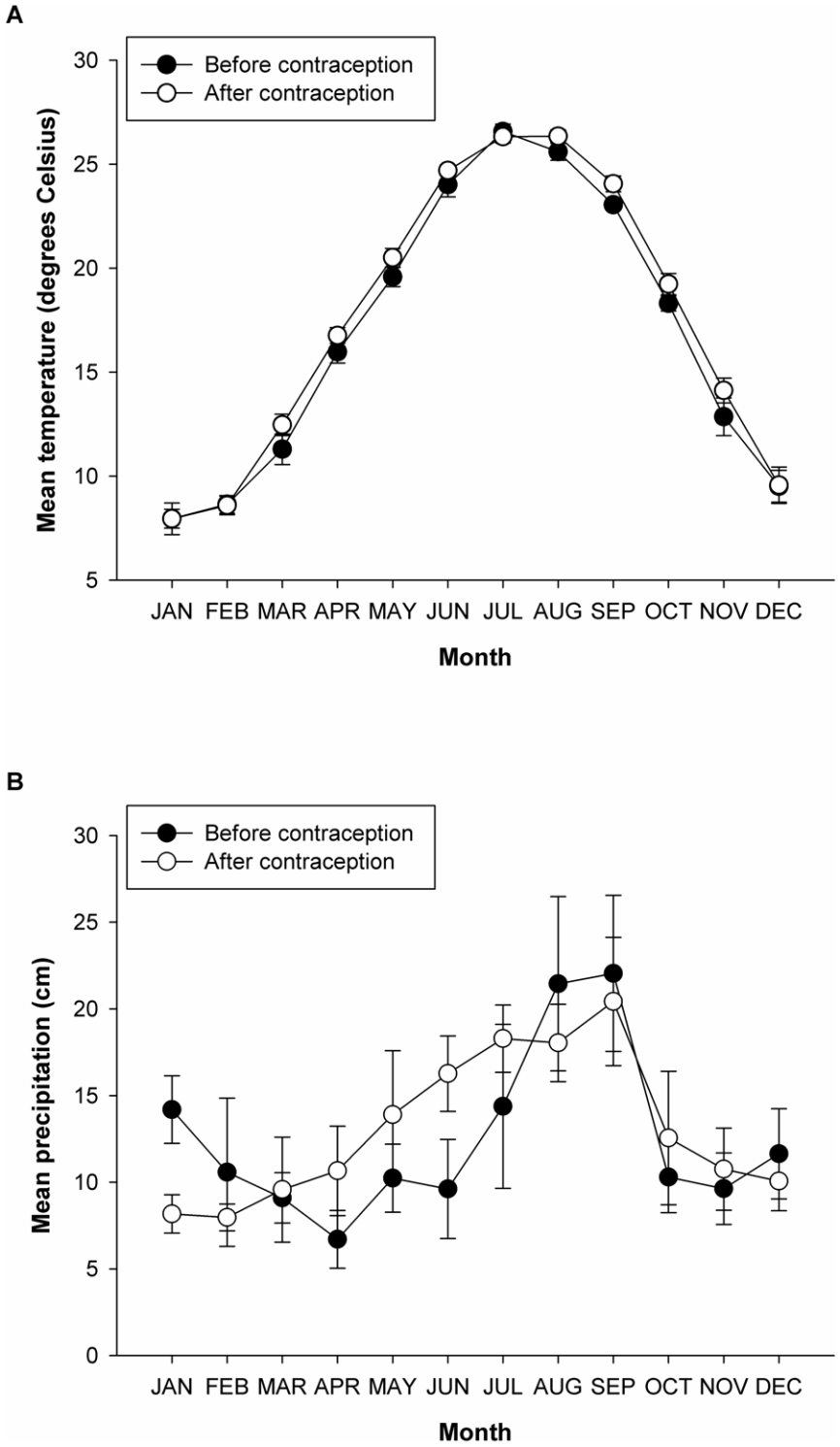


Figure 3. Weather data for six years pre-contraception (1995–2000) and eight years post-contraception (2001–2008) management. Data were collected from Morehead City, North Carolina, approximately 8 km from the study site (Shackleford Banks, North Carolina). Temperatures (A) were marginally warmer post-contraception than they had been pre-contraception. Overall rainfall (B) did not differ before and after contraception, though the seasonal patterns were marginally different pre- and post-contraception.
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birth later in the year than prior recipient and non-recipient mares. Given that gestation in wild horses lasts approximately 11 to 12 months [30], this change indicates a corresponding change in the schedule of ovulatory cycling. Contraception with porcine zona pellucida is popular amongst managers specifically because it effectively reduces the odds of conception without the application of exogenous steroids [2]. Long-term studies on Assateague Island have reported that PZP has little to no effect on reproductive

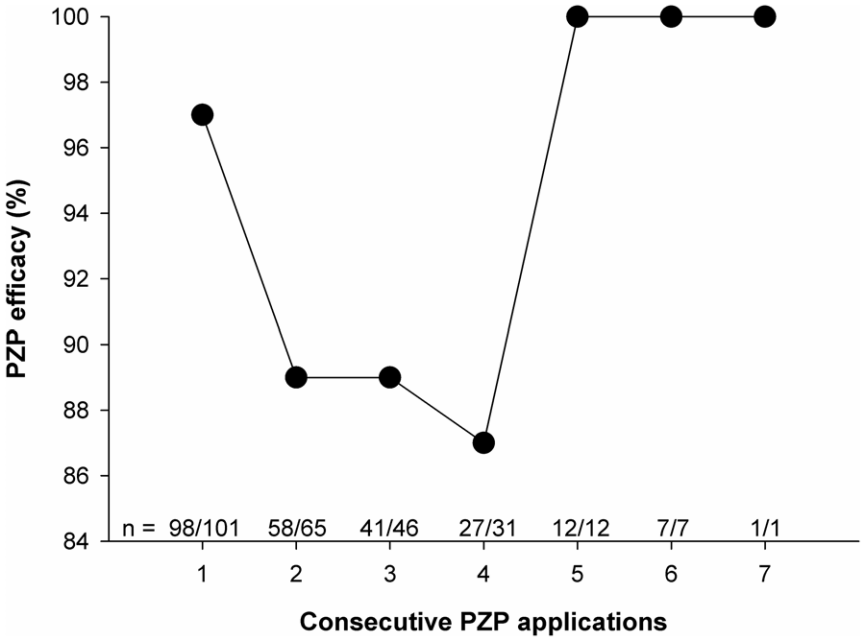


Figure 4. PZP efficacy and number of consecutive PZP applications. PZP efficacy was defined as the number of recipient mares that did not become pregnant divided by the total number of mares receiving the vaccine. Across the first four consecutive applications, PZP efficacy declined, returning to 100% after five or more consecutive applications (5–7 applications have been shown to result in ovulation failure and decreased oestrogen levels [5,40]).
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hormone levels, the schedule of reproductive cycling, or the social behaviors of recipient animals [4]. However, studies in other wild horse populations have shown that recipient mares both initiate and receive more instances of reproductive behavior during both the breeding [39] and non-breeding seasons [9]. This study provides the first evidence that mares treated with PZP can extend ovulatory cycling beyond the normal breeding season. This suggests that populations of wild ungulates can vary in their response to similar contraceptive treatment. Careful consideration of baseline population dynamics should be made prior to treatment in order to fully assess possible PZP effects.

Foaling Date

Mares receiving PZP at any point during their lifetime gave birth over a broader time period than did non-recipient animals. This larger variance among PZP mares is likely driven by the fact that current recipients gave birth later than did prior recipients (see Results, Fig. 1). Moreover, mares receiving more consecutive applications foaled later in the season than did mares receiving fewer applications. Increases in the average interbirth interval for recipient mares did not seem to be driving this result, as foaling date was not affected by the number of years (cumulative or consecutive) that mares failed to conceive. This discrepancy may be due to high variability in the conception and foaling dates of treated mares. First, it is less likely that an animal vaccinated with PZP will conceive at all, thus reducing sample size. Second, due to contraceptive failure, some treated mares will conceive during the normal breeding season, further increasing variability. Interestingly, prior to the application of PZP, the average month of birth did tend to increase with interbirth interval (Linear Mixed Effects Model: estimate = 0.30, SE = 0.17, t = 1.82, $r^2 = 0.06$, $P = 0.07$) [22], demonstrating at least some plasticity in the scheduling of reproductive cycling in Shackleford mares. On Assateague, PZP recipients experience normal reproductive cycling and mate at rates similar to non-recipients [40]. However, when such behavior fails to result in conception over several years, it follows that individuals extending reproductive cycling will be able to achieve conception later in the year if the contraceptive effects of PZP have decreased sufficiently [28,29].

Because feral horses are highly social, such changes can have cascading effects on other group members and throughout the population. Our research has shown that after contraception management, PZP recipients both attract and initiate more instances of reproductive behavior [9] and are more often the harem male's nearest neighbor during the fall/winter (Nuñez, unpublished data), indicating that group spreads are reduced. Such changes represent an increase in energy expenditure and a potential decrease in nutrient intake during a time of year when sufficient energy reserves are at a premium [27]. Moreover, early foal development in unmanaged populations typically occurs during the spring and summer when resources are plentiful [11,27]. Offspring born in the fall/winter months face nutritional and thermoregulatory challenges not experienced by their counterparts born during the normal foaling season, potentially making developmental benchmarks difficult to achieve [27].

Such predictions are not consistent with data from Assateague Island where mares show increased survival, only minimal physiological side effects, and no behavioral or demographic changes [4,5,6]. In addition, foal survival does not differ between foals born in or out of the normal foaling season [41]. However, on Shackleford Banks, recipient mares change groups more often, elicit and receive more instances of reproductive behavior, and receive more harassment from harem males [9,42]. Given these differences in mare response to PZP management in the two populations, it follows that predictions based on the data from one site are not necessarily applicable to the other.

These population differences may be due to the scheduling of PZP administration at the two sites. When the contraception

program on Assateague began in 1994, the priorities for treatment followed a hierarchical approach based on the previous breeding success of the population, ensuring that all mares were given an opportunity to reproduce [3]. Females for which there was a high priority for treatment included those that had produced at least one surviving offspring. Low priority females included those that were less than four years of age. Females greater than four years old that had not produced surviving offspring did not receive treatment. In addition, the plan stipulated that only mares that had produced at least three surviving offspring or two generations of offspring would receive more than three consecutive years of treatment. Foals were not to be removed as removal increases a mare's reproductive success in the subsequent year [43,44,45]. Finally, it was recognized that this plan was subject to change as the population numbers decreased [46]. In the present study, Shackleford mares were contracepted between 1.5 and 2 years of age and received an average of 3.4 ± 0.2 (mean $\pm$ standard error) consecutive years of contraception, regardless of their productivity. To further control population numbers, foals born to these mares (due to contraception failure or changes in the application schedule), were likely to be removed. This difference in PZP administration and subsequent discrepancy in early life experience may contribute to the behavioral differences between the populations, as the ability to conceive with a harem male is likely critical to establishing lasting harem fidelity [16] and the retention of foals (until at least two years of age) is important to maintaining normal reproductive function [43,44,45].

Possible Mechanisms

Although the effect was more pronounced in recipients of PZP, both recipients and non-recipients showed a wider range of foaling dates after contraception management (after 2001). While relatively rare, such extended periods of estrous have been documented in several equine species. Tropical species, for example, have been observed to reproduce throughout the year [12,13,47]. Similarly, studies of temperate species have shown that individuals can vary significantly in reproductive timing [14] and estrous behaviors during the non-breeding season [15]. Our data show that Shackleford mares exhibit at least some plasticity in their reproductive cycling. This plasticity enables mares to time their reproductive cycling according to ecological, sociological, and physiological cues.

For example, our results show that the reproductive changes exhibited by Shackleford mares correlate with warmer temperatures occurring later in the calendar year, after contraception management. Increases in rainfall late in the breeding season also correlate (albeit weakly) with later births. Both warmer temperatures and increased rainfall could result in higher resource availability [27] and afford females the additional reserves necessary to extend reproductive cycling into what is typically the non-breeding season.

The physical condition of mares may also play an important role in the extension of reproductive cycling. On Shackleford Banks, recipient mares are currently in better physical condition than are non-recipients. This is likely due to the fact that successfully contracepted mares are unconstrained by the costs of pregnancy and lactation [48]. Recipient mares will therefore have more resources to allocate to additional reproductive cycles. This effect of PZP, coupled with warmer temperatures occurring later in the year, may act to increase a mare's chances of conceiving later in the calendar year, if PZP antibody titers are sufficiently low [29].

Additionally, extended cycling in non-recipient mares could be influenced by the physiology and behavior of recipients. Shackleford males exhibit higher rates of sexual behavior towards recipient females during both breeding and non-breeding seasons [9,42]. These overt social stimuli may entrain some non-recipients to continue reproductive behaviors and cycling into the early fall. Such stimuli are commonly used to induce receptivity in several domestic species including horses [49], pigs [50], and cows [51]. In the wild, courtship signals from conspecifics advance gonadal cycles or maturation in several taxa, including mammals [52,53,54], birds [55], amphibians [56], and reptiles [57]. Given the importance of social cues in the timing of reproduction among such diverse species, this possibility warrants further investigation in Shackleford mares.

Finally, the declining efficacy of PZP with increased consecutive applications is likely a contributing factor to the later foaling dates of recipient mares. Lyda and colleagues' research with captive, wild mares has shown that antibody titers against PZP remain high for up to ten months after initial treatment [28]. In addition, research with both Shackleford and Assateague horses has shown that initial applications of PZP are often effective over multiple years [9,58], suggesting that antibody titers can remain high for longer. However, laboratory research has shown considerable variability in anti-PZP titers [29], as did Lyda and colleagues' work in which half the mares treated with PZP and Freund's Complete Adjuvant fell below contraceptive levels within the ten months of study [28]. Our data show that increasing the number of consecutive applications can reduce the single year efficacy of PZP by roughly 10%, indicating that either antibody titer or reactivity can decrease more rapidly with consecutive applications. Such patterns could result from the induction of immunological tolerance [59], which reduces responsiveness to self-tissues or repeatedly encountered, non-pathogenic antigens [60]. PZP is designed to mimic host tissue and induce an immune response against self tissue: the recipient's own zona pellucida [2]. As such, it seems reasonable that at least some animals would mount tolerance mechanisms to combat this autoimmunity. In addition, the repeated application of a specific antigen generates an antibody response that is increasingly more specific to that particular antigen [29]. The antibodies produced by mares against porcine zona pellucida should, therefore, become less cross reactive with horse zona pellucida over time. Of course, PZP efficacy will vary depending on mare age and timing of inoculation [61]. Regardless, if PZP recipients extend reproductive cycling and behavior into the non-breeding season, any decrease in efficacy that leaves them fertile in the fall/winter will help drive increases in late season conception.

Although the removal of offspring can induce estrous cycling in ungulate species [62], it is unlikely that the removal of foals has influenced foaling date among PZP-treated mares on Shackleford Banks. Thirty-nine foals (conceived due to contraception failure or administration scheduling) have been removed from the island. Approximately 55% of these foals were born to non-recipient animals. The majority of foal removals were conducted in the January following foal births. Given that non-recipient animals did not give birth later than September and most recipient animals gave birth before December, it is unlikely that foal removals in January induced late-season estrus in Shackleford mares. It is equally unlikely that increases in mare condition due to the alleviation of lactation costs resulted in early resumption of estrus the following spring [27]. If that were the case, during the early spring months we would expect to see an increase in the number of foals born to mares subjected to offspring removal. This is not borne out by the data. Still, the removal of foals is ill-advised as it increases mare fecundity the following year [43,44,45].

Management Implications

When the alternative (gather and removal) is considered, PZP is currently managers' most humane and effective option for population control. However, careful study of the animals' demography, physiology, and behavior is necessary prior to and during treatment to ensure that a) the potential effects of PZP can be assessed accurately, and b) within managerial constraints, unintended effects of PZP are ameliorated. Differences in habitat, resource availability, and demography among conspecific populations will undoubtedly affect their physiological and behavioral responses to PZP contraception, and need to be considered. For instance, while Assateague horses show no behavioral and only minor physiological responses to PZP, horses on Shackleford Banks [9,42] and in the western United States [39] alter social and reproductive behaviors in response to PZP. Our data suggest that mare condition and warming trends may present additional complications. Increases in physical condition and changes in average temperature may interact with management regimes, enabling mares to alter their reproductive physiology even further. Moreover, these data emphasize the importance of study during both the breeding and non-breeding seasons. Much of the research showing little to no effect of PZP on feral horse behavior or physiology has been performed exclusively during the breeding season [4,5,10], potentially missing important differences in recipient response.

If population numbers are managers' primary concern, our data show that giving five or more consecutive applications of PZP will result in 100% contraception efficacy. This is consistent with data from Assateague where mares receiving 5–7 consecutive PZP applications exhibited ovulation failure and decreased urinary oestrogen concentrations [5,40]. However, if managers are tasked with the maintenance of natural behaviors and foaling schedules, consecutive PZP applications should be avoided. Research has shown that one application of PZP is often effective over multiple years, exhibiting yearly efficacy declines similar to that of 2–4 consecutive treatments (on Shackleford) [9,58]. Our data show that current recipients gave birth later than both prior recipient and non-recipient animals. However, prior recipients of PZP gave birth on schedules similar to non-recipients, suggesting that breaks between treatments can ameliorate unintended behavioral and physiological changes in recipient animals. Contraception on such schedules will still maintain lower pregnancy rates, but will allow for the birth of a manageable number of offspring which are also important to the maintenance of normal behaviors [9]. These foals should be allowed to remain in the population for at least two years as earlier removal has been shown to increase a mare's reproductive success in the subsequent year [43,44,45]. Additionally, subadult, dispersing females should be allowed to settle into harems and have at least one foal before receiving contraception [16]. Management regimes such as this would of course necessitate a higher minimum population level. Additional research is needed to determine whether these larger, but still limited population sizes could achieve management goals. If so, this could prove a cost-effective means of controlling animal numbers while maintaining their natural physiology and behavior.

The broader implications of this research are considerable. As this study suggests, the physiological and behavioral effects of PZP are not fully understood. Still, the vaccine is currently administered to many different species including white-tailed deer, *Odocoileus virginianus* [7], elk, *Cervus elaphus* [8], black bears *Ursus americanus* [63], and African elephants, *Loxodonta Africana* [64]. As with conspecific equid populations, habitat, resource, and demographic differences among species will affect their responses to PZP contraception and need to be considered. For social species like the horse, a proper balance between managing population size and maintaining a more natural physiological and behavioral regime is particularly important.

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Author Contributions

Conceived and designed the experiments: CMVN. Performed the experiments: CMVN. Analyzed the data: CMVN JSA. Wrote the paper: CMVN JSA DIR.

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Linking social environment and stress physiology in feral mares (*Equus caballus*): Group transfers elevate fecal cortisol levels

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ABSTRACT

Feral horses (*Equus caballus*) have a complex social structure, the stability of which is important to their overall health. Behavioral and demographic research has shown that decreases in group (or band) stability reduce female fitness, but the potential effects on the physiological stress response have not been demonstrated. To fully understand how band stability affects group-member fitness, we need to understand not only behavioral and demographic, but also physiological consequences of decreases to that stability. We studied group changes in feral mares (an activity that induces instability, including both male and female aggression) on Shackleford Banks, NC. We found that mares in the midst of changing groups exhibit increased fecal cortisol levels. In addition, mares making more group transfers show higher levels of cortisol two weeks post-behavior. These results offer insights into how social instability is integrated into an animal's physiological phenotype. In addition, our results have important implications for feral horse management. On Shackleford Banks, mares contracepted with porcine zona pellucida (PZP) make approximately 10 times as many group changes as do untreated mares. Such animals may therefore be at higher risk of chronic stress. These results support the growing consensus that links between behavior and physiological stress must be taken into account when managing for healthy, functional populations.
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1. Introduction

Social mammals typically organize themselves into coherent groups that feed, rest, and travel together (Alexander, 1974). Social living affords several benefits, but can also accrue costs, including increased competition for resources (Sterck et al., 1997), exposure to parasites and disease (Côté and Poulin, 1995), and stress (Sapolsky, 1983). Social interactions, dominance rank, and variations in population density due to territorial intrusion, predation risk, and food availability are all associated with increases in glucocorticoid levels in several vertebrate species (Creel et al., 2013).

The mammalian stress response occurs when adverse situations trigger the adrenal glands, resulting in increased secretion of glucocorticoids and/or catecholamines (Mostl and Palme, 2002). Short-lived stressful situations are commonplace for a wide range of species (Sapolsky, 2005), and the stress response is adaptive in that it helps organisms escape these dangerous or otherwise taxing situations (Mostl and Palme, 2002; Sapolsky, 2005). For example, increases in glucocorticoids in response to adverse conditions can facilitate facultative migration (Ramenofsky et al., 2012), stimulate immune responses (Martin, 2009), and regulate food intake (Wingfield et al., 1998). When experienced chronically, however, the stress response can become pathogenic (Sapolsky, 2005). Animals that are more persistently stressed exhibit higher basal glucocorticoid concentrations (but see Dickens et al. (2009)), enlarged adrenal glands, and impaired sensitivity of the adrenal system to regulation by negative feedbacks (Sapolsky, 2005). Continued stress can also adversely affect cardiovascular function, inhibit reproduction, compromise immune function, and result in a number of adverse neurobiological effects including decreased neurogenesis, dendritic atrophy, and diminishing synaptic plasticity in the hippocampus (Sapolsky, 2005).

Feral horses (*Equus caballus*) have a complex social structure: social groups, or bands, typically consist of one (though sometimes more than one) male(s), one to several female(s), and their offspring. Excepting dispersal of both male and female offspring, stable bands will remain together for several years (Klingel, 1975). Mares will often remain in the same band for most of their adult lives and will form close associations with one another (Cameron et al., 2009). Reductions in band stability have been correlated with decreases in mare fitness, including increases in parasite load, and

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declines in body condition, fecundity, foal survival, and time spent engaged in preferred behaviors (Klingel, 1975; Linklater et al., 1999; Rubenstein, 1986). Such decreases in stability, whether due to increased male and/or female harassment (including increases in aggressive and/or reproductive behaviors) or the addition/subtraction of various group members, could induce a physiological stress response in mares, especially if stability is not restored. However, the potential effects of such behavior on mare stress physiology have not been documented. Such studies are important as they give managers and researchers a method to more quantitatively measure the physiological effects of behavioral stressors on individual health (Wikelski and Cooke, 2006). In addition, a greater understanding of the physiological responses to social instability may shed light on how the social milieu affects overall female fitness.

Here we seek to elucidate the links between social behavior and physiology in a highly social ungulate, the feral horse. Specifically, we investigate the effects of band transfers on mare stress physiology via fecal cortisol concentrations, a demonstrated indicator of individual stress level (Mostl and Palme, 2002; Wasser et al., 2000). In addition, we investigate the more general effect that increased frequency of band transfer has on mare stress physiology.

2. Materials and methods

2.1. Study area

We conducted this study on Shackleford Banks, a barrier island located approximately 3 km off the coast of North Carolina, USA. The island is 15 km in length, and varies between 0.5 and 3 km in width. The specific study area extended approximately 7.7 km from the center to the eastern end of the island.

The horse population on Shackleford Banks has been co-managed by the National Park Service and the Foundation for Shackleford Horses since 1996. The Shackleford Banks Wild Horses Protection Act stipulated that the Shackleford population consist of no less than 100 and no more than 110 horses (United States Congress, 1997), similar to the population occurring in 1981 (104 horses) (Rubenstein, 1981; Rubenstein and Dobson, 2000). At the time of this study, the population consisted of 124 and 118 horses during the breeding and non-breeding seasons, respectively. The National Park Service and the Foundation for Shackelford Horses maintain the population at this level through contraceptive management with porcine zona pellucida (PZP).

2.2. Study subjects

The social groups of Shackleford horses are typical of feral equids. They consist of coherent bands of one or sometimes two or three stallion(s) with one to several mare(s) and their offspring. Although the bands are predominantly non-territorial and the animals move freely within overlapping home ranges (Rubenstein, 1981), individual bands are spatially distinct from one another and individuals of particular bands rarely interact (Feist and McCullough, 1976; Rubenstein, 1981, 1986). When interactions do occur, they typically involve younger individuals engaging in play and/or exploration, the dispersal of sub-adult individuals (both male and female), and the transfer of adult females from one band to another (personal observation).

Historically, the bands on Shackleford Banks were long-lasting with most changes involving the dispersal of immature individuals (Nuñez, 2000; Rubenstein, 1981). Males sometimes fought to acquire mares from other groups, but stallions almost always retained their mares (Nuñez, 2000; Rubenstein, 1981). During a five-year study (Rubenstein, 1981), only 10.8% of the mares studied transferred groups, as is typical in other feral horse populations (Berger, 1977; Feist and McCullough, 1976). More recently, mares treated with PZP contraception have been shown to change groups more often, making approximately 10 times more group changes than untreated mares and visiting up to 5 times as many groups (Madosky et al., 2010; Nuñez et al., 2009). During a 2.5-month study in 2008, 44% of the mares studied (in a population of 121 horses) transferred groups. Group changes typically involve aggressive herds, chases, and increased reproductive activity by both the band and new stallions, and/or aggression from the new females (personal observation). Population densities at the time of the study were equivalent to those of the 1981 population (see Section 2.1), indicating that these increases in group changing behavior are not due to significant increases in encounter rates between/among bands. All of the mares in this study received PZP at some point during their lifetime (Stuska, 2000–2010) also, see Table 1.

2.3. Animal welfare

All sampling was conducted in accordance with National Research Council standards (National Research Council, 2011). Given the non-invasive nature of this study, neither the Princeton University Institutional Animal Care and Use Committee nor the National Park Service deemed permitting necessary.

2.4. Behavioral and demographic sampling

This study was conducted by three observers during the breeding season (June–August, 2009, J. Smith, J.J. Schurle, and C.M.V. Nuñez) and two observers during the non-breeding season (December, 2009, C.M.V. Nuñez and J.S. Adelman) totaling over 181 hours of behavioral observation (84.67 h, breeding season; 96.75 h, non-breeding season). Horses were identified individually by color, sex, age, physical condition, and other distinguishing markings, including freeze brands. Ages are known from long-term records for the identified horses of Shackleford Banks (Rubenstein and Nuñez, 2009) and from National Park Service data (S. Stuska, unpublished data).

Table 1
Contraception history for mares from 2000–2009.

Mare ID	Total number of years treated	Year last treated	Treatment status for 2009
Bo	2	2005	Untreated
Carrot	1	2009	Treated
Daisy	8	2009	Treated
Darcy	4	2005	Untreated
Donoma	2	2009	Treated
Dusty	6	2007	Untreated
Hardee	5	2009	Treated
Helena	6	2008	Untreated
Hercules	1	2009	Treated
Hezakiah	7	2007	Untreated
Jaquincoke	5	2006	Untreated
Kelty	1	2009	Treated
Larissa	4	2006	Untreated
Liani	4	2009	Treated
Noir	2	2009	Treated
Penelope	4	2005	Untreated
Sadie	8	2009	Treated
Sawathu	2	2009	Treated
Serenac	7	2009	Treated
Shag	5	2009	Treated
Slug	7	2008	Untreated
Texas	6	2006	Untreated
Wallace	1	2009	Treated
Zelda	2	2004	Untreated

Table 2
Breakdown of sample sizes for entire study.

	Breeding (Br)	Non-breeding (NBr)	Total
Fecal samples meeting inclusion criteria	90	36	126
No. unique mares with fecal and behavioral samples	8 in Br only 12 in both seasons	4 in NBr only	24
Fecal samples/mare mean ± 1SE (range)	4.50 ± 0.84 (1–13)	2.25 ± 0.42 (1–6)	5.28 ± 0.81 (1–14)
No. unique mares changing groups	10 in Br only 3 in both seasons	3 in NBr only	16
Group changes/mare mean ± 1SE (range)	2.30 ± 0.32 (1–4)	1.50 ± 0.34 (1–3)	2.43 ± 0.29 (1–4)

We located each study group an average of 1.76 and 1.54 times per week in the breeding and non-breeding seasons, respectively. We recorded each group's GPS location and composition, noting the presence or absence of individual mares. We observed a total of 24 mares (see Table 2). Twelve mares were observed in both seasons; the remaining mares were observed in either the breeding ($n = 8$) or non-breeding ($n = 4$) seasons, representing 53% and 40% of the study population, respectively. Of these, 16 made group transfers (breeding season, $n = 13$ mares; non-breeding season, $n = 6$ mares).

Mare transfer activity was rarely witnessed directly (breeding season, $n = 1$; non-breeding season, $n = 1$); therefore, mare absence from a band was an important metric with which to measure the number of transfers between groups. We remained with each group for a minimum of 30 min to ensure that individuals recorded as absent were not actually nearby, but out of our sight. Transfer behavior was confirmed by the mares' presence in new bands, usually within 1–7 days of mare absence (breeding season, $n = 28/30$; non-breeding season, $n = 8/9$). The remaining instances were confirmed within 16 and 8 days in the breeding non-breeding seasons, respectively.

2.5. Non-invasive hormonal sampling

Fecal samples were collected *ad libitum* (Altmann, 1974). We only collected samples when we were certain of the mares' identity and the location on the ground. Fecal samples were collected within minutes of defecation and stored in 20 ml vials at a 2.5:1 ratio of 95% ethanol to feces (Khan et al., 2002). Samples were stored in a cooler (from 1–5 days) until they could be frozen at −20 °C (June–August) or frozen at −20 °C on the day of collection (December).

Three of the aforementioned observers (J. Smith, C.M.V. Nuñez, and J.S. Adelman) collected fecal samples. We collected samples from a total of 24 mares (see Section 2.4 and Table 2). Limitations in staff, travel logistics, and the rates of mare defecation precluded us from sampling all mares in the study area. A total of 126 samples met our timing criteria and were included in our analyses (breeding season, $n = 90$; non-breeding season $n = 36$). Mares contributed a total of 4.50 ± 0.84 (range = 1–13) and 2.25 ± 0.42 (range = 1–6) samples in the breeding and non-breeding seasons, respectively (see Table 2). We collected an average of 1.52 samples (breeding season) and 2.25 samples (non-breeding season) per day.

2.6. Hormone analysis

In the laboratory, ethanol was evaporated from the samples which were then freeze-dried and sifted to remove vegetative matter. 0.2 g fecal powder was extracted into 2 ml 90% methanol and then run through a prepped Oasis cartridge for solid phase extraction (Beehner et al., 2006; Khan et al., 2002). All samples were stored at −20 °C prior to assay.

Fecal glucocorticoids were quantified using modified protocols of the Immuchem Double Antibody Corticosterone RIA Kit for Rats and Mice, MP Biomedicals, LLC, Orangeburg, NY (Beehner et al., 2006; Wasser et al., 2000), in eight separate assays. This specific method has been validated in a closely related, wild equid, the Grevy's zebra (*Equus grevyi*): the anti-corticosterone antibody detects a rise in cortisol metabolites after an individual is presented with an ACTH challenge (Franceschini et al., 2008). Moreover, fecal metabolites have been shown to increase with circulating glucocorticoids in the domestic horse (*Equus caballus*) (Merl et al., 2000; Mostl et al., 1999). We validated the assay as in Khan et al. (2002). A serial dilution of a horse fecal pool showed parallelism to the corticosterone standard curve (Linear Model; estimate = 6.30, $t = 0.70$, $P = 0.50$). Intra- and inter-assay coefficients of variation (%CV = [mean/SD] × 100) were 5.7 ± 0.6% (mean ± SE) and 9.6% for the fecal extract pool, and 7.4 ± 2.9% and 13.9% for the high concentration controls ($n = 8$). All samples were run in duplicate and any duplicate with a CV >15% was rerun. Mean assay accuracy (observed/expected × 100) was 105 ± 2.2.

Recently, concerns have been raised about the use of fecal metabolites in determining the physiological state of free-living species (Goymann, 2012). Individual, sex, seasonal, and dietary variation can significantly affect how hormones are metabolized. Here, we examine changes in fecal cortisol within individual, female, feral horses. The mares lived in similar ecological conditions (see Section 2.1), and we analyzed changes within season, thereby controlling for such potential differences.

2.7. Statistical analysis

2.7.1. Test 1

We analyzed the effects of group transfers on mare cortisol levels with Linear Mixed Effects Models in R, version 2.13.0 (R Development Core Team, 2011). For each female, cortisol level was analyzed with regards to its timing around group transfers, i.e., before, during, and after group transfers. These categories were defined as follows (also see Fig. 1):

- Before–cortisol levels exhibited before any group transfer(s) occurred (weeks 1–2).
- During–cortisol levels exhibited while the group transfer(s) were occurring (weeks 3–4).
- After–cortisol levels exhibited after the final group transfer occurred (weeks 6–10); these samples were collected a minimum of 7 days after the mares' last group transfer.

For this analysis, mares contributed 3.92 ± 0.82 samples (range = 1–10) and 3.83 ± 0.75 samples (range = 3–6) in the breeding and non-breeding seasons, respectively (also see Table 3 and Supplementary material, Fig. S1). The model included mare ID as a random effect and the following fixed effects: season (breeding/non-breeding), the timing of fecal deposition relative to the group transfer (before, during, or after), and the interaction between season and the timing of fecal deposition. We conducted this analysis in two ways.

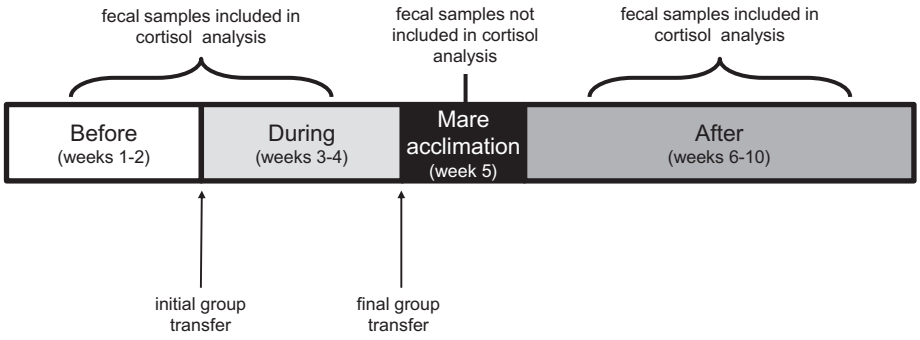


Fig. 1. Timeline for fecal sampling (Test 1).

Table 3
Breakdown of sample sizes for timing relative to group transfer analysis (Test 1, Fig. 4).

	Breeding (Br)	Non-breeding (NBr)	Total
Fecal samples meeting inclusion criteria	51	23	74
No unique mares with fecal and behavioral samples	10 in Br only 3 in both seasons	3 in NBr only	16
Fecal samples/mare mean ± 1SE (range)	3.92 ± 0.82 (1–10)	3.83 ± 0.75 (3–6)	4.63 ± 0.81 (1–11)
No unique mares changing groups	10 in Br only 3 in both seasons	3 in NBr only	16
Group changes/mare mean ± 1SE (range)	2.69 ± 0.26 (1–4)	1.16 ± 0.16 (1–2)	1.81 ± 0.26 (1–4)

- Model 1: included all mares for which appropriate samples were available; not every mare contributed to all time points ($n = 16$ mares; 74 samples; see Supplementary material, Fig. S1).
- Model 2: included only mares for which samples from all time points were available ($n = 6$ mares; 37 samples; see Supplementary material, Fig. S1). Samples from mares for all time points were obtainable during the breeding season only.

2.7.2. Test 2

Cortisol levels were also analyzed with respect to the number of group transfers females made. Samples for this analysis were collected two weeks after the final group transfer. Mares contributed 4.39 ± 0.89 samples (range = 1–13) and 1.36 ± 0.17 samples (range = 1–3) in the breeding and non-breeding seasons, respectively (also see Table 4). The model included mare ID as a random effect and the following fixed effects: season (breeding/non-breeding), the number of group transfers made prior to fecal collection, and the interaction between season and the number of group transfers.

All of the mares in this study received PZP at some point during their lifetime (Stuska, 2000–2010); therefore, tests regarding the possible effects of infertility were not possible.

3. Results

As the breeding season progressed, we detected an increase in mare transfers, with a peak in the middle of the study period, after which group transfers decreased to levels exhibited earlier in the season (see Fig. 2). 65% of the mares studied transferred groups at least one time, with 45% transferring groups 2–4 times (see Fig. 3). During the non-breeding season, we detected more group transfers earlier in the study period (see Fig. 2). 37.5% of the mares studied transferred groups, with 12.5% transferring groups 2–3 times (see Fig. 3). Subsequently, 86% and 60% of the bands observed were affected (i.e. mares left/joined the bands) during the breeding and non-breeding seasons, respectively.

3.1. Timing of group transfers and cortisol levels (Test 1)

Timing of fecal deposition relative to group transfer(s) affected cortisol levels (Linear Mixed Effects Model (LME); overall model: Likelihood ratio (compared to null model) = −290.78, $P = 0.0008$; $F_{2,53} = 3.23$, $P = 0.05$). Season did not have a significant effect on this pattern (Season: $F_{1,53} = 2.75$, $P = 0.10$; Season*Timing of fecal deposition relative to group transfer(s): $F_{2,53} = 1.23$, $P = 0.30$). On

Table 4
Breakdown of sample sizes for number of group changes analysis (Test 2, Fig. 5).

	Breeding (Br)	Non-breeding (NBr)	Total
Fecal samples meeting inclusion criteria	79	19	98
No unique mares with fecal and behavioral samples	7 in Br only 11 in both seasons	3 in NBr only	21
Fecal samples/mare mean ± 1SE (range)	4.39 ± 0.89 (1-13)	1.36 ± 0.17 (1-3)	4.67 ± 0.84 (1-14)
No unique mares changing groups	8 in Br only 2 in both seasons	2 in NBr only	12
Group changes/mare mean ± 1SE (range)	1.90 ± 0.31 (1-4)	1.50 ± 0.50 (1-3)	2.08 ± 0.31 (1-4)

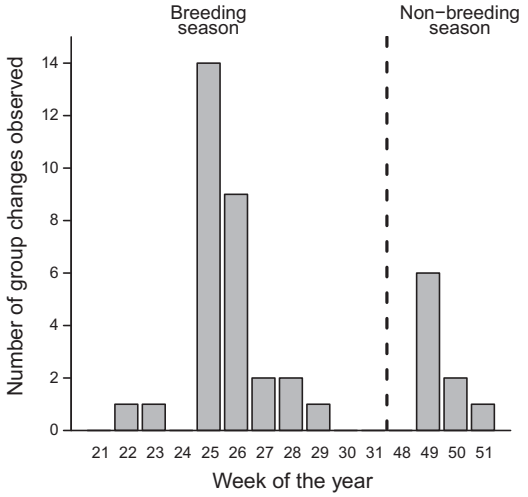


Fig. 2. Distribution of group transfers in the breeding and non-breeding seasons.

average, mares exhibited higher cortisol levels during group transfer(s) than before group transfer(s) (LME: estimate = 7.22, $t = 2.33$, $P = 0.02$; Fig. 4). Cortisol levels exhibited after group transfer(s) did

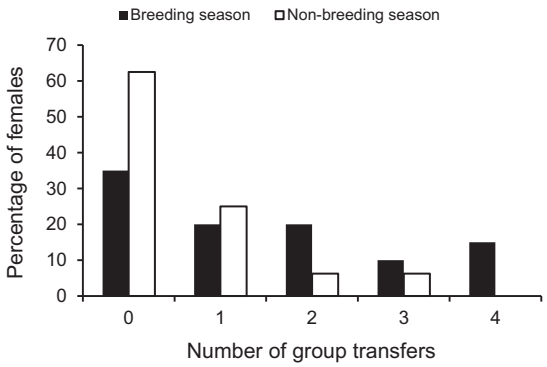


Fig. 3. Percentage of females making 0, 1, 2, 3, and 4 group transfers. During the breeding season (■), more mares transfer groups than do not; the majority of transferring mares make more than 1 transfer. During the non-breeding season (□), there is less transfer activity with fewer mares transferring groups; the majority of mares transferring groups make 1 transfer.

not differ from those exhibited before or during transfer(s) (before: LME: estimate = 1.90, $t = 0.54$, $P = 0.59$; during: F-test for linear combinations, $F_{1,56} = 2.83$, $P = 0.10$; Fig. 4).

Analysis of our second model (including only mares for which we had all samples) yielded equivalent results (Linear Mixed Effects Model (LME); overall model: Likelihood ratio (compared to null model) = −150.90, $P = 0.0003$; $F_{2,29} = 4.14$, $P = 0.05$).

3.2. Number of group transfers and cortisol levels (Test 2)

Mares engaging in this behavior more frequently showed higher cortisol levels two weeks post-transfer(s) (LME; overall model: Likelihood ratio (compared to null model) = −382.24, $P < 0.0004$; $F_{2,72} = 3.51$, $P = 0.03$). Season did not have a significant effect on this pattern (Season: $F_{1,72} = 0.92$, $P = 0.34$; Season*Timing of fecal deposition relative to group transfer(s): $F_{2,72} = 0.67$, $P = 0.51$). Two weeks after their final group transfer, mares making 2+ transfers exhibited higher cortisol levels than mares making 0 transfers (LME: estimate = 9.61, $t = 2.58$, $P = 0.01$; Fig. 5) and marginally higher cortisol levels than mares making only 1 group transfer

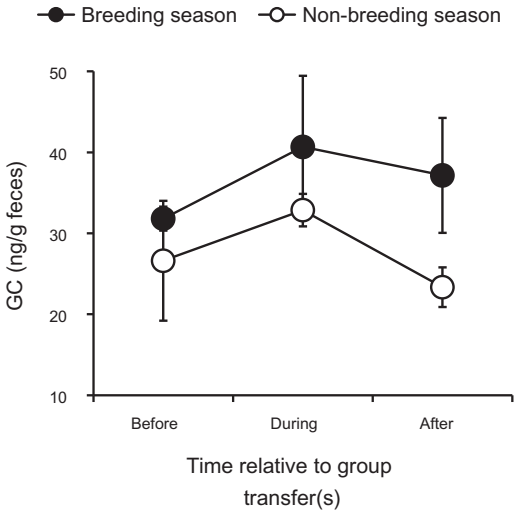


Fig. 4. Mare cortisol level and the timing relative to group transfer(s) in the breeding (●) and non-breeding (○) seasons. Mares showed increases in cortisol during group transfer behavior.

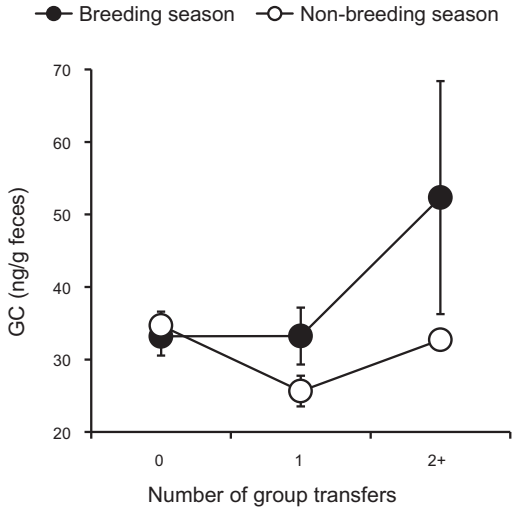


Fig. 5. Mare cortisol level 2 weeks post-group transfer(s) during the breeding (●) and non-breeding (○) seasons. Mares making more group transfers exhibited higher cortisol levels 2 weeks post-behavior than did mares making fewer group transfers.

($F_{1,73} = 3.32$, $P = 0.07$; Fig. 5). Mares making only 1 group transfer exhibited cortisol levels similar to mares making 0 changes (LME: estimate = 0.24, $t = 0.07$, $P = 0.94$; Fig. 5).

4. Discussion

Here we show for the first time that social instability has significant impacts on stress physiology in feral mares. Specifically, we show that mare cortisol levels increase during group transfers (Test 1) and that mares making more band transfers exhibit higher cortisol levels two weeks following transfer behavior (Test 2). Although we did not detect an effect of season on this pattern, the data show trends towards higher cortisol levels during the breeding season (see Figs. 4 and 5). This is not surprising as certain elements of stallion/mare aggression are elevated at this time (Stevens, 1990), but see Romero (2002). Regardless, the patterns of cortisol increase are consistent across season, indicating that the changes in mare cortisol levels are due to their group transfer behavior and not to season alone. Our results show that social

instability and the resultant behaviors, including increased reproductive interest and aggression, are integrated into a mare's physiological phenotype.

Many social changes are associated with group transfers: mares changing groups will often experience behavioral stressors in the form of harassment from both the male(s) (including herds, chases, and increased reproductive interest) and resident females (including kicks, chases, and bites) (Kearns, 2009; Madosky, 2011; Monard and Duncan, 1996; Rutberg, 1990). We cannot precisely determine which of these behaviors drive the patterns recorded in this study. However, the discrete increases in fecal cortisol occurring during group transfers indicate a direct link between the mares' social environment and their physiological response (Test 1). Moreover, the fact that higher cortisol levels are maintained for at least two weeks post-transfer behavior suggests that decreases in social stability have at least some lasting effect on mare physiology (Test 2). We know much about the effects of social environment on mare behavior and fitness (Cameron et al., 2009; Kaseda et al., 1995; Linklater et al., 1999). Our data give insights into how that social environment may be translated into fitness costs for mares via their stress physiology.

The mares in this study exhibited increases in cortisol (7.22 ng/g) comparable to the highest levels exhibited by Grevy's zebra (~10 ng/g) during captivity after capture and relocation (Franceschini et al., 2008). These data indicate that group transfer behavior incurs a significant cost to mares. Although such stress in itself is not inherently detrimental to animal fitness or well-being (Moberg, 2000), the stress response can become pathogenic when experienced chronically (Sapolsky, 2005). On Shackleford Banks, N.C., mares transferring bands more frequently are often subject to a negative feedback loop: their behavior leads to harassment which induces additional group changes, resulting in further harassment and so on (Madosky, 2011), also see Linklater et al. (1999). Results from the present study suggest that such mares are likely experiencing increased stress levels at more regular intervals, and may therefore be at higher risk of chronic stress.

Moreover, mares on Shackleford that are contracepted with porcine zona pellucida (PZP) have been shown to make up to 10 times more group transfers than untreated mares (Madosky, 2011; Nuñez et al., 2009). Direct tests of treated versus untreated mares were not possible in this study (see Section 2.2). However, mares contracepted for the first time in January 2009 and those receiving repeated applications (range = 2–8) over several years (Stuska, 2000–2010) exhibit the same increases in cortisol levels with group transfer behavior (see Supplementary material for analysis and Fig. S2). These results show that PZP treatment itself does not increase cortisol levels in recipient animals. However, changes in the frequency of group transfer behavior by consistently infertile mares (Madosky et al., 2010; Nuñez et al., 2009) may put them at higher risk of chronic stress. PZP contraception has also been conducted on Assateague Island National Seashore (Kirkpatrick, 1995), a site ecologically similar to Shackleford Banks. Currently, no systematic studies of group changing behavior with PZP treatment have been conducted there, thus limiting our ability to compare the behavior of Shackleford and Assateague mares. A study addressing potential changes to treated mares' activity budgets, aggression, and spatial relationships with the stallion on Assateague found no differences (Powell, 1999), indicating little to no effect of PZP treatment on mare behavior. However, it is worth noting that control mares in the Assateague study had been treated with PZP for three consecutive years before testing, and that different behavioral effects have been found in populations other than Shackleford Banks (Ransom et al., 2010, 2013).

Our results may seem to contrast those showing that contracepted mares (on both Assateague Island and Shackleford Banks) live longer and are in better body condition than uncontracepted mares (Kirkpatrick and Turner, 2007; Nuñez et al., 2010). However, longevity and body condition are not the only measures of animal health. High condition scores and long life can often be maintained despite cumulative changes to other physiological systems in response to recurring stressful events (McEwen and Wingfield, 2003). For example, repeated stressors can result in more chronically dysregulated glucocorticoid secretion, chronically elevated food consumption, insulin resistance, and increased deposition of fat which, in some species, can contribute to high condition scores (Leibowitz and Hoebel, 1997; McEwen and Wingfield, 2003; Sapolsky, 2005). Moreover, research with crimson finches (*Neochmia phaeton*) has shown that accepted condition measures do not reliably predict reproductive success or survival (Milenkaya, 2013), suggesting that the value of condition scores for assessing integrative animal health may be insufficient.

How then are we to understand the relevance of increased cortisol in mares that change groups more often? The benefits of lower stress environments (to animals in general) and band stability (to feral horses in particular) have been well documented. Persistently stressed animals show a range of deleterious effects (Sapolsky, 2005), and in feral horses, band instability has been associated with decreased time spent in preferred behaviors, increased offspring mortality, and increased parasite load (Kaseda et al., 1995; Linklater et al., 1999). All of these factors can be explored further; however, future studies investigating parasite burden more closely may yield the most relevant information (Rubenstein and Hohmann, 1989). Because parasites can have important effects on host fitness, such measures could reveal much about overall animal health and ability to fight off infection (Booth et al., 1993). In addition, this metric would offer managers an important, non-invasive tool with which to further quantify animal well-being.

Our results present new evidence that should be considered when evaluating management tools. We show that social stressors are integrated into an animal's physiology via increased cortisol which, when experienced at increased frequency, can increase the risk of chronic stress (Sapolsky, 2005). In addition, such behavioral changes can decrease the mares' ability to form the stable social relationships with one another that are important to decreasing overall male harassment (Cameron et al., 2009). Furthermore, given the high degree of sociality in feral horses, the behaviors of these individuals have the potential to affect the population as a whole. For example, on Shackleford, increases in reproductive behavior (by mares) in the post-breeding season has resulted in increased male attentiveness (Nuñez et al., 2009), which in turn, limits mare movement and foraging efficiency (Rubenstein, 1986; Sundaresan et al., 2007) during a time of year when the conservation of resources is of utmost priority. Similar shifts in the reproductive behavior (Ransom et al., 2010) and subsequent parturition in feral mares (Ransom et al., 2013) have been shown in the Little Book Cliffs, McCullough Peaks and Pryor Mountains populations in the western United States. In the former study, treated mares received 54.5% more reproductive behaviors than did their untreated counterparts (Ransom et al., 2010). In addition, factors that usually determine rates of reproductive behavior directed toward untreated mares (by the stallion) did not exist for treated mares. Typically, stallions engage in higher rates of reproductive behavior with mares aged 9–14 years that are more likely to produce viable offspring. This preference did not hold with treated mares; harem males showed higher rates of reproductive behavior with these females, regardless of mare age and survival probability of the subsequent offspring. In the latter study, Ransom et al. (2013) investigated the effects of prior PZP treatment(s) on the timing of parturition in feral mares after fertility was regained. Time to regain fertility ranged between 1.5–8 years and was highly dependent upon the number of treatments received. On average,

these post-treated mares exhibited peaks in parturition 31.5 days later than did untreated mares (but see Kirkpatrick and Turner (2003)). Subsequently, post-treated mares were more likely to give birth as forage was declining, resulting in fewer resources available to mares and offspring during late term pregnancy and lactation. Such cascading behavioral effects are not uncommon in social species. For example, alterations to female song preference in brown-headed cowbirds (*Molothrus ater*) induce increased solicitation of males by other females, changes in male dominance structure, and a less stable and connected social network (Maguire et al., 2013).

While the changes to feral horse behavior and physiology discussed here could help reduce population numbers, the mechanisms by which such reductions are achieved (decreased social stability and social connectedness amongst mares, increased male/female aggression) also affect the welfare of these animals. Such effects may be of limited concern when population reduction is an urgent priority. However, their consideration is vital if managers are to maintain healthy, functional populations, particularly in social species like the feral horse, in which the manipulation of individuals can have implications for the population as a whole.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2013.11.012.

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Synthesis

Consequences of porcine zona pellucida immunocontraception to feral horses

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Abstract: Porcine zona pellucida (PZP) immunocontraception was developed to provide a more humane, effective, and inexpensive method of population regulation for wildlife species. It has been used to regulate populations of several species including white tailed deer (*Odocoileus virginianus),* elk (*Cervus elaphus*), black bear (*Ursus americanus*), and the feral horse (*Equus ferus caballus*) with varying levels of success. Early studies on Assateague Island National Seashore, Maryland, USA, suggested PZP was as an ideal form of fertility control because it reduced the likelihood of conception to <10%, could be delivered remotely, was thought to be reversible, lacked debilitating physiological side effects, could not pass through the food chain, and showed minimal effects to social behaviors in a closed population of feral horses. However, research on Shackleford Banks, North Carolina, USA and on 3 western populations located in Little Brook Cliffs (Grand Junction, Colorado, USA), McCollough Peaks (east of Cody, Wyoming, USA), and the Pryor Mountains (Lovell, Wyoming, USA) has revealed behavioral and physiological side effects of long-term PZP use. When compared to untreated mares (those that have never received treatment), treated mares demonstrated decreased fidelity to the band stallion, increased and prolonged reproductive behavior, and an increased likelihood of extending reproductive cycling into the nonbreeding season. These effects were more pronounced in animals receiving more total and/or consecutive contraception treatments and can persist even after several years of treatment cessation. Finally, new data indicate that these changes to previously treated mares can affect the behavior and stress physiology of their band stallions, demonstrating the potential for the contraception of individuals to have population-level effects. These results are important to consider if we are to achieve both the effective management of feral horse populations in addition to the maintenance of their overall health and well-being.

Key words: behavior, *Equus ferus caballus*, feral horse, immunocontraception, physiology, porcine zona pellucida (PZP), reproductive, stress, treatment schedule

Feral horses (*Equus ferus caballus*) are a beloved icon of the American West. In 1971, the U.S. Congress, with the passage of the Wild Free-Roaming Horse and Burro Act (WFRHBA), designated feral horses as "wild" horses that are "living symbols of the historic and pioneer spirit of the West" (United States Congress 1971). The WFRHBA designation, the shift in public thinking that sparked it, and conflicting views on the status of feral horses as native or non-native wildlife (Weinstock et al. 2005, Mihlbachler et al. 2011), have likely contributed to the controversy surrounding feral horse management (Garrott et al. 1991). However, with few predators capable of taking feral horses (Eberhardt et al. 1982, Turner et al. 1992, Turner and Morrison 2001) and no legal hunting, unregulated feral horse populations can adversely impact both the local environment (Furbish 1990, Levin et al. 2002) and other wildlife (Ostermann-Kelm et al. 2008, Gooch et al. 2017, Hall et al. 2018) across the United States.

Feral horse population increases on Shackleford Banks, North Carolina, USA resulted in reduced vegetation, higher bird and crab diversity, and lower fish density and species richness in horse-grazed versus ungrazed marshes (Furbish and Albano 1994). In Nevada and Southern California, USA, feral horses outcompete bighorn sheep (*Ovis* spp.) and pronghorn (*Antilocapra americana*) for water; such competition can have serious implications in these desert habitats. Finally, conflict between humans and feral horse populations are particularly acute in the western United States, where the degradation of rangeland plants, soils, and water quality has been documented (Bureau of Land Management 2013).

Despite these impacts, the status of feral horses and their federal protection means

that managers have few options to regulate population numbers. Gathers, in which animals are rounded up by helicopter and either re-released or removed from the range, divide social groups (commonly referred to as bands) and decrease band stability (Kirkpatrick et al. 1997). In addition, the use of hormonal contraception (in both male and female animals) proved problematic due to the dosage needed to achieve infertility, the issue of possible consumption by non-target species, and the subsequent changes to recipient behavior (Turner and Kirkpatrick 1991).

On the other hand, immunocontraception proved easier to administer, did not pass through the food chain, and because of its mechanism of action (in comparison to hormonal contraception in which treatment-induced changes to recipient hormone levels directly affect behavior [Turner and Kirkpatrick 1991]), immunocontraceptive treatment itself caused little to no change to recipient behavior (Kirkpatrick 1990, Turner and Kirkpatrick 1991, Kirkpatrick 1995, Kirkpatrick and Turner 2002). Immunocontraception induces the production of antibodies against structural or functional molecules necessary for reproduction. These antibodies obstruct the normal cell biology of reproduction, reducing the likelihood of pregnancy (Turner and Kirkpatrick 1991).

In the case of the porcine zona pellucida (PZP) vaccine, antibodies bind sperm receptors on the egg's surface, preventing sperm attachment and fertilization over the course of 1 breeding season (Sacco 1977). Though hugely promising, initial work researching the potential effects of PZP on recipient animals did not consider the potential for behavioral effects of prolonged subfertility (Kirkpatrick and Turner 1991). Today, PZP is used with varying levels of success to control reproduction in several free-ranging ungulate species, including white-tailed deer (*Odocoileus virginianus;* McShea et al. 1997), elk (*Cervus elaphus;* Heilmann et al. 1998), and the feral horse (Kirkpatrick 1990, Turner et al. 2002).

Initially, most of the research examining the effects of PZP-induced subfertility on feral horses stemmed from 1 long-term study on Assateague Island National Seashore, Maryland, USA. Researchers reported no debilitating side effects to PZP recipients and only minor ovulation failure and depressed urinary oestrogen concentrations with repeated applications (Kirkpatrick et al. 1996). In addition, the contraceptive effects of PZP were shown to be reversible, safe for pregnant females, and did not adversely affect the survivorship or subsequent fertility of offspring born to treated individuals (Kirkpatrick and Turner 2002).

However, an important caveat was made; on average, mares receiving 4–5 consecutive treatments took 4.4 years to return to fertility (range = 1–8 years), and those receiving 7 consecutive treatments did not return to fertility during a 7-year, treatment-free interval (Kirkpatrick and Turner 2002). These results should have served as a warning to managers that more aggressive treatment could grossly affect future fertility in treated mares (potentially limiting future management options); however, such caution seems to have been taken only rarely (Ransom et al. 2013, Nuñez et al. 2017).

Early on, very little investigation was devoted to the potential for behavioral effects of PZP contraception. Powell (1999) found little to no effect; however, true controls (i.e., animals that had never been treated) were not available for comparison. More recent studies on feral horses living on Shackleford Banks and in the western United States demonstrate important differences in recipient behavior and physiology (Nuñez et al. 2009, Madosky et al. 2010, Nuñez et al. 2010, Ransom et al. 2010). For a gregarious species like the horse, such changes may have serious social and demographic consequences.

Herein, I provide a synthesis of the literature regarding the potential effects of PZP contraception on mares and band social structure. I compare the effects of PZP in different populations and use results from a 17-year dataset to offer management suggestions that may prevent or ameliorate these effects. The research reviewed here was conducted on horse populations in which the individual identity and treatment history of mares was known; therefore, these recommendations will likely not be applicable to all situations (Turner et al. 2001, Killian et al. 2008). However, the potential benefits to feral horse health and well-being and the assurance that future management options remain available (Nuñez et al. 2017) indicate that the suggestions proposed should be applied whenever possible.

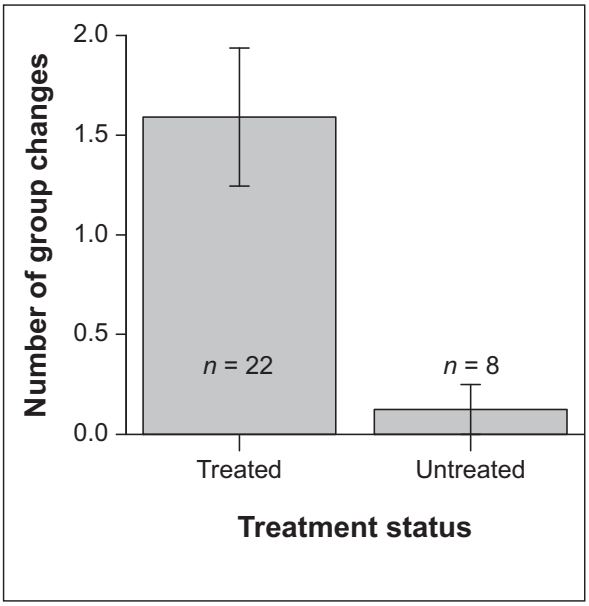


Figure 1. Number of group changes for treated and untreated mares. Even when controlling for the effect of age, treated mares changed groups more often than did their untreated counterparts. Adapted from Nuñez et al. (2009).

Effects of PZP-induced subfertility

PZP-induced subfertility can have important behavioral and physiological consequences for recipient animals (Nuñez et al. 2009, Madosky et al. 2010, Nuñez et al. 2010, Ransom et al. 2010, Ransom et al. 2013). This was especially the case with mares receiving ≥4 total and/or consecutive treatments (Nuñez 2005, unpublished data; Nuñez et al. 2010). Moreover, changes to mare behavior and physiology have the potential to become long-term (especially in mares that received ≥4 total treatments; Nuñez et al. 2017). Finally, such changes in recipient animals can significantly affect the behavior and physiology of their band stallions (Jones and Nuñez 2017, unpublished data). Although such aggressive treatment may be deemed necessary when rapid reductions in animal numbers are of paramount importance, the resultant changes to mare behavior and physiology can limit future management options (Nuñez et al. 2010, Ransom et al. 2014, Nuñez et al. 2017).

Conversely, animals receiving fewer treatments exhibited behavior and reproductive physiology more typical of untreated animals. Moreover, animals receiving fewer treatments were more likely to return to fertility after the suspension of treatment (Nuñez et al. 2017), thereby maintaining flexibility should future management goals change. Although such considerations will not be practical with all populations (Turner et al. 2001, Killian et al. 2008), more careful consideration to feral horse behavior and physiology can help ensure more effective, flexible population management.

Behavior

On Shackleford Banks, PZP-treated mares were up to 10 times more likely to change bands than their untreated counterparts (Nuñez et al. 2009; Figure 1). Moreover, mares receiving 4–6 total treatments were more likely to engage in the behavior than those receiving 1–3 or 0 treatments (Nuñez 2005, unpublished data). This is an important shift in behavior, as band stability, which is affected by the degree of female loyalty to the band stallion (Kaseda et al. 1995), is important to overall animal health. Decreases in band stability have been associated with decreased physical condition and female fecundity, increased parasite load and offspring mortality, and less time spent in preferred behaviors (Linklater et al. 1999). Moreover, increased mare turnover can disturb resident females (Monard and Duncan 1996), causing an overall increase in their levels of aggression (Rutberg 1990, Monard and Duncan 1996). In addition, repeated changes to band composition may prevent the establishment of stable female dominance hierarchies, which are critical to maintaining social cohesion among mares and overall group stability (Berger 1977, Houpt and Wolski 1980, Heitor et al. 2006).

Evidence suggested that such decreases in mare fidelity to the band stallion were related to PZP-induced subfertility: mares that conceived more often over a 5-year period (i.e., mares that were left untreated or for which treatment had failed), were less likely to engage in group changing behavior (Nuñez et al. 2009; Figure 2). Though these results were confounded by PZP treatment (see Figure 2), given the vaccine's mechanism of action, and the demonstrated effects of treatment regime, it seems more likely that prolonged subfertility is the causal mechanism behind decreases in mare fidelity.

Similar to white-tailed deer does (McShea et al. 1997) and free-ranging elk cows (Heilmann et al. 1998), treated mares were also more likely to solicit and receive reproductive behaviors from males (Nuñez et al. 2009, Ransom et al. 2010).

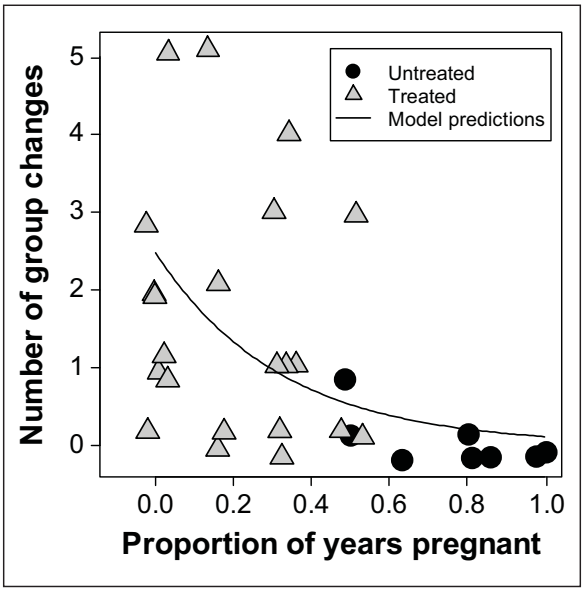


Figure 2. Number of group changes and the proportion of years pregnant from January 2000 to January 2005. Points have been jittered to allow clear visualization of every individual. The number of changes mares made decreased with the proportion of years they were pregnant. Adapted from Nuñez et al. (2009).

In addition, stallions in western populations were more likely to breed PZP-treated versus untreated mares regardless of their age or the probability of producing viable offspring, 2 factors that normally affect male mate choice (Ransom et al. 2010; Figure 3). In the absence of pregnancy, the increased frequency of reproductive cycling demonstrated by treated mares was undoubtedly a factor driving male behavior. In addition, there was the potential for a perceived increase in reproductive "failures" (by mares) to drive female reproductive behavior.

Stress physiology

Because stallions will vigorously attempt to retrieve and maintain resident mares, mares attempting to change groups are often subject to direct male harassment, including aggressive herds, chases, and increased reproductive activity by either the band or new stallion (Madosky 2011), and/or find themselves in close proximity to highly escalated male–male contests (Feist and McCullough 1976, Rubenstein 1981). In addition, if mares are successful at leaving their current band to join a new one, they are often subject to harassment by resident mares establishing their dominance (Rutberg 1990).

On Shackleford Banks, this increased social instability affected mare stress physiology. During group changing behavior, mares exhibited increased fecal cortisol (Nuñez et al. 2014; Figure 4), a reliable measure of stress in several mammalian taxa (Wasser et al. 2000, Mostl and Palme 2002). This effect was not fleeting; mares changing groups more often continued to show elevated cortisol levels for up to 2 weeks post their group changing behavior (Nuñez et al. 2014; Figure 5).

These studies indicate that group transfer behavior can incur a significant cost to mares. Although stress in itself is not inherently detrimental to animal fitness or well-being (Moberg 2000), the stress response can become pathogenic when experienced chronically (Sapolsky 2005), resulting in adverse effects to cardiovascular and immune function, reproduction, and, in extreme cases, neurological functioning. On Shackleford Banks, mares changing bands more frequently can be subject to a negative feedback loop: their behavior leads to harassment, which may induce additional group changes, resulting in further harassment (Madosky et al. 2010; also see Linklater et al. 1999). It is unlikely that the mares observed in our study experienced chronic stress as defined by Sapolsky (2005). However, our data suggest that mares making more group changes experienced increased stress levels at more regular intervals than did mares making fewer group changes. There can be little doubt, therefore, that group changing behavior incurs a physiological cost to mares.

These results have important implications for feral horse management. On Shackleford, PZP-induced subfertility was associated with increased group changing behavior (see Behavior). Though the lack of any untreated mares at the time of our study precluded a direct comparison of the cortisol levels of treated versus untreated mares (Nuñez et al. 2014), the logic was clear: group changing behavior and the associated decreases to social stability induced a physiological stress response in mares. Mares that engage in this behavior more often may be at higher risk for the deleterious effects of the repeated stressor.

Contracepted mares consistently demonstrated improved physical condition and increased longevity when compared to uncontracepted mares (Kirkpatrick and Turner 2007, Nuñez et al. 2010). However, the potential effects of

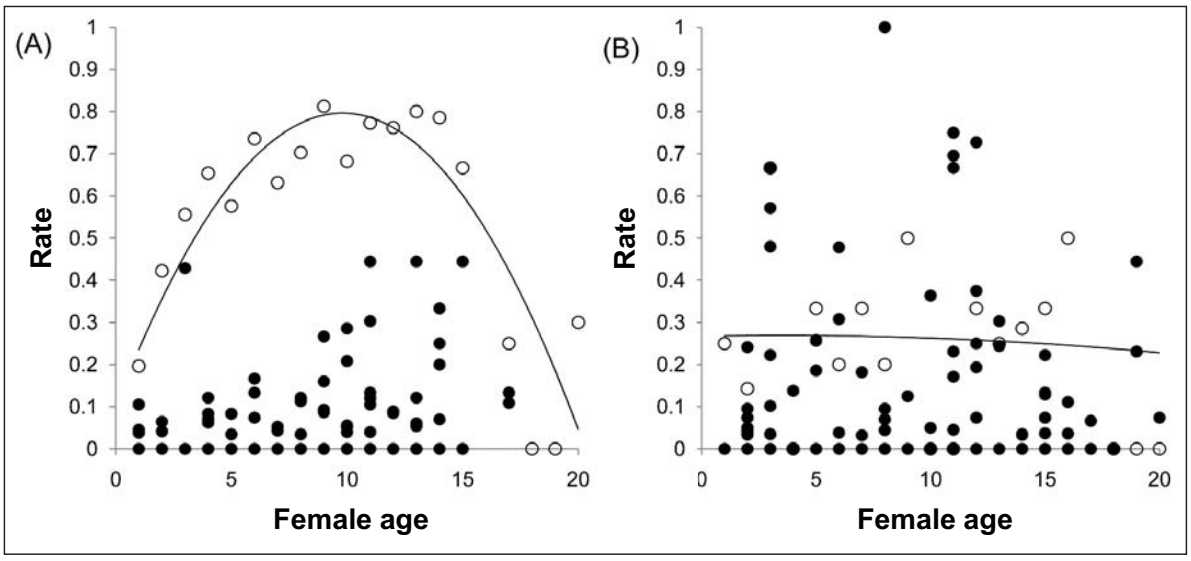


Figure 3. Rate of stallion reproductive behaviors exhibited per hour (●) as a function of mare age, and mean rate of viable foal production (○) as a function of mare age at conception, for repeated observations of 99 untreated mares (A) and 56 treated mares (B) at Little Book Cliffs Wild Horse Range, Grand Junction, Colorado, USA; McCullough Peaks Herd Management Area (HMA), east of Cody, Wyoming, USA; and Pryor Mountain Wild Horse Range, Wyoming and Montana, USA, 2003–2006. The rate of reproductive behavior expressed toward females (by males) generally followed the relationship between viable foal production and age of the female at the time of conception for untreated mares (A). This relationship deteriorated in treated mares (B) and the rate of reproductive behaviors expressed was both higher than control females and exhibited no clear pattern among age cohorts. Adapted from Ransom et al. (2010).

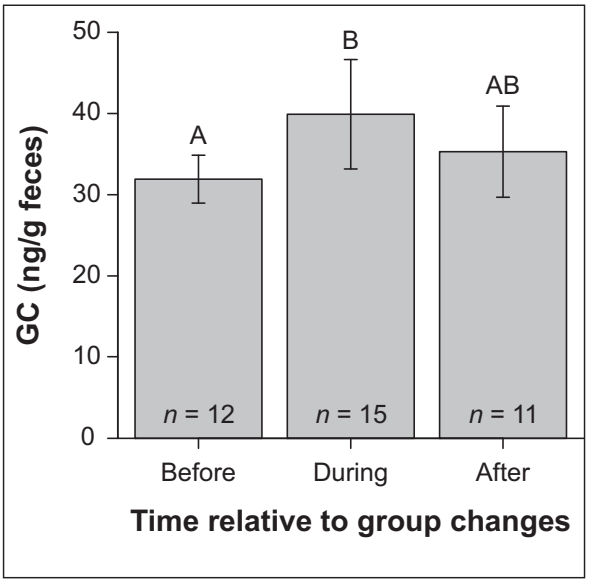


Figure 4. Mare cortisol level and the timing relative to group change behavior. Mares showed increases in cortisol during group changes. Adapted from Nuñez (2014).

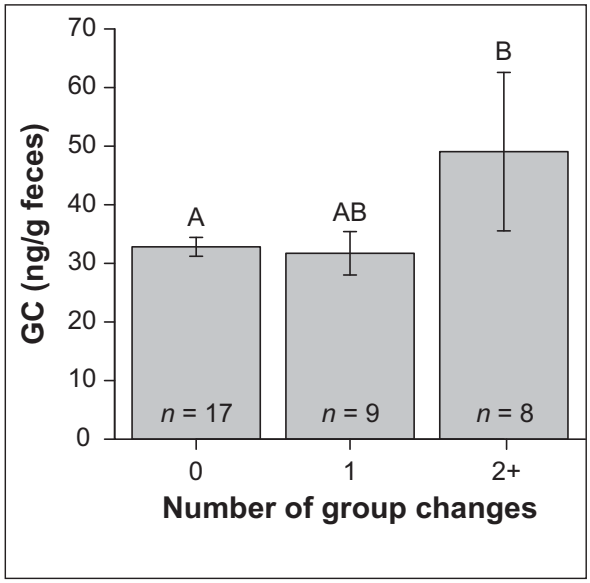


Figure 5. Mare cortisol level 2 weeks post group changing behavior. Mares making more group changes exhibited higher cortisol levels 2 weeks post behavior than did mares making fewer group changes. Adapted from Nuñez et al. (2014).

repeated stressors to treated mares is not trivial. For instance, repeated stress can result in more frequently dysregulated glucocorticoid secretion, elevated food consumption, insulin resistance, and increased fat deposition, which, in some species, can contribute to high condition scores (Leibowitz and Hoebel 1997, McEwen and Wingfield 2003, Sapolsky 2005). Moreover, recent research in other taxa suggests that accepted condition measures do not reliably predict animal survival (crimson finches [*Neochmia phaeton*]) or other constituents of body condition, including

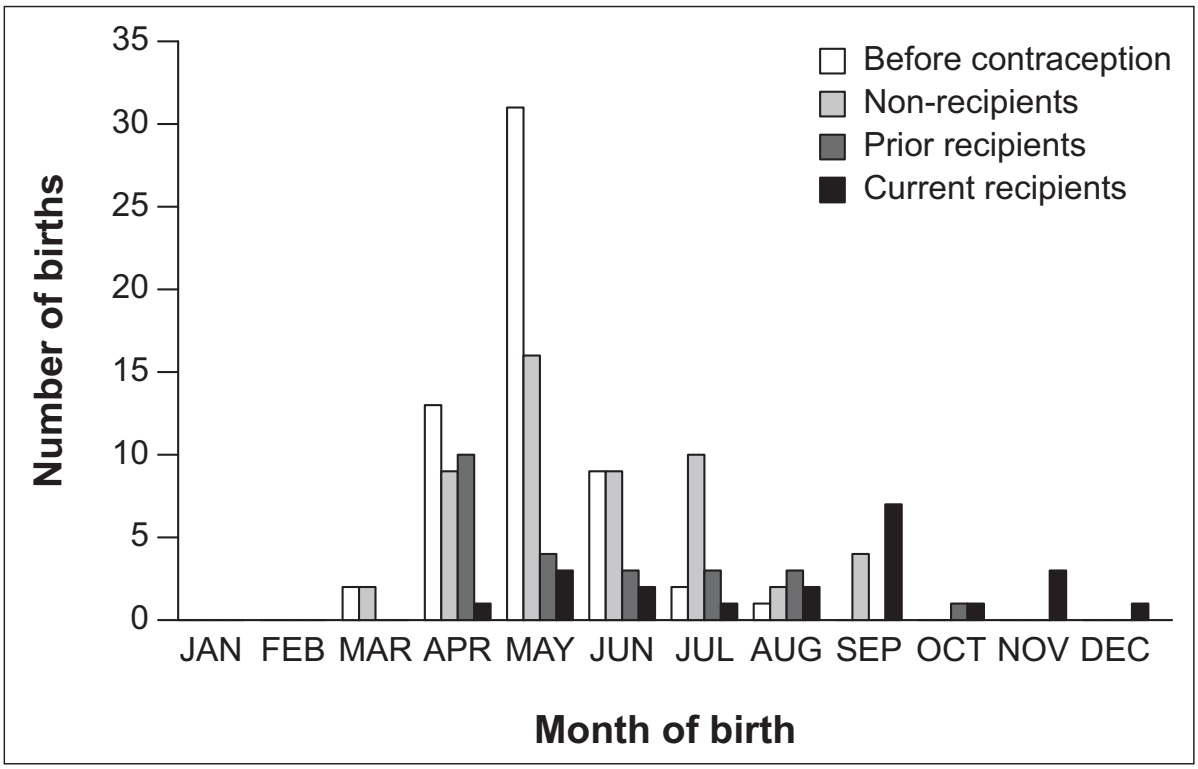


Figure 6. The distribution of births for mares on Shackleford Banks, North Carolina, USA, pre-contraception and post-contraception management. Mares gave birth over a wider range of months and later, on average, after the onset of contraception; this effect was more pronounced in PZP recipients than non-recipients. Adapted from Nuñez (2010).

fat and lean mass (lizards [*Anolis sagrei*]), suggesting that such scoring methods, regardless of what they may indicate, may not be sufficient for the assessment of integrative animal health (Milenkaya et al. 2015, Warner et al. 2016).

Reproductive physiology

In both the Shackleford and western populations studied, mares treated with PZP were more likely to extend reproductive cycling into what is typically considered the non-breeding season (fall and winter) than were untreated mares. This resulted in an increased number of fall and winter births to treated, compared to untreated, mares (Nuñez et al. 2010, Ransom et al. 2013; Figures 6 and 7). On Shackleford, this effect was especially pronounced in mares receiving a greater number of consecutive applications. Each additional consecutive treatment resulted in a 25-day delay in foaling (Nuñez et al. 2010).

These changes constituted an important shift in reproductive physiology, as mares living in temperate climates are typically anovulatory during the non-breeding season (Asa 2002). Gestation lasts approximately 11–13 months in feral horses (Asa 2002). Consequently, mares give birth to foals in seasonal conditions similar to those in which they conceive (i.e., they give birth during the same season the following year). A cessation of ovulatory cycling during the non-breeding season is adaptive in that it precludes conception and subsequent birth during a time of year when resources are at their lowest (Stevens 1990).

Continued cycling into the non-breeding season and the associated fall and winter births could contribute to higher foal mortality; however, management interventions (such as the removal of "at risk" animals) has made the accurate analysis of this possibility infeasible on Shackleford Banks. In areas like the western United States, harsher climatic conditions will likely exacerbate the potential effects of extended cycling to offspring and mare health (Ransom et al. 2013).

Potential for long-term effects of PZP treatment

The potential for long-term effects of PZP-induced subfertility is highly dependent upon the number of treatments females receive during

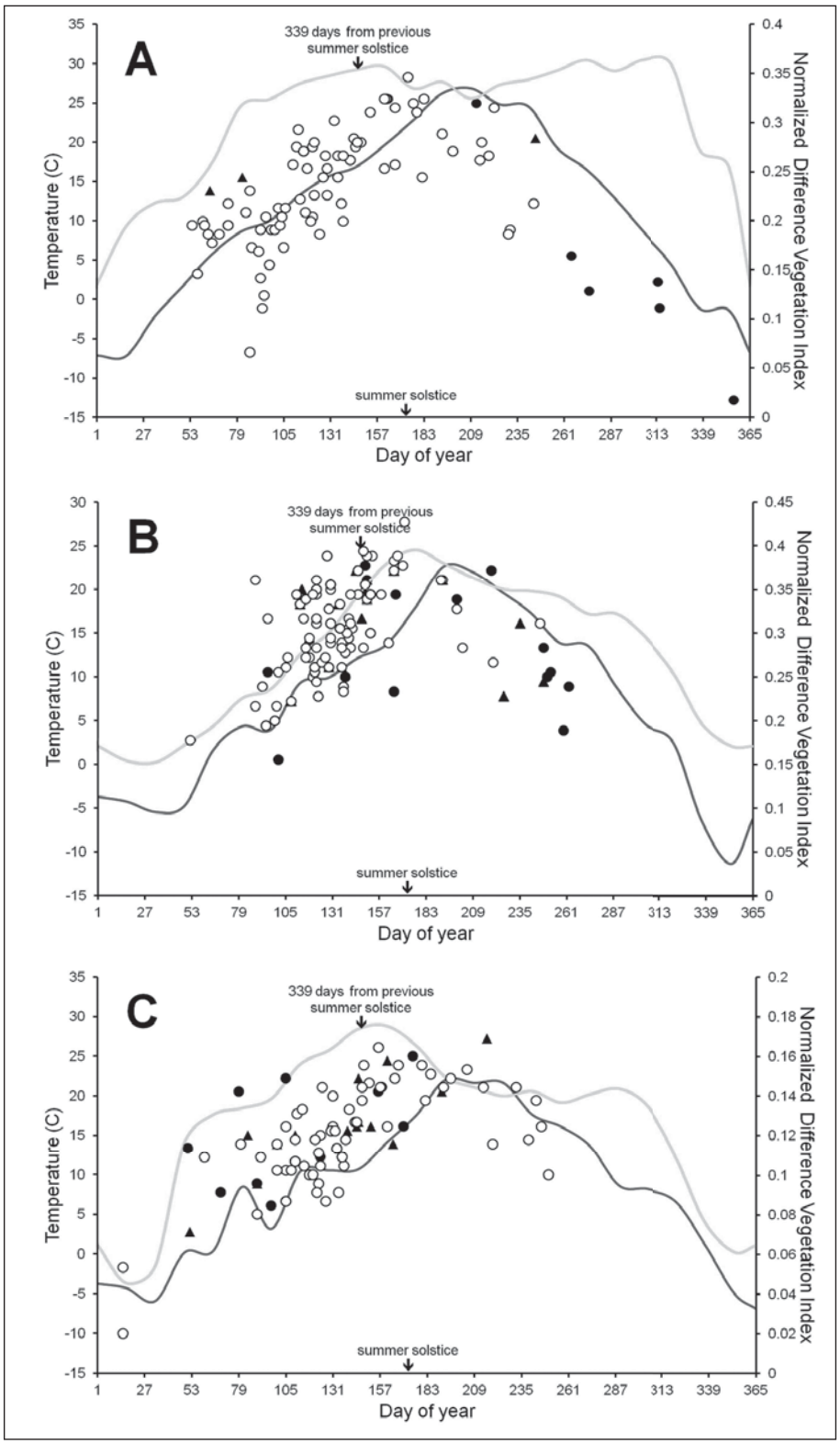


Figure 7. Birth phenology of untreated (○) and post-treated mares (● = first post-treatment birth, ▲ = birth subsequent to ●). Births are shown as a function of temperature at approximate conception date for populations at (A) Little Book Cliffs Wild Horse Range, Grand Junction, Colorado, USA, 2005–2011; (B) Pryor Mountain Wild Horse Range, Wyoming and Montana, USA, 2005–2011; and (C) McCullough Peaks Herd Management Area (HMA), east of Cody, Wyoming, USA, 2007–2011. Post-treated females were previously inoculated with the immunocontraceptive porcine zona pellucida (PZP). Mean Normalized Difference Vegetation Index (shown in lighter gray) represents temporal availability of forage. Mean daily surface temperature is also included (shown in darker gray). Adapted from Ransom et al. (2013).

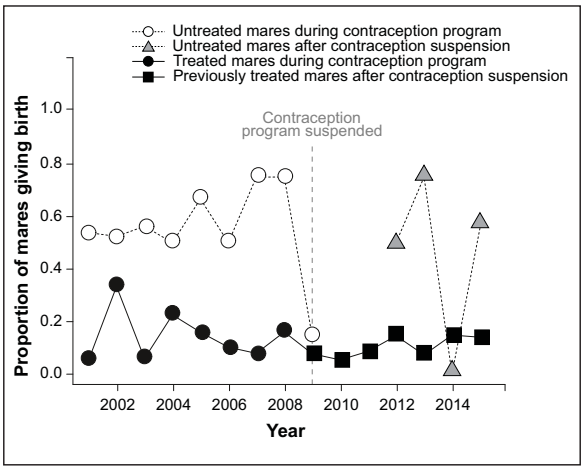


Figure 8. Proportion of mares giving birth in the years during and after contraception management. During the contraception program, treated mares showed markedly lower birth rates than did untreated mares. In 2009, all untreated mares received PZP, leaving no untreated animals of reproductive age on the island in 2010 and 2011. Even after the suspension of treatment, previously treated mares did not return to a level of fertility comparable to untreated mares before 2009 or to younger, untreated animals that became reproductive between 2012 and 2015. Adapted from Nuñez et al. (2017).

their lifetime (Nuñez et al. 2017). In 2009, after 9 years of intensive contraception management on Shackleford Banks, the program was largely but temporarily suspended to allow for controlled population growth (S. Stuska, National Park Service, personal communication). Even when controlling for the effects of mare age on fertility, previously treated mares were less likely to foal than were untreated mares during 5 years of contraception suspension (Figure 8). However, for mares that previously received fewer total treatments (1–3 applications), foaling probability increased with time since their last treatment before declining at approximately 6 years post-treatment. This was not the case for mares that previously received more intensive treatment (≥4 [range = 4–9] total applications); for these mares, there was no increase in foaling probability after contraception suspension. Again, these effects were significant even when controlling for the effects of mare age on fertility.

Moreover, previously treated mares receiving any level of treatment continued to conceive later into the year than did untreated mares, demonstrating prolonged reproductive cycling, and mares previously receiving ≥4 treatments continued to change groups more often than did untreated mares (even after 4–6 years without treatment), demonstrating a prolonged decrease in loyalty to their band stallions (Nuñez et al. 2017).

These results demonstrate the potential for long-term behavioral and physiological effects of PZP-induced subfertility (even after the cessation of treatment). They also show that such effects can be ameliorated with less intense application schedules. When at all possible, careful consideration to the frequency of PZP treatment is important to the maintenance of more typical feral horse behavior and physiology. Moreover, and perhaps just as important, the likelihood that mares will return to fertility (and thus, the ability to maintain future management options) may be compromised if females are kept subfertile for extended periods of time.

Finally, it is critical to note that these results are not anomalous. Similar effects have been demonstrated in western populations of feral horses (Ransom et al. 2013), in other free-ranging species including bison (*Bison bison*; Duncan et al. 2017) and white-tailed deer (Miller et al. 2001), and in several captive species including Asian elephants (*Elephas maximus*), African elephants (*Loxodonta africana*), Seba's bats (*Carollia perspicillata*), big cats (*Felidae*), wildebeest (*Connochaetes*), white rhinoceros (*Ceratotherium simum*), and stingrays (*Myliobatoidei*; Songer 1996).

Effects to untreated associates

In social species, the effects of prolonged PZP-induced subfertility are likely to affect the behavior and physiology of their untreated associates. For instance, increased female group changing behavior could result in increased male–male and male–female aggression (Madosky 2011). Such changes to behavior could subsequently impact the endocrinology of untreated associates, including increases in cortisol and/or testosterone levels. Such impacts have been demonstrated in several taxa including primates, birds, and fish (Wikelski et al. 1999, Cavigelli and Pereira 2000, Hirschenhauser and Oliveira 2006).

Preliminary work on Shackleford Banks strongly suggested such patterns (Jones and Nuñez 2017, unpublished data) indicating that when managing social species, the changes we

effect in target animals are likely to affect the behavior and physiology of their non-target associates. When at all possible, we should attempt to minimize such changes to target animals.

The studies reviewed here show that mares receiving fewer (1–3) total and/or consecutive treatments demonstrate more typical behavior and physiology than do mares receiving more (≥4) treatments. If the primary management goal is to reduce population size, it is unlikely (and perhaps less important) that managers achieve a balance between population control and the maintenance of more typical feral horse behavior and physiology. However, in areas or situations in which the social and physiological functioning of animals is of concern and mare identity and treatment history can be tracked, the application of fewer treatments, including "breaks" during which mares are allowed to reproduce, is recommended. Such treatment will better ensure the health and overall well-being of the population and, depending on managerial goals, more effective, flexible management.

Management implications

Feral horse management via direct population regulation will continue to be a difficult task. With every decision, managers and policymakers must consider the efficacy, feasibility, costs, and benefits of different strategies, the potential effects to feral horse health and welfare, and the impacts of public opinion on management implementation. In the absence of natural predators to regulate feral horse populations, any method we choose will likely necessitate ongoing human intervention. Obtaining balance between the costs and benefits to both feral horse and human populations and the ecology of western rangelands remains challenging. However, results from the studies reviewed here show that with fewer total and/or consecutive treatments, we can maintain more natural behavior and physiology in feral horses.

Mares receiving fewer treatments are less likely to engage in group changing behavior and give birth at times of the year that more closely approximate those of untreated mares. Moreover, these mares are more likely to return to fertility after treatment suspension, maintaining future management options, more natural behavior and physiology, and more typical band stallion behaviors. Finally, given the fact that group changing behavior induces a physiological stress response in mares, the data reviewed here strongly suggest that mares receiving fewer total and/or consecutive treatments will be at lower risk for repeated stressors. Such refinements to contraception scheduling are likely to apply regardless of the methods or vaccines used, as long as it is mare subfertility and not the treatment itself that causes changes to mare behavior and physiology. Lastly, though such methods may not be possible or practical in all areas, in smaller, closed populations, where the identification of individuals and the careful tracking of treatment history is plausible, care should be taken to maintain more typical animal behavior and physiology.

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Immunocontraception decreases group fidelity in a feral horse population during the non-breeding season

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ABSTRACT

The behavioral effects of the immunocontraceptive agent porcine zona pellucida (PZP) have not been adequately studied. Important managerial decisions for several species, including the wild horse (*Equus caballus*), have been based on this limited research. We studied 30 horses on Shackleford Banks, North Carolina, USA to determine the effects of PZP contraception on female fidelity to the harem male. We examined two classes of females: contracepts, recipients of the PZP vaccine ($n = 22$); and controls, females that have never received PZP ($n = 8$). We conducted the study during the non-breeding season from December 2005 to February 2006, totaling 102.2 h of observation. Contracepted mares changed groups more often than control mares ($P = 0.04$). Contracepts also visited more harem groups than did control mares ($P = 0.02$) and exhibited more reproductive interest ($P = 0.05$). For both contracepted and control females, the number of group changes ($P = 0.01$) and number of groups visited ($P = 0.003$) decreased with the proportion of years mares were pregnant. Our study shows that the application of PZP has significant consequences for the social behavior of Shackleford Banks horses. In gregarious species such as the horse, PZP application may disrupt social ties among individuals and inhibit normal social functioning at the population level.

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1. Introduction

Due to the extirpation of their natural predators, ungulate populations in North America have expanded, necessitating their regulation through culling or contraception management (Eberhardt et al., 1982). Immunocontraceptives are widely used to control reproduction in free-ranging ungulates (Kirkpatrick et al., 1990; Turner et al., 1992). In females, the most common form of immunocontraception, porcine zona pellucida (PZP), stimulates the production of antibodies that bind sperm receptors on the egg's surface, thereby preventing sperm attachment and fertilization (Sacco, 1977). While PZP effectively inhibits conception in several different mammalian species (Kirkpatrick et al., 1996), little is known about its potential effects on recipient behavior. Studies in free-ranging elk (*Cervus elaphus*) and white-tailed deer (*Odocoileus virginianus*) indicate that females receiving PZP extend reproductive behaviors into the post-breeding season (McShea et al., 1997; Heilmann et al., 1998). Authors suggest that in response to repeated unsuccessful mating attempts, females continue cycling in an attempt to gain additional reproductive opportunities. Such changes in behavior can have serious consequences for social species, particularly for those that are polygynous with males defending and retaining several females.

Several studies have examined the effects of PZP application to wild horses (*Equus caballus*). These studies have focused primarily on the physiological effects (both

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reproductive and otherwise) of the vaccine (Kirkpatrick et al., 1992, 1997; Turner and Kirkpatrick, 2002). Researchers have reported no debilitating side effects to PZP recipients and only minor ovulation failure and depressed urinary oestrogen concentrations with repeated applications (Kirkpatrick et al., 1996). In addition, the contraceptive effects of PZP have been shown to be reversible, safe for pregnant females, and do not adversely affect the survivorship or subsequent fertility of offspring born to treated individuals (Kirkpatrick and Turner, 2002).

Researchers claim that the contraceptive has no effect on recipient behavior (Kirkpatrick et al., 1996, 1997; Powell and Monfort, 2001), but present no quantitative data to support their conclusions. In fact, no systematic study has specifically addressed this issue with true controls, animals that have never received PZP during their lifetime (Powell, 1999). Additionally, no study on wild horses has focused on the post-breeding period, when the effects of PZP appear most pronounced in other ungulates (McShea et al., 1997; Heilmann et al., 1998).

In wild horse societies, the harem is the core social group, consisting of usually one, but sometimes two or three harem male(s), one to several female(s), and their offspring (Feist and McCullough, 1976; Rubenstein, 1981, 1986; Linklater et al., 2000). Harem groups are typically stable units, showing very few changes in composition over months or years (Klingel, 1975). Female loyalty to the harem male and the male's ability to retain females is paramount to maintaining this stability (Feist and McCullough, 1976; Rubenstein, 1981; Goodloe et al., 2000). Decreases in harem stability have been shown to affect several aspects of mare well-being, resulting in lower overall reproductive success (Kaseda et al., 1995), less time for preferred behaviors, decreased body condition and fecundity, elevated parasite levels, and increased offspring mortality (Linklater et al., 1999).

For the most part, wild horses are non-territorial, with several harems sharing both feeding and water resources (Feist and McCullough, 1976; Rubenstein, 1981; Cameron et al., 2003). Given this ecology, decreases in the stability of individual harems have the potential to affect the interactions and social relationships of neighboring harems and thereby, may affect significant change at the population level. As such, understanding the potential effects of PZP on individual behavior is of broad importance to maintaining a functional population of feral horses.

In this study we investigate the behavioral effects of PZP on the horses of Shackleford Banks, North Carolina, USA during the non-breeding season. Specifically, we examine female propensity to switch harems, the number of harem groups visited, and the occurrence of reproductive behavior. Since the first application of the contraceptive in January 2000, a reduction in the fidelity of resident mares to their harem males has been noted, albeit anecdotally (C. Mason, personal observation). Based on this information, we hypothesized that contracepted females would change groups more often, would visit more groups, and would exhibit reproductive behaviors more often than would control mares (those never having received the vaccine).

2. Materials and methods

2.1. Study area

Shackleford Banks is a barrier island approximately 3 km off the coast of North Carolina, USA located at 34°40′04.94″N and 76°35′39.39″W. The island stretches 15 km in length, and varies between 0.5 and 3 km in width. The specific study area extended approximately 7 km and was located in the center of the island. This site contained all study animals.

Daylight hours, measured from sunrise to sunset times, ranged from 9 h and 53 min at the beginning of the study on 10 December 2005 to 10 h and 35 min at the conclusion of the study on 3 February 2006 (U.S. Naval Observatory Data Services, 2008). In Beaufort, NC, 7.8 km from the study site, average daily temperatures ± 1 S.E. for the past 20 years for December, January, and February were 7.93 ± 0.40 °C, 7.08 ± 0.28 °C, and 7.95 ± 0.26 °C, respectively. During the present study average daily temperatures ± 1 S.E. in Beaufort, NC for December, January, and February were 6.86 ± 1.65 °C, 7.19 ± 0.48 °C, and 7.26 ± 0.30 °C, respectively (National Climate Data Center, 2008).

2.2. Study subjects

The reproductive units of Shackleford horses are typical of feral equids, consisting of coherent harem groups of one or, sometimes two or three stallion(s) with one to several mare(s) and their offspring (Rubenstein, 1981). While multi-male harems are more common in some populations (Linklater and Cameron, 2000), they occur less frequently on Shackleford Banks, accounting for only 19% of all harems on the island at the time of this study. For the most part, these social units are not territorial, and the animals move freely within their overlapping home ranges.

Normally, harem groups are long lasting with most changes involving the dispersal of immature individuals (both male and female). Harem males will sometimes fight to acquire mares from other groups, but stallions almost always retain their mares (Feist and McCullough, 1976; Rubenstein, 1981).

The application of PZP for the purposes of immunocontraception was begun by the National Park Service in January 2000. At that time, eight control mares were identified; one from each of the distinct genetic lineages on the island. These mares would not receive the vaccine at any point during their lifetime. Females younger than 2 years of age were not considered for control status. These procedures determined the current age distribution of control and contracepted animals on Shackleford Banks. The authors of this study were not involved in establishing the number of control and/or contracepted animals.

We observed 30 females that organized themselves into 13 harem groups. Twenty-two mares were treated with PZP at least once between January 2000 and January 2005; the remaining control animals had never been treated. Six of the harem groups investigated contained contracepted females only; two groups contained control females only; the remaining five groups contained both contracepted and control females (see Table 1). All harems considered in this study contained only one harem male. At the time of the study, five of the control mares were pregnant; three of which were nursing foals. An additional control mare nursed a foal, but was not pregnant. Three contracepted mares were pregnant. Two of these females had not received PZP treatment the previous spring; the remaining mare's pregnancy suggests a failure of the treatment. Two other contracepted mares nursed foals; these mares had not received treatment the previous spring. The inoculation, pregnancy, and foaling records for all study animals are shown in Tables 2 and 3.

2.3. PZP contraception

The National Park Service administers PZP from late February through April each year. Mares are first treated at 2 years of age. Each injection contains 100 μg of PZP plus an adjuvant (mixed at the darting site). Initial doses contain Freund's Complete Adjuvant, Modified, *Mycobacterium butyricum* (Calbiochem, Gibbstown, NJ, USA, #344289). All subsequent doses contain Freund's Incomplete Adjuvant (Sigma, St. Louis, MO, USA, #F5506).

For the animals in this study, PZP deterred pregnancy in 97% of cases when administered during the same year. This efficacy dropped to 76% in

Table 1
Initial harem configurations of focal animals.

Harem male	Group type	Control females	Contracepted females	Focal females
Adam	Control	1	0	1
Edge	Control	1	0	1
Axl*	Contracept	0	2	1
Duchovny	Contracept	0	1	1
Pacino	Contracept	0	6	6
Phinius*	Contracept	0	4	1
Teddy*	Contracept	0	5	1
Toro*	Contracept	0	4	4
Clint	Mixed	1	1	2
Dionysis	Mixed	1	1	2
Satellite	Mixed	1	2	3
Stobbs	Mixed	1	2	3
Winston	Mixed	2	2	4

Four of the harems listed reside outside of the study area and were not observed as focal groups (*). However, at least one female from each of these harems moved into focal groups during our study. The focal females column reflects only the number of individuals from each harem that were observed systematically during the study. Other columns show the initial group composition of each harem at the study's outset.

the second year after PZP inoculation. These values are similar to those published for Assateague horses, 94% and 86%, in the first and second years, respectively (Turner et al., 2007).

While we were unable to obtain blood samples for mares during this study, anti-PZP antibody titers in domestic mares remain above control levels for up to 40 weeks post-injection when using similar doses and adjuvant mixtures (Willis, 1994). The National Park Service routinely inoculates mares from February through April. Therefore, in animals inoculated in 2005, anti-PZP antibody levels would have been high during the breeding season, but were likely approaching control levels at the time of this study.

2.4. Pregnancy testing

Fecal samples are collected by the National Park Service in January of each year. All pregnancy testing is completed by enzymeimmunoassay of fecal material at the Science and Conservation Center at ZooMontana in Billings, MT, USA. Using the methods of Kirkpatrick et al. (1991), water extracts of fecal samples are assayed for estrone conjugates and nonspecific progesterone metabolites. Foaling records from the summers following testing were used to supplement assay results.

2.5. Behavioral and demographic sampling

The study was conducted by one observer (C.M.V. Nuñez) during the non-breeding season from December 2005 to February 2006, totaling 102.2 h of observation. Horses were identified individually by color, sex, age, physical condition, and other distinguishing markings including freeze brands. Ages are known from long-term records for the identified horses of Shackleford Banks (Nuñez, 2000).

We located each harem and noted its composition an average of four times each week. We recorded its GPS location and composition, paying particular attention to the presence or absence of females. These data allowed us to assess female willingness (or ability) to remain with their harems. The following measures were analyzed:

- Number of changes that females made, i.e. how many times females switched groups during the study.
- Number of different groups that females visited, i.e. the total number of groups in whom a female was seen during the study.
- The age of the harem male with whom a female was most often associated.
- The size of the group in which the female was most often found.

All incidences of reproductive interest (including copulation, mounting, genital sniffing, and rump rubbing) directed to and initiated by mares were recorded *ad libitum* during scan sampling (Altmann, 1974). Behaviors of reproductive interest were defined as follows:

- Mounting—male places forelimbs around a female's flank; does not include insertion of the penis.
- Copulation—male mounts female; insertion of penis achieved.
- Genital sniffing—animal (male or female) actively places the snout to the genitals of another animal of the opposite sex.
- Rump rubbing—the initiator (male or female) places the chin and/or neck on the rump of a recipient of the opposite sex; initiator rubs its neck back and forth horizontally over recipient's rump.

2.6. Statistical analyses

We analyzed the effect of contraception on the number of group changes, the number of different groups females visited, and the occurrence of reproductive interest (either received or initiated by mares) using generalized linear models in R (version 2.7.1). All variables were poisson distributed and were analyzed using models with a quasipoisson error distribution and a log link function. All models were weighted by the number of times a mare was observed.

Many factors in addition to PZP treatment may affect the number of group changes, the number of groups visited, and the occurrence of reproductive interest. Such factors include mare and harem male age, group size, pregnancy status, the presence of a foal, and the percentage of females contracepted in each group (Feist and McCullough, 1976; Rutberg and Greenberg, 1990; Linklater et al., 2000). We included mare age, PZP treatment, and their interaction in the initial, maximal generalized linear models discussed above. As PZP treatment was correlated with harem male age, group size, pregnancy status, the presence of a foal, and the percentage of contracepted mares in a group, these latter terms were not included in our models to avoid multicolinearity. Non-significant terms were removed from the

Table 2
Pregnancy and foaling histories for control mares.

Mare	2000	2001		2002		2003		2004		2005	
	Pr	F	Pr	F	Pr	F	Pr	F	Pr	F	Pr
Biff	+	**	+	+	+	**	+	+	+	+	+
Carrot	+	+	—	—	+	+	+	+	+	+	+
Damigo	+	+	+	+	—	—	+	**	+	+	+
Hercules	+	**	+	+	—	—	+	+	—	—	—
Julie	+	**	+	**	+	+	+	**	—	—	+
Kelty	+	+	—	—	+	+	—	—	+	+	—
Laurie	+	+	—	—	+	**	+	**	+	**	—
Wallace	—	—	+	**	+	+	—	—	+	**	+

Column headings: Pr, pregnant during post-breeding season (fall) of the listed year; F, foal present (was conceived in prior year). "+" indicates the presence of a foal or that the animal was pregnant; "—" indicates the absence of a foal or that the animal was not pregnant; "**" indicates that an animal foaled, but the offspring died before reaching 1 year of age.

Table 3
Inoculation, pregnancy, and foaling histories for contracepted mares.

	2000		2001			2002			2003			2004			2005-6		
Mare	**PZP**	**Pr**	**F**	**PZP**	**Pr**	**F**	**PZP**	**Pr**	**F**	**PZP**	**Pr**	**F**	**PZP**	**Pr**	**F**	**PZP**	**Pr**
Alexa	+	–	–	–	–	–	–	–	–	+	–	–	+	–	–	+	–
Bo	–	+	+	–	+	+	–	–	–	+	–	–	–	–	–	+	–
C'susha	n/a	n/a	n/a	n/a	n/a	n/a	n/a	–	–	+	–	–	+	–	–	+	–
Darcy	–	+	+	–	+	+	+	–	–	+	–	–	+	–	–	+	–
Doobie	+	–	–	–	+	+	+	–	–	+	–	–	+	–	–	+	–
Dotu	–	–	–	+	–	–	+	–	–	+	–	–	–	–	–	+	–
Dumé	–	–	–	–	+	+	–	+	+	+	–	–	+	–	–	–	–
Dusty	–	–	–	+	–	–	+	–	–	+	–	–	–	+	+	+	–
Hardee	+	–	–	–	–	–	+	–	–	+	+	**	–	+	+	–	–
Helena	+	–	–	–	–	–	–	–	–	+	–	–	+	–	–	+	–
Jaqui	–	–	–	+	–	–	–	–	–	+	–	–	+	–	–	+	–
Juniper	+	–	–	–	–	–	+	–	–	+	–	–	+	–	–	–	–
Larissa	n/a	n/a	n/a	n/a	n/a	n/a	n/a	–	–	+	–	–	+	–	–	+	–
Noir	–	–	–	–	–	–	–	+	+	–	–	–	–	+	**	+	–
Paula	–	+	+	–	+	+	–	+	+	+	–	–	+	–	–	+	–
Serenac	–	–	–	+	–	–	–	+	+	+	–	–	+	–	–	+	–
Shag	+	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	+
Slug	–	+	+	–	–	–	+	–	–	+	–	–	+	–	–	+	+
Sydney	–	+	+	–	–	–	+	–	–	+	–	–	–	–	–	–	+
Tatya	n/a	n/a	n/a	n/a	n/a	n/a	n/a	–	–	+	–	–	+	–	–	+	–
Wire	–	+	**	–	+	+	–	+	+	+	–	–	+	–	–	+	–
Zelda	–	+	**	–	+	+	–	+	+	+	–	–	+	–	–	–	–

Column headings: F, foal present (was conceived in prior year); PZP, contraception during the pre-breeding season (spring) of the listed year; Pr, pregnant during post-breeding season (fall) of the listed year. "+" indicates the presence of a foal, that the animal was pregnant, and/or that the animal was inoculated with PZP; "–" indicates the absence of a foal, that the animal was not pregnant, and/or that an animal was not inoculated with PZP; "**" indicates that an animal foaled, but that the offspring died before reaching 1 year of age; "n/a" indicates that an animal was 0–2 years old and not eligible for contraception. PZP administration began in January 2000; foals present that year are not indicative of PZP efficacy and are not included. The status for the animals during the study period is highlighted.

models by backwards elimination. As sample sizes were limited, terms were retained if their *P*-value was less than 0.10.

To address whether harem male age, group size, pregnancy status, the presence of a foal, and the percentage of contracepted mares in a group had a significant influence on mare behavior, we analyzed them separately for control and PZP groups using Spearman rank correlations against the following variables: number of group changes, groups visited, and occurrences of reproductive interest (see Table 4).

Table 4
Spearman's rank correlations between response variables and predictor variables that correlated with contraceptive treatment.

Predictor variable	Response variable		
	Total changes among groups	Number of groups visited	Instances of reproductive behavior
Male age	*Controls*: $\rho = -0.58$, $P = 0.13$ *Contracepts*: $\rho = -0.16$, $P = 0.47$	*Controls*: $\rho = -0.58$, $P = 0.13$ *Contracepts*: $\rho = -0.18$, $P = 0.43$	*Controls*: $\rho = 0.08$, $P = 0.85$ *Contracepts*: $\rho = -0.03$, $P = 0.88$
Group size	*Controls*: $\rho = -0.44$, $P = 0.28$ *Contracepts*: $\rho = -0.28$, $P = 0.22$	*Controls*: $\rho = -0.44$, $P = 0.28$ *Contracepts*: $\rho = -0.22$, $P = 0.35$	*Controls*: $\rho = 0.01.$, $P = 0.99$ *Contracepts*: $\rho = 0.02$, $P = 0.95$
Percentage of group members contracepted	*Controls*: $\rho = 0.18$, $P = 0.68$ *Contracepts*: $\rho = 0.11$, $P = 0.67$	*Controls*: $\rho = 0.18$, $P = 0.68$ *Contracepts*: $\rho = 0.09$, $P = 0.71$	*Controls*: $\rho = 0.62$, $P = 0.10$ *Contracepts*: $\rho = 0.13$, $P = 0.59$
Pregnant or with foal during study	*Controls*: $\rho = -0.66$, $P = 0.08$ *Contracepts*: $\rho = -0.41$, $P = 0.06$	*Controls*: $\rho = -0.66$, $P = 0.08$ *Contracepts*: $\rho = -0.41$, $P = 0.06$	*Controls*: $\rho = -0.66$, $P = 0.08$ *Contracepts*: $\rho = -0.12$, $P = 0.58$

Each correlation was performed separately for control ($n = 8$) and contracepted ($n = 22$) groups.

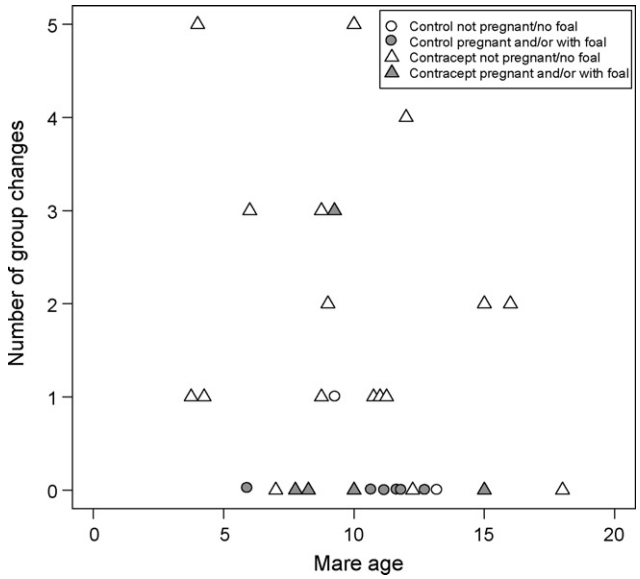


Fig. 1. Number of group changes during the study period by mare age for control ($n = 8$) and contracepted mares ($n = 22$). Even when controlling for the effect of age, contracepted mares change groups more often than do controls. Filled symbols represent mares that were either pregnant or nursing a foal at the time of the study. In the events of ties, mare age has been jittered by 0.2 years to allow clear visualization of every individual.

3. Results

3.1. Number of group changes

A generalized linear model shows that PZP treated mares changed groups significantly more often than did controls, even when accounting for mare age (analysis of deviance, overall GLM: $F_{2,27} = 6.73$, $P = 0.004$; PZP treatment: estimate = 1.99, $t = 2.11$, $P = 0.04$; mare age: estimate = −0.13, $t = -1.92$, $P = 0.07$, see Fig. 1). Pregnancy and/or the presence of a foal seemed to have a marginal effect (see Section 3.3). Spearman rank correlations within treatment groups show that harem male age, group size, and the percentage of contracepted mares in the group had no effect on the number of group changes (see Table 4), suggesting that their influence was not substantial.

3.2. Number of groups visited

A separate generalized linear model shows that contracepted females visited significantly more groups than did control mares, again controlling for mare age (analysis of deviance, overall model: $F_{2,27} = 6.83$, $P = 0.004$; PZP treatment: estimate = 0.49, $t = 2.42$, $P = 0.02$; mare age: estimate = −0.06, $t = -2.39$, $P = 0.02$, see Fig. 2). As above, pregnancy and/or the presence of a foal seemed to have a marginal effect (see below). Spearman rank correlations within treatment groups show that harem male age, group size, and the percentage of contracepted mares in the group had no effect on the number of males consorted with (see Table 4).

3.3. Pregnancy and foal presence

Both control and contracepted mares that were pregnant and/or had foals tended to change groups less often and visit fewer groups than did other mares (Spearman rank correlation: controls, $\rho = -0.66$, $P = 0.08$; contracepts, $\rho = -0.41$, $P = 0.06$; also see Table 4). Given this trend, we investigated whether a mare's history of pregnancy or foaling (over multiple years) affected behavior. For each female, we calculated the proportion of years pregnant and the proportion of years with a foal from January 2000 to January 2005, considering only those years in which the mare was sexually mature. A generalized linear model shows that mares pregnant for a greater proportion of years changed groups less often (overall model: $F_{1,28} = 10.75$, $P = 0.003$; % years pregnant: estimate = −3.11, $t = -2.79$, $P = 0.01$, see Fig. 3A) and visited fewer groups (overall model: $F_{1,28} = 11.77$, $P = 0.002$; % years pregnant: estimate = −1.03, $t = -3.31$, $P = 0.003$, see Fig. 3B). Mare age did not contribute significant explanatory power to these models and was thus removed. The proportion of years that mares had foals from 2000 to 2005 did not affect mare behavior (Group changes, overall model: $F_{2,27} = 2.64$, $P = 0.09$; % years with foal: estimate = −1.96, $t = -1.16$, $P = 0.25$. Groups visited, overall model: $F_{2,27} = 4.63$, $P = 0.04$; % years with foal: estimate = −1.39, $t = -1.44$, $P = 0.16$).

3.4. Reproductive interest

Contracepted mares received and exhibited more reproductive interest (see Section 2.5) than did control mares (analysis of deviance, overall GLM: $F_{2,27} = 6.46$, $P = 0.005$; PZP treatment: estimate = 2.04, $t = 2.03$, $P = 0.05$; mare age: estimate = −0.13, $t = -1.91$, $P = 0.07$, see Fig. 4). Spearman rank correlations within treatment groups show that harem male age, group size, the presence of a foal, and the percentage of contracepted mares in the group had no effect on the occurrence of reproductive

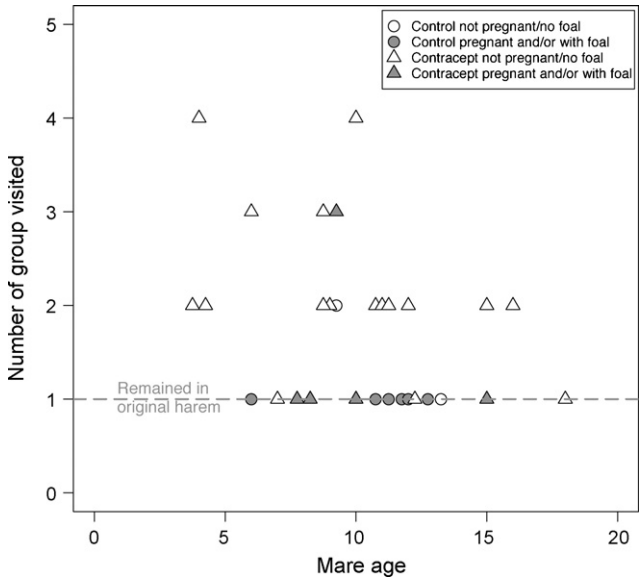


Fig. 2. Number of different groups visited during the study period by mare age for control ($n = 8$) and contracepted mares ($n = 22$). Even when controlling for the effect of age, contracepted mares visit more groups than do controls. Individuals on the dotted line did not change groups during the study. Filled symbols represent mares that were either pregnant or nursing a foal at the time of the study. In the events of ties, mare age has been jittered by 0.2 years to allow clear visualization of every individual.

interest (see Table 4). Pregnancy may have had a marginal effect on the reproductive interest received by control mares (Spearman rank correlation: $\rho = -0.66$, $P = 0.08$). This result is not conclusive however, since only one non-pregnant control mare received any reproductive interest. Pregnancy had no effect on the reproductive interest received or initiated by contracepted mares (Spearman rank correlation: $\rho = -0.12$, $P = 0.58$).

4. Discussion

According to past research, contraception with PZP has little to no effect on the behavior of wild horses (Kirkpatrick et al., 1996, 1997; Powell and Monfort, 2001). The results of this study refute that assertion. Much of the aforementioned research has been based on a single island population, all studies have been conducted

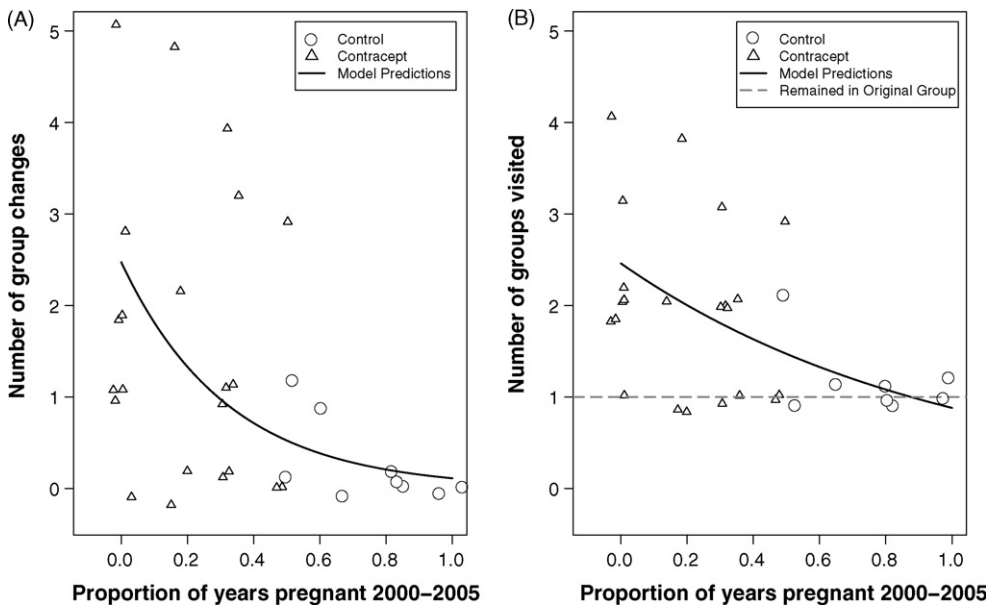


Fig. 3. Number of group changes (A) and groups visited (B) by the proportion of years pregnant from January 2000 to January 2005. Lines show that according to the generalized linear model of the data (see Section 3.3), the number of group changes and groups visited decrease with the proportion of years mares are pregnant. Points have been jittered to allow clear visualization of every individual.

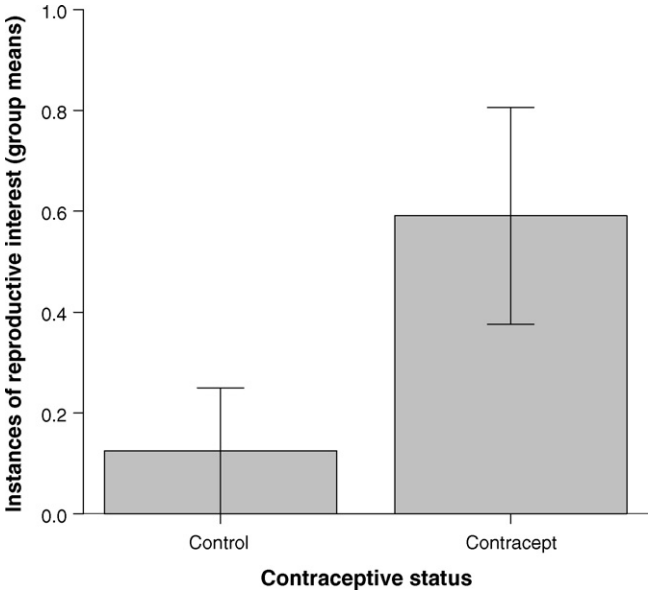


Fig. 4. Instances of reproductive interest during the study period by PZP treatment (means ± S.E.). Contracepted mares exhibit and receive more reproductive interest than do control mares.

solely during the breeding season, and no study has had adequate controls against which to compare PZP-treated females (Kirkpatrick et al., 1997; Powell, 1999). Here, we studied horses during the non-breeding season on Shackleford Banks, North Carolina, making use of animals that had never received contraception as controls. In our study, PZP treatment increased the number of group changes, the number of different groups visited, and the occurrence of reproductive interest, both received and initiated by females. In addition, our results show that even 10 months after PZP inoculation, when anti-PZP antibody titers are likely low, the indirect behavioral effects on recipient animals remain strong. The potential implications of these results for feral horse management are of substantial importance and need to be investigated further.

4.1. Fidelity to the harem male and reproductive interest

Contracepted mares are more likely to switch harem groups and visit more groups than are control mares. Decreases in mare fidelity to the harem male have debilitating consequences for harem stability (see Section 1). Resident females are often disturbed by the addition of new mares, especially if they are strangers (Monard and Duncan, 1996; Parker, 2001), and will become increasingly aggressive in their presence (Rutberg, 1990; Monard and Duncan, 1996). In addition, frequent changes to a harem's composition are likely to prohibit the establishment of a stable female dominance hierarchy, which is paramount to maintaining social cohesion among mares and overall group stability (Berger, 1977; Houpt and Wolski, 1980; Heitor et al., 2006). Moreover, the instability caused by these switching females may adversely affect the resident females' relationship with the harem male, reducing group cohesion even further. Because contracepted females do not simply switch repeatedly between two well-known groups, but rather interact with several different groups, these detrimental effects of harem instability may be felt throughout the entire population.

Contracepted mares both receive and initiate more instances of reproductive interest than do control mares. Reproductive behavior is energetically costly (Galimberti et al., 2000). Repeated bouts of male harassment have been shown to reduce total time foraging in equid species (Rubenstein, 1986; Sundaresan et al., 2007). The relative cost of such behaviors may be especially high during the post-breeding season when resources are scarce (Stevens, 1990). In addition, the costs of this behavior may outweigh the potential benefits, i.e. increased reproductive success. Gestation in wild horses lasts approximately 11–12 months (Asa, 2002). Offspring conceived during the winter months are therefore subject to higher mortality due to the cold temperatures and poor quality forage available at birth.

The differences we observed in harem fidelity and reproductive behavior may result from prolonged estrous cycling into the post-breeding season in response to repeated failures to conceive. This hypothesis has been proposed to explain reproductive behavior during the post-breeding season in both PZP-treated elk (Heilmann et al., 1998) and white-tailed deer (McShea et al., 1997). In equids, reproductive behaviors including copulation, mounting, clitoral winking, and tail raising occur most frequently during estrous when the mare is nearing ovulation (Asa et al., 1979). Additionally, Asa et al. (1979) have shown that mare approaches to, and follows of the harem male are excellent predictors of the transition between estrous and diestrous. We propose that group changes may reflect a similar pattern, with PZP-treated mares approaching non-harem males more frequently during prolonged estrous cycling.

Extended periods of estrous, while relatively rare, have been documented in equids. Tropical species, for example, are less strictly seasonal, with some reproducing throughout the year (Grubb, 1981; Churcher, 1993). In addition, substantial variability in the cycling schedules and receptivity of individual mares (Asa et al., 1979), and the performance of estrous behavior and copulatory activities during the non-breeding season (Asa et al., 1980) have been documented in temperate species. Since the implementation of contraception, at least one winter birth, and therefore winter copulation, has occurred on Shackleford Banks (Susan Stuska, National Park Service, Cape Lookout National Seashore, personal communication). These variations in receptivity, ovulatory schedules, and foaling suggest that the seasonality of reproductive behaviors in *E. caballus* females has the potential to be quite plastic. As contracepted animals have experienced a significant alteration to their physiological state, extended cycling is even more feasible. Future work on Shackleford will test for additional estrous periods in PZP-treated mares by assaying total estrogens and progestins in fecal samples (Asa et al., 2001) during the fall and winter months.

Alternatively, mares may perceive the failure to conceive as a problem with the harem male. This perception alone may be sufficient to cause the observed differences in behavior, regardless of differential estrous cycling. Mares that did not conceive during the prior summer may then switch groups more often during the winter in an effort to prepare for the upcoming breeding season. Such group changes are likely to be less costly in the post-breeding season, as the spacing between band members increases and male herding and aggression decline during this period (Stevens, 1990). This seasonal decrease in harem male attentiveness may have contributed to the observed numbers of group changes and groups visited during this study. Given the strong relationship between contraceptive status and mare fidelity, however, it is unlikely that season is the sole cause of mare behavior. Still, additional study during the breeding season (April–August) is recommended to assess whether the changes in mare behavior result from an interaction between season and contraceptive status.

4.2. The effects of pregnancy

Mare movement between groups is normally rare (see Sections 1 and 2.2). The results of this study strongly suggest that pregnancy and, possibly lactation, are important components to that stability. Regardless of contraception status, pregnant and/or nursing females tend to change groups less often, and over time, mares with a greater proportion of years pregnant are less likely to change groups. These decreases in pregnancy (and possibly lactation) may be the mechanism by which PZP treatment increases the propensity to change groups.

Additionally, decreased pregnancy and increased group switching have the potential to feedback on each other, resulting in even lower overall stability. Increased group switching has the potential to decrease mare fertility via male harassment. The more moves females make, the more male harassment they tend to receive (Rubenstein and Nuñez, 2008). Such harassment can lower female reproductive success, as measured by the number of offspring surviving to independence (Rubenstein, 1986; Rubenstein and Nuñez, 2008). As evidenced by our results, such decreases in pregnancy increase the likelihood that females will change groups. These cascading effects have the potential to adversely affect entire populations (see Section 1) and are worth serious consideration when making management decisions.

4.3. Management implications

If feral horse populations are to be maintained in the most natural state possible, we suggest that a small population of mares never be inoculated with PZP. Although the control mares' effects on group structure on Shackleford Banks have yet to be fully determined, the results clearly demonstrate that they are more faithful to their harem males than are contracepted mares. Mare fidelity to the harem male is important to overall harem stability. As such, it is likely, especially when one considers the sociality of these animals, that control females afford a stabilizing influence not only to individual harems, but also to the entire herd.

We also suggest that the subset of animals designated for control status be more fully representative of female demography. For example, at the time of this study, all control mares on Shackleford were between 8 and 15 years of age (see Figs. 1 and 2). This distribution does not currently afford for the behavior of very young or very old animals. Time can always provide for older individuals, but younger controls are needed to approximate the female population's age structure and natural behavior. For instance, contracepted, dispersing, subadult females likely move between more harems than they would naturally. Therefore, this demographic may adversely affect the entire herd's stability level. Allowing some portion of these animals to disperse, join harems, and reproduce normally could help to stabilize population behavior and structure.

Reevaluating the scheduling of PZP administration may also prove beneficial. An inoculation schedule that allows mares to conceive and give birth may help to ameliorate the most deleterious behavioral effects of PZP. Inoculating females every second and even every third year significantly reduces pregnancy in Shackleford Banks horses (see Section 2.3) and other wild populations (Turner et al., 2007). Contraception on such schedules will keep pregnancy rates low, but will allow for the birth of a manageable number of individuals which, according to this study, have a stabilizing influence on female behavior. Additional research is needed to determine if such contraception schedules will limit population size effectively. If so, this could provide a cost-effective means of controlling animal numbers while maintaining their natural behavior.

The broader management implications of this research are substantial. PZP has been reported to have little to no effect on the behavior of wild horses, specifically, but also wild ungulates in general (Kirkpatrick et al., 1996, 1997; Powell, 1999). The results of this study refute those claims, and in fact, highlight the pitfalls of generalizing recipient and group responses to PZP from one population to another. Moreover, these data emphasize the necessity of

study during all stages of the animals' reproductive cycle to determine the effects of contraception on social behavior. Managers of feral horse and other ungulate populations must use caution in basing contraceptive decisions upon data collected only during the breeding season and from a few, separate populations. Regardless of the ecological and sociological similarities between sites, subtle differences in factors such as demography, ready access to resources, and, as this paper suggests, seasonality, may prove important. Among different populations, such factors may shape the physiological and behavioral effects of PZP in unique and potentially unpredictable ways. Finally the trade-offs between managing population size and maintaining animal health and well-being are worth serious consideration. For social species such as the horse, such consideration is crucial if managers are to maintain behaviorally functional populations.

5. Conclusion

In this study, mares contracepted with PZP behaved differently from control mares. They changed groups more often, visited more groups, and both exhibited and initiated more reproductive interest. These differences in behavior have the potential to adversely affect the stability not only of individual harems, but the entire population on Shackleford Banks, North Carolina. Additional study into the mechanism behind these behavioral differences and into the scheduling of PZP administration will help ameliorate these effects.

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Lingering effects of contraception management on feral mare (*Equus caballus*) fertility and social behavior

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Due to the extirpation of their natural predators, feral horse populations have expanded across the United States, necessitating their management. Contraception of females (mares) with porcine zona pellucida (PZP) is a popular option; however, effects to physiology and behavior can be substantial. On Shackleford Banks, North Carolina, USA, treated mares have exhibited cycling during the non-breeding season and demonstrated decreased fidelity to the band stallion, but PZP's long-term effects on mare physiology and behavior remain largely unexplored. After the contraception program was suspended in this population, we examined how prior exposure to varying levels of PZP treatment impacted (1) foaling probability and foaling dates (a proxy for ovulatory cycling) from 2009 to 2014 and (2) mare fidelity to the band stallion and reproductive behavior during 2013 and 2015. Additionally, we evaluated the effects of time since the mares' last treatment on these factors. Mares receiving any level of prior PZP treatment were less likely to foal than were untreated mares. Among mares that received 1–3 PZP applications, foaling probability increased with time since last treatment before declining, at ~6 years post-treatment. Mares that received 4+ applications did not exhibit a significant increase in foaling probability with time since last treatment. Moreover, previously treated mares continued to conceive later than did untreated mares. Finally, mares previously receiving 4+ treatments changed groups more often than did untreated mares, though reproductive behavior did not differ with contraception history. Our results suggest that although PZP-induced subfertility and its associated behavioral effects can persist after the cessation of treatment, these effects can be ameliorated for some factors with less intense treatment. Careful consideration to the frequency of PZP treatment is important to maintaining more naturally functioning populations; the ability to manage populations adaptively may be compromised if females are kept subfertile for extended periods of time.

Key words: Contraception, feral horse, immunocontraception, management, prolonged subfertility

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Introduction

The extirpation of predator species in North America has precipitated the expansion of free-ranging ungulate populations (Eberhardt *et al.*, 1982; McCullough *et al.*, 1997), escalating human–wildlife conflict (Conover, 1995). Perhaps the most well-known example is that of increasing white-tailed deer (*Odocoileus virginianus*) populations across the United States.

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These deer damage crops, feast on suburban ornamentals, spread disease, and are involved in both vehicle and train collisions (Conover, 1997), resulting in human injury, the loss of human life and costs amounting to more than 1 billion USD of property damage per year (Mastro *et al.*, 2008). Elk (*Cervus canadensis*) populations have also increased in some areas, with severe ecological impacts. In the Yellowstone ecosystem, unfettered elk populations have been associated with an ecological cascade of diverse effects including a reduction in available forage (Ripple and Beschta, 2003) and subsequent decreases in various vertebrate species including grizzly bears (*Ursus ortcos*) (Ripple *et al.*, 2014) and various songbird species, including the Common Yellowthroat (*Geothlypis trichas*), Lincoln's sparrow (*Melospiza lincolnii*), and Warbling Vireo (*Vireo gilvus*) (Baril *et al.*, 2011). Feral horse populations have also greatly expanded across the United Sates. In the west, feral horses and livestock compete for forage and water (BLM, 2011), while in the east, feral horses can negatively impact barrier island plant (Furbish, 1980) and estuarine (Levin *et al.*, 2002) communities. Managers have few options with which to control these populations (e.g. culls, removals), and fewer still that are acceptable to the public (Hobbs *et al.*, 2000; Porton, 2005). Consequently, contraceptive management has become increasingly popular (Hobbs *et al.*, 2000; Porton, 2005).

A commonly used contraceptive in female ungulates, porcine zona pellucida (PZP), stimulates the production of antibodies that bind sperm receptors on the egg's surface, thereby blocking fertilization while still allowing ovulation and the associated cycles of sex hormones (Sacco, 1977). Because PZP does not affect hormone levels directly, it was previously suggested that PZP treatment did not significantly affect recipient behavior or physiology (Kirkpatrick *et al.*, 1996, 1997; Powell and Monfort, 2001; Turner *et al.*, 1997). However, research on white-tailed deer, elk and feral horses has shown that there can be unintended effects on reproductive behavior (McShea *et al.*, 1997; Heilmann *et al.*, 1998; Nuñez *et al.*, 2009; Madosky *et al.*, 2010; Ransom *et al.*, 2010) and physiology (Nuñez *et al.*, 2010; Ransom *et al.*, 2013); in feral horses, these effects can be exacerbated in mares receiving higher numbers of total and/or consecutive PZP applications (Madosky *et al.*, 2010; Nuñez *et al.*, 2010). Briefly, studies investigating the response of free-ranging elk and white-tailed deer to PZP treatment indicate that recipient females extend reproductive behaviors into the post-breeding season (McShea *et al.*, 1997; Heilmann *et al.*, 1998). Authors suggest that in response to repeated unsuccessful mating attempts, females continue cycling in an attempt to gain additional reproductive opportunities (McShea *et al.*, 1997; Heilmann *et al.*, 1998). In feral horses, PZP-induced subfertility was also correlated with reproductive cycling in the non-breeding season (Nuñez *et al.*, 2010; Ransom *et al.*, 2013), increases in the amount of reproductive behavior directed towards mares by band stallions (Ransom *et al.*, 2010), and an increase in group transfer and reproductive behavior by mares (Nuñez *et al.*, 2009; Madosky *et al.*, 2010). Few studies have documented how permanent such side-effects may be (Kirkpatrick, 1995; Madosky *et al.*, 2010; Ransom *et al.*, 2013) and to our knowledge, none have investigated whether the intensity of PZP management can influence female physiology and behavior after the suspension of treatment in managed populations.

Here, we examine the rate at which mares on Shackleford Banks, North Carolina, USA are able to return to their physiological and behavioral baselines after varying levels of contraception management and with increasing time since treatment. Specifically, we monitored per capita births and the effects of varying levels of PZP treatment on the likelihood of conception in individual mares, and foal birth dates from 2009 to 2014, during which time the contraception program was largely suspended. In addition, we examined mare group transfer and reproductive behavior in the summers of 2013 and 2015 to determine whether PZP-induced subfertility had any long-term behavioral effects on previously treated mares. This information is critical to understanding the full effects of long-term subfertility on individuals and populations, and will be integral to designing best-management practices for PZP contraception.

Methods

Study area

Shackleford Banks is a barrier island ~3 km off the coast of North Carolina, USA. The island measures ~15 km in length, and varies between 0.5 and 3 km in width. The Shackleford horses are of Spanish origin and were likely first introduced ~400 years ago (Conant *et al.*, 2012). Currently, the population is co-managed by the National Park Service and the Foundation for Shackleford Horses (National Park Service, 2004). In 1997, federal law was instituted mandating the maintenance of at least 100–110 horses on Shackleford Banks (United States Congress, 1997). The National Park Service and the Foundation for Shackelford Horses limit the population to ~120 individuals, to reduce damage to the ecosystem.

Study subjects

The reproductive units of Shackleford horses are typical of feral equids, consisting of coherent bands of one, or sometimes two or three stallion(s), with one to several mares and their offspring (Rubenstein, 1981). Double-male bands are present, though they make up a small portion of the Shackleford population (5–10% of bands during the course of this study). In addition, differences in the degree of band territoriality have been associated with the island's varying ecology, making the region in which animals reside a reliable proxy for social variation in this population (Rubenstein, 1981). Though the bands are predominantly non-territorial, individual bands are spatially distinct from one another (Feist and McCullough, 1976; Rubenstein, 1981, 1986). Interactions that occur between different bands typically involve younger individuals engaging in exploration and/or play, the dispersal of sub-adult individuals

(both male and female), male–male assessments and/or contests, and the transfer of mares from one band to another (personal observation). Historically, band membership has been long lasting with most changes involving the dispersal of immature individuals. Band stallions sometimes fought to acquire mares from other groups, but stallions almost always retained their mares (Feist and McCullough, 1976; Rubenstein, 1981). More recently, PZP-induced subfertility has been associated with decreases in mare fidelity to the band stallion, increases in mare reproductive behavior, and mare cycling in what is typically the non-breeding season for these mares (Nuñez *et al.*, 2009, 2010; Madosky *et al.*, 2010).

PZP contraception

The National Park Service began the application of PZP for the purposes of immunocontraception in January 2000. In 2009, the program was provisionally suspended because the population was deemed stable (Mason, 2017). The contraception of all but five previously treated mares was halted, and all previously untreated mares of reproductive age were given one dose and booster of PZP. All treatments were administered by the National Park Service from late February through April each year. Each injection contained 100 micrograms of PZP plus an adjuvant (mixed at the darting site). Initial doses contained Freund's Complete Adjuvant, Modified, *Mycobacterium butyricum* (Calbiochem #344 289). All subsequent boosters contained Freund's Incomplete Adjuvant (Sigma #F5506).

For the animals in this study, PZP deterred pregnancy in 99.91% of cases when administered during the same year (Stuska, 2014). These values are similar to those published for Assateague horses, in which PZP deterred pregnancy in 94% of cases (Turner *et al.*, 1997). Over the course of the contraception program, ~19% of pregnancies were due to contraception failures. The contraception histories for the mares considered in this study are displayed in Table S1.

Pregnancy and foaling

Fecal samples are collected by the National Park Service in January of each year. All pregnancy testing is completed by enzyme immunoassay of fecal material at the Science and Conservation Center at ZooMontana in Billings, Montana. Using the methods of Kirkpatrick *et al.* (1991), water extracts of fecal samples are assayed for estrone conjugates and nonspecific progesterone metabolites. In addition, the National Park Service has tracked the foaling records, including foal birth dates, for each mare since 2000 (Stuska, 2014). These data were used to supplement assay results.

Behavioral and demographic sampling

Behavioral and demographic sampling was conducted primarily by two observers (C. Alvarez in 2013 and H. Carr in 2015) and was supplemented with data from three additional observers (B. Streater, 2013; M.A.F. Kent and K.E. Monroe, 2015). All observers were trained by C.M.V. Nuñez. We observed behavior in the majority of reproductive mares (aged 4 years and older) present during the study. We recorded group changing and reproductive behavior for 73% (43/59) and 95% (55/58) of mares in 2013 and 2015, respectively. The number of bands we observed comprised 68% (13/19) and 95% (18/19) of bands on the island in 2013 and 2015. Comparable to previous studies (Nuñez *et al.*, 2009; Ransom *et al.*, 2010), we observed mares for 7 weeks each year from mid-June to late August, totaling 350.15 hours of observation, averaging 3.13 h per mare (range = 1–6.05, SE ± 0.12). Horses were identified individually by color, sex, age, physical condition and other distinguishing markings including freeze brands. Ages are known from long-term records for the identified horses of Shackleford Banks (Nuñez, 2000).

We located each band an average of 0.87 times each week (range = 0.28–1.83, SE ± 0.03). We recorded its GPS location and composition, paying particular attention to the presence or absence of females. Group transfers were recorded if a mare was (a) not in the band she had been seen with previously, or (b) in a different band than she had been seen with previously. Mares did not need to remain with the 'new' group for a minimum length of time for the behavior to be considered a group transfer since even short-lived changes can affect band stability (Madosky *et al.*, 2010) and mare stress physiology (Nunez *et al.*, 2014). We remained with each group for a minimum of 30 min to ensure that individuals recorded as absent were not actually nearby, but out of our sight; when compared to 60-min field observations, 30 min ensured accurate data collection and allowed for larger sample sizes (Nuñez *et al.* 2010). We rarely witnessed mare transfer activity directly ($n = 1$); therefore, mare absence from a band was an important metric with which we measured the number of mare transfers between groups. On average, transfer behavior was confirmed by the mares' presence in new bands within 12.32 days of mare absence (range = 2–31, SE ± 1.44).

All incidences of reproductive interest (including copulation, mounting, genital sniffing, and rump rubbing) directed to and initiated by mares were recorded *ad libitum* during scan sampling (Altmann, 1974) as in Nuñez *et al.* (2009).

Statistical analyses

We analyzed data in R version 3.1.1. We used a linear mixed effects model (nlme package; Pinheiro and Bates, 2000) to determine whether the number of births had increased after the suspension of the contraception program ($n = 157$ total births; 112 births during the program; 45 births after program suspension). All births, regardless of mare age and treatment status at the time of foaling, were included in this analysis. Models included year as a random effect and the following fixed effects: mare treatment (previously treated/untreated), timing (during/after the contraception program), the interaction between mare treatment regime and timing, and a function allowing different variances across groups. We analyzed our data in both raw and arcsine-square root

transformed formats as per Ahrens *et al.* (1990). The arcsine-square root transformation has been shown to correct deficiencies in the normality of error terms, homogeneity of variance, and additivity of percentage data (Ahrens *et al.*, 1990).

To determine whether a mare's contraception history (number of total treatments received) affected her ability to foal in the years following contraception suspension, we used a generalized linear mixed model with a penalized quasi-likelihood (PQL) function (package MASS (Venables and Ripley, 2002)). The GLMM accounts for intra-subject correlation and individual heterogeneity (Jang and Lim, 2006) while the PQL function has proven reliable for the analysis of independent counts (Dean *et al.*, 2004). Models included mare identity as a random effect and the following fixed effects: linear and second-order polynomial functions of mare age (to account for both linear and non-linear effects on the likelihood of foaling), mare contraception history (0 treatments/1–3 treatments/4+ treatments (Kirkpatrick and Turner, 2002), n = 68 mares, Table S1), and island region.

To test for differences in mares' average foaling date with contraception history, we used linear mixed effects models, including a function allowing different variances across groups, and the following fixed effects: linear and second-order polynomial functions of mare age, mare contraception history (0 treatments/1–3 treatments/4+ treatments), and island region. We considered 37 births from 24 mares for this analysis (see Table S1).

To examine mare group changing and reproductive behavior, we analyzed the number of group changes mares made and the number of instances of reproductive interest both initiated and received by mares (n = 59 mares) over two field seasons conducted in 2013 and 2015 (see Table S1). We used linear mixed effects models including mare as a random factor, and the following fixed effects: linear and second-order polynomial functions of mare age, mare contraception history (0 treatments/1–3 treatments/4+ treatments), and island region. All behavioral data were Poisson distributed and log-transformed for analysis.

Parameters were removed from models by backwards elimination if $P > 0.05$. For each analysis, final models were run including linear and second- order polynomial functions of time since last treatment; for this analysis, only mares receiving at least one PZP treatment were considered (see Table S1). Mares less than 4 years of age and the 5 individuals that received treatment between 2009 and 2014 were excluded from all analyses save our per capita birth analysis (see above). In addition, where applicable, models were run both with and without mares belonging to double-male bands. Excluding these mares did not significantly change our findings; therefore, the models presented here include mares belonging to both single- and double-male bands. Where appropriate, we selected best fit models using Akaike's Information Criterion adjusted for small sample size (AICc) (Burnham and Anderson, 2002).

Results

Births

Not surprisingly, yearly per capita births were considerably lower for treated mares than for untreated mares during the contraception program (Linear Mixed Effects Model: mare treatment regime (previously treated); estimate = −0.41, SE = 0.08, t = −5.30, P = 0.0003). This pattern did not change from 2009 to 2014 when the contraception program was largely suspended; previously contracepted mares continued to exhibit subfertility even after the cessation of treatment (Linear Mixed Effects Model: treatment group (previously treated) * timing (after contraception suspension); estimate = 0.07, SE = 0.13, t = 0.50, P = 0.62, Fig. 1). Results were similar with arcsine-square root transformed data (Ahrens *et al.*, 1990) (Linear Mixed Effects Model: mare treatment regime (previously treated); estimate = −0.46, SE = 0.10, t = −4.61, P = 0.0007; treatment group (previously treated) * timing (after contraception suspension); estimate = 0.13, SE = 0.17, t = 0.74, P = 0.50). AICc analysis suggested that models without a variance function were a better fit to the data (AICc without variance function = 5.97 (raw), 24.19 (arcsine transformed); AICc with variance function = 8.89 (raw), 28.20 (arcsine transformed)).

Contraception history and foaling after contraception suspension

Even while controlling for the effects of mare age (Generalized Linear Mixed Model, mare age: estimate = 0.59, SE = 0.27, t = 2.20, P = 0.03; mare age^2: estimate = −0.02, SE = 0.01, t = −2.18, P = 0.03), previously treated mares were less likely to give birth during the study period, regardless of the number of prior treatments received (Generalized Linear Mixed Model, number of treatments (1–3 treatments): estimate = −3.00, SE = 1.24, t = −2.40, P = 0.02; (4+ treatments): estimate = −4.19, SE = 1.27, t = −3.30 P = 0.001, Fig. 2). Island region had no effect on the probability of mare foaling (all $P > 0.20$). These results cannot be explained by inherent subfertility in mares slated for treatment; prior to contraceptive treatment, all mares exhibited equivalent fertility (Generalized Linear Mixed Model, treated vs. untreated: estimate = 0.36, SE = 0.43, t = 0.84, P = 0.41, Table S2).

In addition, we sought to determine whether time since last treatment impacted the probability of foaling in previously treated mares. We found that mares previously receiving 1–3 treatments exhibited an initial increase in foaling probability, followed by a subsequent decrease (Generalized Linear Mixed Model, time since last treatment: estimate = 2.03, SE = 0.43, t = 4.67, $P < 0.00001$; time since last treatment^2: estimate = −0.18, SE = 0.04, t = −4.07, P = 0.0001, Fig. 3A). Conversely, mares receiving 4+ treatments did not exhibit a discernable increase in foaling probability even after several post-treatment years (Generalized Linear Mixed Model, time since last

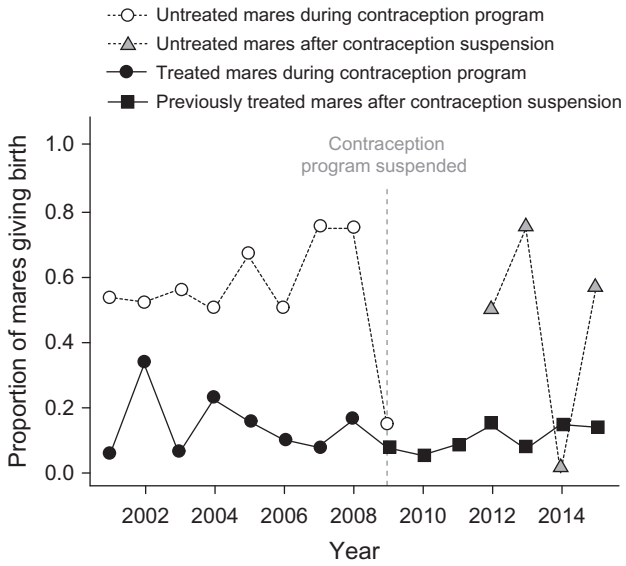


Figure 1: Proportion of mares giving birth in the years during and after contraception management. During the contraception program, treated mares (black circles) showed markedly lower birth rates than did untreated mares (open circles). In 2009, all untreated mares received PZP, leaving no untreated animals of reproductive age on the island in 2010 and 2011. Even after the suspension of treatment, previously treated mares (black squares) did not return to a level of fertility comparable to untreated mares before 2009 (open circles) or to younger, untreated animals that became reproductive between 2012 and 2015 (gray triangles).

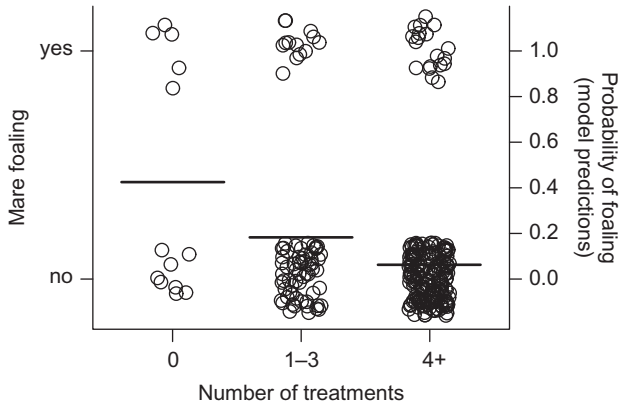


Figure 2: Probability of foaling and contraception treatment; circles represent individual births. Mares previously receiving any level of treatment were less likely to give birth than were untreated mares.

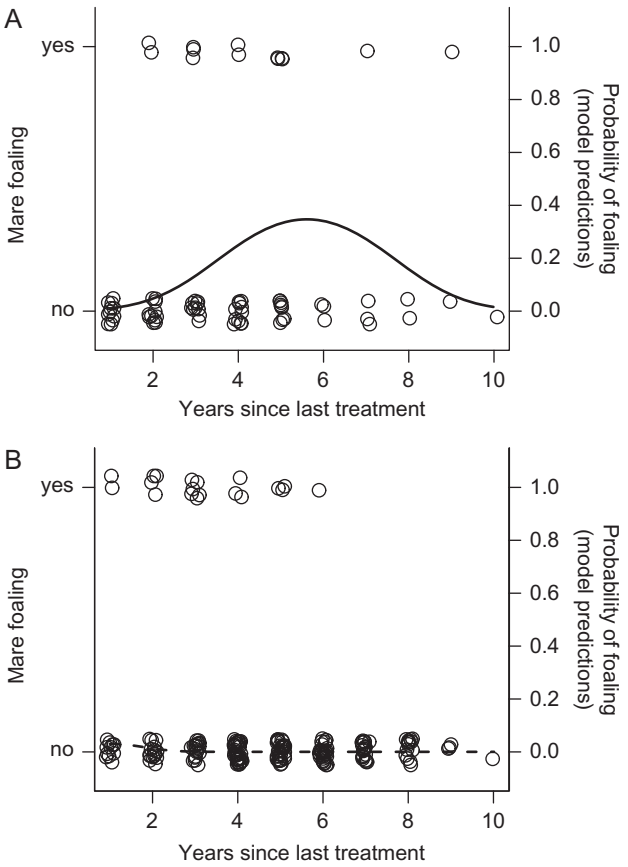


Figure 3: Probability of foaling after contraception suspension and mare treatment; circles represent individual births; solid and dashed lines indicate model predictions. Mares previously receiving 1–3 treatments (**A**) showed a gradual increase in foaling probability, peaking at ~6 years post-treatment, followed by a subsequent decrease. Mares previously receiving 4+ treatments (**B**) did not show a significant increase in foaling probability regardless of time since last treatment.

treatment * number of treatments (4+treatments): estimate = −0.79, SE = 0.25, t = −3.14, P = = 0.002, Fig. 3B).

Foaling date

Mares previously receiving any level of treatment gave birth later in the year than did untreated mares (Linear Mixed Effects Model, number of treatments (1–3 treatments): estimate = 2.29, SE = 0.78, t = 2.92, P < 0.00001; (4+ treatments): estimate = 3.50, SE = 0.90, t = 3.88, P < 0.0001, Fig. 4). Mares having received 1–3 treatments and 4+ treatments did not differ in their average foaling date (Tukey Honest Significant Difference Test: estimate = 1.21, SE = 0.87, z = 1.39, P = 0.34, Fig. 4). Island region and linear and second-order polynomial functions of both time since last treatment and mare age proved insignificant (all P-values > 0.08). Results suggested that models including a variance function were a better fit to the data (AICc with variance function = 155.56; AICc without variance function = 163.02).

Mare fidelity and reproductive behavior

Contraception history had a significant effect on mares' group changing behavior. Even after controlling for the effects of mare age (Linear Model: estimate = −0.04, SE = 0.01, t = −3.04, P = 0.004), mares that previously received 4+ contraceptive treatments changed groups more often than did

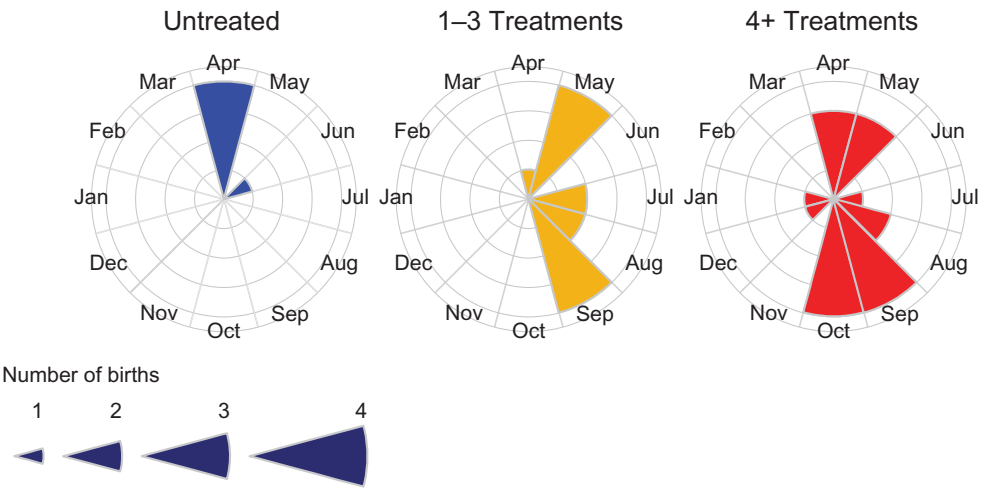


Figure 4: Average foaling date for untreated mares vs. previously treated mares. Mares previously receiving any level of treatment foaled later in the season than did untreated mares. Mares previously receiving 1–3 and 4+ treatments did not differ in their average foaling date.

untreated mares (Linear Model: estimate = 0.75, SE = 0.27, t = 2.80, P = 0.007, Fig. 5). Mares that previously received 1–3 treatments were equivalent to both untreated mares (Linear Model: estimate = 0.43, SE = 0.26, t = 1.66, P = 0.10, Fig. 5) and those receiving 4+ treatments (Tukey Honest Significant Difference Test: estimate = 0.32, SE = 0.15, z = 2.04, P = 0.10, Fig. 5). Island region, linear and second-order polynomial functions of time since last treatment, and second-order polynomial functions of mare age proved nonsignificant (all P-values > 0.08). Results suggested that models without variance functions were better fits to the data (AICc without variance function = 206.55; AICc with variance function = 207.62).

Contraception history did not affect mare reproductive behavior (Linear Mixed Effects Model: all P-values > 0.10). Results suggested that models without variance functions were better fits to the data (AICc without variance function = 131.49; AICc with variance function = 133.76). Island region, linear and second-order polynomial functions of both time since last treatment and mare age proved insignificant (all P-values > 0.07).

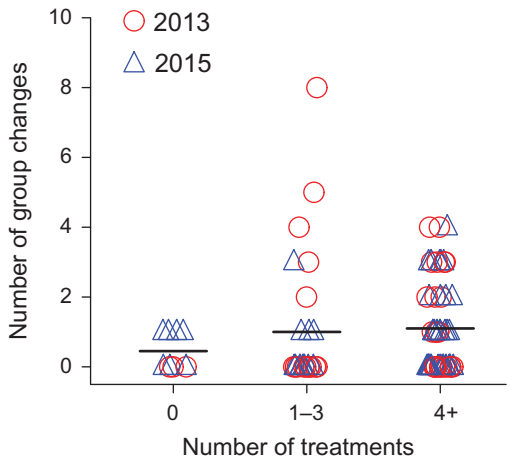


Figure 5: Number of group changes for untreated mares vs. previously treated mares in 2013 (red circles) and 2015 (blue triangles). Mares previously receiving 4+ contraceptive treatments made more group changes than did untreated mares. Mares previously receiving 1–3 treatments did not differ from untreated mares or those receiving 4+ treatments. Segmented lines indicate mean values.

Discussion

We investigated the likelihood that previously treated mares on Shackleford Banks, North Carolina USA, would return to baseline fertility, foaling dates and behavior after the cessation of contraceptive treatment with PZP. Mares received varying numbers of total PZP inoculations, allowing us to more closely investigate the effects of treatment intensity on mare fertility and subsequent effects to mare behavior. This contraceptive has been administered to several mammalian species including, white-tailed deer (*O. virginianus*) (Miller *et al.*, 1999), elk (*C. canadensis*) (Shideler *et al.*, 2001), black bears (*Ursus americanus*) (Lane *et al.*, 2007), bison (*Bison bison*) (Duncan *et al.*, 2013), and African elephants (*Loxodonta africana*) (Fayrer-Hosken *et al.*, 2000); these species have evolved an array of reproductive behaviors and social systems, which are vital to their survival and well-being. A more complete understanding of PZP contraception's potential long-term effects on recipient physiology and behavior is, therefore, of utmost importance if we are to manage and conserve species in the most effective and ethical manner possible. Feral horses have evolved a complex social structure, the maintenance of which is paramount to the overall health of group members

(Kaseda *et al.*, 1995; Linklater *et al.*, 1999). Careful study of mare physiological and behavioral responses to long-term contraception management can serve as a model for other social species for which the formation and maintenance of social bonds is necessary for survival.

Even when controlling for the effects of mare age on fertility, previously treated mares were less likely to give birth than were untreated mares during the study period. Moreover, the number of treatments that mares previously received affected the likelihood that mares would return to fertility after treatment suspension. Regardless of age, mares that previously received 1–3 treatments were more likely to return to fertility than were mares that received 4+ treatments. These results cannot be explained by inherent subfertility in mares slated for treatment: prior to contraceptive treatment, all mares exhibited equivalent fertility. In addition, time since last treatment was an important predictor of foaling probability for mares that received 1–3 treatments, but not 4+ treatments. Mares receiving 4+ treatments remained largely subfertile even after several post-treatment years. Conversely, mares that received fewer treatments exhibited a gradual increase in foaling probability until ~6 years post-treatment, after which, foaling probability decreased. This decrease was likely due to the typical age-specific reproduction in feral mares (Garrott *et al.*, 1991; Carnevale *et al.*, 1994). Feral mares more than 15 years of age exhibit decreased foaling rates (Garrott *et al.*, 1991) and pony mares at or older than 20 years of age are less likely to ovulate at regular intervals (Carnevale *et al.*, 1994). In this study, mares that previously received 1–3 treatments were, on average, 12.42 years of age in 2009, when they received their last inoculation. At 6 years post-treatment, these mares would have been old enough to exhibit the reproductive senescence typical of the species. However, the fact that mares receiving fewer treatments were able to return to fertility while mares receiving more treatments were not is telling; these results demonstrate that mares can retain more natural physiological functioning with less intense contraceptive treatment.

Foaling date was also was also affected by contraception history. Mares previously receiving any level of treatment exhibited later foaling dates than did untreated mares. The persistence of this effect after treatment suspension may be related to increases in mare condition commonly associated with contraception treatment (Turner and Kirkpatrick, 2002). In domestic mares, increased body condition or 'fatness' has been associated with higher circulating levels of leptin (Fitzgerald and Bates, 2000). Mares exhibiting higher circulating leptin were more likely to exhibit reproductive activity during the winter months, suggesting that leptin may modify mare reproductive response to inhibitory photoperiod (Fitzgerald and McManus, 2000). Treated Shackleford mares exhibit the increased physical condition typically demonstrated by contracepted mares (Nuñez *et al.*, 2010). It may be, therefore, that increases in body condition and concomitant increases in circulating leptin have contributed to prolonged effects on mare cycling even after contraception treatment suspension.

Potential effects of late birth dates to foal survival are difficult to assess on Shackleford Banks. In the past, animals perceived to be in poor condition have been removed from the island and adopted off, complicating the analysis of foal survival. As such, we cannot look to our data with confidence to determine if foal survival is compromised by later birth dates. Notably, foal survival is affected by birth date in western feral horses (Ransom *et al.*, 2013). In these populations, the probability of foal survival decreases as parturition date moves further away from the summer peak in resources (Ransom *et al.*, 2013), though these animals are undoubtedly subject to harsher environmental conditions than are Shackleford horses.

Contraception history also had an effect on mare fidelity to the band stallion. Mares that previously received 4+ treatments changed groups more frequently than did untreated mares. Mares that previously received 1–3 treatments did not differ from either untreated mares or those that previously received 4+ treatments. Combined with our findings on fertility, these results demonstrate that unintended side-effects of PZP treatment have the potential to become long term, i.e. they persist with continued subfertility, even if treatment, per se, has ended. However, our results also show that with less frequent treatment, some of these effects can be ameliorated with time, enabling more flexible population management.

Interestingly, our mare fidelity results are similar to those demonstrated in previous studies conducted during the contraception program (Nuñez *et al.*, 2009; Madosky *et al.*, 2010). In those studies, treated mares transferred groups more often than did untreated mares. These behaviors were linked to the presence of a foal (Nuñez *et al.*, 2009): mares with foals, regardless of treatment status, tended to change groups less often, indicating that mares, having successfully reproduced with a stallion, were more likely to remain with said stallion and/or, that mares with foals required a more stable environment in which to raise offspring. In the present study, we found that mares that had previously received 4+ treatments changed groups more often than did untreated mares. The lower foaling probability exhibited by these mares, even after several post-treatment years, is likely the proximate mechanism behind this behavior, rather than PZP treatment, per se. These results show that changes in mare behavior formerly associated with active contraception treatment have persisted with prolonged subfertility, lending additional support to the assertion that such unintended side-effects can persist, even after active contraceptive treatment has ended. Moreover, such behavior represents a significant decrease in mare fidelity by previously treated mares. Prior to the contraception program, Rubenstein (1981) reported that across the entire island, only 10.8% of mares transferred groups, indicating high band fidelity by females. This is in stark contrast to

what was found in our previous study, during which 53% of mares changed groups (Nuñez *et al.*, 2009), in the 2 years of the Madosky study during which 66% and 73% of mares changed groups in 2007 and 2008, respectively (Madosky *et al.*, 2010), and in our current study, during which 42% and 49% of mares changed groups, in 2013 and 2015, respectively.

Contrary to our results from an earlier study (Nuñez *et al.*, 2009), previously treated mares did not engage in more reproductive behavior than did untreated mares. The disparity in our results may be due to differences in the timing of the two studies: the current study was conducted during the breeding season, when breeding behavior is relatively frequent amongst reproductive individuals; our previous study was conducted during the non-breeding season when breeding behavior is more variable. It is worth noting that in the Madosky study (2011), also conducted during the breeding season, Shackleford mares treated with PZP received more harassment from band stallions than did untreated mares. Taken together, these results could suggest that the amount of reproductive behavior in previously treated mares has returned to baseline levels. However, it is not clear whether 'harassment' (Madosky, 2011) is indicative of reproductive behaviors as we have defined them here. As such, our ability to compare results across time is limited.

Our results demonstrate the potential for long-term physiological and behavioral effects to PZP-treated individuals and that these effects can persist with continued subfertility, even after the cessation of treatment. However, our results also suggest that with less frequent treatment, these effects can be ameliorated with time, enabling more flexible population management.

Our results also contrast with claims that PZP is 'completely reversible'; claims upon which feral horse management decisions have been defended (BLM, 2010). Similar long-term subfertility with intense PZP treatment has been found in feral horse populations living on Assateague Island National Seashore, MD (Kirkpatrick and Turner, 2002), in the closely related, but critically endangered Przewalski's horse (*E. ferus przewalskii*) (Feh, 2012), and in feral horses living in the western United States (CO and WY) (Ransom *et al.*, 2013), indicating that these effects are not limited to specific sites or populations, but are pervasive amongst equids. In the Assateague study, nearly 70% of mares receiving three consecutive PZP treatments failed to demonstrate fertility for 1–4 years post-treatment (Kirkpatrick and Turner, 2002). In the Przewalski's horse, only 45% of 20 mares that had been treated with PZP for three consecutive years demonstrated fertility; those that did foal, did not do so until 6.7 years after their last treatment, on average. Moreover, four of nine females receiving only one treatment (one primer injection and one booster injection) did not produce offspring for 4+ years post-treatment (Feh, 2012). Though the mechanism behind the prolonged subfertility in Przewalski's mares is unknown, some of the Assateague mares demonstrated ovulatory failure (Kirkpatrick *et al.*, 1992; Kirkpatrick and Turner, 2002). In western feral horse populations, previously treated mares were less likely to give birth and gave birth later into the calendar year for up to six post-treatment years, again demonstrating the potential for long-term side effects of prolonged, PZP-induced subfertility (Ransom *et al.*, 2013).

The present study demonstrates that the intensity of treatment is also important: mares previously receiving 4+ treatments are less likely to return to fertility and exhibit decreased fidelity to the band stallion. Mares receiving any level of treatment are more likely to give birth later into the calendar year. Conversely, mares receiving 1–3 treatments are more likely to return to fertility and demonstrate behavior more typical of the species (Feist and McCullough, 1976; Rubenstein, 1981; Berger, 1986). These results largely support our previous assertion that more natural physiology and behavior can be maintained in feral horses with less aggressive contraception schedules (Nuñez *et al.*, 2009).

Results similar to those demonstrated in this study have been documented in several captive species (Penfold *et al.*, 2014). A retrospective analysis, combined with published literature and reliable anecdotal reports, demonstrated that extended periods of infertility in females can induce reproductive changes that negatively impact future fertility. This was the case for individuals treated with a contraceptive agent and also for those left untreated, but separated from male counterparts (Penfold *et al.*, 2014). The mechanisms underlying these impacts are varied, but include endometrial hyperplasia [(Asian (*Elephas maximus*) and African elephants, Seba's bats (*Carollia perspicillata*), felids], uterine insufficiencies [wildebeest (*Connochaetes*)], asymmetric reproductive aging [white rhinoceros (*Ceratotherium simum*)], and enlarged ovaries and exuberant uterine responses [stingrays (*Myliobatoidei*)] (Penfold *et al.*, 2014).

The close association with PZP-induced subfertility and mare behavior demonstrated here points to an important link between animal physiology and behavior that should be considered when implementing any management program. Alterations to an animal's reproductive physiology—for whatever managerial gain—will likely be associated with concomitant changes in behavior (McShea *et al.*, 1997; Heilmann *et al.*, 1998; Nuñez *et al.*, 2009; Gilman *et al.*, 2010; Madosky *et al.*, 2010; Ransom *et al.*, 2010). The effects of these subsequent changes—both to management program efficacy and to animal welfare—ought to be weighed against the perceived advantages of our physiological manipulations.

The broader implications of this research are notable. Because other methods for controlling feral horse populations can have pronounced side effects and are often unpopular with the public, PZP is undoubtedly an important option in a manager's toolkit (Hobbs *et al.*, 2000; Kirkpatrick and Turner, 2003; Porton, 2005). However, our results suggest

that the best use of this tool will balance attaining target population sizes against over-treating individual animals. Although the prolonged effects of PZP contraception outlined here could actually assist managers in keeping population sizes low (in feral horses and other over-abundant wildlife), the use of PZP with small populations and/or those of conservation concern should be approached with caution (Ransom *et al.*, 2014). For instance, the fact that mares do not easily return to fertility after the cessation of treatment could limit the ability of populations to rebound in the face of stochastic declines. In the case of Shackleford Banks horses, prolonged PZP-induced subfertility has likely reduced the ability to adaptively manage the population; stopping treatment has not resulted in increased fertility, particularly for mares that previously received 4+ treatments. Given these results and the prolonged effects of PZP-induced subfertility on foaling schedules and behavior (and the potential for additional effects to non-recipient animals (Ransom *et al.*, 2010)), we suggest that monitoring individual treatment histories, or modeling the statistical probability of multiple treatments per individual, could prove critical in maintaining more natural physiological and social dynamics, all while allowing for maximal flexibility in attaining management objectives.

Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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Preliminary Evaluation of Porcine Zona Pellucida (PZP) Immunocontraception for Behavioral Effects in Feral Horses (*Equus caballus*)

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Successful management of captive populations of wild animals requires effective control of reproduction. Contraception is one tool for controlling reproduction of animals in zoos; however, the options available to the animal manager are limited. Contraceptives vary in efficacy, reversibility, and side effects, and thus may not be suitable for widespread use. One consideration when selecting a contraceptive is its potential for side effects on behavior, especially given the fact that reproduction plays such a prominent role in the biology of any species. To date, there have been few evaluations of contraceptives for behavioral effects, and those that have been conducted have focused on hormone-based contraceptives. This study sought to evaluate a novel method of population control, immunocontraception, for behavioral effects in a population of feral horses. Porcine zona pellucida (PZP) immunocontraception prevents fertilization of ova and does not alter normal hormone secretion patterns. It therefore should leave the animal behaviorally intact in terms of reproductive behavior. The study examined the behavior of 43 sexually mature mares on Assateague Island during the 1997 breeding season and, with help from Earthwatch volunteers, collected observations over a 3-month period. The study found no significant differences between treated and untreated mares in general activity budget, aggression given or received, and spatial relationships relative to the stallion. These preliminary findings indicate that PZP contraception seems to have no acute behavioral effects on the behavior of individuals. The study findings also suggest that PZP could be a desirable

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and effective management tool for captive species in which social behavior plays an integral role in group dynamics. Analyses of group level effects and population level effects are continuing.

One of the many challenges facing zoological parks and aquaria is the management and maintenance of genetically viable populations of endangered species. In theory, these populations will eventually serve as reservoirs of genetic diversity for the reinforcement or reestablishment of "wild" populations of nonhuman animals. To meet this goal, animal populations must be managed efficiently and effectively, given the constraints of space and resources. Breeding must be managed to maximize the production of genetically valuable individuals and to minimize the numbers of either overrepresented or genetically inferior individuals. In cooperation with the American Zoo and Aquarium Association, zoos and aquaria have developed a variety of tools to facilitate the development and execution of successful breeding programs. These tools include studbooks, population management plans, and species survival plans (SSPs) that, in one way or another, contribute to making recommendations about allocation of captive space and pairing of individuals for reproduction. Coordination of breeding programs often involves many animals distributed over many institutions. In general, the institutions participating in a breeding program agree on the means by which to achieve the program's objectives; however, unplanned pregnancies do occur, and variation in litter or clutch sizes produces surplus individuals.

In most cases, the number of offspring required from an individual to maintain a genetically healthy captive population is significantly lower than the potential number of offspring it could produce. Thus, by their very design, programs like SSPs require animal managers to have a variety of tools available to allow for compliance with breeding recommendations (Asa, Porton, Baker, & Plotka, 1996). These tools often must be applied to nonendangered species as well, because they occupy valuable space or consume resources that may be needed for endangered species programs.

Two methods by which breeding can be managed are separation of the sexes and establishment of same-sex groups of individuals. Separating the sexes may lead to negative effects on animal health and behavior (Conway, 1976). The practice of establishing same-sex groups is in its infancy in zoological parks, though some groups have been established and maintained with success (T. Stoinski, personal communication, May 1, 1997). Given the current limitations of these methods, contraception seems to be the best tool available for effective management of reproduction.

NEED FOR RESEARCH AND SHARED INFORMATION

Research involving various contraceptive methods has traditionally been aimed at humans, companion animals, and wildlife. Much less is known about the effi-

cacy and safety of contraceptives in captive exotic species (Knowles, 1986). In 1989, the American Zoo and Aquarium Association (AZA), responding to this lack of knowledge, formed a contraception task force, later formalized as the AZA Contraception Advisory Group (CAG). Its goals include (a) the collection and dissemination of data on the efficacy and safety of contraceptives and (b) the coordination and recommendation of research on contraceptive use in captive exotic species (Wemmer, 1989). The CAG has conducted surveys of zoos to assess the extent of use, efficacy, and effects of various contraceptives in primates, carnivores, and ungulates. These surveys are ongoing and updated as new data come in on an annual basis. A database has been created to find taxon-specific recommendations about which contraceptives should be used (Porton, Asa, & Baker, 1990; Porton & Hornbeck, 1993). Despite the number of different contraceptives available, a captive manager's available options for a given species remain limited because of the inherent characteristics of the contraceptive, its mode of action, and the unique biology of the species. A tremendous need remains for both contraceptive research in exotic animals and data sharing on contraceptive use among institutions (Asa et al., 1996).

Full Understanding of Potential Effects

One aspect of contraceptives about which we know comparatively little is how they affect the behavior of the contracepted individual and the conspecifics with which it interacts. By comparison, our knowledge of how contraceptives affect the physiology of the reproductive process is much greater. Clearly, for recommendations to be made about contraception, we must have a complete understanding of all its potential effects. In general, we know more about how hormone-based contraceptives affect behavior because of the history of research on behavioral endocrinology. However, research in this area is restricted to relatively few taxa and has rarely focused on exotic species. In addition, we know virtually nothing about the potential effects that non-hormone-based contraceptives (e.g., immunocontraception) may have on behavior. I provide a brief discussion of some studies that have examined the effects of contraception on the behavior of captive exotic wildlife. For a more general discussion of contraception in exotic species, see Asa et al. (1996).

Hormone-Based Contraception and Behavior of Captive Exotic Animals

Portugal and Asa (1995) assessed the effects of melengestrol acetate (MGA) implants on behavior in Hamadryas baboons (*Papio hamadryas*). Hayes, Feistner,

and Halliwell (1996) assessed the implants' behavioral effects on Rodrigues fruit bats (*Pteropus rodricensis*). Portugal and Asa's first experiment compared the behavior of female baboons immediately before and following implant insertion. They documented significant decreases in open-mouth grin (an aggressive behavior) and time spent foraging during MGA treatment. In addition, they reported nonsignificant trends toward decreased locomotion and increased time spent immobile during treatment. Portugal and Asa also noted that perineal tumescence did not recede entirely in treated females. In their second experiment, they compared the behavior of female baboons after long-term (2 years) treatment with their behavior after implant removal. They found a significant increase in affiliative behavior received by the females following treatment and a trend toward increased affiliative behavior initiated by the females. All females had gained weight during the treatment period (ranging from a 17.3% to 47.6% increase over starting weights). They also found that perineal swellings never fully subsided, indicating that some tissue hypertrophy may have been permanent. In a study of stumptail macaques (*Macaca arctoides*), Linn and Steklis (1990) found that during treatment with depo-medroxyprogesterone (Depo-Provera), another synthetic progestin, there was a decrease in rates of affiliation and a tendency for higher rates of aggression.

Hayes et al. (1996) observed the behavior of control and MGA-implanted Rodrigues fruit bats during a preimplantation period, 1 month postimplantation, and 6 months postimplantation. During the preimplantation period, there were no differences in activity budget between control and treated bats. At 1 month postimplantation, they found that implanted bats moved significantly more than control bats. At 6 months postimplantation, this trend had reversed; however, both groups showed decreased movement compared to 1 month postimplantation. In addition, Hayes et al. noted within-group changes during these study periods. In an analysis of spatial relationships, they found that at 1 month postimplantation, treated bats spent significantly more time with a nearest neighbor than control bats who tended to roost apart from others. Hayes et al. found no effects of treatment on the age–sex structure of neighbors or in the number of displacements given or received. They saw no effect of treatment on the rate of copulation. The authors suggested that the changes in activity budget between groups were most likely due to changes that occurred within groups and not to implants. They found that implanted females had gained weight (more than 8% of body weight), and 7 of 9 treated bats showed poor regrowth of hair and some scarring over the implant insertion site.

Porcine Zona Pellucida (PZP) Immunocontraception

Immunocontraception relies on the body's production of antibodies that interfere with some critical event in reproduction. Immunocontraceptive vaccines have so far been developed against neuropeptides, gonadotropins (Moudgal et al., 1986),

steroid hormones (Al-Kawafi, Hopwood, Pineda, & Faulkner, 1974), sperm (Primikoff, Lathrop, Woolman, Cowan, & Myles, 1988), and ova (Sacco, 1987). To date, the most promising of these vaccines are those directed against the zona pellucida (ZP; Aitken, Richardson, & Hulme, 1984; Sacco & Yurewicz, 1989).

The ZP is a noncellular layer surrounding all mammalian oocytes. The ZP contains two glycoproteins (designated ZP3 and ZP4) that are thought to serve as sperm receptor molecules (Florman & Wassarman, 1985; Hasagawa, Koyama, Inoue, Takemura, & Isojima, 1992). These glycoproteins are so highly conserved in structure across mammals that the ZP of pigs has been used to block fertilization in various nonhuman primates (Sacco, 1987), rabbits (Wood, Liu, & Dunbar, 1981), dogs (Mahi-Brown, Yanagimachi, Hoffman, & Huang, 1985), and humans (Sacco, Pierce, Subramanian, Yurewicz, & Dukelow, 1987). The basic mechanism of action for these ZP vaccines depends on this structural similarity across taxa. When injected, ZP from pig ova causes the host's immune system to produce antibodies to the ZP. These antibodies bind to the PZP as well as to the ZP on the host's oocytes, effectively blocking sperm binding and fertilization.

Emphasizing Variability

To date, PZP has had variable effects on health and reproductive function, depending on the taxon (AZA Contraception Advisory Group, 1995; Kirkpatrick, Naugle, Liu, Bernoco, & Turner, 1995). Among feral horses, PZP contraception appears to be completely reversible and to have no ill effects on ovarian function when animals are contracepted for fewer than 3 consecutive years (Kirkpatrick, 1995; Kirkpatrick, Liu, Turner, Naugle, & Keiper, 1992). After 3 years of contraception with PZP, effects on ovarian function were noted. Treated mares showed lower ovulation rates and depressed estrogen levels (Kirkpatrick et al., 1992). In order for this contraception to be reversed, antibody titers have to decrease to levels that permit successful ovulation and fertilization. Mares treated for 3 consecutive years and then taken off contraception for breeding have at least a 2-year latency to reproduction (Unpublished U.S. National Park Service data). Seven consecutive years of contraception with PZP severely alters ovarian function and endocrine production (Kirkpatrick et al., 1995). It should be emphasized that immune response is variable across individuals and species, and this variability may be manifested in magnitude or duration of response (Fraser, 1980). Further research on immunocontraceptive techniques should include assessments of factors affecting immune response to vaccines.

McShea, Monfort, and Munson (1997) evaluated the efficacy of PZP immunocontraception and its impact on behavior of white-tailed deer (*Odocoileus virginianus*). Does were divided into one control group and two treatment groups. The two treatment groups differed in the total dose of PZP. Treatment with the

smaller of two doses in this study did not prevent parturition, but it significantly delayed parturition dates in treated does. As in the MGA studies discussed previously, treated does showed significantly more weight gain than control does during the study period. McShea et al. found that males exhibited longer breeding seasons during the first year of their study; in the second year, they noted that the breeding season was not longer but had started over 2 weeks earlier. Among does, contracepted females were more active (exhibited more running or walking) than untreated does, though this difference was only evident during estrus and may have been due to the treated does undergoing one to two nonconceptive cycles before getting pregnant. Treated does also exhibited estrous periods later in the season than control does (McShea et al., 1997).

Heilmann, Garrott, Cadwell, and Tiller (1998) analyzed behavioral responses of free-ranging elk to PZP immunocontraception. They found that sexual interaction rates of treated and untreated cows were similar during the normal breeding season for this species but that rates of sexual interaction remained at breeding season levels for treated cows after the end of the normal breeding period. They also found no differences in proportion of time spent feeding, idling, or moving between contracepted and uncontracepted cows.

Hypotheses Tested

I generated a series of hypotheses about how contracepted and uncontracepted mares might differ in their behavior. I first hypothesized that, if contraception was having a severe impact on behavior, I would expect to find differences in the overall activity budgets of contracepted and uncontracepted mares. My second hypothesis predicted differences in spatial relationships between contracepted and uncontracepted mares relative to the stallion in the harem band. One could imagine that contracepted mares either might (a) be close to the stallion more often because they are continuously cycling during the breeding season and hence would show repeated periods of estrous behavior or (b) be far from the stallion and on the periphery of the group because the stallion might find them unsuitable as mates. Finally, I hypothesized that contracepted and uncontracepted mares would differ in social rank and the amount of aggression they gave and received.

METHODS

Study Animals

With the assistance of Earthwatch volunteers, I conducted behavioral observations on a population of feral horses (*Equus caballus*) living on Assateague Is-

land National Seashore, a 33-mile long barrier island running along the coasts of Maryland and Virginia. Observations were restricted to the Maryland herd, which contains approximately 173 individuals and is essentially unmanaged, except for population control.

Though equid social organization appears closely tied to ecology (Rubenstein, 1981), the typical social group in feral horses is the harem band, which contains one breeding male, several reproductive mares, and their dependent offspring. Harem bands on Assateague occupy overlapping home ranges and are not characterized by territoriality. A dominance hierarchy exists within each harem band, and the stallion may not be the alpha animal (Houpt & Keiper, 1982). Among mares, rank correlates most highly with tenure (Seligsohn, 1987) but also very highly with age (Keiper & Sambraus, 1986; Rutberg & Greenberg, 1990). Female hierarchies tend to be stable with few reversals (Berger, 1986; Keiper & Sambraus, 1986). In 1997, nine bands of horses were observed from June 1 to September 5. These bands contained 43 sexually mature mares.

National Park Service personnel and outside researchers had documented significant impacts by the horses on the island's vegetation during the 1980s. Concern over the size of the herd led to the development of a plan to reduce the population using the immunocontraceptive, PZP. Widespread inoculation of the herd began in 1994, though some mares had been treated previously as part of research on PZP's reversibility. Of the 43 mares observed in this study, 24 were contracepted and 19 were uncontracepted during the breeding season of 1997, though all mares in the study had been contracepted for some period in the past. Contracepted mares had been on contraception for an average of 2.54 consecutive breeding seasons (range = 1 to 5). Eighteen of the 19 uncontracepted mares had been on contraception for the previous 3 breeding seasons (1994–1996) and were taken off treatment either to decrease potential side effects of longer term treatment or to allow for breeding.

Behavioral Observations

Observations were conducted from 7 a.m. until 7 p.m. and were approximately balanced across time of day. Each band was observed twice per week on average. Observers received an average of 2.5 days of training in behavioral observation techniques specific to this project. Observers were left to work on their own only after they had achieved a criterion of 85% reliability with the principal investigator. At least two observers were assigned to observe a band during an observation period.

Focal animal observations (Altmann, 1974) were conducted with scan sampling of each sexually mature mare in the band—defined as 2 years old and beyond (Keiper, 1985). Mares were observed in random order. Every 5 min, the identities

of the nearest neighbor to the focal mare and the stallion were recorded. The nearest and most distant adult mare to or from the stallion was also recorded on the 5-min mark. Every 10 min the activity of every group member was recorded. Some of the behavioral categories used in these observations were grazing, standing, resting, locomotion, grooming, and social. Social included any behavior in which the focal animal was interacting with any other animal (including sexual behavior, aggression, mutual grooming, and nursing). In addition, on the 10-min mark, the identities of the most distant animal and most distant sexually mature mare in the group were recorded. Determining which individual had the greatest distance between him or herself and his or her nearest neighbor (who could be any age or sex) scored this. In cases where distances between horses appeared equal, we tried to determine which individual was most distant from the *core* of the group. Core was defined as the subgroup containing the most individuals. All occurrences of sexual, aggressive, and affiliative behaviors were recorded.

Data on aggressive encounters and their outcomes were used to rank individuals in a hierarchy according to the method of Schein and Fohrman (1955). A dominance index was then calculated for each mare that would allow for comparison of mares across differently sized bands (Clutton-Brock, Guinness, & Albon, 1982). Each band, except for 1 band of 3 uncontracepted mares, contained both contracepted and uncontracepted mares. There was no relation between age and contraception status (Mann–Whitney $U = 233, p = .5144$) nor between group size and proportion of mares in the group that were contracepted ($r_s = .1952, n = 8, p = .643$). For these reasons, data for contracepted and uncontracepted mares were pooled across bands. Nonparametric statistical tests were used, and significance was assessed when $p < .05$.

RESULTS

Contracepted and uncontracepted mares had virtually identical activity budgets for most behaviors scored. There was a trend for contracepted mares to be engaged in more social behavior than uncontracepted mares; however, there were no statistically significant differences for any of the behavior categories scored (Table 1).

A mean percentage of scans in which a female was the nearest mare to the stallion and the most distant female from the stallion was calculated for each mare. These were then scaled by group size. There were no significant differences for either measure between contracepted and uncontracepted mares (stallion's nearest female: $U = 116, p = .1661$; most distant female from stallion: $U = 144, p = .6105$; Figure 1).

Mean dominance index was compared between contracepted and uncontracepted mares. Each of three mares who switched bands during the study was

TABLE 1
Activity Budgets of Contracepted and Uncontracepted Mares

	Percent Time		
Behavior	*Uncontracepted*	*Contracepted*	*p*[a]
Graze	51.42	54.26	.1012
Rest	33.35	31.87	.2764
Stand	4.98	4.42	.3654
Locomote	8.88	7.15	.1388
Groom	0.86	1.19	.3517
Social	0.47	1.10	.2688

[a]Mann–Whitney *U* Test.

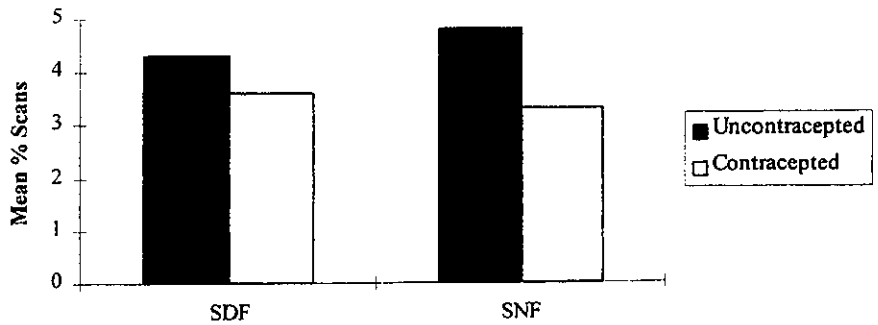


FIGURE 1 Mean percentage of scans in which a mare was the stallion's nearest mare (SNF) and most distant mare (SDF), scaled by group size.

treated as two separate data points because her social rank could potentially change on entering a new group (Seligsohn, 1987). There was no significant difference in mean dominance index between treated and untreated mares ($U = 253, p = .8339$). There also was no significant difference in aggression received ($U = 206.5, p = .7338$) or aggression given ($U = 179, p = .3016$) between contracepted and uncontracepted mares (Figure 2).

DISCUSSION

Previous studies of MGA contraception and one study of PZP contraception have found differences in activity budgets between contracepted and uncontracepted animals (Hayes et al., 1996; McShea et al., 1997; Portugal & Asa, 1995). I found no differences between treated and untreated feral horse mares in this study. There was a nonsignificant trend for treated mares to spend more time en-

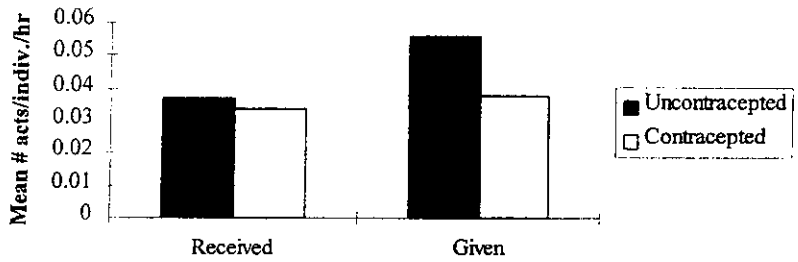


FIGURE 2 Comparison of rates of aggression between contracepted and uncontracepted mares.

gaged in social behavior. This finding might be expected, given that treated mares undergo repeated nonconceptive cycles and thus demonstrate estrous behavior at regular intervals. Heilmann et al. (1998) demonstrated that PZP-treated elk cows showed higher rates of sexual interaction outside the normal breeding season, though their rates were similar to those of untreated cows during the breeding season. Their findings of no significant impact on overall activity budget are in agreement with the results of this study, although fewer categories were scored. Studies of horse behavior on Assateague before implementation of the contraception program indicated that the horses spent an average of 50% of their time foraging (Keiper, 1985). Studies of other populations of feral equids have found that the percentage of time spent grazing ranges from roughly 35% to 70% during the summer months (June, July, August), depending on the habitat (Berger, 1977; Rubenstein, 1981). The results of these studies indicate that both the contracepted and uncontracepted mares in this study have activity patterns that are similar, at least in terms of grazing, to feral horses that have never been exposed to contraception.

Spatial Relationships Unaffected

PZP immunocontraception did not appear to affect spatial relationships between mares and stallions in this study. Contracepted and uncontracepted mares were equally as likely to be the closest or most distant mare from the stallion in the harem band. This may indicate that the contraception status of a mare is not discernable to other individuals in the group. PZP treatment did not appear to affect social rank or rates of aggression given or received in this study. Rutberg and Greenberg (1990) reported aggression rates ranging from .6 to 1.9 aggressive acts per sampling hour in this population before contraception. During the 1997 breeding season, I observed an average of 1.94 aggressive acts per sampling hour.

As mentioned earlier, current data for this population of horses indicate that a mare who has been on PZP for 3 years probably requires 2 years to become fertile again. To date, all mares who have been on PZP for 3 consecutive years and then taken off have become pregnant within 3 years post-treatment. Kirkpatrick (1995) reported that antibody titers only remained high enough to provide contraception for 1 year; thus an annual booster must be given to ensure continued contraception. All but one of the uncontracepted mares in this study had been off PZP only a little more than 1 year. If antibody titers were still high, however, these mares may still have been behaviorally contracepted. Currently, it is unclear how high titers must be to suppress reproduction without affecting behavior. Although much is known about how behavior affects the immune system, there is a paucity of information on how the immune system might affect behavior. Most studies addressing this topic have focused on the effects of exposure toxins rather than non-infectious proteins, and these studies have used neonates of selectively bred individuals as test subjects (Granger, Hood, Ikeda, Reed, & Block, 1996).

A control group of untreated (unexposed) mares was not available for this study. At the beginning of observations, virtually every mare on the island was either on PZP or had been exposed to the vaccine previously. On Assateague, young females are treated in either October of their first year or March of their second year to prevent conception during their second summer. Observations of these females before treatment would not serve as a proper control because they would not have been sexually mature. For most of the behavioral variables reported here, similar values have been reported either for this population before contraception or for other populations of feral horses. However for more complex analyses, valid comparisons probably cannot be made. This underlines the need to conduct baseline studies before interventions of any kind are taken with populations of animals either in the wild or in captivity. A baseline study that addresses behavior and, in many cases, ecology is necessary to identify unambiguously the effects of intervention later. National parks and refuges typically do not have the resources in terms of staff and time to conduct these kinds of studies, which require many hours of observation. Therefore, it is imperative that they solicit local universities, zoos, and other research institutions for assistance so that correctly controlled studies can be done.

Duration of Breeding Behavior Extended

These data should not be taken to mean that PZP contraception has absolutely no effects on the behavior of feral horses. By default, this contraceptive very likely extends the duration of breeding behavior. Normally, mares would get pregnant during a foal heat or in one to two cycles; however, PZP-treated mares continually undergo nonconceptive cycles and thus demonstrate estrous behav-

ior throughout the season. Similarly, stallions continue to tend and mate with mares until they cease to cycle in the fall. Whether this extension of the breeding season has negative effects on behavior remains to be seen. For Hamadryas baboons, it has been suggested that contraceptive methods that allow for continued, nonconceptive cycles should be avoided (Portugal & Asa, 1995). The veterinary literature has also documented the negative effects of repeated exposure to a female's own endogenous hormone cycles associated with ovulation. Immunocontraceptives that do not affect hormone levels (e.g., PZP) and allow for normal ovulatory cycles should be used with caution.

Effects on Sociosexual Behavior

Contraceptives may also have more complex effects on sociosexual behavior. Females who fail to become pregnant after repeated ovulations may seek out new mates (e.g., subordinate males), thus disrupting group stability and order. In situations where only a portion of the females in a group are contracepted, a contraceptive that suppresses estrous behavior may intensify male–male competition for those females who show normal sexual behavior (Asa et al., 1996). Contracepted females may also be aggressive toward other females and their infants.

This is a preliminary evaluation of this contraceptive. Data are currently being analyzed to determine if activity budgets differ between treated and untreated mares during the estrous period. Longitudinal fecal samples were collected from a sample of mares to assess ovarian function and reversibility. Behavioral data will be matched with hormonal data to determine if contracepted and uncontracepted mares exhibit similar patterns of sexual behavior. Data are also being analyzed to assess group stability. Membership in feral horse bands tends to be stable; however, females do change bands occasionally. These membership changes may be temporary or permanent. The National Park Service has conducted a monthly census of the herd for the past 9 years. During the census, membership in each band is recorded. I am currently trying to determine if contracepted mares are more likely than uncontracepted mares to change bands. One could envision a scenario in which a mare leaves a band because she repeatedly fails to get pregnant by a certain stallion. Similarly, a stallion might expel a seemingly barren mare from the harem. Finally, data from the 1997 and 1998 breeding seasons are being analyzed to determine if there are within-individual differences in behavior that are attributable to contraception.

Research Participation and Information Exchange

We clearly need more studies of contraception in wild and captive exotic animals. Many of the methods currently used are still considered experimental—

even for humans. The current data strongly indicate that there is considerable individual and species variability in contraceptive dose, efficacy, and safety (Asa et al., 1996). A contraceptive that effectively halts reproduction while disrupting social behavior may not be desirable to animal managers or humane to the treated animals. For this reason, it is also important for behavioral effects to be included in assessments of contraceptives. If we are to improve and validate our recommendations for the use of contraceptives in exotic wildlife, there must be greater participation in research trials coordinated by the Contraception Advisory Group, and there must be more exchange of information among zoos, wildlife agencies, and university researchers.

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Assessment: Effects of Porcine Zona Pellucida Immunocontraception on Estrous Cyclicity in Feral Horses

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The study used noninvasive fecal hormone monitoring to assess whether contraception with porcine zona pellucida (PZP) had an impact on estrous cycle characteristics in a herd of feral horses living on Assateague Island, Maryland. The study assayed longitudinal fecal samples, collected over a 2-year interval, for total estrogens (estradiol and estrone) and progestagens. Follicular (6.4 ± 2.4 days) and luteal (11.2 ± 3.2 days) phase durations were within the range reported for domestic horses, with an average estrous cycle duration of 18.6 ± 2.5 days. There were no differences ($p > .05$) in phase durations between currently treated and currently untreated mares in either year of the study. Endocrine data suggested that ovulatory failure occurred frequently in Assateague mares; however, we could not link this phenomenon directly to contraception. Overall, no estrous cycle characteristics differed between currently treated and currently untreated mares. However, all mares in this study had been treated with PZP at some point in the previous 10 years. Because PZP has been shown to induce decreased ovulation rates in several *mammalian taxa*, including Assateague horses, we speculate that in addition to blocking sperm binding PZP may adversely impact ovarian function in mares.

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The zona pellucida (ZP) and its potential role in contraception have received substantial attention (Skinner, Timmons, Schwoebel, & Dunbar,1990). Immunocontraception relies on the body's production of antibodies that interfere with critical events in reproduction. To date, vaccines have been developed against neuropeptides, gonadotropins (Moudgal et al., 1986), steroid hormones (Al-Kawafi, Hopwood, Pineda, & Faulker, 1974), sperm (Primikoff, Lathrop, Woolman, Cowan, & Myles, 1988), and ova (Sacco, 1987). The most promising vaccines are those directed against the ZP (Aitken, Richardson, & Hulme, 1984; Sacco & Yurewicz, 1989). Injection of foreign ZP into a host stimulates the production of antibodies that prevent sperm binding to ZP sperm receptors (Florman & Wassarman, 1985; Hasegawa, Koyama, Inoue, Takemura, & Isojima, 1992). The structure of these glycoprotein receptors has been so highly conserved among mammalian species that porcine ZP (PZP) can be used to immunize various taxa such as nonhuman primates (Sacco, 1987), rabbits (Wood, Liu, & Dunbar, 1981), dogs (Mahi-Brown, Yanagimachi, Hoffman, & Huang, 1985), and ungulates (Kirkpatrick, Turner, Liu, & Fayrer-Hosken, 1996). Subsequent contraception is achieved when host-produced antibodies physically block fertilization by binding to ZP sperm receptors.

PZP-induced immunocontraception is an attractive option for controlling wildlife populations because, in theory, this form of contraception does not alter hormone secretion and, therefore, is presumed to leave the treated individual behaviorally and physiologically intact. However, many studies of laboratory and free-ranging animals have shown that PZP treatment can cause abnormalities in ovarian steroid production and histology (Bamezai, Das, & Talwar, 1986; Gulyas, Gwatkin, & Yuan, 1983; Hasegawa et al., 1992; Kirkpatrick, Liu, Turner, Naugle, & Keiper, 1992; Kirkpatrick, Naugle, Liu, Bernoco, & Turner, 1995; Mahi-Brown et al., 1985; Sacco, Pierce, Subramanian, Yurewicz, & Dukelow, 1987; Skinner, Mills, Kirchick, & Dunbar, 1984; Wood et al., 1981). Among horses, no abnormalities in ovarian histology were documented after 1 year of treatment with PZP (Liu, Bernoco, & Feldman, 1989); however, feral mares on PZP for 3 consecutive years demonstrated diminished urinary estrogen conjugate levels and lower ovulation rates than untreated mares (Kirkpatrick et al., 1992). The decline in estrogen excretion and ovulation rate continued in mares that had been on PZP for up to 7 years (Kirkpatrick et al., 1995). These data suggest that, in addition to preventing sperm binding, PZP immunocontraception also may have an adverse impact on ovarian follicular development or function, or both.

In this study, estrous cycle characteristics were analyzed in a population of Assateague horses who were part of a long-term contraceptive field trial (Kirkpatrick et al., 1992, 1995). Young mares (age 2 years) are given two injections of PZP 2 to 4 weeks apart to begin contraception (Kirkpatrick et al., 1992). Liu et al. (1989) determined that PZP immunocontraception lasts for 1 year in mares; thus, on Assateague contraception is prolonged by administering an annual booster of vac-

cine. Noninvasive fecal hormone monitoring was used to facilitate consistent sampling in these feral, free-ranging horses. Fecal sampling also permitted behavioral monitoring to be conducted without animal disturbance. Cyclic fluctuations in fecal estrogen and progesterone metabolites are known to reflect ovarian follicular and luteal dynamics, respectively, in a diversity of wildlife species, including horses (Brown, Wasser, Wildt, Graham, & Monfort, 1997). Specific comparisons between currently treated and currently untreated mares in this study focused on examining differences in follicular and luteal phase lengths and ovulation rates. We assumed that mares who did not receive an annual booster prior to the onset of our study during the 1997 breeding season no longer were effectively contracepted. We tested the hypothesis that estrous cycle parameters would differ in currently treated and currently untreated mares because PZP is an immunocontraceptive that should not interfere with estrous cycles or ovarian hormone production. Thus, any effects that PZP may have while a mare is treated in the 12-month period following initial or booster injection should not be evident once treatment has expired—more than 12 months after initial or booster injection.

METHOD

Sample Collection

The study site and population was described previously (Powell, 1999). Fresh fecal samples were collected from individually identified mares (see Table 1) every 2 to 5 days during the following intervals: July 3 to September 4, 1997 (n = 13 mares); and June 9 to July 23, 1998 (n = 12 mares). The terms *currently treated* and *currently untreated* refer to mares that did or did not receive an inoculation with PZP for contraception during the breeding season.

Fecal samples were collected off the ground within 1 to 3 min after animals defecated. A composite sample of each fecal pile was collected to ensure a homogenous sample, and samples were collected only from animals who could be positively identified. No samples were taken from fecal piles on which the stallion urinated. Feces were stored in polypropylene tubes at –20 °C or –80 °C until assay.

Fecal Extraction and RIA

We conducted fecal hormone metabolite assays according to the methods of Wasser, Monfort, Southers, & Wildt (1994). Briefly, ~ 0.025 g of dried, pulverized feces were boiled in 100% ethanol for 20 min and centrifuged for 18 min at 1500 revolutions per minute. The supernatant was decanted into a clean tube, evaporated to dryness, reconstituted in 1 ml methanol, and then diluted 1:10 with phosphate-buffered saline for the progestagen radioimmunoassay (RIA; Brown, Wasser, Wildt, & Gra-

TABLE 1
Age, Reproductive History, and Contraceptive Status of Feral Mares Sampled for Behavior and Fecal Hormone Metabolites on Assateague Island

ID	1998 Age	Foaled Previously	Year Sampled	On PZP	On PZP During Sampling
N6BIQ	6	N	1997	1994–1996	N
N6BP	7	N	1997, 1998	1994–1996	N
T5AFP	7	N	1997, 1998	1994–1996	N
N9BFO	8	N	1997, 1998	1994–1996	N
N6BKO	8	Y	1997, 1998	1994–1996	N
N9BFN	9	Y	1997, 1998	1994–1996	N
T6M	10	Y	1997, 1998	1994–1996	N
T6	28	Y	1997	1994–1996	N
T5AF	17	Y	1997, 1998	1994–1996, 1998	N/Y
N6BKOS	4	N	1997, 1998	1996–1998	Y
N9BFNS	4	N	1997, 1998	1996–1998	Y
N2BR	5	Y	1998	1995–1998	Y
M6IM	10	Y	1998	1992–1998	Y
N6BK	12	Y	1997, 1998	1994–1998	Y
N6E	18	Y	1997	1988, 1992, 1994–1998	Y

Note. PZP = zona pellucida.

ham, 1994) and diluted 1:10 with steroid diluent buffer for the total estrogens RIA (see following). Tritiated estradiol (~ 6,000 d.p.m.) and ^{14}C-labeled progesterone (~ 1,700 d.p.m.) were added to each fecal sample before extraction to monitor procedural losses. The ^{125}I-progestagen RIA employed a broad-spectrum monoclonal antiserum that cross-reacts with progesterone, 100%; 5α-pregnane-3β-ol-20-one, 96%; 5α-pregnane-3α-ol-20-one, 36%; 17β-hydroxyprogesterone, 15%; pregnenolone, 13%; 5β-pregnane-3α-ol-20-one, 7%; 5β-pregnane-3α, 17α-diol, 20α-one, 5% (Brown, Wasser, et al., 1994; Wasser et al., 1994). Fecal extracts were assayed for total estrogens (unconjugated estradiol-17β [E_2] and estrone [E_1] using a commercially available, double antibody ^{125}I kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA) that cross-reacts with estradiol-17β, 100%; estrone, 100%; estriol, 9%; estradiol-17α, 7%; and equilin, 2.5%. Assays for fecal progestagens and total estrogens were validated using standard techniques to confirm specificity, sensitivity, accuracy, and precision.

Estrous Cycle Duration

We calculated follicular and luteal phase duration estimates using an iterative process described by Brown, Citino, Shaw, & Miller (1994) in which high values for total estrogens or progestagens (considered separately) for each mare

within a sampling year (1997 or 1998) were excluded if they exceeded the mean hormone concentration for all fecal samples plus 2 standard deviations. After exclusion of these values, calculations were iterated until no further outliers (i.e., values that exceeded baseline) were identified. The remaining values were used to approximate baseline estrogen or progestagen concentrations. Follicular and luteal phase duration estimates were obtained by regressing second-order polynomial curves through each outlying point and the adjacent baseline points on either side. The resulting equation was solved for x (day) when y (hormone level) exceeded a 95% confidence interval for the mean baseline value for that hormone. The values obtained were measured to the nearest 0.1 day. The regressions proved to be a good fit for the data (r = .98 for estrogens and .89 for progestagens) and provided more precise estimates for phase lengths than could be obtained by using the fecal samples alone. Phase length estimates are presented as mean ± standard deviation.

Behavioral Data Collection

All occurrences of sexual behavior were recorded using an ethogram modified from Asa, Goldfoot, & Ginther (1979) as part of a larger study of social behavior in this population of horses. For each instance of sexual behavior, we recorded the actor, behavior(s), recipient(s), and any responses. When fecal samples were collected from a group, behavioral data were not formally collected because sampling sometimes produced disturbance in the group (e.g., moving away from the observer); however, ad lib notes were taken on any sexual behavior observed, and a mare was characterized as "in heat" if she demonstrated frequent urination, presenting, or if she allowed the stallion to mount.

Statistical Analysis

Estrous cycle characteristics were compared between years using t tests or Mann–Whitney U tests when the assumption of normality could not be met.

RESULTS

Fecal Extraction and RIA

Fecal progestagens RIA. Serial dilutions of pooled fecal extracts (neat to 1:16,000) yielded displacement curves parallel to those obtained with standard preparations of progesterone. Recovery of unlabeled progesterone (3.75–480 pg/tube) from pooled fecal extracts was 102.0% ± 3.7%. Linear regression analysis of the amount of progesterone measured compared to the amount added, after subtraction of endogenous hormone, yielded a correlation coefficient of 0.98 for the fe-

cal extract ($y = -3.2 + 1.3x$). Assay sensitivity was 1.8 pg/tube, whereas interassay coefficients of variation for high- (~ 30% binding) and low- (~ 60% binding) mass internal controls were 9.8% and 11.1% ($n = 9$), respectively. Intraassay coefficients of variation were less than 10%. Hormone concentrations were expressed as mass equivalents of hormone per gram of dry feces.

RIA of fecal extracts after high performance liquid chromatography (HPLC) revealed at least nine immunoreactivity peaks. One peak (40.7% of total immunoreactivity) coeluted with progesterone, whereas each of the other minor peaks constituted 4.4% to 10.8% of total immunoreactivity.

Fecal total estrogens RIA. Serial dilutions of pooled fecal extracts (neat to 1:16,000) yielded displacement curves parallel to those obtained with standard preparations of estradiol (E_2). Recovery of unlabeled E_2 (1.25–100 pg/tube) from pooled fecal extracts was 106.0% ± 4.5%. Linear regression analysis of the amount of E_2 measured compared to the amount added, after subtraction of endogenous hormone, yielded a correlation coefficient of 0.99 for the fecal extract ($y = 0.16 + 0.97x$). Assay sensitivity was 0.63 pg/tube, whereas interassay coefficients of variation for high (~ 25% binding) and low (~ 67% binding) mass internal controls were 8.5% and 16.8% ($n = 5$), respectively. Intraassay coefficients of variation were less than 10%.

RIA of HPLC-separated fractions of fecal total estrogens showed immunoreactive peaks at fractions 37 to 40 that coeluted with the ^{3}H-E_2 tracer, and this peak accounted for 91.3% of the total immunoreactivity in the fecal pool.

Estrous Cycle Characteristics

In 1997, mean estrous cycle duration was 17.9 ± 2.1 days ($n = 6$ mares showing a follicular phase and subsequent luteal phase) compared to 19.7 ± 3.3 days in 1998 ($n = 3$), giving a composite mean of 18.6 ± 2.5 days. Mean follicular phase duration in 1997 was 6.1 ± 2.3 days versus 6.6 ± 2.5 days in 1998. Mean luteal phase estimates were 11.2 ± 3.3 and 10.5 ± 4.4 days in 1997 and 1998, respectively. A typical estrous cycle is shown in Figure 1.

There was no significant difference in follicular phase durations between currently treated and currently untreated mares in 1997 or 1998: 1997, $t(11) = -0.68$, $p = .51$; 1998, Mann–Whitney $U = 28$, $n = 5, 8$, $p = .35$ (see Figure 2). Luteal phase durations between currently treated and currently untreated mares were not different in either year: 1997, $t(10) = -1.89$, $p = .09$; 1998, $t(5) = 0.27$.

The occurrence of estrous behavior generally coincided with periods of high total estrogens or low progestagens, or both (see Figure 1). The majority (80%, $n = 15$) of mounts and observations of estrus occurred within 1 to 3 days of an estrogen peak or progestagen nadir. All observations of low-intensity sexual receptivity (approaching male, sniffing, proximity) also occurred during these periods. Of the

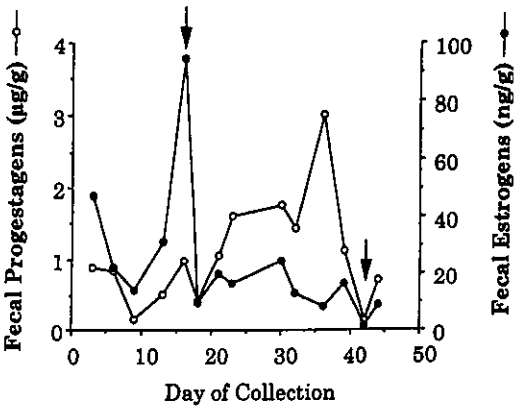


FIGURE 1 A typical pattern of fecal total estrogen and progestagen excretion in an Assateague, Maryland mare treated with porcine zona pellucida for 7 consecutive years. Black arrows indicate observations of estrous behavior.

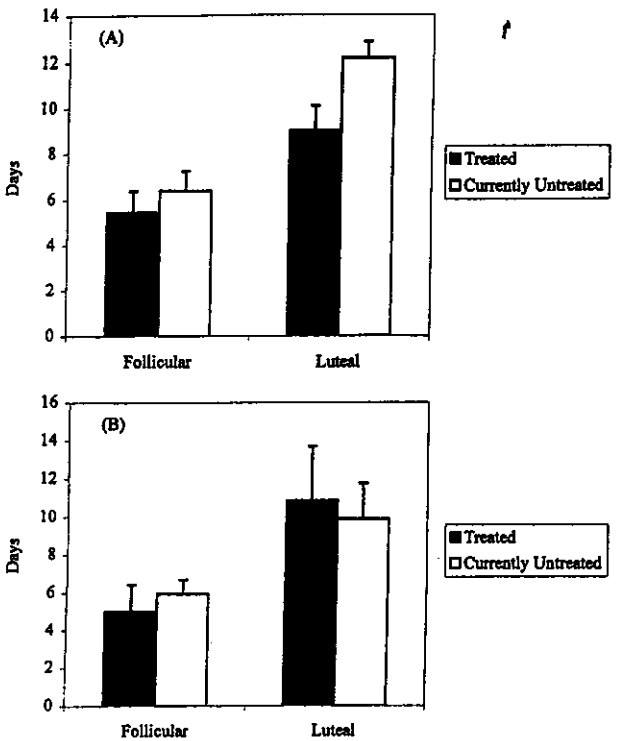


FIGURE 2 Follicular and luteal phase durations in porcine zona pellucida treated and currently untreated Assateague, Maryland mares in the 1997 (A) and 1998 (B) breeding seasons.

15 mares sampled, 7 (47%) showed signs of high-intensity sexual receptivity (frequent urination, presenting, allowed mounting) during the fecal sampling period. Using a logistic regression model, Powell (2000) demonstrated that contraception has no effect on performance of sexual behavior.

Apparent ovulatory failure. Overall, endocrine data from 11 (52%) of the 21 hormone profiles examined (1997 and 1998) suggested ovulatory failure. Presumed anovulatory cycles were characterized by a significant ($p < .05$) increase in total estrogen excretion that was not followed by a corresponding increase in luteal progestagen excretion. The incidence of ovulatory failure was 41.7% in 1997 (5/12): 66.6% (2/3) of currently treated mares and 33.3% (3/9) of currently untreated mares (Fisher exact $p = .60$). Additionally, there was no significant difference in the number of years on PZP treatment between mares that demonstrated ovulatory failure and mares that did not (Mann–Whitney $U = 37.5, n = 5, 7, p = .43$).

The ovulatory failure rate in 1998 (66.6%, 6/9) was not significantly different from the rate observed in 1997 (Fisher exact $p = .71$). Because there was a 7-day gap in fecal sampling in 1998, cases in which the peri-ovulatory estrogen peak immediately preceded the sampling gap were not included because a portion of a luteal phase could have been missed. In 1998, the incidence of ovulatory failure was 60% (3/5) for currently treated mares versus 75% (3/4) for currently untreated mares (Fisher exact $p = 1.0$). As in 1997, there were no significant differences in the number of years on PZP treatment between mares who demonstrated ovulatory failure and mares who did not (Mann–Whitney $U = 16, n = 3, 6, p = .91$).

One mare, N6BP, experienced ovulatory failure in both years, and although luteal activity appeared to precede an anovulatory cycle in 1997, there was no evidence of a luteal progestagen excretion in 1998 (see Figure 3). This mare had been treated for 3 consecutive years but was not treated in 1997. No other females, whether they were treated or not, experienced ovulatory failure in both years.

Of the 11 profiles indicating ovulatory failure, 6 (55%) of these profiles showed significant rises in progesterone at some other point during the sampling period within that year. The 5 remaining profiles were obtained from five different mares, and each of these mares demonstrated normal cycles during the other of the two sampling periods (1997 or 1998). None of the mares in our samples, treated or not, gave birth during the study period.

DISCUSSION

These results and those of Barkuff, Carpenter, & Kirkpatrick (1993) demonstrate that fecal hormone monitoring is useful for tracking ovarian cyclicity and identifying follicular and luteal phases of the estrous cycle in feral horses. Fecal steroid analysis has also been used to track ovarian function in a variety of mam-

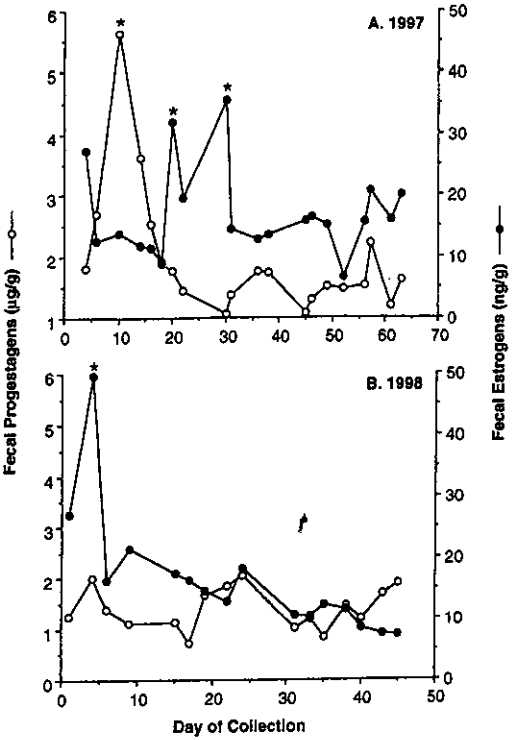


FIGURE 3 Instances of apparent ovulatory failure in mare N6BP in 1997 (A) and 1998 (B). Asterisks indicate data points significantly different from baseline.

malian taxa, including primates, perissodactyls, artiodactyls, and carnivores (Schwarzenberger, Mostl, Palme, & Bamberg, 1996). Assay of fecal progestagens and total estrogens in this population of horses produced patterns of hormone excretion that were similar to those reported for equids using blood or urine (or both) hormone analyses (Ginther, 1992). Lag time between hormone secretion in blood circulation and excretion in the feces was not determined in this study; however, analyses of digesta passage in equines have found an average retention time of 23 hr (Uden, Rounsaville, Wiggans, & Van Soest, 1982); therefore a minimum lag time of approximately 1 day might be expected.

Progesterone accounted for only a small proportion of the total immunoreactive progestagens in feral horse feces, and, based on HPLC results, at least eight other metabolites were detected by our antibody. Schwarzenberger et al. (1996) found that unmetabolized progesterone was virtually absent in feces and generally is metabolized before excretion to several 5α- and 5β-reduced pregnanes with the 20-oxo, 20α-, or 20β-hydroxylated group. In scimitar-horned oryx (*Oryx dammah*) feces, 5α- and 5β-reduced pregnanes were measured, and unmetabolized progesterone contributed a small proportion of total progestagen

immunoreactivity (Morrow & Monfort, 1998). In contrast to progesterone, estradiol and estrone accounted for nearly all of the immunoreactivity in horse feces, and this is in agreement with studies conducted in numerous mammalian species (Schwarzenberger et al., 1996; Brown et al., 1997).

Average estrous cycle lengths reported in this study were generally shorter than those reported in studies of domestic horses, although they were within the range of reported variability (Ginther, 1992). Follicular phase durations for the Assateague mares were very similar to those of domestic mares; however, luteal phase durations were at the lower end of the range reported for domestic mares. These discrepancies may be related to variability introduced by less than daily fecal sampling. Among-individual variability in phase durations in Assateague mares was similar to variation observed in domestic mares (Ginther, 1992). There were no significant differences in phase durations between years or between currently treated and currently untreated mares, although there was a nonsignificant trend toward longer luteal phases in currently untreated mares.

The Assateague mares were contracepted with PZP for 1 to 7 years consecutively, and all showed some evidence of ovarian cyclicity during the 2-year study. Assateague mares appeared to experience a high incidence of ovulatory failure. However, the sample size precluded making a direct link between contraceptive treatment and ovulatory failure in this study. It appears that the apparent ovulatory failure seen in some mares in this study is episodic. Our data show that all mares who demonstrated ovulatory failure did have significant luteal increases in progesterone or normal estrous cycles (or both) at some time during the 2-year sampling period; however, none of the sampled mares foaled. Longer term and more frequent sampling would be required to determine conclusively the rate of occurrence of apparent ovulatory failure in these mares.

Kirkpatrick et al. (1992) found that Assateague mares treated with PZP for 3 years excreted reduced concentrations of urinary estrone conjugates compared to currently untreated mares, and the absence of a luteal phase increase in progesterone metabolites suggested that some treated females were anovulatory. In a later study, Kirkpatrick et al. (1995) found that the percentage of mares excreting normal range concentrations of urinary estrone conjugates declined from 80% for mares treated with PZP for 1 year to 0% for mares treated for 7 years. Hormonal assessments of mares conducted after 1, 2, 3, 6, and 7 years of annual PZP administration revealed that 50% of the treated mares excreted reduced concentrations of urinary estrone conjugates, although most females continued to cycle at least intermittently (Kirkpatrick et al., 1995). Here we present data from a sample of mares assessed in 2 consecutive years. We have demonstrated that the apparently anovulatory condition of some mares is episodic and that the duration of these episodes is variable. Some mares showing ovulatory failure did show evidence of a normal cycle in the same breeding season. Other mares did not show a normal cycle during one breeding season but did so in the other. Contrary to Kirkpatrick et al (1995), we found no association between the number of years of PZP treatment

and the incidence of ovulatory failure. We also have documented one case of a mare showing ovulatory failure in 2 consecutive years.

In summary, ovarian activity in PZP-treated mares (for 1 to 7 consecutive years) ranged from anovulation to intermittent cycles to completely normal estrous cycles (Kirkpatrick et al., 1995; Liu et al., 1989). To date, all mares who have been treated with PZP for 3 years and then taken off contraception have produced healthy foals within 3 years posttreatment (unpublished National Parks Service raw data). However, our data suggest that PZP immunocontraception may not be reversible in all mares after only 1 year. In addition, our data indicate that ovulatory failure appears to be episodic as opposed to chronic in these mares because all mares showed evidence of a luteal phase at some point during the 2-year study.

Ovulatory failure, defined as an increase in estrogen not followed by a sustained increase in progesterone secretion, has been documented in several other mammalian species after treatment with PZP (Bamezai et al., 1986; Gulyas et al., 1983; Mahi-Brown et al., 1985; McShea et al., 1997; Skinner & Dunbar, 1984; Skinner et al., 1984). In the only study to examine PZP-mediated ovarian pathology in mares, Liu et al. (1989) found no histological abnormalities in females ovariectomized 1 year after PZP immunization. However, significant ovarian pathology has been associated with PZP treatment in several other species (Gulyas et al., 1983; Hasegawa et al., 1992; Mahi-Brown et al., 1985; Sacco et al., 1987; Sacco, Subramanian, Yurewicz, DeMayo, & Dukelow, 1983; Skinner & Dunbar, 1984; Skinner et al., 1984; Wood et al., 1981). Histopathological findings range from decreased numbers of oocytes or various stage follicles (or both) to decreased ovarian size and the appearance of abnormal cellular masses. The degree and range of these abnormalities was variable across individuals and species (Sacco et al., 1987), and this may be related to individual or species-specific (or both) differences in immune responsiveness to PZP administration (Liu et al., 1989; Mahi-Brown et al., 1985).

The immune-mediated mechanisms whereby PZP has an impact on ovarian function are not well understood; however, it has been suggested that PZP may interfere with communication between developing oocytes and surrounding follicular cells—dogs (Mahi-Brown, Huang, & Yanagimachi, 1982) and rabbits (Skinner et al., 1990)—or that antibodies to ZP attack the developing occyte when certain ZP glycoproteins are secreted early during occyte development, causing oocyte death: guinea pigs (Hasegawa et al., 1992), dogs (Mahi-Brown et al., 1985), nonhuman primates (Sacco et al., 1987), and rabbits (Skinner et al., 1984; Wood et al., 1981).

A review of the literature suggests that, compared to other mammalian species, horses may be relatively insensitive to the adverse effects of PZP on ovarian function. We are unable to conclude that ovulatory failure in Assateague mares was directly related to PZP contraception because untreated controls were not available when this study was conducted. It is possible that antibody titres were still high enough in our currently untreated mares to affect ovarian func-

tion, making them indistinguishable from treated mares in terms of fecal hormone metabolite excretion. Ovulatory failure is relatively common in domestic horses and may be related to a variety of factors (Ginther, 1992). How common this phenomenon is in feral horses not exposed to PZP remains unknown. However, substantial evidence in other mammalian species provides incentive for conducting additional controlled studies in equids to determine the mechanism of action of PZP and its potential for inducing adverse side effects (ovarian pathology). Without such evidence, it is impossible to evaluate the actual potential of PZP for managing reproduction in wildlife species.

Immunocontraception is an attractive option for population management. Vaccines appear to be long acting and effective at stopping reproduction (Kirkpatrick et al., 1996). PZP does not appear to affect activity budgets of mares, their levels of aggression and sexual behavior, or their spatial relationships with the stallion (Powell, 1999, 2000). Despite these benefits, more research is required before PZP immunocontraception can be recommended for widespread use in wildlife. Long-term (up to 7 years) effects of PZP have only been assessed in equids, and these have been based on hormonal analyses only and not on an examination of ovarian histopathology. Behavioral effects have been assessed in a few studies, but these are restricted to ungulates: moose (Heilmann, Garrot, Cadwell, & Tiller, 1998), white-tailed deer (McShea et al., 1997), and feral horses (Powell, 1999, 2000). Clearly, more integrative research in horses is needed to addresses putative changes in reproductive–endocrine patterns, behavior, ovarian histology, and anatomy.

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Effects of Gonadotropin-Releasing Hormone Immunization on Reproductive Function and Behavior in Captive Female Rocky Mountain Elk (*Cervus elaphus nelsoni*)[1]

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ABSTRACT

Fertility control is a potential method for managing overabundant wildlife populations; however, current technology is limited by duration of treatment efficacy and unacceptable side effects. The objective of this study was to determine the efficacy of a single immunization with gonadotropin-releasing hormone (GnRH) vaccine to suppress reproductive function in pregnant female elk and to evaluate potential behavioral and pathological side effects of treatment. Eighteen captive adult female elk were randomly allocated to one of two experimental groups. Ten females were administered a conjugated and adjuvanted GnRH vaccine intramuscularly, and eight elk received an adjuvant sham vaccine without conjugated GnRH. We compared success of existing pregnancy, neonatal survival, subsequent fertility, reproductive behavior rates, and side effects of treatment between January 2006 and January 2010. The GnRH vaccination did not affect existing pregnancy or calf survival during the year that it was applied; however, it reduced the proportion of pregnant females for 3 yr. Male precopulatory behavior rates exhibited toward GnRH-vaccinated females tended to be greater than those directed at sham-vaccinated females during the second half of the breeding season, when GnRH vaccinates continued to be proceptive. Strong immune and inflammatory responses, including robust GnRH antibody concentrations in GnRH vaccinates, and sterile pyogranulomatous injection site abscesses in both groups, were consistent with vaccination. In conclusion, this GnRH vaccine resulted in prolonged, albeit reversible, impairment of fertility, and is associated with extended reproductive behaviors and partial suppression of hypothalamic-pituitary-gonadal axis function in captive female elk.

elk, fertility control, follicular development, GnRH/GnRH receptor, gonadotropin-releasing hormone, immunocontraception, immunology, ovary, pregnancy, reproductive behavior

INTRODUCTION

Regulating the abundance of elk populations has become a significant issue for natural resource managers in many areas of North America [1]. This is particularly true for protected environments, such as national parks and conservation areas, where unregulated populations, if left unchecked, can have adverse effects on natural and human-dominated systems. Hunting, culling, and trapping have traditionally been used to regulate animal numbers, but there are a growing number of circumstances where these methods pose significant liability [2], and as a result resource managers are seeking alternative approaches to population control [3].

Fertility control offers a potential nonlethal method for controlling the growth of overabundant elk populations, and considerable research has been directed toward the development of different contraceptive technologies [4, 5]. Models have been developed to characterize effects of fertility control on population dynamics of wild ungulates, including elk [6]. Yet, current technologies for altering wildlife fertility suffer from a variety of technical, physiological, and regulatory challenges [4]. As a result, only modest successes have been achieved in developing a safe, practical, and feasible method of controlling reproduction in free-ranging wild ungulate populations.

A potential alternative to current fertility control products, and one that may overcome some of these challenges, involves active immunization against gonadotropin-releasing hormone (GnRH), a small, 10-amino acid neuropeptide produced in the hypothalamus that signals via receptors on gonadotroph cells in the anterior pituitary. Gonadotropin-releasing hormone, along with ovarian- and pituitary-derived endocrine, paracrine, and autocrine signaling, is responsible for gonadotroph function [7, 8], and ultimately gametogenesis [9]. Gonadotropin-releasing hormone is not generally immunogenic but can be made so by conjugation to a large, highly immunogenic carrier protein. When combined with a potent adjuvant, this vaccine stimulates a persistent immune response, resulting in prolonged antibody production against GnRH, the carrier protein, and the adjuvant [4]. Although there are competing theories of action [10], the prevailing hypothesis suggests that antibodies to GnRH induce transient infertility by binding to endogenous GnRH in the hypothalamic-pituitary portal vessels, thus preventing attachment to receptors on gonadotrophs, as well as suppression of pulsatile luteinizing hormone (LH) secretion. Gonadotropin-releasing hormone-antibody titers are correlated with suppression of the reproductive system and infertility in a variety of species [4].

Numerous GnRH vaccines have been developed and are successful in interrupting the hormonal cascade that controls ovulation. These vaccines have been used in physiologic research for decades. However, the use of GnRH vaccines as contraceptive agents in wildlife management has been limited by effective duration, multiple treatments to stimulate adequate antibody response, and deleterious side effects associated with the use of the controversial Freund complete adjuvant (FCA) [4, 5]. Recently, an alternative adjuvant (AdjuVac) containing

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mineral oil and mycobacteria derived from a Johne disease vaccine (Mycopar; Fort Dodge Animal Health, Fort Dodge, IA) has been developed and may be as effective as FCA but is associated with fewer lesions [11].

Based on results from studies in captive and free-ranging wild or feral ungulates, a single application of this GnRH vaccine (GonaCon-B) may prove to be a safe and effective multiyear immunocontraceptive agent for free-ranging wildlife. A single application, formulated with the same or a similar GnRH-protein conjugate, has been shown to provide multiple years of decreased fecundity in several ungulate species, including elk [4, 5, 12]. However, few studies have rigorously investigated the secondary effects of this vaccine on social behaviors or ecological consequences in any wildlife species [5].

Elk are polyestrus short-day ovulators, with the peak of breeding season in North America between mid-September and mid-October [13]. Waves of follicle development occur during the ovulatory [14], anovulatory [15], and transitional [16] seasons. Ovulation occurs approximately every 20–21 days [14], and the ovulatory season can persist as late as April, although it is, on average, 142 days, ending in mid-February [16]. Pregnancy rates for mature females (2–12 yr) can approach one calf per female per year in populations that are not forage limited [17]. Approximately 50% of yearling females become pregnant at 16–18 mo of age if an appropriate body mass is achieved (~220 kg, 10% body fat) [13, 17, 18]. Reproductive senescence is not well described, but fertility may decline by 13–17 yr of age [17]; however, effects of body condition are likely more important to fertility than age [18, 19]. Elk are monotocous, and twinning is exceptionally rare [13]. Calving occurs from late May to early July after a gestation of 247–262 days and coincides with spring nutrient flush in most northern latitudes [13, 20].

Application of GnRH vaccine would be most practical during the winter period (December–February), when elk are concentrated in large, primarily single-sex herds [21], and temperatures and snow cover are conducive to chemical or physical capture [22]. At this time, most adult female elk are in midgestation to late gestation, and any contraceptive treatment must be demonstrated to be safe for the developing fetus and health of the neonate [23].

In light of these issues we examined the contraceptive efficacy and potential side effects of GnRH vaccination by testing the following four hypotheses using captive female elk. A single immunization with GnRH vaccine during midgestation in elk will: 1) not affect existing pregnancy; 2) suppress future fertility, with contraceptive effects waning as GnRH antibody concentrations decrease; 3) suppress reproductive behaviors; and 4) be associated with localized inflammatory reactions but not other pathological side effects.

MATERIALS AND METHODS

Animals and Vaccine

This study was reviewed and approved by the Colorado State University (no. 05-187A-01) and the Colorado Division of Wildlife (no. 07-2005) Institutional Animal Care and Use Committees. Animals were housed at the Colorado Division of Wildlife, Foothills Wildlife Research Facility, in Fort Collins, CO (40°35′46″N, 105°9′29″W). Eighteen female elk (1.5–12 yr of age at study onset; 220–275 kg) and two reproductively proven mature male elk (5–7 yr of age; 400–450 kg) were used for this experiment. The majority of experimental elk were long-term residents of the facility, most having been born in captivity. To meet sample size requirements, two free-ranging 3-yr-old females from a local wild population were captured and brought into the captive herd in the spring of 2005. Female elk were trained for repeated handling in isolation pens, alleyways, and a handling chute, and for blood sampling and ultrasound imaging procedures. Female elk were maintained throughout the experiment in two fenced paddocks (5.0 ha) with minimal native forage and were fed a diet of ad libitum alfalfa-grass hay mix, trace mineral blocks, and water, as well as limited pelleted grain supplement. Male elk were similarly maintained in a paddock, physically removed but within sight of females. During breeding seasons (late September–late November), males were maintained with females. All biological samples were collected and hands-on measurements made while female elk were lightly sedated using xylazine hydrochloride (30–250 mg per animal, i.m.; TranquiVed; Vedco Inc., St. Joseph, MO) in a nonsqueeze chute. Tranquilizer effects were reversed after each sampling session with either yohimbine hydrochloride (30 mg per animal, i.v.; Wildlife Pharmaceuticals, Fort Collins, CO) or tolazoline hydrochloride (600 mg per animal, i.m.; Tolazine; Akorn Inc., Decatur, IL).

Experimental vaccines were prepared as previously described [24]. Briefly, the GnRH vaccine consisted of multiple copies of synthetic GnRH peptide linked to hemocyanin protein (Blue Carrier; Biosonda, Santiago, Chile) from the Chilean mollusk (*Concholepas concholepas* [CCH]) and combined with a water-in-oil adjuvant containing killed *Mycobacterium avium* ssp. *avium*. The sham vaccine was similarly prepared but without conjugated GnRH.

Experimental Protocol

Data were collected between January 2006 and January 2010. At the start of the experiment, all 18 females were pregnant (80–100 days), as determined by serum pregnancy-specific protein B assay [25], rectal palpation [26, 27], and/or transrectal ultrasound [28]. One sham-vaccinated female (20 mo of age) aborted her pregnancy by late February 2006.

Approximately half of the females had been previously exposed to a *Brucella abortus* strain 19 vaccine. To avoid potential confounding anamnestic response effects, which may arise from antigenic stimulation with similar intracellular pathogens (*B. abortus* and *M. avium*) [29] or age-related fertility effects [17], we blocked sample units (female elk) with respect to brucella vaccination history and age (1, 2–10, or ≥11 yr of age) and then randomly assigned animals to either the GnRH vaccine (n = 10) or sham vaccine (n = 8) group. The GnRH-vaccinated females were administered 1.5 mg of GnRH-Blue Carrier protein conjugate with adjuvant (1.5 ml) into the left biceps femoris muscle using a handheld 3-ml syringe and a 3.8-cm, 18-gauge needle. Sham-vaccinated females received a similar volume of carrier protein and adjuvant but without conjugated GnRH. Injection site location was similar in all animals, with placement consistently at the leading edge of the cream-colored rump patch and at the same height as the tuber ischii. To facilitate pretreatment ultrasound examination as described below, hair was removed from the injection site and surrounding skin using electric clippers with a no. 40 blade, and the area was cleaned using isopropyl alcohol.

Females were divided into two pens (each pen with five GnRH vaccinates and four sham vaccinates) and placed in separate paddocks. In late September of each year (2006–2009) one male was placed in each pen for 62–65 days. Females remained in the same pen throughout the study, whereas males were rotated between pens each year to minimize effects due to potential random differences in male fertility and individual male-female interactions.

Pregnancy and Ovarian Measurements

We investigated the effects of GnRH immunization on the existing corpora lutea, pregnancy, and calving (2006) by comparing monthly (January–May 2006) serum progesterone concentrations, calving success, and calf survival and growth. Calves were weighed 12–24 h after birth, and then at 2- to 4-wk intervals for the first 3 mo of life. Calves remained with their dams until weaning on 1 September 2006. Subsequent pregnancy rates were determined each January during a 4-yr period (2007–2010) using methods described above. Once pregnancy was confirmed, abortion was induced using two doses of prostaglandin $F_{2\alpha}$ 6 h apart (25 mg, i.m.; Lutalyse; Pharmacia & Upjohn, Kalamazoo, MI) [30, 31].

We measured ovarian follicular and luteal structures in GnRH- and sham-vaccinated females at single time points the first year after vaccination during the early ovulatory (mid-September), late ovulatory (early January), transitional (late February), and anovulatory (late April) seasons using transrectal ultrasound imaging (5-MHz linear array transducer; Televet 1000; Classic Universal Ultrasound, Tequestra, FL) [14–16]. January imaging occurred coincident with pregnancy diagnosis; at all other time points females were not pregnant. Images were saved on a laptop computer for later evaluation. We measured the total number of antral follicles, follicular diameter (mm), and the presence or absence of ovarian structures consistent with luteal tissue. We then grouped follicles into small (<4 mm), medium (4 to <7 mm), and large (≥7 mm) classes, and calculated total follicular volume combining data from both ovaries for each individual.

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Analysis of Hormone Concentrations and Antibody Titer

Blood samples (10–30 ml) were collected via jugular venipuncture using a 20-gauge blood collection needle, tube holder, and 10-ml blood tubes without anticoagulant (BD Vacutainer SST; Becton Dickinson and Co., Franklin Lakes, NJ). Blood was allowed to clot at room temperature and was centrifuged for 10 min at 1500 × *g*, and serum was decanted to polypropylene tubes and stored at −80°C until assays were performed. Monthly (January–May 2006) serum concentrations of progesterone were measured by radioimmunoassay [32], a technique previously validated in elk [33]. All samples were run in duplicate in a single batch. The range of the standard curve was 0.39 ng/ml (80% ligand-labeled progesterone) to 15.0 ng/ml (20% ligand-labeled progesterone). Intraassay coefficients of variation were 14.1% for the low reference sample and 7.1% for the high reference sample.

Serum GnRH antibodies were measured prior to vaccination, monthly until calving, and prior to introducing males into female pens each year (September 2006–2009). The concentration of unbound GnRH antibodies was estimated by measuring ^{125}I-GnRH binding capacity in peripheral serum [34, 35]. Serum samples were diluted between 1:2 and 1:100 000 using 0.05 M ethylenediaminetetraacetic acid (EDTA) in 0.01 M PBS with 0.1% gelatin (EDTA-PBS gel). A total of 200 μl of diluted test sera, 100 μl of EDTA-PBS gel, and 100 μl of ^{125}I-GnRH (specific activity ~1850 Ci/mmol; ~60 000 dpm) were added to 5-ml glass tubes. Solutions were vortexed and incubated for 24 h at 4°C. One milliliter of 20% polyethylene glycol solution (6000 mw; diluted with PBS) was added, and tubes were vortexed. Tubes were incubated for 20 min at 4°C, then centrifuged for 30 min at 980 × *g*. Supernatants were decanted and tubes placed in a gamma counter (efficiency ~80%; Micromedic Systems, Horsham, PA) to record radioactivity (cpm). Total radioactivity was measured in 100 μl of ^{125}I-GnRH. Nonspecific binding was measured by adding 100 μl of ^{125}I-GnRH to 300 μl of EDTA-PBS gel and handling similarly to test samples. High antibody titer-positive control sera from rabbits [35] and elk previously vaccinated against GnRH (data not shown) as well as negative control elk sera were included with each batch. All samples were analyzed in duplicate. To provide a similar comparison between animals, we selected the 1:1000 dilution to estimate antibody concentration (pmol/ml) and reanalyzed all samples in a single batch. The intraassay coefficient of variation was 3.5%. Gonadotropin-releasing hormone antibody response is reported as picomoles of ^{125}I-GnRH bound per milliliter of serum.

Pathological Side Effects

Mean serum chemistry and hematology parameters, *M. avium* antibody status, and prevalence of injection site reactions were compared between groups. Blood was collected as described above but with the addition of a 10-ml blood tube with EDTA anticoagulant for hematology. Samples were taken prior to vaccination for each assay and then at 30, 90, and 340 days after vaccination for hematology, chemistry, and *M. avium* assays, respectively. Whole blood and serum were submitted to the Colorado State University Veterinary Diagnostic Laboratory (Fort Collins, CO) for analysis. Hematology parameters measured included: total nucleated cells, segmented neutrophils, lymphocytes, monocytes, eosinophils, platelets (all $\times 10^3$/μl), plasma protein (g/dl), red blood cells ($\times 10^6$/μl), hemoglobin (g/dl), packed cell volume (%), mean corpuscular volume (fl), mean corpuscular hemoglobin concentration (g/dl), and fibrinogen (mg/dl). Chemistry profile parameters included: glucose, creatinine, phosphorus, calcium, magnesium, total protein, albumin, globulin, and total bilirubin (all mg/dl); enzymes, including creatinine kinase, aspartate aminotransferase, gamma-glutamyltransferase, sorbitol dehydrogenase (each IU/L); and electrolytes, including sodium, potassium, chloride, and bicarbonate (each mEq/L). *Mycobacterium avium* antibody concentrations were measured using ELISA (HerdChek ELISA; IDEXX Laboratories, Westbrook, ME) and reported as optical density of the sample.

We used palpation and ultrasonography to evaluate injection sites for evidence of localized inflammation prior to vaccination, at monthly intervals until calving (February–May 2006), and prior to each breeding season (September 2006–2009). Injection sites and surrounding skin were shaved as described above. Qualitative changes in skin temperature, erythema, and swelling were assessed by comparing the injection site with adjacent skin. We used ultrasonography to evaluate muscle tissue for subcutaneous and intramuscular signs of inflammation and injury, including lesions such as hematoma, abscess, scar tissue, cellulitis, and myositis, which are associated with characteristic changes in muscle echogenicity and architecture [36–38]. Longitudinal and transverse ultrasound images of skin, fat, fascia, and muscle at the site of injection were collected using a 5-MHz linear array transducer to a depth of 80 mm with a focal zone at 40 mm and were stored on a computer for future analysis. Ultrasound images were described to indicate qualitative degree of change between prevaccination and postvaccination sampling dates in both muscle echogenicity (e.g., hyperechogenic, hypoechogenic) and architecture (e.g., fiber length, fiber pattern). All ultrasound evaluations were performed and read by a single technician. When images were consistent with a well-defined abscess accessible by percutaneous needle aspiration or if a soft, fluid-filled swelling was palpated, it was aspirated, and aerobic, including mycobacterial, cultures and anaerobic cultures were performed. Two abscesses were lanced, drained, and flushed because the welfare of the animal was a concern.

Each animal was observed for the presence or absence of lameness (e.g., limping, gait alterations, reluctance to stand or bear weight on a limb) each time it was moved from pens to the handling chute. Additionally, caretakers observed each female daily during feeding and noted if swelling or discharge was evident at the site of injection and if there were overt signs of lameness.

During the course of the experiment, three GnRH-vaccinated females died because of handling difficulties (n = 1; February 2006) or chronic wasting disease (n = 2; August 2007). Chronic wasting disease is endemic within the facility and was diagnosed in study animals using immunohistochemistry of rectal mucosa-associated lymphoid tissue, retropharyngeal lymph nodes, and brainstem at the level of the obex [39, 40]. Complete necropsies were performed by a veterinary pathologist, and injection sites along with standard tissues (e.g., representative tissues of each organ system) were removed and preserved in 10% neutral-buffered formalin for histopathological evaluation.

Reproductive Behaviors

We compared rates of reproductive behavior interactions (behavior events per hour) of male and female elk from 25 September–12 November 2006 as previously described [33]. Individually identifiable numeric/color-coded collars were placed on each female in both treatment groups. Behaviors were observed during the morning (0400–0600 h), evening (1600–2000 h), and at night (2200–2400 h) from a tower that provided good visibility of both pens. An infrared night vision monocular was used to observe behaviors during dark hours, and a spotlight was used to confirm collar identification. Four technicians similarly trained in behavior identification and blinded to treatment group performed observations. Based on previously reported elk reproductive behaviors, we identified and recorded 13 different interactions associated with harem tending, proceptivity, receptivity, and mating (Table 1) [21, 33, 41, 42]. Only male-female interactions were recorded. All behaviors were attributed to the individual female displaying or receiving the behavior. If the male displayed behavior directed at more than one female (e.g., herd tending) it was recorded as a separate behavior for each female. Because of small sample size, we grouped these specific behaviors into four general behavior categories: general breeding, male precopulatory, female precopulatory, and copulatory (Table 1). A total of 112 two-hour sampling periods were recorded in 47 days during 38 morning (74.1 h; 35%), 43 evening (81.4 h; 38%), and 31 night (58.5 h; 27%) sampling periods. Length of behavioral interactions was typically short compared with sampling interval, so each interaction was recorded as an event. Behavior rates were calculated as events per female per hour, and estimated mean weekly behavior rates were compared between treatment groups.

Statistical Analysis

Yearly pregnancy data are reported as the proportion of pregnant female elk in each treatment group (number of pregnant females in each treatment group/number of females exposed to bulls from late September to late November each year). Pregnancy proportions were compared with one-tailed Fisher exact tests. The difference in proportions was used to estimate treatment effect. We used normal approximations to binomial distributions to compute confidence limits for the differences between proportions.

To evaluate the probability of pregnancy for a given GnRH antibody concentration, we used simple logistic regression with pregnancy status, breeding season, and treatment group as classification variables, and GnRH antibody concentration as the continuous variable (PROC PROBIT; SAS 9.1; SAS Institute Inc., Cary, NC). The probability of pregnancy after treatment with the GnRH vaccine was calculated for a theoretical sample population with an intrinsic pregnancy rate of 1.0 calf per female per year, and for this study's sham-vaccinated control population, which had an intrinsic pregnancy rate of 0.96 during the four experimental years. Using a cutoff value of $\geq$20 pmol GnRH antibody per milliliter of serum to indicate infertility, we estimated the type 1 (false-positive) and type 2 (false-negative) error rates by calculating the specificity (proportion of correctly classified nonpregnant females) and sensitivity (proportion of correctly classified pregnant females) of using antibody concentration as a diagnostic indicator of pregnancy status. Specificity was calculated as the number of true nonpregnant animals indicated by the antibody concentration divided by the total number of nonpregnant females. Sensitivity was calculated as the number of truly pregnant females indicated by the antibody concentration divided by the number of total number of pregnant females. Pregnancy data were combined over 4 yr for specificity and sensitivity calculations.

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TABLE 1. Thirteen individual reproductive behaviors and associated behavior categories observed and recorded 8 to 10 mo after immunization in GnRH-vaccinated and sham-vaccinated elk (*Cervus elaphus nelsoni*).

Behavior category	Reproductive behaviors
General breeding	Male behavior related to establishing, maintaining, and defending a group or harem of female elk (i.e., herding, guarding).
Male precopulatory	Male courtship behavior directed toward an individual female to induce or detect estrus (i.e., Flehmen for urine testing, chivy, sniff and/or lick body, rub body, precopulatory mount).
Female precopulatory	Female courtship behavior directed toward dominant male to arouse copulatory behavior (i.e., sniff and/or lick body, rub body, circle, mount, lordosis).
Copulatory	Male behavior directed toward a receptive female (i.e., intromission).

We tested the hypothesis that mean behavior rates between treatment groups were different using a mixed linear ANOVA model to account for both random (individual animal) and fixed (treatment, date, male, and time of day) effects in a repeated-measures structure (PROC MIXED; SAS 9.1). For each of the four behavior categories, a global model was constructed which included treatment group, time of day, and male, along with all of their first-order interactions. Date was included as an additive trend factor. Next, variance structures, heterogeneous versus homogeneous both within and between individual females, were added to the model, and Akaike Information Criterion adjusted for small sample size (AICc) was used to select the best fit model. Similarly, three covariance structures—none, compound symmetry, and spatial power—were analyzed, and AICc was used for model selection. Finally, after fitting variance and covariance structures, six reduced models were run to find the most parsimonious model to estimate treatment effects. Using the best model, mean behavior rates (±SEM) were estimated using least squares analysis, and hypothesis tests were based on type III generalized estimating equations, which account for sample size imbalance.

Similar methods were used to compare mean (±SEM) concentrations of progesterone, as well as ovarian follicle number, size, and volume. Fixed effects in the analyses were treatment status and date, and individual females were evaluated as a random effect. Differences in calf weight during the first 3 mo of life were analyzed in the same way, with dam treatment status, sire, sex, and age as fixed effects. Differences in proportions of small-, medium-, and large-size follicles were analyzed using a chi-square test with treatment status and date as classification variables (PROC CATMOD; SAS 9.1). A paired Student *t*-test (PROC TTEST; SAS 9.1) was used to evaluate differences in mean optical density readings from *M. avium* ELISA. Descriptive statistics were used to explain the similarity of hematology profiles, presence or absence of luteal tissue during early ovulatory and transitional periods, and occurrence of lesions at the site of injection.

RESULTS

Pregnancy and Calving

Gonadotropin-releasing hormone vaccination did not affect calving success in female elk treated at approximately 80–100 days of pregnancy. Serum progesterone concentrations during the second half of gestation did not differ between treatment groups ($P = 0.849$; Fig. 1). Progesterone concentrations varied by month ($P < 0.001$), but there were no treatment by month interactions ($P = 0.619$). All females, except the single 20-mo-old sham vaccinate that had midgestation pregnancy loss, delivered full-term calves. One calf born to a GnRH-vaccinated dam died during parturition due to dystocia. All calves born alive (n = 15) survived the neonatal period and were weaned at approximately 3 mo of age prior to the 2006 breeding season. Dam vaccination exposure did not affect calf weight at any time point prior to weaning ($P = 0.448$; data not shown).

GnRH Vaccine Efficacy and Duration

Pregnancy proportions in GnRH-vaccinated females were lower ($P \leq 0.05$) than in sham-vaccinated females during the first 3 yr of the experiment (Table 2). Treatment effect decreased between Year 1 and Year 4 ($P = 0.009$), from a high of 0.90 in 2007 to a low of 0.12 in 2010 (Table 2). Antibody concentrations in GnRH-vaccinated elk were detectable 1 mo after inoculation, peaked 3–8 mo after vaccination, and waned during the course of the study (Fig. 2). Gonadotropin-releasing hormone antibodies were not detectable in sham-vaccinated females (data not shown). There was a strong inverse relationship between GnRH antibody concentration and the probability of becoming pregnant ($P < 0.001$; Fig. 3). At 30 pmol/ml free GnRH antibody binding capacity in the peripheral serum, the logistic model predicted a pregnancy rate of 0.10 in a population whose intrinsic pregnancy rate approached 1.0 (Fig. 3). Only one GnRH-vaccinated female with lower than 20 pmol/ml concentrations of antibody (approximately 2–17 pmol/ml) did not become pregnant during the first three breeding seasons. Conversely, one female with consistently high serum antibody concentrations (>20 pmol/ml) did become pregnant during the third and fourth breeding seasons. Using a GnRH antibody concentration of 20 pmol/ml during September as the cutoff point, the assay had a sensitivity of 0.85 and specificity of 0.87 to predict whether a female elk would be infertile during the current breeding season.

Reproductive Behaviors

General breeding and male precopulatory behaviors were the most prevalent interactions recorded. Copulatory behaviors were observed too infrequently for meaningful analysis. Although differences in mean male precopulatory behavior rates only approached significance ($P = 0.073$), those directed toward GnRH-vaccinated females (0.45 ± 0.06 behaviors per hour) were 30% greater than those directed toward sham-

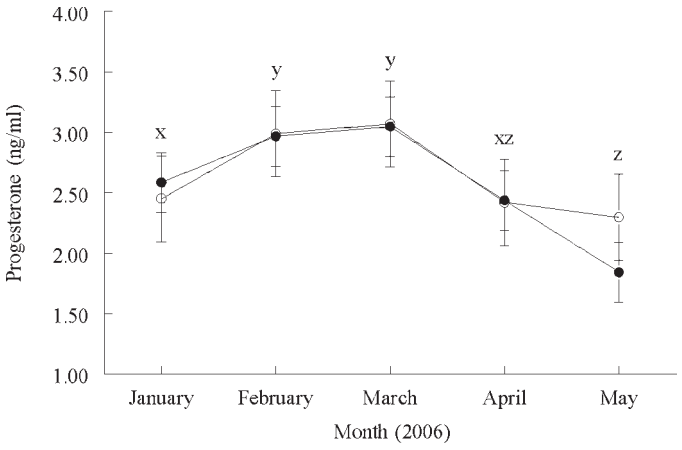


FIG. 1. Mean ± SEM monthly serum progesterone concentrations in GnRH-vaccinated (filled circles; n = 10) and sham-vaccinated (open circles; n = 7) female elk (*Cervus elaphus nelsoni*) between the time of GnRH immunization (January 2006) and parturition (May–June 2006). Means with different letters indicate differences between months ($P < 0.05$).

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TABLE 2. Mean yearly pregnancy proportions (no. pregnant/no. exposed to fertile bull) and estimates of treatment effect size (difference in proportions) with 95% confidence intervals for GnRH-vaccinated and sham-vaccinated female elk (2006–2010).

	Proportion pregnant*		Treatment effect
Yr after treatment†	GnRH-vaccinated [n]	Sham-vaccinated [n]	Difference (95% CI)
0	1.0 [10]^{ax}	1.0 [8]^{ax}	0.0
1	0.10 [10]^{by}	1.0 [7]^{ax}	0.90 (0.71–1.0)
2	0.25 [8]^{byz}	1.0 [7]^{ax}	0.75 (0.50–1.0)
3	0.50 [8]^{byz}	1.0 [7]^{ax}	0.50 (0.15–0.85)
4	0.75 [8]^{az}	0.86 [7]^{ax}	0.12 (0.0–0.29)

* Proportions with different superscripts are significant ($P \leq 0.05$). Letters a and b are between treatment groups within a given year; letters x, y, and z are between years.
† Year 0, 2006 (before treatment); Year 1, 2007; Year 2, 2008; Year 3, 2009; Year 4, 2010.

vaccinated females (0.33 ± 0.06 behaviors per hour). This response became apparent after the initial 2 wk of observation (Fig. 4). Individual males were a significant covariate in male precopulatory behavior ($P = 0.002$), as were date and time of day ($P < 0.001$), and there was an interaction between bull and time of day ($P < 0.001$). Female precopulatory behaviors were not different between treatment groups ($P = 0.720$); however, precopulatory behavior of GnRH-vaccinated females persisted throughout the sampling period, whereas this behavior rate dropped to nearly zero in sham-vaccinated females after the first 3 wk of observation (Fig. 4). Date tended to be an important covariate in female precopulatory behavior ($P = 0.055$). There were no differences in mean general reproductive behavior rates between treatment groups ($P = 0.794$). Time of day and date were significant ($P < 0.001$) covariates for general reproductive behaviors.

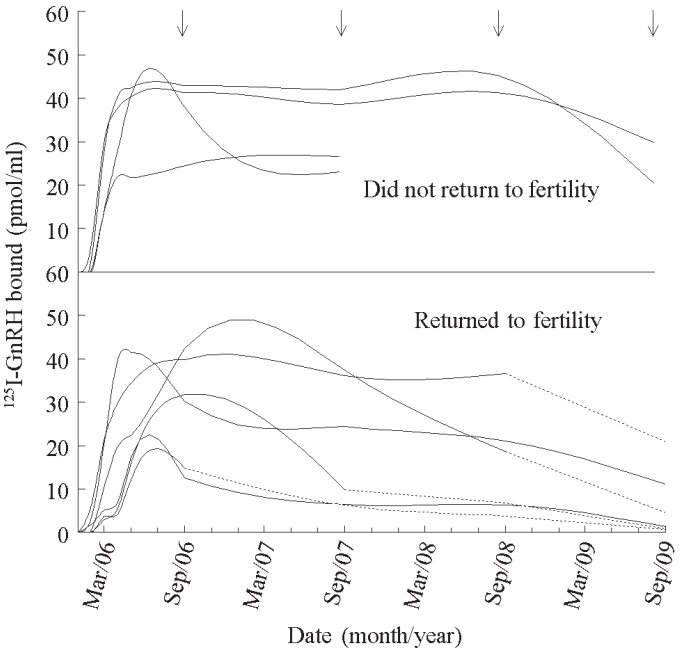


FIG. 2. Persistence of GnRH antibodies measured via ^{125}I-GnRH binding capacity in peripheral blood of GnRH-vaccinated female elk. Top panel shows antibody concentrations in females that did not become pregnant after vaccination (n = 4 of 10) throughout the 4-yr study. Two animals were lost from the study after Year 2. Lower panel shows antibody concentrations from females that either did not experience infertility (n = 1 of 10) or returned to fertility during the study (n = 5 of 10). Solid lines indicate nonpregnant females. Dotted lines indicate pregnant females. Two females became pregnant after antibodies were measured in September 2009. Arrows indicate date when males were placed in female pastures (September 2006–2009).

Pathological Side Effects

Most biochemistry and hematology parameters were within clinically normal ranges for all elk [13]. Two females, one from each treatment group, demonstrated leukocytosis (22×10^3 to 26×10^3 nucleated cells per microliter) 1 mo after injection, which was attributable to neutrophilia (9×10^3 to 12×10^3 per microliter) and lymphocytosis (9×10^3 to 11×10^3 per microliter). The GnRH-vaccinated female demonstrated mild hyperfibrinogenemia (300 mg/dl) and thrombocytopenia (57×10^3 per microliter). An additional four GnRH-vaccinated females had mild hyperfibrinogenemia (300–400 mg/dl). Although all globulin levels were within the clinically normal range (1.9–4.3 mg/dl), serum globulins increased between 0.1 and 0.6 mg/dl at 4 mo after inoculation in every female in both groups.

All animals were seronegative for Johne disease prior to vaccination. One year after injection *M. avium avium* ssp. *paratuberculosis* (MAP) antibody concentrations tended to be greater than zero ($P = 0.059$) in both treatment groups. With one exception, all females showed an increase in MAP antibody concentrations, indicated by an increase in optical density, and 3 (18%) of 17 females had sufficiently robust responses to be classified as seroconverted and Johne disease positive.

Estimated mean (±SEM) number of follicles was greater in GnRH-vaccinated females (5.6 ± 0.44) than sham-vaccinated females (2.46 ± 0.48; $P = 0.005$). Mean size of all imaged follicles in GnRH vaccinates (2.35 ± 0.29 mm; range, 0.3–12.6 mm) was smaller than that of sham vaccinates (4.73 ± 0.84 mm; range, 1.2–16.9 mm; $P = 0.014$). There was no difference in total follicular volume between groups ($P = 0.240$). Date was not a significant covariate ($P \geq 0.05$) in any of the analyses, and there were no date by treatment interactions. The GnRH-vaccinated females had more small follicles (<4 mm) but fewer medium (4 to <7 mm) and large (≥7 mm) follicles ($P < 0.001$) than sham vaccinates. Corpora

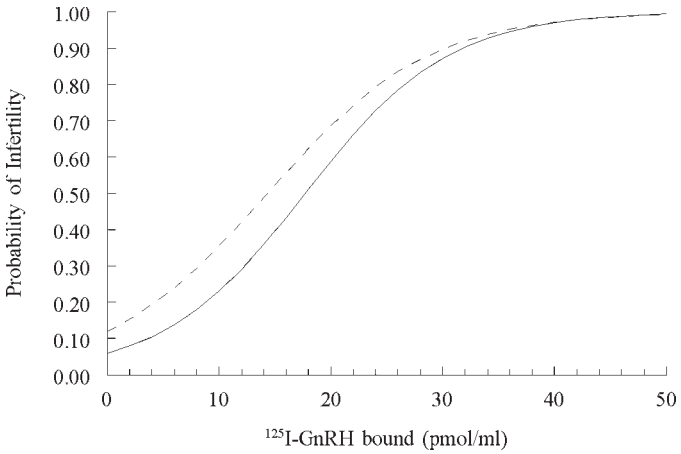


FIG. 3. Predicted relationship between ^{125}I-GnRH binding capacity (antibody concentration) and the probability of infertility in GnRH-vaccinated female elk, modeled with (dashed line; probability of pregnancy if untreated = 0.96) and without (solid line; probability of pregnancy if untreated = 1.0) intrinsic infertility.

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lutea were observed in 6 (27%) of 22 images collected from sham-vaccinated females during early ovulatory and transitional season periods. In contrast, corpora lutea were never observed in GnRH-vaccinated females during the same time periods. Luteal tissue was not observed in any study animal during anestrus.

Between 15 and 52 mo after injection, 6 (35%) of 17 females (n = 4 GnRH vaccinated and n = 2 sham vaccinated) developed clinically apparent abscesses (e.g., large soft swelling with purulent material) at the site of injection. No aerobic, including mycobacterial, or anaerobic bacterial growth was detected in cultures (n = 4). Two of the four GnRH-vaccinated animals described above died of causes unrelated to vaccination (i.e., chronic wasting disease) 19 mo after vaccination. Both possessed large (~500 ml), purulent, multiloculate, encapsulated abscesses at the site of injection. Histopathology revealed pyogranulomatous inflammation and multiple acid-fast bacilli within the capsule and surrounding muscle tissue. The acid-fast bacilli were consistent with mycobacteria. We did not observe lameness at any time point in study animals.

Muscle architecture and/or echogenicity was altered in 10 (~60%) of 17 females (n = 5 from each treatment group). The earliest changes in echogenicity were observed 2 mo after injection; however, the most severe changes, including images consistent with abscessation were seen 5 to 30 mo after vaccination. Regardless of treatment group, there was a wide variation in severity of change, from muscle fiber disruption and diffuse fiber hyperechogenicity to large, hypoechogenic multiloculated areas within the muscle or between fascial planes. All of the animals with clinical abscesses had evidence of muscle fiber disruption in one or more ultrasound images prior to observing external manifestations of the abscess.

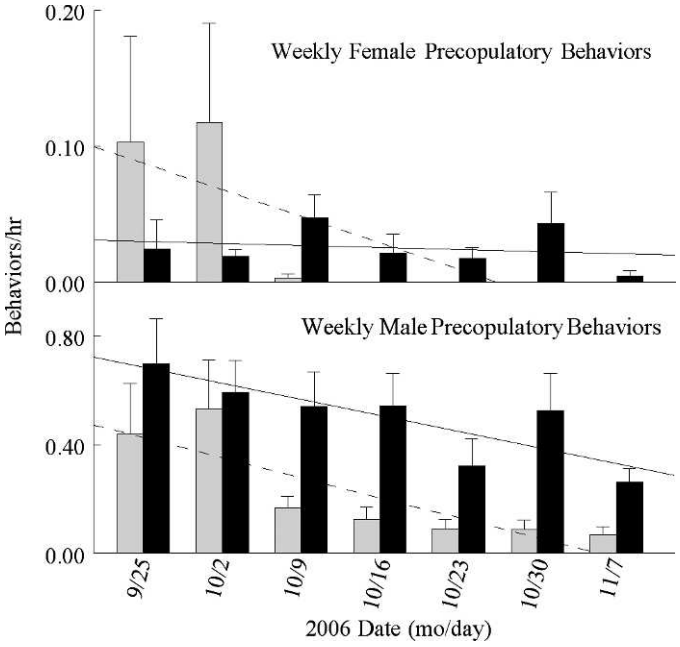


FIG. 4. Mean ± SEM weekly female precopulatory behavior rates demonstrated by GnRH-vaccinated (black bars and solid lines; n = 10) and sham-vaccinated (gray bars and dashed lines; n = 7) female elk (top panel) and mean weekly male precopulatory behavior rates received by female elk (n = 10 GnRH vaccinated; n = 7 sham vaccinated; bottom panel) during the 2006 breeding season (25 September–12 November). Regression lines illustrate persistence of precopulatory behaviors demonstrated and received by GnRH- and sham-vaccinated females.

DISCUSSION

A single vaccination against GnRH during midgestation did not disrupt pregnancy but did decrease the proportion of pregnant female elk for 3 yr after treatment. The efficacy of the GnRH vaccine decreased each year following treatment. These findings supported our predictions that this GnRH vaccine delivered to pregnant female elk reduced fertility, with decreasing efficacy over time. These findings were also consistent, in part, with those reported for nonpregnant female elk treated with GnRH vaccine using keyhole limpet hemocyanin as the carrier molecule rather than CCH [12]. However, in contrast to our measurements, Killian et al. [12] showed a weak inverse relationship between contraceptive efficacy of the vaccine and antibody levels: increasing effectiveness with decreasing antibody titers. Their result is difficult to explain biologically in light of our observations and others [24, 43] that support a strong positive association between infertility and antibody levels. Their finding may be an artifact of small sample size. Despite the association between antibody titer and contraception, we did not identify a GnRH antibody concentration cutoff that predicted contraceptive effects with high sensitivity and specificity. The most useful diagnostic assays have wide separation between negative and positive cutoff values [44, 45]. Because there was overlap in pregnancy status at given antibody concentrations, GnRH antibody concentration as a diagnostic test may be a good indicator of herd or population level of infertility, but a less reliable predictor of individual animal fertility.

The wide variation in antibody concentrations observed in our study was not surprising, given that individual humoral responses to a foreign antigen are known to be influenced by many physiological factors, including nutrition, previous exposure to the same or similar antigens, age, persistence of the antigen, current immune stimulation by other immunogens, and genetics [44]. We attempted to control for the first four potentially confounding factors; however, the latter two factors were not measured or controlled and may have influenced individual responses. Of more interest was the persistence of GnRH antibody concentrations during the course of 4 yr in GnRH-vaccinated animals. Most commercially available vaccines require an initial series of two or three vaccinations with annual revaccination to maintain significant serum antibody concentrations [44]. Previous wildlife immunocontraceptive vaccines have required similar vaccination strategies [46–48]. Only recently have single-dose applications been effective at inducing long-term antibody production and corresponding infertility [43, 49, 50]. It has been suggested that a combination of depot effect produced by a nonbiodegradable oil in water-based emulsion along with an optimized concentration of immunostimulatory killed mycobacteria is responsible for the prolonged antibody effect in GnRH-vaccinated deer [24, 51]. Our finding of extensive localized inflammation at the site of injection nearly 4.5 yr after vaccination supported this hypothesis of a depot effect, particularly given the retained and apparently dead mycobacteria within the sterile lesions. Additionally, *M. avium* antibody concentrations that were often sufficiently elevated to indicate disease status seroconversion supported the assertion that a generalized and robust humoral immune response to the vaccine was critical to its efficacy.

Both GnRH-vaccinated and sham-vaccinated groups had follicles ranging in size from less than 2 mm to more than 12 mm in diameter during the early ovulatory, transitional, and anestrous seasons. Additionally, during transitional seasons ultrasound evidence of luteal tissue was only observed in sham-

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vaccinated females. Although we did not intensively measure follicular development and document ovulation, these data suggested that 12–18 mo after vaccination with GnRH, at least some females had large preovulatory-sized follicles that likely did not ovulate. The range of follicular size was consistent with earlier data from untreated elk in the transitional and anestrous periods [15, 16]. Although a full range of follicular sizes was observed in our study, mean follicular size was smaller but antral follicles were more numerous in GnRH-vaccinated compared with sham-vaccinated females. Our observations are similar, although less extreme, than those of Seekallu et al. [34]. Those authors found complete cessation of follicular waves and development of large-sized follicles in domestic sheep after a series of two vaccinations against GnRH. Differences in experimental methodology may account for this inconsistency. Our measurements were made 12–18 mo after immunization after a single vaccination, whereas they made measurements 26–66 days after booster vaccination. Their more intensive vaccination and monitoring schedule may explain the more complete hypothalamic-pituitary-gonadal axis suppression observed.

Our data indicated fewer follicles developed to the preovulatory stage, which was likely due to incomplete gonadotropin support. Attenuation of follicle development into large antral stages likely resulted in less negative feedback from dominant follicles secreting inhibin and estradiol. An increase in small follicle recruitment due to less negative feedback from dominant follicles and early regression due to incomplete gonadotropin support could account for the increase in numbers of small follicles observed in GnRH-vaccinated elk. Although follicles were on average smaller in GnRH-vaccinated females, antral follicle development persisted and suggested that gonadotropins, particularly follicle-stimulating hormone (FSH), continued to stimulate early follicular development. Although LH secretion is closely regulated by GnRH [52, 53], FSH synthesis is only partially controlled by the interaction of GnRH with its receptor [54–56]. The primary regulator of FSH synthesis is negative feedback from estradiol and inhibin, and secretion is primarily constitutive [54, 56]. It has been suggested that a component of basal LH secretion is similarly free from GnRH regulation [57]. In the current study, antral follicle development was consistent with FSH stimulation and possibly basal LH signaling, albeit at a decreased level. Absence of observed luteal tissue in GnRH-vaccinated females was consistent with a probable lack of sufficient estradiol to initiate an LH surge and ovulation. Experiments to intensively measure individual animal follicular dynamics and concurrent gonadotropin concentrations are required to test these speculative hypotheses.

Contrary to our prediction, reproductive interactions during the breeding season were not eliminated in vaccinated females. Estrous behavior in domestic ruminants is influenced not only by the presence or absence of progesterone and estradiol, but also the concentration and timing of these steroid hormones [58]. Continuous, high concentrations of progesterone (e.g., during pregnancy) usually inhibit expression of estrous behavior regardless of estradiol concentrations [58]. In contrast, sexual receptivity can be triggered by estradiol alone in most species, albeit often in the pharmacological range, as long as progesterone is not inhibitory [59, 60]. In this study, despite the apparent absence of ovulation, both female attractiveness, measured by male precopulatory behaviors, and female proceptivity, measured by female precopulatory behaviors, were maintained throughout the first breeding season after immunization in GnRH-vaccinated, and consequently nonpregnant, animals.

Interestingly, domestic male cattle will spend equal time with young nulliparous females in estrus or in the luteal phase, finding them equally attractive [61]. Male elk may similarly investigate nonpregnant females regardless of estrous status. A combination of low progesterone due to lack of ovulation, and the presence of limited estradiol due to altered follicular development, may have contributed to the continued expression of precopulatory behaviors in female elk. These mechanisms could account for pregnancy status being a stronger indicator of reproductive behavior than vaccination status. Because we observed too few copulatory behaviors to evaluate differences between treatment groups, it is unclear if vaccinated females were receptive to copulation or were only displaying proceptive behaviors. Because GnRH-vaccinated animals did not likely experience progesterone priming prior to estradiol exposure, which is important for the display of full estrous behaviors in domestic sheep [60, 62], we speculate that copulation failed to occur in nonpregnant GnRH-vaccinated animals. Regardless, it is apparent that in the relatively small captive environment of this experiment, with intensive male-female interactions, GnRH vaccination was associated with persistent breeding behaviors. Although these observations may be an artifact of captivity, the consequences in free-ranging populations are unclear but could be significant and deserve further investigation into potential ecological consequences prior to use in a management application.

In accordance with our hypothesis regarding physiological side effects, GnRH vaccination did not significantly affect blood chemistry or hematology parameters, but it did result in considerable injection site reactions. Two females showed evidence of a systemic inflammatory response, and four additional animals had mildly elevated fibrinogen, an indicator of inflammation. Every animal had increased serum globulins. These findings indicated a robust immune response to both the GnRH conjugate and sham vaccines. Abscesses were confirmed in 35% of females, which was consistent with others using a similar formulation of this vaccine in white-tailed deer [43, 63]. However, ultrasound changes indicative of myositis, trauma, and abscessation [36–38] suggested a higher incidence of injection site inflammatory lesions. It was unclear whether the pyogranulomatous inflammation at the site of injection would have resolved with time. Evaluation of the long-term physiological consequences of injection site reactions in animals treated with this vaccine will be essential for managers making choices based on animal welfare concerns.

In addition to antibodies produced in response to GnRH, antibodies were also produced to other elements of the vaccine. Mycobacteria and their cellular components are particularly immunostimulatory [64]. Antibodies produced in response to *M. avium* ssp. *avium* appear to cross-react well with commercial assays for Johne disease, a gastrointestinal disease of domestic cattle and occasionally wild ruminants, such as elk [65]. Vaccination with GonaCon-B and/or the adjuvant components increased mycobacterial antibodies in nearly every animal and induced clinical seroconversion in 18% of animals. Managers concerned with regulatory issues or Johne disease management should be aware of this finding prior to using this vaccine in free-ranging ungulates.

In conclusion, a single vaccination during midgestation with the described GnRH vaccine decreased pregnancy rates for 3 yr after treatment without compromising the existing pregnancy. This result extended potential practical use of contraception by providing multiple years of decreased fertility when applied to either pregnant or nonpregnant female elk. Hematology and serum chemistry parameters were typically normal, and vaccinated females appeared clinically healthy. Furthermore,

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the vaccine appeared to be safe for the developing fetus and neonates born to vaccinated females. By contrast, the vaccine prolonged reproductive behaviors during the breeding season, a finding that has potential ecological effects that require further study. The most apparent pathological side effect of the vaccine was related to the development of sterile abscesses at the site of injection. Although lameness was never observed, most GnRH and sham-vaccinated females showed some level of tissue inflammation or abscess. Finally, we demonstrated that GnRH vaccination inhibited, but did not eliminate, follicular development despite the absence of pregnancy, possibly because of continued FSH secretion, indicating continued partial function of the hypothalamic-pituitary-gonadal axis. Our findings extend both the fundamental and practical understanding of GnRH vaccination in pregnant elk, which may assist wildlife managers in their pursuit of science-based population management.

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Field Evaluation of the Immunocontraceptive GonaCon-B in Free-Ranging Horses (Equus caballus) at Theodore Roosevelt National Park, North Dakota

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became pregnant. The records prior to treatment showed that in 24 doe-years, 34 fawns were born (1.4 fawns/doe-year). In contrast, during 54 doe-years posttreatment with SpayVac®, only one fawn was born (0.019 fawns/doe-year). SpayVac has also been proven to provide multi-year efficacy with a single dose in Fallow Deer (*Dama dama*), White-tailed Deer (*Odocoileus virginianus*), horses (*Equus caballus*), and Gray Seals (*Halichoerus grypus*).

Field Evaluation of the Immunocontraceptive, GonaCon-B, in Free-ranging Horses (*Equus caballus*) at Theodore Roosevelt National Park

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From Colorado State University, Animal Reproduction and Biotechnology Laboratory, Fort Collins, Colorado, USA (Baker, Nett); National Park Service, Biological Resource Management Division, Fort Collins, Colorado, USA (Powers, Ransom); Theodore Roosevelt National Park, Medora, North Dakota, USA (Oehler); and USDA/National Wildlife Research Center, Fort Collins, Colorado, USA (Gionfriddo)

Unchecked free-ranging horse populations can have adverse effects on native plant communities and ecosystem processes, and may lead to conflict regarding appropriate resource allocation between sympatric grazing ungulate species. In response, many land management agencies have reduced free-ranging horse populations through periodic roundups and adoption or sale of excess animals. These methods are time and resource intensive, and can result in injuries to animals and humans. Consequently, more efficient, effective, and humane approaches are desired. One alternative is controlling the fertility of female horses through immunocontraception. We are currently in the final year of a 3-year study at Theodore Roosevelt National Park (ND) to evaluate the effects of the gonadotropin-releasing hormone vaccine, GonaCon-B, on fertility, behavior, and injection site reactions in female horses. In October 2009, park horses were captured via helicopter round-up. All mature mares and band sires were retained to avoid disrupting existing herd dynamics, whereas foals, yearlings, and most satellite males were removed. Mares were blocked according to age and pregnancy status and randomly assigned to a treatment ($n = 29$; 2.0 ml GonaCon-B) or control ($n = 28$, 2.0 ml 0.9% NaCl) group. During March and July 2010 there was no difference between treatment groups in the number of foals observed with mares ($P = 0.65$). During the same time period in 2011 treated mares were three times less likely to have a foal by side than control mares (odds ratio 0.11–1.01, 95% CI). Estimated foaling rate in 2011 was higher ($P = 0.04$) in control mares (0.74, 0.56–0.88) than treatment mares (0.48, 0.31–0.66). Between 25 and 280 days posttreatment nearly 80% of treated mares had visible swelling at the site of injection and a purulent draining abscess was observed in one animal. By 380 days posttreatment approximately half of these swellings were no longer visually detectable. No treatment effects were observed on mare activity budgets, and sociosexual behavior data are currently being analyzed. Our preliminary examination of the data indicate that vaccination with GonaCon-B during gestation does not affect foaling success, decreases fertility for one year posttreatment, is associated with injection site swelling, and does not affect general activity budgets in free-ranging female horses.

Effects of Gonadotropin-releasing Hormone Vaccination (GonaCon-B) on Fertility in Free-ranging Female Rocky Mountain Elk (*Cervus elaphus nelsoni*)

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Fertility control is one method, among a suite of management tools, which may assist in mitigating conflicts associated with locally overabundant wildlife. Rocky Mountain elk (*Cervus elaphus nelsoni*) are resilient, adaptable cervids that can have ecological as well as socio-political impacts on the coupled human-natural environment. We evaluated the safety and efficacy of the gonadotropin-releasing hormone vaccine, GonaCon-B, in free-ranging elk. In January 2008, 120 mature (>2 yr) female elk were captured in Rocky Mountain National Park (CO) and randomly assigned to treatment ($n = 60$; 1.5 ml GonaCon-B) or control ($n = 60$; 1.5 ml 0.9% NaCl) groups. All injections were given with hand-held syringes in the biceps muscle. Location of the injection was recorded in reference to anatomic landmarks. Transrectal palpation and pregnancy specific protein B analyses were used to diagnose pregnancy at the time of application. During the following three winters females were recaptured and euthanized in conjunction with park management culling operations (2009, $n = 20$; 2010, $n = 25$; 2011, $n = 34$). At necropsy pregnancy status was determined. Tissues were collected and preserved from organs associated with the hypothalamic-pituitary-gonadal (HPG) axis. Injection sites were relocated, muscle tissue collected, and microbial culture performed when gross evidence of inflammation was present. There were no differences in pregnancy rates between groups at the time of treatment ($P = 0.558$). In the following three years saline administered control females ($n = 36$) had a mean pregnancy rate of 0.83 (0.71 - 0.95, 95% CI). Pregnancy rate in the treatment group was 0.0 (0.0 - 0.22, $P = 0.0001$) year one, 0.33 (0.12 - 0.61, $P = 0.05$) year two, and 0.65 (0.43 - 0.83, $P = 0.17$) year three. Study animals showed no evidence of lameness or external swelling at the injection site prior to euthanasia; however, at the time of necropsy all females in the treatment group had pyogranulomatous inflammation at the site of injection.

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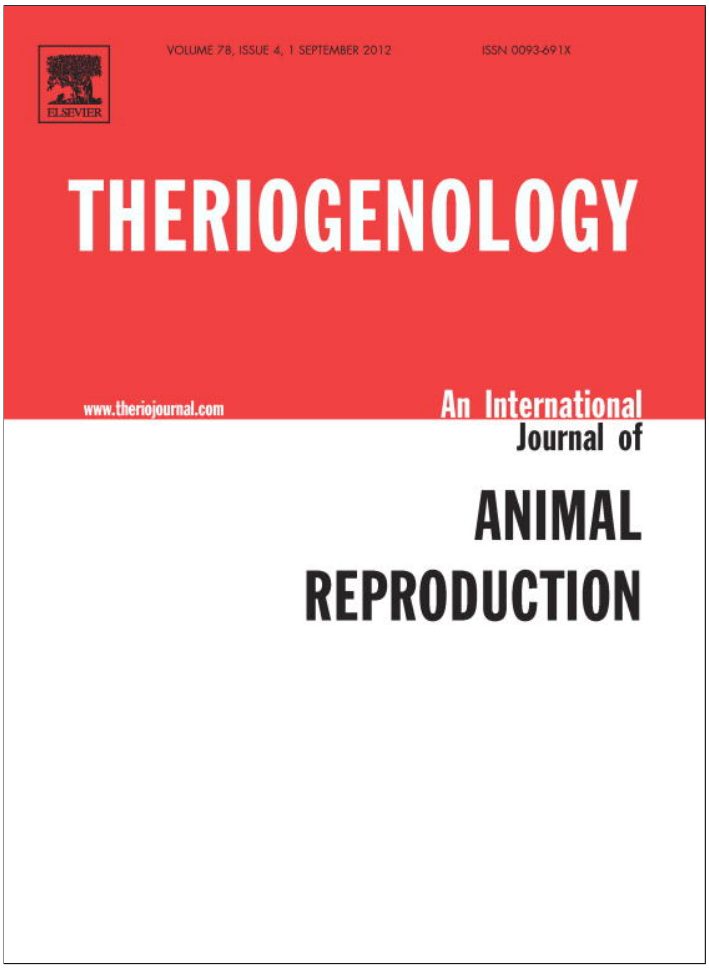


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Passive transfer of maternal GnRH antibodies does not affect reproductive development in elk (*Cervus elaphus nelsoni*) calves

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Abstract

Gonadotropin-releasing hormone is intermittently released from the hypothalamus in consistent patterns from before birth to final maturation of the hypothalamic-pituitary-gonadal axis at puberty. Disruption of this signaling via GnRH vaccination during the neonatal period can alter reproduction at maturity. The objective of this study was to investigate the long-term effects of GnRH-antibody exposure on reproductive maturation and function in elk calves passively exposed to high concentrations of GnRH antibodies immediately after birth. Fifteen elk calves (eight males and seven females) born to females treated with GnRH vaccine or sham vaccine during midgestation were divided into two groups based on the concentration of serum GnRH antibodies measured during the neonatal period. Those with robust (>15 pmol ^{125}I-GnRH bound per mL of serum) titers (N = 10; four females and six males) were designated as the exposed group, whereas those with undetectable titers (N = 5; three females and two males) were the unexposed group. Onset of puberty, reproductive development, and endocrine function in antibody-exposed and unexposed male and female elk calves were compared. Neonatal exposure to high concentrations of GnRH antibodies had no effect on body weight ($P = 0.968$), endocrine profiles ($P > 0.05$), or gametogenesis in either sex. Likewise, there were no differences between groups in gross or histologic structure of the hypothalamus, pituitary, testes, or ovaries. Pituitary stimulation with a GnRH analog before the second potential reproductive season induced substantial LH secretion in all experimental elk. All females became pregnant during their second reproductive season and all males exhibited similar mature secondary sexual characteristics. There were no differences between exposure groups in hypothalamic GnRH content ($P = 0.979$), pituitary gonadotropin content ($P > 0.05$) or gonadal structure. We concluded that suppressing GnRH signaling through immunoneutralization during the neonatal period likely does not alter long-term reproductive function in this species.
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Keywords: Elk; Fertility control; GnRH vaccine; Gonadotropin; Hypothalamic-pituitary-gonadal axis; Wildlife

1. Introduction

Episodic secretion of GnRH from the hypothalamus and signaling at gonadotropic cells of the anterior pituitary gland is required for normal steroidogenesis and gametogenesis in reproductively mature mammals [1]. Increases in pulse frequency mark the shift from reproductive quiescence to activity during puberty and tran-

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sition from anestrus to breeding condition in seasonal breeders [2]. The hypothalamic-pituitary-gonadal (HPG) axis is functional and active during the neonatal period in domestic ruminants [3,4], but is not fully mature until the prepubertal period [5–8]. There is a biphasic increase in gonadotropins during prepubertal maturation in female sheep and cattle [9]. Similar to females, prepubertal males have a critical period of HPG axis maturation during the neonatal period [10,11]. Attainment of reproductive maturity can be hastened by administration of GnRH during early postnatal life [12]. Alternatively, puberty can be delayed by inhibiting GnRH secretion during this early period of development [13,14]. Gonadotropin-releasing hormone signaling during the postnatal/prepubertal time period is important for final maturation of the HPG axis in domestic ruminants.

Vaccination against GnRH is one method of functionally removing the hormone and its effects in vivo. The effects of GnRH exclusion on reproductive system function have been investigated using active and passive GnRH vaccination in mature [15,16] and juvenile [17,18] ruminants. In adults, reproductive function generally returns as antibody titers wane [19,20]. In contrast, immunization of neonatal or prepubertal animals can have a prolonged effect on reproductive function, despite progressive decline in GnRH-antibodies [21–25]. In fact, Brown and colleagues [22,23] reported that active vaccination against GnRH during the neonatal period caused permanent suppression of reproductive function in a subset of mature domestic sheep of both sexes, despite a lack of measurable GnRH antibodies after the prepubertal period. Few studies have investigated the long-term (>1 yr) reproductive effects of GnRH vaccination during the neonatal or prepubertal period.

Gonadotropin-releasing hormone vaccination has been proposed as a method of wildlife contraception [26]. In a previous study, we actively vaccinated midgestation female elk to evaluate the safety, efficacy, and duration of a recently registered GnRH vaccine (GonaCon, National Wildlife Research Center, United States Department of Agriculture, Fort Collins, CO, USA) intended for use in wild ungulates as a contraceptive [27]. Calves born to these females experienced neonatal passive transfer of GnRH antibodies. Before management application of this vaccine, it is imperative to understand the first-generational effects of vaccination in pregnant females to prevent unintended population-level consequences [28].

The purpose of this study was to investigate the long-term reproductive effects of maternal passive transfer of GnRH antibodies to male and female elk calves after birth. Passive vaccination during the neonatal period may delay the onset of puberty and could permanently suppress function of the HPG axis, leading to decreased fertility during adulthood.

2. Materials and methods

2.1. Animals and experimental approach

This study was reviewed and approved by Colorado State University (#07–146A–01) and the Colorado Division of Wildlife (#09–2007) Institutional Animal Care and Use Committees. All animals were housed at the Colorado Division of Wildlife, Foothills Wildlife Research Facility in Fort Collins, CO, USA (40°352046“ N, 105°92029”W). This work builds upon a previous study investigating the effects of active vaccination against GnRH in mature female elk during midgestation [27]. The current study used calves born during the previous work. Fifteen elk calves (N = 8 male; N = 7 female) born to females immunized with GnRH vaccine or a sham vaccine (carrier and adjuvant but no GnRH) during midgestation (approximately 100 days of 255 ± 7 days gestational period [29]) were divided into two groups, based on the presence of serum GnRH antibodies measured during the neonatal period (1–30 days). Dam vaccination status was not necessarily indicative of calf GnRH antibody exposure status, due to nursing from multiple females early in the neonatal period. Calves with robust (>15 pmol ^{125}I-GnRH bound per mL of serum) neonatal titers (N = 10; six males and four females) were designated as the exposed group, whereas those with undetectable titers (N = 5; two males and three females) the unexposed group. Calves were dam-raised in mixed exposure groups until 3 to 4 mo of age when they were weaned (September 1, 2006). One male calf exposed to GnRH antibodies was <30 days at weaning and was euthanized due to welfare concerns. At weaning, calves were separated into same sex groups, maintained in fenced paddocks (approximately 5 hectares), and fed a diet of ad libitum alfalfa-grass hay mix, limited supplement, trace mineral blocks, and water. Before experiments, calves were trained to repeated handling, blood sampling, isolation pens, alleyways, and a handling chute. All biological samples, with the exception of semen collection which required complete immobilization, were collected while elk were standing and lightly sedated using xylazine hydrochloride (30–150 mg per animal im; TranquiVed; Vedco, Inc., St. Joseph, MO, USA) in a nonsqueeze handling chute. Tranquilizer effects were

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reversed after each sampling session with either yohimbine hydrochloride (30 mg per animal iv; Wildlife Pharmaceuticals, Fort Collins, CO, USA) or tolazoline hydrochloride (600 mg/animal im; Tolazine; Akorn, Inc., Decatur, IL, USA). During the experiment (May 2007), one male calf in the GnRH antibody-exposed group died because of a venomous snake bite. The study was conducted from birth (May to August 2006) to approximately 3 yr of age (March and April 2009).

2.2. Antibodies and growth rates

To measure maternal antibody transfer to calves, blood was opportunistically collected from calves before first nursing (N = 2; one from each exposure group) and from every calf 24 h after birth, then at approximately 2-wk intervals for the first 2 mo and monthly until 6 mo. Calves were weighed (kg) at the same time points. Thereafter, similar measurements were made sporadically, but not less than once every 6 mo. Final antibody concentration measurements were made before the 2008 reproductive season (mid September to mid November). Blood samples (10 to 30 mL) were collected via jugular venipuncture using a 20-gauge blood collection needle, tube holder, and 10-mL blood tubes without anticoagulant (BD Vacutainer SST; Becton, Dickinson, and Co., Franklin Lakes, NJ, USA). Blood was allowed to clot at room temperature, centrifuged for 10 min at 1500 × *g*, and serum was decanted to polypropylene tubes and stored at −80 °C until assays were performed. Peripheral GnRH antibody concentrations (pmol of ^{125}I-GnRH bound per mL serum at 1:1000 dilution) were measured using a modified radioligand binding assay, as described previously [27].

2.3. Puberty and fertility

2.3.1. Males

We measured and compared mean monthly serum concentrations of testosterone (ng/mL) between exposure groups from 14 to 21 mo [30]. Blood was collected as described above at single time points at the beginning of each month between August 2007 and March 2008. In addition, we measured secondary sexual characteristics, including antler length (cm), scrotal circumference (cm), and neck girth (cm) at monthly intervals from 9 to 21 mo (March 2007 to March 2008) as well as before the 2008 reproductive season (August 2008). Finally, we measured antler complexity (number of branch points), and weight (g) before the first and second potential breeding seasons (August 2007, 2008) when hardened antlers were removed for herd management purposes. Antlers were consistently removed (using a Giglis wire saw) 1 cm above the ridge of bone where the antler and pedicle meet.

To estimate fertility and confirm pubertal maturation, we collected semen samples at monthly intervals from August 2007 to March 2008 (14 to 21 mo). Semen samples were collected via electroejaculation [31] using a 60-mm diameter rectal probe (Pulsator III, Lane Manufacturing, Inc., Denver, CO, USA) while elk were chemically immobilized (18 to 23 mg butorphanol tartrate, 15 to 19 mg azaparone tartrate, and 6 to 8 mg medetomidine hydrochloride im; Wildlife Pharmaceuticals). An endotracheal tube (20 mm internal diameter) was placed during the procedure to prevent laryngeal collapse and hypoxemia. No supplemental oxygen was administered. Sedative effects were reversed when procedures were completed (10 mg atipamazole hydrochloride im; Pfizer Animal Health, Exton, PA, USA; and 500 mg tolazoline hydrochloride im). Total and progressive sperm motility (%), as well as components of velocity (straight line [VSL], curvilinear [VCL], and average path [VAP]; μm/sec), were evaluated immediately using computer assisted sperm analysis (IVOS, Hamilton, Thorne Biosciences, Beverly, MA, USA) [32,33]. If necessary, samples were diluted with semen extender (E-Z Mixin "BF", Animal Reproduction Systems, Chino, CA, USA) before motility analysis. Undiluted semen was smeared, stained with eosin-nigrosin (Hancock Stain; Animal Reproduction Systems), and slides were stored at room temperature for future evaluation. Sperm morphology (% normal) was evaluated by a single technician according to standards used for bovine semen [34].

2.3.2. Females

Onset of puberty was estimated in females by measuring peripheral serum progesterone concentrations (ng/mL) every 10 days (estrous cycle length = 21 to 22 days, luteal phase = 13 to 17 days [35–38]) from August 2007 to April 2008. Serum concentrations of progesterone ≥1.0 ng/mL were considered indicative of a functional CL and signified females were postpubertal [35,39]. Female fertility was evaluated by assessing pregnancy after exposure to proven herd sire males for 63 days during their second potential breeding season (September to November 2008) at 2.5 yr. Pregnancy status was determined using pregnancy specific protein B assay [40], transrectal palpation [41], and transrectal ultrasound [42] 45 days after bulls were removed from paddocks. For management purposes, when pregnancy was confirmed, abortion was induced using two doses of prostaglandin $F_{2\alpha}$ 6 h apart (25 mg

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im; lutalyse, Pharmacia & Upjohn, Kalamazoo, MI, USA) [43,44].

2.4. Hypothalamic-pituitary-gonadal axis

Pituitary responsiveness to GnRH was measured before the second breeding season (August 2008). All elk were fitted with nonsurgical indwelling jugular catheters (14-gauge 14 cm Abbocath; Abbott Laboratories, Abbott Park, IL, USA) and administered the GnRH analog d-Ala^6-GnRH-Pro^9-ethylamide (1 μg/50 kg body weight iv; Sigma, Chemical Co., St. Louis, MO, USA). Blood samples were collected before treatment then at hourly intervals for 8 h [36,45]. Blood was handled as described above and serum was stored at −20 °C for subsequent analysis of LH [46]. All blood samples collected from males were also analyzed for testosterone [30]. Progesterone was measured in pretreatment serum samples collected from females [47]. Gonadotrope response to GnRH analog challenge was assessed in two ways: (1) mean maximum concentration of LH over all time points (ng/mL); and (2) total amount of LH secreted (ng/mL per min), estimated by calculating the area under the curve for LH response [36]. To avoid confounding female pregnancy estimates, stimulation with GnRH analog was repeated in males but not females during the breeding season (early November 2008).

In addition to challenge with GnRH, we evaluated potential changes to the HPG axis by collecting hypothalamus along with the median eminence, pituitary, and gonads on March 25 (males) and April 14, 2009 (females). We assessed morphologic and morphometric differences in gonads between exposure groups. Gonad mass (g) was determined for each animal. Transverse sections of testes were cut, weighed, and frozen until sperm per gram of testes was measured [48]. Remaining testes tissue and all ovarian tissue was preserved in Bouin's fixative (Polysciences, Inc., Warrington, PA, USA). Pituitary and hypothalamus were hemisectioned through the midsagittal plane. One half of each brain was preserved in 4% paraformaldehyde and the other half wrapped in aluminum foil and frozen at −80 °C. Frozen sections of hypothalamus were assayed for total GnRH content (ng) [49]. Frozen pituitary sections were processed as described by Hart and colleagues [49], and analyzed for LH and FSH content (mg/g tissue) [46,50]. Fixed tissues were trimmed, placed in cassettes, maintained in 10% neutral buffered formalin, and sectioned using standard histology techniques at the Colorado State University Veterinary Diagnostic Laboratory. Hematoxylin and eosin stains were used for histologic examination. A veterinary pathologist (Spraker) qualitatively examined sections of gonad, pituitary, and hypothalamus (magnification × 2 to × 40) for pathologic changes and evidence of differences in morphology.

2.5. Hormone assays

Concentrations of progesterone [47], testosterone [30], LH [46], FSH [50], and GnRH [49] were measured using RIA. Samples were assayed in duplicate in single batches for each hormone at each time point. Intra-assay coefficients of variation for the upper and lower reference standards (20% and 80% ligand-labeled hormone-bound, respectively) were 4% to 15% for progesterone, 3% to 17% for testosterone, 3% to 11% for LH, 3% and 6% for FSH, and 5% and 10% for GnRH. Interassay coefficients of variation were 2% for progesterone, and <20% for testosterone and LH. Both FSH and GnRH were measured in single assays. Mean limits of detection for each hormone (1 SD of the assay) are as follows: progesterone, 5 pg; testosterone, 2.5 pg; LH 30 pg; FSH 0.2 ng; and GnRH 0.5 pg.

2.6. Statistical analysis

Dependent response variables, including body weight, concentrations of progesterone, testosterone, and LH measured at multiple time points, sperm parameters measured after electroejaculation, and secondary sexual characteristics were analyzed using one-way ANOVA models for a nonrandomized design with a repeated measures structure (SAS 9.2, Proc MIXED; SAS Institute, Cary, NC, USA). The independent variables exposure status, sire, date, and time, were included as fixed effects. Sex was also treated as a fixed effect if a response variable was measured in both sexes. Individual animal was included as a random effect. We first modeled the variance-covariance structure for each dependent variable using the restricted maximum-likelihood method, with the most global model of fixed effects (response variable = exposure status + sire + date or time as categorical variables ± sex). We modeled the following variance-covariance structures appropriate for unequal time intervals; variance components (VC), compound symmetric (CS), and spatial power [SP(POW)]. We selected the most appropriate variance-covariance structure using Akaike's Information Criterion with correction for small sample sizes (AICc) and then used the top-ranked structure for subsequent modeling of fixed effects. The best structure for all models had constant variance within an exposure group; some models allowed heterogeneous variance between exposure groups, whereas others were homogeneous for all study

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animals. The best covariance structure for all variables except body weight was variance components, which implies no covariance between repeated measurements. The best covariance structure for body weight was spatial power. Because date and time were the only significant fixed effects in the model describing total LH released after GnRH analog stimulation, a Student *t* test was used to compare differences between sexes and between months for males.

Single time point concentrations of LH, FSH, and GnRH at necropsy, as well as gonad mass, and sperm per gram of testis were analyzed using generalized linear ANOVA models (Proc GLM), with exposure status, sire, and sex (if appropriate) as classification variables. Arcsine transformation was performed for all data expressed as percent. Means and standard errors were estimated using least squares analysis and tests for differences between GnRH antibody-exposed and unexposed groups were based on type III generalized estimating equations. Descriptive statistics were used to explain concentrations of GnRH antibody.

3. Results

3.1. Antibody concentrations and growth

Antibodies to GnRH were measured in exposed calf serum between 1 and 200 days after birth (Fig. 1). Antibodies were undetectable before nursing in blood samples taken from two calves, one with a GnRH vaccinated dam and the other with a sham-vaccinated dam. Maximum antibody concentrations (± SEM) in exposed calves were often higher (37.7 ± 4.1 pmol/mL; range, 15.2 to 54.1 pmol/mL) than those previously measured in the GnRH vaccinated dam cohort at similar time points (May 2006) (28.8 ± 3.8 pmol/mL; range, 12.2 to 42.3 pmol/mL [data not shown]). Antibodies declined exponentially over time in exposed calves with a half-life of approximately 7 days (Fig. 1). Exponential antibody decay was represented by the following equation: $[A] = 67.1e^{-0.1(d)}$ where A = serum GnRH-antibody concentration and d = calf age in days ($r^2 = 0.64$).

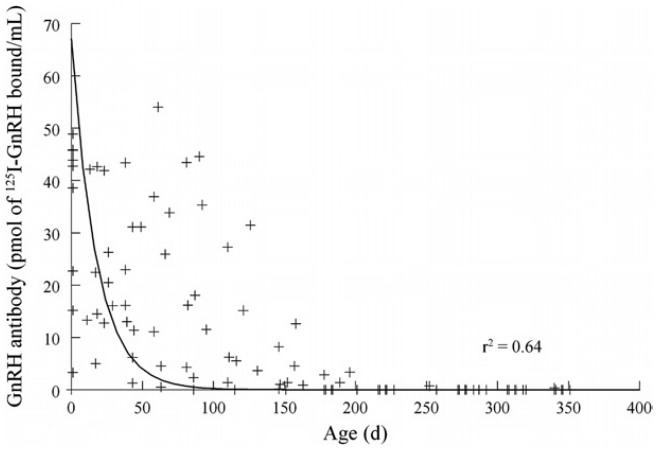


Fig. 1. Persistence of maternal antibody from birth to 1 yr in elk calves (N = six to eight males, two died August 2006 and May 2007; N = 4 females) exposed to colostral transfer of GnRH antibodies during the first 24 h of life. Calves unexposed to GnRH antibodies had undetectable antibody titers (data not shown).

There was no effect of neonatal exposure to GnRH antibodies on body weight (P = 0.968) between birth and 3 yr. Although males were heavier than females at birth (P = 0.024) and at 3 yr (P < 0.001), neither sex (P = 0.905) nor sire (P = 0.913) were significant variables in the model. Age was the most important variable describing body weight (P < 0.001) and there was an age by sex interaction (P = 0.001) with males gaining weight faster than females. Males gained approximately 0.83 kg per day during the first 100 days and then gained more slowly at 0.2 kg per day between 200 and 800 days. Females gained an average of 0.75 kg per day during the first 100 days but decreased their growth rate to 0.1 kg per day between 200 and 800 days.

3.2. Puberty and fertility

3.2.1. Males

There was no effect of exposure on mean monthly concentrations of testosterone (P = 0.659) between 15 and 21 mo. Likewise, sire did not affect concentrations of testosterone (P = 0.275); however, concentration did vary by month (P = 0.001; Fig. 2). Highest concentrations occurred in October and November. With the exception of one calf exposed to GnRH-antibodies, which had a maximum concentration of 1.7 ng/mL during November, all males had at least one testosterone measurement ≥2 ng/mL.

Similar to endocrine results, in male calves neither neonatal antibody exposure nor sire had an effect on semen parameters or most secondary sexual characteristics, including antler mass, neck girth, and scrotal circumference (Table 1). Date was an important variable describing differences in antler length (P = 0.002), antler mass (P = 0.007), neck girth (P = 0.0003), and scrotal circumference (P < 0.0001), but not semen parameters (P > 0.05). Only antler length was affected by exposure status (P = 0.037) with a single male exposed to GnRH antibodies contributing most of the variance. This animal had an unusually short antler, which may have been physically damaged, during his

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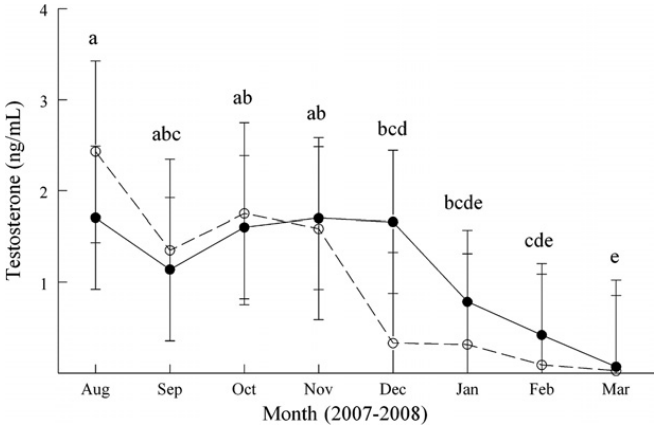


Fig. 2. Mean monthly testosterone concentrations with 95% confidence intervals, between 14 and 21 mo, in male elk exposed (N = 4; ● solid line) and unexposed (N = 2; ○ dashed line) to neonatal GnRH antibodies. Letters denote differences between months ($P < 0.05$); there were no differences between exposure groups within months.

second reproductive season but had a similar number of branch points (five) as other males (five or six per antler). Regardless of exposure group, all males had at least one semen sample which met criteria for adequate progressive motility (≥30%) and morphology (≥70%) of sperm in yearling male domestic cattle [34] and was consistent with acceptable sperm parameters from red deer (*Cervus elaphus*) (>40% motility, >40% normal morphology) [51]. Scrotal circumferences were consistent with those reported for 2-year-old elk [31].

3.2.2. Females

Monthly serum concentrations of progesterone in females were not affected by presence of neonatal antibody titer ($P = 0.727$) or sire ($P = 0.805$); however, concentrations varied by month ($P = 0.002$). All females had evidence of CL formation and regression, based on progesterone profiles, suggesting puberty had been reached by November 2007 (Fig. 3A). All had cyclic changes in serum concentrations of progesterone with multiple samples >1 ng/mL until March 2008 (Fig. 3B). Females were exposed to proven herd sire bulls between September and November 2008 at 28 to 32 mo. All seven females were pregnant when examined in January 2009. Transrectal palpation, ultrasound examination, and pregnancy specific protein B results were concordant.

3.3. Hypothalamic-pituitary-gonadal axis function and structure

In August 2008, before their second potential reproductive season, both males and females responded to GnRH analog stimulation with an acute LH release that peaked between 2 and 5 h and approached baseline levels within 8 h. Although mean maximum concentration of LH was not different between sexes ($P = 0.069$), females (16.1 ± 2.3 ng/mL) had nearly twice the maximum release of LH as males (9.4 ± 2.4 ng/mL; Figs. 4 and 5). Exposure to GnRH-antibodies during the neonatal period did not affect total secretion of LH ± SEM (ng/mL per min) in either males (33.3 ± 7.9 exposed, 35.3 ± 4.1 unexposed; $P = 0.668$) or females (69.7 ± 8.5 exposed, 61.2 ± 8.8 unexposed; $P = 0.333$). Neither mean maximum concentrations of LH nor testosterone were different between months ($P > 0.05$) for males; however, total LH and testosterone released amongst all males in August (42.3 ± 9.2 and

Table 1
Measures of semen quality and secondary sexual characteristics in male elk between 15 and 22 mo of age.

Response variable	Exposed (N = 4)			Unexposed (N = 2)		
	Mean	SEM	Range	Mean	SEM	Range
Normal morphology (%)	59.0	1.6	15–86	67.5	2.4	35–91
Total motility (%)	60.9	1.5	7–98	83.0	1.8	48–98
Progressive motility (%)	39.1	1.3	1–79	64.7	1.5	32–81
Sperm velocities (um per sec)						
VAP	74.5	10.0	31–116	97.6	11.1	76–116
VSL	62.6	10.0	19–100	83.1	11.1	67–104
VCL	119.2	121.2	52–174	153.8	13.5	115–178
Scrotal circumference (cm)	18.7	2.4	13.6–24.7	19.4	2.4	14.8–24.8
Antler length (cm)	148.8	3.1	123–187	166.3	3.8	129–197
Antler mass (g)	2805.5	244.4	1040–4420	2622.3	299.3	1330–4500
Neck girth (cm)	72.7	0.5	62–80	74.8	0.7	68–80

An arcsine transformation was performed on frequency data before analysis. The only end point that differed between groups was antler length ($P < 0.05$).
VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

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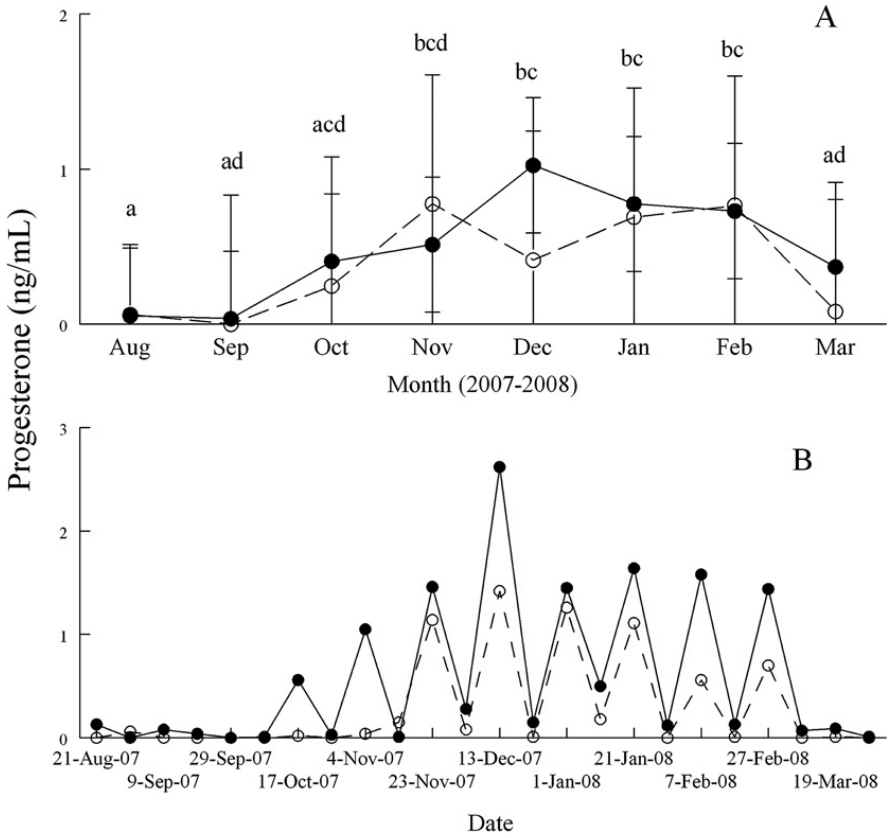


Fig. 3. Top panel (A): Mean monthly progesterone concentrations with 95% confidence intervals, from August 2007 to March 2008, in female elk calves exposed (N = 4; ● solid line) and unexposed (N = 3; ○ dashed line) to GnRH antibodies during the neonatal period. Letters denote differences between months ($P < 0.05$); there were no differences between exposure groups within months. Bottom panel (B): Representative progesterone profiles from female elk calves exposed (● solid line) and unexposed (○ dashed line) to GnRH antibody during the neonatal period. Sampling occurred at approximately 10-day intervals between 15 and 22 mo.

81.8 ± 7.7 ng/mL per min, respectively) was greater than in November (25.6 ± 3.2 and 56.7 ± 6.3 ng/mL per min; $P < 0.001$). A single male with unusually high concentrations of LH (14–22 ng/mL) accounted for most of the variability. Concentrations of progesterone in all females were below the limit of detection at the time of GnRH stimulation in August 2008 indicating lack of luteal tissue.

Endocrine profiles in serum and tissue at the time of necropsy were similar between exposure groups and sexes. There was no effect of exposure to GnRH antibodies on pituitary concentrations of LH ($P = 0.525$) or FSH ($P = 0.349$) in either sex (Table 2). Content of GnRH in hemihypothalmi was not different between exposure groups ($P = 0.979$) or sexes ($P = 0.980$). Serum concentrations of testosterone in males and progesterone in females were nearly undetectable and did not differ between exposure groups ($P > 0.05$). Gonad mass (g) did not vary by exposure status or sire ($P > 0.05$; Table 2).

There were no observed differences in gross or histologic structure of the hypothalamus, pituitary, testes, or ovaries between antibody-exposed and unexposed elk. There was no evidence of overt inflammation or change in structure in the median eminence of any study animal (data not shown). Although gonadotropes were not specifically identified, adenohypophesial structure was similar between exposure groups and was within normal limits for ruminant pituitaries. All ovaries and testes had evidence of gametogenesis. Ovaries had primordial through Graffian follicles, and testes had seminiferous tubules which contained spermatocytes. The only sign of inflammation was infiltration of atretic follicles with eosinophils in four of seven (N = 2 from each exposure group; approximately 60%) females.

4. Discussion

4.1. Antibody effects

Passive transfer of maternal GnRH-antibodies to elk calves shortly after birth did not affect long-term reproductive development in our study elk. There were no significant differences in growth, time of pubertal

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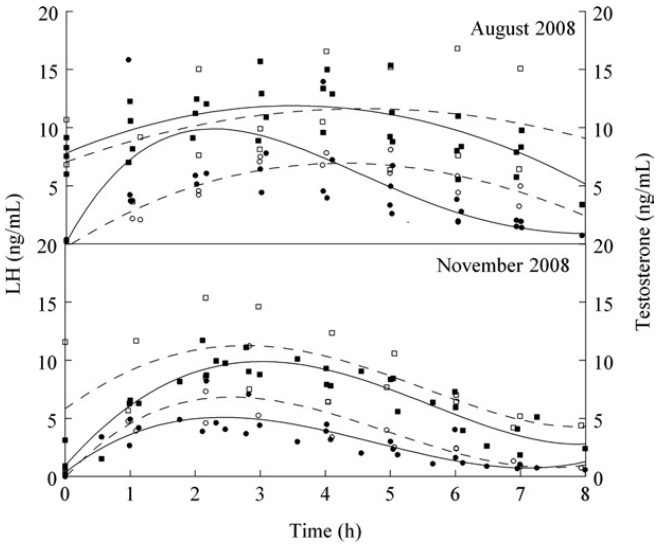


Fig. 4. Serum LH (○ ●) and testosterone (□ ●) concentrations after GnRH agonist treatment at time zero, in male calves (27 to 30 mo) exposed (filled solid line, N = 4) and unexposed (open dashed line, N = 2) to GnRH antibodies during the neonatal period. Total hormone released (ng/mL per min) represented by the area under the curve.

Table 2
Hormone concentrations and gonadal measurements in GnRH antibody-exposed and unexposed male (m) and female (f) elk at the time of necropsy (March/April 2009; approximately 3 yr of age).

Response variable	Exposed (N = 4 m, 4 f)		Unexposed (N = 2 m, 3 f)	
	Mean	SEM	Mean	SEM
FSH (ug/g pituitary)	(m) 1368 (f) 2262	268 268	(m) 1660 (f) 1200	328 568
LH (ug/g pituitary)	(m) 70 (f) 215	67 67	(m) 105 (f) 309	82 142
Hypothalmic GnRH (ng)	(m) 7.1 (f) 5.3	2.2 2.2	(m) 5.3 (f) 6.9	2.7 4.7
Serum testosterone (ng/mL)	0.41	0.73	0.00	0.00
Serum progesterone (ng/mL)	0.18	0.07	0.07	0.08
Testes mass (g)	48.5	5.5	42.6	4.5
Ovary mass (g)	3.1	0.5	2.2	0.2
Number of sperm × 10^6 (per g of testis)	79.9	7.0	77.1	0.6

Gonadotropins and GnRH were measured in both sexes, testosterone in males, and progesterone in females.

onset, or structure and function of the HPG axis between exposure groups in males or females. Therefore, we inferred that the presence of high concentrations of passively transferred GnRH antibodies during the first 60 days of life did not permanently alter the reproductive function of elk. However, because our sample sizes were small, particularly for the unexposed male group, we must be somewhat circumspect about our results. Due to small sample variation, a repeated experiment with relatively large sample sizes (e.g., ≥30) would be more likely to find differences, if they existed. Nevertheless, we consider our results relevant because there are multiple time points per elk for most response variables, and for at least one of those time points, each animal produced a reproductively mature response.

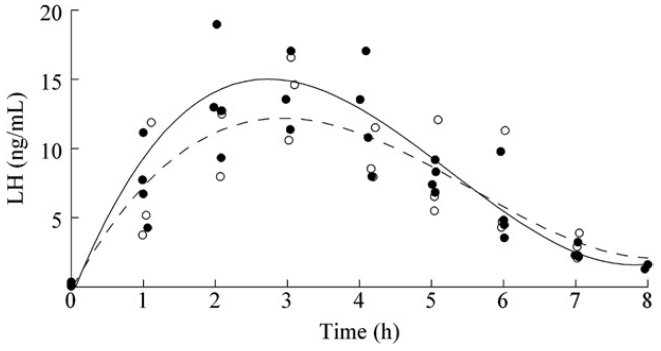


Fig. 5. Serum LH concentrations, after GnRH agonist treatment at time zero, in female elk calves (27 to 28 mo) exposed (● solid line, N = 4) and unexposed (○ dashed line, N = 3) to GnRH antibody during the neonatal period. Total LH released (ng/mL per min) represented by the area under the curve.

Our results were partially in contrast to findings in domestic sheep actively vaccinated against GnRH at 2 wk [21–23] and male rats passively immunized 5 days after birth [24,25]. Clarke et al. found that active vaccination against GnRH during the neonatal period caused permanent HPG axis suppression during adulthood, in a subset of vaccinated males and females, despite a lack of GnRH antibodies. This was caused by decreased GnRH secretion, rather than a decrease in GnRH content of the median eminence [21]. Vogel et al. [24] and Bercu and Jackson [25] found permanent suppression of rat testes development after neonatal passive vaccination, despite normal postpubertal gonadotropin concentrations during adulthood. They suggested that there is a critical window of testes development in rodents at this time that is permanently altered without the timely support of gonadotropins. In our study, we investigated both the potential for passive neonatal vaccination to permanently decrease GnRH secretion and the potential for transient removal of GnRH signaling to alter the structure or function of the pituitary or gonad, even if long-term GnRH secretion was unimpaired during adulthood. We found neither of

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these mechanisms altered reproductive function in elk from this study.

Gonadotropin-releasing hormone is secreted from hypothalamic neurons originating within the blood-brain barrier, but terminating near blood vessels of the hypothalamic-pituitary portal system outside of the blood-brain barrier. After active GnRH vaccination in male pigs at 10 and 18 wk and subsequent necropsy at 26 wk, Molenaar and colleagues found lesions, including fibrosis and scar tissue formation within the median eminence which were positively correlated with GnRH-antibody titer and testes atrophy [52]. They suggested that T-cell-mediated autoimmune reactions directed at GnRH neurons or retrograde transport of GnRH antibodies might result in destruction of the neurons or their processes. They demonstrated inflammatory lesions of the median eminence, consistent with the speculation that anti-GnRH immunoglobulin G, in addition to interleukin cytokines, may be responsible for permanent changes to function of GnRH neurons. Their study was different from ours in two important ways. First, the lesions they described were present in animals which had concurrently high concentrations of GnRH antibodies, which may have indicated the inflammatory processes were in progress at the time of death, but the lesions could have been transient. Second, pigs were actively vaccinated with Freund's complete and incomplete adjuvants, potent stimulants of both humoral and cell-mediated components of the immune response [53]. In contrast, elk in our study did not have detectable antibody concentrations by 6 mo and most response variables were measured between 9 and 30 mo. More importantly, elk in the current study were passively immunized with maternal antibodies, rather than stimulating in vivo humoral and cell-mediated immune responses. Although concentrations of GnRH antibodies were certainly higher in our study 24 h after birth, due to reporting inconsistencies, it was not possible to compare concentrations of antibody between the two studies at later time points. Regardless, our elk were not exposed to a full complement of humoral and cell-mediated components of the immune response, whereas pigs exposed to Freund's complete adjuvant along with GnRH conjugated to keyhole limpet hemocyanin (KLH) likely had robust stimulation of the entire immune system. Although we did not examine the histologic structure of the median eminence to the same degree of Molenaar et al. [52], there was no apparent evidence of inflammation or fibrosis in hypothalmi and there was no clinical evidence of HPG axis dysfunction in our elk. Therefore, we inferred that elevated antibody titer alone was not sufficient to induce a permanent decrease in GnRH secretion.

In altricial species, such as the rat, maturation of the HPG axis is incomplete at birth [54]. In precocious species, such as sheep and likely elk, the fetal HPG axis is functional by midgestation [3]; however, hypothalamic signaling is required for development of gonadotrophic cells during the last month of gestation and puberty is reliant on an adequate gonadotrope population [55]. It is unknown exactly when the permanent structure or function of the HPG axis is complete in elk, but we inferred that elimination or at least a decrease in GnRH signaling during the first 60 to 180 days after birth does not delay the process. Because we did not measure gonadotropin secretion during the neonatal period, we do not know if there were adequate concentrations of GnRH antibodies to completely remove GnRH signaling. In our previous study in adult female elk with similar or lower concentrations of GnRH antibodies, there was suppression of fertility, but not complete inhibition of final stages of follicular development, indicating a partially intact HPG axis [27]. Alternatively, ewes with lower concentrations of GnRH antibodies, but with a more aggressive active vaccination schedule, had complete cessation of follicular wave development [56]. It is likely that antibody concentrations were sufficient to influence GnRH signaling in the current study. This finding added strength to our speculation that antibodies alone do not account for long-term suppression of the HPG axis documented in previous studies.

4.2. Comparative reproductive ecology

Our investigation of endocrine changes, direct and indirect measures of fertility, and development of secondary sexual characteristics through puberty in male elk provided results similar to those in untreated and closely related red deer. For male red deer, the highest mean testosterone concentrations occur in late summer and early fall when antlers are hard and velvet has been shed [57–59]. Conversely, in the spring, at the time of antler casting, testosterone is nearly undetectable and testes are at their smallest [58,59]. Male elk in this and other studies [31] appeared to follow a similar seasonal pattern, with testosterone concentrations increasing to a peak in late summer/fall (September to October) then gradually declining through the winter period (December through March). Scrotal circumference mirrored testosterone concentrations, being minimal in late spring and attaining maximum size in late summer. However, as expected, scrotal circumference did not

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regress to prepubertal dimensions. Male elk pituitary response to stimulation with GnRH analog before and during the second reproductive season was similar to that reported in red deer stags [57,58]. Maximum concentrations of both LH and testosterone, induced by GnRH stimulation, were higher in August than in November. Interestingly, the single outlier male with an unusually large maximum concentration of LH had a relatively modest corresponding maximum concentration of testosterone. This pattern of large LH release with little secretion of testosterone, was characteristic of the nonreproductive season in red deer [57]. It was our observation that this individual was socially the least dominant male and did not produce a satisfactory semen sample until December 2007.

In elk populations that are not nutrient-limited, males are generally reproductively competent at 14 to 16 mo [31] with similar fertility rates as those seen in older more mature bulls [60]. Although we did not directly measure fertility of males in this experiment, there were no significant differences in semen quality or secondary sexual characteristics associated with fertility between exposure groups. Sperm motility, velocity, and morphology parameters met or exceeded standards for domestic bulls [34] and were similar to findings in red deer [33]. Semen evaluation and sperm analysis have remained elusive as a reliable indicator of fertility in domestic animals [61,62]; however, these assays may be more useful in wild species where strong artificial selection pressures for increased fertility are not in effect [63]. Swimming velocity and sperm morphology account for differences in fertility rates of Iberian red deer (*Cervus elaphus hispanicus*) [33]. Additionally, antler size and complexity are associated with testes size and sperm velocity [64]. Finally, despite seasonal testicular atrophy, all males in our study had maturing spermatocytes within the seminiferous tubules during the early spring at approximately 33 mo. Therefore, we inferred that all males were reproductively mature and likely fertile by the end of their first reproductive season at 15 to 21 mo.

Puberty and ultimately fertility in female elk is a function of body condition. In that regard, 50% of yearling females are fertile at 16 to 18 mo if a minimum body mass (approximately 220 kg, 10% body fat) is achieved [65–67]. The first estrous cycle of the reproductive season often has a short interovulatory interval [68], followed by regular ovulations every 20 to 24 days [38]. In our study, every female had regular increases in serum progesterone concentrations at approximately 20-day intervals. Most females had evidence of functional CL by mid-October and all were cycling by early November. This is later than that reported for mature female elk in North America [68], but is within the range observed in other studies with 2-yr-old females [66]. Three of seven females continued to display elevated concentrations of progesterone into late March when sampling was terminated. This was in accordance with previous research which demonstrated estrous cycling in mature females can occur as late as March but typically ends in late February [68]. Pituitary stimulation with GnRH analog before the breeding season induced an LH release similar to that in nonpregnant mature female elk during the reproductive season [36]. In our confirmatory test of fertility, all females became pregnant. These results provided further evidence for the lack of an effect of GnRH antibody exposure on maturation of the HPG axis or fertility in female elk calves in this study.

4.3. Conclusion

There was no apparent effect of exposure to high concentrations of maternally transferred GnRH-antibodies on the long-term structure or function of the hypothalmo-pituitary-gonadal axis of male or female elk. The elk HPG axis is likely structurally mature at birth and transient disruption in GnRH signaling through antibody neutralization was not sufficient to permanently change function in our study elk. Our findings provide important knowledge to inform wildlife management decisions regarding the application of fertility control in free-ranging populations of elk. Additionally, we have advanced the understanding of the mechanism(s) of how GnRH vaccination may affect long-term fertility in ruminant species. The presence of neonatal antibodies alone did not appear to account for changes in fertility later in life.

Acknowledgments

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Quantifying Equid Behavior—A Research Ethogram for Free-Roaming Feral Horses



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U.S. Department of the Interior
U.S. Geological Survey

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Quantifying Equid Behavior—A Research Ethogram for Free-Roaming Feral Horses

By Jason I. Ransom and Brian S. Cade

Introduction

Feral horses (*Equus caballus*) are globally distributed in free-roaming populations on all continents except Antarctica and occupy a wide range of habitats including forest, grassland, desert, and montane environments. The largest populations occur in Australia and North America and have been the subject of scientific study for decades, yet guidelines and ethograms for feral horse behavioral research are largely absent in the scientific literature. The U.S. Geological Survey (USGS) Fort Collins Science Center conducted research on the influences of the immunocontraceptive porcine zona pellucida (PZP) on feral horse behavior from 2003–2006 in three discrete populations in the American west (see Ransom and others, 2007; Ransom, 2009). These populations were the Little Book Cliffs Wild Horse Range in Colorado, McCullough Peaks Herd Management Area in Wyoming, and Pryor Mountain Wild Horse Range in Montana; the research effort included over 1,800 hours of behavioral observations of 317 adult free-roaming feral horses. The following ethogram was developed during the course of this study to facilitate accurate scientific data collection on feral horse behavior, which is often challenging to quantify. By developing this set of discrete behavioral definitions and a set of strict research protocols, scientists were better able to address both applied questions, such as behavioral changes related to fertility control, and theoretical questions, such as understanding social networks and dominance hierarchies within social groups of equids.

Background

Feral horses in North America are descendents of animals that escaped domesticity or were intentionally released by settlers, explorers, and military personnel, and because of their highly adaptive nature were able to thrive in a multitude of environments. In the United States most feral horses are federally protected by the Wild Free-Roaming Horses and Burros Act of 1971, which refers to them as "living symbols of the historic and pioneer spirit of the West," stating that "they contribute to the diversity of life forms within the Nation and enrich the lives of the American people" (Public Law 92-195, 85 Stat. 649, as amended). Roughly 30,000 feral horses are distributed across 10 western States in the United States on 21.4 million ha of federally designated horse range. With annual population growth rates reported at 15–25 percent (Eberhardt and others, 1982; Wolfe and others, 1989; Garrott and Taylor, 1990; Garrott and others, 1991) and very limited natural depredation, the influence of feral horses on native flora, fauna, and ecosystem processes varies greatly (see Smith, 1986; Fahnestock and Detling, 1999; Coughenour, 2002; Levin and others, 2002; Beever and Herrick, 2006; Beever and others, 2008). Understanding animal behavior can be an important tool in addressing a species' influences and roles in an ecosystem and can lead to better management and conservation practices (Sutherland, 1998; Buchholz, 2007). Additionally, when management practices such as fertility control alter natural biological processes, it is important to consider the ethical implications, such as influences on animal behavior and social structure (Nettles, 1997; Asa and others, 2005; Porton, 2005).

Social Organization

Feral horses arrange themselves in family groups known as bands, which range in size from 2 to 20 or more animals. Band size is variable and may be a response to both the environment the horses inhabit and population density. Animals can reduce risk of predation by living in groups (Pusey and Packer, 1997), and in open habitats without cover in which to hide, animals may form larger groups (Molvar and Bower, 1994). The latter was found to be true in the USGS study,

where mean band size in the sage steppe habitat found at McCullough Peaks was 8.01 ± 0.38 (SE) horses, while habitats with trees and dense vegetation had smaller mean band sizes—4.45 ± 0.16 horses at Little Book Cliffs and 4.16 ± 0.17 horses at Pryor Mountain.

Feral horse bands typically consist of a single polygynous stallion, adult females, foals of the year, and yearling males and females. Bands with multiple stallions do occur and were first reported by Denniston (1979) and Miller (1979). Miller (1979) found that multiple stallions were present only in bands consisting of greater than 9.3 horses. This finding was supported by the USGS study, during which multiple-stallion bands were recorded only at McCullough Peaks; multiple stallions occurred in five bands of horses consisting of 9–18 animals each. At 2 years of age, subordinate males typically disperse the band into bachelor groups of two to several horses, but occasionally 2- and 3-year-old males remain with the band if the stallion does not drive them away. Rutberg and Keiper (1993) found that the age at which male horse dispersal occurs is strongly correlated with the number of peers in the natal group.

Feral horse bands are not geographically territorial and home ranges overlap with those of other bands in the population. This geographically non-territorial, harem-type social structure is known as Type I organization and also is used by the Przewalski horse (*Equus ferus przewalskii*), Plains zebra (*Equus burchellii*), and Mountain zebra (*Equus zebra*) (Klingel, 1975; Rubenstein, 1989). The other organizational structure found in equids, Type II organization, involves adult males establishing geographic territories and females moving between them. Type II organization is used by the Grevy's zebra (*Equus grevyi*), African wild ass (*Equus africanus*), and Asiatic wild ass (*Equus hemionus*). While feral horses do not exhibit geographic territoriality, the USGS study did document resource-oriented territoriality whereby stallions (with the entire band present) defended mineral sources repeatedly and often with elevated agonistic interactions when other bands approached.

Ethological organization within horse bands is characterized by a dominance hierarchy, but the literature has conflicting functional explanations for hierarchical structures in equids. Houpt and others (1978) found that body weight affected social rank but age did not; however, other researchers (Keiper and Sambraus, 1986; Rho and others, 2004; Heitor and others, 2006) found age to be linearly correlated with social rank. Reproductive status of females has been shown to influence their social dominance in a band, as well as their rate of interchange between bands (van Dierendonck and others, 2004). Most studies, however, indicate that agonism is strongly correlated with dominance rank among females (Houpt and others, 1978; Waring, 1983; Keiper and Receveur, 1992; Weeks and others, 2000). The USGS study found that the most dominant members of feral horse bands were female, and stallion rank varied by band. This ethological structure is reported in much of the existing literature (Houpt and Keiper, 1982; McCort, 1984; Keiper and Sambraus, 1986; Klimov, 1988; and Keiper and Receveur, 1992). Lack of stallion dominance has been attributed to the amount of time stallions spend away from the band while recruiting new females or engaging in agonistic interactions with other stallions; females in a band, however, can establish stronger hierarchical relationships because they continuously remain in close proximity to one another (Keiper and Receveur, 1992). Social rank in bachelor groups, which are often unstable, has only been attributed to individual temperament (Tilson and others, 1988).

Ethological Data Collection and Analyses

It is possible to quantify behavior through the systematic enumeration of discretely defined categorical behaviors in terms of counts of occurrence or proportions of time exhibited. Collection of ethological data may be accomplished through several techniques, and the ultimate decision rests on the precisely defined research objectives. The reader is referred to Altmann (1974) for a definitive overview of sampling methodology for observational data. In the last 30 years, many insights have contributed to refining these methods, such as how to determine the appropriate time period between sampling when using the instantaneous scan sampling method and a better research design to capture infrequent behavioral expressions. In sampling feral horse behavior, the USGS study found that instantaneous scan sampling (the method of recording the categorical behavior of each focal animal at precise moments in time) at 1-min intervals was ideal for quantifying time budget data. Continuous sampling is an alternative to this technique and involves recording a focal animal's behavior continuously and noting the point in time that a new behavior begins. Continuous sampling was impractical for our study due to the number of animals and observers involved. Mitlohner and others (2001) found no significant difference between behavioral data recorded with instantaneous scan sampling versus continuous sampling when intervals were 15 minutes or less, and found a high correlation between the techniques when using 1-min intervals.

Collecting data on time budgets using instantaneous scan sampling is fairly straightforward, but behaviors that are infrequent and expressed briefly are not likely to be captured adequately (Houpt, 1991; Doran, 1992). Two relatively good options exist for collection of such data: the all-occurrence method and focal sampling. All-occurrence data collection records every behavior every time it is observed (Altmann, 1974) and can be a good technique when the number of animals in the focal band is not too large and the behaviors of interest are easy to detect. Although less efficient in terms of data collection, the focal sample method may be more reliable since the observer's focus is directed at only one animal (Altmann, 1974; Houpt, 1991). This method involves randomly selecting one individual in the band and continuously observing and recording all behavioral expressions. In feral

horse research, capturing briefly occurring expressions is most important for reproductive, social, and agonistic behaviors. Feeding, resting, and locomotion typically occur in periods long enough to be captured by most techniques.

Ethological protocols must be established prior to data collection and should be defined by research objectives. The discrete behavioral categories presented in this ethogram do not preclude the exhibition of simultaneous behaviors. For example, it is typical in feral horses for a comfort behavior such as masturbation to occur while the animal is feeding, so a protocol should be explicitly defined stating which behavior takes precedence in the data record. If the objective of your research is to determine the amount of time horses are feeding in relation to some covariate, then feeding is the appropriate category, and if expression of comfort behavior is the focus, then it should be recorded as such (or in this case, one might wish to subdivide comfort into several behaviors, since comfort is generally a short-duration behavior with many specific expressions). Regardless of the approach, notations of simultaneous occurrences are invaluable in retrospect.

Simultaneous behavioral expressions are not the only observational data collection pitfall. Some behaviors, such as olfactory investigation, can be ascribed to several categories. This behavior may fall under comfort, reproduction, or harem social behavior, but the correct categorization of such expressions is not subjective: each expression does have an absolute definition. In such cases, the context of the behavior will define its true characterization. When such potentially confusing behaviors are prevalent, they are addressed specifically in the ethogram.

Considerably more in-depth analyses of behavior are possible when covariates associated with individual focal subjects are collected reliably. Many factors may influence behavior, such as age, body condition, dominance rank, presence of dependent foal, climate, and habitat. Fortunately, many feral horses have unique natural markings, making identification of individual animals possible without the use of radio-collars or numbered tags. Pelage color; facial patterns such as snips, strips, blazes, and stars; and leg markings such as coronet bands, socks, and stockings collectively make cataloging and referencing study animals feasible (Sponenberg and Beaver, 1992; Gower, 2000; Green 2001). Properly cataloging and identifying individual horses in a research effort can provide the foundation from which to build in-depth covariate data and thus lead to a better understanding of animal behavior.

Analyses of behavioral data may incorporate many traditional statistical methods, but time budget data presents a particularly challenging dilemma. These analyses must consider the compositional nature of a time budget: models exhibiting strong support for one behavioral category must also be considered for each other category. Proportions of time spent in behavioral categories are compositional dependent variables because the sum of the proportions in all behavioral categories sum to 1. In other words, there is a unit sum constraint (Aitchison, 1986) by which more time spent in one or more behavioral categories must be associated with less time spent in one or more other behavioral categories. These compensatory shifts in estimates are absolute, leading to a dependent relationship among behaviors constituting the time budget being analyzed (Elston and others, 1996). Many compositional data-analysis procedures have been developed (for example, log-ratio procedures; Aitchison, 1986, 1992), but few are appropriate for data that exhibit real values of 0 or 1. It may be important that the amount of time an animal exhibits a given behavior is truly 0 percent or 100 percent. Many methods, such as log-ratio analysis, require data transformations that eliminate values of 0 and 1 by assigning very small or very large values (greater than 0 or less than 1) (Bakeman and others, 1992; Clark and Messina, 1998). This process ultimately alters the distribution of data and may adversely affect conclusions drawn about individual behaviors in the composition (Bingham and Brennan, 2004). Once transformed, the common method of log-ratio analysis also produces only quantitative statements about the overall distribution of the composition in comparison to another and the sampling distribution of the difference; it does not specify explicit contributions of each piece of the composition to those differences (Aitchison, 1986). If your data are not composed of real values of 0 and 1, log-ratio analyses may be appropriate. It is best suited for compositions with only two elements since conclusions about the change in allocation may be drawn easily (Elston and others, 1996), but most behavioral studies aim to address multiple behaviors. Some extensions of the algebra of log-ratio compositional measures have been made by Billheimer and others (2001), but currently these procedures are limited to three-part compositions and still suffer the restriction of not allowing zero proportions.

One solution to the problem of comparing time-budget compositions with several behaviors and true values of 0 and 1 is the use of a multi-response permutation procedure (MRPP; Mielke and Berry, 2007). This method does not require data-transformation and analyzes compositional differences by approximating probabilities of a test statistic based on average Euclidean distances for detecting distributional differences. In other words, the test is focused on differences in multivariate cumulative distributions rather than means. This approach may yield useful results for drawing conclusions about how different animals or treatment groups allocate their time, but it still does not provide insightful conclusions about specific behaviors within the composition of interest. No standardized way to approach this problem currently exists, but one solution is simply to model each behavior to query further covariates and relationships of interest: analyze each part of the behavioral composition as a univariate dependent variable. For such results to be interpretable, however, the same models must be applied and presented for all individual behaviors in the composition.

In simple compositions with few behaviors, the trade-off in time allocation may be evident. It is likely, however, that such clear trade-offs will not always be evident and multiple models must be presented for each behavior. For example, consider a simple composition of three horse behaviors

(Y_f = feeding, Y_r = resting, and Y_l = locomotion, where $Y_f + Y_r + Y_l = 1.0$). If Y_f has a strong linear increase with the predictor variable X_1 (for example, 0/1 indicator variable of sex) and a weak linear increase with predictor variable X_2 (for example, herd size), then Y_r, Y_l, or both must decrease with X_1, but the actual magnitude of that decrease will depend on both behaviors and the degree to which they also are influenced by X_2 (model 1 is $Y = f(X_1 + X_2)$). It could easily be that the strongest model for Y_r might include X_2 and another predictor (say X_3, and model 2 is $Y = f(X_1 + X_3)$). Thus, it is possible for model 1 to be strongly selected for analyzing the Y_f part of the Y compositional vector of behaviors but model 2 to be strongly selected for the Y_r part of the composition. Likewise, one model may be strongly supported for several behaviors and a complement model may be strongly supported for the remaining behaviors in the composition (see Ransom, 2009); however, because the behavioral composition really induces a relationship among the parts of the composition, statistical model results will be uninterpretable if a selected model is not considered to apply to all parts of the behavioral composition (for example, Y_f, Y_r, and Y_l).

The increasing use of maximum-likelihood and Bayesian approaches for solving complex problems may ultimately lead to the development of more lucid guidelines for many problems indicative of behavioral data. These methods can provide powerful tools for analyzing scientific data and can lead to a deeper understanding of the processes and errors involved (Hobbs and Hilborn, 2006). Regardless of the statistical approach, the path toward proper model selection for individual dependent behaviors in a composition has not been described in the literature. The use of model weights and model averaging may assist in drawing coherent conclusions about parameters of interest (Burnham and Anderson, 2002), but all supported models must be considered simultaneously for all dependent compositional variables to avoid violating the inherent statistical assumptions. A reasonable approach to assessing strength of evidence for compositional models, without violating assumptions of dependence, is to sum the minimum Akaike's Information Criteria (AIC: Burnham and Anderson, 2002) scores for the individually-modeled constituent behaviors in a composition; subsequently, each sum then forms an aggregate AIC score for each compositional model. The strength of evidence for the overall composition may then be assessed using AIC weights as Burnham and Anderson (2002) describe for individual models.

Ethogram

This ethogram was developed to investigate the influences of fertility control on feral horse behavior; thus, the behavioral groupings are broad in some contexts, such as feeding behavior, and much more detailed in areas of specific interest to the research objectives, such as agonism. Behavioral researchers often consolidate an array of behavioral expressions into broad categories since data collection on every specific behavior expressed is usually impractical and makes analyses excessively difficult. Therefore, this ethogram divides behavioral expressions into 13 discrete categories that cover the range of expressions a feral horse may exhibit in free-roaming conditions. Two additional categories (human awareness and out-of-sight) are used to account for biased observations. These 13 categories can easily be further aggregated *a posteriori* if desired, but categorizing these behaviors separately *a priori* allows for a wider range of possible analyses while still being practical for data collection. An example of a broader aggregation of these categories is the consolidation of grooming, comfort, standing attentive, and elimination into *maintenance*; herding, reproduction, harem tending, harem social, agonism, and submission into *social*; and human awareness and out-of-sight (unavailable to the observer) into *unknown* (fig. 1). At any given time during the course of observation, a focal animal will be exhibiting behavior that can be described by one of these categories. Developing a research ethogram from the several hundred behavioral expressions documented in equids can be a daunting task; therefore, this basic ethogram for feral horses is presented as a starting point for behavioral studies. For an excellent ethogram resource detailing complete equid behavior expressions, the reader is referred to McDonnell (2003). For a comprehensive work on equine behavior, the reader should also see Waring (1983).

Feeding

Feeding behavior occupies roughly half of the daily time budget of feral horses (fig. 1) and usually entails grazing. Grazing occurs as a horse bites off and ingests grasses and forbs close to the ground (fig. 2). The feeding category also includes browsing on woody plants and trees, eating snow, drinking, mineral licking, coprophagy (eating feces), and pawing at food resources. The latter is critical in defining feeding as a mutually exclusive category in that a horse may be pawing at soil, plants, or snow, but if the action is directly related to acquiring and ingesting a food resource, then it should be considered as feeding behavior. Also, horses move as they graze; therefore, as long as the horse is feeding while it is moving, it should be considered as feeding rather than locomotion.

Resting

The second largest amount of time in the daily time budget of feral horses is allocated to resting, which may occur during 25–35 percent of the daylight hours (fig. 1). Resting behavior is characterized by a general lack of attention and relaxed state and may occur in a standing position or in recumbency (fig. 3). This includes both relaxation and sleeping. Horses are able to sleep standing up due to the stay apparatus that allows their body to be supported without active muscular control (Dallaire, 1986). This behavior is typically observed

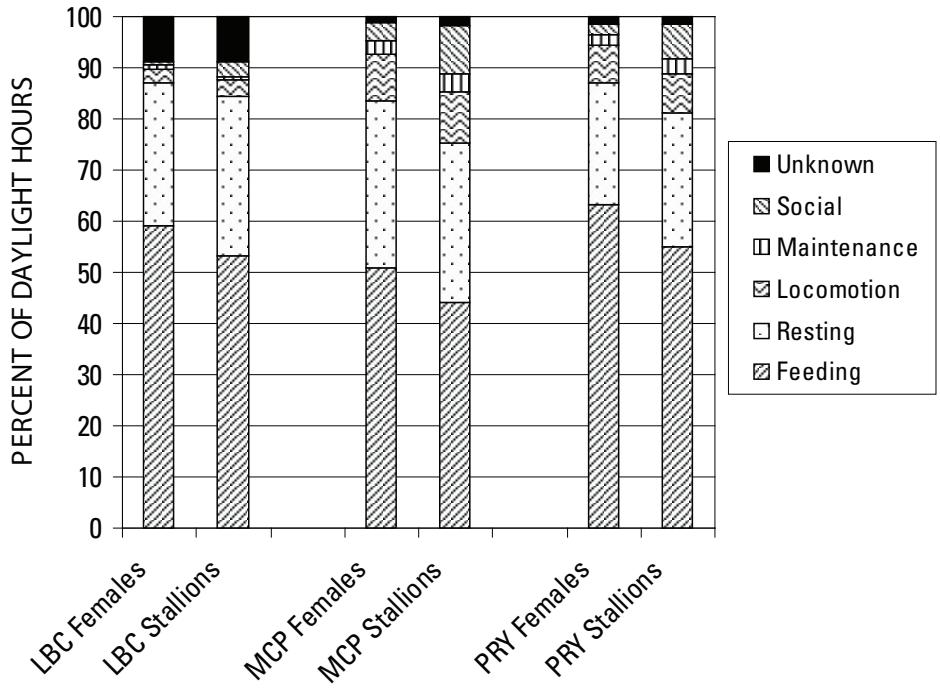


Figure 1. Observed daytime activity budgets of adult control female feral horses (n = 207, mean age = 7.79 yrs ± 0.33 SE, range = 2–21 yrs) and harem stallions (n = 110, mean age = 11.63 yrs ± 0.43 SE, range = 5–26 yrs) at Little Book Cliffs Wild Horse Range in Colorado (LBC), McCullough Peaks Herd Management Area in Wyoming (MCP), and Pryor Mountain Wild Horse Range in Montana (PRY), 2003–2006.



Figure 2. Feral horses grazing, the most typical *feeding* behavior.



Figure 3. *Resting* behavior in a band of feral horses is often characterized by huddling. Aside from the physical protection received by this behavior, the head-to-tail orientation of band members in this type of resting behavior also facilitates grooming. This allows for insect control around the head by the tail-swishing effect of neighbors.

as a body position with the head lowered, eyes closed, and one rear foot slightly elevated. Resting behavior in a band is often characterized by huddling, which facilitates insect control by the tail-swishing of close neighbors. Recumbent rest can be sternal or lateral. The comfort behavior of sun-basking is expressed in a laterally recumbent state, so a protocol must be defined *a priori* characterizing this state as rest or comfort, based on research objectives.

Locomotion

The third largest amount of the daily time budget is spent on locomotion. This behavior includes walking, trotting, cantering, galloping, jumping, and swimming and in feral horses is typically used for moving from one resource to another (fig. 4). Since most feral horse populations in North America occupy arid or semi-arid environments, movement to the few and scattered water sources is often the impetus for daily occurrences of extended locomotion (typically every 12 to 24 hrs). Otherwise, feral horses do not expend vast amounts of energy in locomotion, with the exceptions of brief social interactions between bands, stallion agonistic expressions, and female recruitment efforts by stallions. In this ethogram, locomotion integral to reproductive, harem tending, or comfort behaviors is considered part of its respective behavioral expression and not recorded as locomotion.

Maintenance

Grooming

Grooming behavior occupies a relatively small but important part of the daily time budget of feral horses and is often observed as rolling. Rolling occurs both on land and in water and is thought to assist with pelage health and insect control (fig. 5) (Waring, 1983). Other grooming behaviors include shaking, nibbling or licking on self, tail-swishing, rubbing, and periodic stomping to displace flies and biting insects. Allogrooming (also known as mutual grooming) in this ethogram is not considered categorically as a grooming behavior since it is also a social interaction that involves more than one animal. Depending on the nature of the research, it may be appropriate to consider allogrooming as part of the grooming category.



Figure 4. *Locomotion* is most typically expressed as walking from one place to another, as seen in this feral horse stallion. The higher energy-expense gaits of trotting, cantering, and galloping are observed far less frequently.



Figure 5. *Rolling* by this feral horse mare is a grooming behavior associated with pelage hygiene and insect control.

Comfort

Comfort behavior takes on a wide variety of forms in feral horses and includes any type of self-enjoyment expression, such as play, investigation, and stimulation (fig. 6). Some examples of comfort behavior are sun-basking, shelter seeking, masturbation, sexual play, object play, locomotor play, play fighting, and some olfactory investigations. Olfactory investigations also may be indicative of reproductive behavior or social behavior and should be included in the appropriate category for data collection based on specific research objectives. This ethogram also includes yawning and stretching as comfort behaviors, though it would not be inappropriate to include those expressions as a form of resting behavior.

Standing Attentive

When horses receive a stimulus that causes alertness, they react by exhibiting a rigid body posture with head upright, ears pointed forward, and eyes open and alert (fig. 7). This may be momentary in the case of a sound or smell causing alertness, but such standing attentive behavior also occurs for extended periods of time when a female is standing guard over a sleeping foal or a predator is nearby. In this ethogram, standing attentive does not include instances when the behavior is a result of human presence (fig. 8). In those cases, human awareness is considered its own category so that the biased data may be properly addressed during analyses.

Elimination

Elimination in horses is expressed as urination or defecation (fig. 9), though depending on research objectives, it should be determined whether any such expression will be included in this category or whether elimination with social implications is considered elsewhere. Socially, feral horse stallions create fecal middens known as stud piles and repeatedly defecate on them. These middens are thought to facilitate communication or ownership status of certain resources (Feist and McCullough, 1976; Rubenstein and Hack, 1992). When female feral horses urinate or defecate, it is common for the harem stallion to cover it with his urine for similar reasons. It has been reported that female horses do not cover the urine of other females in this fashion (McDonnell, 2003), though this was documented periodically during our study and may have implications in the female dominance hierarchy.



Figure 6. A typical *comfort* behavior for feral horse males is masturbation, which is expressed when a horse flexes his erect penis against his abdomen. This behavior is not expressed in conjunction with a nearby female and should not be confused with the penis drop observed when a male is reproductively tending a female.



Figure 7. Feral horse mares are focused on an external stimulus (in this case, a foal distress vocalization in the distance) and are exhibiting *standing attentive* behavior. Note the directionally pointed ears, rigid body position, and focused eyes characteristic of this expression. This behavior should not be confused with *human awareness*.



Figure 8. *Human awareness* is an important behavior to record when conducting any behavioral research so that biased data can be properly omitted from analyses of naturally occurring behaviors. Here, an entire band has paused because they noticed the photographer in the distance.



Figure 9. A female feral horse is exhibiting *elimination* by urination. Urination behavior in horses is sexually dimorphic: female horses urinate in a posterior direction and male horses urinate in an anterior direction.

In this ethogram, random urination and defecation are considered elimination, whereas systematic covering of urine or feces from the female by a harem stallion is considered as a harem tending behavior. The systematic covering of urine from a female by a female in the same harem is considered harem social behavior.

Social

Harem Social

Harem social behavior is considered to be social interaction among band members that is not specifically indicative of reproductive, harem tending, or agonistic behavior. Allogrooming is considered a harem social behavior in this ethogram (fig. 10), as is olfactory investigation (from one horse to another, fig. 11, or the systematic smelling of urine deposits among band members) and pair-bonding among juveniles (fig. 12).

Herding

When a feral horse stallion actively drives females of his harem together, it is considered herding behavior. This involves the stallion moving systematically behind the females, posturing with his head held low and ears laid back, and controlling the direction and movement of the band members (fig. 13). Waring (1983) termed this motion with the stallion's head moving side-to-side as snaking. Some researchers may choose to consider this as an agonistic behavior, but it is considered here as its own category since the function of the behavior is maintaining the harem rather than aggression toward specific females.

Harem Tending

Harem tending consists of stallion behaviors that are directed at maintaining the harem and include the defense and recruitment of females. The defense of females is expressed as a stallion positioning himself between his harem females and a perceived threat (typically another stallion) (fig. 14). It is also expressed by covering a female's urine or feces with his own, or by depositing feces on a stud pile (fig. 15). Recruitment, or stealing, of females by a harem stallion from another stallion is also considered harem tending behavior in this ethogram.

Reproduction

Reproductive behavior in feral horses is characterized by a series of stallion-initiated behaviors and a series of mare-initiated behaviors. The stallion reproductive sequence often



Figure 10. One of the most common *harem social* behaviors is allogrooming (also known as mutual grooming). It is expressed by the lateral parallel body position of two horses that allows for nibbling along the back or withers of each horse. While this behavior can be considered grooming, it is also thought to facilitate pair-bonding and dominance structure between band mates (Waring, 1983).



Figure 11. Olfactory investigation in the *harem social* behavior context is often observed as prolonged olfactory engagement of a female and her foal in a bonding regimen. This also may include licking and nudging.



Figure 12. Pair-bonding among juveniles within a band is also a common *harem social* behavior. In this example, female foals are closely engaging each other, which is thought to facilitate development of the female-female ethological relationships critical to band structure and stability later experienced as reproductive members of a family group (Crowell-Davis and others, 1986).



Figure 13. A feral horse stallion (leftmost horse) is *herding* females in his band by posturing and actively driving his females back into a cohesive group. Herding is the most common social behavior expressed by the dominant male toward females in his harem.



Figure 14. The blue roan stallion (foremost) is *harem tending* by expressing a posturing behavior and positioning himself between his harem females (several additional females out of the photo to the left) and an intruding male (right). Depending on the persistence and signals of the intruder, this behavior may develop into *agonism*.



Figure 15. Male feral horses also express *harem tending* by maintaining fecal middens, or stud piles, in which feces are aggregated at strategically located positions within the band's home range.

(but not always) begins with herding or reproductive tending behavior. Reproductive tending is the close following of a female by the male, without the directional driving observed in herding behavior. Following this tending behavior, the stallion often vocalizes and exhibits the olfactory response known as flehmen (fig. 16) (Stahlbaum and Houpt, 1989). He then proceeds by rubbing his head on the female's flanks and (or) resting his chin on her back, and extends his penis out of the prepuce (fig. 17). Some of these behaviors out of sequence are not necessarily associated with a stallion reproductive sequence; therefore, protocols to determine how to categorize the observed behavior are specified in figure 18.

Female reproductive behavior is signified by estrous, which can sometimes be challenging to detect from typical field observation distances. A mare initiates her reproductive sequence by presenting herself facing away from the stallion, lifting her tail, and vocalizing (though sometimes the female faces the stallion first). These behaviors alone do not necessarily signify estrous, since the female could simply be vocalizing and preparing to defecate. If in estrous, it is typically accompanied by posturing her body with hind legs slightly apart (an apparent squat) and often turning her head toward her posterior. The mare also will frequently emit small streams of urine, a behaviour that is often concurrent with rhythmic 'winking' of her vulva: winking can be observed by the periodic flashes of pink from the inner coloration of her vaginal membranes (McDonnell, 2003). These contrasting pigments can be seen in the rolling female in figure 5, simply because of her posture. The urine emitted from female horses in estrous consists of specific variations of urinary volatile compounds that may facilitate chemical communication during reproductive behavior (Ma and Klemm, 1997). Female horses may express estrous behavior during the anovulatory period (typically winter) as well as during pregnancy (Crowell-Davis, 2007). Such anomalies to our conventional understanding may lead to misinterpretation of estrous behavior in the field and care should be taken to correctly and systematically identify this behavior.

Once both male and female are exhibiting reproductive behaviors, copulation may occur with the stallion mounting the female from behind, neck arched over her back and forelegs resting on her sides (fig. 19). Several events could occur in this situation and should be categorized separately if reproductive behavior is a focus of the research. These include successful copulation, unsuccessful copulation (the stallion cannot adequately mount the female for some reason), mare acceptance, mare rejection (the mare kicks and moves away from the stallion), and forced copulation (the mare is not presenting or accepting the stallion's advance, but he relentlessly mounts her anyway).



Figure 16. Flehmen response in this feral horse stallion is displayed by the elevated head, and raised, inverted upper lip. This posture is a response to particularly exciting chemicals and olfactory signals that are detected by the main olfactory epithelium and concentrated directly into the vomeronasal organ, which in part controls sexual activity (Estes, 1972; Mills and Nankervis, 1999).



Figure 17. A feral horse stallion is exhibiting penis extension and rubbing of the female's flanks as part of the male reproductive behavior sequence.

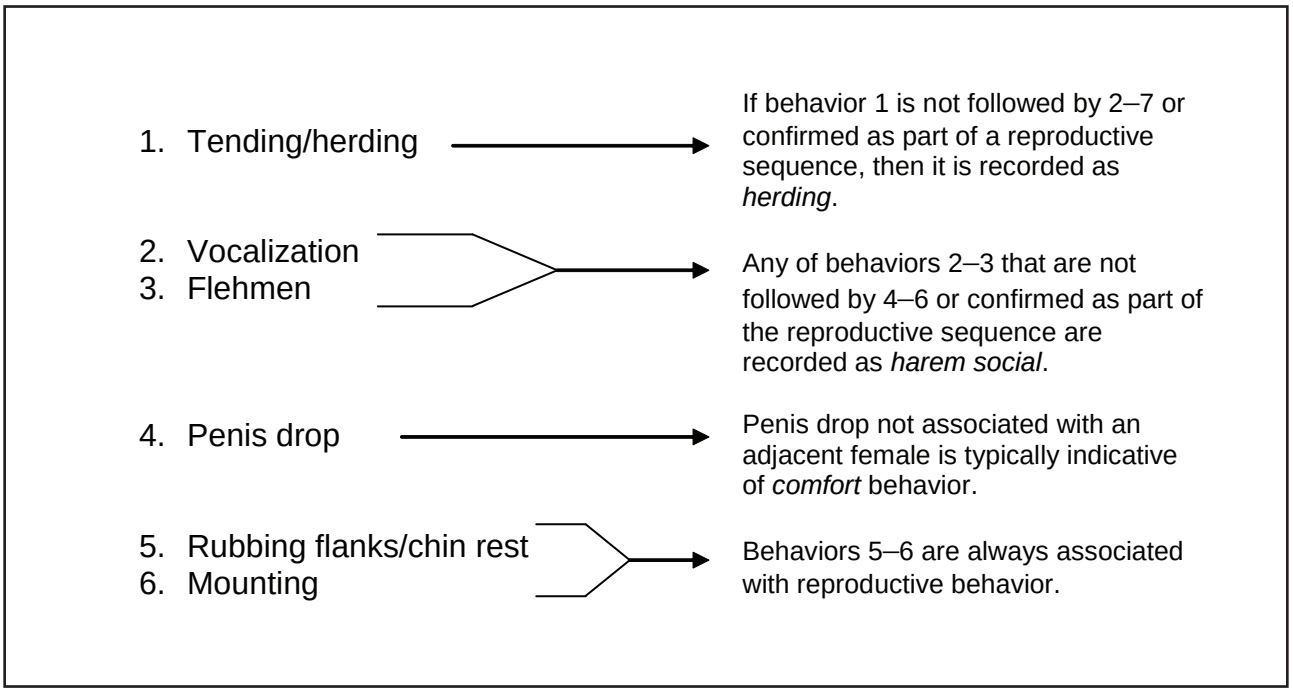


Figure 18. Basic guidelines for distinguishing reproductive and non-reproductive behaviors in stallions.



Figure 19. *Successful copulation* is occurring between this feral horse male and female. This reproductive behavior is characterized by a maintained mounting position on the female for the male and a relatively stationary, relaxed position for the female. *Unsuccessful copulation* is often a result of *mare rejection*, with the female expressing *agonistic* behaviors such as kicking toward the male and a brief mounting position for the male.

Parturition may also be included in the reproductive category or designated as its own behavioral category, depending on research focus.

Agonism

Agonism in feral horses is exhibited by a wide range of behaviors and in this ethogram is put into seven categories arranged by increasing intensity of the interaction. Note that any or all of the following behaviors may be exhibited in play by young animals, in which case they should be considered comfort behavior rather than agonism. The first four categories of agonism may occur between males only, females only, or between males and females, but the remaining levels of agonism are typically observed only between males.

The most common and least intense form of agonism is the threat. Threats are characterized by laterally pinned back ears, arched neck, and (or) a movement of the head toward the opposing horse, but with no physical contact (fig. 20). Most conflicts and dominance interactions among feral horses are resolved by these gestures alone.

The next agonistic expression is the bump or push, which is expressed by the aggressor making forceful contact with another horse using its head, neck, or shoulder (fig. 21).

As agonism escalates, the aggressor may chase its adversary at a gallop to displace the animal from the immediate area or with the intent of engaging the animal in more agonistic behavior (fig. 22). Chasing behavior is typically brief (seconds) or may last several minutes; however, the USGS study documented this behavior occasionally persisting over long time periods (greater than 1 hour) and great distances (greater than 3 km).

The fourth level of agonism involves biting an opponent or kicking with the hind legs (fig. 23). This level involves physical contact, though serious injury is rare.

Rearing occurs when the horse lifts its forelegs off the ground and elevates its body into a more vertical position, thus looking larger to the opponent (fig. 24). While technically only a threat, this body position typically signifies a more intense agonistic interaction and provides the initial position for commencement of stomping, striking, boxing, and dancing behaviors.



Figure 20. *Agonism* is expressed by both of these feral horses in the form of a *threat*. This is the most common form of agonism between feral horses and is most easily recognized by the laterally positioned, posterior pointing direction of the ears (shown in both animals here).



Figure 21. *Pushing* is an agonistic expression shown here by the foremost horse pushing toward the more distant horse.



Figure 22. *Chasing* is a moderately intense form of agonism and is shown here along with an expression of imminent biting behavior.



Figure 23. *Biting* is a common expression of agonism in equids and is expressed here between feral horse females to assert dominance.



Figure 24. *Rearing* is an expression often signifying a potentially intense agonistic interaction, typically between feral horse stallions.



Figure 25. *Striking* or *stomping* is being expressed by the feral horse stallion on the left. This behavior is typically indicative of highly agonistic expression and is expressed by the directed extension and downward motion of one or both front feet.

Stomping and striking occur when a horse uses one or both front feet to attack an opponent by extending the legs out and downward. This behavior has the potential to seriously injure the recipient (fig. 25).

The most intense form of agonism between feral horse stallions is boxing and dancing. These behaviors involve the engagement of the agonists in vertical body positions and the use of the front legs in striking (boxing) or closer engagement that results in head and neck biting (dancing) (fig. 26). On very rare occasions, feral horse stallions have been observed engaging in prolonged fights resulting in considerable injury and sometimes fatal outcomes.

Submission

Submission is exhibited by the loser of the agonistic encounter. This may be expressed by simply running away, but also may be expressed with laid down ears (distally or posteriorly), lowered head posture, lowering of the hindquarters, and sometimes jaw snapping (especially in juveniles) (fig. 27).

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Figure 26. *Boxing* and *dancing* between these two stallions is illustrative of the highest level of agonism that feral horses typically express.



Figure 27. *Submission* is being expressed by a young male horse (grullo [dark grey] horse in center) and directed toward the larger, dominant male (foremost), who is displaying *threat* behavior associated with dominance.

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Original Article

Foaling Rates in Feral Horses Treated With the Immunocontraceptive Porcine Zona Pellucida

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ABSTRACT Locally abundant feral horses (*Equus caballus*) can rapidly deplete available resources. Fertility control agents present promising nonlethal tools for reducing their population growth rates. We tested the effect of 2 forms of the immunocontraceptive porcine zona pellucida (PZP) on foaling rates in 3 populations of feral horses in the western United States. A liquid form requiring annual boosters was administered at Little Book Cliffs Wild Horse Range, Mesa County (CO), and Pryor Mountain Wild Horse Range, Bighorn County (WY) and Carbon County (MT), and a time-release pellet form designed to produce 2 yr of infertility was administered at McCullough Peaks Herd Management Area, Park County (WY). Average foaling rates (foals born/mare-yr) from direct observation of untreated and treated female horses (mares), 2004–2008, were 60.1% ($n = 153$ mare-yr) versus 6.6% ($n = 91$ mare-yr) at Little Book Cliffs, and 62.8% ($n = 129$ mare-yr) versus 17.7% ($n = 79$ mare-yr) at Pryor Mountain, respectively. At McCullough Peaks, mean annual foaling rates from 2006 to 2008 were 75.0% ($n = 48$ mare-yr) for untreated mares and 31.7% ($n = 101$ mare-yr) for treated mares. Controlling for age of mares and pretreatment differences in fertility, PZP reduced foaling rates in all 3 herds. The pellets used at McCullough Peaks (produced by cold evaporation) were less effective than pellets used in a previous trial and produced by heat extrusion. Immunocontraception with PZP may be a useful tool in reducing fertility rates in some western United States feral horse herds, but population growth reduction will depend on timely access to mares for inoculation and the proportion of mares that can be successfully treated. Published 2011. This article is a U.S. Government work and is in the public domain in the USA.

KEY WORDS *Equus caballus*, feral horse, fertility control, immunocontraception, PZP, western United States, wildlife contraception.

Tools for regulating animal populations are needed when habitat and resources are inadequate to sustain local abundance. Traditionally, regulation of wildlife populations has focused on lethal methods. Fertility control has emerged as an increasingly viable alternative to such methods and may be a particularly valuable tool when managers are faced with regulating protected species that are locally overabundant (Hobbs et al. 2000, Porton 2005). Such is the case with rapidly reproducing feral horses (*Equus caballus*) that roam large areas of public land in the western United States that are managed by the Bureau of Land Management (BLM) and U.S. Forest Service (Eberhardt et al. 1982, Garrott et al. 1991).

Many fertility control techniques have been proposed or tested for managing feral horses in the United States, ranging from hormone implants (Plotka et al. 1992) and intrauterine devices in females (Daels and Hughes 1995, Killian et al. 2008) to testosterone propionate treatment of males (Turner and Kirkpatrick 1982). These efforts produced tools that were publicly unacceptable, expensive to use, or impractical on a large scale (Kirkpatrick and Frank 2005). In recent decades, immunocontraceptive vaccines incorporating antigens such as porcine zona pellucida (PZP) or gonadotropin-releasing hormone (GnRH) have been used to limit reproduction in feral horses (Killian et al. 2008, Kirkpatrick and Turner 2008, Gray et al. 2010). Manager interest in these agents increased because of the relative ease of their application, expected efficacy and duration, and anticipated lack of side-effects.

Porcine zona pellucida acts as a vaccine against pregnancy by stimulating the production of zona pellucida antibodies in female mammals (Sacco 1977, Liu et al. 1989). These antibodies provide a barrier that prevents sperm from binding to the surface of an ovum and results in limited penetration of

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the zona pellucida and subsequent limited pregnancy in horses (Liu et al. 1989, Kirkpatrick et al. 1990). The most widely used formulations of PZP include a liquid form with a reported effectiveness of 7–10 months (Liu et al. 1989, 2005; Lyda et al. 2005), a time-release, heat-extruded (H/X) pellet form with a reported 22-month effectiveness (Turner et al. 2008), and a liposome-encapsulated liquid form known as SpayVac® (Immunovaccine Technologies, Halifax, NS, Canada), targeted for multiple-year effectiveness (Brown et al. 1997, Killian et al. 2008).

Detected physiological side-effects of PZP treatment have included a decrease in ovarian function when treatment is applied for >3 yr (Kirkpatrick et al. 1995), and injection-site reactions that appear to be nondebilitating in feral horses (Roelle and Ransom 2009). Reproductive behaviors were also found to be affected during the breeding season (Ransom et al. 2010) and band fidelity and reproductive behaviors were found to be affected outside of the breeding season (Nuñez et al. 2009).

Access to animals for timely inoculation and other management constraints may affect the utility of PZP as a management tool for western feral horse populations. We hypothesized that effectiveness of the treatments would be less than that previously demonstrated clinically. To evaluate this hypothesis, we applied PZP in liquid and pellet form in 3 western feral horse populations managed by BLM and assessed the effectiveness of the agent on individual female horses (hereafter, mares) from 2002 to 2008. Our objectives were to quantify the effectiveness of multiple years of contraception with liquid PZP and a single application of PZP pellets produced by an alternate, and more efficient, method (cold evaporation; Turner et al. 2008) on individual free-roaming mares in the context of age-specific foaling rates and actual management constraints.

STUDY AREAS

We inoculated mares with PZP at the Little Book Cliffs Wild Horse Range, the McCullough Peaks Herd Management Area, and the Pryor Mountain Wild Horse Range. The Little Book Cliffs Wild Horse Range, located in Mesa County, Colorado, USA (latitude 39°12′N, longitude 108°25′W), consisted of approximately 14,600 ha of sloping plateaus, sagebrush (*Artemisia* spp.) parks, and steep shale and sandstone cliffs dissected by 4 major canyon systems. Elevations ranged from 1,500 m to 2,250 m. The study area also exhibited dense stands of Colorado piñon (*Pinus edulis*) and Utah juniper (*Juniperus osteosperma*). Population size varied from 122 to 204 horses during the course of this study. Snow accumulation limited access to much of this study area during winter months, though horses that migrated into lower canyons could sometimes be accessed. The horses could only be approached close enough to dart (<60 m) when they were in open sagebrush parks.

Located in Park County, Wyoming, USA (latitude 44°35′N, longitude 108°40′W), the McCullough Peaks Herd Management Area consisted of 44,400 ha of open sagebrush park with badlands along the western edge. Vegetation consisted of large expanses of small shrubs, grasses, and forbs. Elevations ranged from 1,200 m to 1,964 m. Population size was as high as 495 horses before a large management removal in 2004, and ranged from 112 to 194 in subsequent years. The horses in this population did not allow humans to approach within darting range and remote delivery of PZP was deemed impractical at this site at the time of study.

The Pryor Mountain Wild Horse Range, located in Bighorn County, Wyoming and Carbon County, Montana, USA (latitude 45°04′N, longitude 108°19′W), consisted of slightly >16,000 ha of low desert, foothill slopes, forested montane slopes, steep canyons, and isolated grassy plateaus. Elevations ranged from 1,190 m to 2,625 m. Three undeveloped roads intersected the study area and were impassable from late autumn to early spring due to snow accumulation and seasonal erosion. Horses in this population were more conditioned to humans and could be approached at close range (<40 m) with little or no evasive behavior throughout the summer and when located at accessible low elevations in winter.

METHODS

Vaccine Preparation

We used the liquid form of PZP at Little Book Cliffs and Pryor Mountain and the cold-evaporation (C/V) pellet form at McCullough Peaks. Personnel from the Science and Conservation Center at ZooMontana (Billings, MT) prepared the liquid agent according to the methods of Dunbar et al. (1980) and delivered both agent and adjuvant to us. We emulsified PZP and adjuvant in alcohol-sterilized syringes prior to use, as described by Kirkpatrick et al. (1990). For hand injections, we loaded the emulsion into alcohol-sterilized syringes fitted with single-use sterile needles. For injections by dart, we loaded the emulsion into 1-cm^3 barbless darts (Pneu-Dart®, Williamsport, PA) as they were removed from the manufacturer's packaging and placed a small dab of petroleum jelly on the tip of the dart to prevent leakage. Both primer and booster injections at Little Book Cliffs and Pryor Mountain contained 0.5 mL (100 μg) of PZP. Primer inoculations at Little Book Cliffs also contained 0.5 mL of Freund's complete adjuvant (FCA; Claassen et al. 1992, Kensil et al. 2004), whereas those at Pryor Mountain contained 0.5 mL of either FCA or Freund's modified adjuvant (FMA). Freund's complete adjuvant can produce false-positive tuberculosis test results in treated animals and has been known to contribute to abscessed injection sites (Broderson 1989); therefore, FMA was substituted for FCA after research indicated no difference in efficacy between the 2 adjuvants (Lyda et al. 2005). Booster inoculations were identical except that we substituted Freund's incomplete adjuvant (FIA). Additional details on darts and delivery methods can be found in Roelle and Ransom (2009).

Personnel at the Medical College of Ohio (now the College of Medicine, University of Toledo) prepared the time-release pellets using the cold-evaporation process described by Turner et al. (2008). All pellets contained a saponin adjuvant, QA-21 (Virbac Corporation, Brussels, Belgium), and were

designed to release PZP in bolus fashion at 1 month, 3 months, and 12 months. The 1-month and 3-month pellets each contained 100 μg of PZP and 200 μg of adjuvant; the 12-month pellets contained 250 μg of PZP and 500 μg of adjuvant. The PZP used in these pellets was obtained from ZooMontana.

Contraceptive Treatments

We initiated treatments at each of the 3 study areas in conjunction with gathers (roundups) conducted by BLM (Pryor Mountain in Sep 2001, Little Book Cliffs in Jul 2002, and McCullough Peaks in Oct 2004) and collected all data in accordance with Colorado State University Animal Care and Use Committee Standards (protocol 03-107A-02). Age classes and numbers of horses treated were in accordance with local plans developed by BLM managers. At Little Book Cliffs and McCullough Peaks, these management decisions were based on the number of mares in each class that needed to be contracepted in order to meet specific population growth goals, as predicted by the WinEquus model (Jenkins 1996). Managers at these sites randomly selected mares within each age class to be treated, and we randomly assigned control status to mares within bands to ensure that each band of horses being observed for a concurrent study of PZP effects on behavior contained both treated and untreated mares (Ransom et al. 2010). Treatment decisions at Pryor Mountain were not random across age classes, but rather all animals in given cohorts were treated, as discussed below. We gave all injections intramuscularly in the hip, regardless of delivery method.

We treated mares of all ages ≥1 yr at Little Book Cliffs. The primer dose was administered by hand to gathered mares while they were restrained in a squeeze-chute. Additional ungathered mares received the primer by dart in order to make up the desired sample size because an insufficient number of mares were captured. We delivered a booster inoculation by dart when the animals could be relocated (never sooner than 24 days), followed by annual boosters by dart. We recorded delivery distance of dart applications using Leica Rangemaster 1200 Scan laser rangefinders (Leica, Solms, Germany). We attempted to schedule the annual boosters such that mares would be continuously immunized. Duration of effectiveness of PZP has not been studied in detail, especially for multiple years of treatment.

At the time we initiated our experiments, available evidence indicated that PZP antibody titers would be maintained at contraceptive levels in many mares for 10 months following a first booster and that duration of efficacy may be extended following subsequent boosters (Liu et al. 1989, Kirkpatrick and Turner 2002). Based on these studies and consultation with the manufacturers of the drug, we assumed 10 months duration of efficacy for the first booster, 11 months for the second, and 12 months for all subsequent boosters. Using this schedule as a guide, most mares at Little Book Cliffs were treated for 4 consecutive yr, though a few were lost to the study for various reasons and 4 mares received 5 booster inoculations. We did not add young animals to the treatment group as they reached 1 yr of age, but we did add them to the control group when they reached 3 yr of age. Precise age (observed as a foal) was available for 73.9% of 88 mares (both treatment and control) studied at Little Book Cliffs. Remaining mares were aged by tooth eruption and wear patterns (American Association of Equine Practitioners 2002).

At Pryor Mountain, treatments were similar to those at Little Book Cliffs except that we inoculated mares 1–2 yr of age (2002–2004) and older mares (≥15 yr, 2002–2004; ≥11 yr after 2004). These age classes were selected by managers to address public input concerning welfare of young and old females. Animals were, thus, added to the treatment group as they reached 1 yr of age (2002–2004) or 15 yr of age (11 yr of age after 2004), and controls consisted of middle-age animals that had never been treated. Treatments at Pryor Mountain also differed from those at Little Book Cliffs with respect to the timing of the first booster. At Pryor Mountain, management constraints (e.g., public appeals timeframe for Environmental Assessments) and weather forced us to deliver a number of first boosters only 14–16 days after the primer, instead of the 3–4 weeks used in previous studies (Kirkpatrick et al. 1997). Of the 83 treated or control mares at Pryor Mountain, ages of 64 (77.1%) were known (observed as a foal) from a long-term demography study (Roelle et al. 2010). We estimated ages of the remaining mares as at Little Book Cliffs.

At McCullough Peaks, we administered all inoculations in October 2004 while gathered mares were individually restrained in a squeeze-chute. We gave each mare a primer inoculation of liquid PZP (FMA as adjuvant), followed immediately by the time-release pellets, which were delivered by jabstick or hand-held trocar syringes and designed to act as boosters. Treated mares were of all ages ≥1 yr. We did not add mares to the treatment group in subsequent years, but we did add recruited mares to the control group when they reached 3 yr of age. We collected foaling data for 1 yr beyond the expected contraceptive effect in order to detect a possible residual effect, as was reported in the only similar study of time-release PZP (Turner et al. 2007). Precise ages were known for 34.4% of the 64 mares studied at McCullough Peaks (born during our study). We estimated ages of the remaining mares as at Little Book Cliffs.

Field Observations

U.S. Geological Survey (USGS) and BLM employees and volunteers recorded all field observations from the ground using optical equipment (binoculars, telescope) as necessary. These observers had completed undergraduate degrees in equine science, veterinary or preveterinary medicine, or biology, or had extensive experience working with equines. All mares and foals were identified and cataloged individually on the basis of pelage color, natural markings, and band association. Observers were trained in identifying individuals using these attributes and were provided with a photo database containing images of each individual for reference.

We located >99% of all mares and foals (when present) at least weekly from April to October of each year, and most

were relocated several times per week. Of those not located weekly, 4–6 mares annually at Little Book Cliffs were difficult to access, but were located at least monthly. Likewise, 1 mare at Pryor Mountain (2004) and 2 mares at McCullough Peaks (2007) were also not found weekly, but were observed at least monthly. Observations during the remainder of the year were opportunistic and largely dictated by weather. It is possible that some foals were born and died without being observed; however, given the intensity of observations, we believe that this rarely occurred. We matched foals with dams based on observations of attachment (e.g., nursing, general proximity) during the early days and weeks of a foal's life (Waring 2003); foaling rate was, thus, defined as the frequency of live births per cohort as detected by direct observation.

Data Analysis

The average gestation period for a horse is 335–342 days (Card and Hillman 1993); thus, mares are often pregnant when they receive a contraceptive agent, and the effect of contraception is not seen until the second foaling season following immunization. For example, a pregnant mare immunized in December of one year will give birth to a foal in the following spring, will be infertile for the subsequent breeding season, and the effect of that contraception will be seen in the next foaling season. We subsequently divided mares into 3 groups: untreated (control) animals that were never immunized; pretreatment animals that had been immunized, but the immunization had not yet taken effect; and treated animals that had been immunized and the immunization had presumptively taken effect. These strata were based on the date of booster treatment for each individual and the presumed duration of efficacy discussed previously.

We modeled foaling probability of mares with mixed-effects logistic regression, where individual mare was included as a random effect on the intercept term to account for the repeated observations (multiple yr) of individuals over time. This was necessary to account for variation that may be present among individuals who were sampled repeatedly, though not always equally over time. Such variation may arise from physiology, behavior, or any number of external factors that may affect conception, pregnancy, and parturition. We used the lmer(·) function in the lme4 package in R version 2.10.0 (The R Foundation for Statistical Computing 2009) to obtain mixed-effects model estimates. If the variance estimate for the random effect of individual mare converged to zero, we re-estimated the model as a simpler fixed-effects logistic regression using the glm(·) function. Foaling rates of feral horses often increase for the first few years after mares reach sexual maturity, remain high through middle age, and decrease in older mares (Garrott and Taylor 1990, Roelle et al. 2010). We, therefore, included linear and quadratic effects of age in all models. We rescaled age in the quadratic effect by subtracting the mean age of horses (8.89 yr across all populations), so that the intercept term of the model corresponded to probability of foaling at mean age. We fitted separate models for each study area.

To assess effects of the immunocontraceptive, we compared untreated mares to pretreatment mares and untreated mares to treated mares. In both comparisons we used only years in common between the 2 groups so that any environmental effects were constant. We used the first comparison (untreated vs. pretreatment) to isolate any preexisting differences associated with potential nonrandom selection of individuals that could have arisen from allocation of controls per band rather than across the population. We modified the estimated odds ratio of foaling rates in the second comparison (untreated vs. treated mares) by the estimated odds ratio associated with the preexisting differences. The odds ratio of producing foals associated with the immunocontraceptive was then equal to $\exp(\hat{\beta}_{\text{treated}} - \hat{\beta}_{\text{pretreatment}})$ where $\hat{\beta}_{\text{treated}}$ was the estimated difference between treated and untreated mares and $\hat{\beta}_{\text{pretreatment}}$ was the estimated difference between pretreatment and untreated mares. We estimated variance for the combined odds ratio using simple formulas for linear combinations of uncorrelated random variables (Neter et al. 1996:1318). Because the estimated differences for pretreatment and treated groups used the same horses, the assumption of uncorrelated estimates was violated to some degree (le Cessie et al. 2008). Other models combining all 3 treatment groups, which ignored our desired balance across years, suggested that the estimated differences between untreated and pretreatment mares and between untreated and treated mares were positively correlated. Excluding a positive covariance in our variance estimates for the combined odds ratio produced a wider interval than would have resulted had we been able to account for the positive covariance. Thus, we consider our variance estimates for the combined odds ratios assuming zero correlation to be conservative. We estimated degrees of freedom for use with a t-distribution for constructing confidence intervals based on Satterthwaite (1946) as provided in Buckland et al. (1993:89).

At Little Book Cliffs and Pryor Mountain, mares received varying numbers of annual boosters: darts did not always function properly (Roelle and Ransom 2009), and it was not always possible to meet the schedule outlined above for keeping mares continuously contracepted. We used mixed-effects logistic regression to estimate probability of foaling among presumptively treated mares as a function of these variables. We included number of boosters received as a continuous variable and assigned a value for dart function (good or poor) based on physical examination of the dart after it was recovered. Darts were classified as poor if the vaccine did not fully discharge from the dart, pieces of the dart separated, or the needle broke on impact, and classified as good if the dart fully discharged and was structurally intact. We assigned a value for booster timing (good or poor) based on the number of days between injections. Consistent with the planned injection schedule for keeping mares continuously contracepted, we considered timing to be poor if >300 days (approx. 10 months) elapsed between the first and second boosters, >330 days (approx. 11 months) between the second and third, and >365 days (12 months) between all subsequent boosters. We also considered timing to be poor if the first booster was delivered during the

breeding season, which we defined based on foaling dates for untreated mares and an average gestation period (approx. 11 months). At Little Book Cliffs, 82.1% of births to untreated mares ($n = 117$ mare-yr) occurred in March–May, so we defined the breeding season as April–June. At Pryor Mountain, 93.5% of births to untreated mares ($n = 108$ mare-yr) occurred in April–June, so we defined the breeding season as May–July. We included linear and quadratic effects of age as in the previous comparisons.

At Little Book Cliffs and Pryor Mountain, we hypothesized that method of primer injection (dart or syringe) might affect foaling rate of treated mares in the first year of contraception. Also, at Pryor Mountain we hypothesized 2 additional factors might have an effect on foaling rate of treated mares in the first year of contraception: adjuvant used with the primer (FCA or FMA) and time between the primer and first booster (good or poor). We considered time between the primer and first booster to be poor if the booster was delivered <21 days after the primer. We examined foaling rates of treated mares as a function of these factors using fixed-effects logistic regression, again incorporating linear and quadratic terms for age of the mare.

RESULTS

At Little Book Cliffs, 25 individual mares composed the treatment group (received a primer inoculation and ≥1 booster inoculation, and were ≥3 yr of age at the time of expected parturition), and the untreated group (≥3 yr of age at the time of expected parturition) comprised 63 mares. At Pryor Mountain, 40 mares were treated (one additional mare was included in pretreatment analyses but died before a treatment effect could be detected) and 42 were untreated. At McCullough Peaks, 36 mares received the pellet form of the vaccine and 28 were untreated. Most mares were observed for foaling in multiple years and, unless otherwise noted, sample sizes reported hereafter represent mare-years.

Mares at Little Book Cliffs demonstrated a cumulative behavioral aversion to darting with first darts being delivered at an average of 28.6 ± 1.5 m (SE) linearly increasing to fourth darts being delivered at an average of 39.4 ± 3.3 m. Pryor Mountain horses were more conditioned to human presence and most received no >2 dart applications. Behavioral aversion to darting was not evident at this site; these horses received first darts at an average of 17.67 ± 2.1 m and second darts at an average of 12.2 ± 1.6 m.

At both Little Book Cliffs and Pryor Mountain, the original protocol called for boosters to be timed such that mares would always be effectively contracepted. This protocol was followed reasonably well at Little Book Cliffs, but at Pryor Mountain other management considerations and weather often intervened, and 72 of 79 boosters (91.1%) were delivered in August, September, or October, irrespective of when the previous booster was delivered. Delivering vaccinations by dart was seldom possible from November through February at either Little Book Cliffs or Pryor Mountain because seasonal access to roads and trails was impeded by drifting snow.

Of the 6 foals born to treated mares at Little Book Cliffs, 3 were born to a single mare. At McCullough Peaks, 20 of the 32 foals born to treated mares were attributed to 4 mares that produced a foal in each of the 3 treatment yr and another 4 mares that produced a foal in each of 2 treatment yr. At Pryor Mountain, 4 of the 14 foals born to treated mares were accounted for by 2 mares that produced 2 foals each. Though the number of foals born to treated mares was relatively small, the timing of these births provides some quantitative evidence that second and subsequent boosters may have a longer contraceptive effect than does the first booster (Fig. 1).

In all 6 comparisons of treatment groups (untreated vs. pretreated and untreated vs. treated at 3 study areas) mare age affected foaling (either the linear or quadratic term, or both; $P < 0.05$). Across all populations in our study, there was a nonlinear relationship ($R^2 = 0.80$, $n = 972$ mare-yr) between age of control and pretreatment mares and foaling rate (Fig. 2). After controlling for mare age, foaling rate of untreated mares as compared to pretreatment mares was lower at Little Book Cliffs, and slightly higher at McCullough Peaks and Pryor Mountain; however, the difference only approached significance ($P = 0.09$) at Little Book Cliffs (Table 1).

The combined odds ratios indicate that PZP treatment reduced the foaling rate at all 3 study areas ($P < 0.05$; Table 1); however, effectiveness of the treatment varied by area. At Little Book Cliffs, the mean foaling rate observed among treated animals was 6.6% (range = 0.0–10.0% over 5 yr), whereas the mean annual foaling rate observed at Pryor Mountain was 17.7% (range = 0.0–35.0% over 5 yr). The mean annual foaling rate observed at McCullough Peaks was even higher (31.7%); interestingly, however, foaling rate in the third year of treatment effect at McCullough Peaks

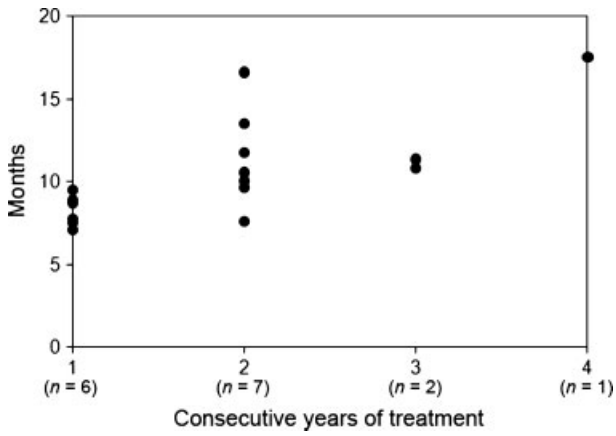


Figure 1. Foals born to feral horse (*Equus caballus*) females treated with the immunocontraceptive porcine zona pellucida (PZP) as a function of time (months) from booster inoculation to conception (calculated from parturition date) and the number of consecutive annual treatments received. Females inhabited Little Book Cliffs Wild Horse Range (Mesa County, CO) and Pryor Mountain Wild Horse Range (SE Carbon County, MT), USA, from 2002 to 2008, and received 1–4 booster inoculations containing a 1-cm^3 emulsion of PZP and Freund's incomplete adjuvant.

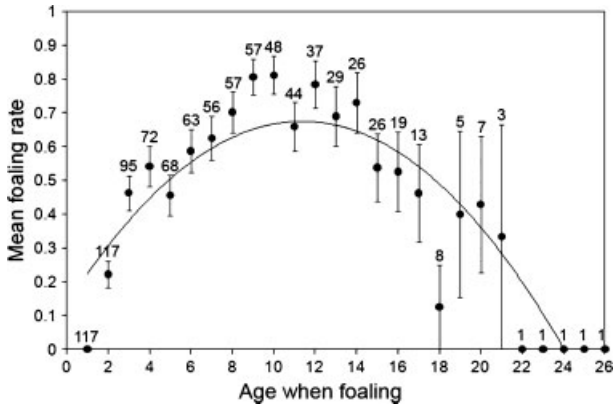


Figure 2. Mean foaling rates (±SE of the mean) for all ages of untreated and pretreatment feral horse (*Equus caballus*) females (*n* given at the top of each error bar) at Little Book Cliffs Wild Horse Range (Mesa County, CO, USA) 2002–2008, McCullough Peaks Wild Horse Management Area (Park County, WY, USA) 2003–2008, and Pryor Mountain Wild Horse Range (SE Carbon County, MT, USA) 2002–2008.

(29.0%, $n = 31$ mares) was nearly identical to that in the first year (27.8%, $n = 36$ mares), even though the pellets used at McCullough Peaks were designed for only 2 yr of efficacy.

When combined in a mixed-effects model with linear and quadratic effects of age and a random effect of individual mare, neither dart function ($z = 1.30$, $P = 0.20$), consecutive years of treatment ($z = 0.49$, $P = 0.62$), nor booster timing ($z = 1.04$, $P = 0.30$) affected foaling at Little Book Cliffs ($n = 91$; Table 2). Similarly, none of these covariates had an effect at Pryor Mountain (dart function, $z = 0.71$, $P = 0.48$; consecutive years of treatment, $z = -0.04$, $P = 0.97$; booster timing, $z = -0.32$, $P = 0.75$; $n = 79$). When combined in a fixed-effects model with linear and quadratic effects of age, time between the primer and first booster also had no effect ($z = 0.38$, $P = 0.70$) at Pryor Mountain, nor did adjuvant used with the primer ($z = 0.15$, $P = 0.88$; $n = 40$ mares). There was no random effect of individual mare in this model because we considered only the first booster for each mare. Lack of significance for the primer adjuvant term, despite the relatively large difference in foaling rates (FCA = 7.4%, FMA = 46.2%; Table 2) may be explained by the fact that all (17) of the mares ≤6 yr of age, most of which would have a low foaling rate in the absence of contraception, received FCA as the primer adjuvant, and none produced foals. An exact Chi-square test on the remaining 23 mares (all ≥12 yr of age) also showed no effect of the primer adjuvant (FCA, 2 of 10 mares foaled; FMA, 6 of 13 foaled; $P = 0.38$). No models could be properly fitted for foaling rate in response to method of primer delivery because of the uneven distribution of delivery methods at each site (Table 2).

DISCUSSION

Feral horses treated using 2 different forms of the immunocontraceptive PZP exhibited decreased foaling rates compared to untreated mares. Fertility was strongly related to age of mares and proved to be critical in understanding the effectiveness of treatment. Both the liquid and C/V forms of PZP were robust in terms of timing and the various management constraints encountered during application periods, though we suspect the difference in foaling rates between the 2 sites where liquid PZP was used relate to such challenges. Few treated mares produced foals in our study, but there is some evidence that repeated annual boosters increased the effective duration of each treatment.

Several investigators have noted that individual mares vary greatly in their ability to raise antibody titers against PZP and that some mares may simply not respond (Liu et al. 1989,

Table 1. Logistic regression comparisons of foaling rates (%) for untreated, pretreatment, and treated females at mean age in 3 feral horse herds in the western United States, 2002–2008. Contraceptive treatments consisted of liquid porcine zona pellucida (PZP) followed by annual boosters at Little Book Cliffs Wild Horse Range (Mesa County, CO) and Pryor Mountain Wild Horse Range (SE Carbon County, MT), and a liquid primer plus time-release PZP pellets at McCullough Peaks Herd Management Area (Park County, WY).

Comparisons and parameter values	Little Book Cliffs	Pryor Mountain	McCullough Peaks
Pretreatment vs. untreated			
Years of comparison	2002–2004	2002–2007	2003–2005
n pretreatment	41	116	88
n untreated	92	153	23
Estimated foaling rate, pretreatment (95% CI)	82.9 (67.3–92.0)	65.9 (51.2–78.0)	83.1 (69.3–91.4)
Estimated foaling rate, untreated (95% CI)	70.3 (57.0–80.9)	71.0 (60.5–79.7)	87.0 (66.3–95.8)
Odds ratio	2.051	0.786	0.734
95% CI for odds ratio	0.878–4.790	0.376–1.647	0.230–2.337
P-value (H_0: foaling rates are equal)	0.094	0.522	0.596
Treated vs. untreated			
Years of comparison	2004–2008	2004–2008	2006–2008
n treated	91	79	101
n untreated	153	129	48
Estimated foaling rate, treated (95% CI)	8.0 (3.4–17.5)	34.0 (13.8–62.3)	30.2 (17.4–47.0)
Estimated foaling rate, untreated (95% CI)	73.7 (62.5–82.5)	73.4 (62.2–82.2)	89.7 (68.8–97.2)
Odds ratio	0.031	0.187	0.049
95% CI for odds ratio	0.011–0.083	0.059–0.598	0.013–0.189
P-value (H_0: foaling rates are equal)	<0.001	0.004	<0.001
Treated vs. untreated (age adjusted)			
Combined odds ratio	0.015	0.238	0.067
95% CI for combined odds ratio	0.004–0.056	0.060–0.950	0.011–0.403

Table 2. Comparisons of covariates potentially affecting foaling rates (%) among treated females in 2 feral horse herds in the western United States, 2002–2008. Rates given are calculated from the observed data and are not controlled for age. Contraceptive treatments consisted of liquid porcine zona pellucida (PZP) followed by annual boosters at both Little Book Cliffs Wild Horse Range (Mesa County, CO) and Pryor Mountain Wild Horse Range (SE Carbon County, MT). FCA = Freund's complete adjuvant, FMA = Freund's modified adjuvant.

	Little Book Cliffs		Pryor Mountain	
Covariate	**Foaling rate (%)**	***n***	**Foaling rate (%)**	***n***
Booster timing				
Good	5.1	59	19.4	31
Poor	9.4	32	16.7	48
Dart function				
Good	5.8	86	16.9	71
Poor	20.0	5	25.0	8
Consecutive years of treatment				
1	8.0	25	20.0	40
2	4.4	23	21.4	28
3	9.1	22	0.0	6
4	5.9	17	0.0	5
5	0.0	4	—	—
Time between primer and first booster				
<21 days	—	—	26.7	15
≥21 days	8.0	25	16.0	25
Primer adjuvant				
FCA	8.0	25	7.4	27
FMA	—	—	46.2	13
Primer delivery method				
Hand injection	10.5	19	0.0	3
Dart	0.0	6	21.6	37

Turner et al. 2002, Lyda et al. 2005). Our results, particularly at Little Book Cliffs and McCullough Peaks, where multiple births to individual mares accounted for a high proportion of the foals born to treated animals, seem to confirm this conclusion. Early investigations also showed that application of PZP does not affect pregnancies in progress (Liu et al. 1989, Kirkpatrick et al. 1990). Our results support this conclusion; foaling rates among pretreatment mares at all 3 study areas were greater than or equal to foaling rates among untreated mares.

Our results at Little Book Cliffs (6.6% observed foaling rate) for mares treated with liquid PZP are similar to those reported previously. Turner and Kirkpatrick (2002) reported that 34 of 340 mares (10.0%) that received a primer followed by annual boosters produced foals on Assateague Island National Seashore, Maryland, USA. Later in the same multiyear study, the authors reported a foaling rate of 3.9% ($n = 861$) among treated mares (Kirkpatrick and Turner 2008; table 1). In a composite sample from multiple western horse herds, Turner et al. (2002) found an 8% foaling rate ($n = 376$) among treated mares. In contrast to the multiyear study on Assateague Island, however, mares in these western herds received only a single booster inoculation.

At Pryor Mountain, the foaling rate observed among treated mares (17.7%) was somewhat higher than that found at Little Book Cliffs and reported in other studies (Turner and Kirkpatrick 2002, Turner et al. 2002, Kirkpatrick and Turner 2008). Although none of the covariates we included in the models were significant at either Little Book Cliffs or Pryor Mountain, we suspect that timing of the boosters, especially in the first and second years, may be responsible for the higher foaling rate at Pryor Mountain. In the only other multiyear study of PZP efficacy in feral horses (Assateague Island), researchers delivered boosters in the spring to maximize the probability that anti-PZP titers would remain high and mares would remain contracepted throughout the following breeding season, rather than attempting to keep mares contracepted throughout the year (Kirkpatrick et al. 1990). At some (unspecified) point in time, however, investigators at Assateague began delivering boosters in August or September if the mares had already been contracepted for ≥2 yr (Turner and Kirkpatrick 2002). Delivery of the booster in August or September allowed more efficient access to the mares and apparently did not affect efficacy of the treatments (Turner and Kirkpatrick 2002). Actual booster timing at Pryor Mountain was, thus, similar to the revised approach at Assateague, except that mares received boosters in August–October irrespective of the number of years contracepted. If the first PZP booster is effective for only 7–10 months (Liu et al. 1989, 2005; Lyda et al. 2005), then first boosters delivered in August–October may not result in effective contraception throughout the following breeding season. The same may be true for second boosters, for which duration of efficacy is not well-known. Apart from this potential effect at Pryor Mountain, however, effectiveness of the liquid form of PZP appears to have been quite robust with respect to timing of boosters.

Foaling rates among mares at McCullough Peaks treated with the C/V pellets were higher than those reported in the only other study where a presumptive multiple-year, time-release form of PZP was used on horses. Using H/X pellets on the Clan Alpine herd in Nevada, USA, investigators found foaling rates of 5.2% ($n = 58$) in the first year of treatment, 14.9% ($n = 68$) in the second year, and 31.6% ($n = 19$) in the third year (Turner et al. 2007:table 4). Foaling rates in the first 2 yr were significantly different

from rates among untreated mares ($P < 0.001$), and investigators felt that there was some residual, though statistically nonsignificant, effect in the third year (31.6% foaling, $n = 19$; Turner et al. 2007). Observed foaling rate in the third year of our study (29.0%) was remarkably similar, suggesting no advantage of the C/V pellets in terms of increased duration of efficacy.

Differences between our results and those from Clan Alpine appear to be related to the pellet formulation (C/V vs. H/X). Subsequent to the initiation of our study, Turner et al. (2008) reported that C/V pellets performed poorly in vitro; >80% of the total PZP was released before the desired window. In white-tailed deer (*Odocoileus virginianus*), C/V pellets were also less effective in the first year (2 of 8 F produced fawns) than H/X pellets (0 of 9 F produced fawns), though sample sizes were small (Turner et al. 2008).

The primer doses for both liquid and pellet applications contained either FCA or FMA, which are widely recognized as being highly immunogenic (Kensil et al. 2004): the PZP dosage in the liquid boosters and the 1-month and 3-month pellets was equal and the 12-month pellet contained 2.5 times more PZP. However, whereas the booster doses of liquid PZP contained FIA, the pellet doses contained QA-21. The adjuvant dosage was much higher for QA-21 than for FIA, and QA-21 is also highly immunogenic (Kensil et al. 2004), but it is possible this difference in booster adjuvants could have contributed to the effectiveness of treatment observed.

Our study also illustrated the importance of considering age-specific differences in foal production when evaluating the effects of a contraceptive. In most previous studies of which we are aware, age has been addressed by selecting only mares in prime reproductive status, usually ages 6–15. This is a reasonable approach in situations where investigators are able to select specific mares for treatment; however, this is not the case in most western United States feral horse populations, where treatments necessarily span a broader range of ages. Age was a significant covariate in all of the models we used to compare treatment groups. Furthermore, we might well have reached an incorrect conclusion regarding importance of the primer adjuvant had we not included mare age in our comparisons. Equivalence of FCA and FMA in raising antibody titers has also been demonstrated in vitro (Lyda et al. 2005) and in vivo at Assateague Island, where the switch from FCA to FMA has apparently not affected effectiveness of the treatments.

Liquid doses of PZP can be delivered by dart or by hand injection. Pellet forms of PZP can currently only be delivered by hand injection in conjunction with gathers, but researchers are developing the technology to apply pellets via dart. The liquid form of PZP appears to reduce foaling rates more effectively than the C/V pellet form, but considerable time can be expended in delivering the drug by dart, depending on wariness of the animals. The additional challenge of cumulative behavioral aversion to dart application may be a long-term concern for repeated vaccinations in areas where horses are not accustomed to human presence. Feral horses in the western United States are often inaccessible at the optimal time for an annual inoculation (late winter); therefore, if PZP is applied remotely in the liquid form, scheduling boosters such that mares are continuously contracepted appears to be a more effective strategy than a static annual date of inoculation.

To date, time-release (pellet) technology appears to offer the greatest promise for a multiyear immunization using PZP and, based on the 2 studies completed thus far, pellets produced by the H/X process appear to be superior to those produced by the C/V process. Both types, however, significantly reduced foaling in treated mares as compared to controls. Pellets produced by a third process referred to as pressure-molding have not yet been tested in vivo in feral horses (Turner et al. 2008). The liposome-encapsulated form of PZP, SpayVac®, may also be useful as a multiyear contraceptive agent, but to date has been tested in only a small number of horses (Killian et al. 2008, Gray et al. 2010).

MANAGEMENT IMPLICATIONS

In herds where feral horse mares can be accessed annually for booster inoculations, the liquid form of PZP delivered by dart can reduce foaling rates and the effective duration of each treatment may increase with repeated annual applications. However, a multiyear contraceptive agent would be preferable for use in most cases, even if horses can be accessed for annual boosters, because of increased efficiency in application and decreased problems with behavioral aversion to treatment and ability to locate individual animals on the range. Ultimate success in limiting population growth will depend not only on the efficacy of the agent, but also on the proportion of mares that can be accessed for treatment (Garrott 1991, 1995; Kirkpatrick and Turner 2008) and the age of mares treated. Porcine zona pellucida appears to be a robust form of fertility control in feral horses and able to withstand a wide array of application challenges in the field, though effectiveness may vary considerably by drug form and application strategy. Managers must also consider the scope and magnitudes of potential side-effects associated with use of the drug and weigh the known effects against the benefit of reducing population growth.

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Contraception can Lead to Trophic Asynchrony between Birth Pulse and Resources

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Abstract

Abiotic inputs such as photoperiod and temperature can regulate reproductive cyclicity in many species. When humans perturb this process by intervening in reproductive cycles, the ecological consequences may be profound. Trophic mismatches between birth pulse and resources in wildlife species may cascade toward decreased survival and threaten the viability of small populations. We followed feral horses (*Equus caballus*) in three populations for a longitudinal study of the transient immunocontraceptive porcine zona pellucida (PZP), and found that repeated vaccinations extended the duration of infertility far beyond the targeted period. After the targeted years of infertility, the probability of parturition from post-treated females was 25.6% compared to 64.1% for untreated females, when the data were constrained only to females that had demonstrated fertility prior to the study. Estimated time to parturition increased 411.3 days per year of consecutive historical treatment. Births from untreated females in these temperate latitude populations were observed to peak in the middle of May, indicating peak conception occurred around the previous summer solstice. When the post-treated females did conceive and give birth, parturition was an estimated 31.5 days later than births from untreated females, resulting in asynchrony with peak forage availability. The latest neonate born to a post-treated female arrived 7.5 months after the peak in births from untreated females, indicating conception occurred within 24–31 days of the winter solstice. These results demonstrate surprising physiological plasticity for temperate latitude horses, and indicate that while photoperiod and temperature are powerful inputs driving the biological rhythms of conception and birth in horses, these inputs may not limit their ability to conceive under perturbed conditions. The protracted infertility observed in PZP-treated horses may be of benefit for managing overabundant wildlife, but also suggests caution for use in small refugia or rare species.

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Introduction

Phenology of nearly all biological phenomena is influenced by natural abiotic events and is reflected in traits evolving to maximize fitness [1]. Births of many large mammal species, for example, occur in annual pulses that are regulated by seasonal cues such as photoperiod and temperature [2]. Increasing sunlight and temperature that accompanies the transition from winter to spring can trigger a physiological response in the pineal gland that initiates reproductive receptivity (Figure 1) [2–4]. This ultimately influences when females may conceive and thus when offspring are born. For example, feral horses (*Equus caballus*) in the northern hemisphere typically begin reproductive cyclicity in early spring and continue until late autumn; consequently, we may posit that conception should naturally peak near the longest day of sunlight (summer solstice) and parturition should peak 335–342 days later (duration of equine gestation [5]). This pattern should result in synchrony of the birth pulse with spring, when climate and forage availability for the dam can contribute to increased neonate survival.

Human actions have the potential to perturb such phenology. Resource managers are increasingly considering transient contraceptives to help limit growth of wildlife populations when species become locally overabundant and risk depleting resources or altering natural communities [6,7]. These agents are especially appealing to managers of protected species and small populations because fertility of treated individuals may return when the transient contraception wears off [8]. However, the transient nature of many fertility control agents and variation of individual responses to those agents introduces uncertainty toward persistence of infertility and the timing of births thereafter. Cascading trophic asynchrony between birth pulses and seasonal resource peaks can have profound effects on the population ecology of wildlife species [9–12]. Observed changes in such phenology and their implications have garnered much attention in climate change science [13,14], but more direct anthropogenic influences, such as fertility control, remain almost completely unstudied. Unintended

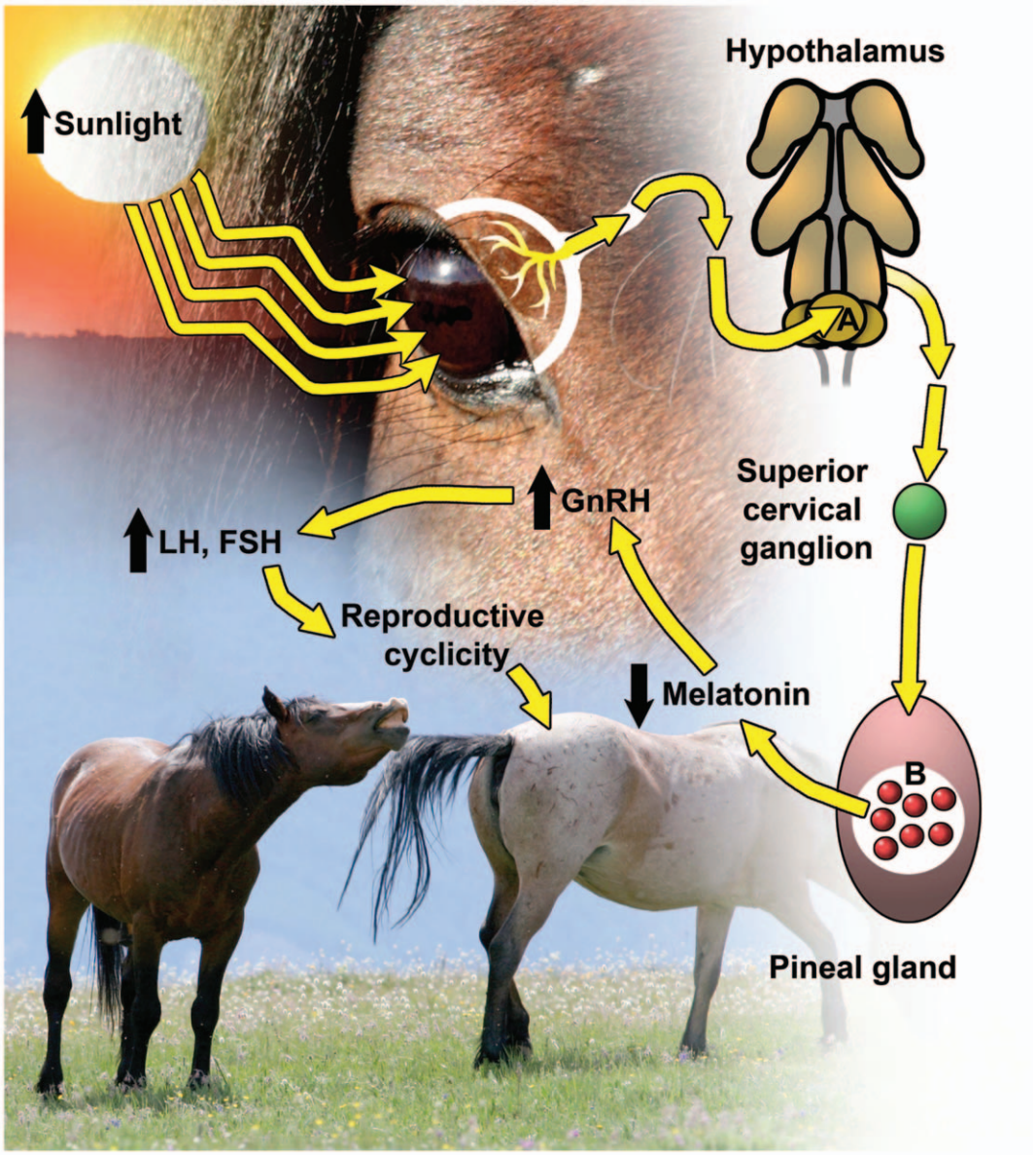


Figure 1. The physiological pathway from photoperiod to reproductive cyclicity in the horse (*Equus caballus*). Increasing sunlight toward the long days of summer strikes the retina, which transmits a neural signal to the suprachiasmatic nuclei (A), across the hypothalamus, and ultimately synapses to the pineal gland to stimulate release of inhibitory neurotranmitters toward the pinealocytes (B). Melatonin production then diminishes, thus increasing release of gonadotropin releasing hormone (GnRH), which increases luteinizing hormone (LH) and follicle stimulating hormone (FSH), and ultimately induces reproductive cyclicity.
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persistence of contraception, and consequent phenological changes in births, may compromise the ability of populations to recover after catastrophic stochastic events and threaten viability of small refugia populations.

The immunocontraceptive porcine zona pellucida (PZP) is becoming an increasingly popular management tool and has been used in at least 76 animal species worldwide [6]. This transient contraceptive may last 10–22 months per application, depending on the formulation and species [6,15,16]. The management appeal of PZP is that it is expected to be reversible, is relatively easy to apply, and has few known contraindications for individuals [17,18]. Population-level contraindications are more uncertain, but some evidence suggests decreased fecundity among untreated females, decreased fidelity in social groups, and increased adult survival can occur in populations containing PZP-treated females [19–22].

The individual-level efficacy and behavioral implications of PZP were previously investigated in three free-roaming feral horse populations in the western U.S. [16,23]. We have now followed each individual female in these same populations past their targeted infertile years to further evaluate return to fertility, phenology of births, and neonate survival. We hypothesized that duration of residual infertility would be a function of cumulative historical treatments. We also hypothesized that due to the expected individual response variability to treatment and the unknown and variable duration of infertility, post-treated females would give birth later in the year compared to untreated females, and those births would be asynchronous with forage availability.

We expected that parturition timing would be constrained by photoperiod and temperature and that offspring born toward the end of this temporal window would have decreased survival.

Materials and Methods

Ethics Statement

All data for this study were collected in accordance with the Colorado State University approved Animal Care and Use Committee protocol 03-107A-02. All data collection was conducted with the permission of the Bureau of Land Management (BLM) on public lands they administer, and involved routine observations of feral horses, which are protected under The Wild Free-Roaming Horses and Burros Act of 1971 (U.S. Public Law 91–195, as amended).

Study Areas

The Little Book Cliffs Wild Horse Range, located in Mesa County, Colorado, U.S.A. (latitude 39°12′N, longitude 108°25′W), consisted of approximately 14,600 ha of sloping plateaus, sagebrush (*Artemisia* spp.) parks, and 4 major canyon systems. Elevations ranged from 1,500 m to 2,250 m. The study area was characterized by dense stands of Colorado piñon (*Pinus edulis*) and Utah juniper (*Juniperus osteosperma*). Population size varied from 131–179 horses during 2005–2011 and was distributed in bands of 2–9 horses. Mean annual temperature was 11.5°C (minimum = −26.7, maximum = 41.1°C). Mean total annual precipitation was 235.4 mm (range = 184.4–300.2 mm), typically falling in a monsoonal pattern of late summer rains.

McCullough Peaks Herd Management Area in Park County, Wyoming, U.S.A. (latitude 44°35′N, longitude 108°40′W), consisted of 44,400 ha of primarily open sagebrush steppe. Elevations ranged from 1,200 m to 1,964 m. Population size ranged from 169–236 horses in bands of 2–17 individuals during 2007–2011. Mean annual temperature was 8.0°C (minimum = −30.0, maximum = 37.8°C) and mean total annual precipitation was 271.2 mm (range = 168.9–389.1 mm).

The Pryor Mountain Wild Horse Range, located in Bighorn County, Wyoming and Carbon County, Montana, U.S.A. (latitude 45°04′N, longitude 108°19′W), consisted of roughly 16,000 ha of low desert, foothill slopes, forested montane slopes, steep canyons, and isolated grassy plateaus. Elevations ranged from 1,190 m to 2,625 m. Vegetation types varied greatly from lower to higher elevations of the range with lower elevations dominated by sagebrush communities, mid elevations dominated by curl-leaf mountain mahogany (*Cercocarpus ledifolius*) and Utah juniper communities, and high elevations dominated by limber pine (*Pinus flexilis*), subalpine fir (*Abies lasiocarpa*), and alpine bluegrass (*Poa alpina*). Mean annual precipitation was 161.4 mm (range = 96.7–233.4 mm) and mean annual temperature was 7.1°C (minimum = −33.9, maximum = 40.0°C). The population ranged 171–233 during the study and was arranged in bands of 2–12 individuals.

Treatments

Free-roaming female feral horses were formerly treated with PZP for 1–5 years at all three study sites [16]. The conventional liquid form of PZP was designed to provide infertility through only a single year per inoculation, and this form was used at Pryor Mountain beginning in 2001 and at Little Book Cliffs beginning in 2002. The date of annual inoculation ranged from Jan. 5–Dec. 4, which reflects the time of year when each female was initially vaccinated, perpetuated by annual efforts to locate and re-vaccinate them remotely. These re-vaccination times varied for each horse and year due to occasional difficulties in accessing the sites as well as individual variation in horses' tolerance of humans.

The time-release form of PZP consisted of a single inoculation of liquid PZP simultaneously applied with 3 pellets designed to release PZP in a bolus fashion at 1, 3, and 12 months, for a total of 22 months of targeted infertility [16]. All females in the treatment group at McCullough Peaks received time-release PZP in October 2004. No females were re-treated with PZP unless they had produced a foal during post-treatment. All females that were re-treated were omitted from our study at that time.

We defined post-treatment for this study as beginning two full parturition seasons after the final inoculation of conventional PZP or after the single inoculation using PZP time-release pellets. For example, if a female received a conventional PZP inoculation in autumn 2004, then she was presumably contracepted in 2005. This meant she should not produce an offspring, but could conceive at some time in 2006. The first post-treatment year would then be 2007. If that 2004 inoculation was time-release PZP, then the first post-treatment year could have been as early as 2007.

Data Collection

We observed post-treated females from 2005 at Little Book Cliffs and Pryor Mountain and from 2007 at McCullough Peaks until they died, were removed, were treated again by managers, or the end of 2011, whichever came first. We omitted data for one female from the Little Book Cliffs and six females from McCullough Peaks because they produced offspring in every treatment year and thus were never effectively contracepted. Females that produced offspring during treatment years, but not in every year, were retained because contraception could have occurred. The untreated female group consisted of all individuals in each population that had never been inoculated with PZP and were at least 4 years old at the first year of this study period. The minimum age of post-treated females at the first year of this study period was 4 years old. The resulting data arose from observations of 88 post-treated females (age 4–24 years) and 119 untreated females (age 4–23 years) (Table 1).

Observation protocols followed Ransom et al. [16] for all three sites. For the Pryor Mountain site in 2010–2011, we also used data provided by the Pryor Mountain Mustang Center, WY, USA; these were collected under the same protocols. Throughout the study, 96.1% of all females and offspring (when present) were located at least weekly from April to October of each year. At Little Book Cliffs, 6–8 females were difficult to access weekly, but were located at least once per month. A single band containing two females at McCullough Peaks was not located weekly, but was observed at least monthly during 2007–2010. In 2011, that band was found only once and both females were observed with neonates. At Little Book Cliffs, we also used motion-activated infrared trail cameras at remote water points to provide supplemental observations. Observations during the winter were irregular and sometimes constrained by weather. It is possible that some offspring were born and died without being observed during the course of this study; however, given the intensity of observations, we believe this rarely occurred. We matched all neonates with dams based on observations of attachment (e.g., nursing, general proximity) during the early days and weeks of a neonate's life [24]. We did not attempt to assess pregnancy in females that may have visually appeared pregnant but did not produce a viable offspring. Parturition probability was thus estimated from the frequency of live births per cohort as detected by direct observation.

Table 1. Parturition data from untreated and porcine zona pellucida (PZP) treated female feral horses (*Equus caballus*) at Little Book Cliffs Wild Horse Range, CO, Pryor Mountain Wild Horse Range, MT, and McCullough Peaks Herd Management Area, WY, USA.

	Little Book Cliffs WHR (2005–2011)	Pryor Mountain WHR (2005–2011)	McCullough Peaks HMA (2007–2011)
Number of untreated females	41	44	34
Number of untreated females never producing offspring	6	11	15
Age of untreated females	4–23 yr	4–15 yr	4–21 yr
Number of post-treated females	22	38	28
Number of post-treated females never producing offspring	15	18	15
Age of post-treated females	6–21 yr	4–24 yr	4–20 yr
Observed parturition range for untreated females	Feb. 23–Sep. 1	Feb. 21–Sep. 3	Jan. 15–Sep. 7
Observed parturition range for post-treated females	Mar. 5–Dec. 22	Apr. 5–Sep. 29	Feb. 20–Aug. 4
Estimated parturition peak for untreated females	May 10 (Apr. 28–May 22)	May 14 (May 4–May 24)	May 19 (May 8–May 30)
Estimated parturition peak for post-treated females	Aug. 24 (Jul. 18–Sep. 29)	Jun. 18 (May 31–Jul 6)	May 4 (Apr. 14–May 24)
Estimated difference in peak parturition date (95%CI)	105.9 (69.7–142.1) days	34.8 (17.0–52.3) days	None

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We visually classified the body condition of each dam when we first detected her neonate in the field. This body condition score increased discretely from 1 to 9 as fatness increased [25]. Neonate data were collected concurrently and the general activity state of each neonate was categorized as vigorous, lethargic, or immobile. If neonates were resting when first observed, they were continually observed until the band became active again and neonate activity could be classified. Neonate date of birth was estimated subjectively by observing presence of an umbilicus, level of activity, and time elapsed since the dam was previously observed pregnant. The median time elapsed before a neonate was detected was only six days during the post-treatment observation years ($n = 328$ neonates).

Data Analyses

Model structure. We used mixed-effects regression models to estimate parturition probability, phenology, and offspring survival in the maximum likelihood framework [26]. Individual female was used as a random effect on the intercept term to account for the repeated observations (multiple years) of individuals over time. This was necessary to account for variation that may be present among individuals who were sampled repeatedly, though not always equally over time. Such variation may arise from the many biotic and abiotic factors that may affect conception, pregnancy, parturition, and neonate care. We also used population as a random effect on the intercept term to account for variation attributed to location and treatment regimen. We used the lmer(·) function in the lme4 package in R version 2.14.1 (R Development Core Team 2011) to obtain all mixed-effects model estimates.

Parturition probability. Parturition history for each female was known (binomial response of the female producing at least one offspring in the past or not) from direct observation of all females <12 years old at Little Book Cliffs and Pryor Mountain, and all females <9 years old at McCullough Peaks. All older females were documented giving birth at least once within that same time period. Parturition rates of feral horses typically increase for the first few years after females reach sexual maturity, remain high through middle age, and decrease in old age [16]; therefore, we included linear and quadratic effects of age in models of parturition probability. We rescaled age in the quadratic effect by subtracting the mean age of horses (9.98 yr across all populations), so the intercept term of the model corresponded to probability of parturition at mean age.

Total annual precipitation for the biological year prior to parturition (spring of conception through spring of parturition) was used as a proxy for forage abundance, and thus potential body condition at the year of conception. Daily surface climate data were obtained from the National Climate Data Center (http://www.ncdc.noaa.gov) for Grand Junction, CO (Station 53488, about 13 km southwest of Little Book Cliffs), Cody, WY (Station 481840, about 32 km west of McCullough Peaks), and Lovell, WY (Station 485770, about 21 km south of Pryor Mountain) for all years of the study. The complete model of parturition probability included the fixed effects of age, treatment, conception year total precipitation, and parturition history. We ran a secondary linear model (lm(·) function) for only the post-treated females that produced offspring in order to investigate the influence of age at first treatment and number of treatments received (1–5 annual inoculations). This secondary model only applied to horses at Little Book Cliffs and Pryor Mountain, where repeated inoculations of conventional liquid PZP were applied.

Parturition phenology. We hypothesized that photoperiod and temperature were critical factors influencing phenology of conception, and thus phenology of parturition. Consequently, the model of parturition phenology included treatment and temperature at the approximate conception date as fixed effects, and individual and population as random effects on the model intercept. A supplemental model was considered for post-treatment females at Little Book Cliffs and Pryor Mountain to assess the interaction of last treatment date and number of consecutive annual treatments as a fixed effect, also using individual and population as random effects. Because only one treatment was applied at McCullough Peaks and the actual decay rate of the time-release pellets was unknown, we could not apply this supplemental model for that site.

Survival. Survival was a binomial response attributed to persistence of a neonate from parturition until the following year April observation or death during that time period. Managers removed 30 neonates from the range during their birth year, and those animals were not included in modeling survival. We hypothesized that survival was a function of treatment, dam age,

dam body condition, mean winter temperature (Nov. 1–Mar. 31), and the temporal difference between birth date and spring peak available forage. We used Normalized Difference Vegetation Index (NDVI) data obtained from the National Aeronautics and Space Administration (http://modis.gsfc.nasa.gov) and reconciled using ArcGIS software (Esri, Redlands, California) to assess temporal variation in forage availability. The phenology product from Tan et al. [27] was used to generate date of mean maximum NDVI in each year across each study area. Mean NDVI was used as a temporal indicator of forage availability, but not forage abundance because tree presence and distribution can strongly influence magnitude of this metric.

Results

Parturition Probability

During the years of observation, 15–44% of untreated females and 47–68% of post-treated females never produced offspring (Table 1). Of all post-treated females in the study, 81% had successfully produced offspring prior to treatment and the remaining 19% were <4 years old when first inoculated. Likewise, 97% of all untreated females in the study had successfully produced offspring prior to or during the study period. The probability of producing offspring was strongly related to treatment ($z = -6.90$, $P<0.001$) and age ($z = -2.93$, $P = 0.003$), and weakly influenced by total annual precipitation during year of conception ($z = 1.79$, $P = 0.074$). At mean age and mean conception-year precipitation, the estimated parturition probability for post-treated females was 25.6% (95% CI = 17.8–35.2%) and for untreated females was 64.1% (53.1–73.9%) if they had produced offspring in any year prior to the study. The estimated parturition probability was only 2.1% (1.3–3.2%) for post-treated females and 10.7% (7.0–15.9%) for untreated females that had never previously produced offspring. Parturition probability increased 0.497% (0.494–0.500%) per year of age for all females until age 10; after which it declined at the same rate. Population contributed to a small amount of variation in the model ($\sigma = 0.33$) but individual identity was more influential ($\sigma = 0.82$).

Of the 52 females that received 1–5 annual injections of conventional PZP (Little Book Cliffs and Pryor Mountain), 27 produced at least one offspring post-treatment. The length of time between last inoculation and first parturition ranged from 565–2,971 days and was strongly influenced by the total number of years a female was treated ($t = 5.18$, $P<0.001$). Estimated time to parturition increased 411.3 (246.5–576.0) days per year of consecutive treatment (Figure 2). Neither age at first treatment ($t = -1.04$, $P = 0.309$) nor fertility history ($t = 0.01$, $P = 0.991$) influenced the length of time to parturition. The secondary model was not run for McCullough Peaks, where all 36 females were treated with the 22-month time-release PZP pellets on the same day. Thirteen post-treated females in that population produced an offspring during the 5 years of observation and the observed length of time between inoculation and first parturition ranged from 530–2,000 days.

Parturition Phenology

Births from all untreated females increased in frequency toward the summer solstice and decreased toward the winter solstice, and those trends corresponded with temperature and forage availability (Figure 3). Parturition from untreated females was similar between populations and ranged from Jan. 15–Sep. 7 (Table 1). Parturition from post-treated females ranged from Feb. 20–Dec. 22, and the estimated peak was 31.5 (17.0–46.0) days later than births from untreated females, after controlling for temperature at conception date. The latest neonate born to a post-treated female arrived 7.5 months after the peak in births from untreated females, indicating conception occurred within 24–31 days of the winter solstice. Among the post-treated females that gave birth late and then did not produce a neonate the next year, there was some evidence that subsequent births began to shift back toward the phenology observed for untreated females (Figure 3).

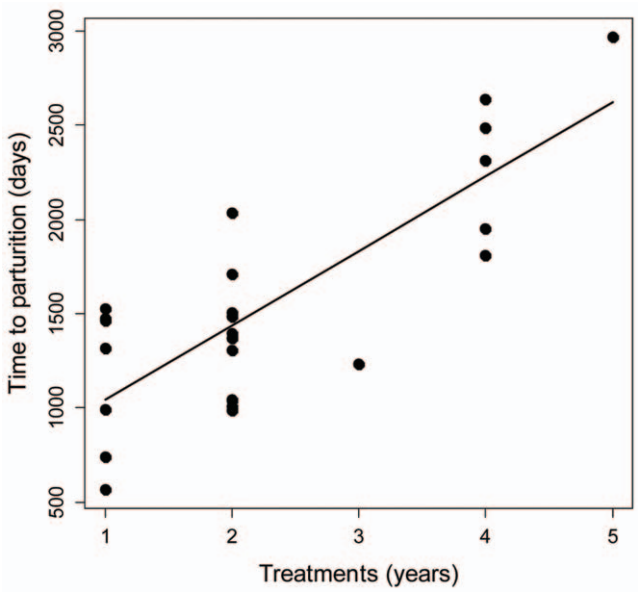


Figure 2. Time between final contraceptive inoculation and first parturition from feral horse (*Equus caballus*) females. Births shown are from horses at Little Books Cliffs Wild Horse Range, CO, USA and Pryor Mountain Wild Horse Range, MT, USA as a function of consecutive annual porcine zona pellucida (PZP) inoculations, 2005–2011.
doi:10.1371/journal.pone.0054972.g002

Survival

Nearly every neonate born was observed in a vigorous activity state, with the exception of four that were immobile (one to a post-treated female) and two that were lethargic (none to post-treated females). All six of those neonates died before the following spring. Body condition of dams at first observation of neonates ranged 3–9 (mean = 5.73, 5.61–5.84). Eighty-five percent of neonates were observed alive in the spring following their birth. The estimated survival probability of a neonate born to a dam in mean body condition at spring peak greenness was 79.9% (67.9–85.4%). This probability decreased 11.4% (10.7–12.0%) with each 1-unit decrease in dam body condition. Survival declined 1.4% (1.4–1.5%) for every 10 days after peak greenness that parturition occurred.

Treatment ($z = -1.49$, $P = 0.137$), band size ($z = 0.67$, $P = 0.500$), dam age ($z = -1.16$, $P = 0.247$), and mean winter temperature ($z = 0.89$, $P = 0.372$) did not influence survival in this model; however, the treatment and winter temperature effects were disparate between sites. At Little Book Cliffs and Pryor Mountain, treatment weakly influenced survival ($z = -1.78$, $P = 0.075$), whereas at McCullough Peaks it clearly had no influence ($z = 0.01$, $P = 0.993$). Likewise, mean winter temperature influenced survival ($z = -2.16$, $P = 0.031$) at Little Book Cliffs and Pryor Mountain, but not at McCullough Peaks ($z = 1.00$, $P = 0.320$).

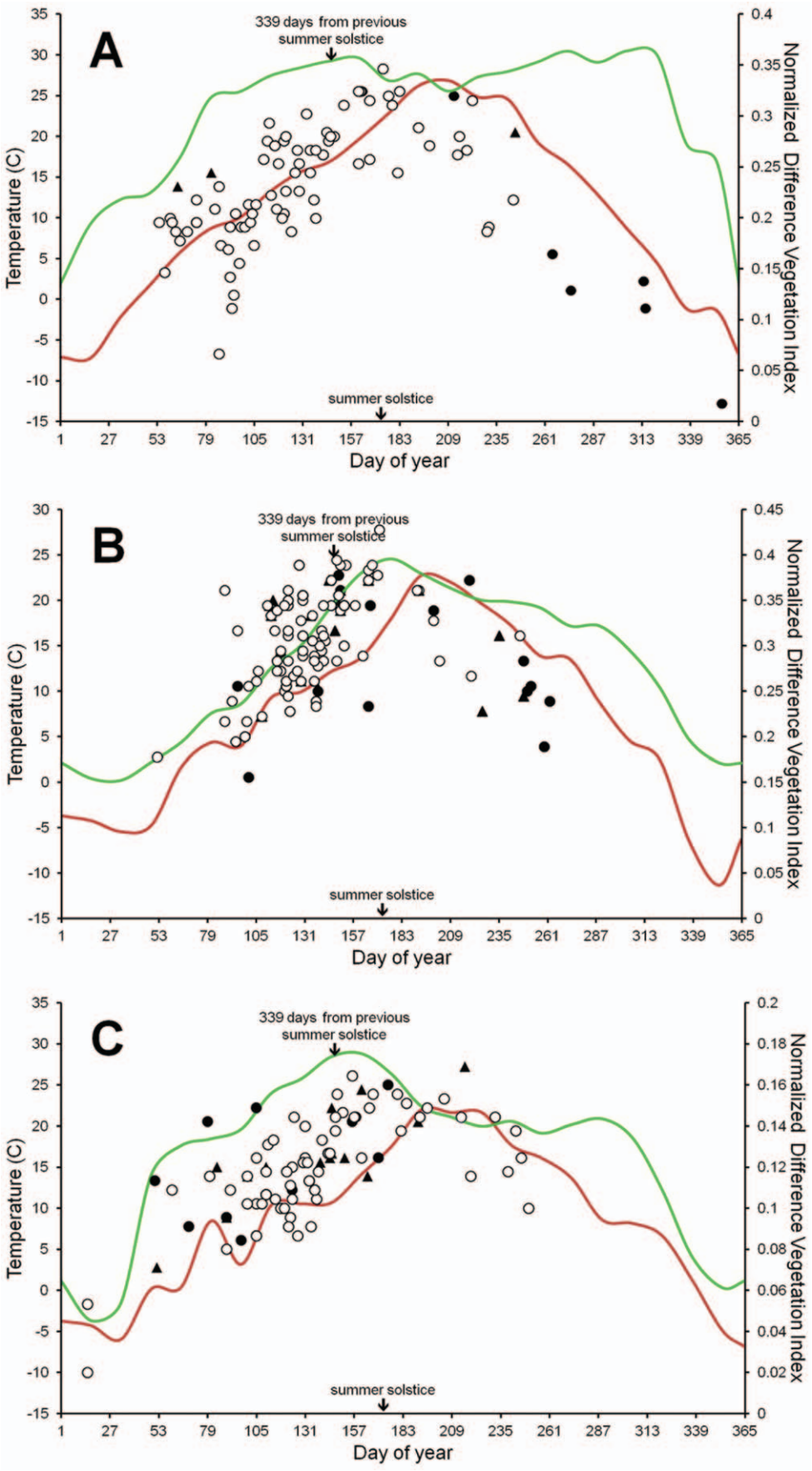

A
339 days from previous summer solstice
summer solstice
Temperature (C)
Normalized Difference Vegetation Index
Day of year
B
339 days from previous summer solstice
summer solstice
Temperature (C)
Normalized Difference Vegetation Index
Day of year
C
339 days from previous summer solstice
summer solstice
Temperature (C)
Normalized Difference Vegetation Index
Day of year

Figure 3. Birth phenology of feral horse (*Equus caballus*) untreated (○) and post-treated (• = first post-treatment birth, ▲ = birth subsequent to •) females. Births are shown as a function of temperature at approximate conception date for populations at Little Book Cliffs Wild Horse Range, CO (A), 2005–2011, and Pryor Mountain Wild Horse Range, MT (B), 2005–2011, and McCullough Peaks Herd Management Area, WY (C), 2007–2011. Post-treated females were previously inoculated with the immunocontraceptive porcine zona pellucida (PZP). Mean Normalized Difference Vegetation Index (green) represents temporal availability of forage. Mean daily surface temperature is shown in red.
doi:10.1371/journal.pone.0054972.g003

Discussion

Parturition Probability

The probability of post-treated females producing offspring was 38.5% lower than for untreated females, after controlling for differences due to age, precipitation during conception year, and fertility history. The return to fertility rates observed were highly variable and this may in part be due to the disparate abilities of individual females to raise therapeutic-level antibodies against PZP and the length of time those antibodies persisted above threshold concentrations [15,28,29]. A similar result was reported for horses at Assateague Island National Seashore, MD and VA, USA, where 68.8% of 32 female horses treated for three consecutive years with PZP became pregnant 1–4 years after the last treatment [8]. Only 3 horses were vaccinated for four consecutive years in that study: one became pregnant 3 years after the last treatment, another became pregnant after 4 years, and the third became pregnant after 8 years. In a similar, but critically endangered species, *E. ferus przewalskii*, only 45% of 20 females that had been inoculated with PZP for 3 consecutive years demonstrated fertility after 6.7 post-treatment years [30]. More surprisingly, 4 of 9 females in that study that had only received a single treatment (one primer injection followed by one booster injection) never produced any offspring after 4–8 years of observation. While the mechanism for explaining this protracted infertility in *E. ferus przewalskii* is unknown, some of the Assateague horses were found to have experienced ovulatory failure [31]. This is not the targeted physiological response of PZP vaccination, and the effect was highly variable between individuals due to the episodic nature of ovulatory failure, as opposed to a chronic condition. Studies of non-equid species have demonstrated atrophic changes in ovarian morphology, folliculogenesis, and reproductive endocrine function, indicating that prevention of sperm binding may not be the only mechanism acting on fertility in PZP-inoculated animals [32–35].

Parturition Phenology

Parturition phenology for North American feral horses has been shown to peak during May [36–38]. We estimated parturition from untreated females peaked May 10–19 across the 3 populations, which was 22–43 days before spring forage availability began to decline, and placed conception roughly 7–15 days before the longest day of sunlight (or 323–332 days from previous summer solstice). This phenology appears synchronized with the most abundant forage during the parturition period while females' metabolic needs should be elevated from late term pregnancy and lactation [39]. Our findings for PZP-inoculated females demonstrated a markedly different phenology. The estimated peak in parturition from post-treated females at two sites (Aug. 24 at Little Book Cliffs and Jun. 18 at Pryor Mountain) occurred as forage availability was declining; however at Little Book Cliffs where births were latest, the monsoonal rains provided a bimodal distribution in greenness (Figure 3A). The second peak in forage availability arrived Nov. 1, which was 69 days after estimated peak in parturition from post-treated females.

This protracted birth phenology resulted in parturition dates spanning 305 days for post-treated females as compared to 229 days for untreated females. Across all females in the 3 populations, the parturition season ranged nearly the entire year (341 days). This indicates surprising plasticity in birth phenology of temperate latitude horses given the physiological mechanisms thought to be driving reproductive cyclicity. At Shackleford Banks, North Carolina, USA, female feral horses exhibited estrus beyond the normal breeding season after being vaccinated annually with PZP for 1–6 years [38]. Based on the observed birth phenology in our study, this phenomenon occurred in our subjects as well; however, we recorded 81.4% of 328 documented births between March 1 and June 21. This suggests that photoperiod and temperature remain fundamentally important in regulating birth phenology, but several other mechanisms may be acting to help explain the wide range of birth dates observed. Follicular activity of the estrous cycle during the first half of the breeding season is characterized by more numerous large follicles and greater incidence of anovulatory waves [40]. Gestation length is variable and has been attributed to seasonal variations that influence nutrition of females [41]. The annual rhythm of luteinizing hormone (LH) secretion is partially regulated by photoperiod, but also has a strong endogenous component [42]. Age and fertility history of females can strongly influence the occurrence of a winter anovulatory period [43], as can melatonin sensitivity of individuals [2]. This litany of influences is compounded by the known variations in PZP immunocontraception efficacy and duration, as well as the uncertain mechanism of action that may be confounded with ovarian pathologies.

The protracted breeding season of PZP-vaccinated females found in our study, as well as by Nuñez et al. [38], indicates that horses that would normally conceive during their first or second reproductive cycle of the year continue to cycle throughout the year and then only conceive when contraception sufficiently decays. This inherently extends the breeding season and challenges the physiological constraints assumed to be regulating fertility. The 'self-correcting' trend we observed provides additional empirical support for the importance of such abiotic factors in birth phenology, but also illustrates that phenological shifts associated with the uncertainties surrounding immunocontraception may be short term. The long term effects of PZP treatment on population ecology are less clear given the behavioral components that can affect social networks, as well as fitness of untreated females in social groups [19,21–23].

It should be noted that the protracted phenology detected at Little Book Cliffs and Pryor Mountain was not observed at McCullough Peaks, where the final bolus release of PZP is assumed to have occurred around July. This should have led to infertility persistence through the following July but appears to have persisted much longer in many individuals. Some variation in parturition phenology likely arose from the unique immune response of each female to the vaccine, but this was not compounded by the widely disparate vaccination dates experienced at the other two sites, and thus a more consistent phenology was observed.

Survival

The ultimate consequence of altered birth phenology is survival. Collectively, survival of all neonates in our study was consistent

with the mean survival rate (73.9%) derived from 12 historic studies on feral horse neonates [44]. Survival probability for neonates in our study was 79.9%, at mean body condition of the dam and at spring peak NDVI. This probability predictably decreased with decreasing dam body condition and with temporal distance between date of birth and spring peak NDVI. Treatment did not influence survival in the global model, but was influential at the two sites where unseasonably late births were observed. The treatment effect on survival at Little Book Cliffs was somewhat ameliorated by the secondary peak in forage availability following the monsoons. Such fine scale ecosystem effects may be important considerations when assessing the potential for fertility control applications on specific populations.

Conclusion

Humans are increasingly attempting to manage the planet's wildlife and habitats with new tools that are often not fully understood. The transient nature of the immunocontraceptive PZP can manifest into extraordinary persistence of infertility with repeated vaccinations, and ultimately can alter birth phenology in horses. This persistence may be of benefit for managing over-abundant wildlife, but also suggests caution for use in small refugia or breeding facilities maintained for repatriation of rare species. These results introduce ethical questions toward regulating populations with tools that may alter ecology of the species, but also offer quantitative insights that can be weighed with the depth of management need. The growing need for humane, non-lethal, population control measures also led us to empirical evidence that illustrates the physiological flexibility of horse reproduction in the race for fitness. Photoperiod and temperature are powerful inputs driving the biological rhythms of conception and birth in horses, but do not limit the ability of horses to conceive under perturbed conditions.

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Author Contributions

Conceived and designed the experiments: JIR. Performed the experiments: JIR. Analyzed the data: JIR. Contributed reagents/materials/analysis tools: JIR NTH JB. Wrote the paper: JIR NTH JB.

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REVIEW

Ecological feedbacks can reduce population-level efficacy of wildlife fertility control

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Summary

1. Anthropogenic stress on natural systems, particularly the fragmentation of landscapes and the extirpation of predators from food webs, has intensified the need to regulate abundance of wildlife populations with management. Controlling population growth using fertility control has been considered for almost four decades, but nearly all research has focused on understanding effects of fertility control agents on individual animals. Questions about the efficacy of fertility control as a way to control populations remain largely unanswered.

2. Collateral consequences of contraception can produce unexpected changes in birth rates, survival, immigration and emigration that may reduce the effectiveness of regulating animal abundance. The magnitude and frequency of such effects vary with species-specific social and reproductive systems, as well as connectivity of populations. Developing models that incorporate static demographic parameters from populations not controlled by contraception may bias predictions of fertility control efficacy.

3. Many population-level studies demonstrate that changes in survival and immigration induced by fertility control can compensate for the reduction in births caused by contraception. The most successful cases of regulating populations using fertility control come from applications of contraceptives to small, closed populations of gregarious and easily accessed species.

4. Fertility control can result in artificial selection pressures on the population and may lead to long-term unintentional genetic consequences. The magnitude of such selection is dependent on individual heritability and behavioural traits, as well as environmental variation.

5. *Synthesis and applications*. Understanding species' life-history strategies, biology, behavioural ecology and ecological context is critical to developing realistic expectations of regulating populations using fertility control. Before time, effort and funding are invested in wildlife contraception, managers may need to consider the possibility that many species and populations can compensate for reduction in fecundity, and this could minimize any reduction in population growth rate.

Key-words: behaviour, demography, ecological process, fertility control, fitness, immunocontraception, population dynamics, population ecology, wildlife contraception, wildlife management

Controlling reproductive capacity of wildlife

Humans have been attempting to control the abundance of animals for over 13,000 years (Diamond 2002). Although regulating the herd size of domestic animals has been a feature of human economies for millennia, people have also sought to regulate populations of wild animals using hunting and culling techniques. Recent societal trends have motivated wildlife managers to seek nonlethal strategies of regulating births using fertility control (Hobbs, Bowden & Baker 2000; Porton 2005). This trend may be attributed to an increasingly mutualistic societal view arising from abundance of wildlife in suburban and urban areas (Teel & Manfredo 2009). In such circumstances, perception of wildlife species may focus less on

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their role as a natural resource and more on how they are part of our social environment (Leong 2010). This may lead humans to be more aware of, and concerned about, the welfare of these species. The balance of human perception and wildlife abundance is precarious because as highly adaptive species increase in density, wildlife–human conflicts increase, and society can then be confounded with the dichotomy of desire for wildlife protection and relief from wildlife conflict (Knuth *et al.* 2001; Lauber & Knuth 2004; Lauber *et al.* 2007; Hadidian 2009).

The practice of using fertility control to manage reproduction in wildlife emerged over 40 years ago (Asa & Porton 2005). Wildlife managers have been attracted to fertility control as a means to regulate overabundant wildlife when animals threaten people's lives, livelihoods or property; when they cause declines in more desirable species; and when their densities are high enough to increase disease transmission or disrupt ecosystem function (Caughley 1981; Hone 2007). The vast majority of empirical knowledge about wildlife fertility control comes from individual-level studies of drug safety and efficacy (Garrott 1995; Kirkpatrick, Lyda & Frank 2011). As a consequence, we know relatively little about how fertility control influences population ecology. It remains uncertain whether findings of research on the efficacy of fertility control at the individual level can allow inferences to populations. There may be compensation in vital rates that allow individuals to maintain fitness, thus offsetting the effects of fertility control agents. If such compensation occurs, then it may be infeasible to control some wildlife populations using fertility control.

Types of fertility control applied to wildlife include products that disturb normal reproductive hormone cascades or interfere with conception, such as immunocontraceptive vaccines, pharmaceuticals, hormone derivatives, agonists, antagonists, mechanical devices and surgical techniques (Asa 2005). All of these methods may prevent births, but they also may induce unintended changes to behaviour and physiology (Nettles 1997; Gray & Cameron 2010). It is not surprising that hormonal derivatives such as melengestrol and levonorgestrel affect behaviour (Gray & Cameron 2010), but it is surprisingly uncertain how variable such behavioural changes can be, even within similar taxa. Stump-tailed macaque *Macaca arctoides* females, for example, can become markedly more aggressive towards conspecifics when treated with a synthetic progestin (Linn & Steklis 1990), but hamadryas baboon *Papio hamadryas* females can become more passive when treated with a similar progestin (Portugal & Asa 1995).

Any fertility control application that changes the reproductive capacity of an individual has the potential to induce individual behavioural changes that can alter family group structure, influence interspecific and intraspecific interactions and ultimately shape population dynamics in unforeseen ways. Fertility control in free-roaming wildlife populations has been associated with changes in immigration (Ramsey 2005; Merrill, Cooch & Curtis 2006), decreased group fidelity (Nuñez *et al.* 2009; Madosky *et al.* 2010), increased survival (Caughley, Pech & Grice 1992; Kirkpatrick & Turner 2007; Williams *et al.* 2007), altered reproductive behaviour (Nuñez, Adelman & Rubenstein 2010; Ransom, Cade & Hobbs 2010) and shifted phenology (Ransom, Hobbs & Bruemmer 2013). Understanding and predicting these sometimes subtle and incremental shifts is difficult, and the challenge of separating the influences of multiple population growth controls can be daunting (Sibly & Hone 2002). To exacerbate these problems, the longitudinal studies needed to detect and quantify long-term population-level effects of fertility control treatments are expensive and time-consuming.

Insights into population-level effects of fertility control have largely been accomplished through efforts that simulate population dynamics (Caughley, Pech & Grice 1992; Hone 1992; Hobbs, Bowden & Baker 2000; Davis & Pech 2002; Merrill, Cooch & Curtis 2006). Simulation models can be useful tools for screening management alternatives; however, widespread behavioural and demographic changes induced by fertility control may subvert the underlying assumptions of demographic parameters in models that fail to consider these changes. The assumptions of many fertility control population models could be wrong if the vital rates informing them are based on the wealth of *a priori* ecological knowledge about species and systems before fertility control was applied.

Here, we synthesize findings from studies on population-level effects of wildlife fertility control and compare some of those outcomes with those from individual-level studies. We specifically investigate the demographic and behavioural components from wildlife fertility control studies, seeking to understand feedbacks that might be influencing population-level outcomes of fertility control strategies. We attempt to identify some of the underlying processes to help inform modelling efforts and future design for empirical population-level fertility control studies, and ultimately try to determine whether, in fact, nature can overcome control efforts at a scale that makes fertility control ineffective as a wildlife management tool.

The feedback dilemma

Considering animal behaviour in broad ecological and conservation contexts is critical to understanding population ecology (Sutherland 1998; Buchholz 2007); yet, a lack of rigorously quantified behaviour data often inhibits our ability to assess the magnitude of its importance. At the individual level, wildlife contraceptive studies have illuminated a wide array of behavioural changes attributed to fertility control applications (Nettles 1997; Gray & Cameron 2010). Studies on captive animals incorporate more experimental control than is usually possible with free-ranging wildlife, but the artificial environments inherent in captive studies often preclude the insights towards ecological feedbacks that studies of free-ranging wildlife

contribute. Consideration of results from both types of studies is needed to understand the potential population-level effects of fertility control acting through birth, survival, immigration and emigration. Feedbacks on population growth may be negative or positive, and the nature of feedback direction may vary with the ecology of the species in question.

Fertility control is designed to reduce the probability that an individual gives birth, so it should be expected that fecundity of treated animals will be less than that of untreated animals; however, indirect effects make the broader influence of treatment on birth rates less clear. Unintentionally protracted contraception has been attributed to physiological changes in ovarian function after fertility control treatments (Kirkpatrick *et al*. 1995; McShea *et al*. 1997; Nettles 1997; Powell & Monfort 2001; Stoops *et al*. 2006). Such residual infertility was observed in feral horses *Equus caballus* and Przewalski horses *E. ferus przewalskii* repeatedly treated with porcine zona pellucida (PZP) and was further complicated by shifts in the seasonal birth pulse when fertility resumed (Nuñez 2009; Feh 2012; Ransom, Hobbs & Bruemmer 2013). Increased frequency of reproductive behaviour has been observed in females treated with PZP vaccines and has the potential to focus attention of breeding males away from fertile females and towards the attractive, but sterile, females (Shumake & Wilhelm 1995; McShea *et al*. 1997; Heilmann *et al*. 1998; Nuñez, Adelman & Rubenstein 2010; Ransom, Cade & Hobbs 2010). Similarly, both elk *Cervus elaphus* and mule deer *Odocoileus hemionus* males demonstrated increased reproductive behaviour towards females treated with gonadotropin-releasing hormone vaccine (GnRH) or leuprolide (a GnRH agonist) after the untreated cohort became pregnant (Baker *et al*. 2002, 2004; Conner *et al*. 2007; Powers *et al*. 2011). All of these studies represent a potential mechanism for decreased fitness in males and could result in a higher net contraceptive effect at the population level (Ransom 2012).

In contrast, fecundity of untreated female mice *Mus domesticus* increased when fertility-controlled females were present, attenuating the contraceptive effect (Chambers, Singleton & Hinds 1999). Many species have been reported exhibiting a compensatory reproduction response when a subset of the population has been perturbed, such as might be the case with large-scale fertility control application (Swenson 1985; Kirkpatrick & Turner 1991; Boyce, Sinclair & White 1999; Williams 1999). How such effects originate through changes in social networks is unknown. Likewise, the effects of fertility control on biochemical and olfactory communication are virtually unknown, but have been implicated in disrupting intraspecific interactions in a primate (Crawford, Boulet & Drea 2010) and could lead to greater uncertainty about fecundity at the population level.

Fidelity to family groups has decreased in conjunction with fertility control applications (Fayrer-Hosken *et al*. 2000; Nuñez *et al*. 2009; Madosky *et al*. 2010), and time spent with conspecifics or potential mates has also changed (Bertrand *et al*. 1996; Poiani *et al*. 2002; Harrenstien *et al*. 2004). Territoriality increased for pikas *Ochotona curzoniae*, and the increase was related to decreased litter size (Liu *et al*. 2012). These types of changes can affect time budgets such that basic maintenance behaviours of feeding and resting decrease, resulting in lower body condition in animals receiving contraception (see Ji, Clout & Sarre 2000; for example). Similarly, female elk treated with leuprolide had comparable maintenance behaviours as untreated elk, but retained less body fat than untreated, pregnant females, possibly due to decreased anabolic hormone exposure during their infertile year (Conner *et al*. 2007).

Increased or decreased agonism between conspecifics has also been attributed to some contraceptive applications (Linn & Steklis 1990; Portugal & Asa 1995; Penfold, Patton & Jochle 2005; Snape, Hinds & Miller 2011). These behaviours can alter dominance hierarchies and thus access to resources (Dublin 1983; Schulte *et al*. 2000; Creel 2001). More directly, some deer treated with hormonal applications lost body condition or stopped eating altogether (Bell & Peterle 1975; White, Warren & Fayrer-Hosken 1994). Low body condition arising from any of these processes can enhance the fertility control effect by changing the ability of individuals to meet the nutritional requirements needed to support pregnancy and lactation (Cook *et al*. 2004; Rhind 2004).

Apart from the many potential direct and indirect effects of fertility control on births, some applications may also influence survival. Body condition and longevity can increase when the costs associated with reproduction decrease. For example, feral horses treated with a PZP vaccine had higher survival probability than untreated controls (Turner & Kirkpatrick 2002; Ransom 2012). Similarly, survival was higher among fertility-controlled rabbits *Oryctolagus cuniculus* (Twigg *et al*. 2000; Williams *et al*. 2007), coyotes *Canis latrans* (Bromley & Gese 2001) and foxes *Vulpes vulpes* (Saunders *et al*. 2002). These results show that reductions in recruitment caused by fertility control can be offset at least in part by enhanced survival of females, thereby attenuating the effect of fertility control on population growth rate.

Feedbacks amplifying the effect of fertility control can also occur. Disease transmission was 28% higher in populations of possums *Trichosurus vulpecula* with sterile females, illustrating the potential for decreased survival in fertility-controlled populations (Caley & Ramsey 2001). Phenological shifts in birth patterns among females that formerly received contraception may also have implications for survival of both females and their neonates if births occur asynchronously with forage resources (Ransom, Hobbs & Bruemmer 2013).

Perhaps, the most challenging impediment to predicting population-level effects of fertility control occurs when populations are open to immigration and emigration, processes that can be accelerated by feedbacks from the

application of fertility control. Male possums, for example, increased in frequency at sites where females received contraception (Ji, Clout & Sarre 2000), and in one study, the rate of immigration equalled the rate of reduction in births attributed to fertility control (Ramsey 2005). A similar feedback was also observed in deer (Merrill, Cooch & Curtis 2006). Home ranges of fertility-controlled female rats *Rattus argentiventer*, koalas *Phascolarctos cinereus* and white-tailed deer were larger than those of fertile females (Jacob, Matulessy & Sudarmaji, 2004; Gilman *et al*. 2010; Hynes *et al*. 2011). This was explained by an absence of range-limiting dependent young, and in deer, this larger home range was positively correlated with increased mortality rates (Gilman *et al*. 2010).

For nearly all of the collateral effects discussed here, there are almost as many cases where no collateral effects were detected when a different fertility control method was used or a different species was studied (Gray & Cameron 2010: Fig. 2). Such variation in individual and species responses emphasizes the need for careful consideration of species biology, reproductive system, physiology, behavioural ecology, population biology and ecological context when considering fertility control management for a population.

Population-level effects of fertility control

Scientific progress towards understanding population-level effects of fertility control has been slow despite an articulate call for population-level research of wildlife contraception nearly two decades ago (Garrott 1995). We reviewed 479 papers in the scientific literature on wildlife fertility control from 1980 to 2011 and found that 90% focused on individual-level effects (Fig. 1). Half of the papers were published in the last decade, reflecting an increasing interest in wildlife fertility control. There was an increasing trend in population-level studies, but still only 10% of the papers reviewed addressed population-level effects.

Only 6·5% of all papers we reviewed considered behavioural or indirect demographic effects in population-level assessment of fertility control (Table 1), and only half of those quantitatively considered such effects in modelling population dynamics with fertility control (Table 2). This is surprising given that such a large number of individual-level studies describe the potential for indirect effects to influence fertility control efficacy at the population level (Nettles 1997; Gray & Cameron 2010). The number of studies that empirically tested fertility control management for achieving long-term reduction in population size was too small for us to conduct a meaningful quantitative meta-analysis. It is worth noting, however, that the studies evaluating fertility control in open populations of promiscuous breeders did not sufficiently reduce population size for long time horizons (Twigg *et al*. 2000; Ramsey 2005; Jacob, Singleton & Hinds 2008), and a study of an open population of deer had mixed results (Merrill, Cooch & Curtis 2006). Efforts to manage relatively small, closed populations of feral horses, deer, elephants and feral cats using fertility control were much more successful (Rudolph, Porter & Underwood 2000; Delsink *et al*. 2006; Rutberg & Naugle 2007; Kirkpatrick & Turner 2008; Mendes-de-Almeida *et al*. 2011; Ransom 2012).

A broad consensus among both empirical and simulated studies is that in most free-roaming wildlife populations, a majority of the individuals in one or both sexes must be infertile to realize meaningful reduction in population growth. Reduction in abundance can require aggressively maintained treatment for long periods of time (Hobbs, Bowden & Baker 2000). This can mean that >50% of females must be treated in closed populations of long-lived, low-fecundity species to achieve moderate reduction in growth (Hone 1992; Hobbs, Bowden &

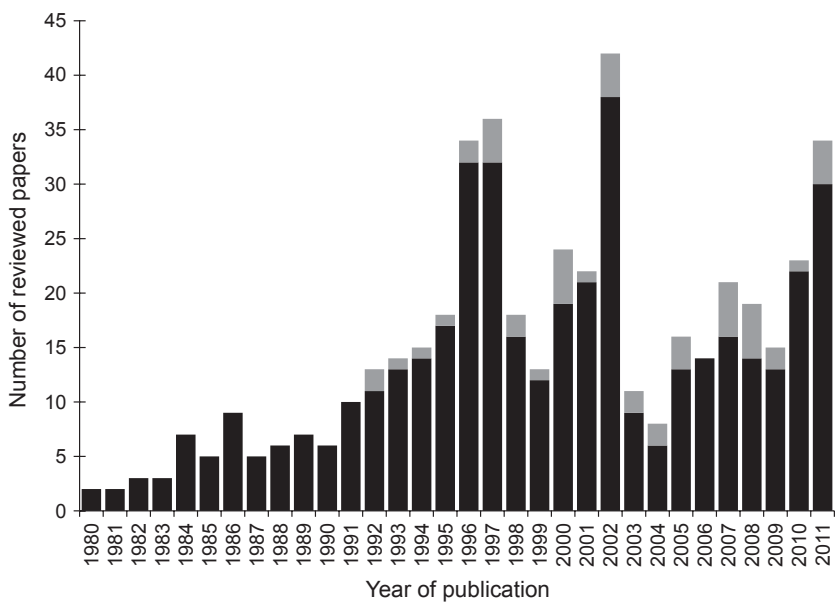


Fig. 1. Trends in publication (n = 479 papers) for research on fertility control in wild and feral fauna from 1980 to 2011. Proportion of each bar in grey depicts investigations that included empirical or simulated population-level effects.

Table 1. Number of reviewed scientific publications from 1980 to 2011 that included population-level effects of fertility control on wild or feral animals in theoretical, simulation or empirical contexts and the number of those publications that considered behavioural or indirect demographic feedbacks on population-level effectiveness of the application

	Type of study			Feedback on fertility control efficacy		
Fauna type	Theoretical	Simulation	Empirical	Negative	Positive	Not considered
Ungulate	1	10	6	4	0	13
Rodent	2	2	6	6	1	3
Marsupial	0	1	3	4	0	0
Pachyderm	2	2	1	2	0	3
Carnivore	0	3	1	0	0	4
Fish	1	0	0	1	0	0
Non-specific	4	3	0	4	0	3
Total	10	21	17	21	1	26

Baker 2000), and perhaps, >90% of females must be treated in open populations where immigration can compensate for birth reductions (Merrill, Cooch & Curtis 2006). Controlling populations of short-lived, high-fecundity animals is even more daunting because the high recruitment rates require fertility control treatments to be applied frequently (Singleton *et al*. 2002; Williams *et al*. 2007; Jacob, Singleton & Hinds 2008). In some applications, the lag time between treatment and effect on birth rates can result in too many new fertile females being recruited into the population for treatment strategies to be efficiently applied, even in long-lived, low-fecundity species (Nielsen, Porter & Underwood 1997; Whyte, van Aarde & Pimm 1998). Achieving population goals using fertility control for highly promiscuous breeders may not be feasible (Caughley, Pech & Grice 1992).

Some simulations have concluded that the response of a population to fertility control is nonlinear, and if survival increases as fecundity declines, then the survival is more influential on population growth than births (Hone 1992; Sinclair 1997; Hobbs, Bowden & Baker 2000; Merrill, Cooch & Curtis 2003). The logical conclusion to this is that fertility control is generally more effective at maintaining populations than actually reducing them, and thus, effective use of fertility control to maintain a population at reduced abundance may require culling to get the population to the desired level within a manageable time frame (Hone 1992; Barlow, Kean & Briggs 1997; Hobbs, Bowden & Baker 2000). Perhaps, most alarming is that many of these simulations assume 100% sterility, which is generally only achieved using surgical applications. Physically treating such large numbers of animals may be prohibitive due to the labour, time and expense necessary for capture, handling and application.

In contrast to these discouraging results, there is some evidence that fertility control can reduce and maintain populations at desired targets for abundance. Female feral horses have a long lifespan and can potentially produce offspring annually for up to 20 years of their life. A fertility control study at Assateague Island, the USA, reported zero population growth in only 2 years, with 42–76% of adult female feral horses treated annually with PZP (Kirkpatrick & Turner 2008). Population decline began 8 years after treatment began, and by year 11, the population had decreased nearly 23%. This sharply contrasts most simulation predictions, but may be explained by the indirect effects of treatment, the population parameters, life-history strategies of the species and the continual accessibility to individuals for treatment. This example reflects a relatively small, closed population of a polygynous species with strong social hierarchies. Thus, the lack of immigration and potentially reduced fecundity in subordinate females may act to supplement the effects of fertility control at the population level. Residual effects of treatment and decreased birth rate among untreated females contributed to a 33% difference in expected and realized births in another study of PZP in feral horses (Ransom 2012). Survival, however, appears to have increased in older females in both studies, which partially offsets the effects of fertility control. This complex system of feedbacks demands a deeper understanding of the influences acting on vital rates in populations proposed for control, and before we categorically dismiss the use of fertility control to functionally reduce populations, we must carefully consider how those feedbacks are operating.

Implications towards fitness

The genetic consequences of controlling any individual's fertility could have long-term effects on population fitness. Such artificial selection can theoretically occur in two ways. First, by purposeful or inadvertent non-random selection of animals targeted for contraception, managers may prevent reproduction in wildlife in ways that change gene flow in a population. This would have genetic effects similar to those that may arise from traditional means of wildlife management, such as non-random lethal removal of individuals from the gene pool. The second and far more uncertain mechanism is that inferred by the animal itself. Behavioural decisions unique to individuals shape their fitness, especially among males (Smith & Blumstein 2008). Such may be the case when polygynous males divert reproductive behaviour or when agonism increases or decreases in response to fertility control in the

Table 2. Population-level effects of fertility control in simulated (S) and empirical (E) studies that explicitly incorporated demographic feedbacks into analyses

Source	Taxa	Data	Individual treatment efficacy	Percent of population treated	Population effect	Feedback	Mechanism
Caughley, Pech & Grice (1992)	Gregarious, stratified by dominance structure	S	Non-specific: 100%	0–100% in social groups of up to 5 females	If female loses dominance at treatment, productivity increases	Negative	Increased births from subordinate females
Hone (1992)	a: long-lived, low fecundity b: short-lived, high fecundity	S	Non-specific: 100%	20–100% of original fecundity of females	Curvilinear response: for b, 80% fertility reduction ≈ 50% abundance reduction	Negative	Increased survival
Seagle & Close (1996)	White-tailed deer (*Odocoileus virginianus*)	S	Non-specific: 100%	0–100% of females	No reduction at <50% of females treated in open population	Negative	Increased immigration
Barlow, Kean & Briggs (1997)	a: monogamous and, b: polygamous	S	Non-specific: 100%	0–100% of females and/or 0–100% of males	Greatest effect in (a) with both sexes treated; less effect in (b)	Negative	Increased survival; increased births in b
Twigg *et al.* (2000); Williams *et al.* (2007)	European rabbit (*Oryctolagus cuniculus*)	E	Tubal ligation: 100%	0%, 40%, 60%, 80% of females	After 4 years, no difference in population size between treatments	Negative	Increased survival, increased immigration, decreased emigration
Singleton *et al.* (2002)	House mouse (*Mus domesticus*)	E, S	Tubal ligation & ovariectomy: 100%	67% of females	75% density reduction after 11 weeks, but compensation by 20–24 weeks	Negative	Increased births per female
Davis & Pech (2002)	Based on red fox (*Vulpes vulpes*)	S	Non-specific: 100%	0–100% of females	Absent density dependence, density increased with up to ~50% treatment	Negative	Increased survival
Merrill, Cooch & Curtis (2006)	White-tailed deer (*Odocoileus virginianus*)	S	Non-specific: 100%	30%, 45%, 60% of females	No population reduction if movement rates were >25% of observed and treatment was <45%	Negative	Increased immigration

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Table 2. (continued)

Source	Taxa	Data	Individual treatment efficacy	Percent of population treated	Population effect	Feedback	Mechanism
Ramsey (2005)	Brushtail possum (*Trichosurus vulpecula*)	E	Tubal ligation: 100%	0%, 50%, 80% of females	60% recruitment for 50% treated/74% for 80% treated: no population effect	Negative	Increased immigration, increased survival
Kirkpatrick & Turner (2008)	Feral horse (*Equus caballus*)	E	PZP: 0–8% birth rate in females	42–76% of females	Population decreased 22·8% in 11 years	Negative	Increased survival
Jacob, Singleton & Hinds (2008)	Ricefield rat (*Rattus argentiventer*)	E	Tubal ligation, progesterone: 100%	Up to 76% of females	No population effect in open population	Negative	Increased births, increased survival
Budke & Slater (2009)	Feral cat (*Felis catus*), closed population	S	Surgical or contraception: 100%	0–30% of females	91% of adult females need to be sterile to stop population growth	Negative	Increased survival

population. The age of individuals also interacts with demographic parameters of the population, as well as environmental variation, to influence fitness (Gaillard *et al*. 2000).

Individual physiology introduces a special consideration in the case of immunocontraceptives, and some authors have questioned the soundness of using a wild animal's immune system to select for the ability to reproduce (Nettles 1997; Cooper & Herbert 2001; Cooper 2004; Cooper & Larsen 2006). Immunocontraceptives are typically <100% effective at preventing pregnancy because they impose their effects through stimulation of the immune system. Depending on the magnitude of heritability, it is possible to select for decreased immune function and likely decreased fitness in relatively few generations. For example, if 10% of females in a population fail to mount a significant antibody response to an immunocontraceptive vaccine, and that phenotype has 80% heritability, then approximately 20% of female progeny will likewise not respond (Cooper & Larsen 2006). Thus, the trait has doubled in a single generation, assuming all females within the population were vaccinated. This has the potential to lead to immune incompetence and resistance to immunocontraception in the population. If immune responses responsible for contraception are mediated through the same or similar genetic pathways as those responsible for responding to pathogens and disease states, it is possible and even likely to select for decreased population fitness.

Alternatively, if a large proportion of the variation in contraceptive response is attributable to the environment, then even intense selection will have little effect on the phenotype of future generations (Magiafoglou *et al*. 2003). Difficulties in estimating heritability and finding reliable, accurate and sensitive indicators of changes in phenotype of immune function, given multigene effects on the system, pose significant challenges to resolve this question. However, there is evidence from studies with mice, chickens and pigs that strongly suggest that antibody production, delayed type hypersensitivity and phagocytic activity are heritable traits (Mouton, Sant'Anna & Biozzi 1988; Sarker *et al*. 1999; Wilkie & Mallard 1999; Pinard-van der Laan 2002). Significant changes in both humoral and cell-mediated immune responses can be achieved in as few as three generations in controlled environments (Sarker *et al*. 1999). Genetic variability in phenotype can change in response to environmental conditions (Hoffman 1999). This emphasizes the need for both laboratory and field studies of immunocontraceptives when investigating variance in immune response.

The proportion of the population targeted for fertility control will also influence selection pressure applied by non-response. From a practical standpoint, managers are likely to treat as many animals as possible when beginning a fertility control programme, particularly in extensively managed populations that are above management objectives. This will maximize non-response selection

pressure (Garrott 1995). Targeting for contraception may be more discriminating in intensively managed feral horse herds by selecting only a proportion of the population and ensuring that each female contributes an offspring to the population prior to contraception (Nuñez, Adelman & Rubenstein 2010). This strategy would result in decreased selection for non-response. Additionally, immigration and emigration will affect gene flow in the population and dilute the selection pressure. Treatment application intensity, non-response rate, change in fitness and migration will all influence the strength of artificial selection. This is one aspect of fertility control that wildlife managers have little, if any, valid data with which to make an informed decision. A cross-disciplinary approach involving immunologists, reproductive scientists, population or conservation geneticists and wildlife biologists may begin to answer these questions.

CONCLUSION

Study of individual effects of fertility control applications is an important step towards technology development of potential tools for managing wildlife, but understanding their ecological effects is a critical prerequisite for using them. Natural systems and processes are strained under anthropogenic pressures and ever-fragmenting landscapes, and resources to address these challenges are often lacking. Actions such as population-level fertility control demand that practitioners weigh not only the biological and ecological costs, but also the economic, political and regulatory costs involved for implementation.

Definitive generalizations about the biological and ecological conclusions of fertility control across taxa and biomes remain elusive. Primary empirical studies are still needed to evaluate population-level effects of fertility control on large wildlife populations over long time periods. Given the resources necessary to conduct such studies, it is likely that most future insight will arise from adaptive management applications. As such, fertility control as a management tool must be applied in thoughtful, calculated ways to achieve the greatest likelihood of success at reducing or maintaining population numbers. Current evidence suggests that population-level management of wildlife using fertility control is more likely to be successful if it is applied to populations (i) that are relatively small, (ii) that have little or no opportunity for an influx of new animals, (iii) that are comprised of a species that exhibits a relatively long gestation period and (iv) where a large proportion of individual animals can be accessed and aggressively treated. Fertility control management of non-native species might be more successful than management of native species because non-natives have not evolved in the ecosystem of concern, and the system is not dependent upon the species for natural function. For these reasons, alterations to life history, natural selection or behaviour may be also more publically acceptable, as long as they do not affect the conservation of native wildlife species. This hypothesis has not been tested to date. Likewise, applications to wildlife species occupying highly disturbed ecosystems may be effective because there may be disproportionately large influences from anthropogenic sources (i.e. where ecosystem drivers are more dependent on human actions than on natural processes), and the effects of fertility control on natural processes may be relatively minor in comparison.

If the necessary biological and ecological conditions can be met for effective use of fertility control to regulate wildlife populations, it is critical to address regulatory, political and economic concerns through public discourse and debate between agency, academic, political and public stakeholders who value their common wildlife resources for a wide variety of reasons. This includes transparent consideration of laws and regulations governing label and off-label use of drugs and vaccines; the welfare of animals subjected to capture, handling and treatment; and public acknowledgement that the targeted population effect and the ability of a management tool to reach such goals are not absolute. Rigorous economic analysis of the cost-effectiveness of fertility control relative to other management options is necessary to economically justify investing in the control actions (sensu Sebastián-González *et al.* 2011). This requires quantitative evaluation of the population-level and ecosystem-level benefits of the proposed actions.

Robust quantitative tools are necessary for modelling outcomes due to the disparate responses observed across species, populations and control options. Feedbacks from fertility control application vary widely and can produce quite unexpected results. In some cases, unintended changes to births, survival, immigration or emigration could provide a formidable barrier to effective management of populations using fertility control. Scientists, managers and practitioners are thus faced with understanding the often confounding nature of the actions implemented to achieve goals for populations. Fertility control can lead to complex population-level effects on the target species as well as cascading impacts on ecosystems. Both population-level and ecosystem-level impacts can and should be explored using a range of tools including empirical studies and simulation modelling. Expanding the prevailing birth-centric research theme to more thoughtful data collection and modelling that considers births, deaths, immigration, emigration, gene flow and ecological interactions will improve the quality and utility of fertility control studies.

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Behavior of feral horses in response to culling and GnRH immunocontraception

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ABSTRACT

Wildlife management actions can alter fundamental behaviors of individuals and groups, which may directly impact their life history parameters in unforeseen ways. This is especially true for highly social animals because changes in one individual's behavior can cascade throughout its social network. When resources to support populations of social animals are limited and populations become locally overabundant, managers are faced with the daunting challenge of decreasing population size without disrupting core behavioral processes. Increasingly, managers are turning to fertility control technologies to supplement culling in efforts to suppress population growth, but little is quantitatively known about how either of these management tools affects behavior. Gonadotropin releasing hormone (GnRH) is a small neuropeptide that performs an obligatory role in mammalian reproduction and has been formulated into the immunocontraceptive GonaCon-B™. We investigated the influences of this vaccine on behavior of feral horses (*Equus caballus*) at Theodore Roosevelt National Park, North Dakota, USA, for a year preceding and a year following nonlethal culling and GnRH-vaccine treatment. We observed horses during the breeding season and found only minimal differences in time budget behaviors of free-ranging female feral horses treated with GnRH and those treated with saline. The differences observed were consistent with the metabolic demands of pregnancy and lactation. We observed similar social behaviors between treatment groups, reflecting limited reproductive behavior among control females due to high rates of pregnancy and suppressed reproductive behavior among treated females due to GnRH-inhibited ovarian activity. In the treatment year, band stallion age was the only supported factor influencing herding behavior ($P < 0.001$), harem-tending behavior ($P < 0.001$), and agonistic behavior ($P = 0.02$). There was no difference between the mean body condition of control females (4.9 (95% CI = 4.7–5.1)) and treated females (4.8 (95% CI = 4.7–4.9)). Band fidelity among all females increased 25.7% in the year following vaccination and culling, despite the social perturbation associated with removal of conspecifics. Herding behavior by stallions decreased 50.7% following treatment and culling ($P < 0.001$), while harem-tending behavior increased 195.0% ($P < 0.001$). The amount of available forage influenced harem-tending, reproductive, and agonistic behavior in the year following culling and treatment ($P < 0.04$). These changes reflected the expected nexus

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between a species with polygynous social structure and strong group fidelity and the large instantaneous change in population density and demography coincident with culling. Behavioral responses to such perturbation may be synergistic in reducing grazing pressure by decreasing energetically expensive competitive behaviors, but further investigation is needed to explicitly test this hypothesis.

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1. Introduction

Gregarious relationships can impart individual benefits such as decreased depredation, increased foraging efficiency, and increased fecundity (Pusey and Packer, 1997). When resources to support populations of social animals are limited and populations become locally overabundant, managers are faced with the daunting challenge of decreasing population size without disrupting core behavioral processes. For example, the limited contiguous habitat and finite resources found on public wildlands cannot sustain overabundant populations of large ungulates, and managers continuously seek innovative and publically-acceptable tools for managing these species (Powers et al., 2011). Historically, such populations have been reduced by culling, using either lethal methods such as hunting, or non-lethal methods such as capture and translocation. Limiting fecundity through the use of immunocontraceptives is becoming a more commonly considered tool for controlling wildlife abundance. However, these tools can be accompanied by physiological changes that may alter behavior and ultimately influence population dynamics in unforeseen ways (Ransom et al., 2014). The paucity of quantitative data on natural behavior of wild free-roaming fauna, especially pertaining to those treated with fertility control agents, impairs our ability to understand species' influences and roles in ecosystems and thus our ability to effectively manage populations.

Elk (*Cervus canadensis*) and feral horses (*Equus caballus*) are increasingly targeted for fertility control management in the United States and both species exhibit complex social behavior and polygynous mating systems. Whereas elk congregate seasonally into mating groups (Geist, 1982), feral horses form bands that persist year-round with distinct associations of individuals sometimes lasting a decade or more (Klingel, 1982). These bands are largely maintained by reproductive, herding, agonistic, and defensive behaviors initiated by the polygynous male in the band, as well as hierarchical relationships among females (Ransom and Cade, 2009). Culling can result in discrete changes to population density and demography, which could perturb social organization. Likewise, effective fertility control agents lead to discrete changes in presence of offspring in the population, and thus may influence social behavior.

Approximately 33,000 feral horses roam 13 million ha of public lands in the western United States and managers have historically relied on nonlethal culling through a process of capture, removal, and public adoption to regulate animal abundance (Garrott and Oli, 2013). This process is unsustainable because demand in the U.S. for feral horses as domestic stock is less than half the number of animals removed, which has left as many horses in federally-subsidized pastures as persist in the wild. Several fertility control techniques have been investigated for use in feral horses, but most of these efforts resulted in tools that were publicly unacceptable, expensive to use, or impractical on a large scale. Among the most promising techniques are immunocontraceptive vaccines such as porcine zona pellucida (PZP) and gonadotropin-releasing hormone (GnRH). These agents are relatively easy to apply, are reasonably efficacious, and are not expected to be accompanied by serious contraindications (Kirkpatrick et al., 2011).

Gonadotropin releasing hormone is a small neuropeptide that performs an obligatory role in mammalian reproduction. When combined with a potent adjuvant, the GnRH vaccine stimulates a persistent immune response resulting in prolonged antibody production against GnRH, the carrier protein, and the adjuvant (Miller et al., 2008). The most compelling hypothesis of vaccine effectiveness suggests that antibodies to GnRH likely induce transient infertility by binding to endogenous GnRH, thus preventing attachment to receptors on gonadotropes and suppression of pulsatile luteinizing hormone (LH) secretion (Molenaar et al., 1993). Gonadotropin releasing hormone antibody titers are correlated with suppression of the reproductive system and infertility in a variety of species (Fagerstone et al., 2010). As anti-GnRH antibodies decline over time, concentrations of available endogenous GnRH increase and treated animals usually regain fertility (Powers et al., 2011).

This immunocontraceptive vaccine, known by the trade name as GonaCon-B^{TM}, has been shown to provide multiple years of infertility in several wild ungulate species including horses (Killian et al., 2008; Gray et al., 2010), bison (*Bison bison*) (Miller et al., 2004), elk (Powers et al., 2011), and white-tailed deer (*Odocoileus virginianus*) (Gionfriddo et al., 2009). Given the physiological mechanism of action, GnRH immunocontraception has the potential to reduce fertility and suppress the reproductive behaviors typically associated with copulation; however, in GnRH-vaccinated elk, such behaviors were not entirely diminished likely due to incomplete suppression of the hypothalamic pituitary gonadal (HPG) axis (Powers et al., 2011). Similar to elk, feral horses are polyestrous and fertility control agents that do not suppress these social behaviors can lead to females exhibiting frequent reproductive behaviors that can cascade into altered behaviors from associated band members and nearby males (Nuñez et al., 2009; Ransom et al., 2010).

Rigorous quantitative investigation into the potential effects of GnRH treatment on feral horse behavior is missing from the assessment of this immunocontraceptive as a potential management tool. Such data are

Table 1
Population metrics for feral horses (*Equus caballus*) at Theodore Roosevelt National Park, North Dakota, USA, 2009 and 2010. Normalized Difference Vegetation Index (NDVI) is shown for only the behavior observation period in each year.

	2009	2010
Number of males	64	26
Number of females	74	59
Number of foals	27	31
Mean male age ± SE (range)	5.0 ± 0.45 (1–15)	7.7 ± 0.68 (1–16)
Mean female age ± SE (range)	5.3 ± 0.50 (1–20)	6.4 ± 0.45 (1–18)
Number of bands	19	16
Mean band size ± SE (range)	8.3 ± 0.07 (3–14)	8.7 ± 0.07 (3–19)
Total population size	165	116
Mean NDVI (95% CI)	0.44 (0.324–0.548)	0.43 (0.346–0.504)
Density (number of horses/ha)	0.009	0.006

also absent in regard to culling, which is arguably the most common feral horse management practice worldwide. We investigated the influences of GnRH treatment on behavior of feral horses at Theodore Roosevelt National Park, North Dakota, for a year proceeding and a year following GnRH vaccination and culling. Our objectives were to quantify effects of these two management tools on time budgets of individuals and social interactions between individuals. Based on the assumed mechanism of action of GnRH vaccine, we posited that inoculation would suppress reproductive behaviors in treated females compared to controls and that the sudden decrease in density and social perturbation caused by population reduction would measurably influence social behaviors of all horses.

2. Materials and methods

2.1. Study area

Theodore Roosevelt National Park is located in southwestern North Dakota, USA (45°55′N/103°31′W). The South Unit of the park, where this study was conducted, comprised approximately 19,000 ha and consisted of eroded badlands with gullies and ravines separated by large upland plateaus, and small buttes. The mixed-grass prairie vegetation was predominantly comprised of needle and thread (*Hesperostipa comata*), threadleaf sedge (*Carex filifolia*), western wheatgrass (*Pascopyrum smithia*), blue grama (*Bouteloua gracilis*) and little bluestem (*Andropogen scoparius*) (Butler et al., 1986). Sympatric ungulates included feral horses, pronghorn (*Antilocapra americana*), bison, mule deer (*Odocoileus hemionus*) and elk. Feral horses were confined to the South Unit by a 1.8–2.4 m high woven wire boundary fence and no other feral horse populations were nearby. All individual horses in this population were previously identified and assigned individual identity numbers by managers, and age and reproductive data for each animal have been maintained since 1993 (National Park Service, unpublished data).

2.2. Treatments and study subjects

In spring/summer 2009, when pre-treatment data were collected, 165 horses occupied the park and were organized into 19 bands (Table 1). All bands contained a single adult stallion and adult females and their offspring, with the exception of one band that also contained a second stallion. No females persisted outside of bands; whereas, bachelor males were distributed across the landscape individually or sometimes in small ephemeral bands.

Horses were gathered into permanent corrals and handling facilities using a helicopter during a scheduled management capture in October 2009. Managers attempted to gather all horses in the population at that time, and removed 78 horses based on the agency's specific management needs. Eighty-one percent of the culled animals were foals, yearlings, and 2- and 3-year olds, and 14% were bachelor males not associated with any band. The remaining individuals removed included three females age ≥16 years, and one 6-year old female; none of which were part of our experimental group. These removals inherently skewed the sex ratio of the population between years because so many bachelor males were removed; however, paternity attributed to bachelor males is thought to be low (Bowling and Touchberry, 1990) and all study bands were subjected to competition pressure equally in each year at the population level. Post-treatment data were collected in spring/summer 2010, when 116 horses (including those born in 2010) occupied the park (Table 1).

We selected 58 adult females (2–17 years of age) using a randomized complete block design consisting of a GnRH-vaccinated treatment group and a saline control group. The resulting experimental female population consisted of 29 GnRH-vaccinated females (treatment) and 28 saline-treated (control) females, with one mature female remaining in the population that could not be captured. Females that were not in either treatment group were excluded from behavior analyses. The GnRH vaccine ultimately did not prevent conception in all inoculated horses (Baker et al., 2013); therefore, to more clearly separate the effects of infertility on behavior, we also excluded from analysis observations of 14 treatment females that became pregnant in 2010 and produced offspring in 2011. Females of both treatment groups were dispersed randomly across 16 bands with no difference in proportion of treated and control females between bands (paired t-test for unequal means: $t = 0.116$, $P = 0.909$). The same adults and bands observed in 2009 were observed in 2010, with none of the research cohort or band stallions removed during the culling operation.

Veterinarians blinded to treatment status assessed the general health, pregnancy, and body condition of each

animal at the time of capture. Pregnancy status and approximate stage of gestation were determined using rectal palpation of the reproductive tract and transrectal ultrasound imaging (Bucca et al., 2005). We visually assessed body condition of all females and scored condition using a numerical index that increased 1–9 with increasing fatness (Henneke et al., 1983). Females in the treatment group received an intramuscular injection in the gluteus muscle, by hand-syringe, containing 2000 μg of GnRH vaccine (2 mL, GonaCon-B™ (National Wildlife Research Center, Fort Collins, CO, USA)). Females in the control group were injected in a similar manner with an equal volume of saline solution. All data for this study were collected in accordance with Colorado State University Animal Care and Use Committee standards (protocol 12-3197A).

2.3. Behavioral sampling

Behavioral observations were conducted during three daylight time periods (08:00–12:00 h, 12:01–16:00 h, and 16:01–20:00 h) from April 18, 2009–July 16, 2009 and April 8, 2010–August 19, 2010. Each observation session included collection of a 20 min instantaneous scan sample of time budgets at 1 min intervals for each adult band member (≥1 years old), and all-occurrence data collection for social interactions, exactly as implemented by a synonymous study of PZP in horses (Ransom et al., 2010). We used a feral horse ethogram that categorized specific behaviors into 15 discrete time budget categories; agonism, comfort, elimination, feeding, grooming, harem-social (e.g., allogrooming, pair-bonding, female-female urine marking), harem-tending (e.g. stallion defense of a band female or recruitment of a new female into the band), herding (e.g., driving or snaking behavior by the stallion), interaction-with-humans, locomotion, out-of-sight, reproduction, resting, standing attentive, and submission (Ransom and Cade, 2009). The infrequency and similar nature of several behaviors in the instantaneous scan samples led us to aggregate them into broader categories for time budget analyses. Grooming, comfort, standing attentive, and elimination were combined into *maintenance*; reproductive, harem-tending, harem-social, agonism, submission, and herding were combined into *social*; and interaction-with-humans and out-of-sight were combined into *unknown*. The time spent in the category *unknown* ultimately did not differ between treatment groups or between years.

We also collected social behavior data at every occurrence throughout each observation session because instantaneous scan sampling has the potential to underestimate rare events (Ransom and Cade, 2009). These all-occurrence behaviors included intraband social behaviors associated with herding, reproduction, agonism, and harem-tending and were retained as discrete categories for social behavior analyses. We observed all horses from the nearest distance that did not elicit attention of horses to the presence of the observer, typically 50–300 m. All observations were conducted using a 15–45 × 600 mm spotting scope or 10 × 42 mm binoculars when the distance between horses and observers was too far to allow unassisted detailed observation.

2.4. Statistical analyses

We modeled the frequency of each behavior using mixed-effects linear regression, where individual female identity and sampling time period (time of day) were included as random effects on the intercept term of each model. This accounted for variation that may have been present among individuals who were sampled repeatedly, though not always equally over time, and for temporal variation in behavior when samples were not equally collected across all times of day. We used the lme4 package of R version 2.14.1 (The R Foundation for Statistical Computing 2011) and SYSTAT 12.02.00 (SYSTAT Software, Inc. 2007) to calculate descriptive statistics and obtain mixed-effects model estimates using restricted maximum likelihood (Harville, 1977).

We fitted separate models for each year to assess the fixed effects of treatment (x_1, pre-treatment or pre-control in 2009, treatment or control in 2010), foal presence (x_2, dependent foal < 1 year of age present with the female, or no foal present with the female), female age (x_3), band size (x_4, total number of animals in the group), and band fidelity (x_5, number of times a female changed bands within the sampling year) on time budget behaviors. In the notation of Gelman and Hill (2007), models took the form of $y_{jk[i]} = \alpha_{0j[i]} + \alpha_{1k[i]} + \beta_0 + \beta_{x_1}x_{1[i]} + \beta_{x_2}x_{2[i]} + \beta_{x_3}x_{3[i]} + \beta_{x_4}x_{4[i]} + \beta_{x_5}x_{5[i]} + \varepsilon_{jk[i]}$, where the random effect intercept of $\alpha_{0j[i]} \sim N(\mu = 0, \sigma_j^2)$ represents time period j (08:00–12:00 h, 12:01–16:00 h, 16:01–20:00 h), for $i = 1,\ldots,n$ observations, and the random effect intercept $\alpha_{1k[i]} \sim N(\mu = 0, \sigma_k^2)$ represents $k = 1,\ldots,n$ individual horses for $i = 1,\ldots,n$ observations. This equation identifies the fixed effect intercept as β_0, x = independent variable with parameter β_x, ε = statistical error, and y = dependent variable. All-occurrence social behavior was modeled in the same framework, but because those data involved a given stallion directing a behavior toward a given female, we also included the additional variable of stallion age as a fixed effect.

In order to gain insight into the effects of removal versus fertility control, we also modeled time budget and all occurrence behavior across both years, replacing treatment status with observation year as a fixed effect. This allowed us to look at the broad paradigm change, independent of trying to separate the inherently related aspects of changing sex ratio, density, and competition. We included Normalized Difference Vegetation Index (NDVI) as a fixed effect in this model in order to estimate the temporal influence of forage availability on behavior that could have varied between years. The NDVI data were obtained from the National Aeronautics and Space Administration (http://modis.gsfc.nasa.gov) and reconciled using ArcGIS software (ESRI, Redlands, California). We also included sex as a fixed effect in this model to gain insight into both male and female time budgets in concert with the culling perturbation.

3. Results

The 2009 pregnancy assessment indicated that 89% of the control group and 86% of the treatment group were pregnant at the time of saline- or GnRH vaccine-injection. In the following year, 71% (95% CI = 53.9–89.3%) of control females and 72% (95% CI = 55.1–89.7) of treated females were observed with a dependent offspring. Mean body condition of pre-control females was 5.1 (95% CI = 4.8–5.4) and of pre-treatment females was 4.9 (95% CI = 4.7–5.2) in 2009. Likewise, mean body condition of control females was 4.9 (95% CI = 4.7–5.1) and of treated females was 4.8 (95% CI = 4.7–4.9) in 2010.

We collected behavioral data on 93 feral horses (23 males, 29 pre-treatment females, 27 pre-control females, 14 other females) for 582.4 h in 2009. The median age of observed stallions was 7 years (range = 3–14 years), median age of observed females was 5 years (range = 2–17), and median band size was 8 horses (range = 3–17). In 2010, approximately 5 months after treatment and culling, we observed 77 of the same feral horses (18 males, 29 treated females, 28 control females, two other females) for 1263.90 h. The median age of observed stallions was 9 years old (range 5–15), median age of observed females was 5 years old (range = 3–18), and median band size was eight horses (range = 3–19). The final data used for GnRH treatment analyses arose from 1331 observations of 29 pre-treated and 29-pre-control females in 2009 and 2299 observations of 15 treated and 28 control females in 2010. The final data used for culling analyses arose from 2115 observations of 23 males, 27 pre-control females and 29 pre-treatment females in 2009, and 3274 observations of 18 males, 28 control and 29 treatment females in 2010.

3.1. Time budgets

Band size and foal presence were influential in how females allocated their time during 2009, but there were no differences in behaviors between pre-treatment and pre-control females (Table 2). However, treated and control females spent their time differently in the year following inoculation. Treated females fed more and moved more than control females, and reciprocated by reducing time in resting and maintenance behaviors (Fig. 1). There were no changes in time spent engaging in social behaviors between treated or control females and their conspecifics (Table 2). Band size influenced feeding time, increasing 0.9 ± 0.40 (SE)% with each additional horse in the band in 2009. This effect was not evident in 2010 after the population was reduced. Time of day influenced feeding behavior in both years. Horses fed an estimated 7.3 ± 4.69% (2009) and 7.5 ± 5.01% (2010) less in the morning, as compared to 7.4 ± 5.00% (2009) and 9.0 ± 5.04% (2010) more in the afternoon.

Time spent feeding was largely reciprocated by time resting among both treated and control groups (Fig. 1). There were no differences between pre-treatment and pre-control females in time spent resting ($t_{1270} = 0.459$, $P = 0.646$), but like feeding, this relationship changed after inoculation, and treated females rested less than their control counterparts (Table 2). Foal presence influenced

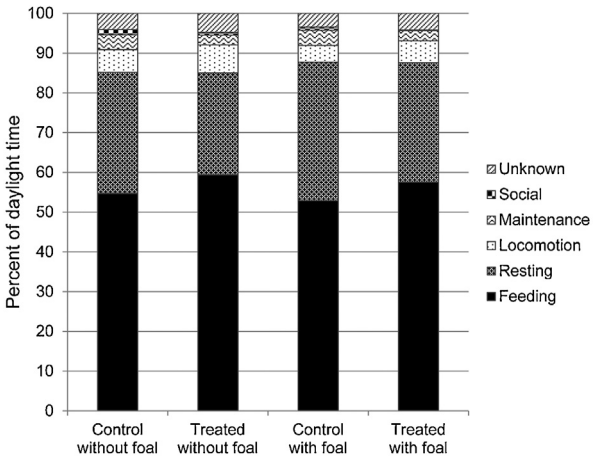


Fig. 1. Mean allocations of daylight time budget behaviors for feral horse (*Equus caballus*) females at median age (5 years old) and median band size (eight horses). Estimates are shown from mixed-effects linear regression considering individual identity and time of day as random effects. Data consisted of 2299 observations of 28 control and 15 treatment females in 2010 at Theodore Roosevelt National Park, USA. Foal presence was defined as a dependent born during the observation year.

resting in both years with independent females resting 4.4 ± 2.27% (2009) and 4.4 ± 1.83% (2010) less than females with a dependent foal (Table 2). Resting occurred temporally opposite of feeding activity, with 7.0 ± 4.43% (2009) and 6.5 ± 4.18% (2010) more resting in the morning and 6.8 ± 4.72% (2009) and 7.2 ± 4.22% (2010) less in the afternoon.

The remainder of horses' time was occupied by locomotion (about 5% of their time), maintenance (about 3% of their time), and social behavior (about 1% of their time) (Fig. 1). Treated females spent less time moving than controls and reciprocated with increased maintenance behavior (Table 2). Age was a statistically significant effect in modeling locomotion in 2010, but because time in locomotion only differed by 0.12% per year of age, this is unlikely to be biologically significant. Foal presence, however, was a strongly supported effect and females with a dependent were on the move 1.5 ± 0.35% more than independent females (Table 2). Maintenance behavior was influenced by band fidelity in 2010 and time spent exhibiting this behavior increased 1.6 ± 0.44% per band change. Six of 43 females (14%) were observed changing bands one to three times during 2010. In contrast, 23 of 58 females (40%) changed bands one to four times in 2009. The decreased band size in 2010 appears to have influenced time spent performing maintenance behavior (Table 2). Like age and locomotion, this effect was statistically significant but with an increase of only 0.1 ± 0.06% per additional band member it is unlikely to have biological meaning. Locomotion, maintenance, and social time budget behaviors were influenced minimally by time of day or individual variation (Table 2).

3.2. Social behaviors

All-occurrence social behaviors associated with herding, harem-tending, reproduction, and agonism from stallions toward females were observed 57% of the time

Table 2
Supported effects in a mixed-effects linear regression of feral horse (*Equus caballus*) time budget behaviors at Theodore Roosevelt National Park, USA. Variance for the random effects of time of day (j) and individual horse identity (k) are shown as σ_j^2 and σ_k^2.

Behavior	Supported effect	t	P	Difference	95% lower confidence limit	95% upper confidence limit	σ_j^2	σ_k^2
2009								
Feeding	Band size	2.217	0.027	0.878	0.101	1.654	0.589	0.163
Resting	Foal presence	−1.923	0.054	−4.370	−8.828	0.089	0.524	0.156
Locomotion	Band size	−2.369	0.018	−0.186	−0.340	−0.032	<0.001	0.009
Maintenance	None	–	–	–	–	–	0.007	0.100
Social	Foal presence	1.936	0.053	0.183	−0.002	0.369	<0.001	<0.001
2010								
Feeding	Treatment	−2.064	0.039	−4.685	−9.136	−0.234	0.716	0.188
Resting	Treatment	2.239	0.020	4.826	0.762	8.890	0.490	0.125
	Foal presence	−2.420	0.016	−4.425	−8.010	−0.840		
Locomotion	Treatment	−3.511	<0.001	−1.319	−2.056	−0.582	0.002	<0.001
	Age	2.059	0.040	0.117	0.005	0.229		
	Foal presence	4.304	<0.001	1.522	0.829	2.217		
Maintenance	Treatment	2.655	0.008	1.266	0.330	2.200	0.006	<0.001
	Band size	2.449	0.014	0.136	0.027	0.245		
	Band fidelity	3.617	<0.001	1.574	0.721	2.430		
Social	None	–	–	–	–	–	0.002	0.013

($n = 331$) during 2009 and 51% of the time ($n = 650$) during 2010. Herding by stallions was the most frequently observed social behavior. Band size influenced this behavior in 2009, with herding increasing 0.16 ± 0.56 times per hour (tph) per additional horse in the band (Table 3). In 2010, however, age of the stallion was the only important fixed effect on this behavior and herding increased 0.18 ± 0.038 tph per year of age. At median stallion age, median female age, and median band size, pre-treatment females received herding behavior 2.24 ± 0.743 tph and pre-control females received this behavior 1.98 ± 0.694 tph ($t_{274} = -0.892$, $P = 0.373$); likewise, treated females received herding behavior 1.06 ± 0.415 tph and control females received this behavior 0.99 ± 0.454 tph ($t_{602} = -0.271$, $P = 0.787$). Time of day was not an important influence on herding in either year and variance of this random effect was similar in both years (Table 3).

Harem-tending occurred less frequently than herding in 2009, but at a similar rate as herding in 2010. The supported fixed-effects in models of harem-tending were band size in 2009 and age of stallion in 2010 (Table 3). As band size increased per horse, harem-tending decreased 0.05 ± 0.020 tph in 2009, and as stallion age increased per year, harem-tending decreased 0.11 ± 0.026 tph in 2010. As with herding behavior, time of day did not influence harem-tending occurrence in either year and there was little individual variance in 2009.

Contrary to our prediction that reproductive behaviors would be reduced in GnRH-treated mares compared to controls, we observed almost exactly the same frequency in both years and between treatment groups. At median stallion age, median female age, and median band size pre-treatment females received reproductive behaviors 0.57 ± 0.374 tph and pre-control females received reproductive behaviors 0.40 ± 0.349 tph ($t_{274} = -1.054$, $P = 0.293$); likewise, treatment females received reproductive behaviors 0.59 ± 0.270 tph and control females received reproductive behaviors 0.56 ± 0.297 tph ($t_{602} = -0.177$, $P = 0.860$). The only supported fixed-effect in either year was female age in 2009, which estimated a 0.05 ± 0.024 tph decrease in reproductive behavior per year of age.

Agonism occurred at similar rates as reproductive behaviors in both years and was influenced by stallion age in both years (Table 3). Agonism from males toward females increased 0.03 ± 0.019 tph per year of male age in 2009 and 0.04 ± 0.017 tph per year of male age in 2010. At

Table 3
Supported effects in a mixed-effects linear regression of feral horse (*Equus caballus*) social behavior at Theodore Roosevelt National Park, USA. Variance for the random effects of time of day (j) and individual horse identity (k) are shown as σ_j^2 and σ_k^2.

Behavior	Supported effect	t	P	Difference	95% lower confidence limit	95% upper confidence limit	σ_j^2	σ_k^2
2009								
Herding	Band size	2.871	0.004	0.159	0.050	0.267	0.072	<0.001
Harem-tending	Band size	−2.731	0.007	0.054	−0.093	−0.015	<0.001	<0.001
Reproduction	Female age	−2.094	0.037	0.051	−0.099	−0.003	<0.001	0.073
Agonism	Stallion age	1.990	0.048	0.038	0.000	0.076	<0.001	<0.001
2010								
Herding	Stallion age	4.906	<0.001	0.184	0.110	0.258	0.047	0.153
Harem-tending	Stallion age	−4.234	<0.001	0.110	−0.162	−0.059	0.007	0.163
Reproduction	None	–	–	–	–	–	<0.001	0.096
Agonism	Stallion age	2.436	0.015	0.042	0.008	0.076	0.026	<0.001
	Band fidelity	2.411	0.016	0.201	0.037	0.365		

median stallion age, median female age, and median band size pre-treatment females received agonistic behaviors 0.26 ± 0.251 tph and pre-control female received agonistic behaviors 0.44 ± 0.234 tph ($t_{274} = 1.744$, $P = 0.082$); likewise, treatment females received agonistic behaviors 0.19 ± 0.184 tph and control females received agonistic behaviors 0.36 ± 0.203 tph ($t_{602} = 1.627$, $P = 0.104$). Band fidelity also influenced the 2010 estimate and agonism increased with number of band changes (Table 3). Neither time of day nor individual variance influenced agonism occurrence in either year.

3.3. Population reduction

Horses allocated time differently by sex and by year (Table 4). Males fed less, rested more, moved more and engaged in social interactions more often than females, but all horses fed less, rested more, moved more, and performed less maintenance behavior and engaged in more social behavior in 2010 as compared to 2009 (Fig. 2). Normalized Difference Vegetation Index was highly correlated between years ($R^2 = 0.90$). The finer scale all-occurrence behavioral data revealed that herding by stallions decreased 50.71% from 2009 to 2010, and appeared to increase in frequency concurrent with the seasonal green-up period (and concurrent reproductive season) (Fig. 3A). Harem-tending behavior increased 195.0% from 2009 to 2010, when both were estimated at median stallion age, median female age, median band size, and mean NDVI. Harem-tending also occurred in concert with forage production, but this relationship was more pronounced in 2010, when the frequency of occurrence was markedly escalated (Fig. 3B). Time of day and individual identity contributed little to variance in these models (Table 4).

4. Discussion

There were no differences in any behaviors between treatment groups before GnRH vaccination and culling. Females vaccinated against GnRH managed their time differently than controls. They fed less, rested more, traveled less, and performed more maintenance behaviors than their control counterparts, but engaged in the same amount of social behavior. The estimates across years, however, indicated that pronounced behavioral changes occurred in how stallions engaged with females in social behaviors. Stallions herded all females less and harem-tended more following the changes in population density, demography, and social perturbation that resulted from culling. The date of observation in relation to forage availability explained some differences between years, while the increased mean age of stallions as well as the decreased band size in 2010 also explained some of the differences. None of the models indicated that herding or harem-tending varied due to the treatment status of females, but rather implied that the paradigm shift in competition influenced how band stallions managed their family groups. Because the same stallions were observed in both years, the effect of age potentially reflected the additional year of experience each male possessed, in the context of competition pressure.

The similarity in female social behavior between treatment groups likely related to estrous behavior, a catalyst of other social behavior. Estrous behavior is influenced by the concentration and timing of progesterone and estradiol, the steroid hormones that fluctuate with occurrence of pregnancy (Katz, 2007). During pregnancy, high concentrations of progesterone usually inhibit estrous behavior (Crowell-Davis, 2007; Fabre-Nys and Gelez, 2007). The GnRH-antibodies stimulated by GnRH vaccination should have interrupted the hormonal cascade in females and ultimately suppressed pulsatile luteinizing hormone (LH) secretion, and thus ovulation (Powers et al., 2011). Consequently, we might expect that similar social behavioral expressions (albeit for different reasons) between pregnant females and GnRH-treated females would be observed. However, a similar study in elk concluded that GnRH was not completely suppressed in vaccinated females and thus some LH stimulation occurred and likely allowed limited production of estradiol (Powers et al., 2011). This permitted reproductive behaviors, at a reduced level, in GnRH-treated individuals. It should also be noted that estrous behavior has been observed with low frequency among both pregnant female and anovulatory female horses (Asa et al., 1983; Crowell-Davis, 2007). This is thought to be a bonding mechanism that assists in the maintenance of stable social groups of horses year-round, rather than ephemeral reproductive groups, such as those observed in elk. Also, the complex social behaviors among feral horses do not entirely center on reproductive receptivity, and other behaviors associated with dominance hierarchies, leadership, and gregarious lifestyles may or may not be as directly influenced by fluctuations in progesterone and estradiol.

Regardless of the similarity in social behaviors between treatment groups, treated females still allocated their time budgets differently than controls in 2010. Most GnRH-vaccinated females in the final dataset were likely not pregnant in 2010 because we categorically omitted the treated females that demonstrated unsuccessful response to vaccination by producing offspring in 2011. Pregnancy influences time spent feeding and resting simply due to the metabolic needs of barren females as compared to pregnant females (National Research Council, 2007). The composite nature of time budget behaviors requires that if any one behavior changes, then other behaviors such as locomotion or maintenance must also change; thus, the small differences between treatment groups were unsurprising.

The estimates of time spent per behavior for all horses in our study fell well within the ranges previously reported for daylight time budgets of horses (Mayes and Duncan, 1986; Boyd, 1998; Berger et al., 1999; Ransom et al., 2010). Ultimately, body condition scores of control and treated females were at parity in 2010. This may indicate that the metabolic demands of pregnant and non-pregnant females were compensated for behaviorally, that the body condition scoring system was not sensitive enough to detect finer-scale differences, or that forage abundance was high enough to obfuscate the effects of behavior on body condition. Foal presence influenced resting in both years with independent females resting less than females with a

Table 4
Between year differences in time budget and social behaviors of feral horses (*Equus caballus*) Theodore Roosevelt National Park, USA, as estimated by mixed-effects linear regression. Variance for the random effects of time of day (j) and individual horse identity (k) are shown as σ_j^2 and σ_k^2.

Behavior	Supported effect	t	P	Difference	95% lower confidence limit	95% upper confidence limit	σ_j^2	σ_k^2
Time budget								
Feeding	Year	2.141	0.032	2.141	1.838	4.178	0.743	0.098
	Sex	5.413	<0.001	6.725	4.289	9.160		
	Band size	3.699	<0.001	0.497	0.234	0.761		
	Age	−3.040	0.002	−0.477	−0.784	−0.169		
	NDVI	−22.407	<0.001	−115.698	−125.820	−105.576		
Resting	Age	2.844	0.004	0.445	0.138	0.752	0.515	0.104
	NDVI	19.903	<0.001	100.605	90.695	110.514		
Locomotion	Year	−5.726	<0.001	−1.280	−1.719	−0.842	0.001	<0.001
	Sex	−4.014	<0.001	−0.917	−1.366	−0.469		
	Band size	−3.720	<0.001	−0.098	−0.150	−0.046		
	NDVI	−5.433	<0.001	−6.307	−8.582	−4.031		
Maintenance	Year	5.122	<0.001	1.754	1.083	2.425	0.014	0.014
	Sex	−2.957	0.003	−1.279	−2.127	−0.431		
	NDVI	15.987	<0.001	27.654	24.263	31.045		
Social	Year	−3.862	<0.001	−0.714	−1.077	−0.352	0.001	0.014
	Sex	−3.095	0.002	−0.991	−1.618	−0.363		
	Band size	2.013	0.044	0.057	0.002	0.113		
	NDVI	6.798	<0.001	0.062	0.044	0.079		
All occurrence								
Herding	Year	7.581	<0.001	1.148	0.851	1.445	<0.001	0.063
	Stallion age	5.226	<0.001	0.026	0.088	0.193		
Harem-tending	Year	−9.483	<0.001	−0.838	−1.012	−0.665	0.004	0.970
	Stallion age	−4.578	<0.001	−0.078	−0.111	−0.044		
	Band size	−4.793	<0.001	−0.057	−0.080	−0.033		
	NDVI	4.918	<0.001	2.385	1.433	3.336		
Reproduction	Band size	−2.242	0.025	−0.025	−0.047	−0.003	<0.001	0.044
	NDVI	2.095	0.037	1.019	0.065	1.973		
Agonism	Stallion age	2.938	0.003	0.035	0.012	0.058	0.009	0.008
	NDVI	−2.736	0.006	−1.032	−1.772	−0.292		

dependent foal. Band size was also an important effect on some behaviors; most notably, herding and harem-tending behavior in 2009 were significantly related to band size, but this was not the case in 2010. This effect on social behaviors was replaced with stallion age in 2010. These contradictions may be in part due to the change in population size that occurred between years or due to changes in band composition. Even though band size did not change notably between years, some young animals were removed from bands.

Density of bachelor males in the population was likely influential in the large changes in herding and harem-tending behaviors observed between years: the number of bands and band size did not differ greatly between

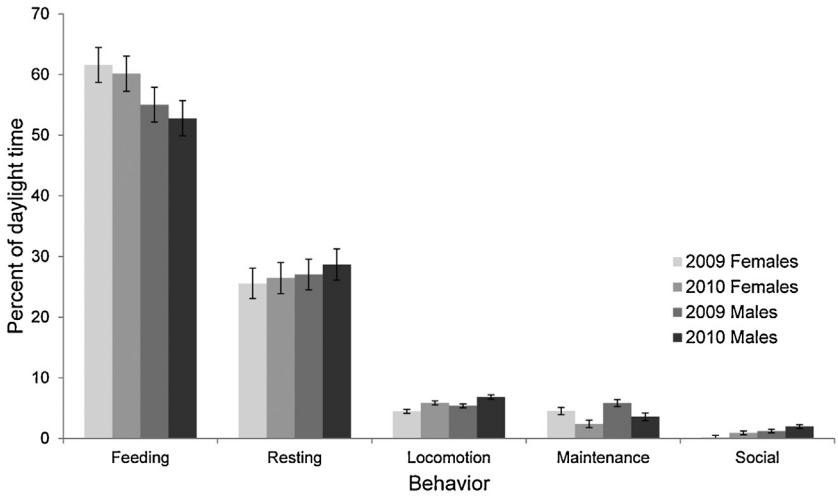


Fig. 2. Comparison of mean ± SE daylight time budget behaviors of feral horses (*Equus caballus*) before (2009) and after (2010) culling and GnRH treatment at Theodore Roosevelt National Park, USA. Estimates are shown at median age, median band size, and mean Normalized Difference Vegetation Index (NDVI) and arose from mixed-effects linear regressions considering individual identity and time of day as random effects.

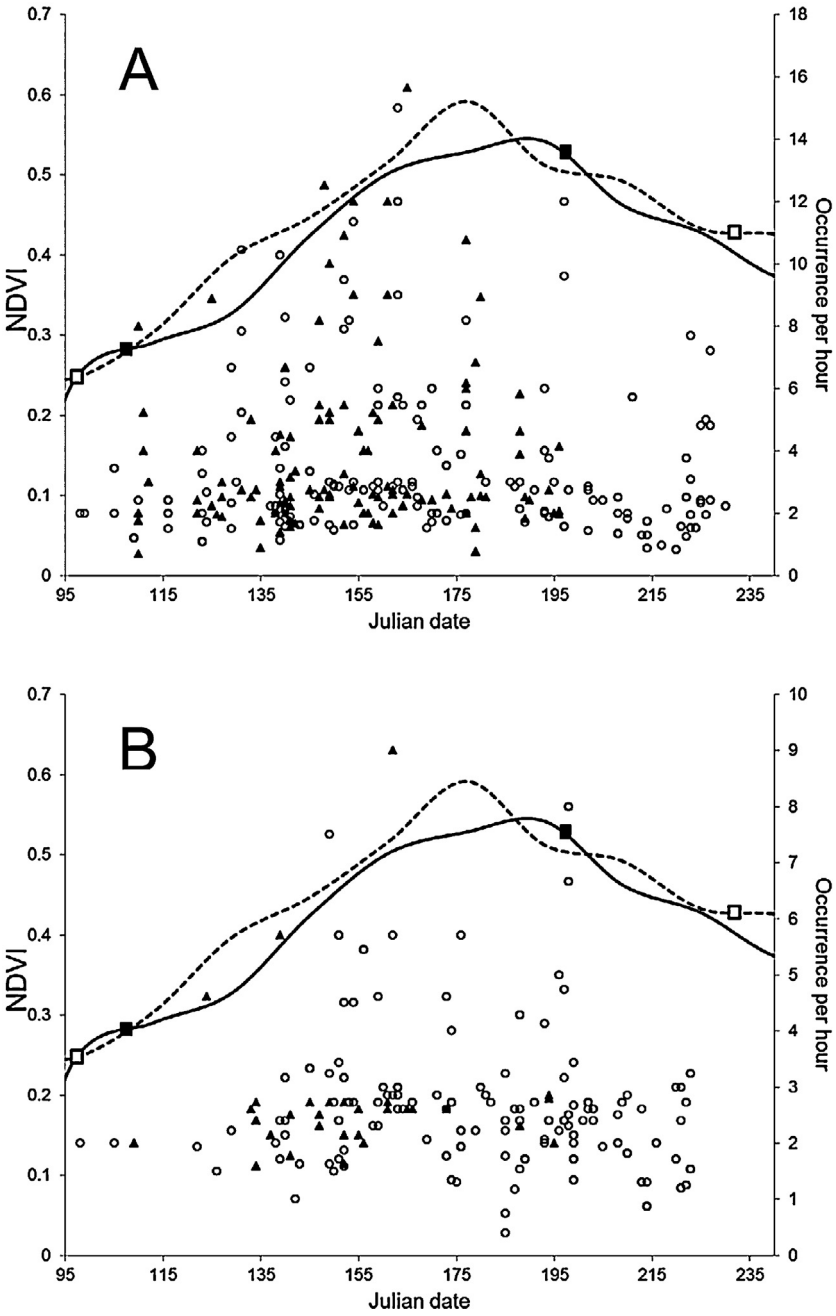


Fig. 3. Temporal occurrence and rate of feral horse (*Equus caballus*) herding (A) and harem-tending (B) behaviors in relation to seasonal trend in forage availability using Normalized Difference Vegetation Index (NDVI) as an index of greenness. NDVI for the pre-treatment year of 2009 is shown as a solid line with behaviors as solid triangles and NDVI for the post-treatment year of 2010 is shown as a dashed line with behaviors as hollow circles. Boxes on the NDVI trend lines designate the beginning and end of each behavior observation season.

years, but the number of available males in the population decreased 41% between years and the mean age of males in the population increased by nearly 3 years or 36%. This combination of effects presents compelling evidence that quickly and non-randomly reducing population density has behavioral implications for individuals and how bands function in the social dynamics of the population.

Herding and harem-tending behavior are important behaviors used by band stallions to maintain bands, but also represent an energetic cost for both the male and female(s) involved. Such costs may have biological meaning in terms of body condition and fidelity of females to the band (Ransom et al., 2010). The decline in herding behavior detected between years is not surprising given the decreased presence of competing males in the

population, which is often what prompts a stallion to actively herd females together (Waring, 2003). This decrease in potential energetic expense lends support for the practice of culling because while it decreases grazing impact by removing animals, it also leads to the remaining animals expending less energy (that would have to be replaced by grazing) by decreasing frequency of behaviors that arise from competition. Consequently, 2.2% less feeding behavior was estimated for stallions in 2010 as compared to 2009 in our study. This is supported by the observed increase in harem-tending behaviors in our study, most of which are likely driven by instinctual behavioral mechanisms more than active presence of a competitor. Such behaviors include stallions marking urine deposits of band members and constructing fecal middens that relate to reproductive status, communication, and band identification (Feist and McCullough, 1976; Rubenstein and Hack, 1992).

Little has been studied on the effects of culling in feral horse populations, and what has been presented in the literature focuses on stress and its effects on reproduction (Hansen and Mosley, 2000; Ashley and Holcombe, 2001). A short term study did collect data on percent of time horses spent feeding, resting, vigilant, traveling, and engaged in agonistic encounters and found no difference between control (horses not gathered or pursued) and simulated (horses that were pursued but evaded capture) groups (Hansen and Mosley, 2000). That study did not investigate the broader effects relating to density and sex ratio changes. We could find no other studies that investigated behavior before and after population removals of feral horses. There are some comparable studies of feral horse behavior in relation to immunocontraception however.

Porcine zona pellucida is the most extensively investigated immunocontraceptive for feral horses, and recent studies have found PZP-treated females exhibit more frequent reproductive behaviors and seasonally prolonged reproductive behaviors than their control counterparts. In a comparable study of three feral horse populations, 54.5% more reproductive behaviors were received by PZP-treated females than by control animals during the breeding season (Ransom et al., 2010). Likewise, in another feral horse population, more frequent reproductive behaviors among PZP-treated females were observed in the non-breeding season (Nuñez et al., 2010). Elevated rates of estrus have been reported in PZP-treated females of other species as compared to control females, which likely explains this increase in observed reproductive behavior (Mahi-Brown et al., 1985; Shumake and Wilhelm, 1995; Heilmann et al., 1998; Curtis et al., 2002). Seasonality of estrus in horses may not be as constrained by abiotic factors as previously thought, so it is possible that any contraceptive that does not completely suppress estrus could be associated with seasonally atypical expressions of reproductive behavior (Ransom et al., 2013). Decreased band fidelity has also been reported among PZP-treated female horses (Nuñez et al., 2009; Madosky et al., 2010), and in our GnRH study the per capita female interchange rate decreased 25.7% from 2009 to 2010. Given that some social reorganization was expected following the culling perturbation, the observed decrease in interchange provides additional evidence that GnRH-vaccinated females were not expressing the frequent reproductive behavior that appears to be coincident with decreased band fidelity.

Studies of the effects of GnRH immunocontraceptive vaccines on behavior of female horses are limited; nevertheless, a few small studies do illustrate some general patterns in responsiveness. In the only other feral horse study reported in the literature, no differences were observed between treatment groups in time spent feeding, resting, or traveling in either the breeding or non-breeding season (Gray et al., 2010). Of the 25 copulations observed in that study, only one was a GnRH-vaccinated female and it occurred during the non-breeding season. Both treated and control females demonstrated reproductive behavior roughly once every 2 h in our study. Our results concur with observations of reproductive behaviors in controlled experiments where captive mares were treated with GnRH vaccine. In those studies, treated mares showed suppressed ovarian activity and ovulation after immunization but continued to show evidence of estrous behavior at irregular intervals and duration (Dalin et al., 2002; Imboden et al., 2006). Similar behavioral responses to contraception induced by GnRH vaccine and GnRH agonist have been reported in other ungulate species as well (Baker et al., 2002, 2004; Conner et al., 2007; Powers et al., 2011). The parity we observed between treatment groups therefore simply reflects the limited reproductive behavior among control females due to high rates of pregnancy and suppressed reproductive behavior among treated females due to GnRH-inhibited ovarian activity. Since GnRH is highly conserved across mammalian species, fertility control technology founded on this hormone may suppress reproduction but does not always prevent the occurrence of sexual behaviors.

Our study was limited to only a pre-treatment and treatment year, and as such inferences should only be extended to the individual level in the short term. Both pregnancy and birth rates between treatment groups in our study were the same, but variation in birth interval among individuals can make longer term trends less certain in terms of births, dependent foals, physiology, and associated behaviors. While some studies have demonstrated females may be less likely to foal in consecutive years, as opposed to alternate years, others have found no differences (Roelle et al., 2010). Probability of foaling can also be strongly related to band size (Roelle et al., 2010), which was equitable between years in our study but may trend downward with effective fertility control over long time periods. For these reasons, the short duration of our study provides some relatively clear inference toward short-term effects of GnRH and culling on individual behaviors; however, long term effects of fertility control on behavior may manifest to all individuals as a population paradigm shifts to lower fecundity, and variable and unknown effects on social structure and dynamics emerge.

5. Conclusion

Our study found only minimal differences in behavior of free-ranging female feral horses treated with GnRH

and those treated with saline. Those differences could be physiologically explained and body condition between the two treatment groups was at parity. Following the same methodological protocols as a similar study of PZP-treatment on behavior of feral horses (Ransom et al., 2010), GnRH appears to lead to fewer behavioral modifications than PZP in this species in the short term. Long term influences of GnRH remain uncertain. The ultimate assessment of any fertility control agent must include the scope and magnitude of all potential contraindications, vaccine efficacy, and weighing the known effects against the benefit of reducing population growth. The differences in how band stallions socially interacted with all females between years provided strong evidence related to the effects of culling. These behavioral responses shed light on the effects of reducing competition and density in a social polygynous species with strong group fidelity. Such perturbation may be synergistic in reducing grazing pressure by decreasing energetically expensive competitive behaviors, but further investigation is needed to explicitly test this hypothesis.

Conflict of interest

We declare no conflict of interest among financial, personal, or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, our work.

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6

Wild and Feral Equid Population Dynamics

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Wild equid populations are shaped by births, deaths, emigration, and immigration, each of which is subject to a suite of biological and ecological inputs across space and time. How these inputs influence population dynamics varies between populations as well as among species. In our rapidly changing world, human actions also increasingly affect wildlife populations (Sanderson *et al.* 2002). Fragmented landscapes, hunting of predators, reintroduction of species, invasive species, competition with humans and their livestock for resources, direct killing, and climate change all can influence population dynamics (see chap. 12). Most wild equids occupy arid or semiarid steppe, grassland, and desert environments, but some feral populations also thrive in forests, montane, and coastal environments, and across hot and cold, xeric and mesic, conditions. The array of factors potentially influencing these populations is great, but some fundamental elements influencing birth, survival, and movement are common to all.

Food can be one of the most important natural factors limiting populations of large herbivores (Fowler 1987; Gaillard *et al.* 1998, 2000; Gaidet and Gaillard 2008). As such, some populations may exhibit density dependence and be limited because of their own impact on forage resources, or because population density itself influences age-specific natality, behavior, or survival (Choquenot 1991; Gaillard *et al.* 1998, 2000; Flux 2001; Linklater *et al.* 2004; Bonenfant *et al.* 2009). When such populations lack resources, body condition of individuals can diminish and disease and parasitism can increase, all with the cumulative effect of reducing fecundity and survival, and ultimately limiting population growth (Eberhardt 1977). Some populations may persist without exhibiting density dependence, changing through time without realized self-imposed resource limitation. Those populations may still be limited by predation or climate events (Ellis and Swift 1988; Georgiadis *et al.* 2003). Over time, any given population may move between exhibiting density dependence and apparent density independence as the influencing factors change. Regardless of the specific paradigm, both deterministic and stochastic mechanisms play important roles in shaping populations (Björnstad and Grenfell 2001).

Attempting to draw broad inferences across equid population states, processes, and inputs is a challenging task, but the wild and feral members of family Equidae are perhaps more similar than not in their equation of life on earth. Some species, notably African wild ass (*Equus africanus*) and kiang (*E. kiang*), are vastly understudied, and considerable gaps persist in our knowledge of even their basic ecology. Other species, such as the feral horse (*E. caballus*), provide rich data sets that can inform us with in-depth ecological perspectives. In this chapter, we have synthesized data from studies on equids across the globe toward understanding the common and diverging natural influences acting to shape their populations. The populations in these studies ranged on 6 continents across >91 degrees of latitude, from savannah to mountains and deserts to islands, and thus were influenced by a diversity of biotic and abiotic factors. We focus here on natality, survival, and population growth. We briefly discuss movement into and out of populations, but largely defer that topic to chapter 13. The influence of humans on these populations is a broad topic that can profoundly affect survival, and we must defer that topic to chapter 12.

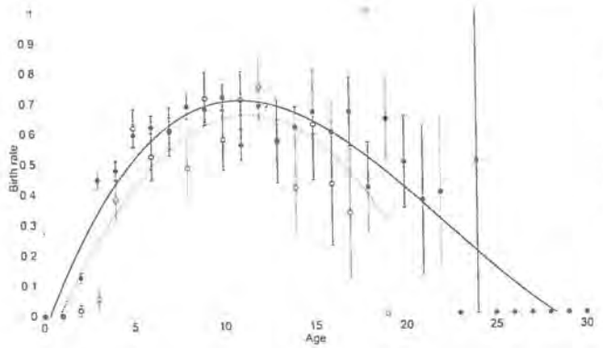


Fig. 6.1 Mean age-specific birth rates of horses ± standard error, as derived from *E. caballus* (feral) in the western United States (solid dots and solid trend line, n = 1,013 births, excluding females that received fertility control treatments) (Ransom *et al.* 2011; J. Ransom, unpublished data), and *E. f. przewalskii* (open dots and dashed trend line, n = 217 births, excluding females in the year of their introduction) (Great Gobi B Strictly Protected Area, Mongolia, unpublished data).

Natality

All species of wild equid appear to follow the same general distribution of age-specific natality that is observed in horses, with primiparous births most typically occurring from females age 2–5 years, and peak birth rates occurring roughly for females age 6–17 (fig. 6.1). There is some evidence that when forage is abundant, female equids are more likely to give birth at an earlier age (Berger 1986; Saltz and Rubenstein 1995; Tatin *et al.* 2009). When forage and water becomes exhausted, age of primiparous birth can increase and birth rates for all females can decline quickly (Ginsberg 1989). The majority of feral asses (*E. asinus*) in Death Valley, USA, for example, were observed giving primiparous birth at age ≥4 years while persisting in an extremely harsh environment (Moehlman 1974). (Note that sexual maturity is reached at 1.5 years; Grinder *et al.* 2006.) In the wild, female plains zebra (*E. quagga*) have been documented giving birth as old as age 18 (Klingel 1969), mountain zebra (*E. zebra*) at age >21 (Joubert 1974*a*), Przewalski's horses (*E. ferus przewalskii*) at age 20 (O. Ganbaatar, unpublished data), and feral horses as old as age 24 (fig. 6.1).

Horses and zebras typically give birth to one offspring annually, though extraordinarily rare cases of twins have been reported (Roelle *et al.* 2010; Vodička 2010). Feral asses have been observed giving birth to either one or two offspring (Grinder *et al.* 2006), as have Asiatic wild asses (*E. hemionus*) (Schook *et al.* 2013). The cases of twins born to Asiatic wild asses all resulted in early mortality of at least one sibling, and it is unknown how frequently twin births occur in the wild. Wild females of all asses are most typically observed with only one offspring of the year. The mean birth rate for equid females ≥2 years old across all species and studies we reviewed ranged from 0.20 in mountain zebras to 0.68 in feral asses, with a mean across species of 0.47 (95% confidence interval, or CI = 0.35–0.59) (table 6.1). Note that the lowest value from individual studies in this range arose from Przewalski's horses in the Gobi Desert following a catastrophic winter climate event (birth rate = 0.04) (Kaczensky *et al.* 2011), and the maximum value occurred in a small Asiatic wild ass (onager) population that started to increase rapidly six years after reintroduction (birth rate = 1.00) (Saltz and Rubenstein 1995). Despite these extremes, however, birth rates among wild equid species are remarkably similar when put in context of the pressures selecting for fitness.

Birth rates in wild populations reflect offspring born and observed, rather than pregnancy rate, which is presumably higher in most cases. Pregnancy rates



Table 6.1. Mean birth, survival, and population growth rates of wild and feral equids

Species	Populations	Mean Adult Birth Rate	Mean Foal Survival Rate	Mean Adult Survival Rate	Mean Population Growth Rate (λ)
E. caballus (feral)[1]	Tornquist Park, Argentina; Garden Station, Australia; Alberta, Canada; Sable Island, Canada; La Camargue, France; Namib Desert, Namibia; Kaimanawa Ranges, New Zealand; Peneda-Gerês, Portugal; Vodnyi Island, Russia; Galicia, Spain; Exmoor, UK; New Forest, UK; Snowdonia National Park, UK; Assateague Island, USA; Garfield Flat, USA; Granite Range, USA; Great Basin, USA; Jicarilla Wild Horse Territory, USA; Little Book Cliffs, USA; McCullough Peaks, USA; Montgomery Pass, USA; Pine Nut and Pah Rah, USA; Pryor Mountain, USA; Red Desert, USA; Salmon, USA; multiple locations across western USA	0.56 ± 0.001 (0.23–0.92)	0.84 ± 0.002 (0.09–0.97)	0.90 ± 0.001 (0.79–0.98)	1.18 ± 0.001 (0.84–1.39)
E. f. przewalskii[2]	Xinjiang, China; Le Villaret, France; Great Gobi B, Mongolia; Hustai National Park, Mongolia	0.36 ± 0.011 (0.27–0.75)	0.61 ± 0.003 (0.59–0.83)	0.86 ± 0.003 (0.83–0.98)	1.13 ± 0.001 (1.12–1.18)
E. asinus (feral)[3]	Dorisvale, Australia; Victoria River, Australia; Bandelier National Monument, USA; Death Valley, USA; Grand Canyon, USA; Mojave Desert, USA	0.68 ± 0.003 (0.41–0.89)	—	—	1.19 (—)
E. africanus[4]	Denkelia Desert, Eritrea	0.30 ± 0.066 (0.00–0.71)	—	—	—
E. hemionus[5]	Qatrouyeh National Park, Iran, Makhtesh Ramon, Israel; Great Gobi B, Mongolia; Badghyz Nature Reserve, Turkmenistan	0.48 ± 0.004 (0.15–0.76)	0.57 ± 0.001 (0.40–0.64)	0.86 (0.85–0.86)	1.04 ± 0.001 (0.95–1.15)
E. kiang[6]	Tibetan Plateau	0.50 (—)	—	—	—
E. zebra[7]	Ai-Ais / Fish River Canyon National Park, Namibia; BüllsPort Guest Farm, Namibia; Gondwana Cañon Park, Namibia; NamibRand Nature Reserve, Namibia; Camdeboo National Park, South Africa; De Hoop Nature Reserve, South Africa; Karoo National Park, South Africa; Mountain Zebra National Park, South Africa	0.20 ± 0.002 (0.16–0.45)	0.98 ± 0.001 (0.87–0.98)	0.92 ± 0.001 (0.91–0.93)	1.13 ± 0.002 (0.85–1.23)
E. grevyi[8]	Barsalinga, Kenya; Dvur Kralove Zoological Garden (translocated from Kenya); Laikipia, Kenya; Lewa, Kenya; Ngare Ndare, Kenya; Samburu Buffalo Springs Game Reserve, Kenya	0.64 (—)	0.57 ± 0.084 (0.27–0.90)	0.85 ± 0.002 (0.77–0.88)	1.02 —

Table 6.1. continued

Species	Populations	Mean Adult Birth Rate	Mean Foal Survival Rate	Mean Adult Survival Rate	Mean Population Growth Rate (λ)
E. quagga[9]	Laikipia, Kenya; Athi-Kapiti Plains, Kenya; Serengeti, Tanzania; Ngorongoro, Tanzania; Hwange National Park, Zimbabwe; iMfolozi-Hluhluwe, South Africa; multiple private reserves, South Africa; Kruger National Park, South Africa; Boland Lanbou, South Africa; Elandsberg Private Reserve, South Africa	0.48 ± 0.056 (0.23–0.75)	0.67 ± 0.120 (0.13–0.96)	0.84 ± 0.025 (0.79–0.88)	0.98 ± 0.028 (0.92–1.01)

Sources:

[1]Pellegrini 1971, Tyler 1972, Feist and McCullough 1975, Nelson 1978, Perkins *et al.* 1979, Boyd 1980, Wolfe 1980, Eberhardt *et al.* 1982, Salter and Hudson 1982, Seal and Plotka 1983, Keiper and Houpt 1984, Berger 1986 Sniff *et al.* 1986, Wolfe *et al.* 1989, Garrott and Taylor 1990, Berman 1991, Turner *et al.* 1992, Baker 1993, Monard *et al.* 1997, Gregor and Romney 1999, Ashley 2000, Gomes and Oom 2000, Goodloe *et al.* 2000, Ashley and Holcombe 2001, Turner and Morrison 2001, Linklater *et al.* 2004, Greyling 2005, Grange *et al.* 2009, Scorolli and Lopez Cazorla 2010, Contasti *et al.* 2012, Dawson and Hone 2012, Ransom 2012, Spasskaya *et al.* 2012, Stanley and Shultz 2012, Lagos 2013.

[2]Chen *et al.* 2008, Tatin *et al.* 2009, Kaczensky *et al.* 2011, Usukhjargal and Bandi 2013.

[3]Moehlman 1974, Norment and Douglas 1977, Mogart 1978, White 1980, Ruffner and Carothers 1982, Johnson *et al.* 1987, Perryman and Muchlinski 1987, Choquenot 1990, 1991.

[4]P. Moehlman, unpublished data.

[5]Solomatin 1973, 1977, Wolfe 1979, Zhirnov and Ilyinsky 1986, Saltz and Rubenstein 1995, Feh *et al.* 2001, Ziaee 2008, Nowzari 2011, Hemami and Momeni 2013, Lkhagvasuren *et al.* in review.

[6]Schaller 1998.

[7]Joubert 1971, Penzhorn 1985, Lloyd and Rasa 1989, Novelle *et al.* 1992, 1996, Smith *et al.* 2007, Gosling 2013, Eastern Cape Parks, unpublished data, H. Hrabar, unpublished data, South African National Parks, unpublished data.

[8]Dobroruka *et al.* 1987, Rubenstein 1989, 2010, Rowen 1992, Williams 1998.

[9]Klingel 1965, 1969, Petersen and Casebeer 1972, Spinage 1972, Smuts 1976*a*,*b*, Ohsawa 1982, Rubenstein 1989, Hack *et al.* 2002, Georgiadis *et al.* 2003, Grange *et al.* 2004, Barnier *et al.* 2012, Grange *et al.* 2012, 2015, H. Hrabar and G. Kerley, unpublished data.

Note: Table includes some semiwild populations in managed reserves. Mean rates from each source study were weighted by sample size (number of individuals sampled) and length of study (years) to generate the mean values shown for each species ± standard error. The range of individual study means is given in parentheses after the point estimate. Birth rate is total number of foals divided by all females at least 2 years of age, and survival rate for adults represents individuals ≥2 years of age. Foal survival represents individuals surviving from birth to age 1.

have not been rigorously measured across equid species in the wild, but the feral horses of Kaimanawa, New Zealand, exhibited pregnancy rates of 0.79–0.94, with an average birth rate observed at only 0.49 (Linklater *et al.* 2004). Similarly, feral horses in Idaho, USA, exhibited an average pregnancy rate (across all ages) of 0.68, with only 8% of 1- to 3-year-olds becoming pregnant, increasing to 93% of 10- to 12-year-olds becoming pregnant (Seal and Plotka 1983). Plains zebra pregnancy rates in the Serengeti were reported ranging from 0.69 in 3- to 5-year-olds to 0.88 in 13- to 16-year-olds (Grange *et al.* 2004), but birth rates in similar systems of east Africa were reported as only 0.26–0.51 (Klingel 1969; Georgiadis *et al.* 2003). It is possible that in many studies the reported birth rates may be negatively biased because offspring aborted, stillborn, or depredated immediately after birth were undetected by observers. Abortion is extraordinarily unlikely after three months of gestation in horses, which implies that the environmental conditions early in pregnancy are perhaps most influential in the successful birth of a foal (Roberts 1986).

Interbirth interval (the time between subsequent offspring) among equids can vary depending on individual-level reproductive history and resource availability. Primiparous feral horses, for example, were less likely to foal in the year following their first foal, whereas multiparous females were more likely to foal if they had foaled the previous year (Roelle *et al.* 2010). The interbirth interval among plains zebra, on the other hand, appeared to be longer if the first foal was male (Barnier *et al.* 2012). Feral horses also demonstrated a protracted interbirth interval after producing a male foal under stressed resource conditions (Monard *et al.* 1997).

Both strong physiological and environmental inputs influence the rate and phenology of conception

and subsequent year births in most wild equid populations. Hormones associated with reproduction (such as estradiol, progesterone, and testosterone) fluctuate cyclically through time in both males and females (Kirkpatrick *et al.* 1977; Nuñez *et al.* 2011; Schook *et al.* 2013), reaching an annual apex of reproductive potential when enough sunlight and warm temperatures have stimulated the hormonal cascade (Ransom *et al.* 2013). This mechanism can help regulate birth cycles, and ultimately produces birth pulses that are often synchronized with forage availability. Estrus, birth rates, and birth phenology have been correlated with rainfall in some populations of zebras (Klingel 1969; Joubert 1974*b*; Williams 1998; Sinclair *et al.* 2000; Rubenstein 2010), feral horses (Siniff *et al.* 1986; Berman 1991; Scorolli and Lopez Cazorla 2010; Ransom *et al.* 2013), and Asiatic wild asses (Saltz *et al.* 2006). This synchrony with forage resources may increase female and neonate survival, especially because nutritional requirements of lactating females are elevated and higher-quality forage can result in increased body condition and nutritional support for neonates (Ginsberg 1989; National Research Council 2007). In a study of three temperate populations of feral horses where birth phenology was perturbed, estimated survival of foals decreased 1.4% for every 10 days after peak forage that parturition occurred (Ransom *et al.* 2013).

Environmental pressures can contribute greatly to birth phenology, and in some cases can truncate the birth season to a short, synchronized pulse. Predation, for example, has been posited to influence the timing of births under the premise that neonates are most vulnerable in their first hours or days of life, and as such predators can only harvest a finite number of young in a short period of time; consequently, if all young are born together, more should survive (Darling 1938; Sinclair *et al.* 2000). Parturition season for Asiatic wild asses in the Mongolian Gobi (where wolves prey on them), for example, typically begins mid-June and concludes around mid-July (P. Kaczensky, personal communication), and similarly the same species in Badghyz State Reserve, Turkmenistan (13° latitude farther south), was reported to foal primarily between 14 April and 14 May, but some foals were recorded as early as late as 28 July (Wolfe 1979). In contrast, a Sri Lanka feral ass population located almost on the equator and with no predators present exhibited no birth seasonality, and offspring were born uniformly across every month of the year (Santiapillai *et al.* 1999). Predation pressure may not be the primary driver of length of birthing season, however; length of gestation, birth interval, and duration of resource availability can all be important factors (Williams 1998). Wildebeest (*Connochaetes taurinus*) sympatric with Grevy's zebra (*E. grevyi*) in Kenya, for example, exhibited a birth season constrained to only two weeks, whereas the Grevy's zebra present in the same environmental conditions gave birth over the course of months (Williams 1998). In that system, rainfall was highly stochastic, and thus seasonal forage availability did not exhibit a pronounced peak.

Gestation period is similar among equid species, though environmental inputs can create plasticity (Howell and Rollins 1951). Average gestation length ranges from 327 to 357 days in horses (Howell and Rollins 1951; Card and Hillman 1993; Monfort *et al.* 1994), 321 to 404 days in asses (Grinder *et al.* 2006; Pagan *et al.* 2009; Schook *et al.* 2013), and 364 to 390 days in zebras (Grubb 1981; Penzhorn 1988; Churcher 1993). Gestation period in kiang is the least quantified, with reports ranging from 210 to 365 days (St-Louis and Côté 2009). Given the nearly yearlong gestation across all equid species, we could expect estrus, reproductive behavior, and conception to peak between the spring equinox and the summer solstice, waning thereafter with subsequent parturition occurring roughly at the same time the following year. Where such empirical data were present in the literature, this pattern was evident across equid species, though it is more variable in populations near the equator (fig. 6.2).

While body condition, resource availability, and biological inputs all contribute to conception and birth success, these factors may also influence sex ratio of young born into the population. Female feral horses in poor body condition at the time of conception have been observed producing more female foals (Cameron *et al.* 1999), for example, aligning with the much-debated Trivers-Willard hypothesis, which posits that when populations are experiencing more stress (and thus individuals have decreased body condition), more female offspring will be produced because they can increase fitness more quickly than inputs of male offspring (Trivers and Willard 1973). Sex ratio of foals across all equid species and populations we reviewed is of course quite variable, precisely because of these environmental inputs, but on average we found that the ratio of males to females born did not differ from parity (1.1 males to 1 female; 95% CI = 0.93–1.29:1). The extreme situations were 2.2 males to 1 female in a population of mountain zebra (n = 16 foals; H. Hrabar, unpublished data), and 2.2 males to 1 female in an Asiatic wild ass population that was experiencing a high growth rate (n = 53 foals; Saltz and Rubenstein 1995). In the latter case, it is interesting that while more males were being produced in a density-independent environment

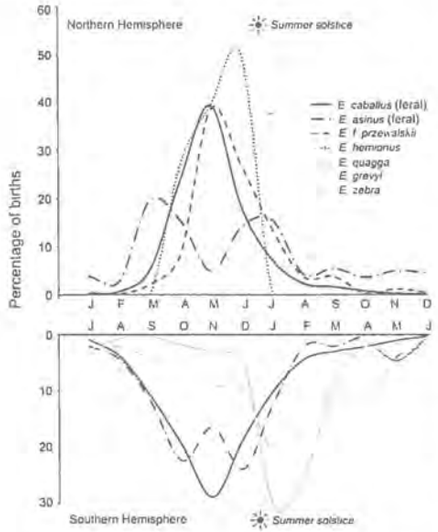


Fig 6.2 Percentage of births by month across equid species. Data are smoothed and shown as the mean across populations. Data for *E. caballus* derived from Tyler 1972, Boyd 1980, Keiper and Houpt 1984, Berger 1986, Berman 1991, Lucas *et al.* 1991, Ashley 2000, Gomes and Oom 2000, Linklater *et al.* 2004, Greyling 2005, Nuñez *et al.* 2010, Scorolli and Lopez Cazorla 2010, Lagos 2013, Ransom *et al.* 2013, and N. Spasskaya, unpublished data; data for *E. asinus* derived from Moehlman 1974, McCool *et al.* 1981, Ruffner and Carothers 1982, and Santiapillai *et al.* 1999; data for *E. f. przewalskii* derived from Chen *et al.* 2008, Prague Zoo 2010 (wild and semiwild populations only), and Usukhjargal and Bandi 2013; data for *E. hemionus* derived from Wolfe 1979 and O. Ganbaatar, unpublished data; data for *E. quagga* derived from Klingel 1965, Smuts 1976*b*, and Sinclair *et al.* 2000, data for *E. grevyi* derived from Dobroruka *et al.* 1987 and Rowen 1992, data for *E. zebra* derived from Joubert 1974*b*, Penzhorn 1985, Westlin-van Aarde *et al.* 1988, and H. Hrabar, unpublished data.

(in concurrence with Cameron and Linklater 2007), the investigators noted that more female foals were being produced by primiparous mothers of age 2 or 3 years as compared to multiparous mothers of age 4 or 5 years. This may reflect an evolutionary disparity in effective maternal investment, but that hypothesis was not specifically tested. The opposite extreme was observed in a resource-stressed population of plains zebra where the sex ratio was 0.27 males to 1 female (n = 19 foals; Hrabar and Kerley 2013) and a feral horse population where the sex ratio of 0.47 males to 1 female was observed (n = 25 foals; Stanley and Shultz 2012).

Survival

Most feral horses, feral asses, and some Przewalski's horses and mountain zebra live in environments without significant predation pressure and exhibit relatively high survival rates across all age classes. Most other wild equid populations are subject to predation from a variety of large predators, and thus experience locally reduced survival rates. Despite this dichotomy of population paradigms, age-specific survival is surprisingly comparable among equid species, with some elevated mortality in foals, relative security in the adult cohort, and then a gradual decrease as senescence prevails (fig. 6.3). The resulting age structure of wild populations is also relatively consistent among species and populations, with foals typically comprising 8–15% of a population, juveniles 13–28% of a population, and adults 71–78% of a population (Berman 1991; Tatin *et al.* 2003; Grange *et al.* 2004; Smith *et al.* 2007). Equids are long-lived animals, and where individuals were followed from birth, known ages for individual wild Przewalski's horses exceeded 25 years old (Prague Zoo 2010) and for feral horses exceeded 29 years old (Ransom 2012). Equids generally live longer in captivity owing to more controlled environmental conditions and applied veterinary care; as such, captive Asiatic wild asses >27 years old have been reported (Volf 2010), as have Przewalski's horses >30 years old (Ballou 1994). Mortalities in wild equid populations arise from a variety of causes. Deaths of adult Przewalski's horses (n = 145) at Hustai National Park, Mongolia, for example, were attributed to blood parasites (2.1%), endoparasites (1.4%), disease (7.6%), stillbirth (9.7%), injury (12.4%), predation (22.1%), starvation (31.1%), and other/unknown reasons (13.8%) (Usukhjargal and Bandi 2013). Nine percent of foal deaths (25 of 278 foals) in the same study were attributed to infanticide by stallions (box 6.1).

Annual foal survival rates reported across the studies we reviewed averaged 0.71 (95% CI = 0.53–0.88), and adult survival rates averaged 0.88 (95% CI = 0.85–0.90) (table 6.1). Much of the variation in annual survival rates of both foals and adults by population may be attributed to localized predation. A large number of predators have been documented killing both young and adult equids, including African lion (*Panthera leo*), leopard (*P. pardus*), cheetah (*Acinonyx jubatus*), and spotted hyena (*Crocuta crocuta*) for feral horses, zebras, and asses in Africa; wolf (*Canis lupus*) and snow leopard (*Uncia uncia*) for Asiatic asses, Przewalski's horses, and kiang in the Middle East and Asia; wolf and brown bear (*Ursus arctos*) for feral horses in Europe; and puma

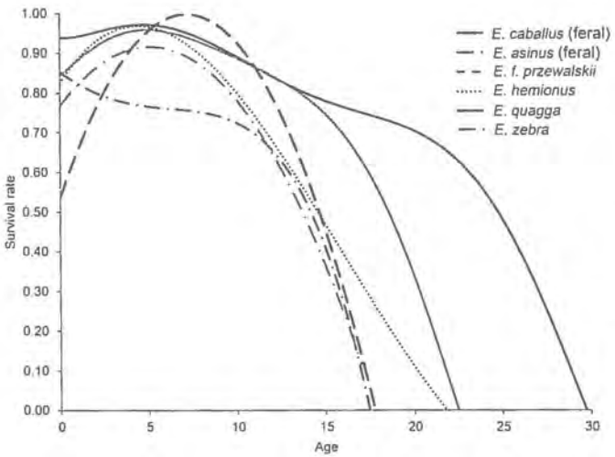


Fig. 6.3 Trend in age-specific survival rates for equid species. Data for *E. caballus* derived from Ransom 2012; data for *E. asinus* derived from McCool *et al.* 1981; data for *E. f. przewalskii* derived from Hustai National Park, unpublished data; data for *E. hemionus* derived from Saltz and Rubenstein 1995 and Lkhagvasuren *et al.* in review; data for *E. quagga* derived from Spinage 1972; data for *E. zebra* derived from Joubert 1974*a*

(*Puma concolor*) and jaguar (*P. onca*) for feral horses and feral asses in the Americas. In one feral horse population in the western United States, foal survival was reported to be only 27%, and 82% of foal carcasses found showed evidence of being killed by puma (Turner *et al.* 1992); however, most feral horse populations coexist with pumas and experience limited depredation. In the Gobi Desert of Mongolia, 23% of wolf scats analyzed contained remains of Asiatic ass (Feh *et al.* 2001), but it is unknown how many of those samples arose from predation as opposed to scavenging.

Analysis of predator diets has provided some evidence of selection for equids, with a wide range of selection to nonselection across species and seasons. Wolves in northwest Spain clearly selected for feral horses (Ivlev index = 0.99–1.00) (Lagos 2013), but a similarly pack-organized canine, African wild dog (*Lycaon pictus*), specifically avoided hunting zebra even when they were relatively abundant (Hayward *et al.* 2006). In Lewa, Kenya, African lions strongly selected Grevy's zebra over plains zebra, even though plains zebra outnumbered Grevy's zebra four to one (Rubenstein 2010). In the Serengeti, Tanzania, 90% of all spotted hyena kills were adult zebra when the available ungulate assemblage was quite diverse (Kruuk 1972). In systems where feral horses and predators most typically coexist, a high percentage of all mortality is attributed to a sole predator as opposed to disease, accident, or other natural causes. Wolves in Peneda-Gerês National Park, Portugal, for example, have been responsible for 89% of horse mortalities, and in nearby Galicia, Spain, that figure rises to 96% (Gomes and Oom 2000; Lagos 2013). In systems with multiple predators or sympatric prey species, the percentage of equid mortality attributed to depredation can be somewhat ameliorated, such as plains zebras in the Serengeti, Tanzania, where 59–74% of all mortality was attributed to African lion and spotted hyena (Schaller 1972), or Przewalski's horses in Hustai National Park, where 13–50% of horse mortalities were attributed to wolves (Van Duyne *et al.* 2009).

At a finer scale, predator species may also select for one sex of prey over the other; thus they may affect prey populations differently at long timescales. Spotted hyenas at Ngorongoro, Tanzania, killed 2.25 female plains zebra for every one male, but lions at Serengeti, Tanzania, killed only 0.83 female plains zebra for every one male (Kruuk 1972). Longitudinal studies indicate that shifts in diet can also occur over time, as in the Serengeti, Tanzania, where 19–20% of spotted hyena diet was composed of plains zebra before the 1970s, but after that time it diminished to <9% (Kruuk 1972). The zebra population remained relatively stable over that time period (Hack *et al.* 2002), but changes in other prey populations or competing predator populations could have contributed to the apparent dietary shift.

Some behavioral traits may also influence survival. Group formation in ungulates has been posited to be a function of open environments where foraging can be optimized because groups of animals are more vigilant for predators than isolated individuals can be (Kie 1999). Equids form groups of various sizes and composition, which may influence predation risk (see chap. 2). Schaller (1972) reported that lions were 13% more successful when plains zebras were alone rather than

BOX 6.1 INFANTICIDE AMONG EQUIDS

Infanticide is not common in equids but has been reported in both captive and wild populations. This unique source of mortality arises when a stallion intentionally kills a foal, an action hypothesized to be a fitness-driven evolutionary behavior (Hrdy 1979). The concept posits that if a novel stallion kills an unrelated foal, then the foal's mother may more quickly return to estrus and be fertilized by the assailant male, thus increasing his fitness faster through reduction of interbirth interval. This concept has been discussed most broadly in the classic case of the African lion (Packer and Pusy 1982), but it has drawn the attention of equine ethologists because of their polyandrous mating systems.

Infanticide among the equids appears to be most prevalent in the harem-forming species and has been reported most frequently in captive conditions where artificial social constructs may elevate frequency (Pluháček *et al.* 2006). In the wild, infanticide has been documented at the highest rates in Przewalski's horses, where 9% (25 of 278 foals born 1993–2011) of all foal mortality in Hustai National Park, Mongolia, was attributed to this phenomenon (Usukhjargal and Bandi 2013). It was also reported in the same species at Kalamaili Ungulate Protected Area, China, where 83.3% of all foal mortalities (7 mortalities out of 24 births) was attributed to infanticide in the 5-year period following reintroduction (Chen *et al.* 2008). Nearly half of those deaths occurred in the first year, lending support to the proximate social stresses presented when novel stallions suddenly become available to social groups.

Infanticide was reported in domestic horses (Duncan 1982) and New Forest ponies (Tyler 1972), and attempted infanticide among feral horses has been documented across populations (Tyler 1972; Berger 1986; Gray 2009). When more than one adult male has been present in a band of feral horses, extraordinary maternal protection has also been reported, potentially minimizing the incidents of infanticide (Cameron *et al.* 2003). While the sample size is limited, there is some evidence that intruding stallions may selectively target male foals (all cases reported by Duncan 1982, with additional cases reported by Berger 1986), which could reflect the potential for reducing future competition from unrelated males, but that hypothesis has never been explicitly tested. Induced abortion has also been documented when novel stallions forcibly copulate with a pregnant female. At the Granite Range, USA, such feticide was reported for 78% of forced copulations by novel feral horse males (Berger 1986).

Harem-forming zebra have also demonstrated infanticide, with three cases being reported in wild mountain zebra (Penzhorn 1975), and multiple cases reported in captive plains zebra (of which several individuals originated from wild populations) (Pluháček *et al.* 2006). In a captive mountain zebra enclosure in Namibia, Joubert (1971) reported an attempted infanticide that failed when the female actively defended her foal, but the stallion then proceeded to kill two sympatric antelope instead. This event illustrates the uncertainties underlying our understanding of mechanisms and motivations related to infanticide (Pluháček *et al.* 2006). It is possible that some cases of infanticide are motivated by a more immediate need: survival. At Hluhluwe Reserve, South Africa, a severely injured plains zebra foal was observed in the center of a band vocalizing continuously and loudly after escaping from a predator attack. All adult members of the band were expressing elevated vigilance behaviors and nervousness, and the band stallion eventually approached and killed the foal with strikes to the head. While the genetic relationship of the stallion and foal was unknown, the silencing of the foal instantly reduced the exposure of the band to local predators (J. Ransom, unpublished data). Infanticide has not been reported for Grevy's zebra, but the territorial organization of this species can result in females not remaining with specific males long enough for the males to benefit from such behavior.



Photo by J. Ransom

in a group. Unlike most equids, where foals closely accompany their dams most or all of the time, Grevy's zebra sometimes leave their foals in aggregated juvenile groups or "kindergartens" (Williams 1998). The source of this behavior may arise from energetic constraints of young, but ultimately it may also place foals at risk of depredation, especially during nocturnal aggregations (Klingel 1974).

Because most equids inhabit arid environments, considerable interannual variance in precipitation and related productivity may also influence survival (Archer *et al.* 1999). One model simulation of zebra populations suggested that survival decreases more rapidly during dry seasons than reproduction increases during wet seasons, which would constrain population growth over time (Georgiadis *et al.* 2003). In contrast, neither wet season nor dry season rainfall at Kruger National Park, South Africa, influenced plains zebra survival in a longitudinal empirical study, though it did affect other ungulates in the system (Owen-Smith *et al.* 2005). Across ungulate species, it appears that interannual variance in precipitation influences juvenile survival more profoundly than it does adult survival (Gaillard *et al.* 1998, 2000). In feral horses, each centimeter of spring rainfall increased foal survival by 1.5%, did not measurably influence survival for animals up to age 19, and then significantly increased survival rates for animals ≥20 years old (Ransom 2012).

Population Growth

Quantifying population dynamics is generally not as simple as adding births and subtracting deaths. Understanding population growth and decline is further complicated by movement into and out of populations, and by catastrophic stochastic events that change rates both instantaneously and longitudinally. In addition to that volume of uncertainty, the size of most wild equid populations is not absolutely known or even estimated robustly (Moehlman 2002). We have reviewed studies where some measure of population growth was reported or could be reasonably derived. To discuss this more clearly, we refer to the theta-logistic model because it accommodates the nonlinear density dependence more indicative of equid populations (Gilpin and Ayala 1973):

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right)^{\theta}$$

In this model, r is the intrinsic rate of increase, N is the population size, K is the carrying capacity, t is time, and θ is the nonlinear modifying parameter for the ratio of

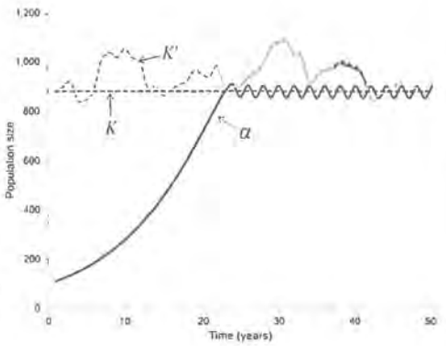


Fig. 6.4 An example of theta-logistic population growth for a simulated equid population over time (black line), where growth rate (r) is fixed and reaches the fixed carrying capacity of K. The same population is simulated with a randomized carrying capacity (K') in gray, illustrating the stochastic effects of resource availability on a single parameter. Point α represents the inflection point where population growth begins to moderate as the population approaches K.

N:K. Conceptually, when $\theta < 1$, r decreases rapidly with population size; likewise, the population will quickly approach K when $\theta > 1$. For equids, this is a steeper curve than the classic logistic sigmoid curve, and the inflection point is much closer to K (fig. 6.4). In this example, a simplified deterministic population trajectory is used to illustrate growth of hypothetical equid population. To add a degree of reality, K is randomized (K') to illustrate how carrying capacity can vary annually with the amount and quality of resources available over time, which in turn can influence the maximum number of individuals that could survive at a given time (fig. 6.4, gray lines). Conceptually, when rapid growth is reported for a population, the theta-logistic model suggests that the population is most likely below α (the inflection point) on the sigmoid curve, and when slow growth is observed, the population might be closer to K at that point in time. Many studies report an instantaneous rate of increase (λ) rather than r. This simply reflects the growth rate of a population from one year to the next as if it had instantly occurred, or $\lambda_t = (N_t / N_{t-1})$ where t represents one year. For synthesis and discussion purposes, we have converted r values from individual studies to λ, using $\lambda = e^r$.

Mean annual population growth (λ) in the studies we reviewed averaged 1.12 (95% CI = 1.04–1.19) across all species (table 6.1). While the reported range of growth rates was similar between species, the average

growth rates for Asiatic wild ass, Grevy's zebra, and plains zebra were notably lower than the other species (1.04, 1.02, and 0.98, respectively). It is unknown whether this finding simply reflects the paucity of data for these species, is an artifact of their different life history strategies, or is indicative of a more complex set of factors. Robust population dynamics metrics for African wild ass and kiang are completely missing from the literature, illustrating critical science needs for better understanding equid ecology.

Some variability in population growth rates over time and between studies and species may be explained by the relationship between abundance and resources. Equids are generally well equipped to persist near K because their longevity and high survival rates, low recruitment rates, and low sensitivity to moderate environmental stressors result in relatively durable populations, barring catastrophic mortality events (Saltz 2002). When mass mortality events occur, equids are quite slow to recover because of their inherently low recruitment rates, but at the longitudinal scale, this is when equids should be most productive. For example, a small, reintroduced Asiatic wild ass (onager) population exhibited its maximum growth rate as abundance expanded to the resources available (Saltz and Rubenstein 1995). In feral horses, heavily managed populations that have persisted near K but then experience a large culling event can be suddenly reduced far below α, prompting a compensatory response in increased growth rate (Choquenot 1990; National Research Council 2013).

Focused predation can impart nonrandom influence on population growth, such as at Pryor Mountain, USA, where predation from specific pumas was increasingly limiting the horse population from 1993 to 2004 to a nadir of $\lambda = 0.97$ before the pumas were killed and the predation pressure was dramatically released (Ransom 2012). In a plains zebra population in South Africa, models of population growth including lion predation were far more supported than models of climate factors, though population growth of only one of the two populations was affected by number of lions present (Grange *et al.* 2012). While food, water, and predation have been widely investigated as agents of population constraint, behavior has also been posited to limit equid populations by reduced fecundity arising from social stress (Linklater 2000; Tatin *et al.* 2009).

Perhaps the most unpredictable natural influence on population growth of equids also carries the greatest magnitude of effect: catastrophic climate events such as extreme drought and storms. Such events can have lasting influence on population dynamics and in some cases can threaten extirpation of populations. The dzud winter (extreme cold with heavy snow) of 2009/2010 in the Mongolian Gobi, for example, suddenly reduced the Przewalski's horse population by 60% in a single season (Kaczensky *et al.* 2011). The population effect from direct mortality was compounded by a severe reduction in per capita births the following year, with only one female foaling. Though not well quantified, the extirpation of Asiatic wild asses from Kazakhstan at the end of the nineteenth century was documented following a similar dzud winter, though hunting by humans and competition with their livestock certainly contributed to the decline (Zhirnov and Ilyinsky 1986). Winter mass mortalities of feral horses in the American West have been documented with a 51% decline in a single winter at Pryor Mountain (Garrott and Taylor 1990) and 12% in a winter at the Granite Range (Berger 1983). The latter study indicated that severe snowstorm mortality might be more common than assumed and also referenced an earlier unpublished event in the Granite Range when 300 horses died (roughly 50% of the population).

Despite the extraordinary capacity of equids to persist in harsh arid environments, drought can still promulgate catastrophe. One severe mass mortality event related to drought was reported for plains zebra at the Tuli Game Reserve in southern Africa in 1983, where 199 carcasses and only 58 live zebra were detected following two years of drought (Walker *et al.* 1987). Hundreds of mountain zebra were reported dead in the Namibian Khomas Hochland during the 1968/1969 drought, with mortalities attributed to starvation, entrapment in mud at waterholes, and behavioral disorientation leading to increased human depredation (Joubert 1971). Large-scale drought-related mortalities are relatively common in the Australian outback, where tens of thousands of feral horses perish during extended drought. Similar events are generally precluded in the United States only by artificial water supplementation.

Severe thunderstorms have also had population-level effects on small equid populations. For example, a thunderstorm overtook Tornquist Provincial Park in Argentina, killing 193 feral horses in 48 hours (28% of the population) (Scorolli *et al.* 2006). Likewise, electrical storms at the high-altitude area of Pryor Mountain have produced fatal lightning strikes to individuals and entire feral horse bands, with an event in 1999 killing 4% of the population with a single lightning strike (US Bureau of Land Management, unpublished data).

Little has been quantified regarding movement of individuals into and out of equid populations in the



context of population growth rate. But one only need consider the migrant case of the plains zebra to understand the ramifications of how migration can quickly influence access to resources and changes in body condition that may influence birth rates, or how such migrations attract predators that might influence survival rates (Naidoo *et al.* 2014). Localized movements of plains zebra in Kruger National Park, South Africa, required travel of 10–15 km every 1–2 days during the dry season for individuals to drink, thus reducing time available for foraging and resting (Cain *et al.* 2012). Feral horses in central Australia also traveled up to 15 km during drought in order to access water, and one population declined 51% in density during a severe drought; it is unknown how many of those animals died as opposed to emigrated from the area (Berman 1991). In a similar xeric environment, feral asses of Death Valley became more prevalent within 1.6 km of a water source in summer (Moehlman 1974). Anthropogenic disturbance at water holes has been implicated in movement of Grevy's zebra, with the energetic costs arising from long-distance movement to and from water being reflected in reduced foal survival (Williams 1998). Whether any of these traveling individuals truly moved from one population to another, affecting population demography, is unknown, but the catalysts for such movement clearly provide an opportunity for immigration and emigration, which ultimately can influence both population dynamics and genetic flow.

Conclusion

The wild and feral equid populations of the world share many common features, and they function similarly for a variety of reasons. Equids can be quite long-lived ungulates and demonstrate relatively low fecundity, meaning that, in the absence of extreme stressors, their populations should be rather stable over time. Unfortunately, this is seldom realized in a changing world where anthropogenic influence permeates nearly all populations (see chap. 12) and climate events can impart severe catastrophes. Recovering from such population decimations requires long timescales for equids. Ultimately, most wild equids persist in a state where extensive conservation measures are needed to prevent extirpation and most feral equids persist at the other end of the curve, where extensive management is necessary to mitigate resource damage.

We aimed in this synthesis to gain a better understanding of equid ecology and what naturally shapes their populations, but such findings are not functionally separate from the effects that humans have on these populations. Direct killing, encroachment, and resource competition remain the largest threats to wild equids and profoundly influence the dynamics of most populations. The scale of human impacts thus confounds attempts to understand the natural influences on these species. What we can conclude, however, is that precipitation and phenological rhythms can strongly influence births, whereas survival is more strongly influenced by predation and mass catastrophe. Equids also exhibit complex social behavior that may drive population dynamics in ways we simply have not quantified or fully understand (see chap. 3). Such uncertainties in both the changing environment and our shortfalls in understanding the depth of equid societies demand forward thinking conservation and careful management for a group of species that nearly all persist on the world's threatened and endangered list.

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Influences of immunocontraception on time budgets, social behavior, and body condition in feral horses

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ABSTRACT

Managers concerned with shrinking habitats and limited resources for wildlife seek effective tools for limiting population growth in some species. Fertility control is one such tool, yet little is known about its impacts on the behavioral ecology of wild, free-roaming animals. We investigated influences of the immunocontraceptive porcine zona pellucida (PZP) on individual and social behavior in bands of feral horses (*Equus caballus*) in three discrete populations and used 14 hierarchical mixed effect models to gain insight into the influences of PZP treatment on feral horse behavior. A model of body condition was the strongest predictor of feeding, resting, maintenance, and social behaviors, with treated females allocating their time similarly to control females. Time spent feeding declined 11.4% from low condition to high condition females ($F_{1,154} = 26.427$, $P < 0.001$) and was partially reciprocated by a 6.0% increase in resting ($F_{1,154} = 7.629$, $P = 0.006$), 0.9% increase in maintenance ($F_{1,154} = 7.028$, $P = 0.009$), and 1.8% increase in social behavior ($F_{1,154} = 15.064$, $P < 0.001$). There was no difference detected in body condition of treated versus control females ($F_{1,154} = 0.033$, $P = 0.856$), but females with a dependent foal had lower body condition than those without a foal ($F_{1,154} = 4.512$, $P = 0.038$). Herding behavior was best explained by a model of treatment and the interaction of band fidelity and foal presence (AIC_c weight = 0.660) which estimated no difference in rate of herding behavior directed toward control versus treated females ($F_{1,102} = 0.196$, $P = 0.659$), but resident females without a dependent foal were herded 50.9% more than resident females with a foal ($F_{3,102} = 8.269$, $P < 0.001$). Treated females received 54.5% more reproductive behaviors from stallions than control mares ($F_{1,105} = 5.155$, $P = 0.025$), with the model containing only treatment being the most-supported (AIC_c weight = 0.530). Treated and control females received harem-tending behaviors from stallions equally ($F_{1,105} = 0.001$, $P = 0.969$) and agonistic behaviors from stallions equally ($F_{1,105} < 0.001$, $P = 0.986$). Direct effects of PZP treatment on the behavior of feral horses appear to be limited primarily to reproductive behaviors and most other differences detected were attributed to the effects of body condition, band fidelity, or foal presence. PZP is a promising alternative to traditional hormone-based contraceptives and appears to contribute few short-term behavioral modifications in feral horses.

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1. Introduction

The size and distribution of human populations has rapidly increased over the last several decades, leading to shrinking habitats and limited resources for many large vertebrates around the world. Managers seek effective tools for limiting population growth in some of these species. Attempts to regulate populations have historically emphasized controlling survival, but more recently, fertility control has emerged as an alternative to lethal methods of population control (Hobbs et al., 2000; Porton,

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2005). Little is known about impacts of fertility control on the behavioral ecology of wild, free-roaming animals; yet, understanding animal behavior can be an important tool in addressing a species' influences and roles in an ecosystem and can lead to better management and conservation practices (Sutherland, 1998; Buchholz, 2007). The ethical considerations of potentially altering animal behavior and social structure through the use of fertility control agents on free-roaming wildlife are also a growing concern among managers and the public, as are concerns for animal welfare, such as potential negative physiological reactions to treatment (Nettles, 1997; Asa et al., 2005; Porton, 2005).

Large populations of ungulates (white-tailed deer (*Odocoileus virginianus*), elk (*Cervus canadensis*), and feral horses (*Equus caballus*)) are targeted for fertility control management in the United States. Feral horses exhibit the Type I mating system found in the family Equidae, which is characterized by family groups (bands) typically consisting of a polygynous male with females and their young (Klingel, 1975). Such social groups are largely maintained by a suite of behaviors consisting of reproductive, herding, agonistic, and defensive expressions initiated by the polygynous male in the band, as well as hierarchical dominance relationships among females (Feist and McCullough, 1976; McCort, 1984; Klimov, 1988; Pickeral et al., 1993; Linklater et al., 1999). With such complex social organization, artificial changes to behavior may have far-reaching implications for the treated individual and other members of the family group, as well as the social dynamics of the entire herd. An ideal fertility control agent for free-roaming populations of such animals would have little influence on behavior. The immunocontraceptive porcine zona pellucida (PZP) has been proposed as such an agent.

PZP acts as a vaccine against pregnancy by stimulating the production of zona pellucida antibodies in female mammals (Sacco, 1977; Liu et al., 1989). These antibodies provide a barrier that prevents sperm from binding to the surface of an ovum and results in limited penetration of the zona pellucida and subsequent limited pregnancy in many taxa (Kirkpatrick and Turner, 1991; Kirkpatrick et al., 1996). PZP treatment has been shown to reduce fertility in horses with only 0–19% of treated mares producing foals (Liu et al., 1989; Kirkpatrick et al., 1996). Unlike most conventional contraceptives, this immunocontraceptive does not appear to affect ovarian function of the inoculated female horse when used for up to 3 years; however, additional treatments have been reported to decrease ovarian function (Kirkpatrick et al., 1995). Some additional potential side-effects of PZP treatment have also been investigated in feral horses: formerly treated mares have successfully returned to fertility (Kirkpatrick and Turner, 2002) and injection site reactions were minimal and non-debilitating (Roelle and Ransom, 2009). It remains unclear how PZP treatment influences the behavior of feral, free-roaming horses.

Many wild ungulate species have complex mating systems maintained by dominance hierarchies within family groups, active recruitment and defensive behaviors by polygynous males, and resource driven behavioral patterns. PZP treatment is not expected to have a direct effect on these behaviors in horses because ovarian function remains intact and artificial hormones are not administered. However, horses are polyestrous and females may exhibit estrous behavior during both ovulatory and anovulatory phases of ovarian follicular development, which is thought to facilitate social structure (Crowell-Davis, 2007). PZP treatment of females has the potential to elevate the exhibition of repetitive estrous behavior beyond that which stallions are accustomed to. We hypothesize that such polyestrous cycling in individual mares may elicit excess reproductive behaviors by both the cycling female and nearby males, prompt more frequent herding and harem-tending behaviors by stallions, and elevate frequency of agonism between stallions and females. We also posit that if individuals allocate more time to reproductive and social behaviors, less time is available for critical feeding and maintenance behaviors and could present long-term concerns for animal health.

Current research on this topic has been limited to a few island populations where time budgets of PZP-treated females were found to be comparable to control females (Powell, 1999; Rogers, 2001); however, it has recently been shown that band fidelity can decrease and rates of reproductive behavior can increase in association with PZP treatment (Nuñez et al., 2009). These results are somewhat conflicting and present concern that animals in disparate populations may exhibit different behavioral responses to PZP treatment, possibly due to percent of females treated, population size, or density. Approximately 37,000 feral horses roam on 13 million ha of public lands in the western United States and represent the vast majority of feral horses in the US. We investigated influences of PZP immunocontraception on the behavior of feral horses in three discrete free-roaming western populations over a 4-year period. Our objective was to quantify effects of immunocontraception on time budgets of individuals and social interactions within family groups, differentiating between control females and effectively contracepted females.

2. Methods

2.1. Study areas

Three study areas, Little Book Cliffs Wild Horse Range, McCullough Peaks Herd Management Area, and Pryor Mountain Wild Horse Range were chosen to represent the wide variety of habitats occupied by feral horses in the western United States. These populations use a mosaic of habitats, including desert, sage steppe, piñon-juniper woodland, coniferous forest, and montane environments (Table 1). A small number of unimproved roads traversed these areas, but the majority of land at all three locations was designated as Wilderness Study Area and closed to motorized vehicles throughout the period of this study.

Little Book Cliffs managers removed 68 horses from the population in 2004 and Pryor Mountain managers removed seven horses in 2003 and 19 horses in 2006, but none of these animals were members of the focal bands

Table 1
Geographic, biologic, climatic, and population data for conditions present during behavioral observations of feral horses (*Equus caballus*) at Little Book Cliffs Wild Horse Range (LBC), McCullough Peaks Herd Management Area (MCP), and Pryor Mountain Wild Horse Range (PRY), USA, April–October, 2003–2006. The number of treated females comprises all individuals vaccinated with the immunocontraceptive porcine zona pellucida (PZP).

Study area	Location	Elevation (m)	Size (ha)	Terrain	Dominant vegetation	Precipitation (% of obs.)	Temperature (mean (°C) ± SE)	No. of treated females	Population size	Band size (mean ± SE)
LBC	39°12′N; 108°27′W	1500–2250	14,614	Sloping plateaus, sagebrush parks, steep shale and sandstone cliffs, 4 major canyon systems	Sagebrush (*Artemisia tridentata*), grasses, Colorado piñon (*Pinus edulis*), Utah juniper (*Juniperus osteosperma*)	4.88	25.12 ± 1.32	27	120–204	4.45 ± 0.16, range = 2–9
MCP	44°35′N; 108°40′W	1200–1964	44,440	Flat, open sagebrush park, rugged badlands along western edge	Sagebrush, grasses, small stands of cottonwood (*Populus* sp.) and willow (*Salix* spp.)	3.78	26.62 ± 1.80	36	112–495	8.01 ± 0.38, range = 2–19
PRY	45°04′N; 108°19′W	1175–2670	16,046	Lowland desert, foothill slopes, forested montane slopes, steep canyons, grassy plateaus	Sagebrush (*A. tridentata*), grasses, Utah juniper, Douglas fir (*Pseudotsuga menziesii*) and limber pine (*Pinus lexilus*)	5.25	20.63 ± 1.17	54	142–168	4.16 ± 0.17, range = 2–11

used in our study. Managers removed 379 animals from the McCullough Peaks population in October 2004. Due to management need, 1-year-old females, and 1- and 2-year-old males were removed from the focal bands used in this study, but all reproductive animals in these bands were captured and released as intact family units. Hansen and Mosley (2000) reported no changes in feeding or reproductive behavior and no effect on fecundity following such removals.

2.2. Study subjects

Horses in the treatment group at all three study areas were given primer injections consisting of 0.5 mL (100 μg) PZP and 0.5 mL (100 μg) Freund's complete adjuvant or Freund's modified adjuvant. Subsequent booster injections at Little Book Cliffs and Pryor Mountain consisted of 0.5 mL (100 μg) PZP and 0.5 mL (100 μg) Freund's incomplete adjuvant. These injections of liquid PZP were designed to provide approximately 1 year of infertility, with annual boosters extending the period of contraception. In addition to the primer dose given to horses at McCullough Peaks, a second (simultaneous) injection delivered booster doses via three time-release PZP pellets (bolus release at 1, 2, and 12 months) designed to provide 2 years of infertility (Turner et al., 2008). A full description of these procedures is described in Roelle and Ransom (2009).

Most female horses were pregnant at the time of their first injection of PZP and some did not exhibit infertility after receiving treatment; therefore, to clarify behaviors associated with effective fertility control, all analyses considered animals to be in the treatment group only when they had received at least the primer and one booster dose, and exhibited non-pregnancy. Three females that received PZP but continued to produce young were omitted from analyses. Observations of treated animals that occurred after prescribed treatment had concluded were omitted from analyses because post-treatment PZP titer levels could not be verified; it was thus unknown if contraception persisted or return to fertility had occurred. Animals in the control group were not injected with PZP and exhibited pregnancy as it naturally occurred.

During the course of this study, 27 horses were treated at Little Book Cliffs, 36 were treated at McCullough Peaks, and 54 were treated at Pryor Mountain, with the number of treatments received by any individual horse varying from 1 to 4. The percentage of treated females >1 year of age in each population therefore varied by year, but averaged 36.0% at Little Book Cliffs, 80.6% at McCullough Peaks, and 25.7% at Pryor Mountain. All focal animals at each site were monitored by trained technicians one to five times per week from April to October each year, 2003–2006. Pregnancy and parturition were primarily determined by visual assessment, with supplemental pregnancy data opportunistically gained through fecal assays of estrone conjugates and progesterone metabolites (Kirkpatrick et al., 1991) or blood estrone sulfate/progesterone analyses (Munro and Stabenfeldt, 1984; Stabenfeldt et al., 1991). All data for this study were collected in accordance with the Colorado State University Animal Care and Use Committee standards (protocol 03-107A-02).

2.3. Behavioral sampling

Eight to ten feral horse bands were chosen in each of the three populations from a stratified random sample consisting of bands with at least one stallion, one treated mare and one control mare, though natural interchange dictated that band compositions were plastic throughout the study. Behavioral observations were separated into three daylight time periods: 08:00–12:00 h, 12:01–16:00 h, and 16:01–20:00 h. We collected behavioral data for 3133 horse hours (total band observation hours times the number of focal animals in each band) at Little Book Cliffs, 4221 horse hours at McCullough Peaks, and 2592 horse hours at Pryor Mountain. Each observation session included collection of climatic (temperature, precipitation, cloud cover, wind speed), geographic (UTM coordinates and elevation), and temporal (time and date) data, a 20-min instantaneous scan sample of time budgets (Altmann, 1974) at 1 min intervals for each band member, and all-occurrence data collection for social interactions (Altmann, 1974).

In order to accurately record behaviors, we defined an ethogram that categorized specific behaviors into a suite of 15 discrete time budget categories (*see* Ransom and Cade, 2009). These categories were agonism, comfort, elimination, feeding, grooming, harem-social (e.g., allogrooming, pair-bonding), harem-tending (e.g., stallion defense of a harem mare or recruitment of a new mare into the harem), herding (driving or snaking behavior by the stallion), interaction-with-humans, locomotion, out-of-sight, reproduction, resting, standing attentive, and submission. The relative infrequency and similar nature of several behaviors in the instantaneous scan samples motivated us to aggregate them into broader categories for time budget analyses. Grooming, comfort, standing attentive, and elimination were combined into *maintenance*; reproductive, harem-tending, harem-social, agonism, submission, and herding were combined into *social*; and interaction-with-humans and out-of-sight were combined into *unknown*. The time spent in the category *unknown* ultimately did not differ between treatment groups in any population.

Instantaneous scan sampling has the potential to underestimate rare events (Houpt, 1991; Doran, 1992); thus, we also collected social behavior data at every occurrence throughout each observation session. These all-occurrence behaviors included intraband social behaviors associated with herding, reproduction, agonism, and harem-tending and were retained as discrete categories for social behavior analyses. We conducted all observations from distances that did not elicit attention of horses to the presence of the observer. McCullough Peaks horses were relatively reactive to the presence of humans and observations were generally conducted approximately 100 m away unless terrain allowed an observer to remain concealed at a closer distance. Horses at Little Book Cliffs and Pryor Mountain were less reactive to human presence and observations were conducted at approximately 50 m or more. All observations utilized 10 mm × 50 mm binoculars or a 16–45 mm × 60 mm spotting scope in situations where unaided observations were not adequate.

2.4. Model covariates

We initially considered age, climate, time of day, band fidelity, presence of dependent young, size of the band, body condition score, and experience of the dominant male within a band as factors that might help explain variation in horse behavior beyond the effects of treatment. We also considered sampling year in the event that behaviors varied temporally due to an effect we did not otherwise explicitly include in our analyses. Age was absolutely known from parturition records for 88.4% of all individuals at Pryor Mountain, 42.1% of all individuals at Little Book Cliffs, and 34.2% of all individuals at McCullough Peaks. An experienced veterinarian estimated ages for the remainder of animals in this study using dental assessments during scheduled management captures. This method provides only approximate age due to the many external factors effecting tooth wear (Martin, 2002).

Climatic data (temperature, cloud cover, wind speed, and precipitation) was relatively homogeneous across locations and observations so we did not further consider climate in behavior analyses. Behavioral observations were distributed equally across daylight time periods so we did not further consider time of day. The total number of all horses present in the band at the time of the observation constituted the band size. Band composition data collected every 7 days, from April to October of each year, determined band fidelity: females were considered transient if they left their band and went to another band at least once during the season and resident if they never left during the season. Presence of young was recorded as a dependent foal from birth to the end of that sampling season or documented death that season. Body condition of individuals was determined using a 10-step index similar to the system of Henneke et al. (1983); however, since these scores were assigned solely by visual assessment (rather than palpation) in the field, the scale was adapted to a 0.0–5.0 score with half-step intervals similar to that described by Rudman and Keiper (1991). This scale facilitated more consistent estimation of body condition in the field. Tenure of males as band stallions in this study was unknown, so we assumed age was a reasonable indicator of band stallion experience. Age range of the observed band stallions was 4–26 years, with mean age = 11.88 (95% CI: 10.43–13.33).

2.5. Statistical analyses

Some horses were born into focal bands, died, or immigrated/emigrated to/from focal bands during the study; therefore, some horses were observed repeatedly over the course 4 years while others were only observed in a single year. As a result, samples were not equally distributed across individuals or across years. Repeated measures of individuals were accounted for by averaging data for each individual or unique pair of individuals per year and including the influence of individual identification on the random effect intercept of each model. This design allowed analyses to include a measure of variability among individuals as well as among years. Observations of individual horses were not averaged over the entire 4-year

period because potentially important variables such as age, band fidelity, presence of a dependent foal, and treatment status often varied annually. Five extreme outlier values were detected in the residual values for time budget behaviors and these observations were omitted from analyses. The final dataset considered in all analyses consisted of 184 control female and 103 treated female observations that were comprised of 99 individual control horses (32 at Little Book Cliffs, 34 at McCullough Peaks, 33 at Pryor Mountain) and 56 individual treated horses (18 at Little Book Cliffs, 16 at McCullough Peaks, 22 at Pryor Mountain).

We formulated a candidate set of 14 hierarchical mixed effects models, which, in the notation of Gelman and Hill (2007), took the form of $y_{hjk[i]} = \alpha_{0hj[i]} + \alpha_{1hk[i]} + \beta_0 + \beta_x x_{[i]} + \varepsilon_{hjk[i]}$, where the random effect intercept of $\alpha_{0hj[i]} \sim N(\mu = 0, \sigma^2_{hj})$ for treatment h (treated or control) in population j (Little Book Cliffs, McCullough Peaks, or Pryor Mountain), for $i = 1, \ldots, n$ observations, and the random effect intercept $\alpha_{1hk[i]} \sim N(\mu = 0, \sigma^2_{hk})$ for h in $k = 1, \ldots, n$ individual horses for i observations. This equation identifies the fixed effect intercept as β_0, with ε = statistical error, and y = dependent variable. The candidate model set included models with the fixed effect parameter (β_x) for fixed effects (x) of age (model 1), treatment (model 2), body condition (model 3), foal presence (model 4), band fidelity (model 5), band size (model 6), sampling year (model 7), and the interactions of treatment and band fidelity (model 8), treatment and body condition (model 9), band fidelity and body condition (model 10), band fidelity and foal presence × treatment (model 11), age and treatment (model 12). Social behavior involved pairs of animals, and the fixed effect of stallion age (model 13) and average distance between the stallion and focal mare (model 14) were also considered for those analyses.

Analyses of individual time budget behaviors and social behavior data were performed using the Hierarchical Linear Mixed Model function of SYSTAT 12® (SYSTAT Software Inc., 2007) with estimates obtained by restricted maximum likelihood (Harville, 1977). We also analyzed body condition as a dependent variable using the candidate model set, excluding Models 3, 9, and 10 because they contained body condition as an independent variable. Probabilities for all tests are reported with a 95% level of confidence.

We used minimum corrected Akaike's Information Criteria (AIC_c) to evaluate the strength of evidence for all mixed models (Burnham and Anderson, 2002). Models achieving the greatest AIC_c weight were considered the best model for the predicted behavior, and all models exhibiting a ΔAIC_c of <2 were considered plausible (Burnham and Anderson, 2002). Time budget behaviors were modeled individually to investigate the influences of fixed effects on each behavior; however, the strength of evidence for each model was ultimately evaluated based on the composition of all behaviors in the time budget (feeding, resting, locomotion, maintenance, and social). This was performed by summing the minimum AIC_c scores from each individually modeled constituent behavior and using the aggregate AIC_c score ($aAIC_c$) as strength of evidence for the overall composition (Ransom and Cade, 2009). This procedure allowed detailed analyses of the individual behaviors without violation of the inherent compositional dependence. Time budgets were analyzed as mean percent of time allocated to each behavior in the composition, per horse, per year and social behavior models were evaluated individually because the data represent frequency-of-occurrence.

3. Results

3.1. Time budgets

Feeding behavior for all horses occupied the largest percent of daylight time budgets and there was no difference detected between treatment groups, with control females feeding an estimated 57.7% ± 3.2 SE of daylight hours and treated females feeding an estimated 55.0% ± 3.3 SE of daylight hours ($F_{1,154} = 0.351$, $P = 0.554$). The most-supported model for feeding behavior was Model 3 (body condition) with an AIC_c weight of 0.947, and no other models were considered plausible. This model estimated an 11.4% decrease in time spent feeding as body condition score increased from 1.0 to 4.0 (Fig. 1, Table 2).

Resting behavior occupied an estimated 27.1% ± 2.0 SE of control female daylight time budgets and 27.9% ± 2.2 SE of treated female daylight time budgets ($F_{1,154} = 0.075$, $P = 0.785$). Models 3 (body condition) and 4 (foal presence) were both plausible for explaining resting behavior, with Model 3 carrying an AIC_c weight of 0.705 and Model 4 an AIC_c weight of 0.209. Body condition in model 3 estimated an increase of 6.0% in time spent resting as body condition increased from 1.0 to 4.0, which partially compensated for the negative trend detected in feeding behavior (Table 2). Model 4 estimated that foal presence best explained resting behavior with independent females resting an average of 29.3% ± 1.6 SE of the time and females with a dependent foal resting 12.9% less ($\bar{x} = 25.5\% \pm 1.6$ SE).

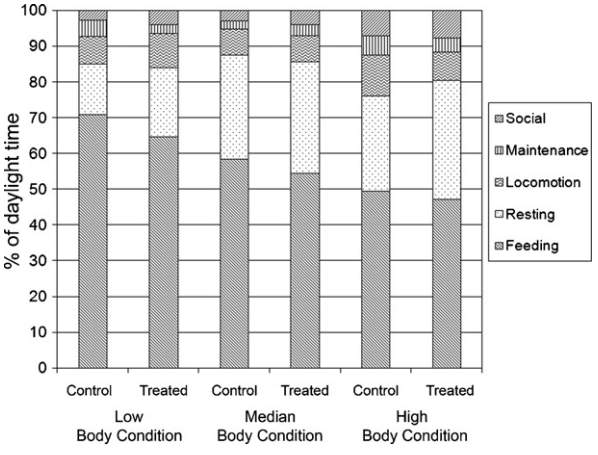


Fig. 1. Mean allocations of daylight time budget behaviors as a function of low (<2.5), median (3.0) and high (>3.5) body condition scores for repeated observations of 99 control female and 56 treated female feral horses (*Equus caballus*) at Little Book Cliffs Wild Horse Range, McCullough Peaks Herd Management Area, and Pryor Mountain Wild Horse Range, USA, 2003–2006.

Table 2
Hierarchical linear mixed model estimates of the difference in means for each fixed effect in the most-supported models of daylight time budget behaviors (feeding, resting, locomotion, maintenance, social) and all-occurrence social behaviors (reproductive, herding, harem-tending, agonism) for repeated observations of 99 control and 56 treated feral horses (*Equus caballus*) at Little Book Cliffs Wild Horse Range, McCullough Peaks Herd Management Area, and Pryor Mountain Wild Horse Range, USA, 2003–2006. Random effect variance is denoted as σ_p^2 for the horse population and σ_i^2 for the individual horse.

Behavior	Model	AIC_c weight	Effect	df_{NUM}	df_{DENOM}	F	P	Difference	95% LCL	95% UCL	σ_p^2	σ_i^2
Feeding	3	0.947	Body condition	1	154	26.427	<0.001	−0.114	−0.158	−0.070	0.0013	0.0001
Resting	3	0.705	Body condition	1	154	7.629	0.006	0.060	0.017	0.104	0.0003	0.0005
	4	0.209	Foal presence	1	154	5.374	0.022	0.038	0.006	0.070	0.0008	0.0007
Locomotion	2	0.461	Treatment	1	154	0.006	0.941	0.002	−0.043	0.046	0.0007	<0.0001
	4	0.250	Foal presence	1	154	2.020	0.157	0.006	−0.002	0.014	0.0006	<0.0001
Maintenance	3	0.782	Body condition	1	154	7.028	0.009	0.008	0.002	0.015	0.0002	<0.0001
Social	3	0.947	Body condition	1	154	15.064	<0.001	0.018	0.009	0.028	0.0002	0.0001
Reproductive	2	0.530	Treatment	1	105	5.155	0.025	−0.060	−0.113	−0.008	0.0005	0.0051
Herding	11	0.660	Treatment	1	102	0.196	0.659	−0.123	−0.443	0.659	0.1091	0.0211
			Band fidelity × foal presence	3	102	8.269	<0.001	–[a]				
Harem-tending	3	0.930	Body condition	1	105	20.608	<0.001	0.141	0.079	0.203	0.0075	<0.0001
Agonism	6	0.925	Band size	1	105	12.080	<0.001	−0.007	−0.011	−0.003	<0.0001	<0.0001

[a] This variable produces a vector of interaction estimates, as described in the text.

Locomotion occupied a relatively small amount of female daylight time budgets, and while model 2 (treatment) best explained this behavior (AIC_c weight = 0.461), it estimated no difference between treatment groups (Table 2). Control females in this model were in locomotion an estimated 6.6% ± 1.6 SE of the time and treated females were in locomotion an estimated 6.4% ± 1.6 SE of the time. Model 4 (foal presence) was also plausible for explaining locomotion behavior and was supported by 25.0% of the AIC_c weight. The effect of foal presence was weak and the model estimated females with a dependent foal were in locomotion an average of 6.2% ± 1.1 SE of the time and independent females were in locomotion an average of 6.8% ± 1.1 SE of the time (Table 2).

Maintenance behavior was expressed by control females an estimated 1.7% ± 0.8 SE of the time and by treated females an estimated of 2.4% ± 0.8 SE of the time ($F_{1,154} = 0.420$, $P = 0.518$). This behavior was best explained by Model 3 (body condition) and while the effect was significant, the amount of time spent in expression of maintenance behavior only increased 0.8% as body condition increased from 1.0 to 4.0 (Table 2). Likewise, social behavior was also best explained by Model 3 (body condition) and this behavior increased 1.8% as body condition score increased from 1.0 to 4.0. No other models were supported for maintenance or social behaviors.

Body condition was clearly the most-supported effect for explaining daylight time budget behaviors, with an $aAIC_c$ weight of 0.996 among the candidate models. Body condition scores of the animals observed ranged from 1.0 to 4.0 with a median score of 3.0. Model 2 (treatment) and Model 4 (foal presence) were the most plausible models for predicting body condition, with AIC_c weights of 0.390 and 0.285, respectively. There was no difference detected in body condition of control females ($\bar{x} = 2.99 \pm 0.08$ SE) compared to treated females ($\bar{x} = 2.97 \pm 0.08$ SE) ($F_{1,154} = 0.033$, $P = 0.856$) in Model 2; however, females with dependent foals in Model 4 exhibited lower body condition ($\bar{x} = 2.91 \pm 0.04$ SE) than females without dependent foals ($\bar{x} = 3.02 \pm 0.03$ SE) ($F_{1,154} = 4.512$, $P = 0.038$). The estimated decrease in time feeding was compensated by an increase in resting, maintenance, and social behavior as body condition increased (Fig. 1); however, body condition data exhibited considerable variation suggesting that other factors we did not measure may also influence time budget behaviors.

3.2. Herding behavior

Herding was the most commonly observed intraband social behavior at all three sites. Stallions were observed herding females at Little Book Cliffs at a mean rate of 0.20 (95% CI: 0.16–0.25) times per hour (tph), McCullough Peaks 0.17 (95% CI: 0.13–0.22) tph, and Pryor Mountain 0.68 (95% CI: 0.52–0.85) tph. Band fidelity contributed strongly to the models of herding behavior and models containing this variable totaled 79.7% of the AIC_c weight. The most-supported model for herding behavior was Model 11 (treatment + the interaction of band fidelity and foal presence), and it attained an AIC_c weight of 0.660 with no other candidate models being plausible.

The effect of treatment in Model 11 was not significant and was accompanied by considerable variation, but the interaction effect of band fidelity and foal presence was strong (Table 2). Resident females without a foal were herded by stallions 0.57 tph ± 0.15 SE whereas resident females with a dependent foal were herded by stallions 50.9% less at an average of 0.29 tph ± 0.15 SE. Transient females without foals were herded by stallions least often, at an average of 0.18 tph ± 0.15 SE, and transient females with a dependent foal were herded similarly to resident females with a foal at an average of 0.27 tph ± 0.16 SE.

3.3. Reproductive behavior

Reproductive behavior was the second most frequently observed intraband social behavior at all three sites. Little Book Cliffs stallions were observed exhibiting reproductive

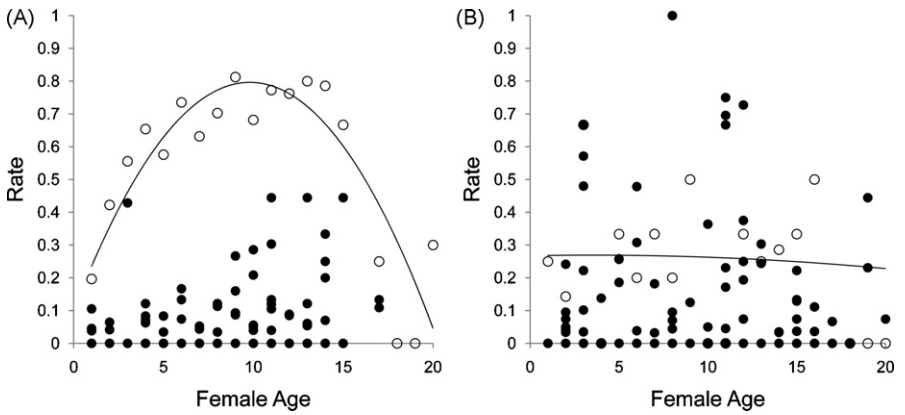


Fig. 2. Rate of feral horse (*Equus caballus*) reproductive behaviors exhibited per hour (●) as a function of female age, and mean rate of viable foal production (○) as a function of female age at conception, for repeated observations of 99 control females (A: $y = -0.007x^2 + 0.142x + 0.101$, $R^2 = 0.89$) and 56 treated females (B: $y = -0.0002x^2 + 0.001x + 0.267$, $R^2 = 0.002$) at Little Book Cliffs Wild Horse Range, McCullough Peaks Herd Management Area, and Pryor Mountain Wild Horse Range, USA, 2003–2006.

behavior toward females at a mean rate of 0.05 (95% CI: 0.02–0.09) tph, with McCullough Peaks stallions exhibiting the same rate of 0.05 (95% CI: 0.03–0.08) tph. Pryor Mountain stallions exhibited this behavior toward females at the rate of 0.10 (95% CI: 0.07–0.13) tph. Copulation (successful and unsuccessful) comprised 34.2% of the reproductive behaviors observed and pre-copulatory behaviors such as reproductive tending, flank-rubbing, chin-resting, flehmen response, and penis extension comprised 65.8% of the observations. The most-supported model for reproductive behavior was Model 2 (treatment), with an AIC_c weight of 0.530, and no other candidate models were plausible.

The effect of treatment was highly significant with control females receiving an average of 0.05 ± 0.02 SE reproductive behaviors from stallions per hour and treated females receiving 54.5% more reproductive behaviors at an average of 0.11 ± 0.02 tph (Table 2). The rate of reproductive behavior expressed toward females generally followed the relationship between viable foal production and age of the female at the time of conception (Fig. 2A), but this relationship deteriorated in the treatment group and the rate of reproductive behaviors expressed was both higher than control females and exhibited no clear pattern among age cohorts (Fig. 2B).

3.4. Harem-tending behavior

Harem-tending behavior was observed with greater frequency at Pryor Mountain than at the other two sites, but was still relatively infrequent in all populations. Stallions exhibited harem-tending behavior toward females at Pryor Mountain 0.17 (95% CI: 0.12–0.22) tph, whereas this was observed at Little Book Cliffs only 0.04 (95% CI: 0.03–0.05) tph and at McCullough Peaks only 0.02 (95% CI: 0.01–0.03) tph. Model selection among harem-tending models revealed body condition as an important effect, with Model 3 attaining 93.0% of the AIC_c weight. Harem-tending behaviors by stallions toward females increased 14.1% as body condition increased from 1.0 to 4.0 (Table 2). There was no difference detected in harem-tending behaviors by stallions toward control females ($\bar{x} = 0.08$ tph ± 0.04 SE) as compared to treated females ($\bar{x} = 0.08$ tph ± 0.05 SE) ($F_{1,105} = 0.001$, $P = 0.969$).

3.5. Agonism

Agonistic behaviors from stallions toward female band members were observed 0.03 (95% CI: 0.01–0.05) tph at Little Book Cliffs, 0.01 (95% CI: 0.01–0.02) tph at McCullough Peaks, and 0.04 (95% CI: 0.03–0.06) tph at Pryor Mountain. In order of increasing intensity, 67.7% of these agonistic expressions were in the form of a threat, 1.7% were bumps or pushes, 26.8% were kicks or bites, and 3.8% were chases. Model 6 (band size) best explained the observed agonistic expressions and was supported by 92.5% of the AIC_c weight. The effect of band size was statistically significant, but only resulted in agonistic behavior decreasing 0.7% as band size increased from 3 to 14 horses. There was no difference in rates of agonistic behavior exhibited by stallions toward control females ($\bar{x} = 0.03$ tph ± 0.01 SE) compared to treated females ($\bar{x} = 0.03$ tph ± 0.01 SE) ($F_{1,105} < 0.001$, $P = 0.986$). Agonism between females occurred too infrequently to conduct meaningful analyses.

4. Discussion

Previous research indicated that immunocontraception with PZP did not lead to behavioral changes in feral horses (Kirkpatrick et al., 1996; Powell and Monfort, 2001); however, more recent work by Nuñez et al. (2009) refuted these assertions with evidence that PZP immunocontraception led to a decrease in band fidelity and an increase in reproductive behaviors during the non-breeding season. Our findings are similar to those of Nuñez et al. (2009), indicating that PZP treatment can affect behavior in feral horses. We found that treated females received significantly more reproductive behaviors from stallions than did control females, and the most-supported model of this behavior was that of treatment. Models containing the fixed effect of treatment were also supported for

locomotion and herding behavior, but no differences were detected between treatment groups. All other behaviors investigated were better explained by models of body condition, foal presence, or band size.

4.1. Time budgets

Estimates of daylight time budget allocations for all age and treatment groups fell within the broad ranges previously reported for equids (Mayes and Duncan, 1986; Boyd, 1988; Berger et al., 1999). The differences detected in time budget behaviors were primarily a function of body condition and are comparable to expected differences in time budgets between barren and pregnant/lactating females. Boyd (1988) found pregnant female horses spent more time feeding than barren females, which corresponds to the respective metabolic needs of animals in these conditions (Henneke et al., 1984; National Research Council, 2007).

These results largely concur with Powell's (1999) finding of no difference in time allocation between PZP-treated and control horses; however, data were not completely comparable because control females in Powell's study were previously treated and PZP titer levels were unknown. Rogers (2001) found that PZP had no effect on time spent walking, grooming, or standing in feral horses. Likewise, Heilmann et al. (1998) found no differences in time spent moving or idling between PZP-treated and untreated free-ranging female elk. Our results are consistent with those from these studies, and it appears that the complex social organization of feral horses does not greatly influence the time allocated to these basic maintenance behaviors. McShea et al. (1997) did note that PZP-treated female white-tailed deer spent more time running or walking than untreated females, but we did not detect this effect in horses.

Models that were most strongly supported for time budget compositions attributed primary differences between treatment groups to body condition and foal presence effects. Body condition contributed to individual time budget behavior with females in lower body condition feeding more than those in higher condition. Few animals exhibited extremely low or high body condition in our study and investigation into this effect may require a wider range of conditions to elucidate more in-depth results. Social dominance in relation to body condition has been attributed to food gain and energy expenditure (Hogstad, 1987; Cuthill and Houston, 1997; Fischoff et al., 2007) and stochastic climate events could cause depressed or elevated states of body condition and lead to greater differences between treatment groups. Turner and Kirkpatrick (horses: 2002) and McShea et al. (white-tailed deer: 1997) found that contracepted females exhibited higher body condition than control females, and Conner et al. (2007) found a seasonal difference in winter body condition of females, but no overall difference between fertility control treatment groups. Likewise, we found no difference in body condition of treated versus control females in our study.

van Dierendonck et al. (2004) reported that mares without foals spent less time resting and a greater percent of time in social interaction than either pregnant or post-partum mares. One plausible model of resting behavior in our study exhibited support for the effect of foal presence, but with contrary results. Mares with dependent foals rested less than their barren counterparts. This conflicting result may be attributed to the social structure (absence of stallions) in van Dierendonck et al.'s (2004) study, compared to the free-roaming natural populations that experienced threats of predation and competition among stallions in our study. Females with dependent young simply had to be more vigilant when faced with these natural constraints on their time.

4.2. Social behavior

Frequency of some social behavior was heterogeneous across the populations we studied, likely resulting from local densities of bands on the landscape. This proximity of groups to one another introduces different levels of social pressure between bands and illustrates that some populations may be more sensitive to fertility control management strategies than others. All-occurrence analyses of social behavior detected 54.5% more reproductive behaviors were received by treated females than by control animals. This is not surprising given the higher rates of estrous reported in PZP-treated females of other species as compared to control females (Mahi-Brown et al., 1985; Shumake and Wilhelm, 1995; Heilmann et al., 1998; Curtis et al., 2002). Nuñez et al. (2009) reported more frequent reproductive behaviors exhibited and received by treated female horses outside of the breeding season; likewise, Heilmann et al. (1998) found that PZP-treated female elk exhibited higher rates of sexual interaction outside of the breeding season than did untreated females. Powell (1999) also found that reproductive tending and mating duration by stallions lasted longer as a result of mares continuing their estrous cycles into the fall, which he proposed may contribute to a disruption in band stability. Similarly, McShea et al. (1997) found that the breeding season behavior of white-tailed deer males was extended, and started earlier in the subsequent year after females were treated. Our study examined only the frequency of reproductive behaviors and did not examine seasonality. The difference between treatment groups in pattern and relationship to age-specific fecundity we detected appears to directly correspond to the treatment applications across age cohorts and efficacy of the vaccine. It may be important to consider additional research to assess the scope of this potential effect as it could have implications for stallion body condition and consequent lifetime fitness.

Herding behavior may be important because of the high energetic cost of locomotion for both the male and female(s) involved and may have biological meaning in terms of stallion body condition and fidelity of females to the band. We detected no significant difference in herding between treatment groups, but there was a strong effect of the interaction between band fidelity and foal presence in predicting this behavior. Rogers (2001) found that the percentage of time females were herded was different between treatment groups and detected significant effects of foal presence and band fidelity. Even when generalized

across 3 populations, we also detected this difference and found that absence of a dependent foal was associated with an increase in herding behavior. Linklater et al. (1999) found that transient mares had poorer body condition than resident mares as a result of greater harassment by stallions and Nuñez et al. (2009) found that immunocontracepted female feral horses exhibited a decrease in band fidelity during the non-breeding season. These findings suggest further investigation into the effects of fertility control on band fidelity would help determine the scope and extent of this relationship in immunocontracepted female horses during the breeding season.

Harem-tending and agonistic behaviors by stallions did not differ among treatment groups, but stallions invested more harem-tending behaviors in mares with higher body condition. Optimality theory may explain this finding by the perceived fitness pay-off the stallion would receive from investing in animals more likely to contribute offspring (MacArthur and Pianka, 1966), but the true nature of this relationship has not been explained by our analyses. Stallions also expressed no difference in frequency of agonistic behaviors toward control and treated females. This is not surprising since agonistic expressions within feral horse bands are often expressed between females in order to establish and facilitate dominance (Houpt et al., 1978; Waring, 1983; Keiper and Receveur, 1992; Weeks et al., 2000). The stallion is generally not the most dominant member of the band, perhaps as a result of greater time spent away from the group while defending or recruiting females (Keiper and Receveur, 1992).

While few studies have investigated the relationship of agonism and contraception in feral horses, agonism related to presence of a foal and band fidelity has been described. Estep et al. (1993) found no change in agonistic behaviors associated with presence or absence of a dependent foal, and Rutberg and Greenberg (1990) found that frequency of aggression did not vary between females of transient and resident status. Our study concurs with these results as the covariates of interest were not present in the most-supported models of agonistic behavior, and they were not significant effects in the candidate models that contained them.

5. Conclusion

Our study found that reproductive behavior was the only acute short-term response attributable to PZP treatment. Most behaviors we measured were better explained by body condition and those findings are consistent with the metabolic needs of barren versus pregnant/lactating females. Foal presence was also an important effect in some behaviors, and while no differences in treatment groups were detected, the efficacy of treatment inherently influences this effect making it important in the long-term consideration of fertility control use. The long-term influence of changes to band fidelity and foal presence on band structure, social networks, dominance hierarchies, pair-bonding, and socialization requires further study. Such effects have broader implications than the results presented in this study and may imply more dynamic changes to the ecology of feral horses.

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Potential Demographic and Genetic Effects of a Sterilant Applied to Wild Horse Mares



Open-File Report 2015–1045

U.S. Department of the Interior
U.S. Geological Survey

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U.S. Department of the Interior
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U.S. Department of the Interior
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U.S. Geological Survey, Reston, Virginia: 2015

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Abbreviations Used in This Report

≥	Greater than or equal to
<	Less than
λ	lambda; annual population growth rate multiplier for a herd
BLM	Bureau of Land Management
K	carrying capacity
N	population size
U.S.	United States

Potential Demographic and Genetic Effects of a Sterilant Applied to Wild Horse Mares

By James E. Roelle and Sara J. Oyler-McCance

Abstract

Wild horse populations on western ranges can increase rapidly, resulting in the need for the Bureau of Land Management (BLM) to remove animals in order to protect the habitat that horses share with numerous other species. As an alternative to removals, BLM has sought to develop a long-term, perhaps even permanent, contraceptive to aid in reducing population growth rates. With long-term (perhaps even permanent) efficacy of contraception, however, comes increased concern about the genetic health of populations and about the potential for local extirpation. We used simulation modeling to examine the potential demographic and genetic consequences of applying a mare sterilant to wild horse populations. Using the VORTEX software package, we modeled the potential effects of a sterilant on 70 simulated populations having different initial sizes (7 values), growth rates (5 values), and genetic diversity (2 values). For each population, we varied the treatment rate of mares from 0 to 100 percent in increments of 10 percent. For each combination of these treatment levels, we ran 100 stochastic simulations, and we present the results in the form of tables and graphs showing mean population size after 20 years, mean number of removals after 20 years, mean probability of extirpation after 50 years, and mean heterozygosity after 50 years. By choosing one or two combinations of initial population size, population growth rate, and genetic diversity that best represent a herd of interest, a manager can assess the likely effects of a contraceptive program by examining the output tables and graphs representing the selected conditions.

Introduction

As the primary agency responsible for management of wild horses (*Equus caballus*) on public lands in the United States (U.S.), the Bureau of Land Management (BLM) has a need for a long-lasting, non-lethal method to control population growth rates. Given the protection afforded by The Wild Free-Roaming Horses and Burros Act of 1971 (P.L. 92–195 as amended) and general lack of natural predators, wild horse populations commonly increase at rates of 15–25 percent per year (Eberhardt and others, 1982; Garrott and others, 1991a) and quickly exceed the carrying capacity of their ranges. In the past, BLM has managed excess horses through a gather, removal, and adoption program, with unadopted horses held in short- and long-term holding facilities until the end of their natural lives. The sustainability of this strategy has recently been called into question, however (U.S. Government Accountability Office, 2008; Garrott and Oli, 2013; National Research Council, 2013). Therefore, BLM has increased efforts to develop a contraceptive that will reduce population growth rates in wild horse herds and thus reduce the number of excess animals that must be removed and cared for.

The contraceptive SpayVac has recently demonstrated the potential for long-lasting efficacy (at least 4 years) in wild horses (Killian and others, 2008). Maximizing the duration of contraceptive effectiveness is especially important in managing wild horses, which in most cases must be captured in order to successfully administer the drug. With extended efficacy (or perhaps even permanent sterility), however, comes increased concern for both the genetic health of a horse herd and the possibility of extirpation. Simulation modeling can be a useful tool in understanding the likely consequences of alternative management actions. We used the VORTEX software package (Lacy and others, 2014; Lacy and Pollak, 2014) to simulate the effects of treating various proportions of mares with a permanent sterilant. Response variables included population size, number of animals removed, average heterozygosity, and probability of local extirpation. (The VORTEX manual (Lacy and others, 2014) uses the term "extinction" rather than "extirpation." We believe that extirpation is more appropriate in this case, as we are simulating local populations, not the entire species.) We chose the VORTEX software because it can be used to simulate both demographic and genetic effects, and because it has been useful in simulating management of the horse population on Assateague Island (Ballou and others, 2008).

VORTEX is an individual-based model in which events happen or fail to happen on the basis of probabilities for each modeled individual. For example, if 100 females are of breeding age, each of which can produce at most one offspring, and fecundity is set to 80 percent, there is no guarantee that exactly 80 offspring will be born. Rather, for each female of breeding age, a random number chosen from a uniform distribution between 0 and 1 is compared to the probability of producing an offspring (in this example, 0.8). If the random number is less than 0.8, then the female is tagged in the model to give birth but otherwise does not. Thus, no two simulations are identical even when they use the same demographic parameters, and multiple iterations must be run in order to examine the possible range of outcomes. In general, we present results in the form of mean values for the multiple simulations; however, it is important to remember that extreme values can also be of interest. In addition, it is important to note that these simulations represent the results of management prescriptions applied consistently through time, without adaptation based on monitoring of results. In the real world, there would be many opportunities to adjust management strategies based on previous results.

Model Parameters

Parameter values and VORTEX options used in our simulations are summarized in table 1 and described in some detail in the sections that follow. Our knowledge of the biology and demography of horse populations and the efficacy of SpayVac and other forms of contraception is incomplete; what is applicable to one herd may not be applicable to all herds. In light of that fact, in cases where specific parameter values were in question, we tried to select the value or option that would have the largest effect on one or more of the response variables. For example, we don't know that SpayVac is a sterilant, but it may be. Furthermore, BLM is considering the use of other techniques (for instance, ovariectomy) that definitely will be permanent. So, for modeling purposes, we assume a permanent sterilant; once a mare is treated and the treatment works, she is infertile for life. This assumption is likely to minimize the number of animals tagged for removal during a simulation, while at the same time maximizing any decline in heterozygosity. We were similarly conservative in our choice of other parameter values and options, always choosing those that were likely to have the most detrimental effect on population demography and genetics.

Table 1. Parameter values and options used in simulations of the effects of a mare sterilant applied to wild horses.

[H_o, observed heterozygosity; ≥, equal to or greater than; BLM, Bureau of Land Management]

Parameter or option	Value selected for simulations
Mating system	Long-term polygyny
Age of first reproduction	Females = 3 years, males = 5 years
Maximum age (life span)	25 years
Maximum age of reproduction	20 years
Basic time cycle	1 year
Maximum litters per year	1
Maximum progeny per litter	1
Density-dependent reproduction	Not used
Reproductive rates	See tables 2 and 3
Mortality or survival rates	See tables 2 and 3
Sex ratio at birth	50 percent males
Mate monopolization	Mean of 3.0 mates per successful sire
Initial population size	100, 250, 500, 750, 1,000, 1,250, or 1,500
Initial age distribution	Stable age distribution calculated from age-specific schedules in table 2 and 3
Carrying capacity	Arbitrarily set at 5× the initial population size, but the contraception and removal programs prevent carrying capacity from having any effect
Initial heterozygosity	Actual allele frequencies at 13 microsatellite loci from the Lost Creek herd in Wyoming (high diversity, $H_o = 0.78$) or the Cibola-Trigo herd in Arizona (low diversity, $H_o = 0.49$)
Removals	When the simulated population reaches ≥150 percent of the initial population size, try to remove enough animals to get back to the initial population size. BLM's age-class removal criteria are used, but are constrained such that no more than 90 percent of any age and sex class are removed.
Efficacy of treatment	Treatment is efficacious in 90 percent of all newly treated mares
Duration of efficacy	If treatment is efficacious, female remains contracepted for life
Proportion of females treated	0 percent–100 percent, in 10 percent increments
Standard deviation due to environmental variability	$S_{EV} = 0.12$ for fecundity; $S_{EV} = 0.06$ for mortality
Concordance	Yes. Breeding and mortality are affected simultaneously.
Probability of catastrophe	10 percent annually
Impact of catastrophe	Reduce reproduction to 50 percent of normal; reduce survival to 75 percent of normal
Distribution of litter sizes	Not used; litter size in horses is virtually always 1
Number of iterations per simulation	100
Number of years per iteration	20 years for population size and removals; 50 years for heterozygosity and probability of extirpation
Number of populations in each simulation	1
Definition of extirpation	The population is considered to be extirpated when it no longer has individuals of both sexes.

Demographic Parameters

Mating System

VORTEX models breeding systems as monogamous or polygamous and short-term or long-term. With monogamous breeding, a male is needed for every breeding female; males may therefore become a limiting factor restricting breeding. In polygamous models, only one male is needed for all females to have an opportunity to breed. However, another parameter (see the section Mate Monopolization) can specify that only a subset of males has an opportunity to breed. For example, a polygynous system can be created in which successful males control harems of, say, five females on average, while the remaining males are excluded from breeding.

If a short-term option is chosen, then VORTEX assumes that mates are randomly reshuffled each year and that all available individuals have an equal probability of breeding. If a long-term option is chosen, then once harem relationships between stallions and mares are formed, those relationships remain intact across years of the simulation until a member dies or disperses to a different population. Demographically, it does not matter whether long-term relationships or rearrangement of harems each year is chosen. Genetically, there may be a small effect on the rate of loss of genetic diversity from the population, with greater loss of diversity expected in populations with higher harem fidelity.

We realize that wild horses do not necessarily mate for life (mares do move between harems) but, of the two choices available in VORTEX, long-term polygyny comes closer to representing the mating system of wild horses. We therefore used long-term polygyny as the mating system in all of our simulations. This is a conservative assumption, in the sense that it represents the greatest opportunity for loss of genetic diversity. Any additional interchange that does occur in the real world will tend to ameliorate effects and result in higher genetic diversity than predicted by our models.

Age of First Reproduction

VORTEX describes reproduction in terms of the age when the first offspring are born, not the age at onset of sexual maturity or the age at first conception. The program also assumes that breeding (and, for that matter, all other events) takes place at discrete intervals, usually years. Thus, age at first reproduction is entered as an integer value. The value used should represent something like the median age of first reproduction, not the earliest age ever observed, because the earliest observed age may not be typical of the population. We therefore used 3 years as the age of first reproduction in females and 5 years as the age of first reproduction in males (Garrott and Taylor, 1990; Garrott and others, 1991b; Roelle and others, 2010).

Maximum Age and Maximum Age of Reproduction

If the species being modeled does not reproduce throughout its entire adult life, the age when the species ceases to reproduce must be specified. For example, humans typically reach reproductive senescence before they die. If the typical life expectancy is 70 years but reproduction ceases at 50 years, then 50 years would be the maximum age of reproduction. VORTEX requires that the maximum age be the same for both sexes, but allows the maximum age of reproduction to vary by sex. Establishing the maximum age of reproduction is difficult because old animals are hard to age (unless they were marked or identified in the year of birth) and because sample sizes of old animals are typically small. Garrott and others (1991b) present information showing that some mares can reproduce at greater than 20 years

of age, whereas Roelle and others (2010) found no evidence of reproduction by females older than 16 years. Roelle and others (2010) also found that very few harems are held by stallions older than age 20. On the basis of available evidence, we used 20 years as the maximum age of reproduction and 25 years as the maximum age.

Maximum Number of Progeny per Year

VORTEX provides a great deal of flexibility in specifying the maximum number of progeny that can be born to a given female in a year (or other time interval), thus allowing species that produce multiple clutches or litters to be modeled. However, most of this flexibility is not needed for modeling a horse population. Given that horses produce only a single litter per year (the average gestation period is 335–342 days; Card and Hillman, 1993) and that twins are virtually unknown, we used 1 for the maximum number of litters per year, 1 for the maximum number of progeny per litter, and a year (365 days) as the basic time cycle.

Density-Dependent Reproduction

VORTEX allows the reproductive rate of the population being modeled to change with changing population size. For example, reproduction could be low when the population is small owing to a difficulty in finding mates (Allee effect) or, conversely, reproduction could drop off when the population is large (more specifically, at high density) owing to limited resources or territories, intraspecific competition, crowding, or stress.

Density-dependent reproduction, also known as compensatory reproduction, has been reported for wild horses (Kirkpatrick and Turner, 1991; Jenkins, 2000; Roelle and others, 2010), but quantifying the magnitude of the effect is difficult, and the information available is insufficient to allow generalization or calculation of some average function. In addition, many of our simulated populations differed only about 50 percent (that is, between N and 1.5 × N) because of the way we implemented the removal function (see section Removal and Contraception Programs). In our estimation, reproductive rates are not likely to change substantially owing to density in that relatively small range. We therefore did not use density-dependent reproduction in our simulations.

Reproductive and Mortality Rates

VORTEX models reproductive rate as the proportion of adult females giving birth each year (or other time interval), where an adult female is defined by the age of first reproduction (see previous discussion). Similarly, mortality rates are modeled as the proportion of animals dying in each age and sex class. From age 0 to the age of first reproduction, each age and sex class can have a different mortality rate; after the age of first reproduction, all animals are considered adults, and the mortality rate can vary only by sex.

While these parameters are fundamental to modeling population dynamics, values are well known for perhaps only a handful of wild horse populations. In our estimation, however, wild horse managers are more likely to have at least some idea of the annual population growth rate multiplier for a herd (λ, lambda), which is just a different way of expressing the growth rate itself. We therefore modeled a set of scenarios in which $\lambda = 1.05$, 1.10, 1.15, 1.20, or 1.25 (that is, annual growth rates of 5 percent, 10 percent, 15 percent, 20 percent, or 25 percent in the absence of contraception). To produce these growth rates, we used reproductive and survival rates (table 2) derived largely from Garrott

(1991a). We arrived at the values for λ = 1.25 by experimenting with parameters in the model. Table 3 provides an example of how we translated these parameters into VORTEX.

Table 2. Annual survival and reproductive rates used to simulate various annual population growth rates.

Population growth rate (percent)	Juvenile survival rate (percent)	Adult survival rate (percent)	Mares foaling (percent)
5	80	91	50
10	80	92	65
15	80	97	65
20	80	99	80
25	90	99	92

Table 3. Translation of the annual rates shown in table 1 into VORTEX parameter values for an annual population growth rate of 20 percent (λ = 1.20).

[λ, annual population growth rate multiplier for a herd; <, less than; ≥, equal to or greater than]

Reproduction		Female Mortality		Male Mortality	
Mare age (years)	Mares foaling (percent)	Age (years)	Rate (percent)	Age (years)	Rate (percent)
1	0	<1	20	<1	20
2	0	1 to <2	1	1 to <2	1
≥3	80	≥2	1	2 to <3	1
				3 to <4	1
				4 to <5	1
				≥5	1

We believe that, in the absence of contraception, growth rates of most wild horse herds will fall within the range shown in table 2. Rather than having to specify reproductive and mortality rates, a manager will need only to choose one or perhaps two values for the annual population growth rate to use the output from the model.

Sex Ratio at Birth

While foal sex ratio can differ from 50:50 in small herds in a given year (Roelle and others, 2010), over time the ratio tends to be fairly even in most herds. For example, Garrott (1991b) found that for a 12-year period 65 of 74 (88 percent) herds sampled in Nevada, Oregon, and Wyoming had a foal sex ratio that did not differ from 50:50. We therefore used 50 percent males in the foal crop in our simulations.

Mate Monopolization

As noted in the previous section, we ran our simulations using a polygynous breeding system. In order to do so, VORTEX requires that one of the following three parameters be specified: percentage of males in the breeding pool, percentage of males successfully siring offspring, or mean number of mates per successful sire. The remaining two parameter values are calculated from the one that is supplied based on the assumptions that some males are excluded from the breeding pool and that breeding

success among males in the pool of available breeders is described by a Poisson distribution. Only the first parameter (percentage of males in the breeding pool) is used by VORTEX.

Of the three ways in which mate monopolization can be described in VORTEX, mean number of mates per successful sire is the one most commonly discussed in the literature on feral horses, but comparisons among studies are hampered by inconsistencies in defining what constitutes a potential mate (that is, the age classes of mares). Roelle and others (2010) defined potential mates as all mares 2 years of age and older, and found a mean harem size of 2.2 mares (range 1.9 to 2.7) during 12 years. Using the same age criteria, Kaseda and Khalil (1996) found average harem sizes of 1.8 to 5.3 mares in many years.

Potential effects of using different values for the mean number of mates per successful sire are not intuitive; to a first approximation, however, one might expect that varying this parameter would affect genetics more than demography. We explored sensitivity of model results to mean number of mates per successful sire in a series of simulations with initial population size = 500, proportion of mares treated = 0.5, lambda = 1.05 (in the absence of contraception and removals), and initial heterozygosity = 0.77. We varied mean number of mates per successful sire systematically between 1.5 and 5.0. The fairly low growth rate and fairly high proportion of mares treated consistently kept the simulated population after 50 years at 200–250 animals; however, final heterozygosity ranged only from 0.74 to 0.75. Given the apparent lack of sensitivity of the model, we decided to set the value of mean number of mates per successful sire to 3.0 for all simulations.

Initial Population Size

Horse herds managed by BLM vary considerably in size, and the size of the herd is potentially very important relative to demographic and genetic results of contraception. We approached this problem by simulating herds with initial populations of 100, 250, 500, 750, 1,000, 1,250, and 1,500 animals. These initial populations should encompass the appropriate management level (AML; population goal) for many herds. We recommend a more detailed, site-specific analysis before a sterilant is applied to any herd with fewer than 100 animals. In addition, we implicitly assume that a herd is at its appropriate size before the results of these simulations can be used to evaluate a fertility control program. For example, the results of the simulations having an initial population and appropriate management level of 500 animals cannot be used if the population in fact contains, say, 1,000 animals.

Initial Age Distribution

VORTEX allows two options for specifying the initial age distribution used in a simulation. A specific age distribution (the number of individuals in each sex and age class) can be entered, or VORTEX can calculate the stable age distribution (proportion of animals) that would result from the age-specific schedules of reproduction and mortality described previously. We used the latter option, and the proportions were applied to the initial population size to determine the number of animals initially in each sex and age class.

Carrying Capacity

Carrying capacity (K) describes a theoretical upper limit to the size of a population. In VORTEX, if the actual population, N, exceeds K at the end of a particular year, then additional mortality is applied equally across all age and sex classes to reduce the population such that N = K. While carrying capacity is an important theoretical construct, values for K are difficult to estimate. We

somewhat arbitrarily set the value for K at 5 times the initial population size. With the contraception and removal programs specified as described in subsequent sections, simulated populations never reach carrying capacity.

Genetic Parameters

VORTEX allows inbreeding depression to be incorporated into the population model as a reduction in first-year survival among inbred individuals. Although some diploid species that have been studied show depressed fitness when inbred (O'Brien and Evermann, 1988; Quattro and Vrijenhoek, 1989), the rate at which inbreeding affects survival and reproduction is not well understood for most species. Therefore, we elected not to use this function in the wild horse model. A better approach for investigating the potential genetic consequences of contraception and gathers (round-ups) is to track the degree of genetic diversity, which can be loosely linked to fitness and an ability to adapt to environmental change.

VORTEX tracks changes in genetic diversity using heterozygosity (the frequency of two different alleles at a given gene) as a metric. Heterozygosity ranges from 0 (all individuals are homozygous and the same at all genes) to 1 (all individuals are heterozygous with different alleles at all genes). It is generally believed that higher levels of heterozygosity are associated with more genetic variation and lower probability of inbreeding depression.

VORTEX can track heterozygosity in two ways. Because VORTEX includes a detailed simulation of genetic change in the populations, one way to measure changes in heterozygosity is to start with maximum heterozygosity (1) by assigning each founder individual two unique alleles at a number of loci (genes). Each offspring is randomly assigned one of the two alleles from each parent at each locus. This method tracks relative loss of heterozygosity through time. Perhaps a more realistic way to examine loss of heterozygosity is to mimic real populations by starting with individuals that have a known level of heterozygosity. VORTEX allows this option by allowing real allele frequencies to be used as a starting point for model simulations. Thus, if we know the level of heterozygosity for a population, we can enter real allele frequencies such that the starting point of the simulations will have exactly that level of heterozygosity. We used the latter of these two approaches and ran the simulations for two different initial levels of heterozygosity (high and low) as reported for two real-world herds (Gus Cothran, Texas A&M University, unpublished reports prepared for BLM). Actual allele frequencies at 13 microsatellite loci were taken from the Lost Creek herd in Wyoming (high genetic diversity, $H_o = 0.78$; report prepared in 2010) and the Cibola-Trigo herd in Arizona (low genetic diversity, $H_o = 0.49$; report prepared in 2008). Absent site-specific information, we typically expect larger herds to have higher genetic diversity.

Removal and Contraception Programs

Removals

Given the limitations of available contraceptive methods, and facing population sizes that greatly exceed the desired number of animals in many herd management areas, BLM currently views contraception as a means of reducing the number of animals that must be removed, and perhaps increasing the interval between gathers, rather than as a replacement for gathers. We therefore simulated a gather-and-removal program that is as similar as possible to the program that BLM uses in the field. We modeled a gather-and-removal event in any year in which the simulated population was 150 percent or more of the initial population, and we attempted to remove sufficient animals to return the population

to its initial size. We removed animals from age classes according to BLM's removal criteria, which specify the following order: ages 4 and under, followed by ages 11–19, followed by ages 5–10. We constrained the number of animals removed, however, such that no more than 90 percent of any group was taken (because it would rarely be possible or desirable to remove every animal from a given age class). Our response variable is the total number of animals removed in 20 years averaged for 100 iterations.

Contraception

We implemented a simulated contraception program using capabilities in VORTEX called "population state" variables and "individual state" variables. Briefly, we used these variables to define the proportion of previously untreated females that are treated in each application and the probability that the treatment works if it is given (efficacy). In most cases, animals in BLM herds need to be gathered in order to apply a fertility control agent; we therefore treated mares following every gather and removal. Killian and others (2008) found that 17 percent of mares treated with SpayVac were pregnant in years 2 through 4 of their study, which implies efficacy of 83 percent. The most aggressive assumption would be that the treatment is 100 percent efficacious. However, it is probably also fair to assume that some mares will not respond to an immunocontraceptive such as SpayVac. We therefore assumed efficacy of 90 percent for all simulations. We also assumed that once a mare is successfully contracepted, she remains contracepted for life without further treatment (we assumed permanent sterility). We varied the proportion of mares treated from 0 to 100 percent in increments of 10 percent to illustrate the range of possible contraceptive effects. For example, when the proportion of mares treated is set at 20 percent, each mare not successfully treated in the past has a 20 percent chance of being caught and treated, and there is a 90 percent chance that the treatment will be effective. Note that high treatment rates (90–100 percent, for example) would likely be very difficult to achieve in the real world because some animals are difficult or impossible to gather.

Random Variation

Environmental Variation in Breeding and Mortality

In addition to the variability inherent in an individual-based model, VORTEX allows specification of variability (standard deviation) in the proportion of females breeding and in the proportion of both sexes dying due to factors external to the population (such as weather, predation, parasite loads). Estimates of variability in these parameters are generally not available for wild horse herds, but sufficient information was available to calculate standard deviations in fecundity and mortality for the Pryor Mountain herd (Roelle and others, 2010).

The standard deviation due to environmental variability, s_{ev}, for either fecundity or mortality, can be expressed as $s_{ev} = \sqrt{s_{tot}^2 - s_{ds}^2}$, where s_{tot}^2 is the total variance in the parameter for all years and s_{ds}^2 is the sampling (binomial) variance for all individual rates. From the Pryor Mountain data set (Roelle and others, 2010), we calculated $s_{ev} = 0.12$ for fecundity and $s_{ev} = 0.06$ for mortality. We combined ages and sexes for both of these calculations and used the resulting values in all simulations.

Concordance of Environmental Variation in Breeding and Mortality

Factors such as those already described (weather, predation, parasite loads) can affect reproduction and survival independently or simultaneously. Simultaneously means that good years for

reproduction are also good years for survival. We know of no information that can be brought to bear on this subject in regard to wild horse demography, but it seems logical that demographic effects would be most pronounced if these parameters vary simultaneously. We therefore opted for concordance in environmental variation.

Number and Types of Catastrophes

The VORTEX software allows specification of catastrophes (floods, forest fires, epidemic diseases) through particular effects on reproduction and survival. Perhaps the most common such event on western ranges is drought, and although droughts occur fairly frequently, we know of no specific information relating drought to reproduction and survival in wild horses. Nevertheless, we felt that it was important to include some form of catastrophe in our simulations. We therefore specified that drought would have a 10 percent probability in any year and that it would reduce reproduction to 50 percent of the normal level and survival to 75 percent of the normal level.

Distribution of Litter Sizes

VORTEX allows for one additional form of random variation if females can produce litters of different sizes. Because wild horses almost never produce more than one offspring, we did not use this parameter.

Simulation Control

Number of Iterations

As noted in the previous section, VORTEX uses a random number generator to simulate random events in an organism's life cycle. Therefore, no two runs or iterations of the model are identical even with a single set of input data. To obtain a more complete picture of demographic behavior, multiple iterations are required. We explored changes in the standard deviation of both mean population size and heterozygosity after 50 years while varying the number of iterations from 25 to 1,000. Because of the complexity of the removal and contraception functions, no consistent pattern emerged in the standard deviations of these parameters. More particularly, increasing the number of iterations to more than 100 did not consistently lead to lower variance about the mean. We therefore chose 100 iterations for all of our simulations.

Number of Years per Iteration

Initial simulations with some basic data indicated that significant changes in heterozygosity are rare in less than 50 years. On the other hand, significant demographic changes can occur in as little as 20 years. We therefore evaluated mean population size and removals at the end of 20 years of simulation, but we used 50 years to evaluate heterozygosity and probability of extirpation.

Number of Populations

VORTEX can be used to model a single, isolated population or a complex metapopulation composed of as many as 50 populations with interchange of animals between them. Although it is clear that some animals move between certain populations of wild horses, we know of no information on

rates of such movement. We therefore modeled only a single population without introduction of animals from outside, which is the worst case from the perspective of genetic diversity.

Extirpation Definition

VORTEX offers several methods for defining extirpation of a population. We used the definition that says a population is extirpated when it no longer contains individuals of both sexes.

Interpreting Model Output

Appendix A contains output from 70 sets of simulations representing two levels of genetic diversity (low or high), seven initial population sizes (100, 250, 500, 750, 1,000, 1,250, or 1,500 animals), and five population growth multipliers (1.05, 1.10, 1.15, 1.20, or 1.25). Each set of simulations is represented by a figure, and each figure contains five graphs and a table of supporting data. The order of the figures varies first by annual growth rate, then by population size, and then by genetic diversity. Thus, figure 1 has low genetic diversity, an initial population size of 100, and a growth multiplier of 1.05, and is followed by figure 2 with low genetic diversity, an initial population size of 100, and a growth multiplier of 1.10, and so on. In each figure, the first four (black and white) graphs and the table represent the same set of simulations, and the graphs and table have identical values. Due to a nuance in the way VORTEX operates, however, the final (color) graph represents a different set of simulations having the same parameter values but differing slightly due to random variation as already discussed. Thus, the value for average population size at 20 years on the color graph will not in general be identical to that in the table, but the two values will usually be very similar.

The black and white graphs present mean values (that is, averaged for 100 iterations) of four response variables on the vertical axes: mean population size after 20 years, mean number of removals after 20 years, mean probability of extirpation after 50 years, and mean heterozygosity in the herd after 50 years. In all cases, the horizontal axis is the percentage of previously untreated mares that are treated following each gather, varying from 0 percent to 100 percent. The colored graph represents the mean trajectory of the population size for a subset of the treatment levels (0 percent, 20 percent, 40 percent, and so on) depicted as lines of different color. It is very important to understand that there is some information that cannot be conveyed by mean values. For example, there are many cases where the table and black and white graphs show that animals were removed, but the colored graph lines never plot higher than 1.5 × the initial population. The appropriate interpretation is that on average the population stayed below the threshold (1.5 × the initial population), but in some individual iterations the population grew sufficiently to trigger a removal.

As an example application of model results, suppose a manager wants to know what proportion of mares can be treated with a sterilant in a herd having low genetic diversity, an initial population size of about 750, and a population growth multiplier of 1.15. This set of conditions is represented by figure 18 in appendix A. The first thing to notice about figure 18 is that no treatment level has any real effect on either mean probability of extirpation after 50 years or mean heterozygosity after 50 years. The graph of mean population size after 20 years, however, shows that treatment levels greater than 50 percent of the mares result in populations somewhat less than 750. We also see that if 50 percent of the mares are treated, the mean number of removals after 20 years is around 900, or about 500 fewer than in the scenario where no mares are treated. The number of removals can be further reduced by treating a higher proportion of mares, but only if average population levels below 750 are acceptable. The average trajectory of the population size through time can be examined by looking at the last (color) graph in the

figure 18, which also shows that treatment rates more than about 50 percent will result in populations that are commonly below the starting point of 750 animals.

Finally, uncertainties about level of genetic diversity, population size, or growth rate can be addressed by examining figures representing nearby values in the parameter space. Thus, in the example in the preceding paragraph, a manager might also wish to look at the figures representing 500 and 1,000 animals in the population and growth multipliers of 1.10 and 1.20 to gain some understanding of how sensitive model results are to these assumptions.

Conclusions

We believe that simulation results presented herein represent a useful starting point for evaluating a management program involving a contraceptive technique that is permanent (or nearly so). In particular, the results show that only in the most extreme circumstances (such as low initial genetic diversity, low population growth rate, high proportion of mares treated, no change in management for 50 years) would there likely be any noticeable effect on genetic diversity or a significant probability of extirpation of a herd. Monitoring and adaptive management would reduce the probability of unacceptable results even further. For example, a simple exchange program in which an animal or two were moved between herds periodically would help to maintain genetic diversity, and the contraceptive program could always be modified or discontinued in the event that the reduction of population was greater than anticipated. In short, we see nothing in our results that should steer wild horse managers away from permanent contraceptive techniques, as long as results are monitored and adjustments are made if necessary.

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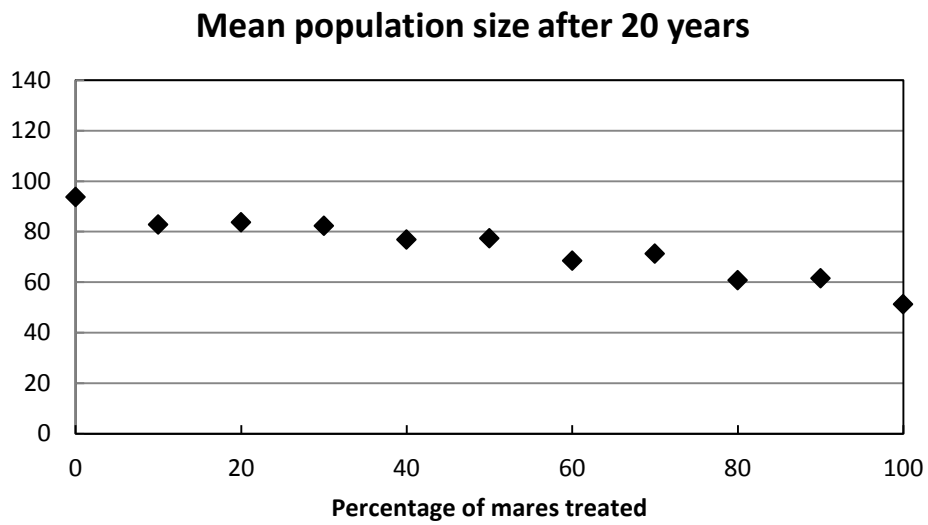
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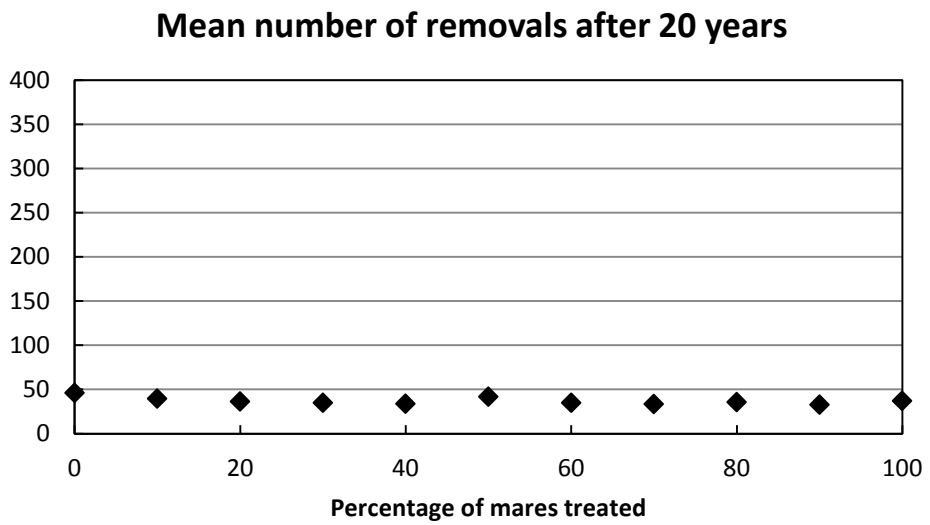
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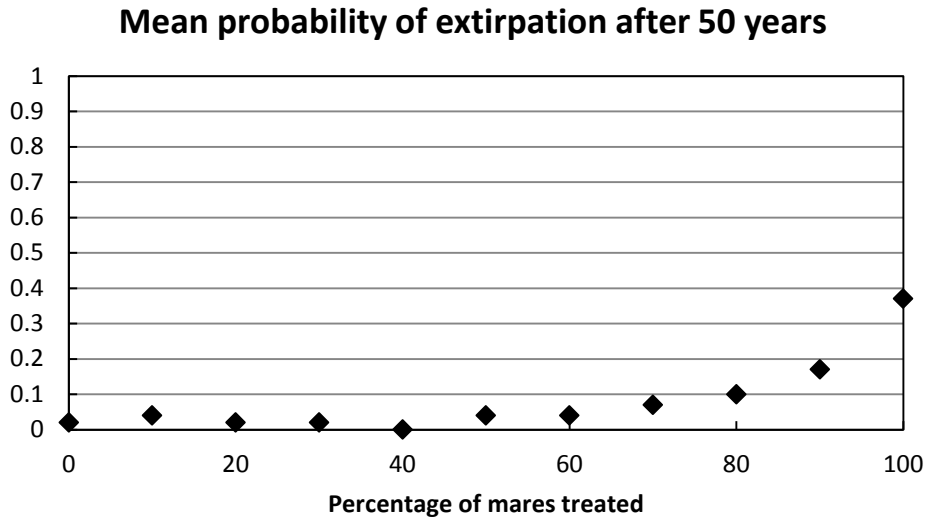
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Appendix A. Simulation Results







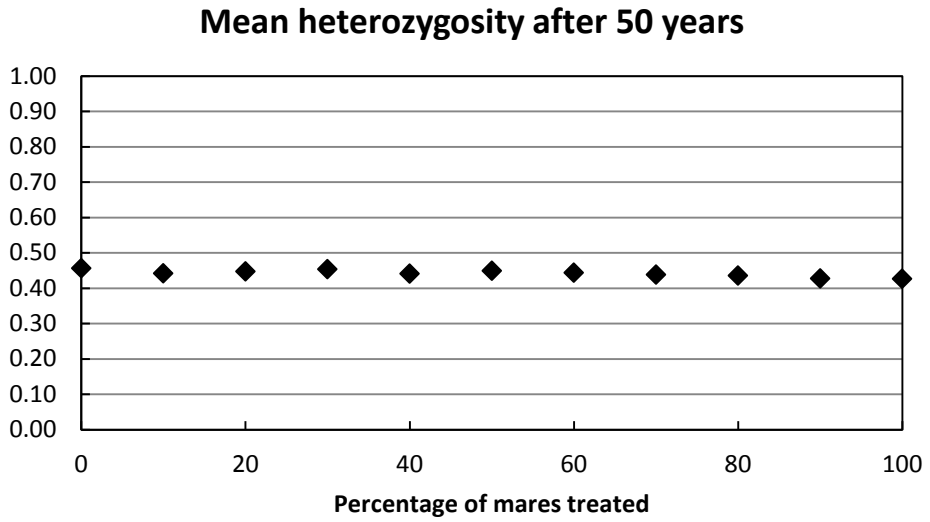


Figure 1. Initial population size = 100, lambda = 1.05, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	93.71	82.73	83.68	82.28	76.81	77.32	68.49	71.19	60.66	61.53	51.22
Mean number of removals after 20 years	45.83	39.52	36.34	34.74	33.65	41.69	34.88	33.26	35.4	32.7	36.94
Mean probability of extirpation after 50 years	0.02	0.04	0.02	0.02	0	0.04	0.04	0.07	0.1	0.17	0.37
Mean heterozygosity after 50 years	0.46	0.44	0.45	0.45	0.44	0.45	0.44	0.44	0.44	0.43	0.43

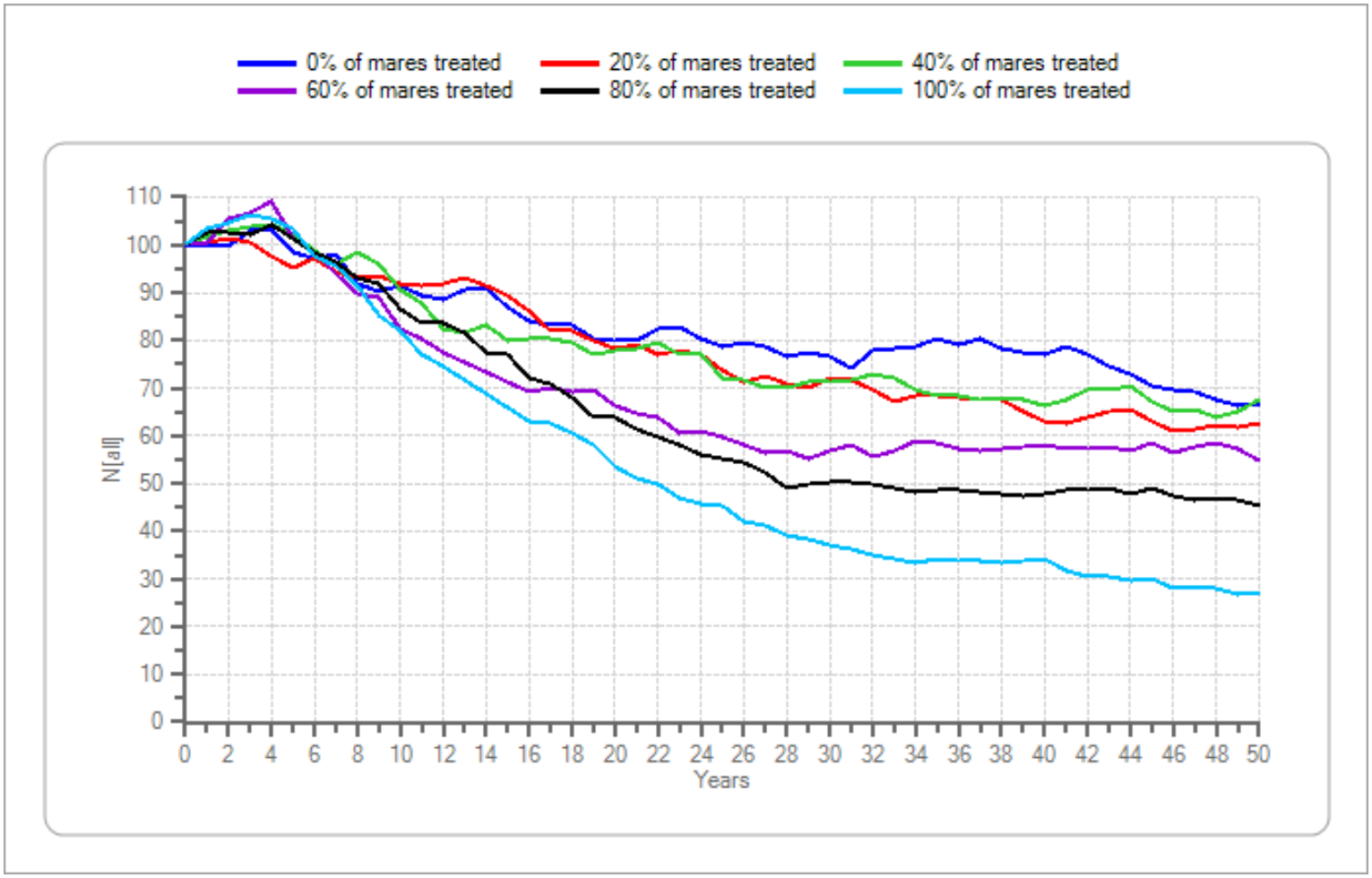


Figure 1. Initial population size = 100, lambda = 1.05, genetic diversity = low.—Continued

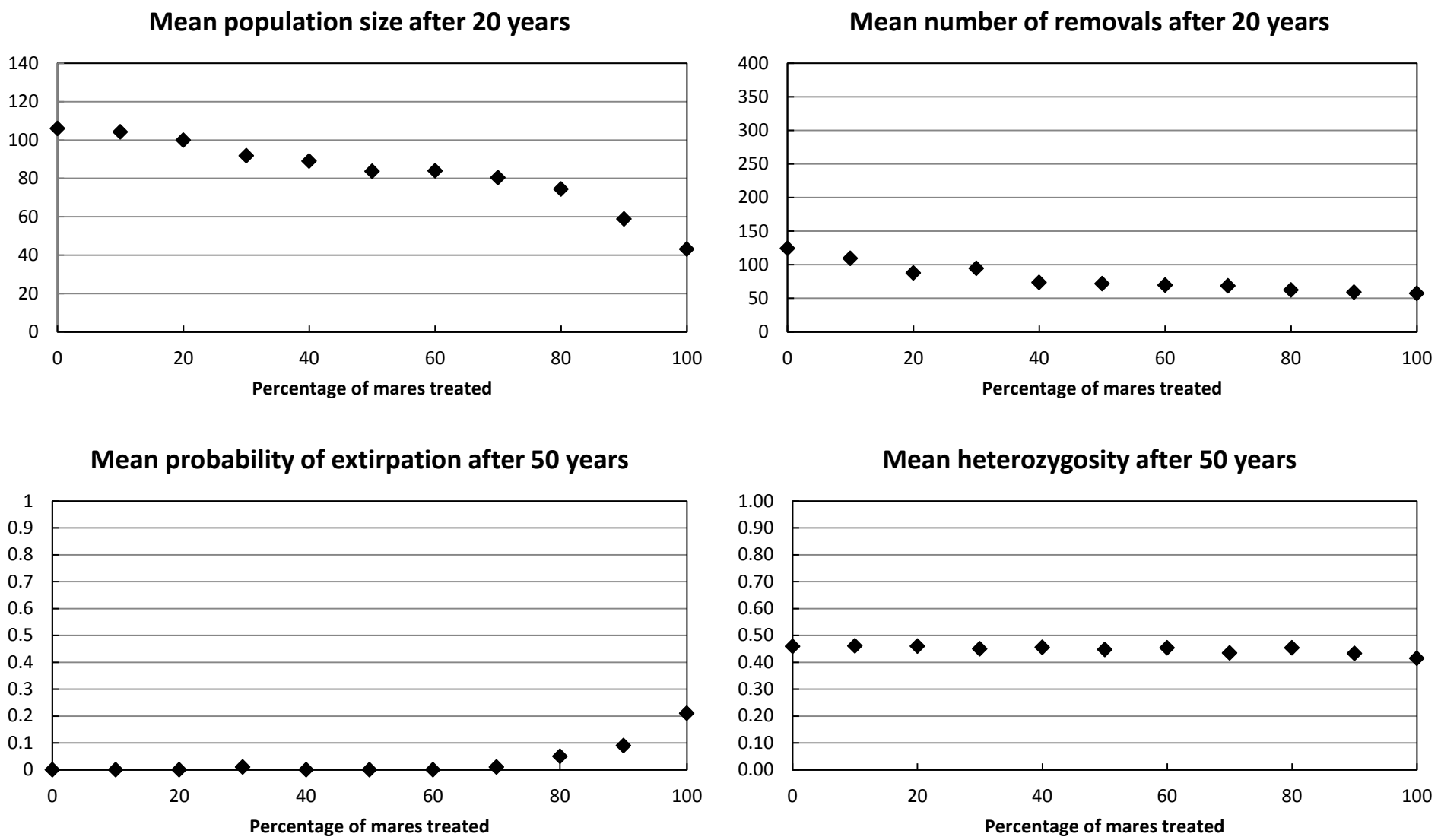


Figure 2. Initial population size = 100, lambda = 1.10, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	105.96	104.13	99.93	91.74	89	83.71	83.91	80.41	74.37	58.79	43.07
Mean number of removals after 20 years	124.34	109.22	87.59	94.47	73.54	71.53	69.56	68.28	62.33	58.98	57.08
Mean probability of extirpation after 50 years	0	0	0	0.01	0	0	0	0.01	0.05	0.09	0.21
Mean heterozygosity after 50 years	0.46	0.46	0.46	0.45	0.46	0.45	0.45	0.43	0.45	0.43	0.41

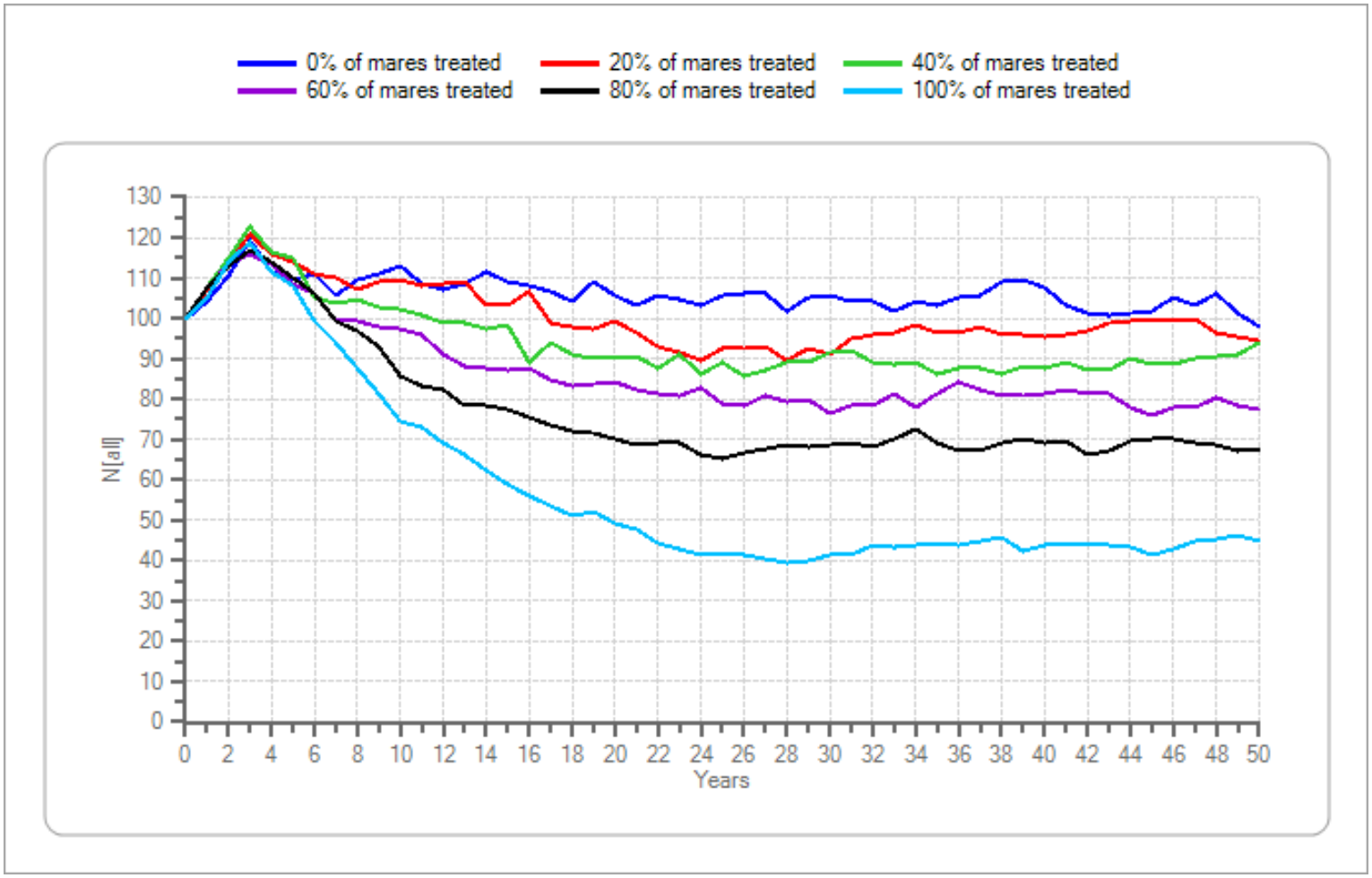


Figure 2. Initial population size = 100, lambda = 1.10, genetic diversity = low.—Continued

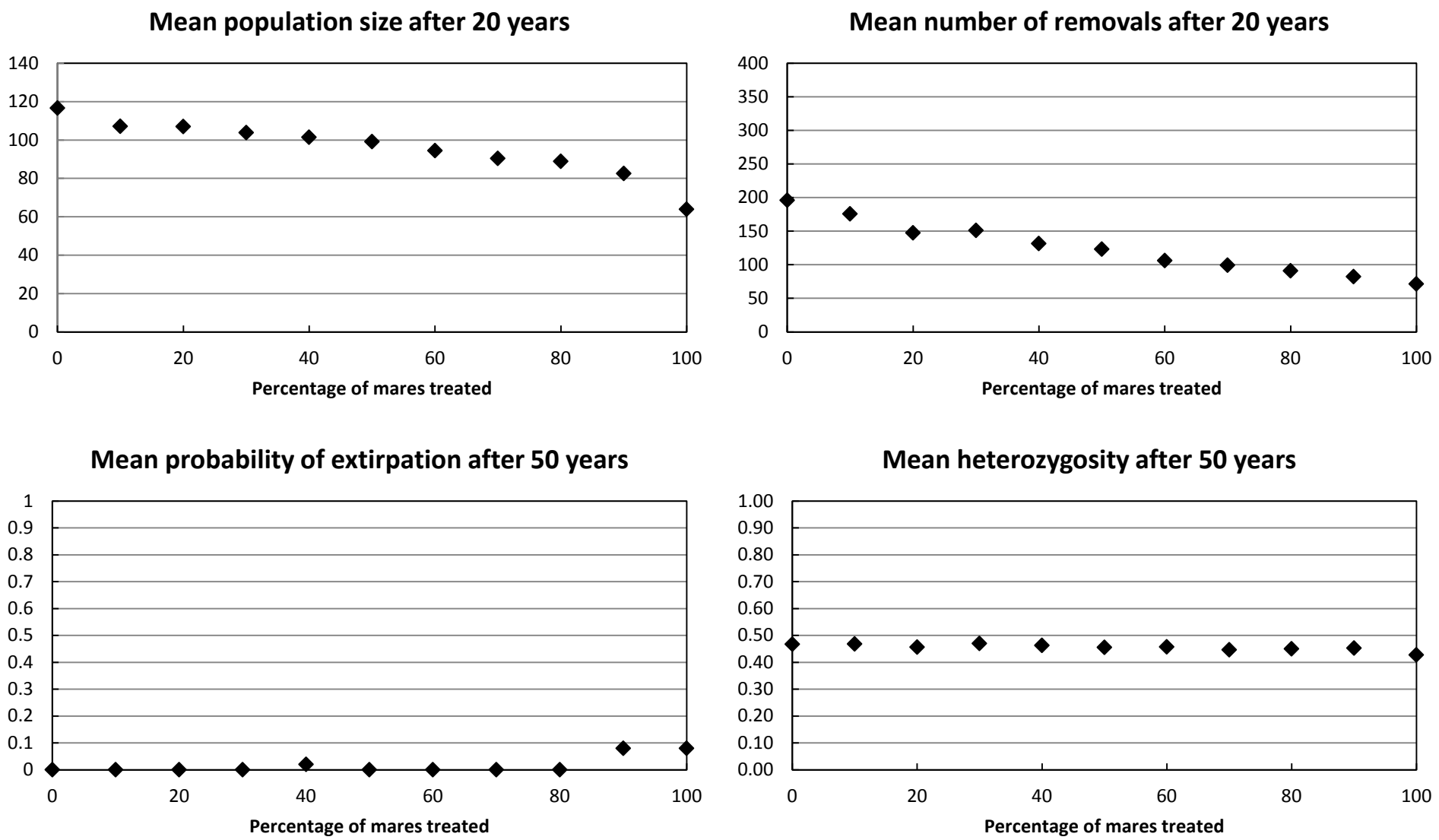


Figure 3. Initial population size = 100, lambda = 1.15, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	116.67	107.04	106.93	103.8	101.36	99.18	94.47	90.36	88.82	82.5	63.9
Mean number of removals after 20 years	196.1	175.66	147.22	150.85	131.53	123.08	106.05	99.21	90.8	82.28	71.23
Mean probability of extirpation after 50 years	0	0	0	0	0.02	0	0	0	0	0.08	0.08
Mean heterozygosity after 50 years	0.47	0.47	0.46	0.47	0.46	0.46	0.46	0.45	0.45	0.45	0.43

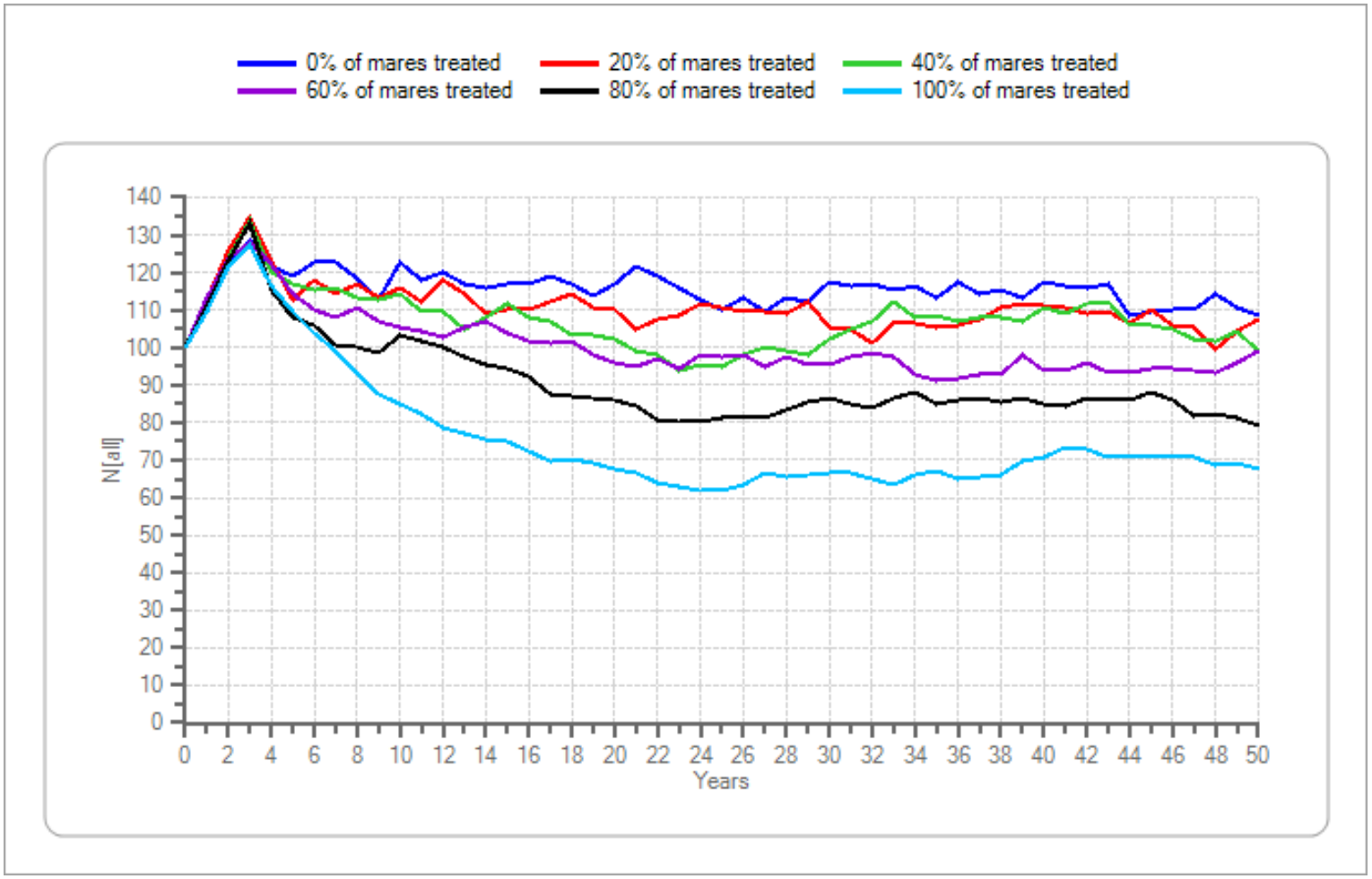
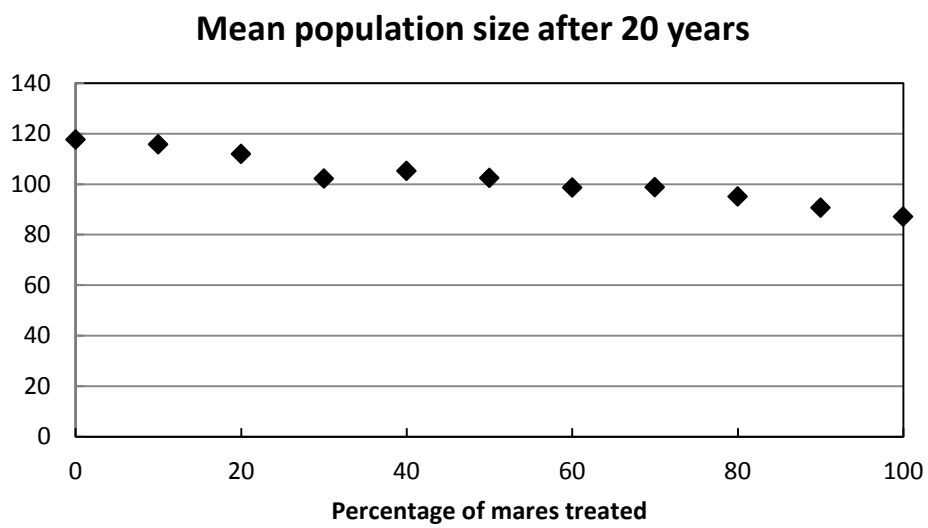
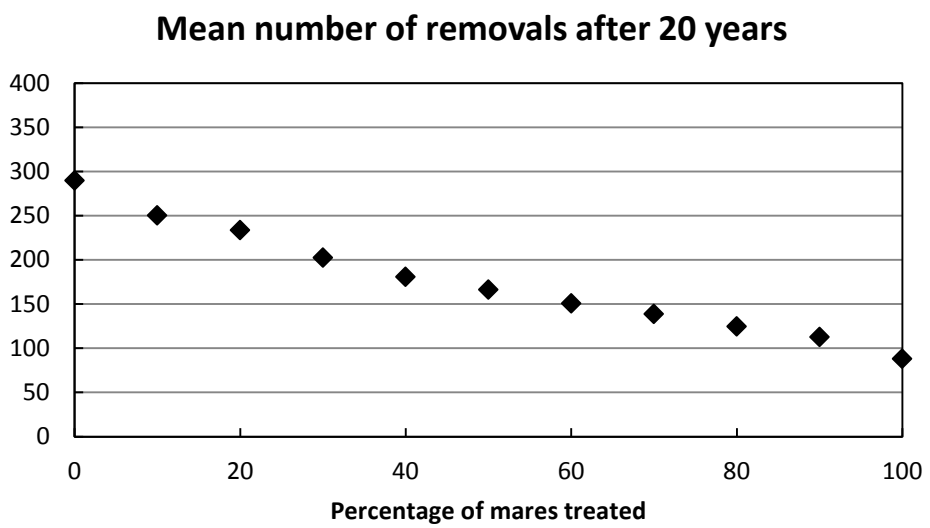
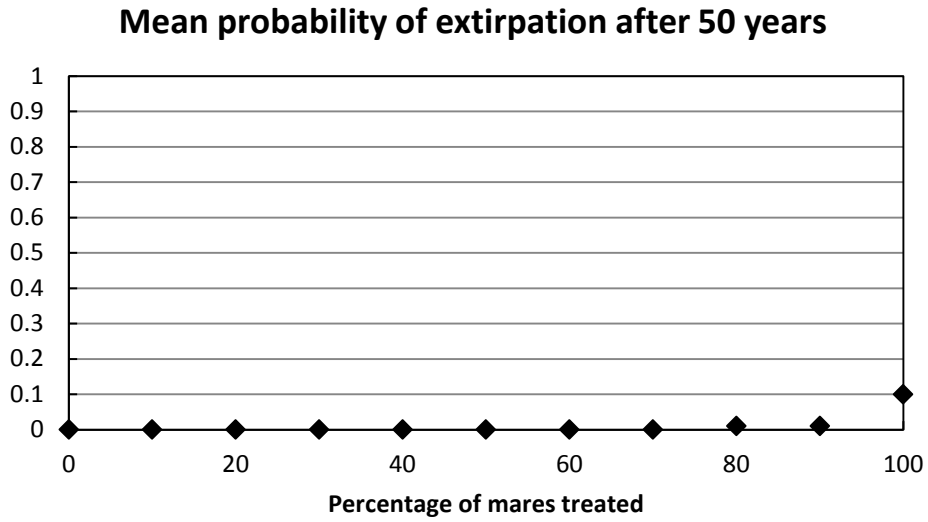


Figure 3. Initial population size = 100, lambda = 1.15, genetic diversity = low.—Continued







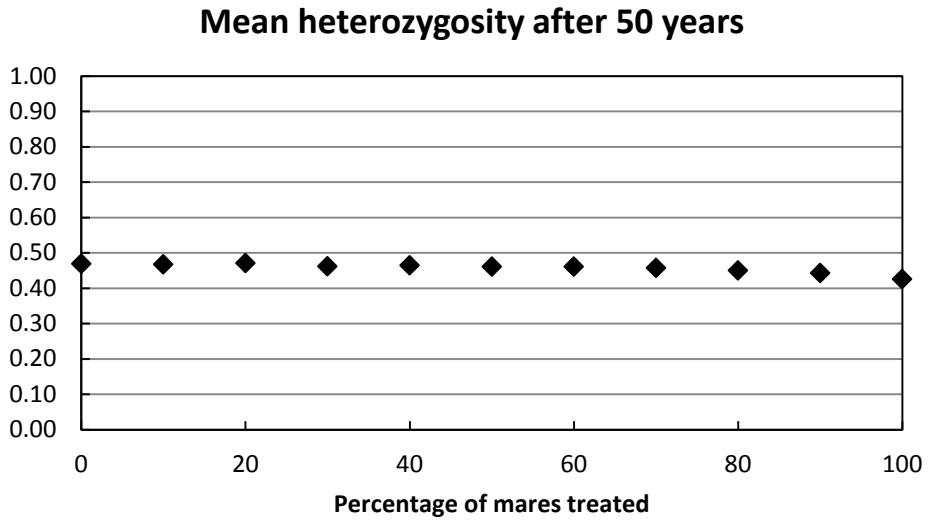


Figure 4. Initial population size = 100, lambda = 1.20, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	117.66	115.72	111.98	102.21	105.14	102.37	98.63	98.73	95.01	90.61	87.04
Mean number of removals after 20 years	289.73	250.34	233.51	202.32	180.67	166.39	150.74	138.75	124.57	112.71	88.13
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0.01	0.01	0.1
Mean heterozygosity after 50 years	0.47	0.47	0.47	0.46	0.46	0.46	0.46	0.46	0.45	0.44	0.43

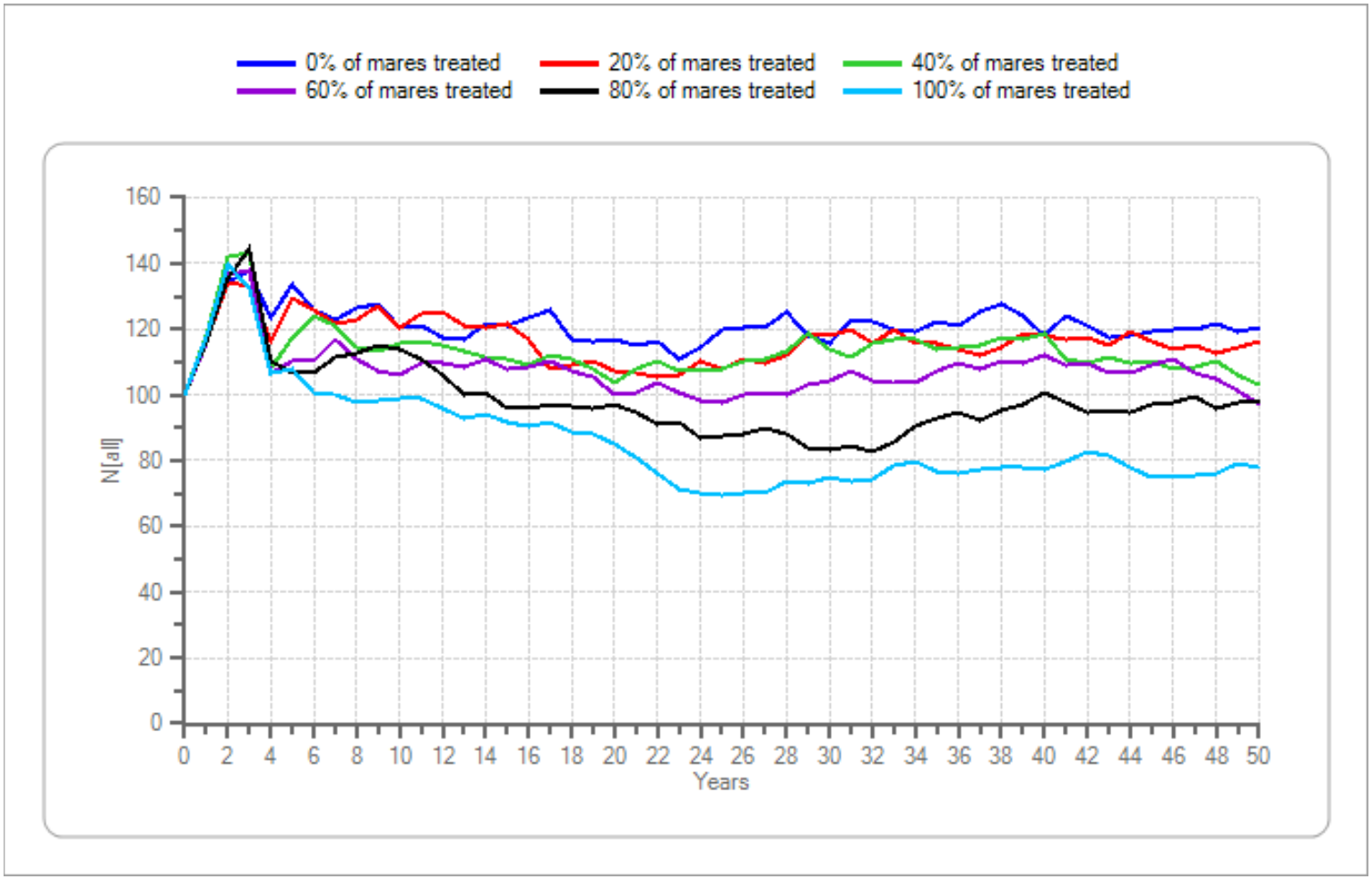


Figure 4. Initial population size = 100, lambda = 1.20, genetic diversity = low.—Continued

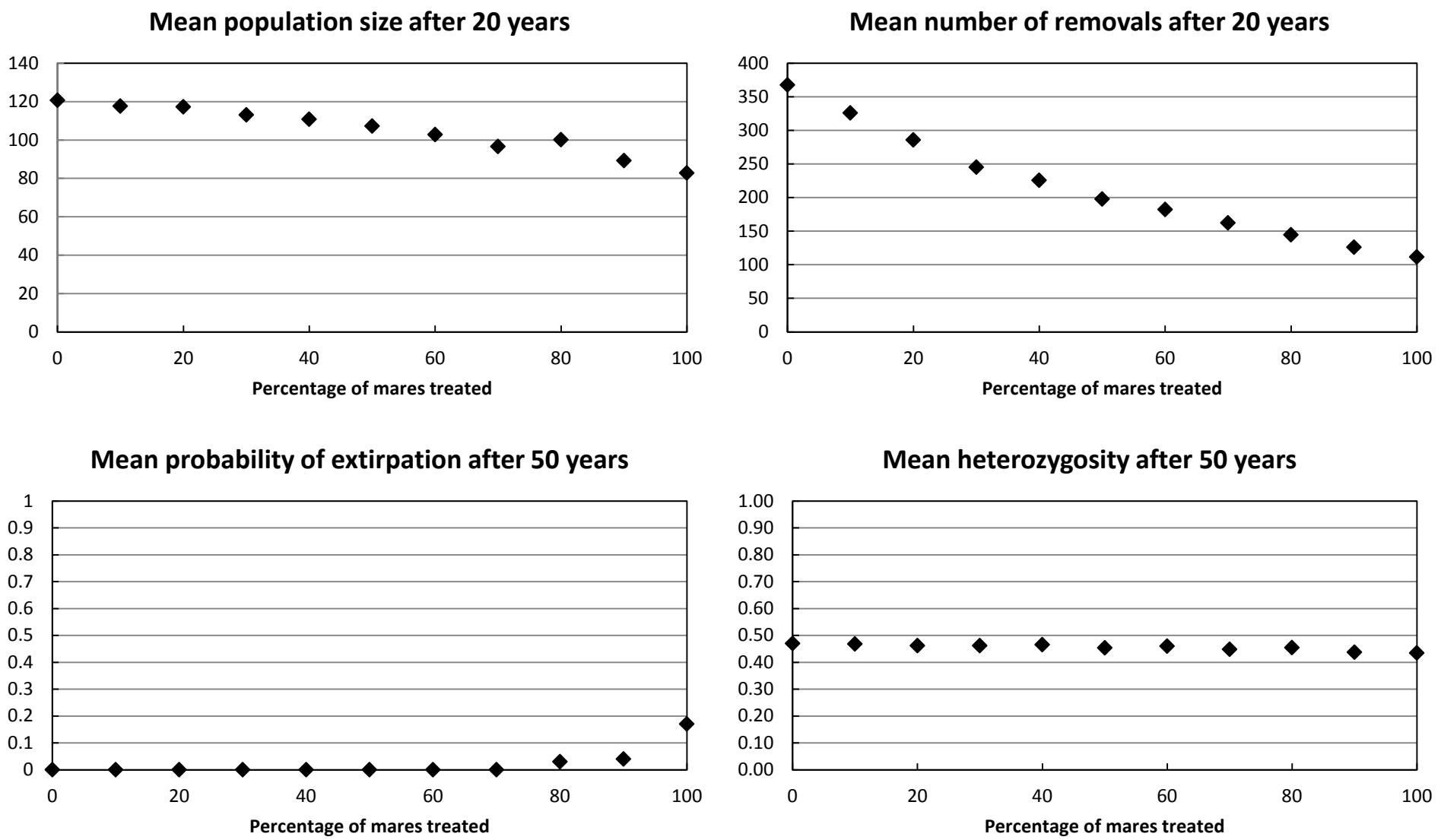


Figure 5. Initial population size = 100, lambda = 1.25, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	120.71	117.59	117.22	113.11	110.75	107.17	102.75	96.64	100.1	89.18	82.72
Mean number of removals after 20 years	367.4	325.91	285.86	245.19	225.62	197.56	182.24	162.3	144.4	126.13	111.72
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0.03	0.04	0.17
Mean heterozygosity after 50 years	0.47	0.47	0.46	0.46	0.47	0.45	0.46	0.45	0.45	0.44	0.43

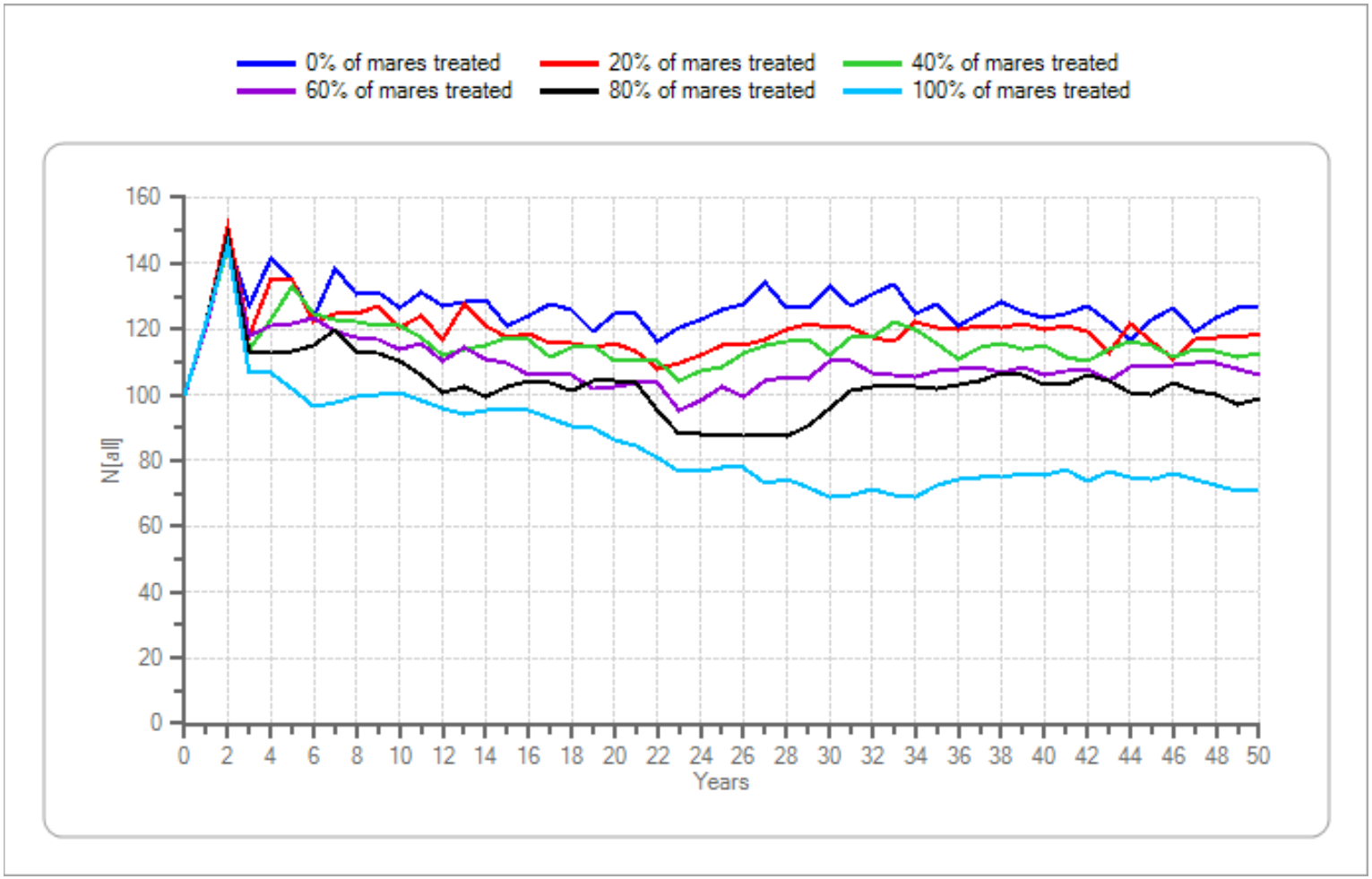
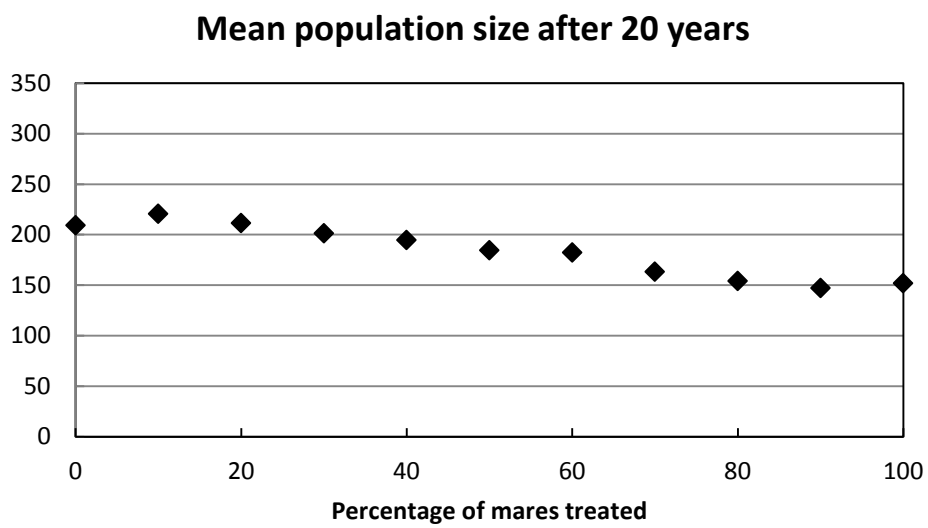
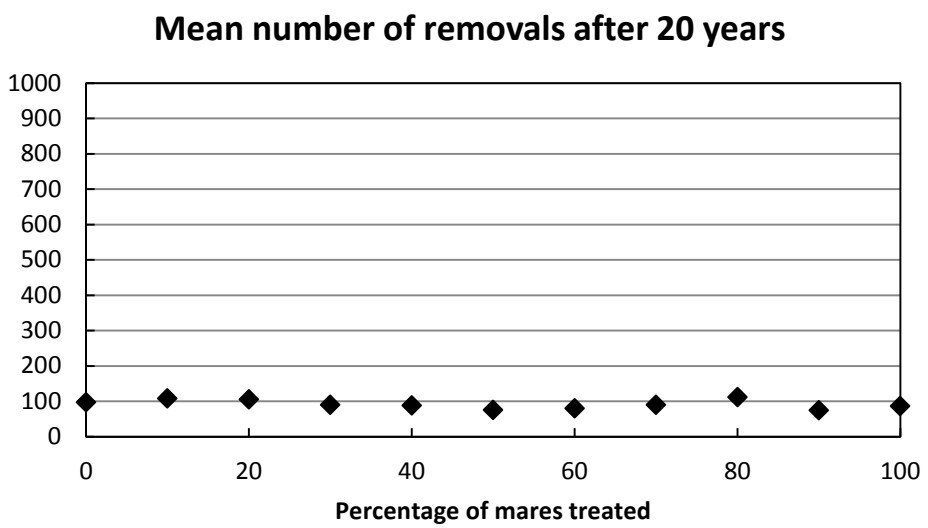
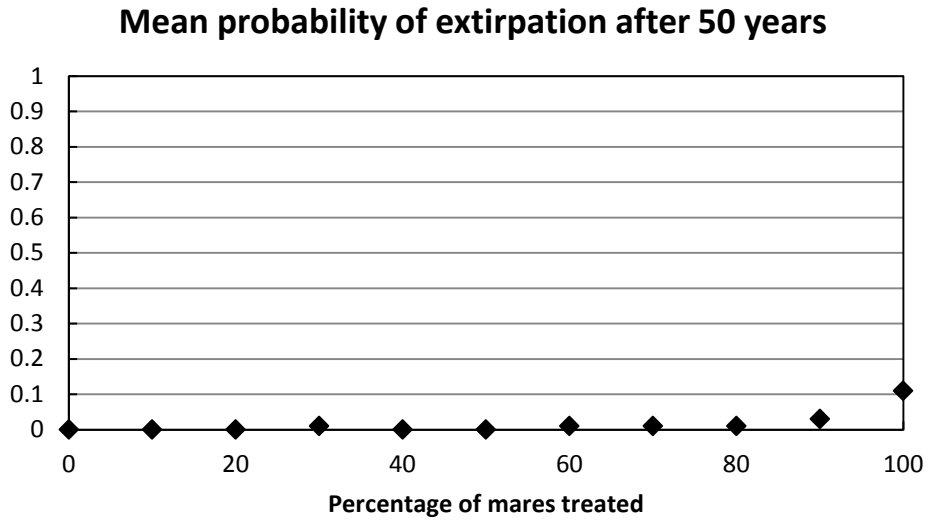


Figure 5. Initial population size = 100, lambda = 1.25, genetic diversity = low.—Continued







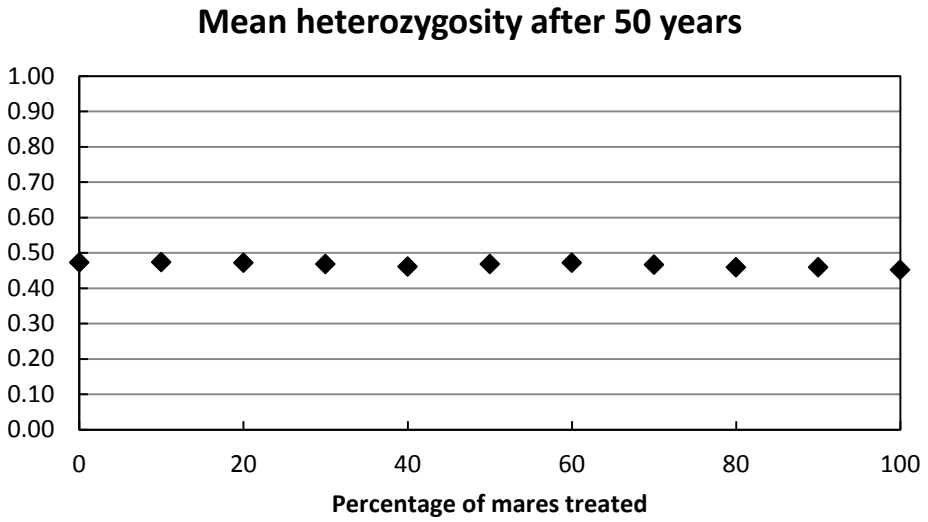


Figure 6. Initial population size = 250, lambda = 1.05, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	209.16	220.46	211.25	201.33	194.71	184.37	182.2	163.33	153.91	147.12	151.88
Mean number of removals after 20 years	96.65	107.54	105.05	89.47	87.49	75.07	79.56	89.29	111.56	74	85.79
Mean probability of extirpation after 50 years	0	0	0	0.01	0	0	0.01	0.01	0.01	0.03	0.11
Mean heterozygosity after 50 years	0.47	0.47	0.47	0.47	0.46	0.47	0.47	0.47	0.46	0.46	0.45

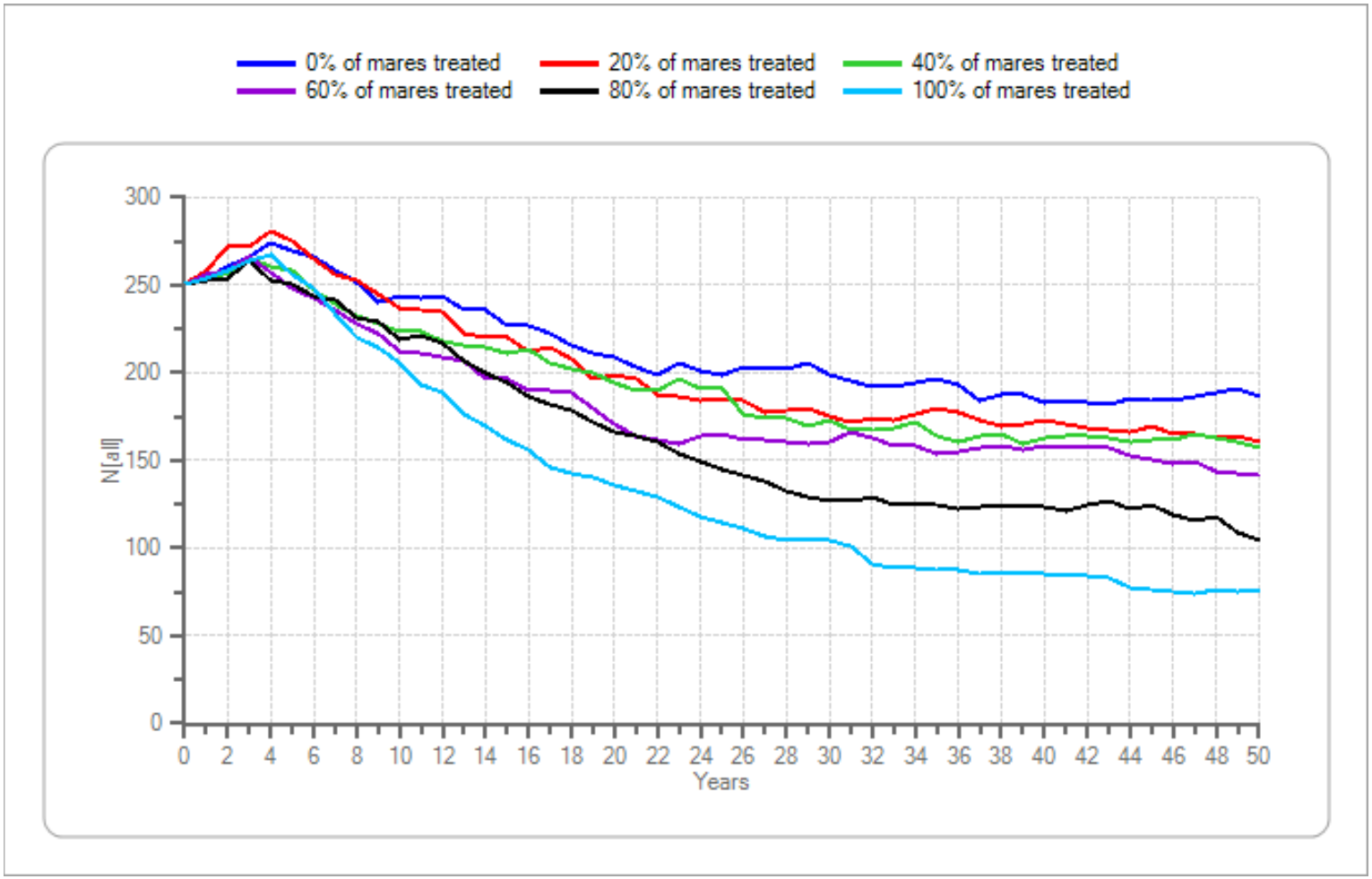
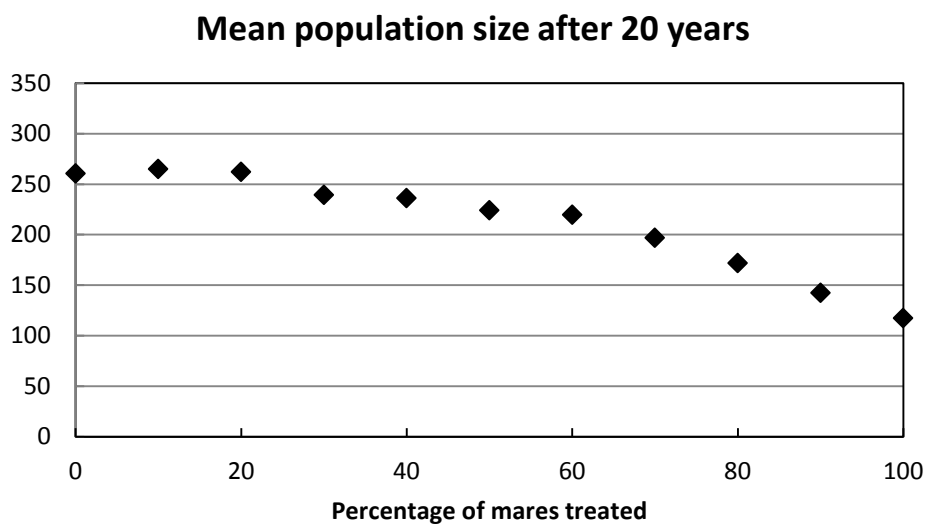
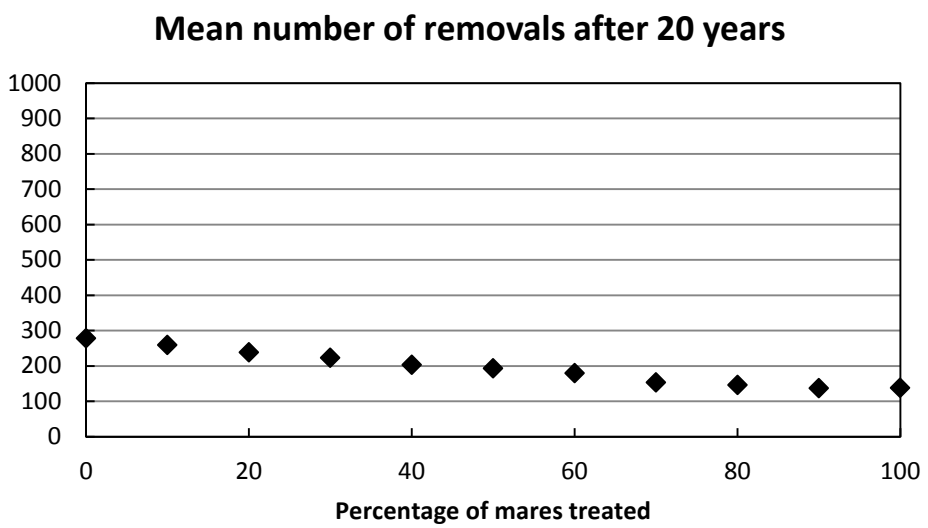
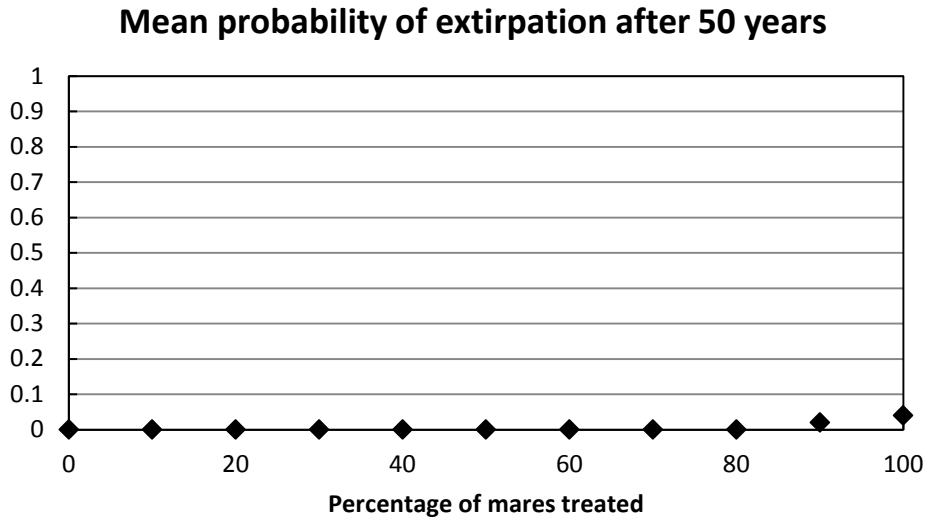


Figure 6. Initial population size = 250, lambda = 1.05, genetic diversity = low.—Continued







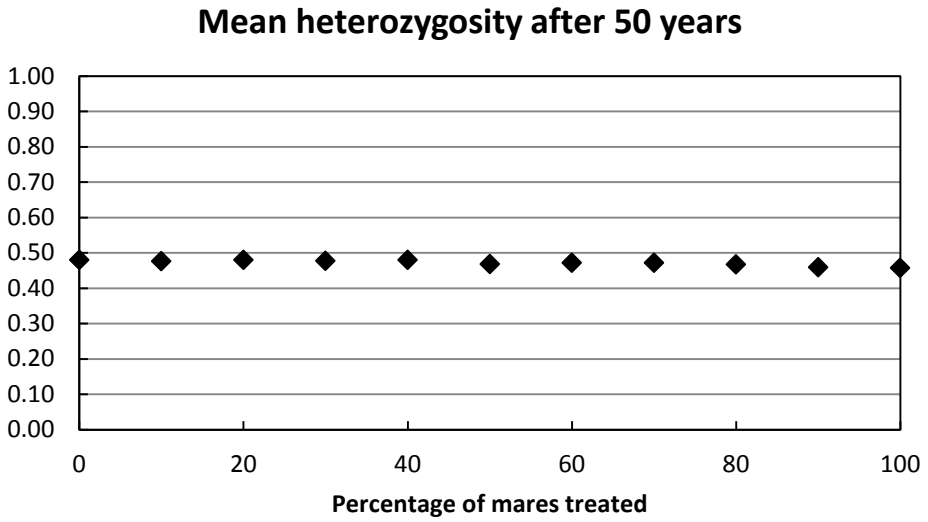


Figure 7. Initial population size = 250, lambda = 1.10, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	260.38	264.77	262.05	239.17	236	224.01	219.74	196.91	171.78	142.36	117.4
Mean number of removals after 20 years	278.3	259.21	237.83	223.07	202.93	192.93	179.33	153.23	145.94	137.16	137.79
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0.02	0.04
Mean heterozygosity after 50 years	0.48	0.48	0.48	0.48	0.48	0.47	0.47	0.47	0.47	0.46	0.46

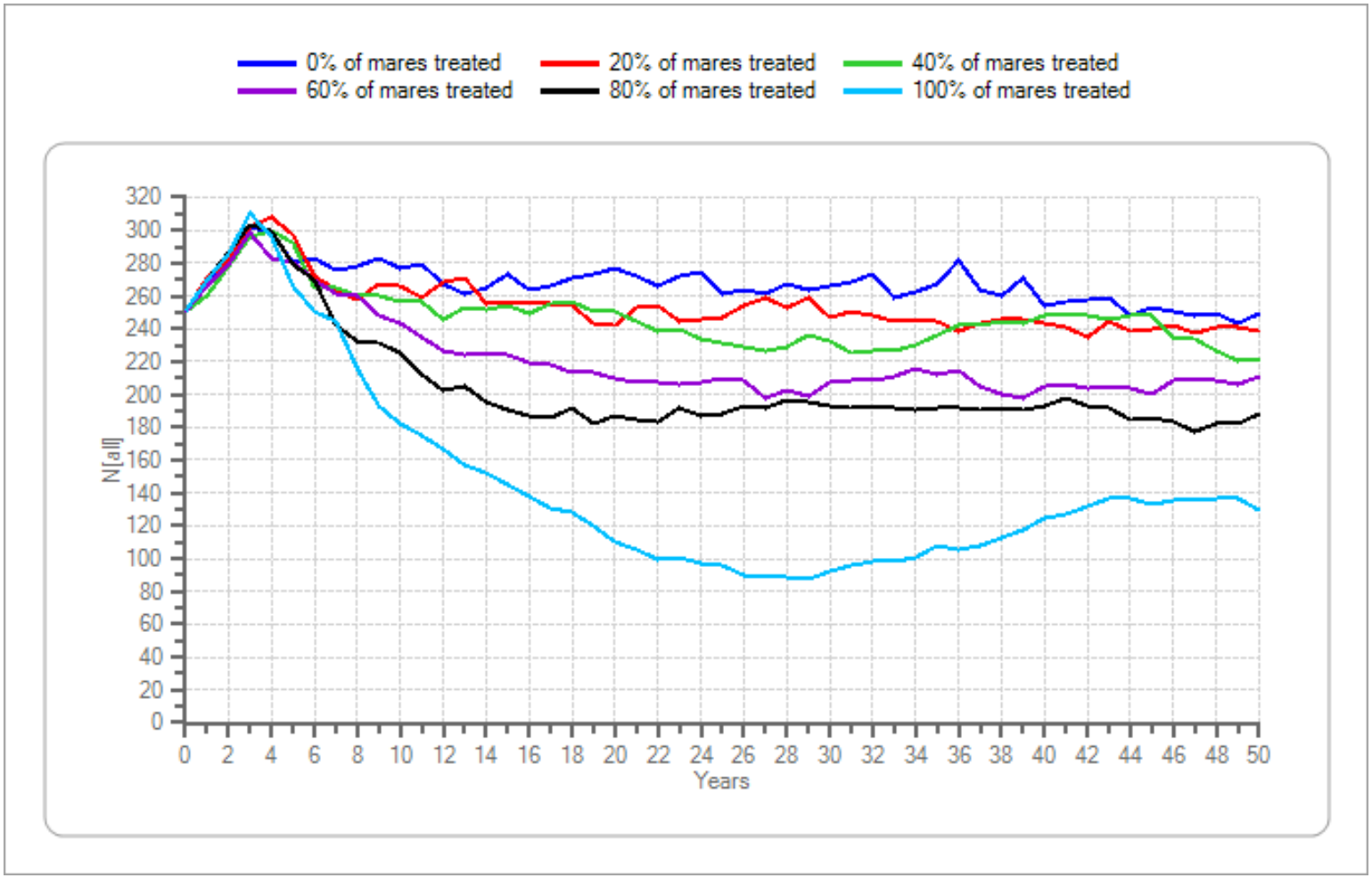
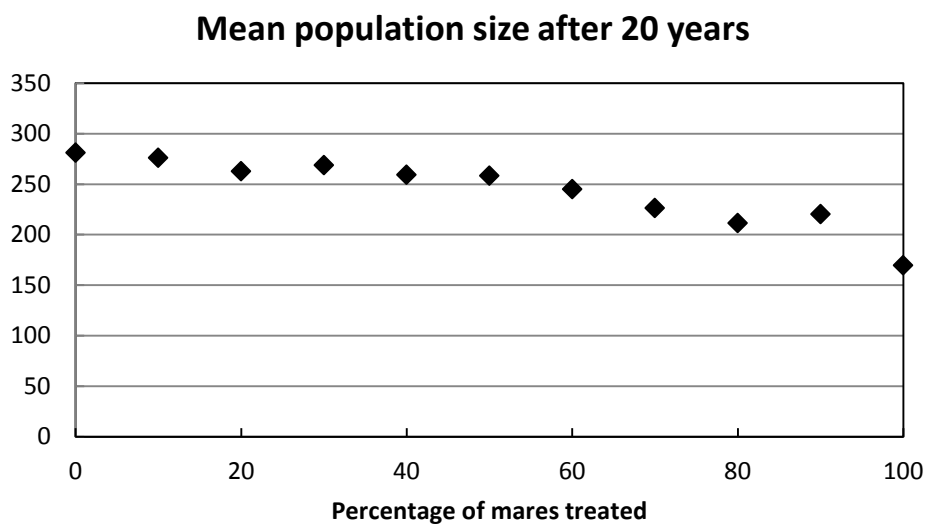
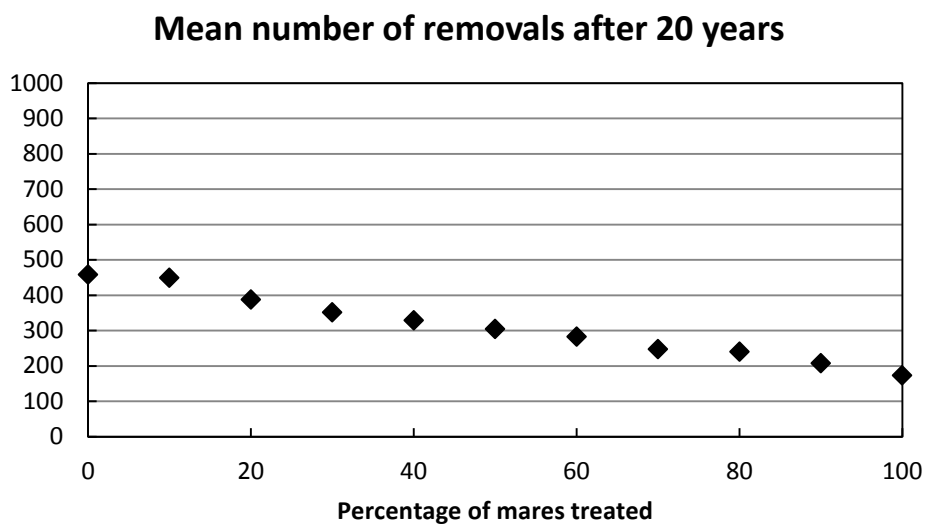
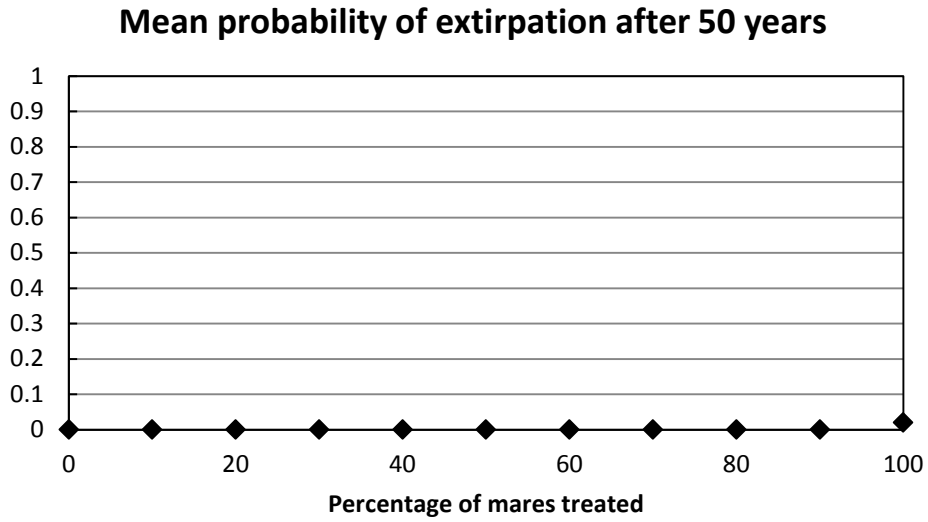


Figure 7. Initial population size = 250, lambda = 1.10, genetic diversity = low.—Continued







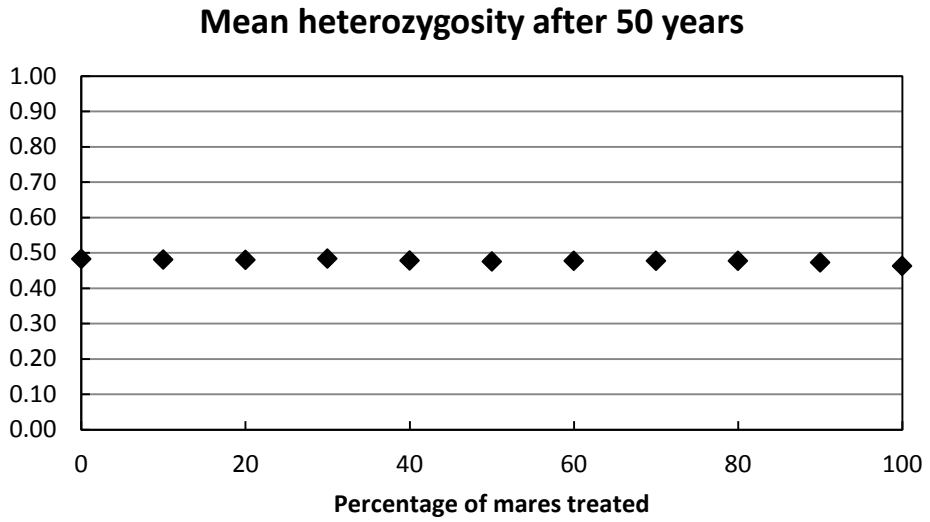


Figure 8. Initial population size = 250, lambda = 1.15, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	281.13	275.97	262.64	268.65	259.12	258.31	244.81	226.37	211.37	220.14	169.63
Mean number of removals after 20 years	458.22	448.92	387.51	351.03	328.21	304.61	282.41	247.07	239.97	207.47	173.26
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0.02
Mean heterozygosity after 50 years	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.47	0.46

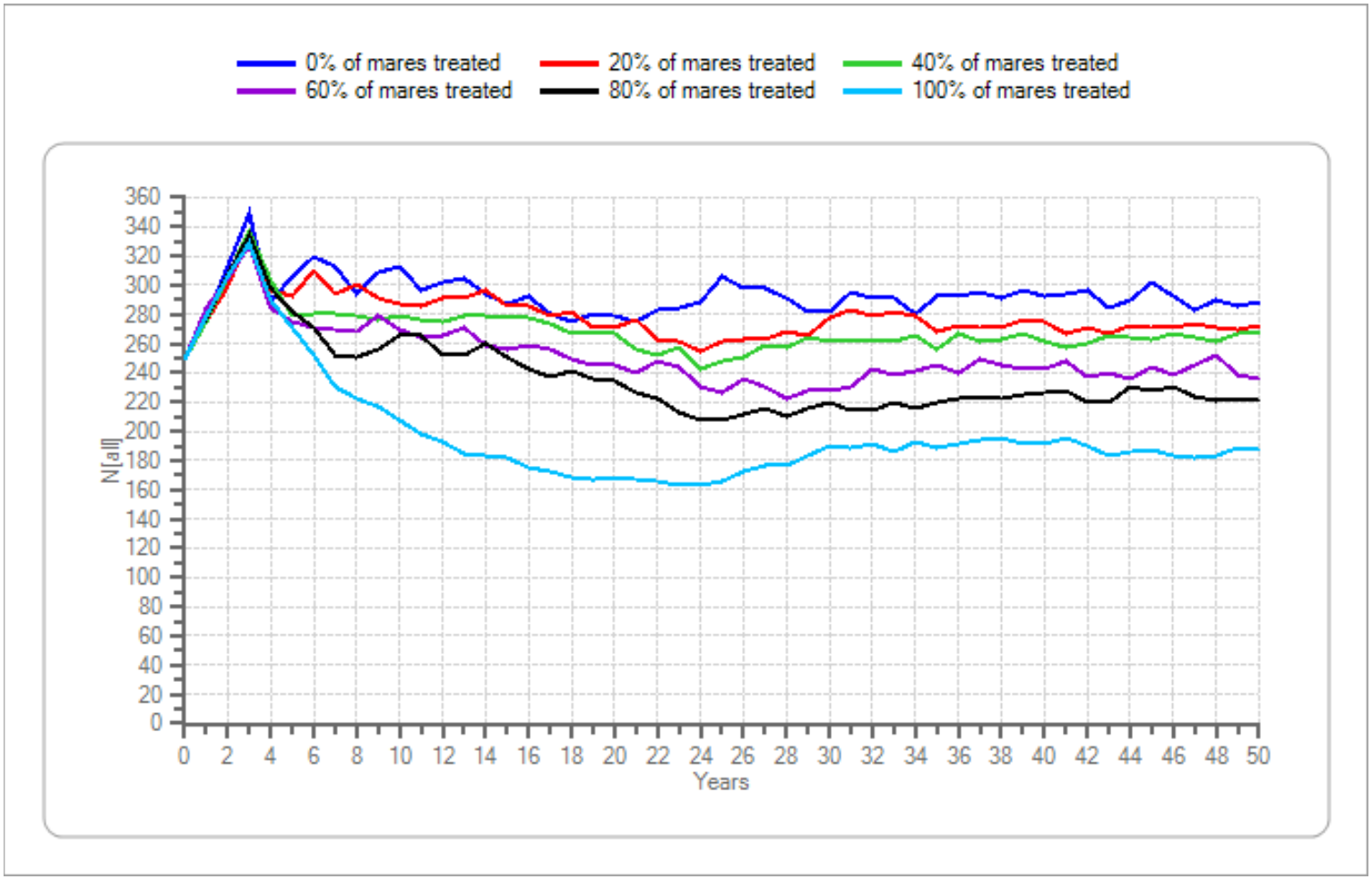
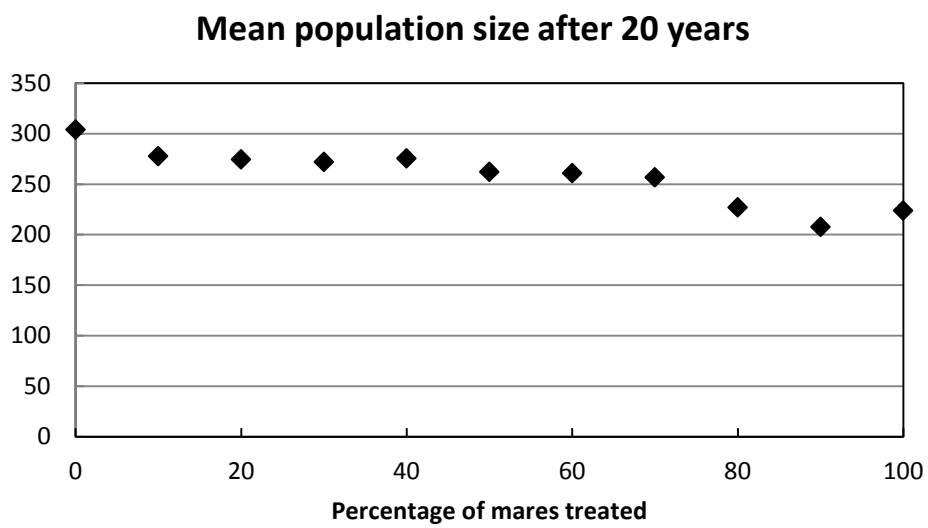
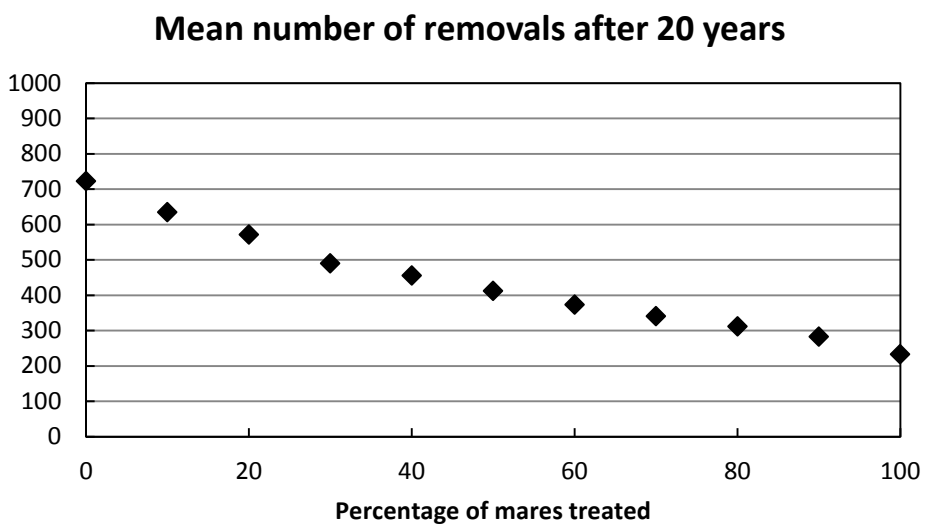
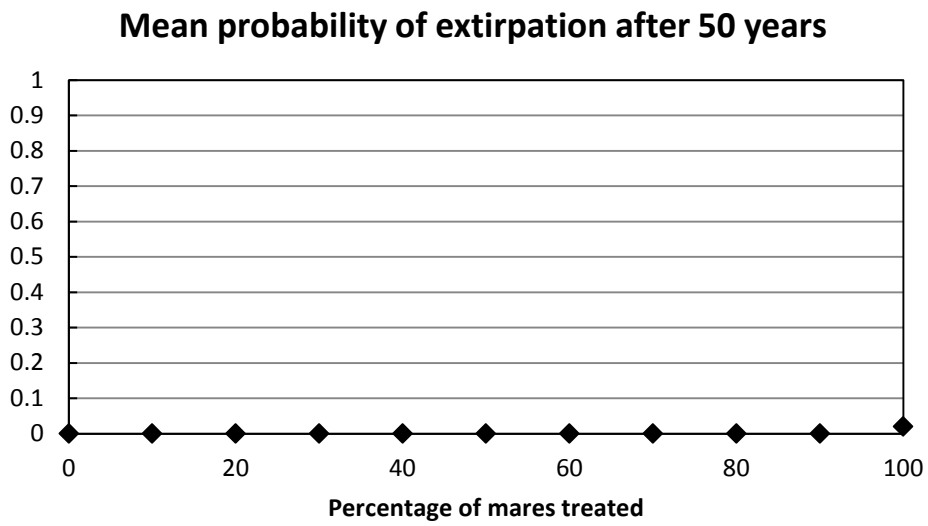


Figure 8. Initial population size = 250, lambda = 1.15, genetic diversity = low.—Continued







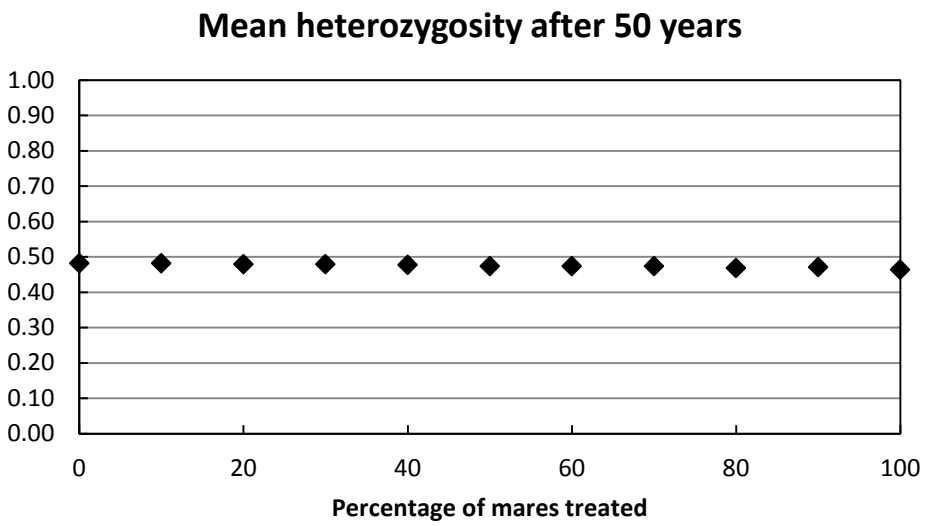


Figure 9. Initial population size = 250, lambda = 1.20, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	303.72	277.47	274.27	271.98	275.36	262.1	260.81	256.62	226.89	207.59	223.76
Mean number of removals after 20 years	722.53	634.9	570.99	490.14	455.85	412	373.4	340.01	311.73	282.63	232.31
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0.02
Mean heterozygosity after 50 years	0.48	0.48	0.48	0.48	0.48	0.47	0.47	0.47	0.47	0.47	0.46

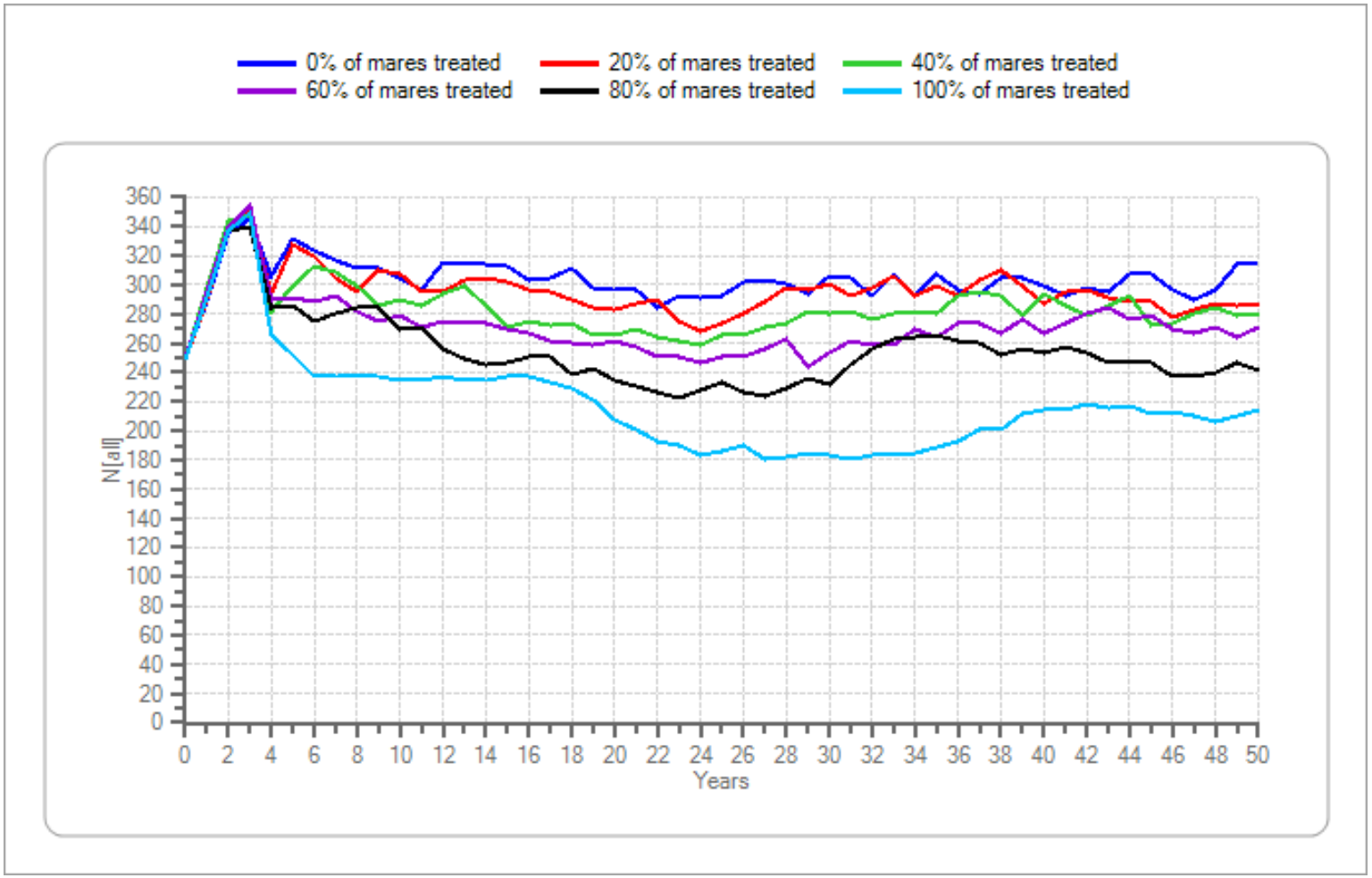
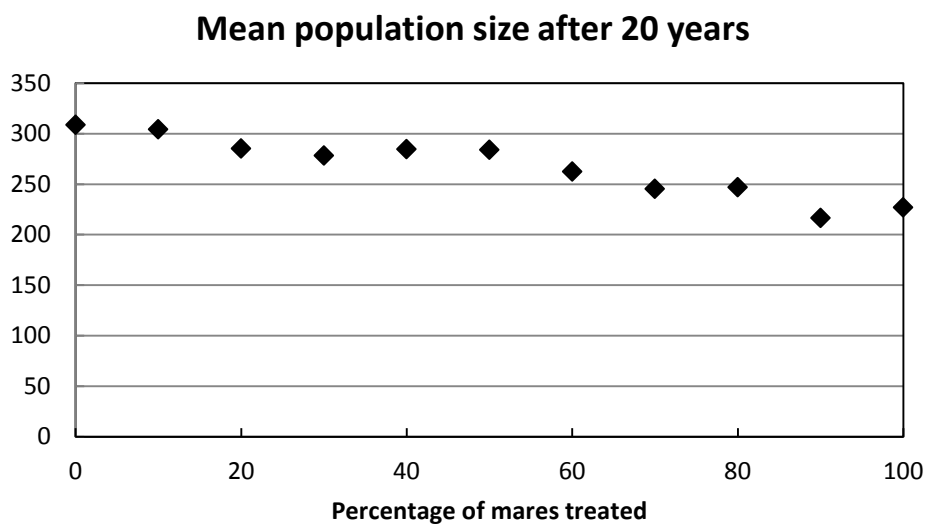
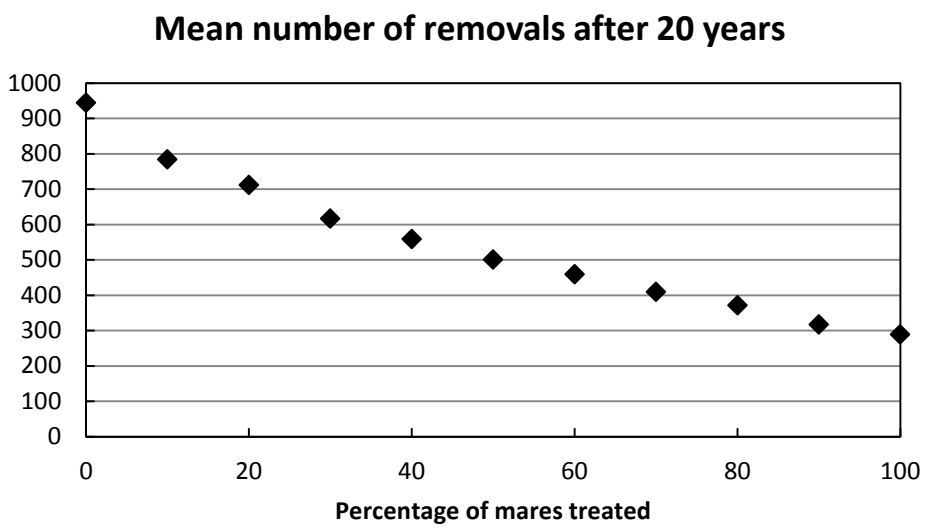
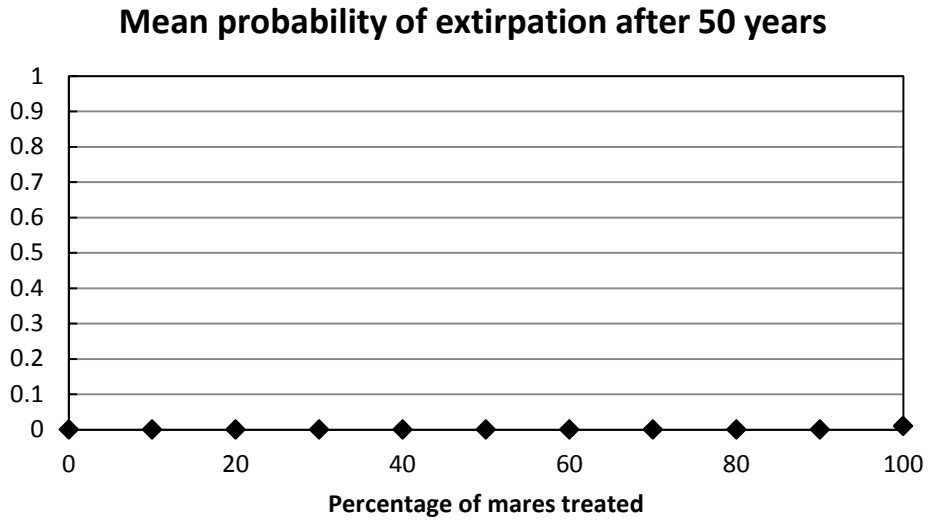


Figure 9. Initial population size = 250, lambda = 1.20, genetic diversity = low.—Continued







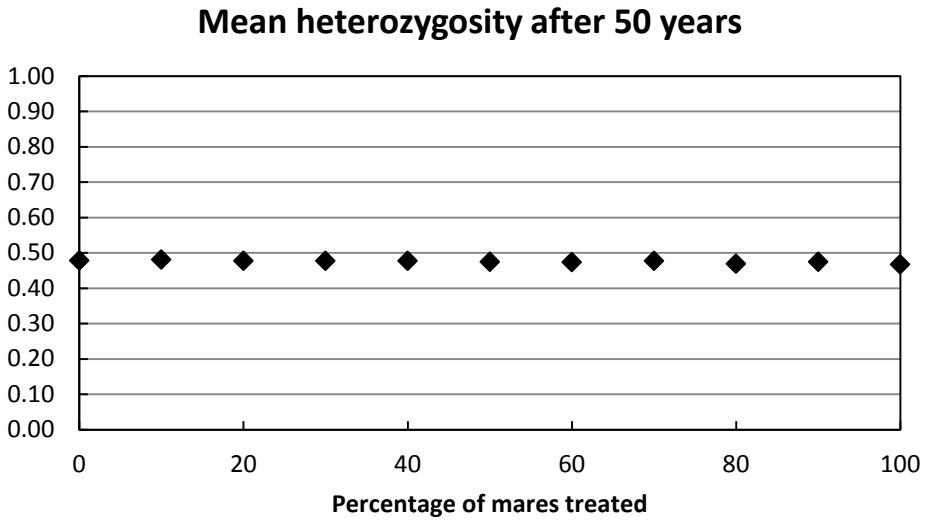


Figure 10. Initial population size = 250, lambda = 1.25, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	308.52	304.15	285.11	278.16	284.69	283.85	262.37	245.28	246.89	216.51	226.76
Mean number of removals after 20 years	944.59	783.74	711.97	616.4	558.71	501.11	459.28	409.28	370.9	316.51	288.81
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0.01
Mean heterozygosity after 50 years	0.48	0.48	0.48	0.48	0.48	0.47	0.47	0.48	0.47	0.47	0.47

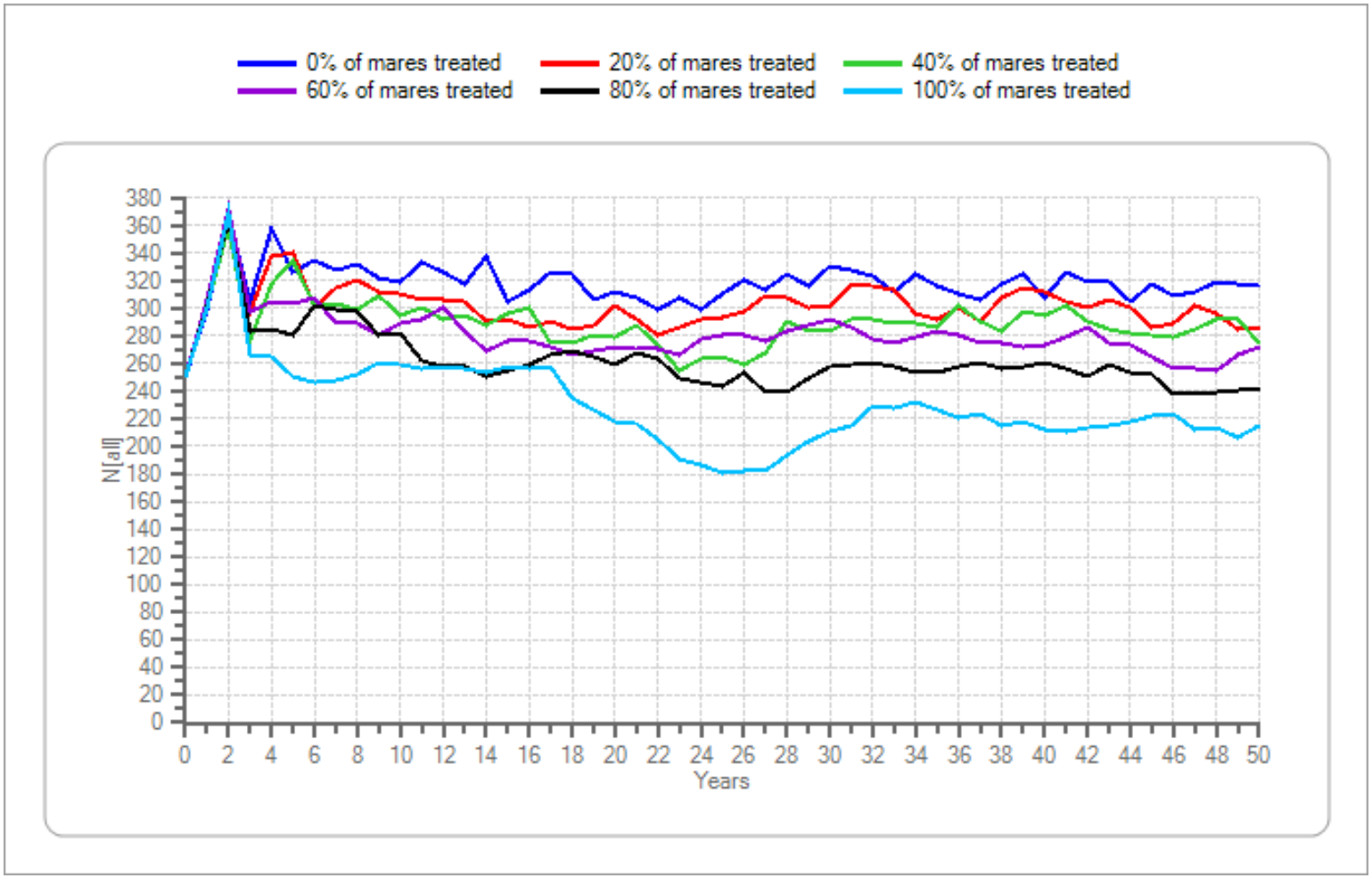
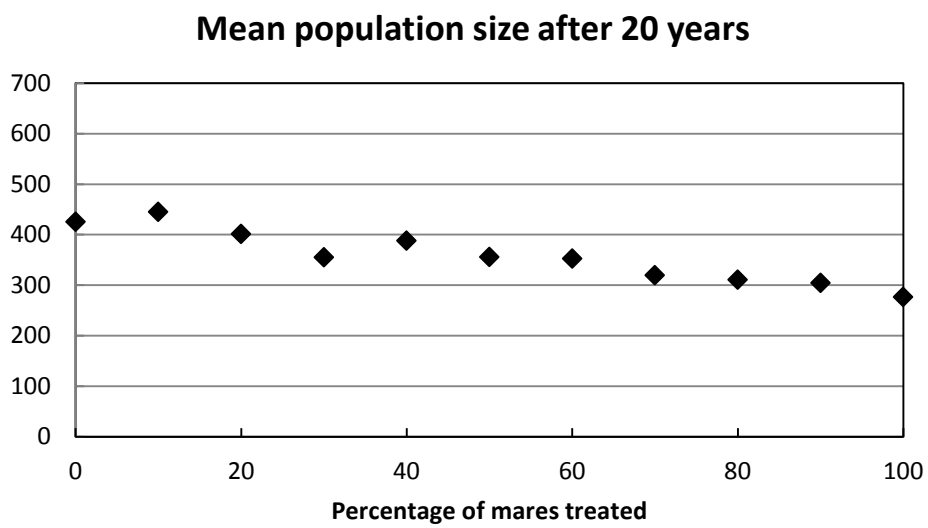
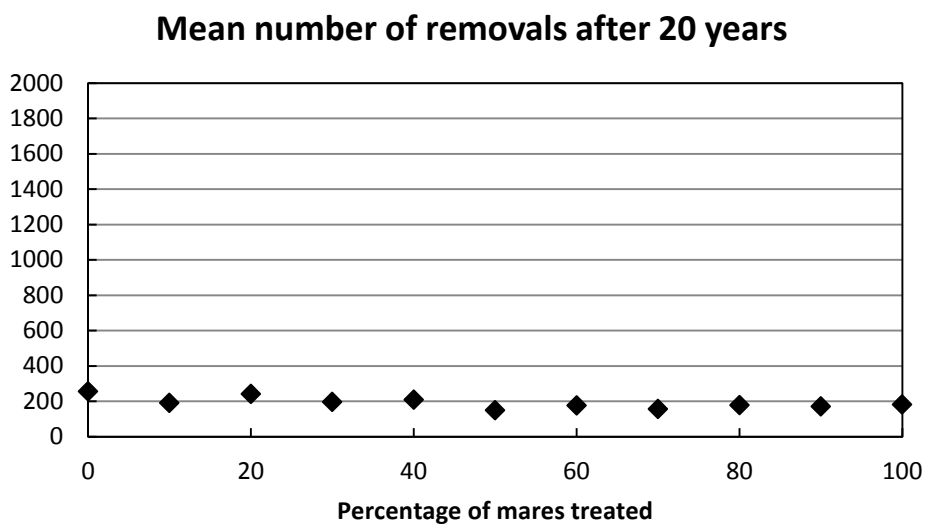
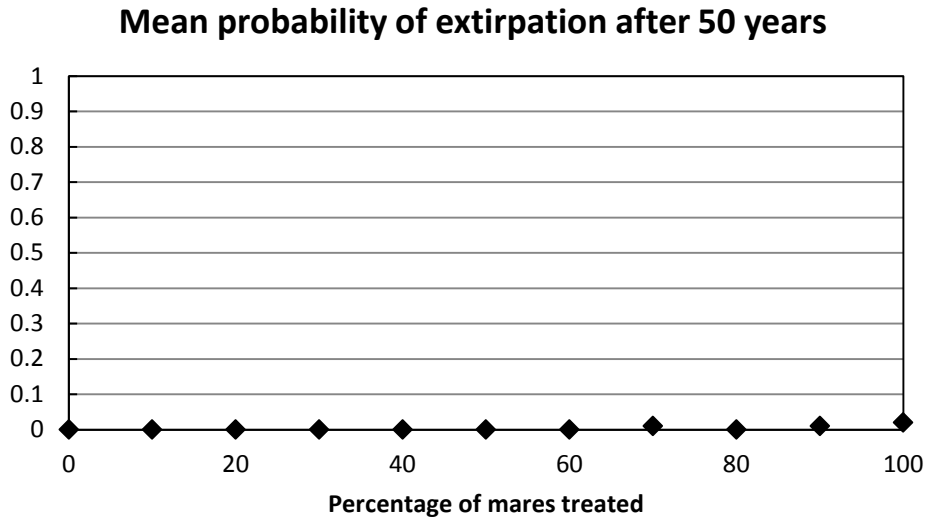


Figure 10. Initial population size = 250, lambda = 1.25, genetic diversity = low.—Continued







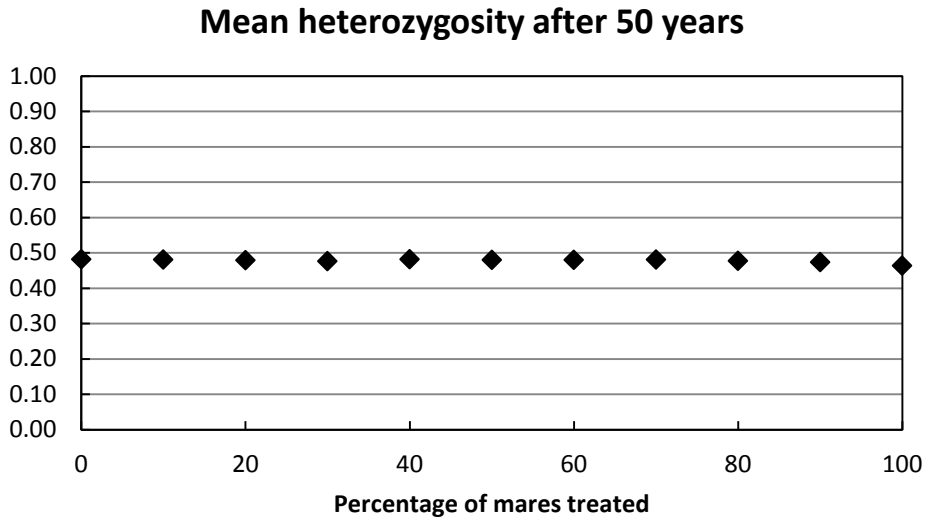


Figure 11. Initial population size = 500, lambda = 1.05, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	425.35	444.78	401.43	354.61	387.64	355.61	352.23	319.32	310.27	304.15	276.61
Mean number of removals after 20 years	255.36	190.95	241.46	196.02	208.87	147.97	176.38	156.08	177.73	169.95	181.87
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0.01	0	0.01	0.02
Mean heterozygosity after 50 years	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.47	0.46

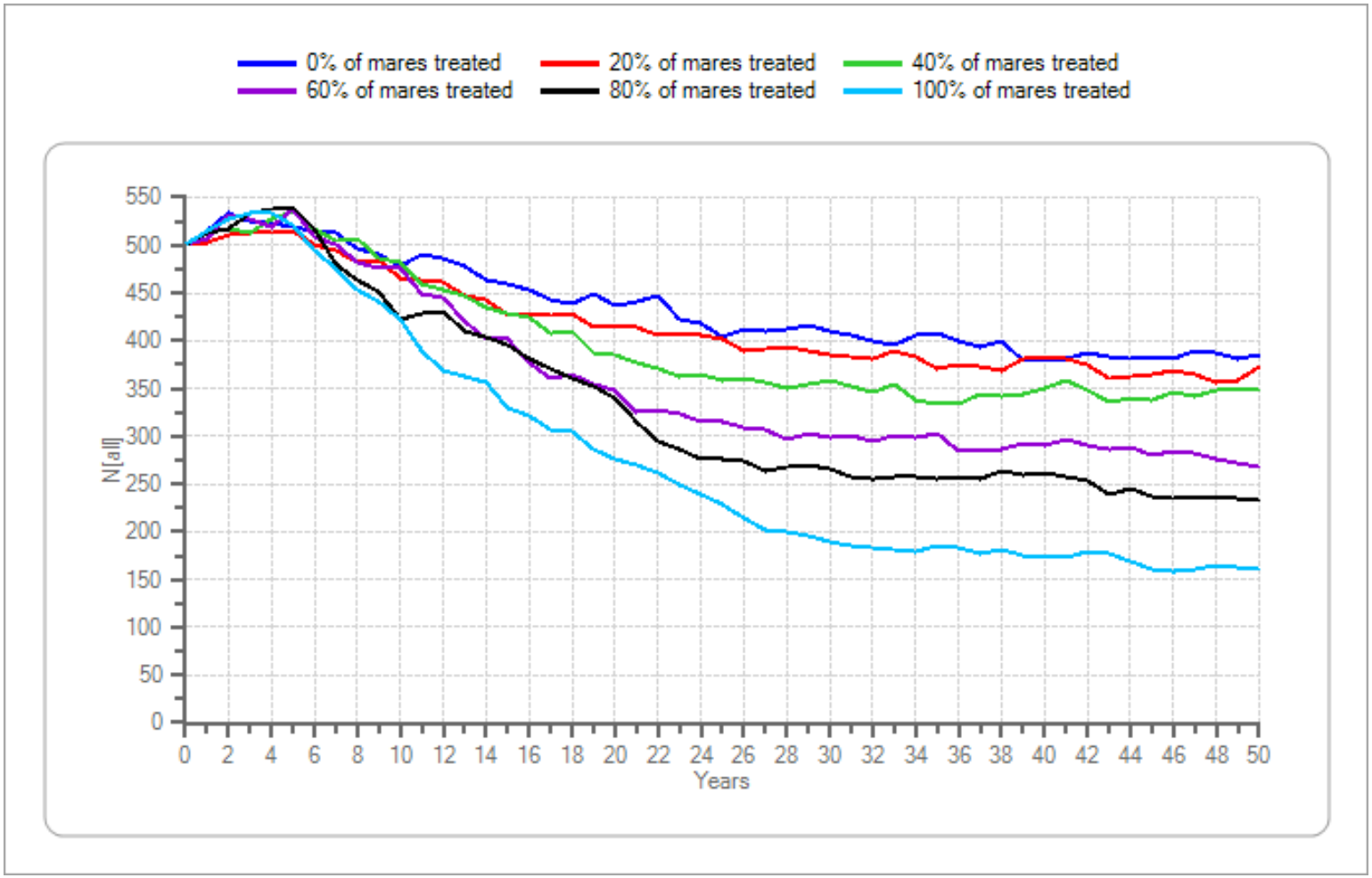
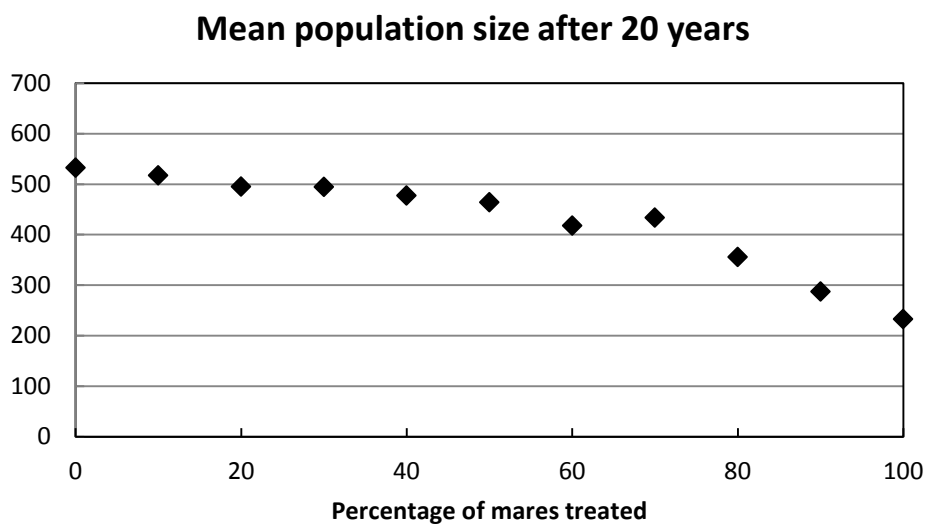
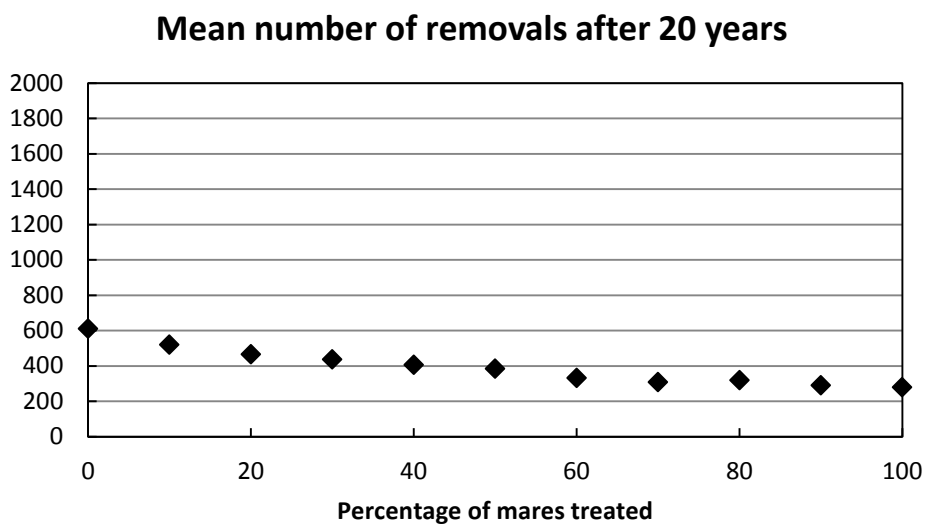
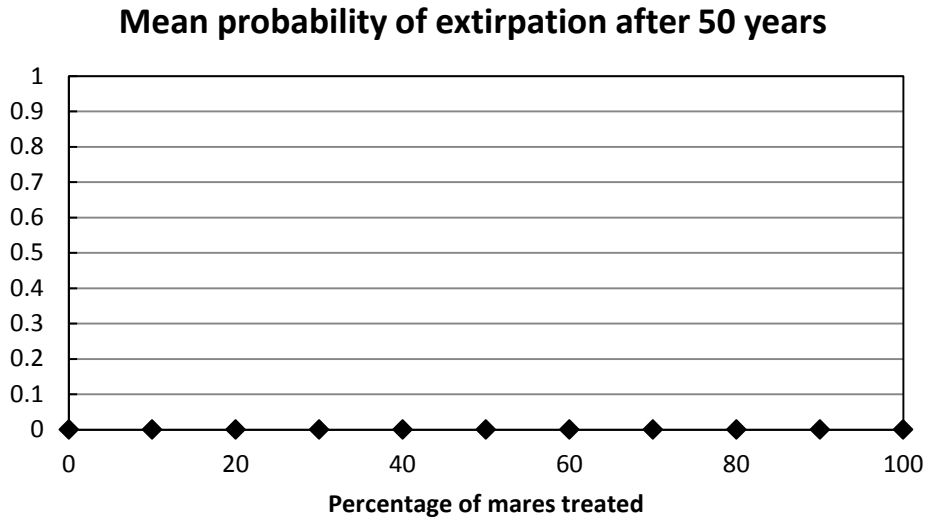


Figure 11. Initial population size = 500, lambda = 1.05, genetic diversity = low.—Continued







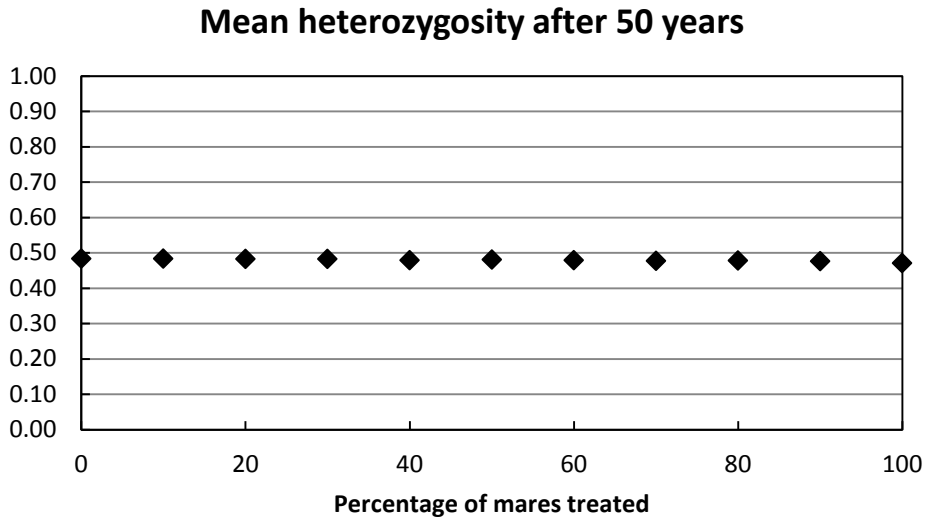


Figure 12. Initial population size = 500, lambda = 1.10, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	532.14	517.32	494.85	494.04	476.94	463.89	417.36	433.64	355.55	286.94	232.49
Mean number of removals after 20 years	610.21	520.05	465.8	436.08	406.01	384.76	332.11	307.18	318.48	289.02	279.16
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.47

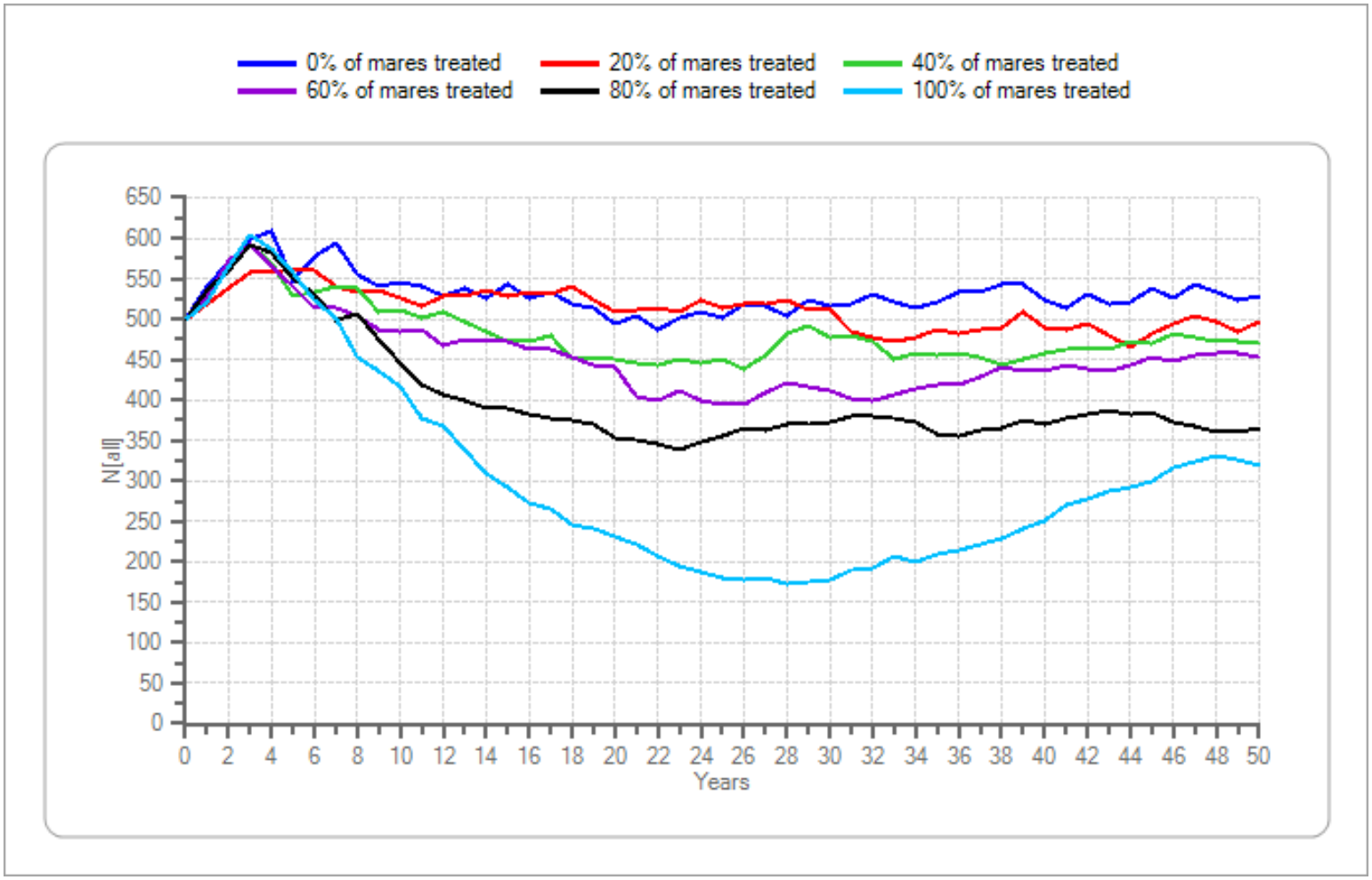
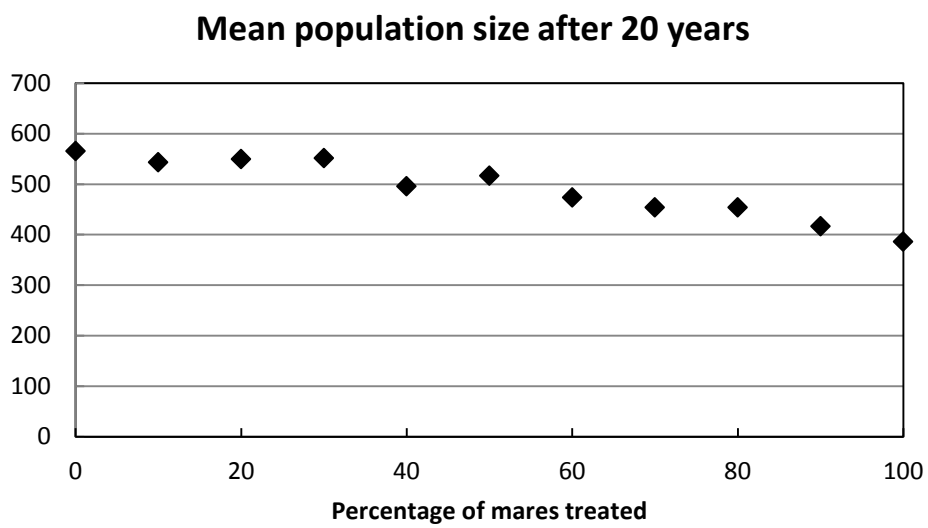
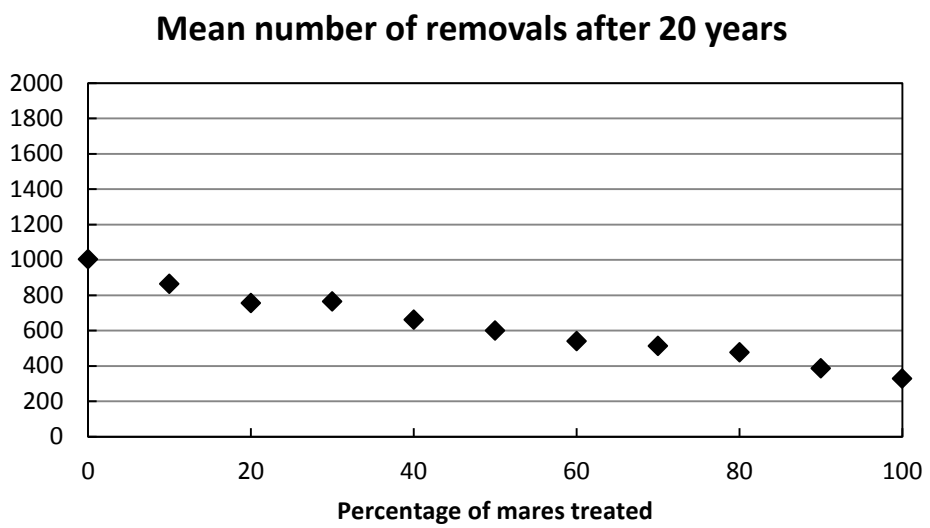
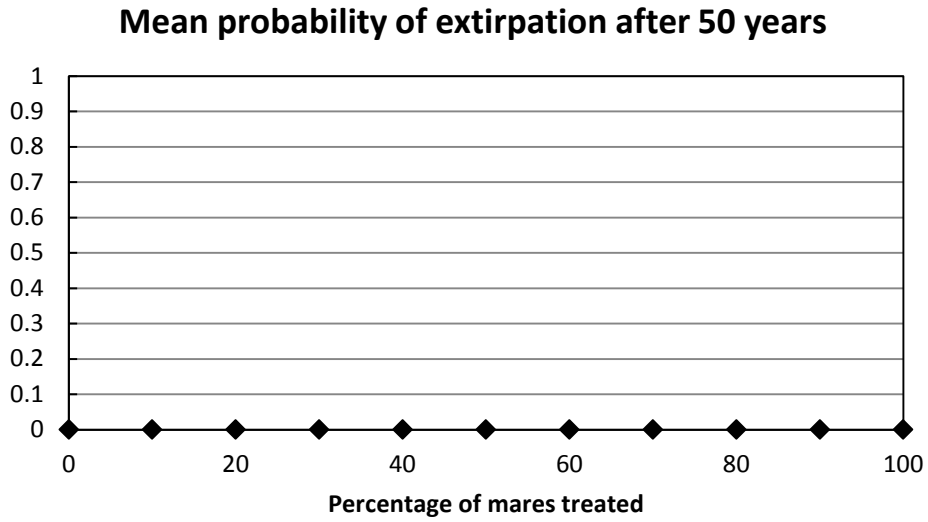


Figure 12. Initial population size = 500, lambda = 1.10, genetic diversity = low.—Continued







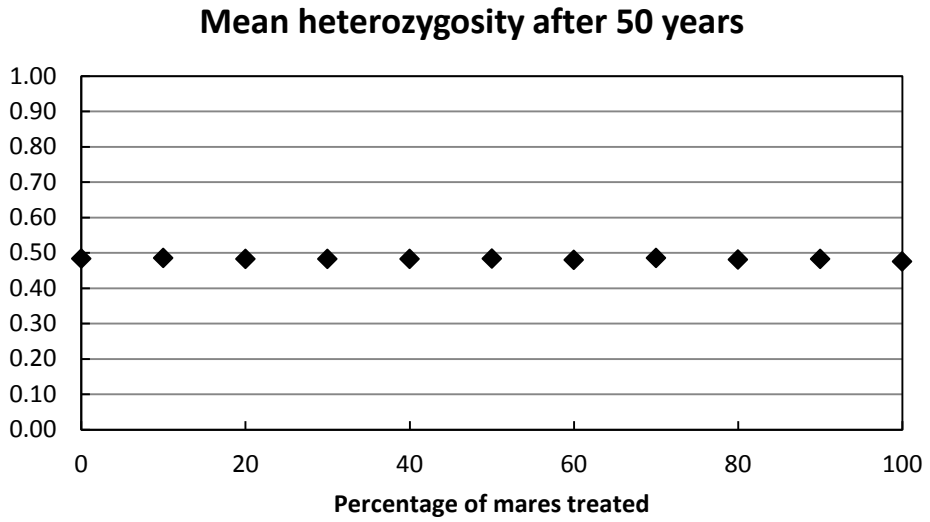


Figure 13. Initial population size = 500, lambda = 1.15, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	565.34	543.12	549.14	551.57	495.37	516.42	473.26	453.73	453.48	416.08	385.94
Mean number of removals after 20 years	1002.92	863.83	755.3	764.15	660.85	598.87	539.63	512.76	475.42	384.89	328.2
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.48	0.49	0.48	0.48	0.48	0.48	0.48	0.49	0.48	0.48	0.48

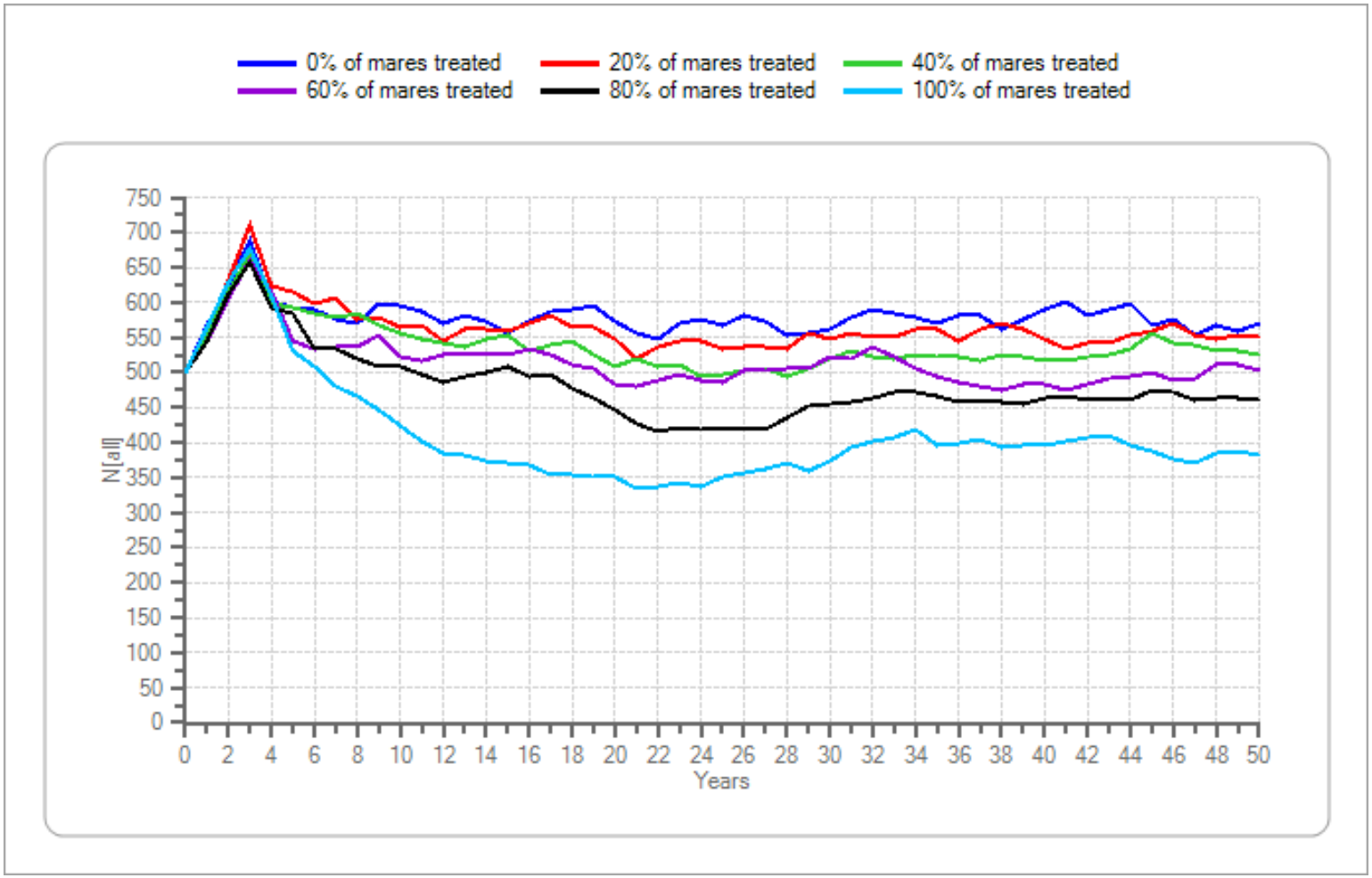
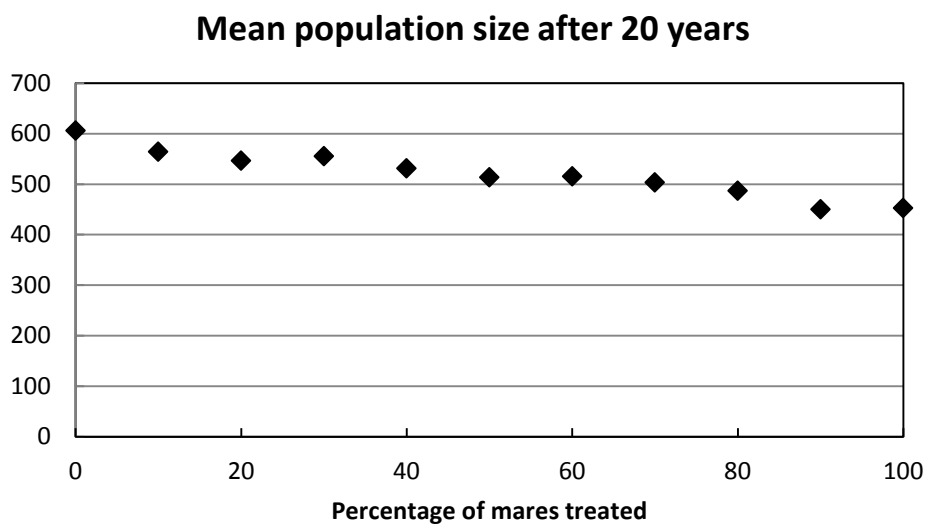
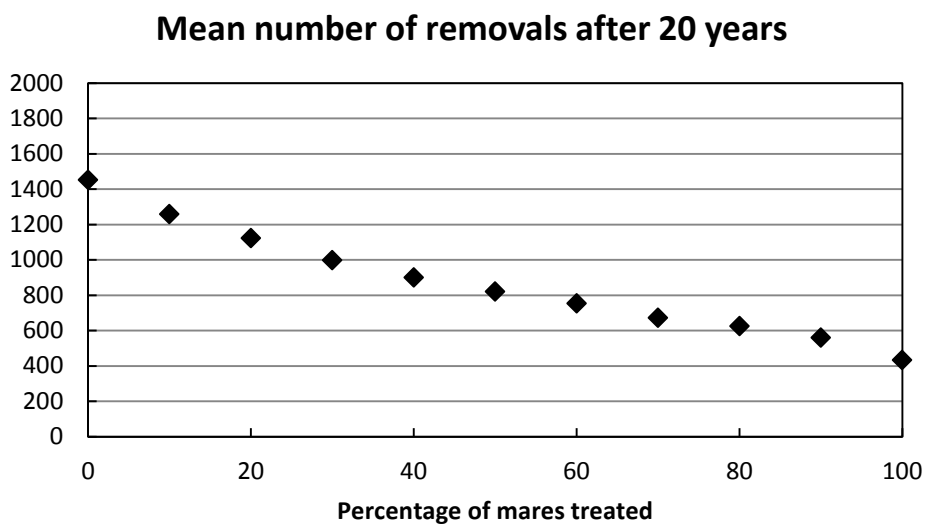
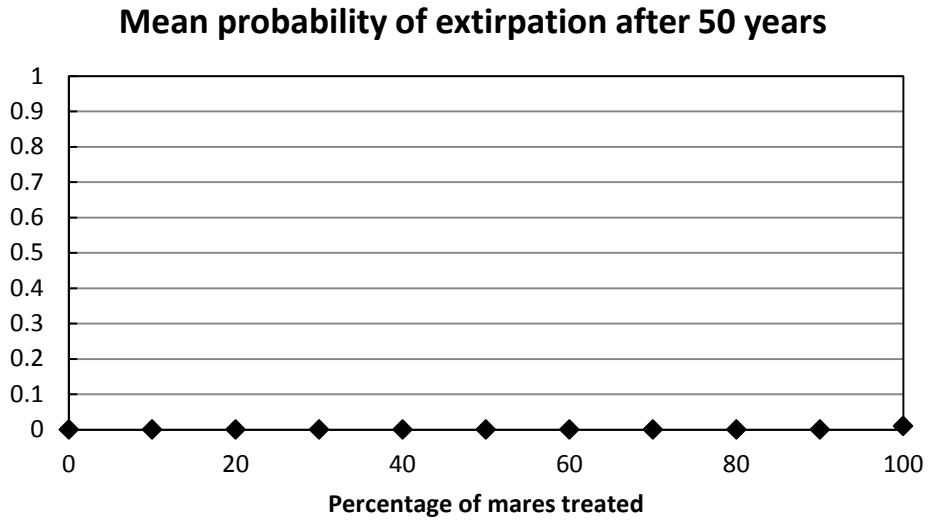


Figure 13. Initial population size = 500, lambda = 1.15, genetic diversity = low.—Continued







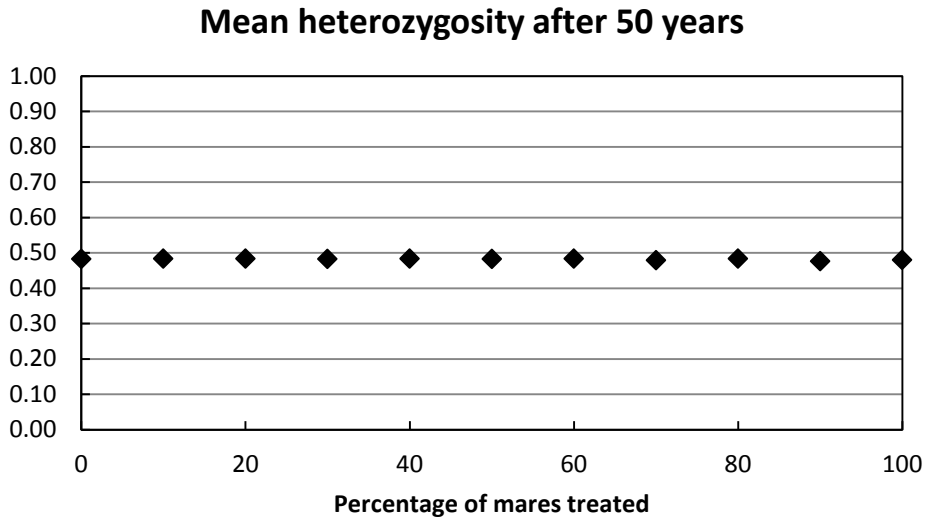


Figure 14. Initial population size = 500, lambda = 1.20, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	606.16	564.12	546.4	555.28	531.1	513.41	515.42	502.94	486.58	450.15	452.21
Mean number of removals after 20 years	1451.75	1258.73	1122.91	997.36	900.23	820.29	752.55	671.84	624.39	559.54	432.37
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0.01
Mean heterozygosity after 50 years	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48

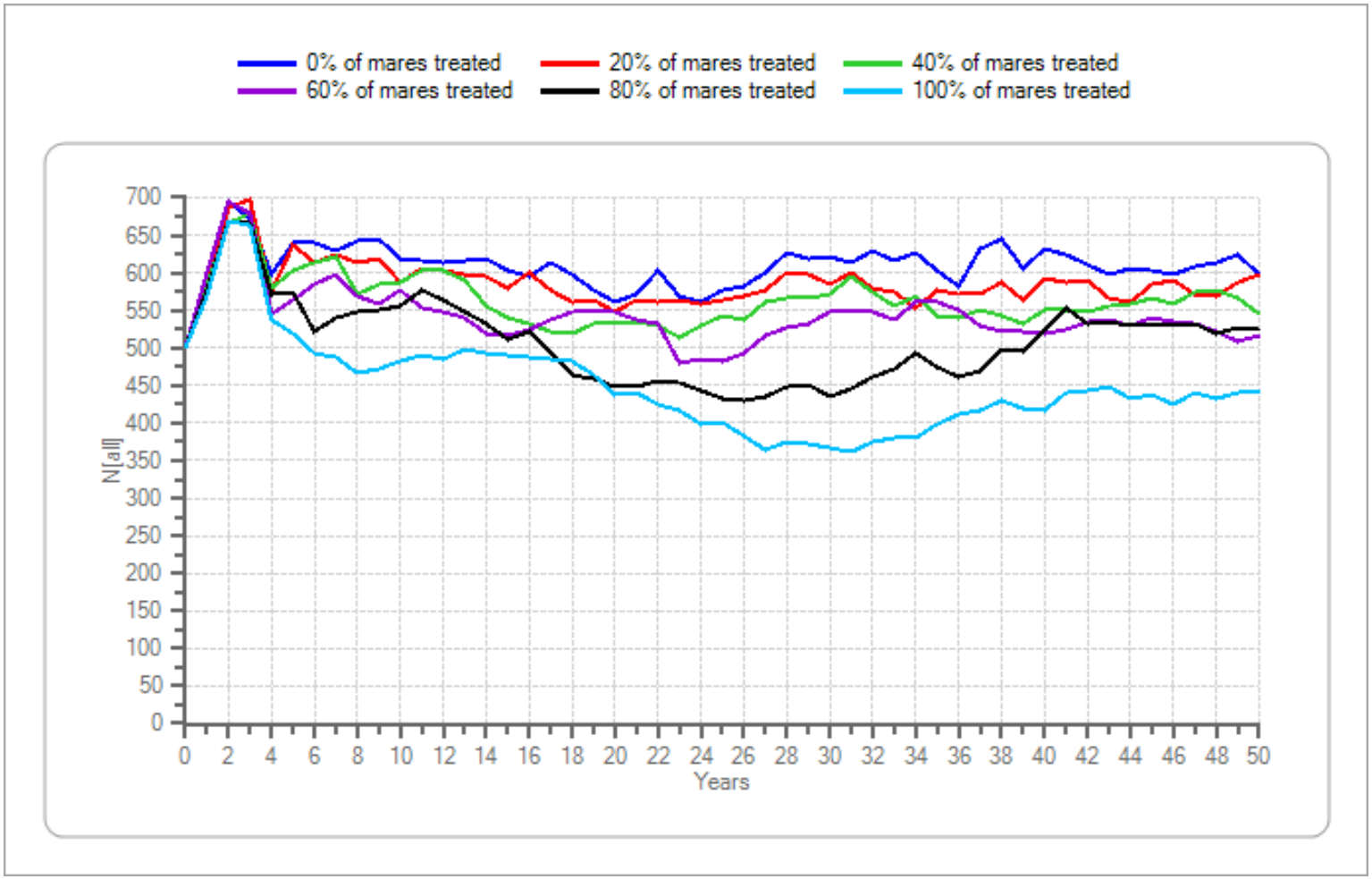
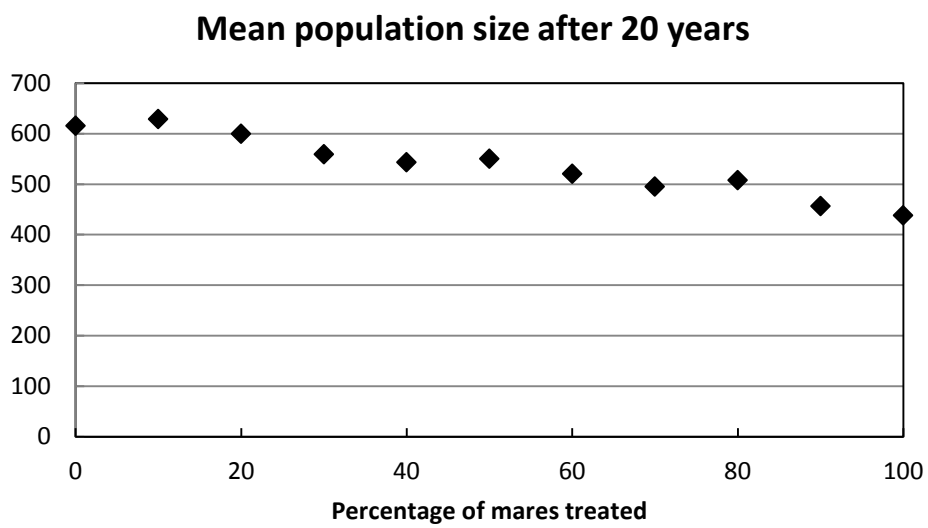
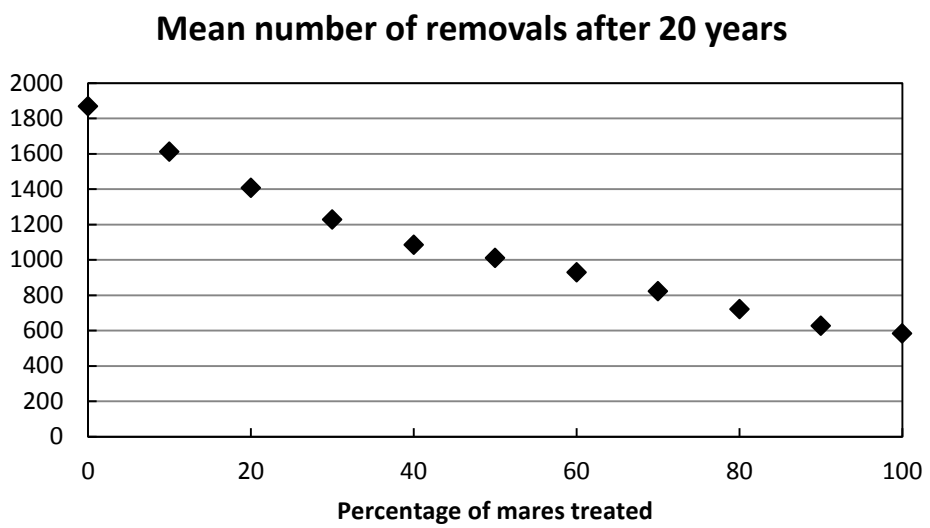
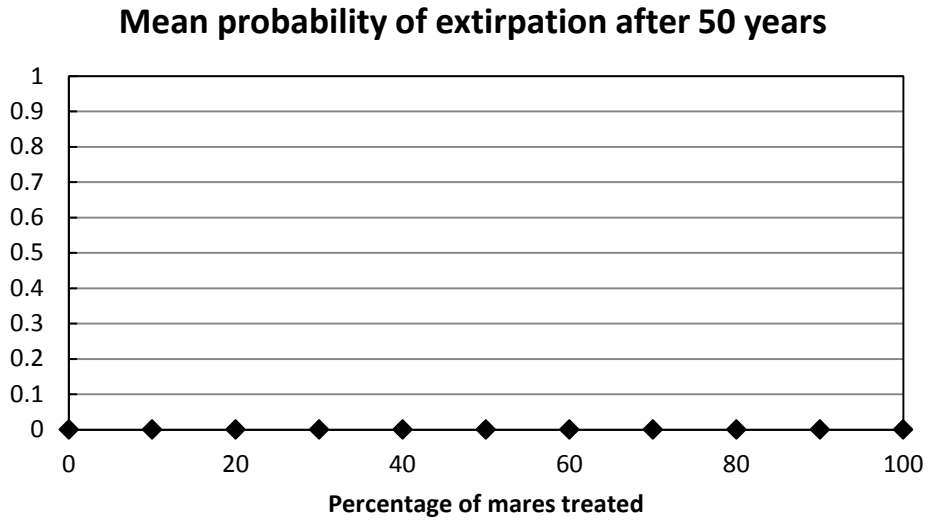


Figure 14. Initial population size = 500, lambda = 1.20, genetic diversity = low.—Continued







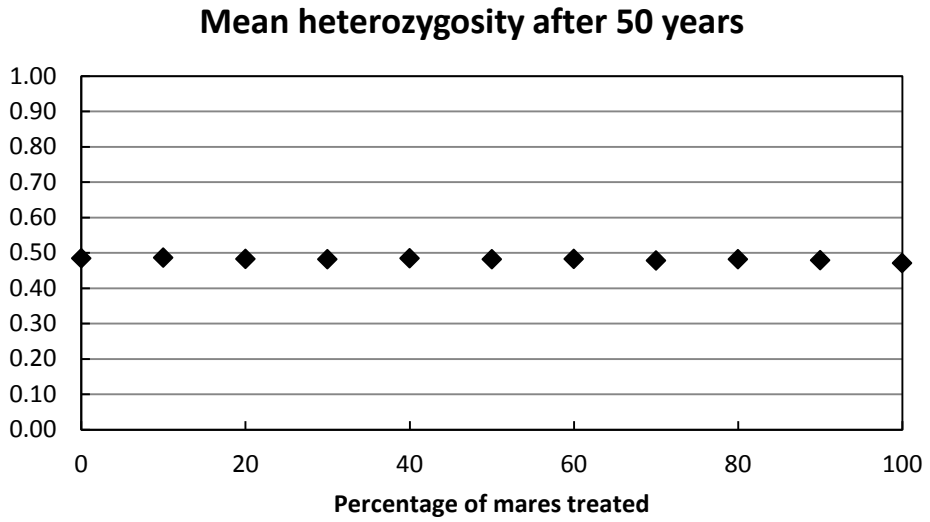


Figure 15. Initial population size = 500, lambda = 1.25, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	615.29	628.52	599.27	559.22	543.09	550.12	520.04	495.03	507.73	456.42	437.97
Mean number of removals after 20 years	1868.95	1612.35	1406.33	1227.35	1083.85	1009.6	928.44	822.54	721.17	626.66	583.8
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.48	0.49	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.47

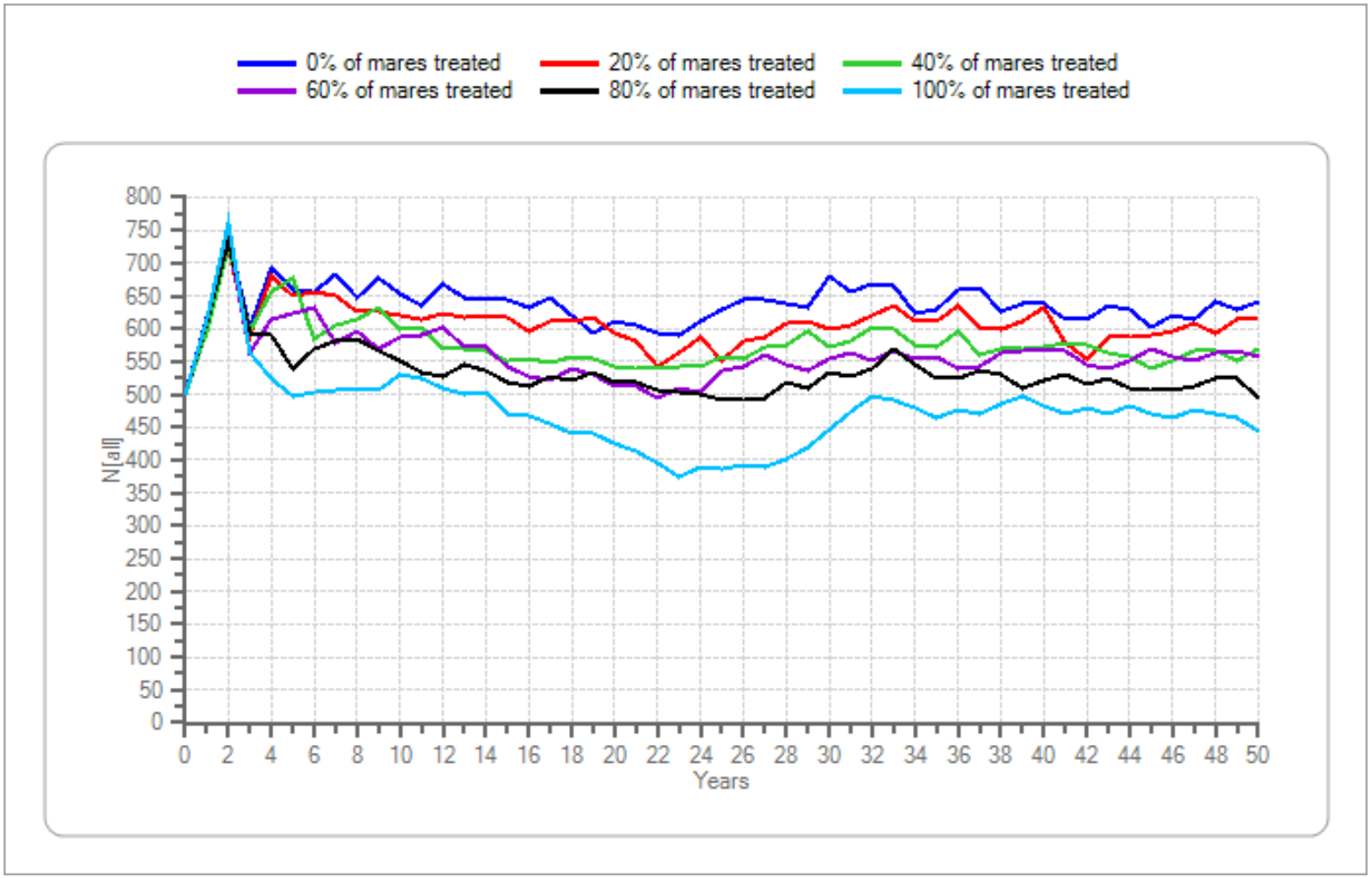
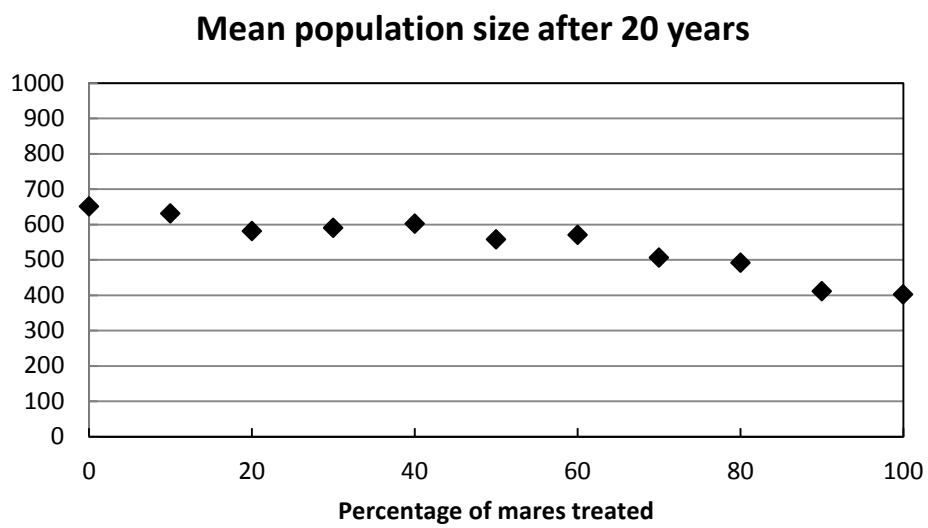
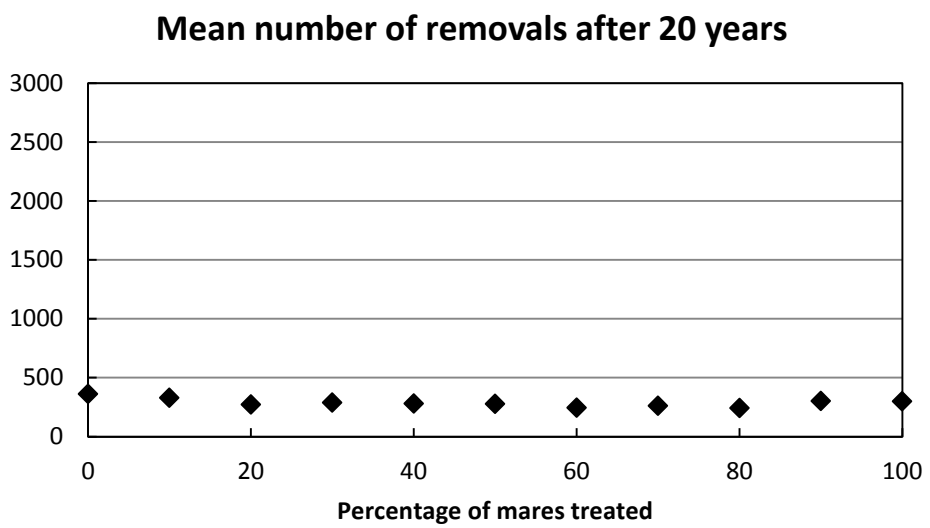
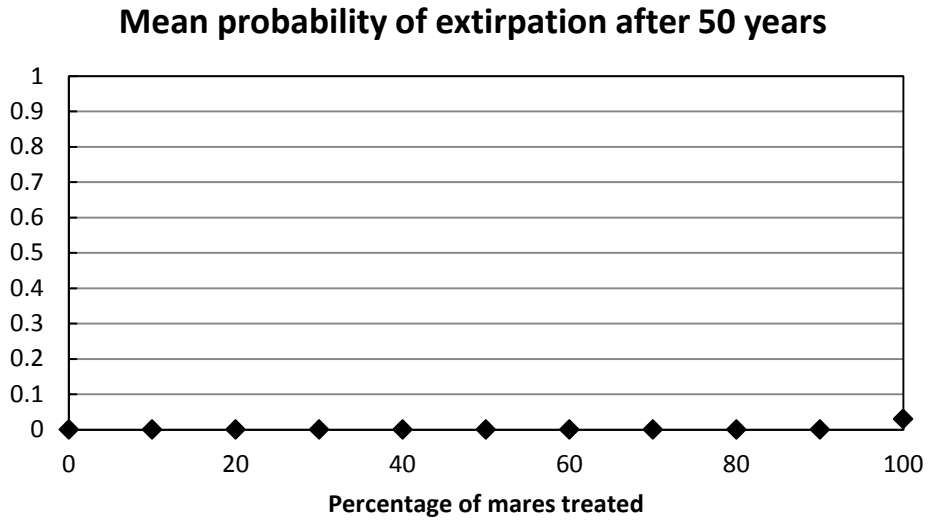


Figure 15. Initial population size = 500, lambda = 1.25, genetic diversity = low.—Continued







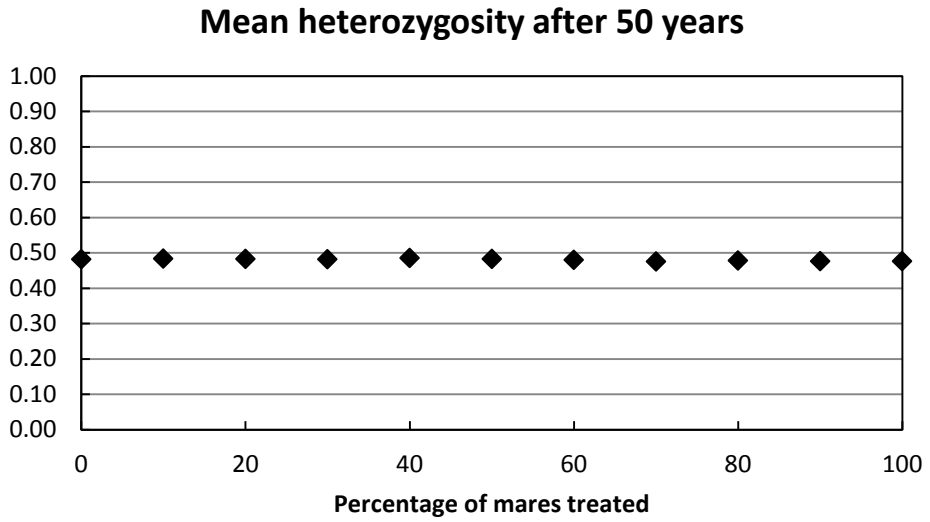


Figure 16. Initial population size = 750, lambda = 1.05, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	650.88	631.43	581.13	590.12	602.15	557.46	570.81	506.51	491.17	411.2	401.89
Mean number of removals after 20 years	361.95	328.24	272.67	288.49	279.12	276.66	243.67	261.64	241.29	302.35	297.91
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0.03
Mean heterozygosity after 50 years	0.48	0.48	0.48	0.48	0.49	0.48	0.48	0.48	0.48	0.48	0.48

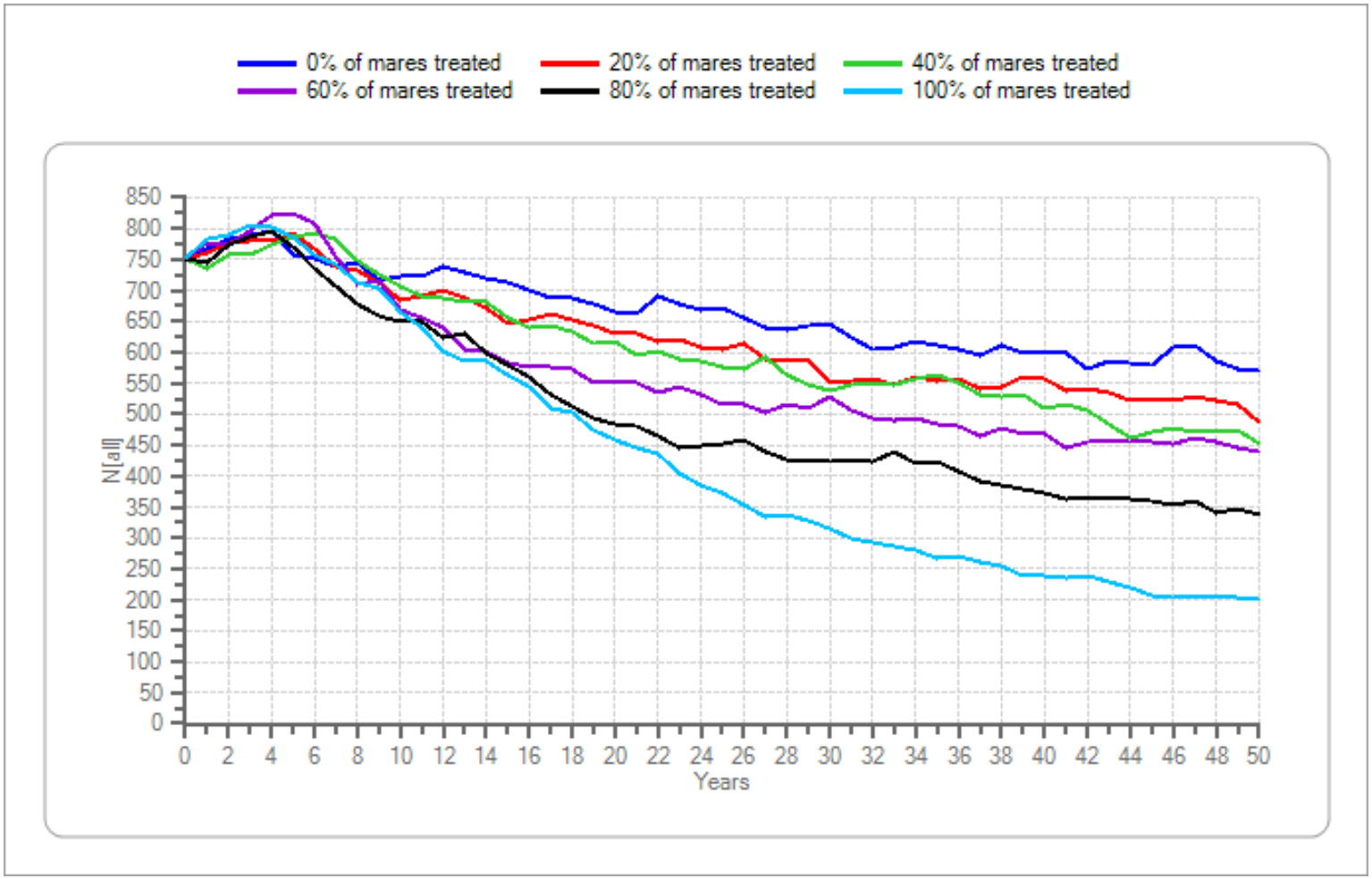
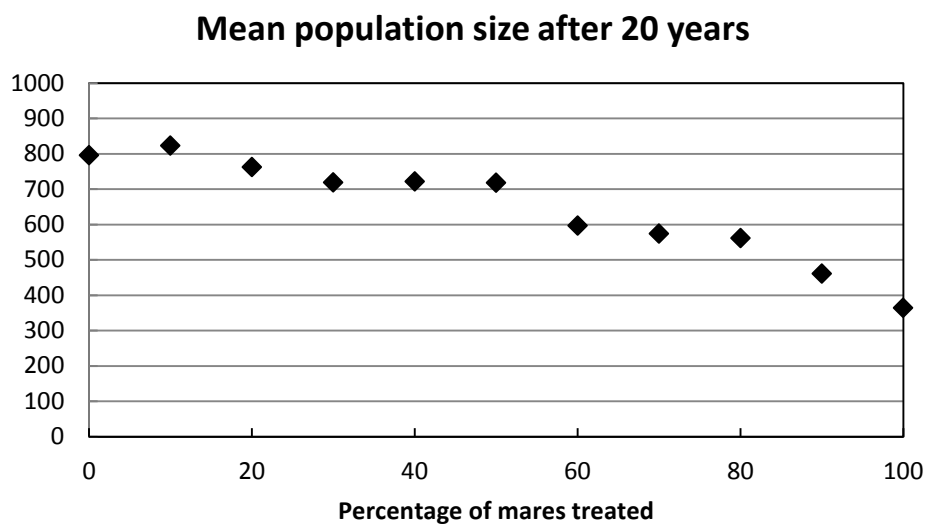
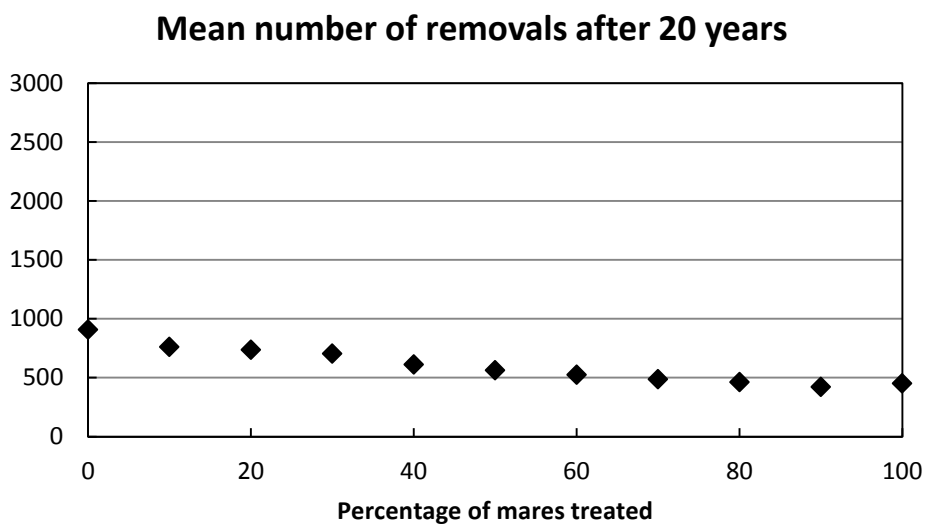
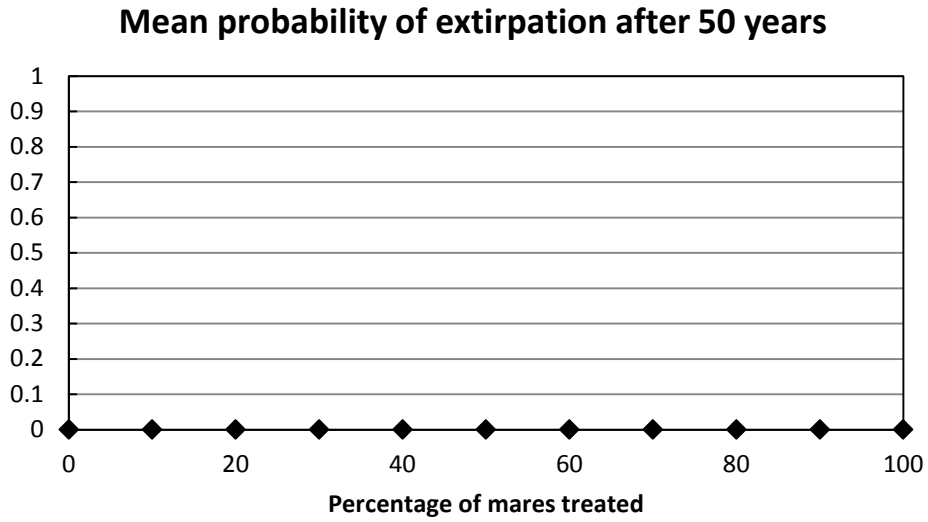


Figure 16. Initial population size = 750, lambda = 1.05, genetic diversity = low.—Continued







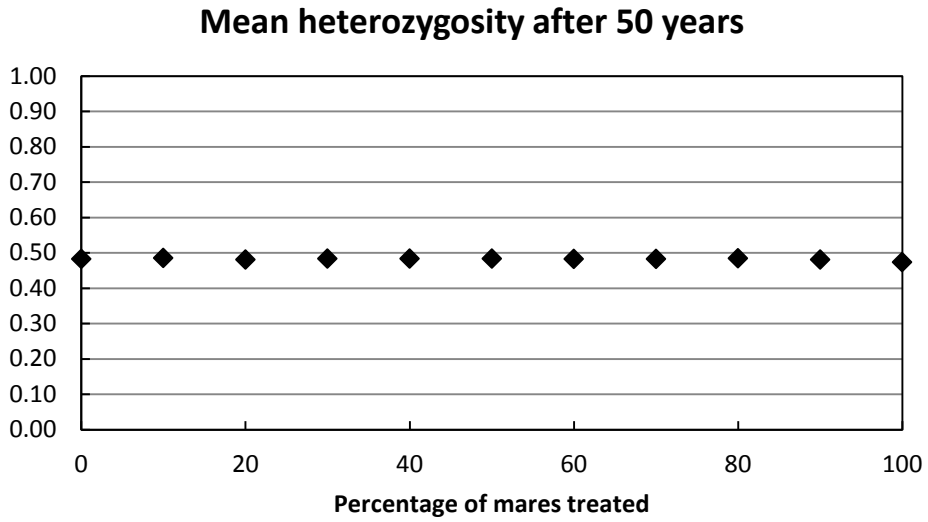


Figure 17. Initial population size = 750, lambda = 1.10, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	795.56	823.24	762.24	718.7	721.89	717.77	597.06	573.89	561.16	460.9	363.9
Mean number of removals after 20 years	908.52	760.43	736.27	703.39	611.56	562.49	523.38	486.72	462.27	420.49	450.7
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.48	0.49	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.47

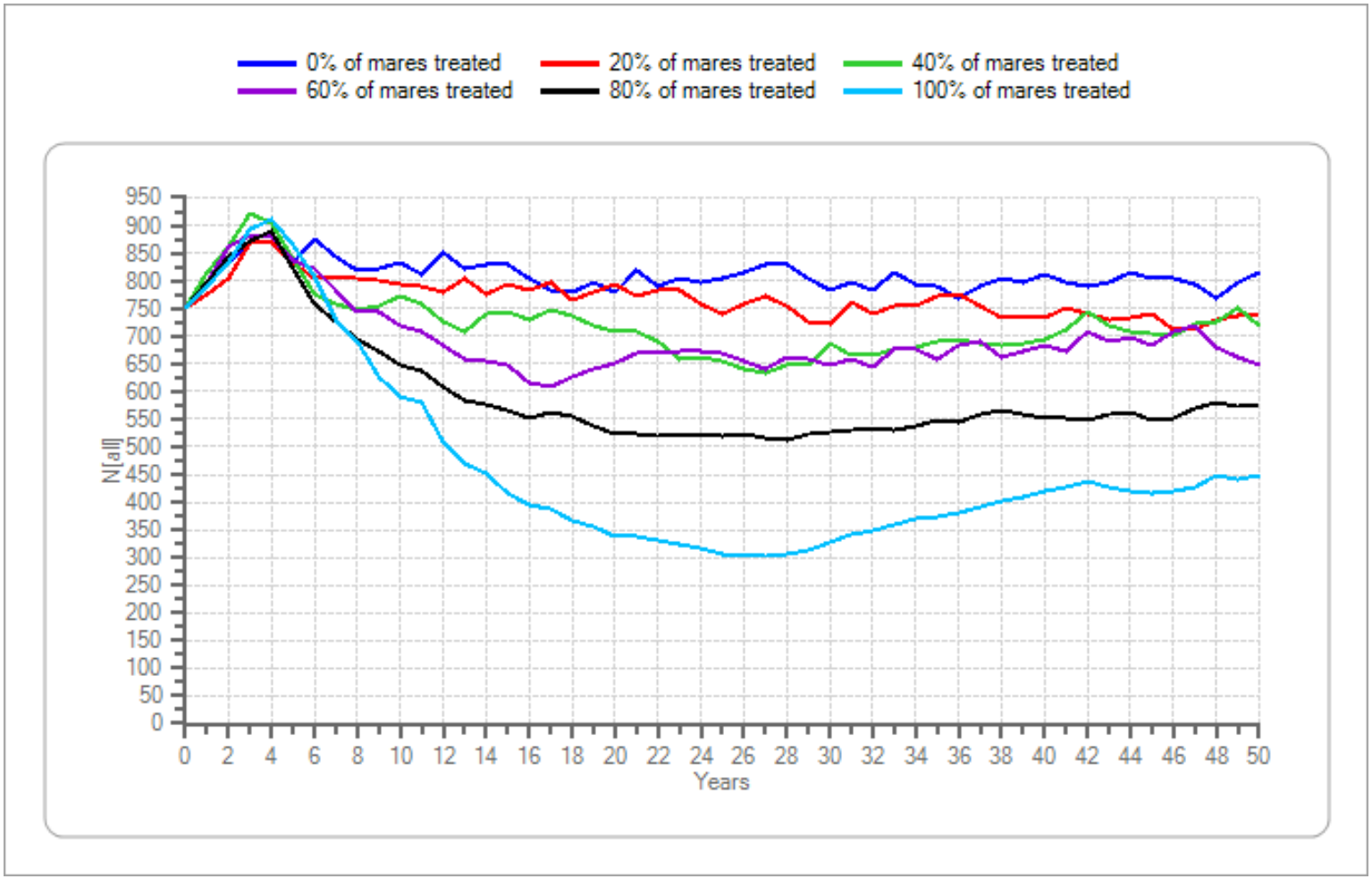
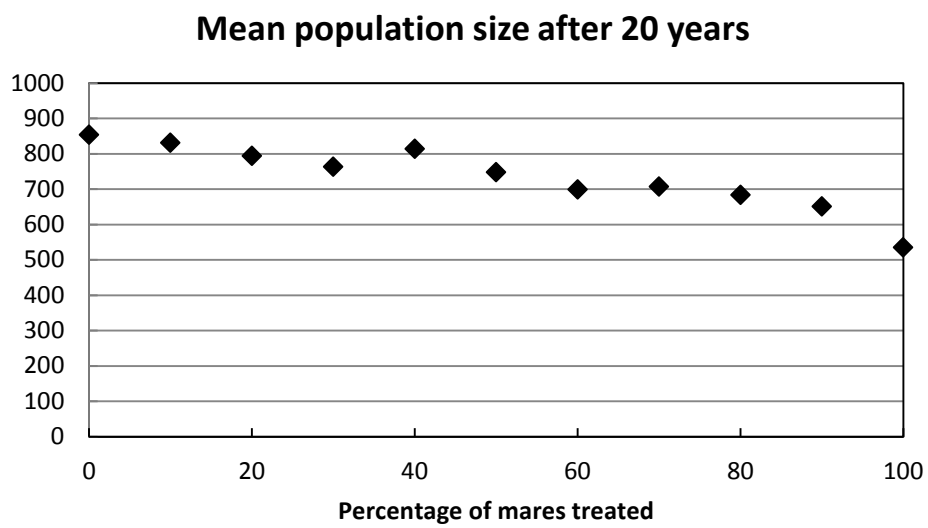
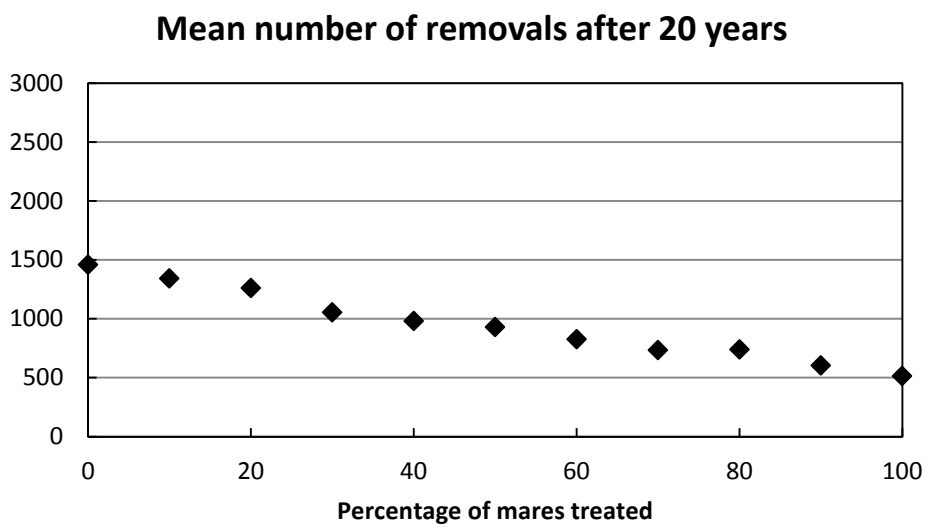
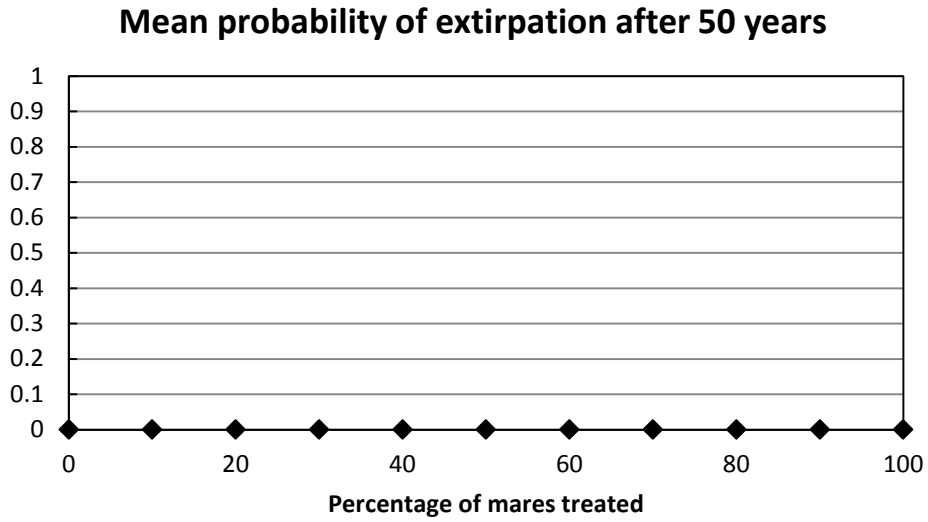


Figure 17. Initial population size = 750, lambda = 1.10, genetic diversity = low.—Continued







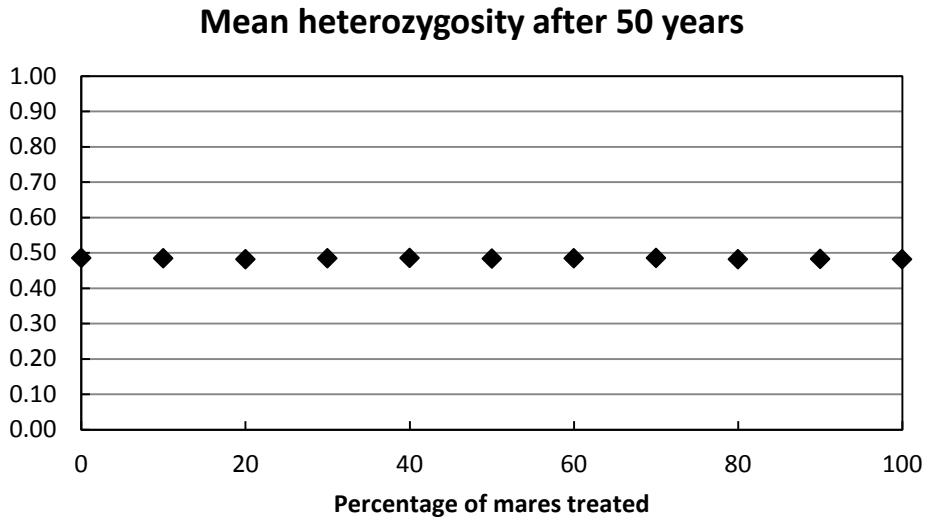


Figure 18. Initial population size = 750, lambda = 1.15, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	853.53	831.08	794.36	763.4	813.88	747.49	698.91	707.4	683.74	650.95	535.33
Mean number of removals after 20 years	1458.62	1341.8	1260.79	1054.92	979.41	928.62	825.18	733.54	738.13	602.52	512.15
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.48	0.48	0.48	0.49	0.48	0.48	0.49	0.48	0.48	0.48

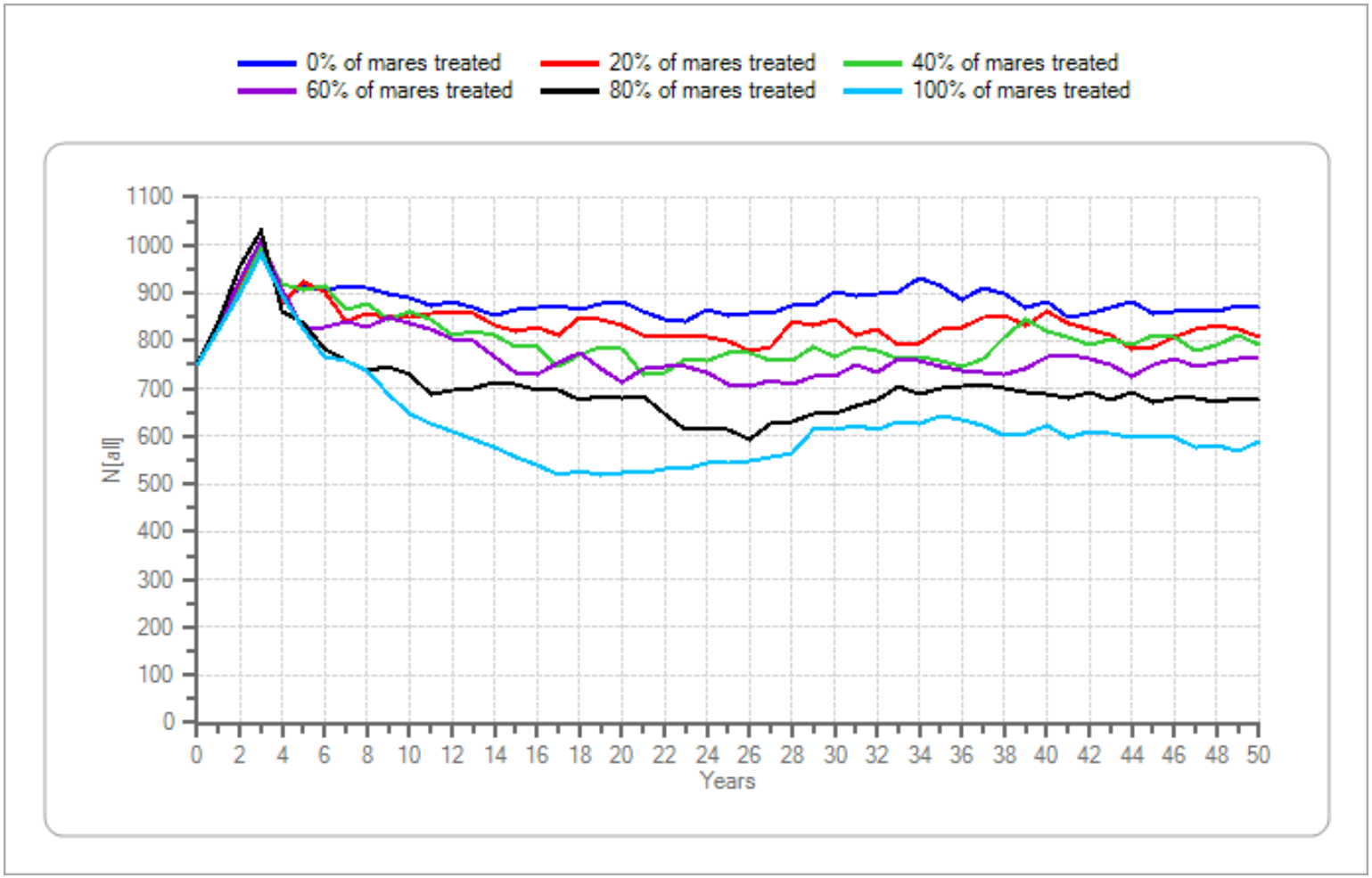
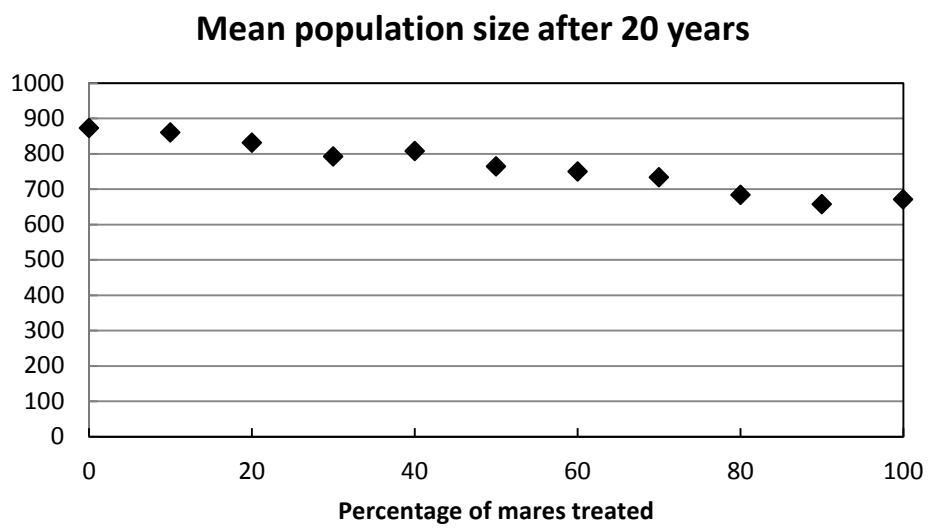
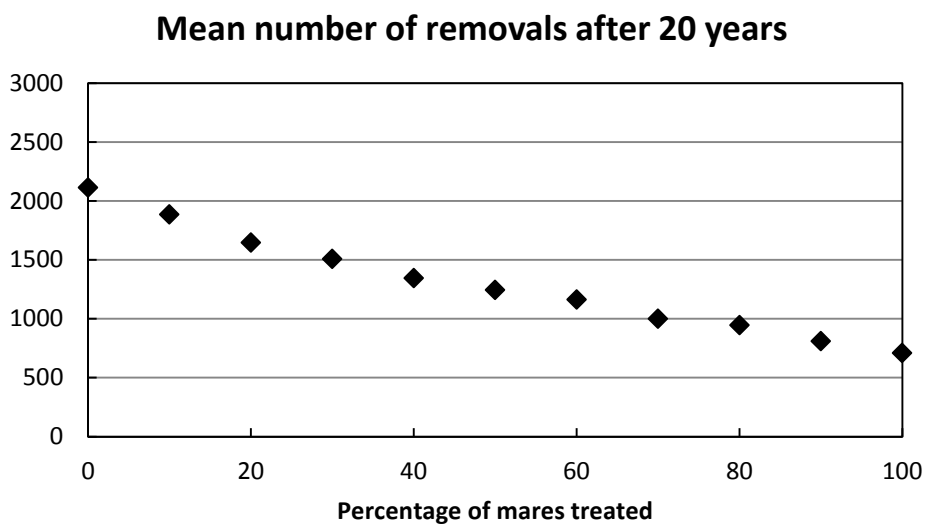
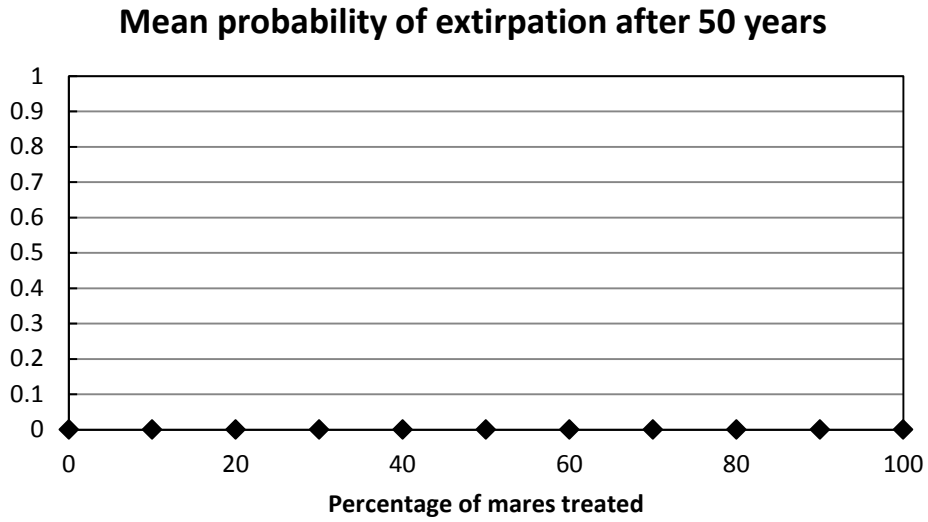


Figure 18. Initial population size = 750, lambda = 1.15, genetic diversity = low.—Continued







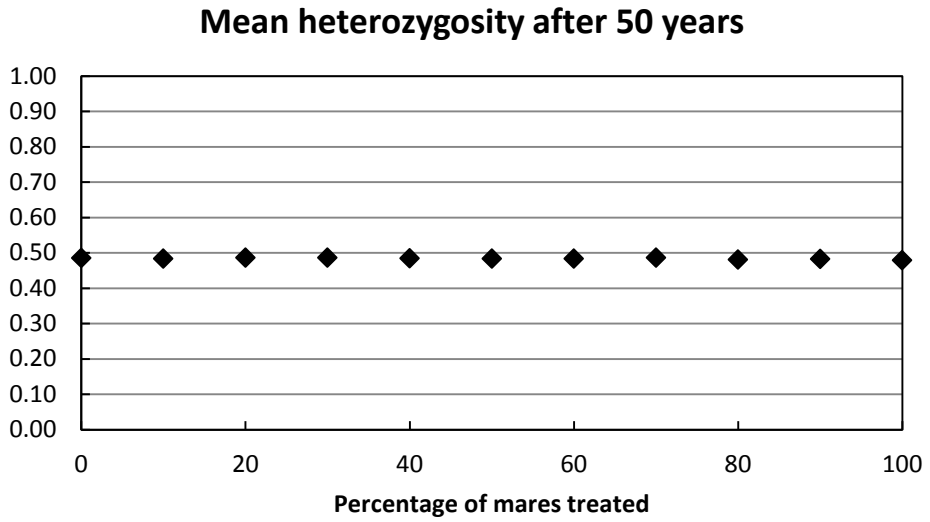


Figure 19. Initial population size = 750, lambda = 1.20, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	872.42	860.25	830.74	792.41	807.75	763.95	749.48	733.6	683.58	657.07	670.54
Mean number of removals after 20 years	2112.42	1885.73	1646.6	1507.2	1345.72	1244.22	1162.02	999.18	944.68	809.02	709.93
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.48	0.49	0.49	0.48	0.48	0.48	0.49	0.48	0.48	0.48

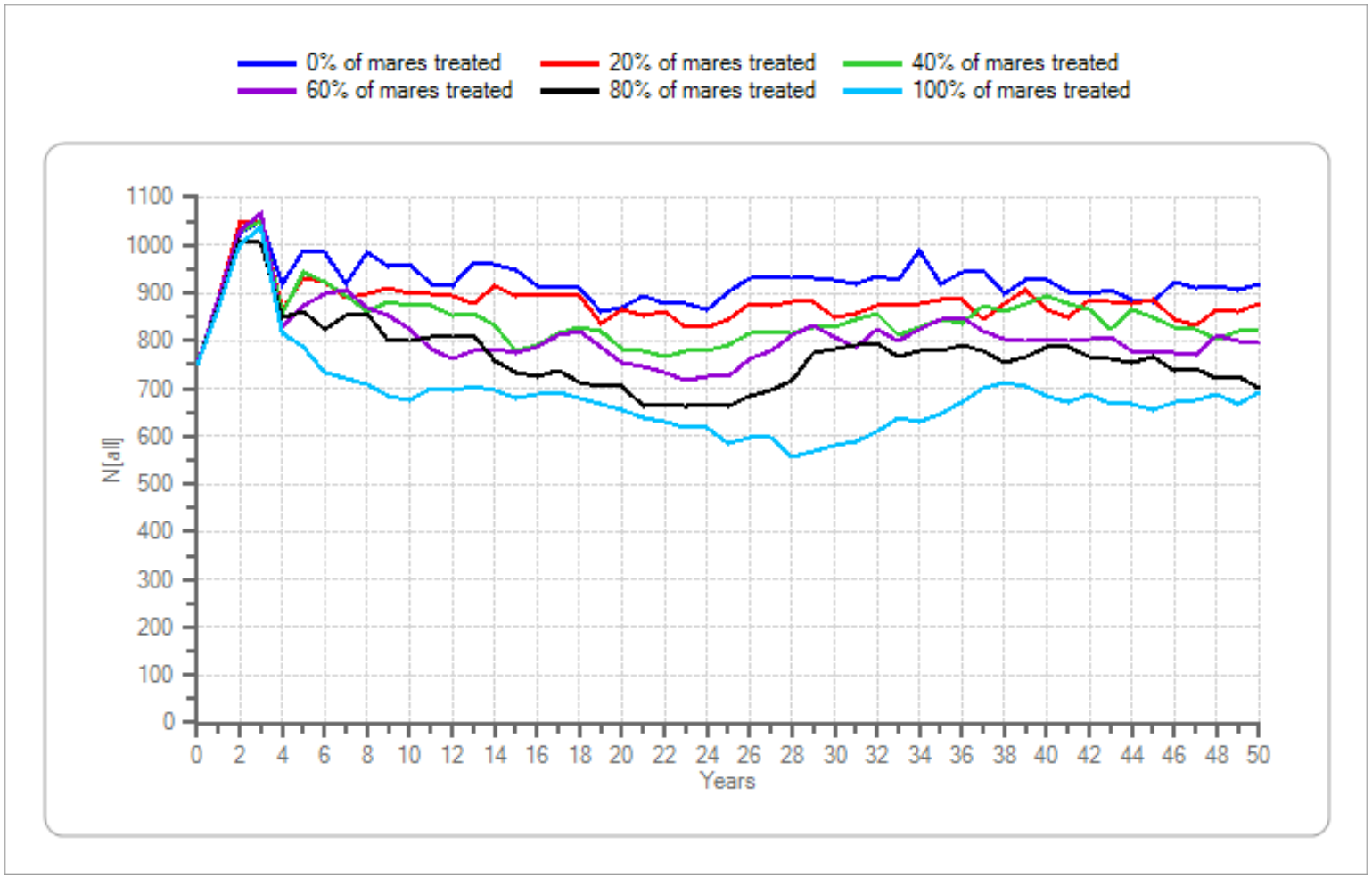
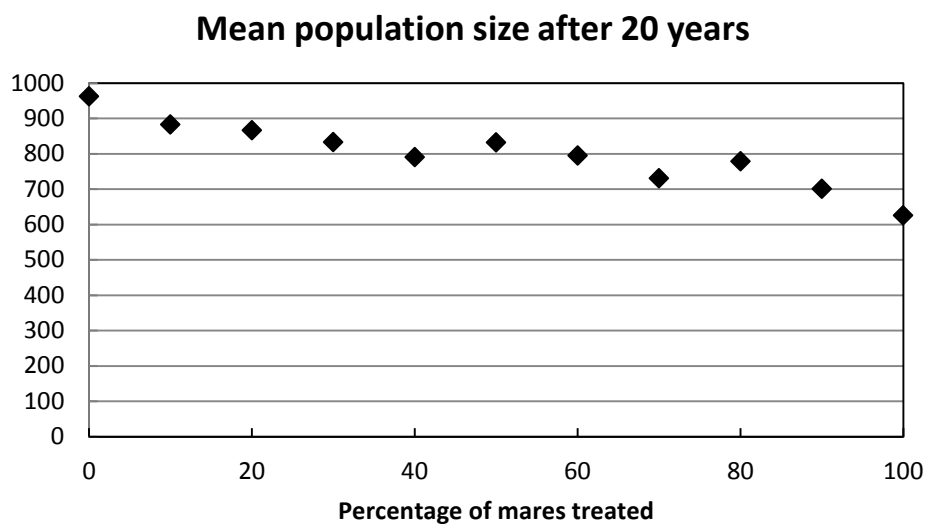
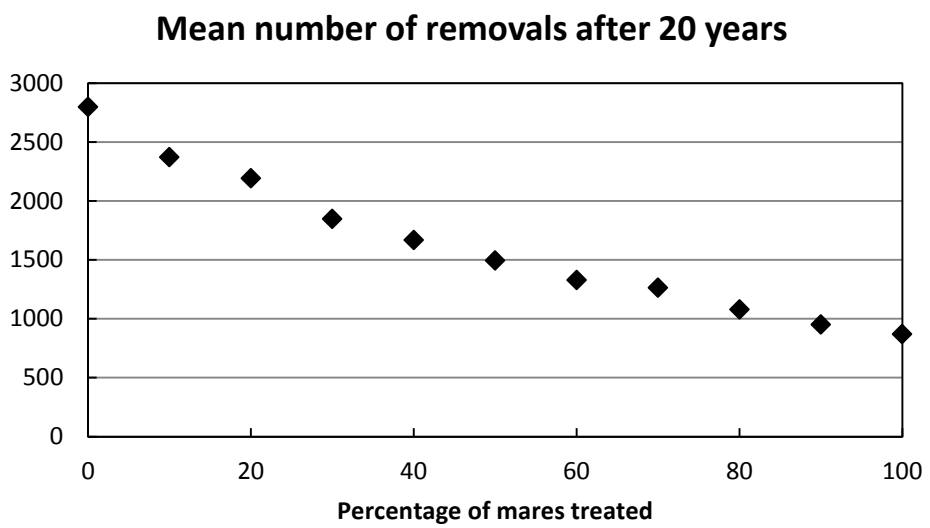
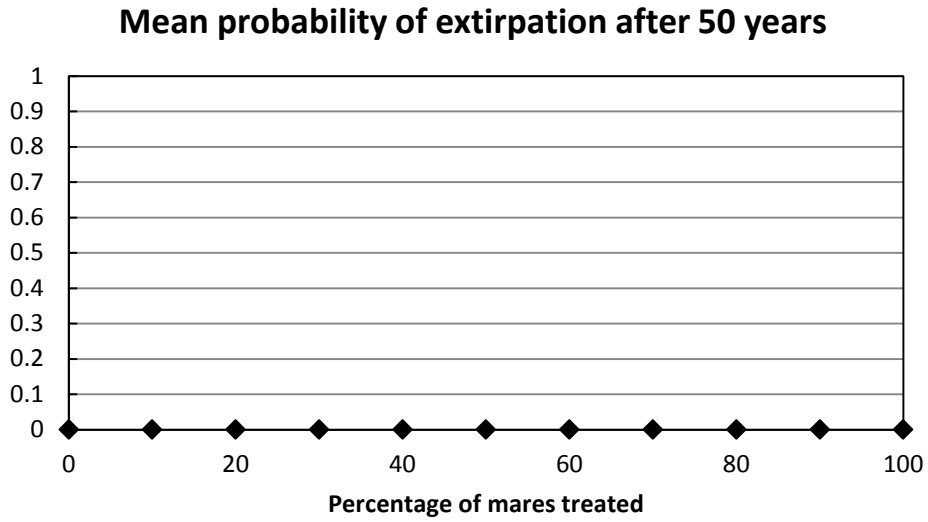


Figure 19. Initial population size = 750, lambda = 1.20, genetic diversity = low.—Continued







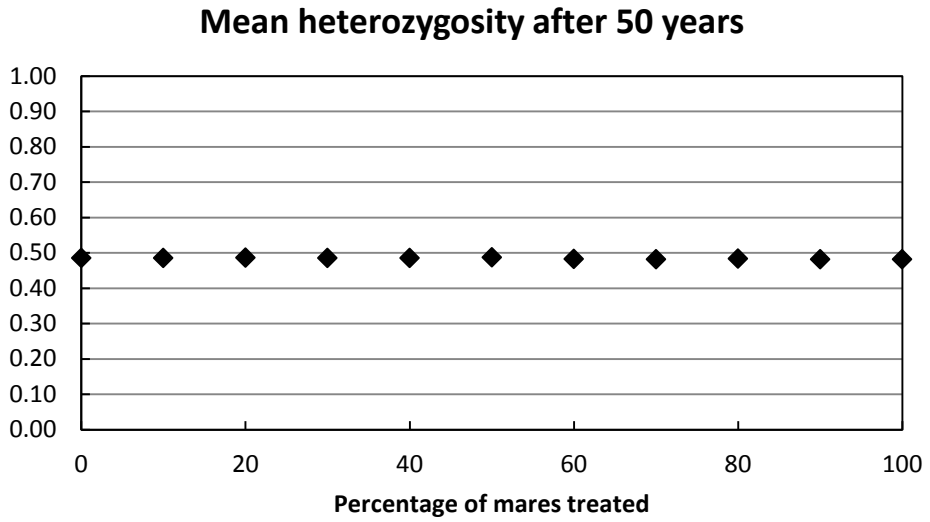


Figure 20. Initial population size = 750, lambda = 1.25, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	962.21	882.4	866.81	832.94	790.31	832.37	794.81	730.52	778.33	701.12	625.35
Mean number of removals after 20 years	2796.8	2371.7	2191.6	1846.0	1667.5	1492.6	1328.4	1264.1	1078.9	951.3	870.4
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.49	0.49	0.49	0.48	0.48	0.48	0.48	0.48

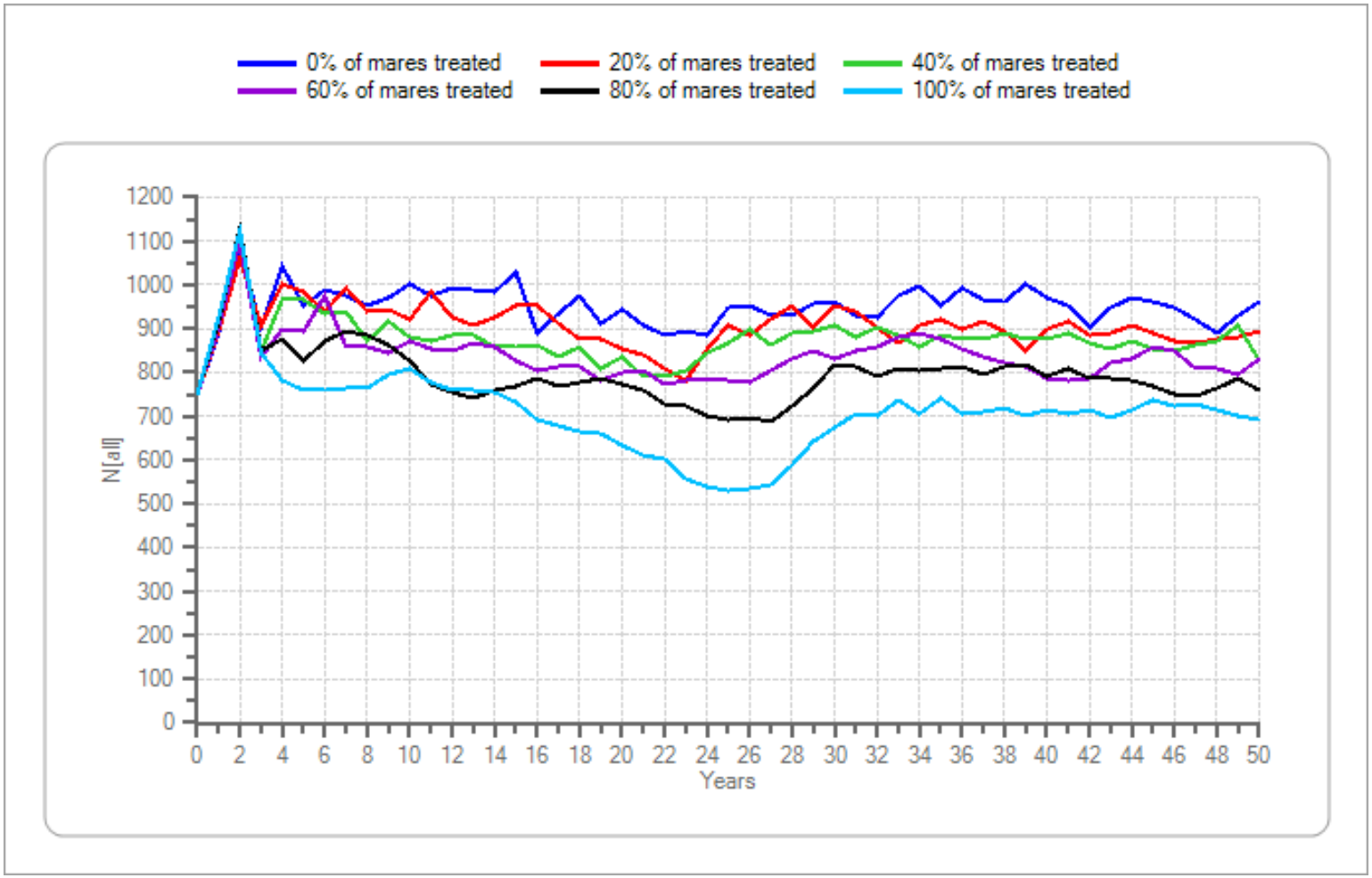
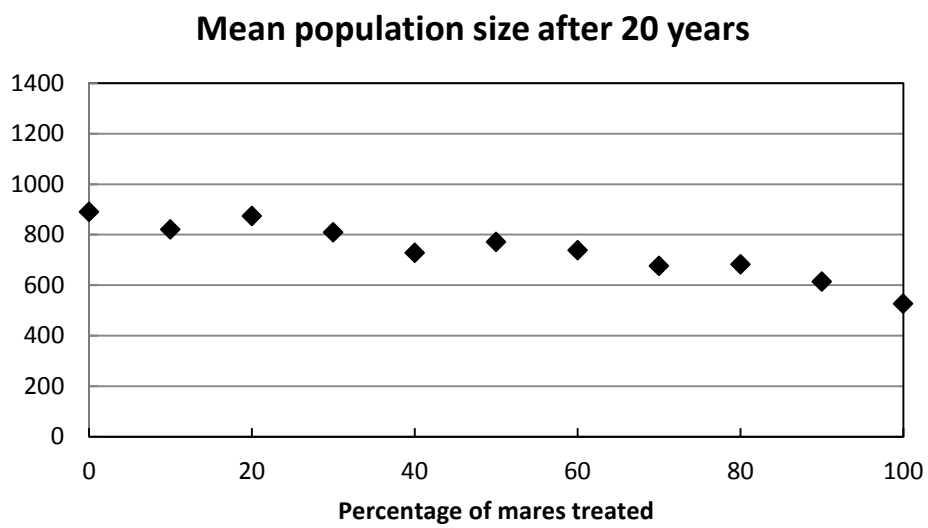
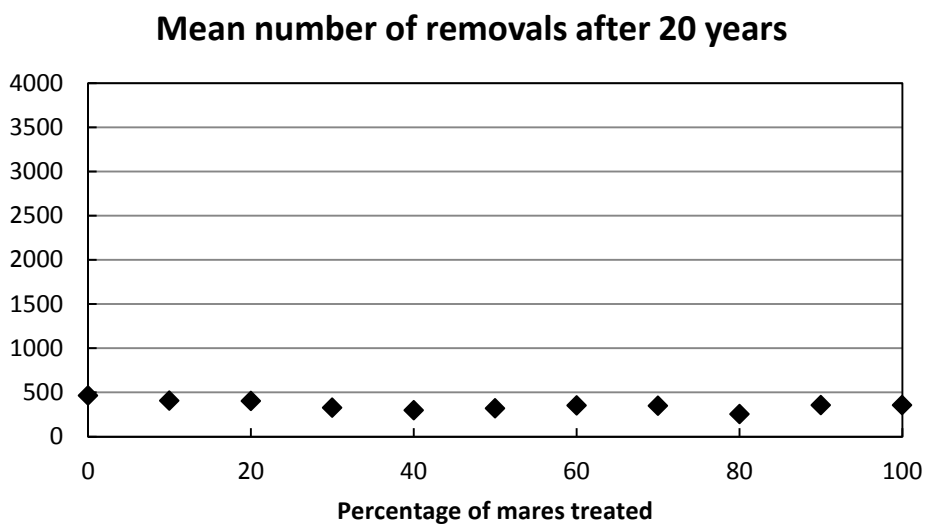
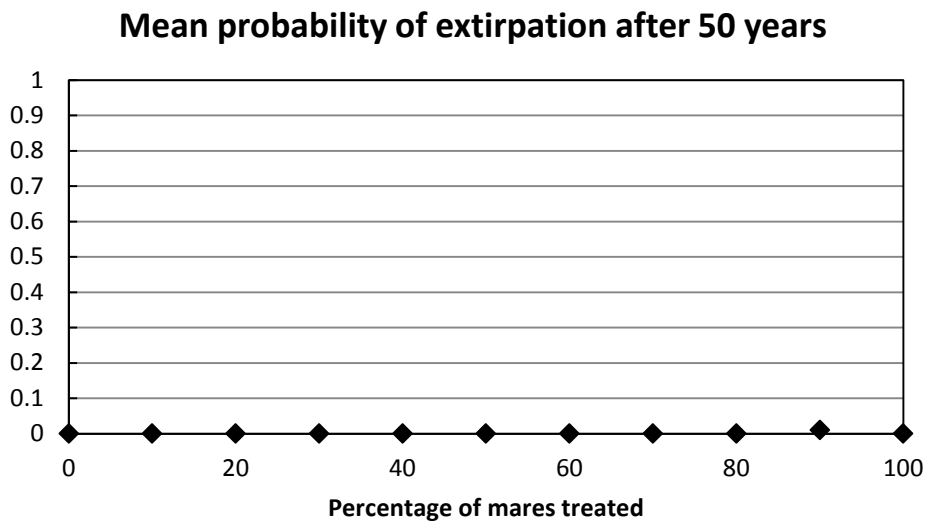


Figure 20. Initial population size = 750, lambda = 1.25, genetic diversity = low.—Continued







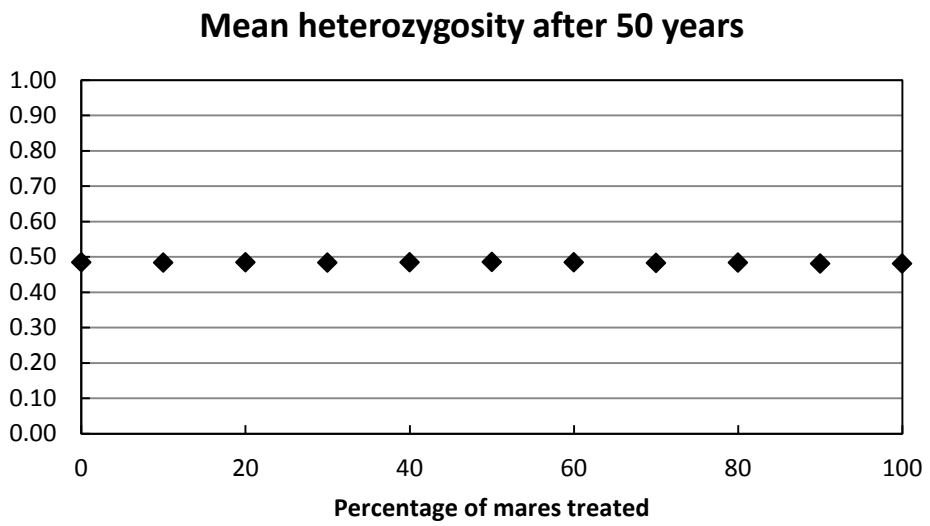


Figure 21. Initial population size = 1000, lambda = 1.05, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	889.31	819.79	873.34	808.05	727.98	770.69	737.69	675.8	681.41	613.88	525.62
Mean number of removals after 20 years	462.82	404.15	400.39	327.35	297.35	318.94	349.73	348.62	252.02	354.25	356.61
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0.01	0
Mean heterozygosity after 50 years	0.48	0.48	0.48	0.48	0.48	0.49	0.48	0.48	0.48	0.48	0.48

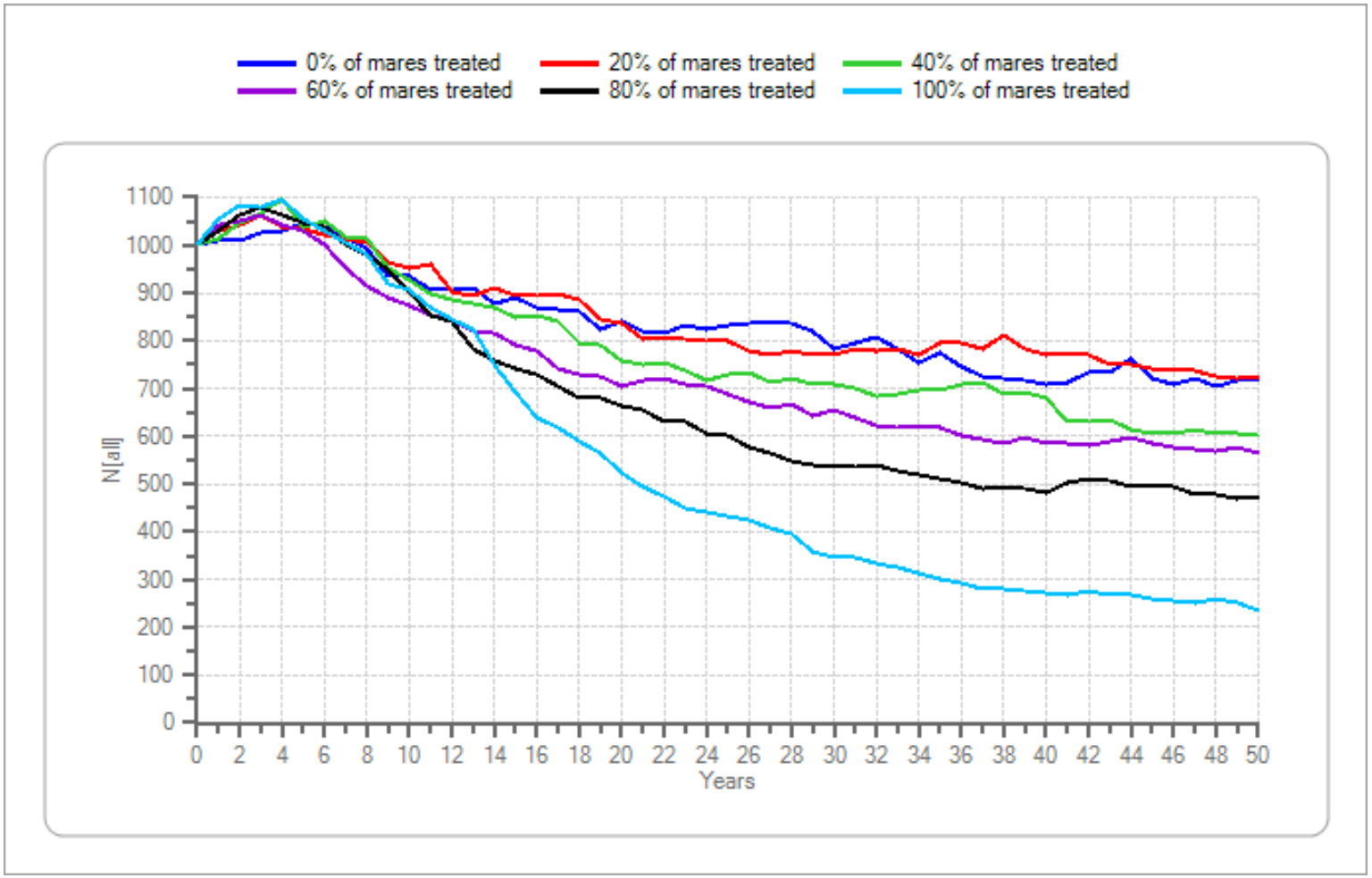
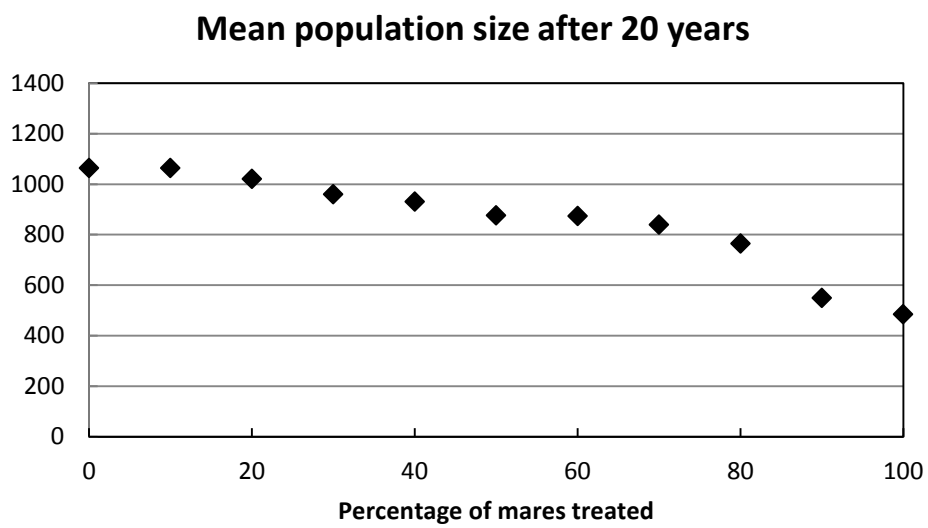
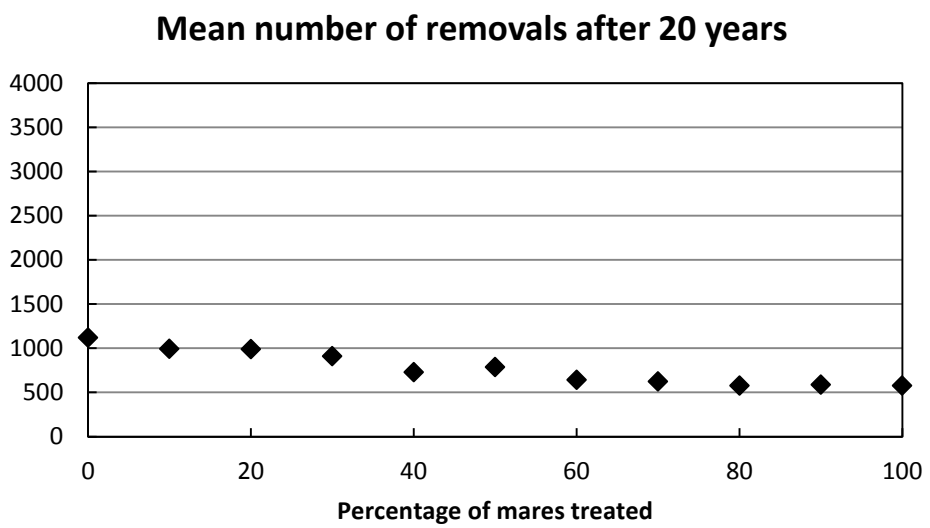
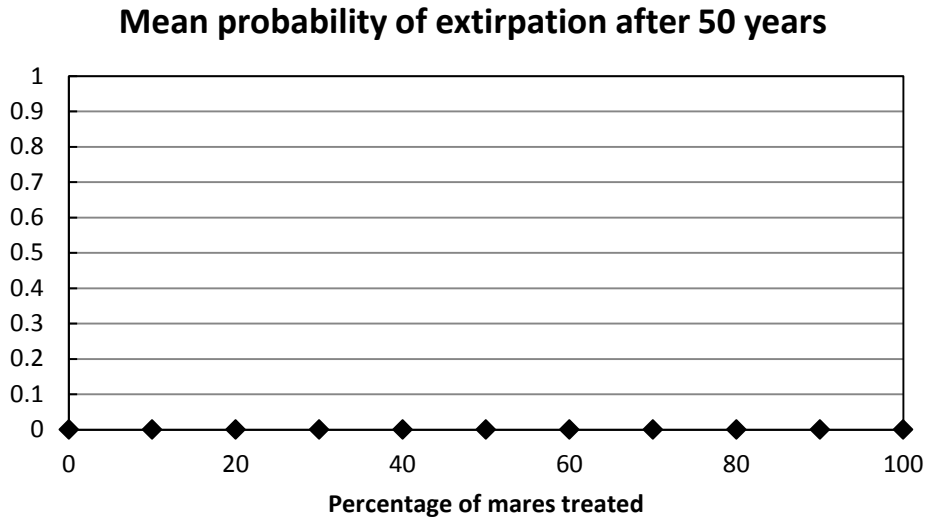


Figure 21. Initial population size = 1000, lambda = 1.05, genetic diversity = low.—Continued







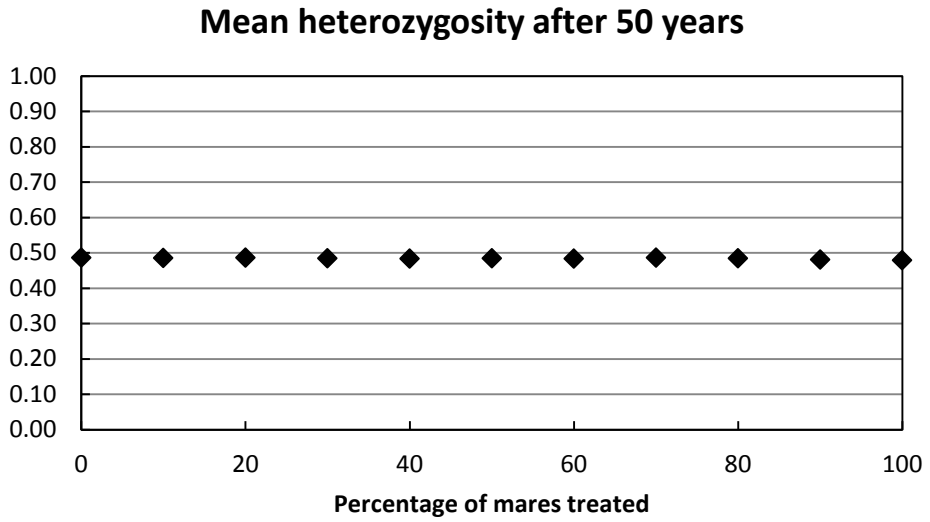


Figure 22. Initial population size = 1000, lambda = 1.10, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1063.82	1064.02	1019.82	959.43	930.43	875.89	872.81	839.08	764.81	549.04	483.72
Mean number of removals after 20 years	1118.7	991.5	988.6	908.4	728.6	785.9	642.2	621.2	577.2	585.4	577.7
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.48	0.48	0.48	0.48	0.49	0.48	0.48	0.48

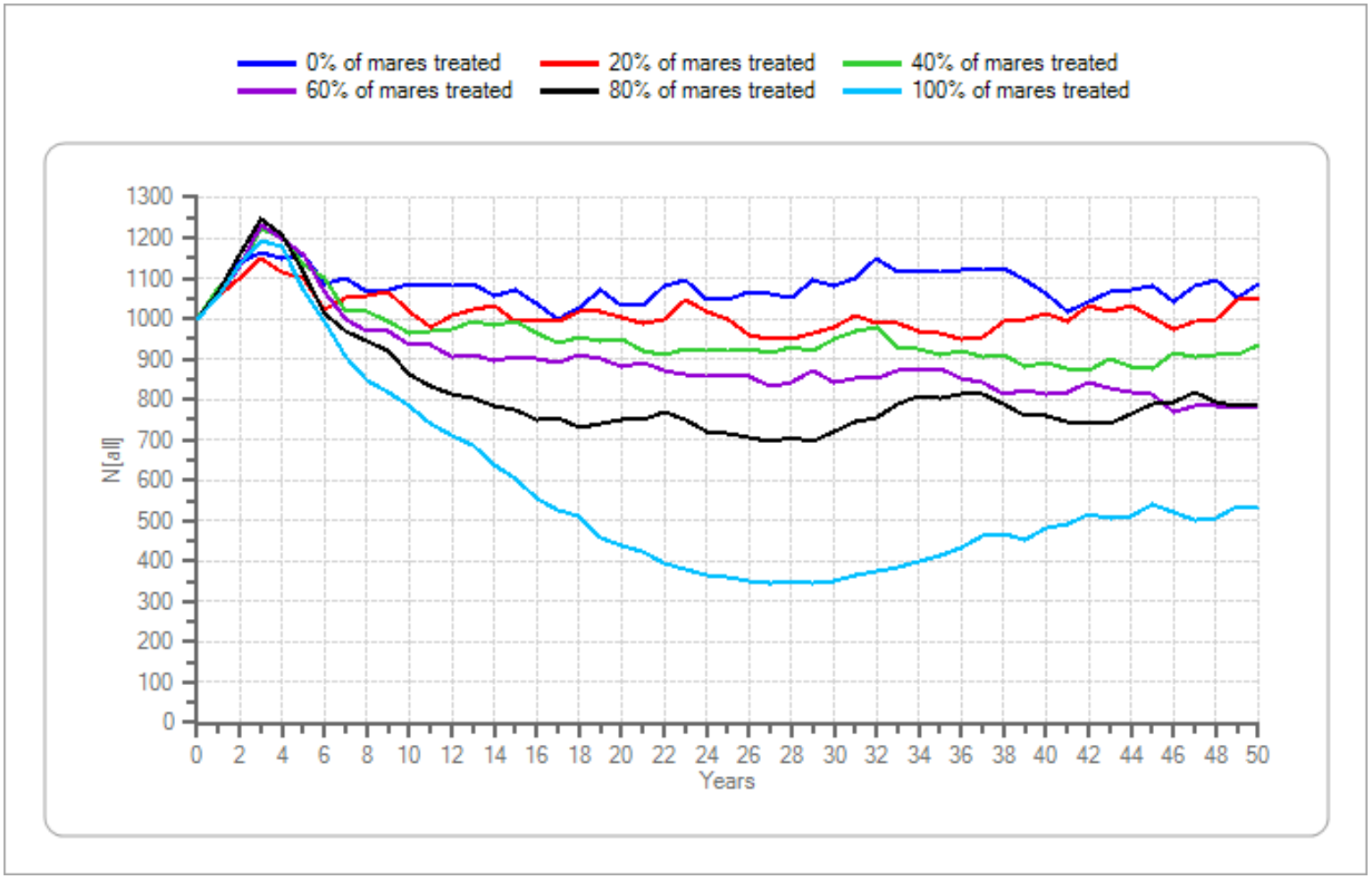
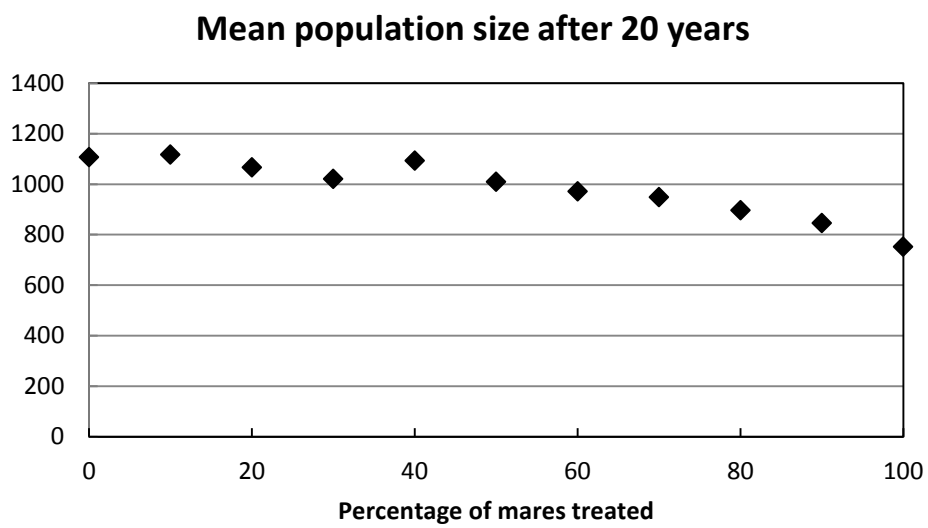
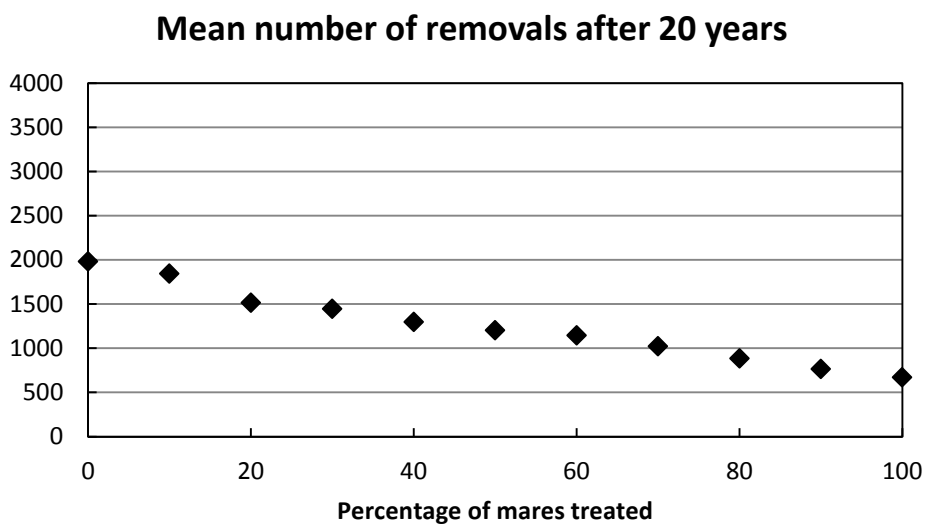
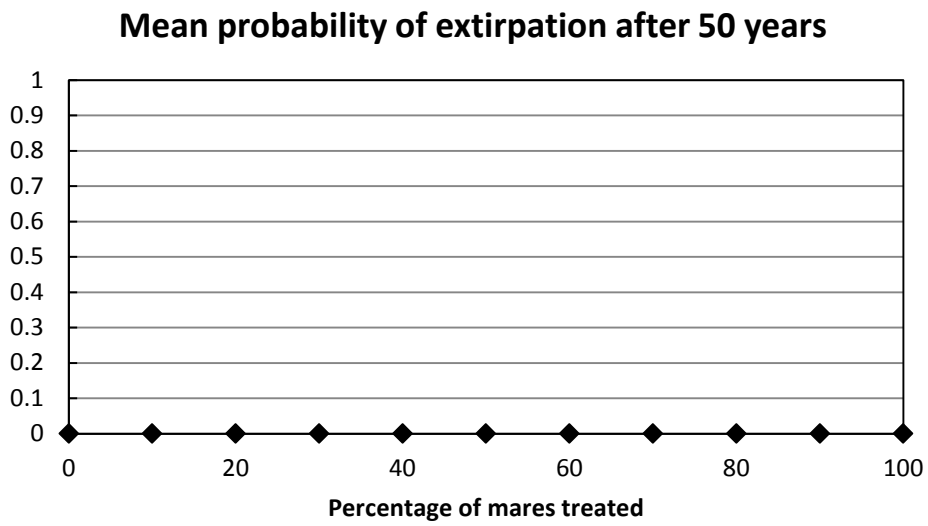


Figure 22. Initial population size = 1000, lambda = 1.10, genetic diversity = low.—Continued







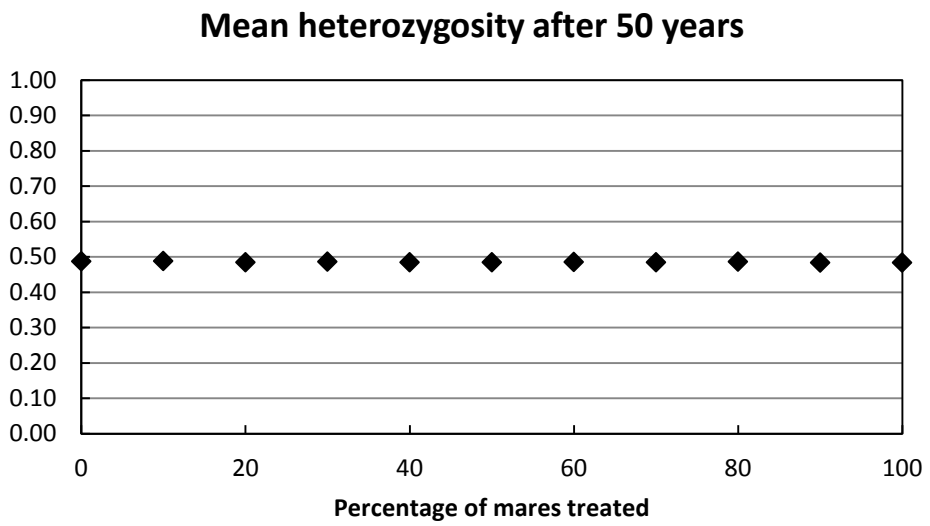


Figure 23. Initial population size = 1000, lambda = 1.15, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1106.82	1117.06	1065.72	1020.7	1093.2	1008.49	970.31	948.69	895.72	845.6	752.22
Mean number of removals after 20 years	1979.8	1841.8	1512.9	1445.2	1296.8	1201.1	1143.0	1020.5	883.9	764.1	669.3
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.48	0.49	0.48	0.48	0.49	0.48	0.49	0.48	0.48

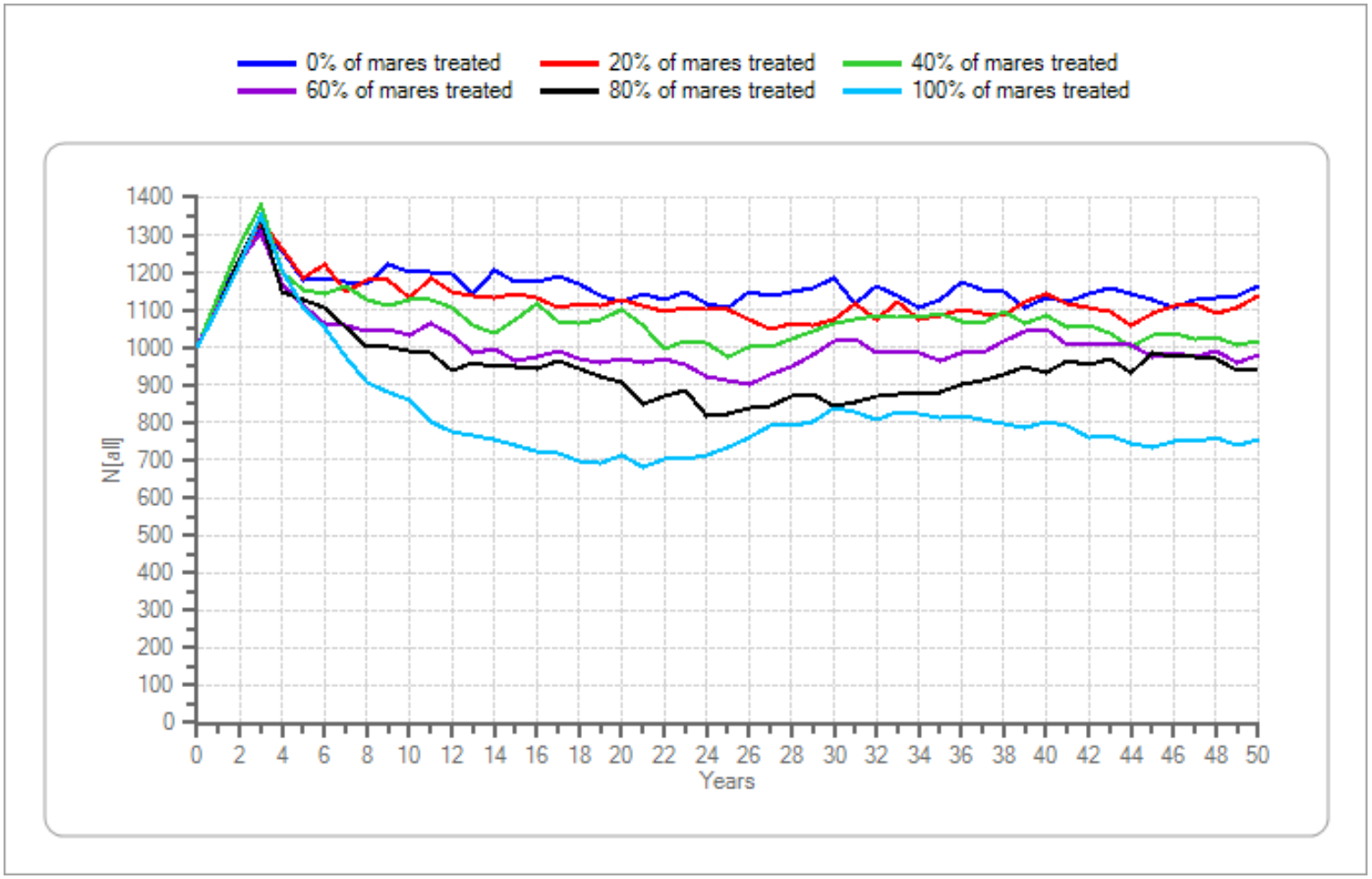
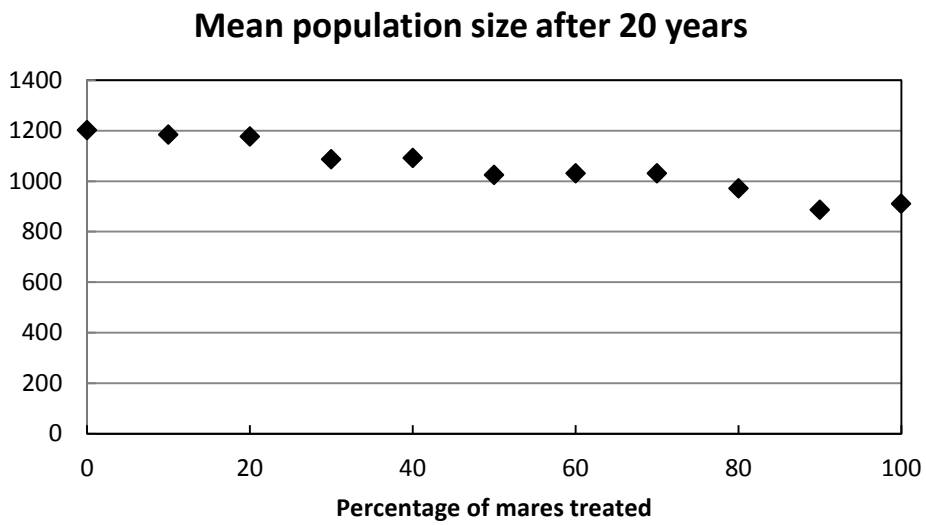
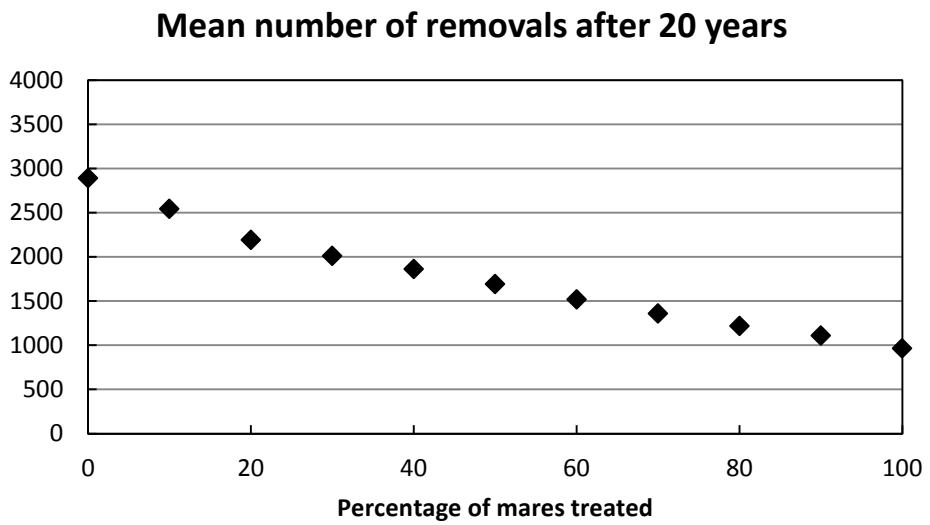
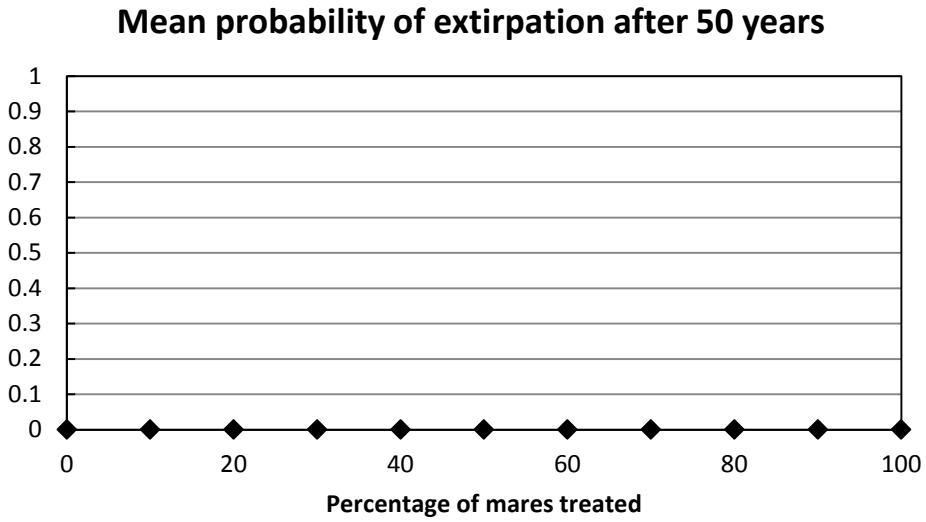


Figure 23. Initial population size = 1000, lambda = 1.15, genetic diversity = low.—Continued







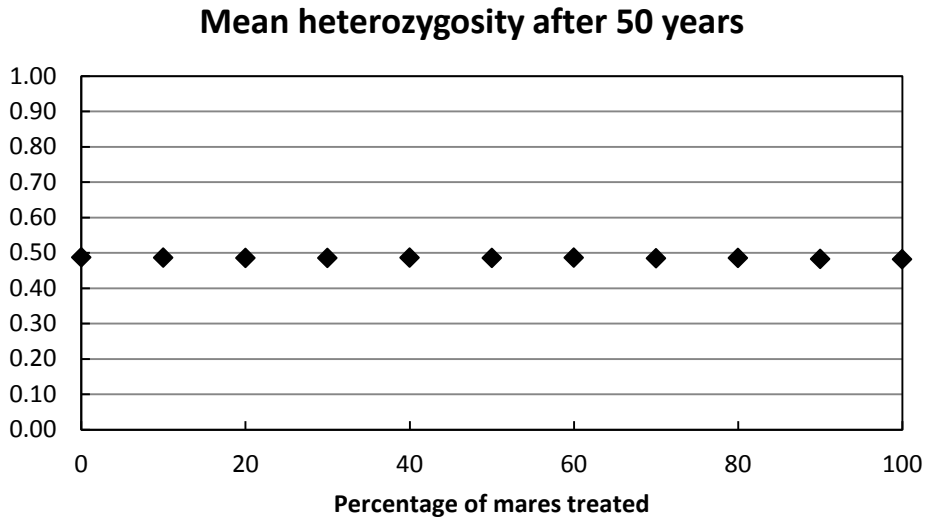


Figure 24. Initial population size = 1000, lambda = 1.20, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1201.08	1184.02	1176.54	1085.77	1090.8	1024.31	1030.98	1029.85	970.74	885.67	910.27
Mean number of removals after 20 years	2890.6	2543.6	2191.6	2009.4	1861.7	1689.8	1515.9	1357.8	1215.4	1107.5	961.8
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.48	0.48	0.48	0.48

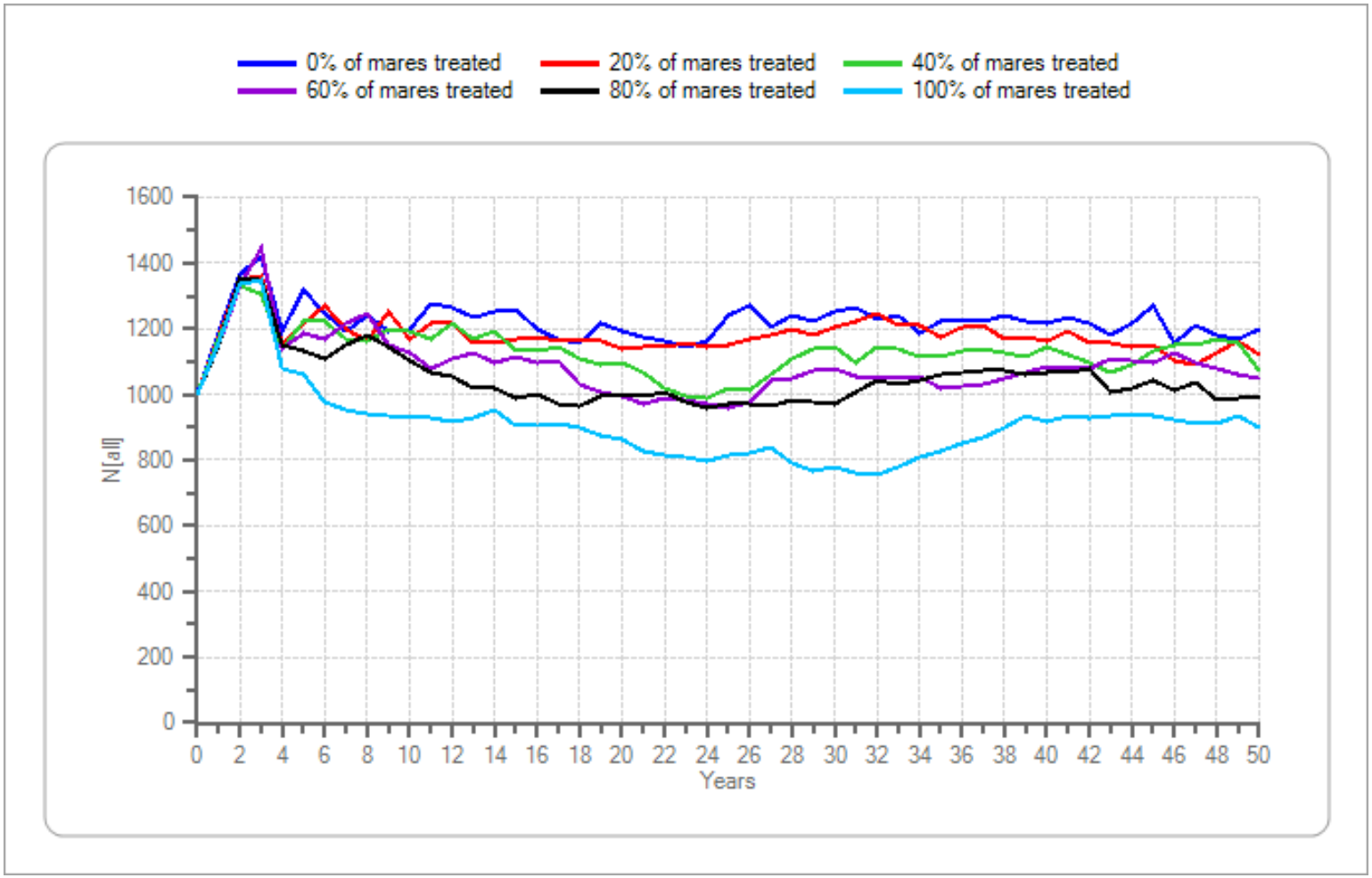


Figure 24. Initial population size = 1000, lambda = 1.20, genetic diversity = low.—Continued

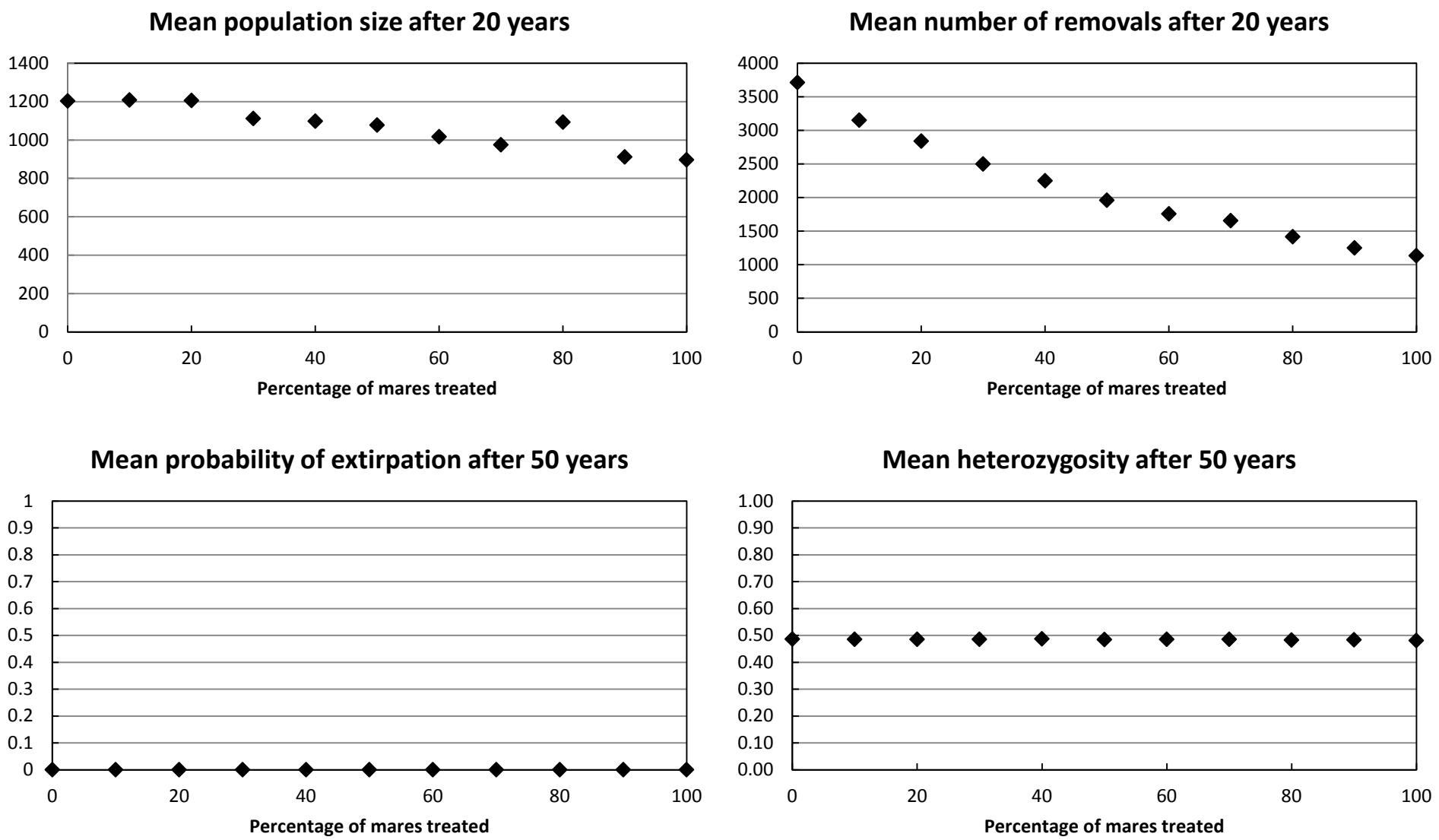


Figure 25. Initial population size = 1000, lambda = 1.25, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1202.9	1207.36	1205.51	1111.97	1097.25	1076.74	1016.51	975.02	1092.2	911.9	896.56
Mean number of removals after 20 years	3713.4	3151.0	2838.8	2498.7	2250.0	1960.2	1756.7	1654.6	1417.1	1247.7	1131.8
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.49	0.49	0.48	0.49	0.49	0.48	0.48	0.48

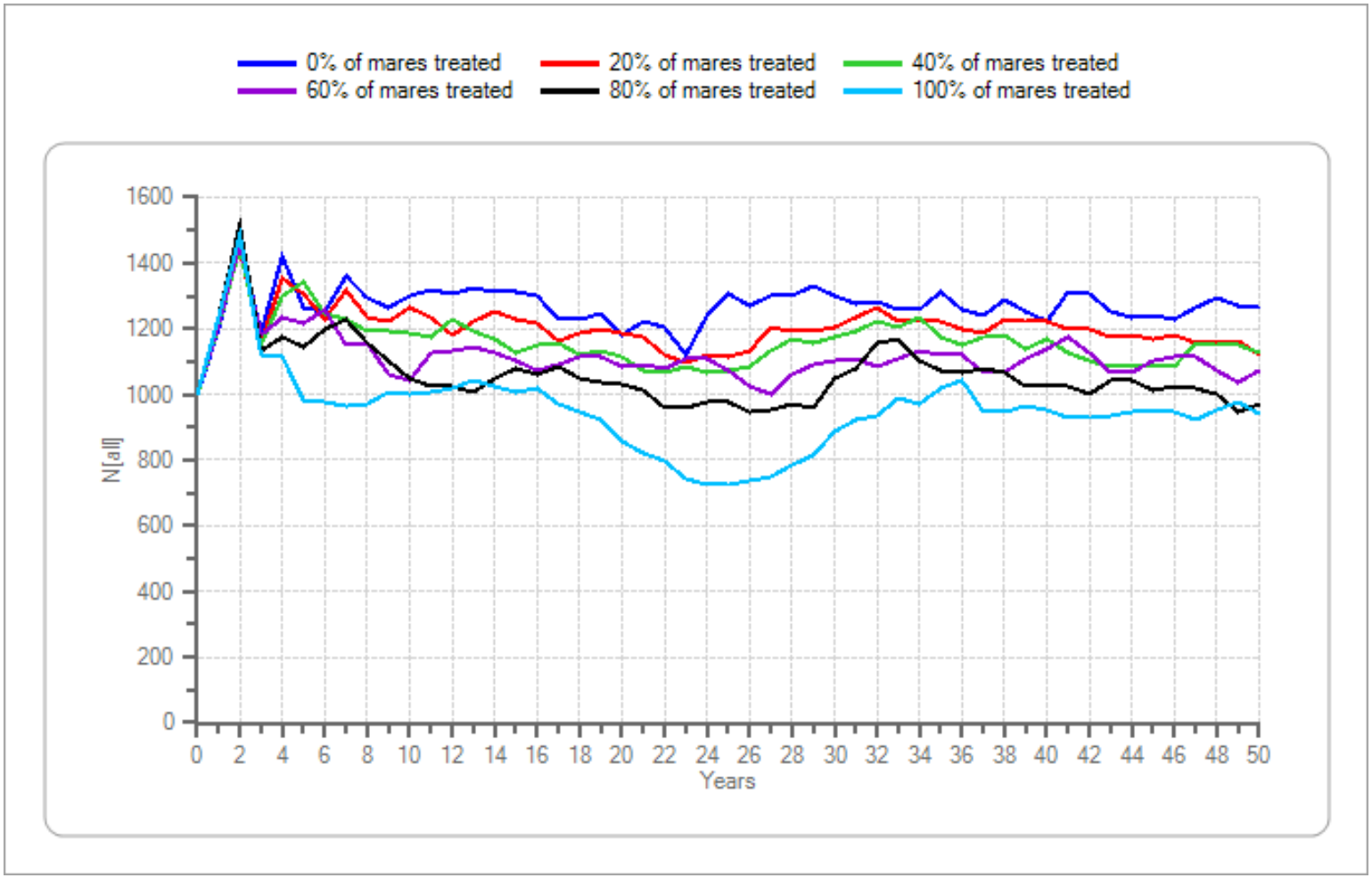
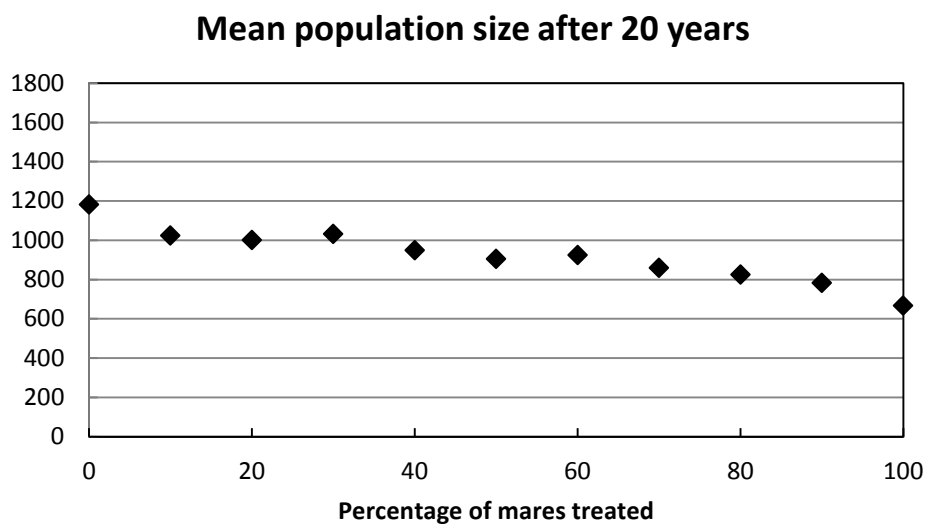
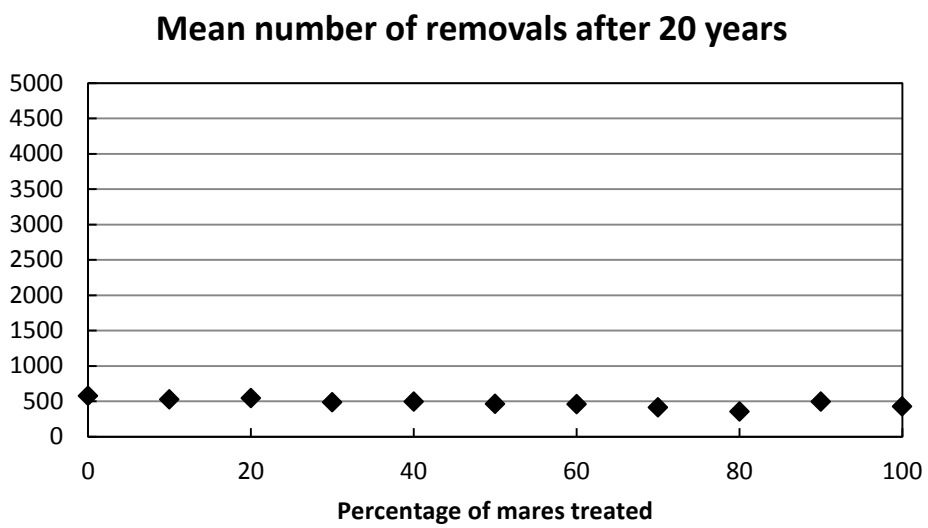
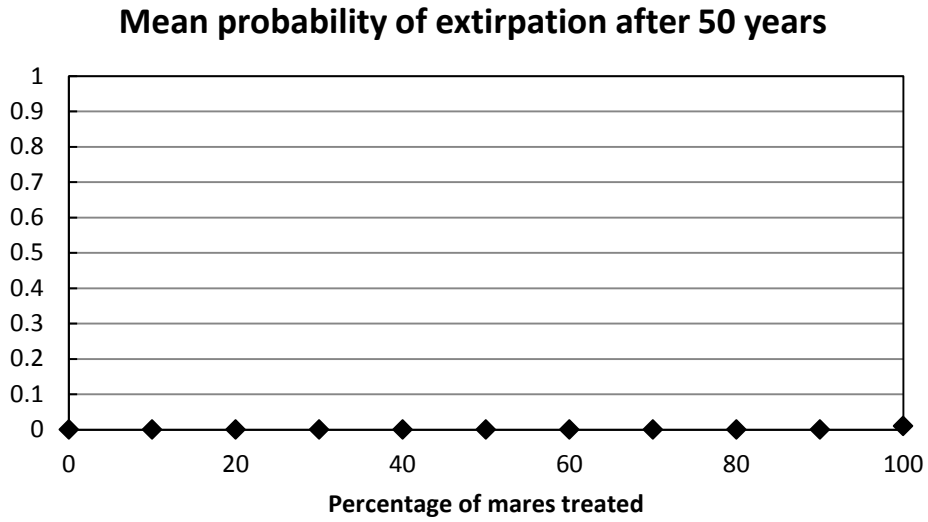


Figure 25. Initial population size = 1000, lambda = 1.25, genetic diversity = low.—Continued







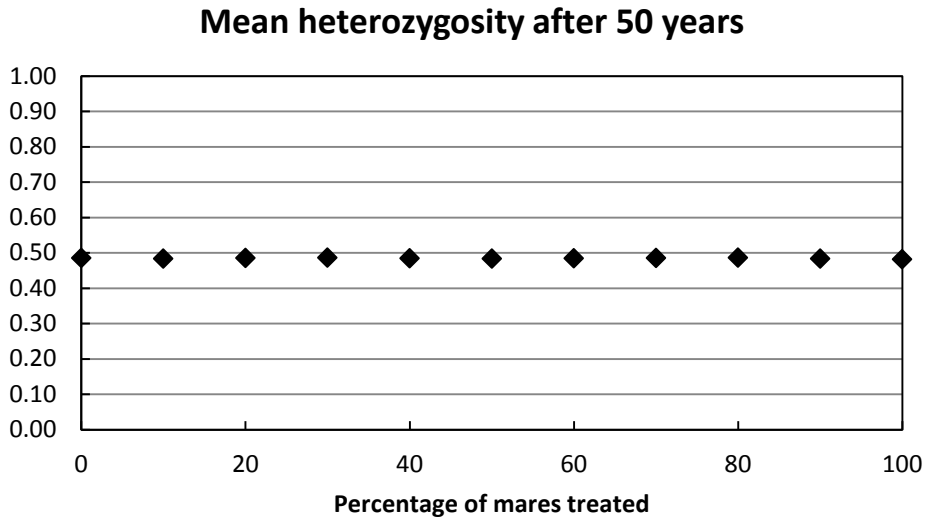


Figure 26. Initial population size = 1250, lambda = 1.05, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1181.85	1024.21	1000.58	1032.15	948.63	904.73	923.82	859.2	824.58	782.46	666.31
Mean number of removals after 20 years	574.25	523.43	544.18	483.72	493.96	463.54	459.15	412.01	352.83	491.73	425.11
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0.01
Mean heterozygosity after 50 years	0.49	0.48	0.49	0.49	0.48	0.48	0.48	0.49	0.49	0.48	0.48

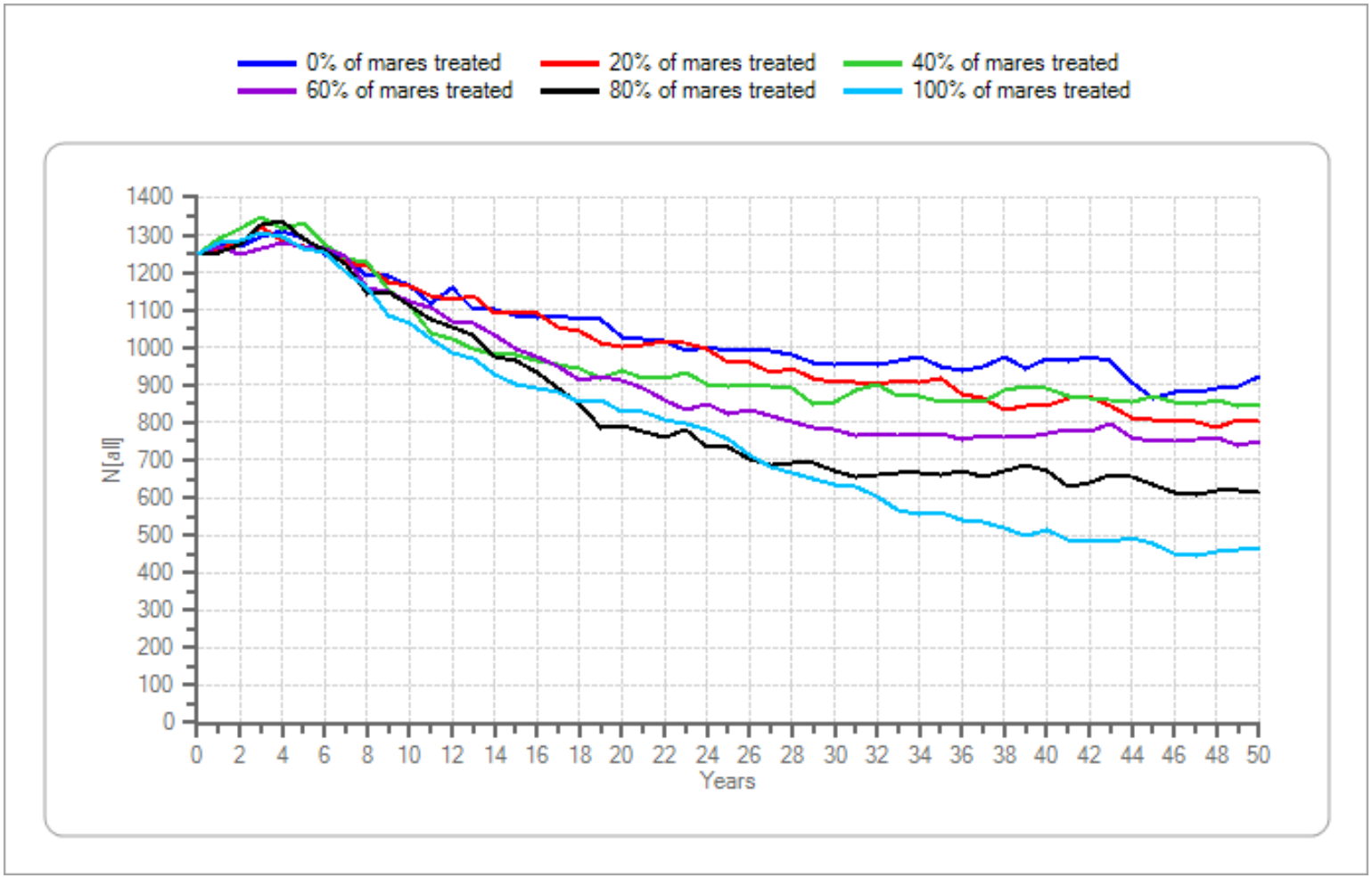
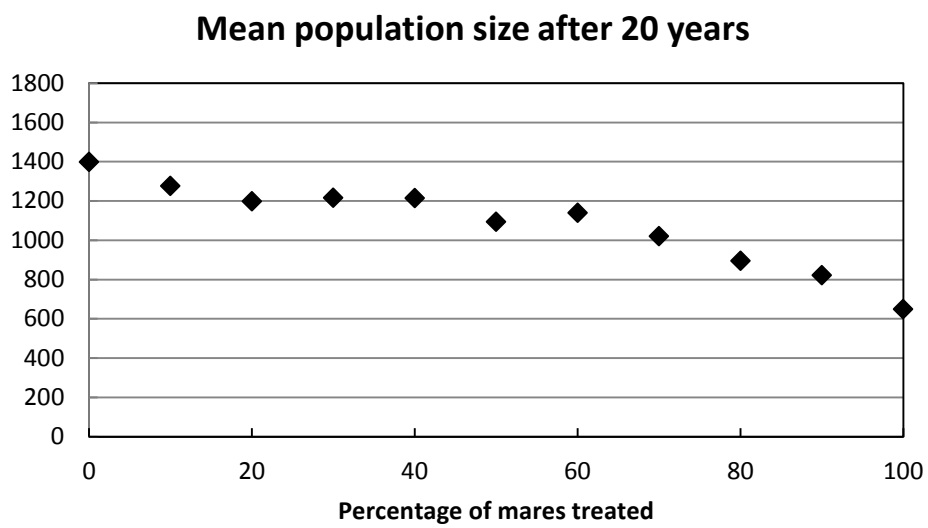
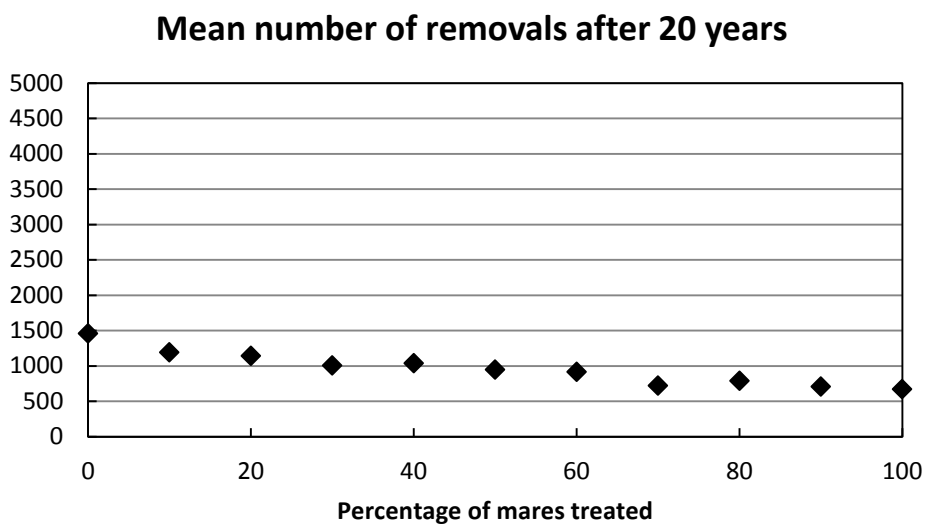
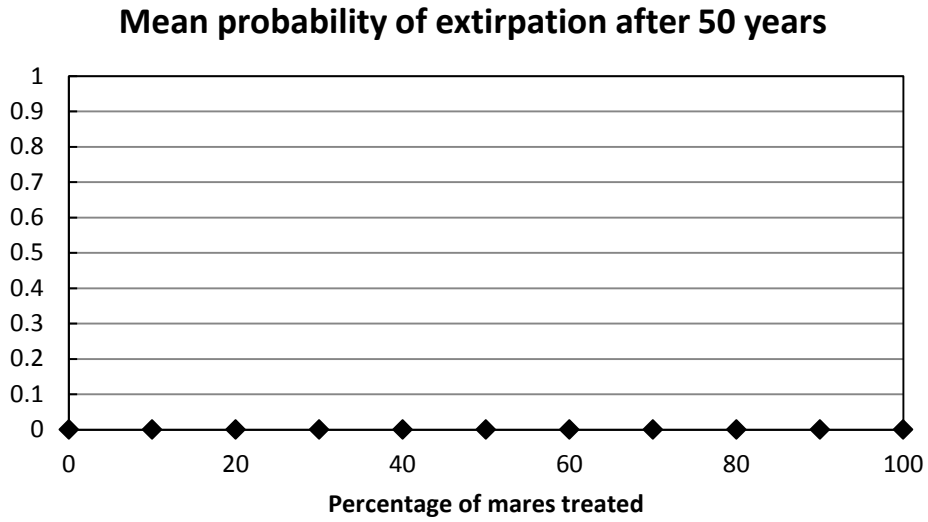


Figure 26. Initial population size = 1250, lambda = 1.05, genetic diversity = low.—Continued







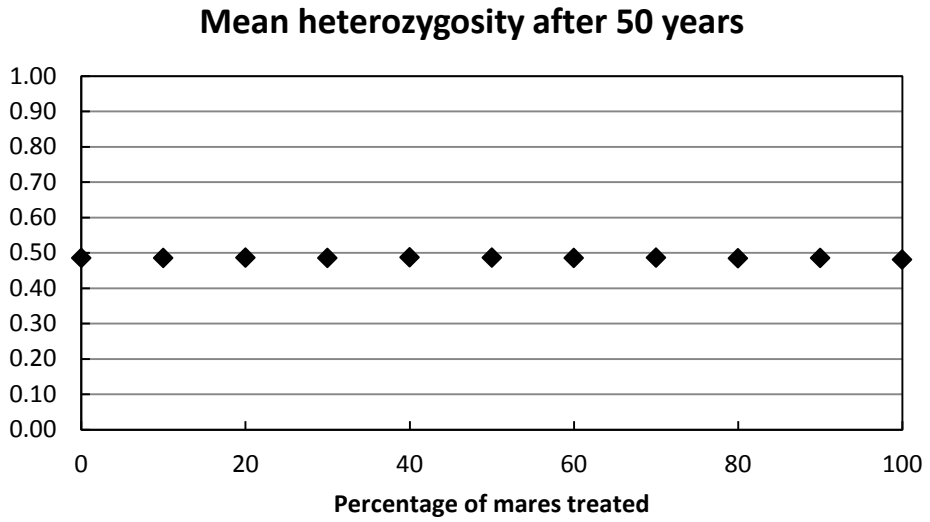


Figure 27. Initial population size = 1250, lambda = 1.10, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1398.94	1275.26	1198.43	1215.27	1214.22	1094.1	1138.43	1019.54	893.99	821.65	649.09
Mean number of removals after 20 years	1458.6	1191.7	1140.2	1004.3	1038.6	948.0	915.8	720.1	788.2	708.3	672.1
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.48	0.49	0.49	0.49	0.49	0.49	0.49	0.48	0.49	0.48

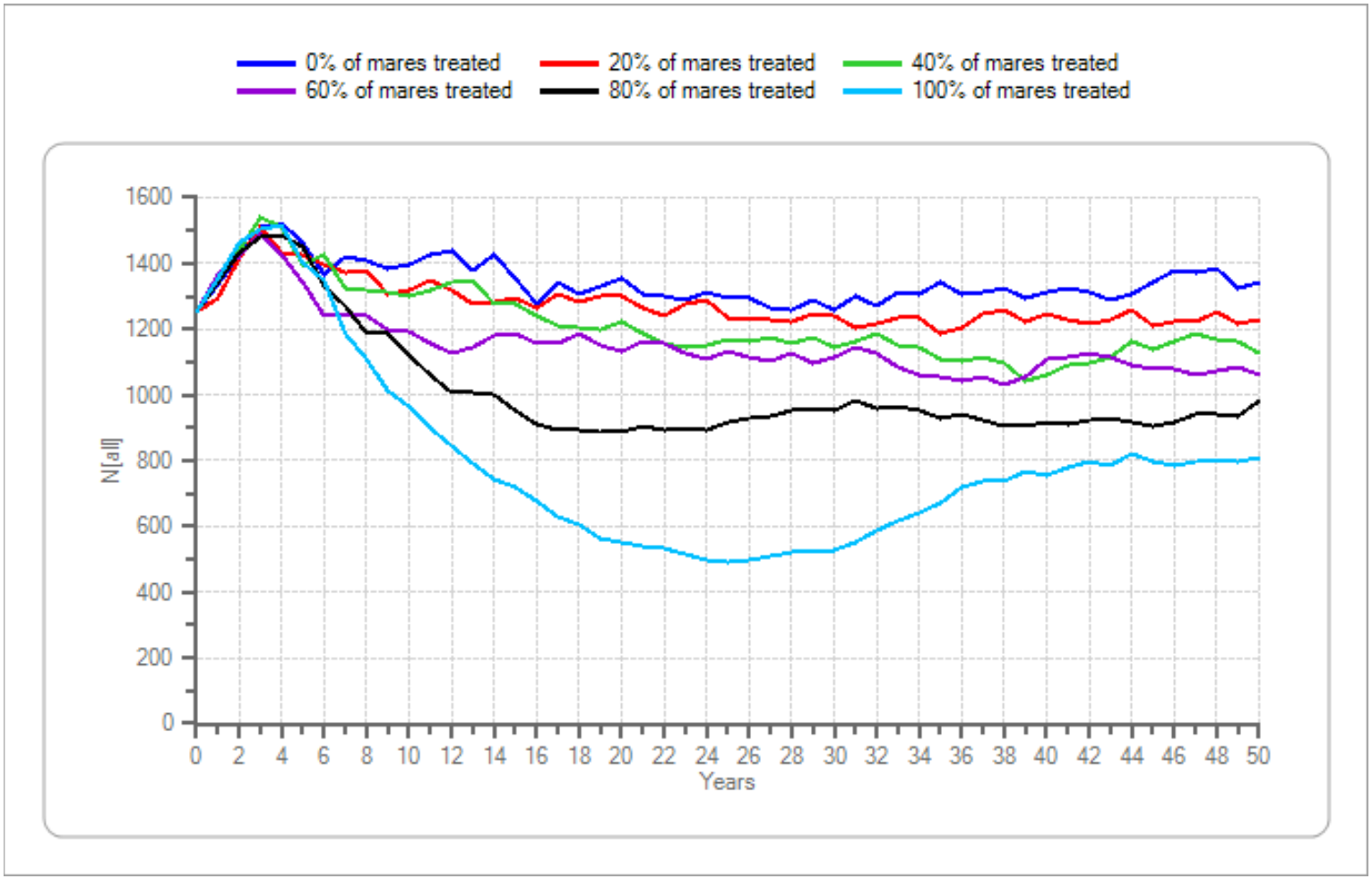
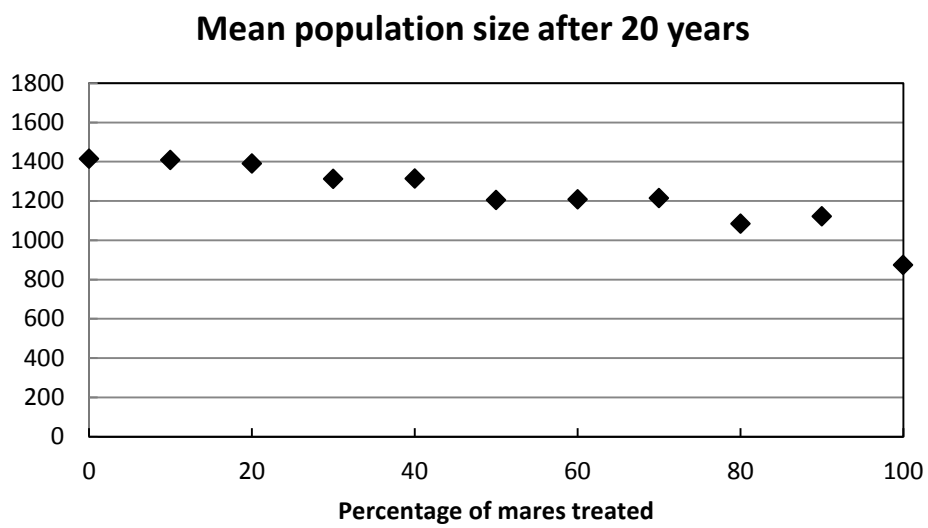
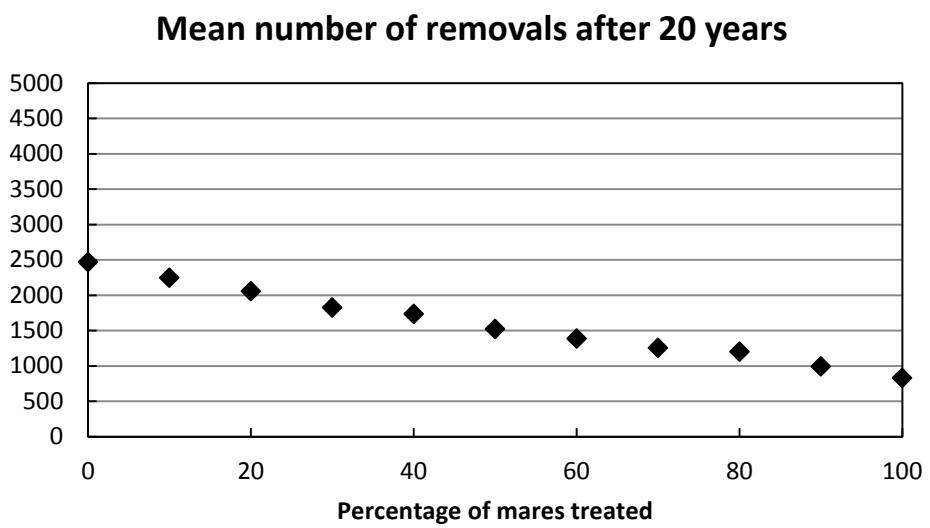
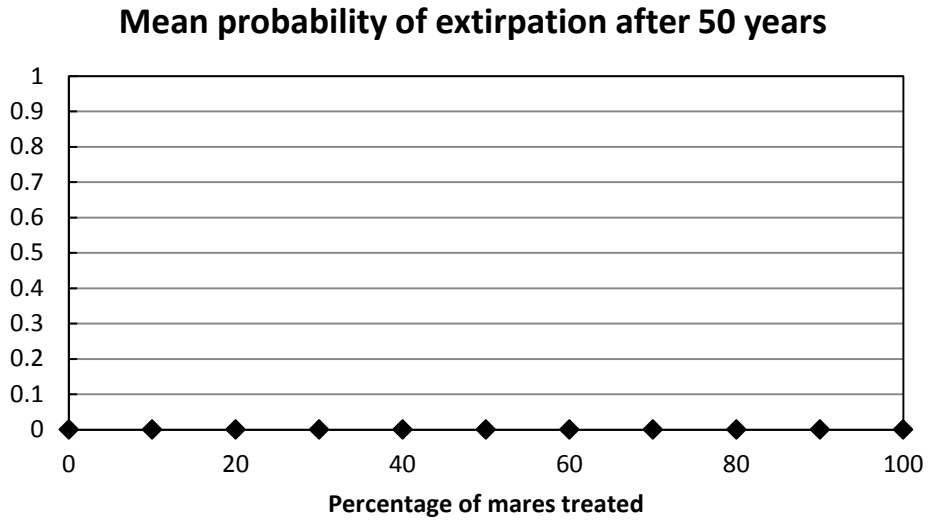


Figure 27. Initial population size = 1250, lambda = 1.10, genetic diversity = low.—Continued







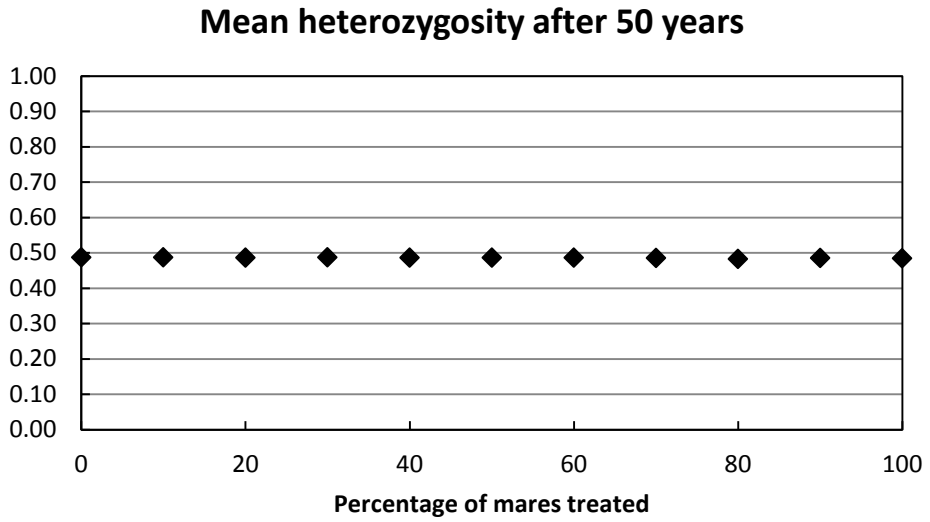


Figure 28. Initial population size = 1250, lambda = 1.15, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1413.8	1407.5	1390.5	1311.6	1313.5	1204.9	1207.4	1213.3	1084.0	1120.9	874.2
Mean number of removals after 20 years	2465.8	2243.6	2057.3	1822.6	1733.3	1522.9	1385.8	1252.5	1198.7	990.3	829.2
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.48	0.49	0.48

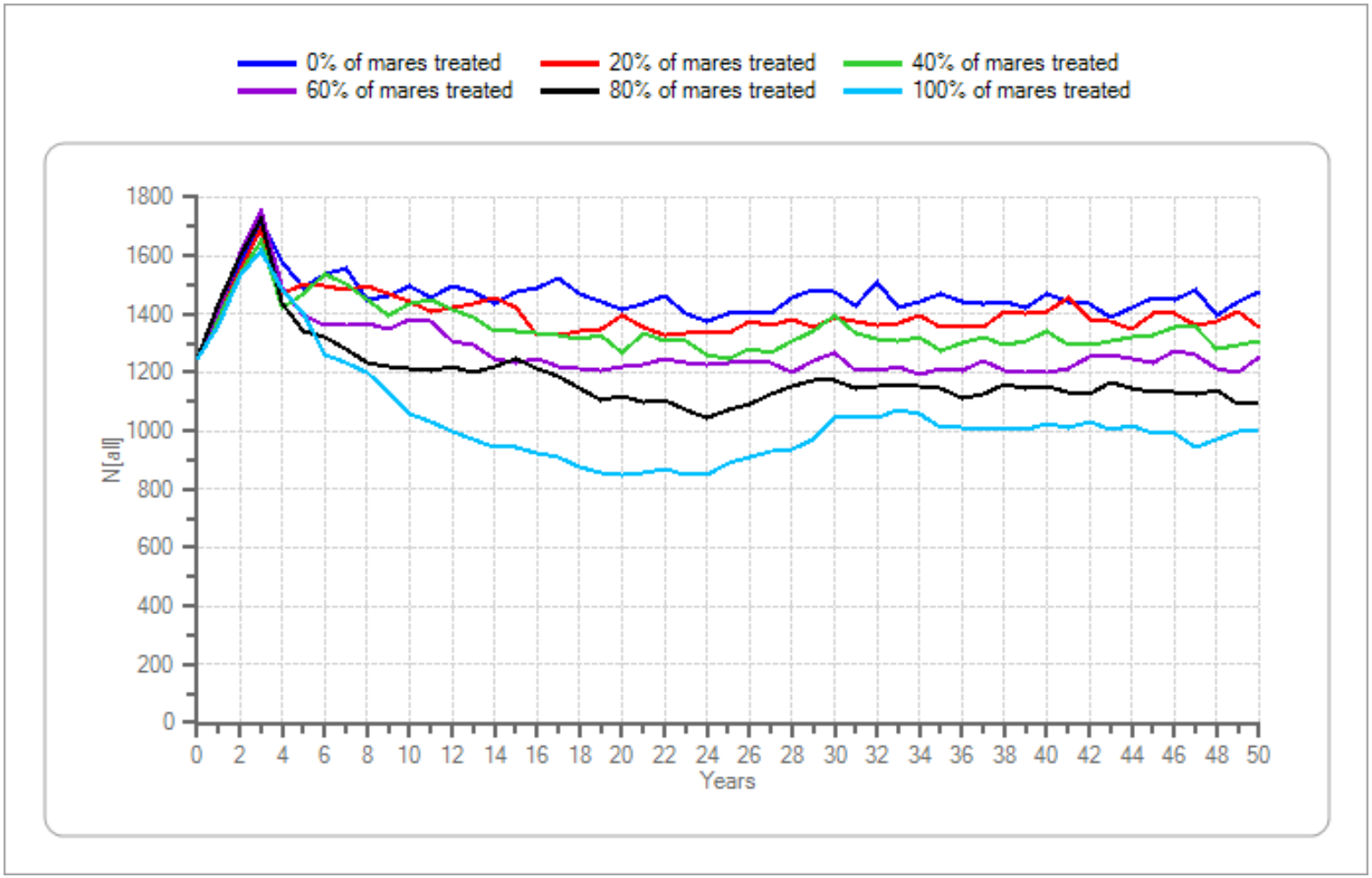
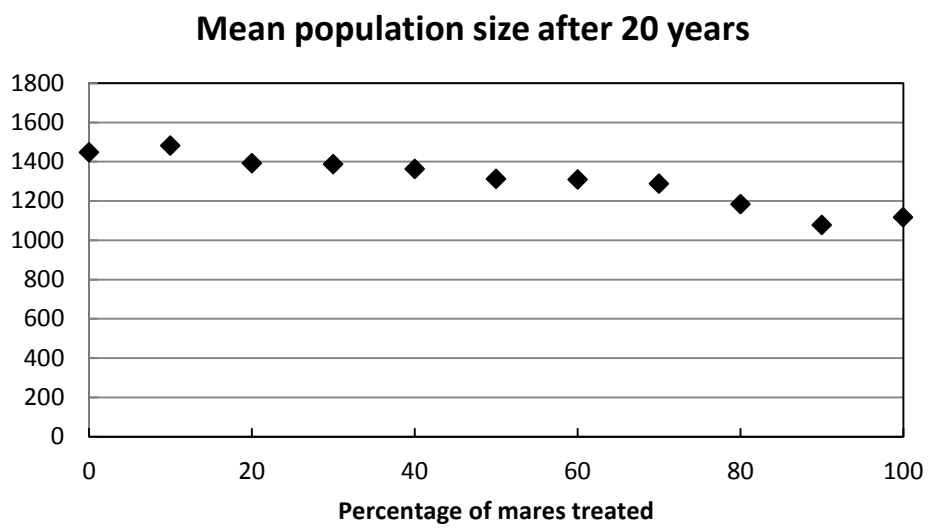
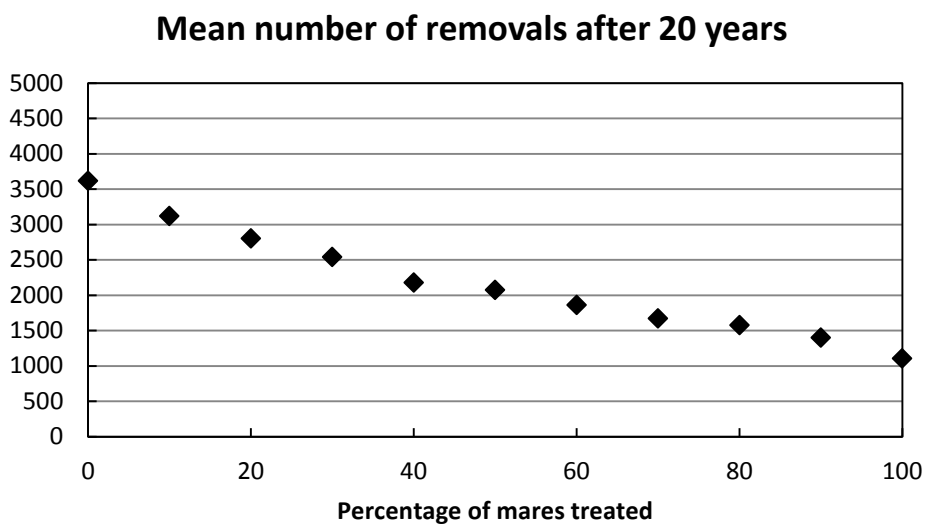
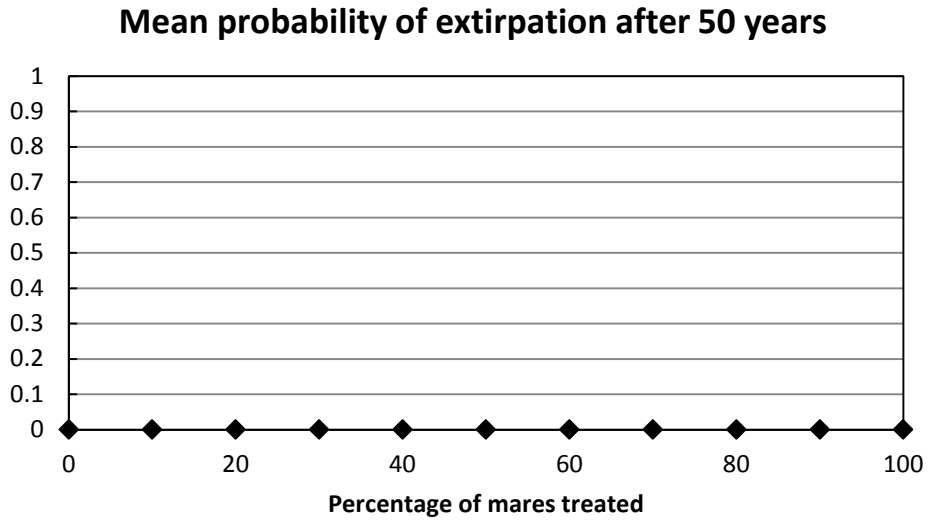


Figure 28. Initial population size = 1250, lambda = 1.15, genetic diversity = low.—Continued







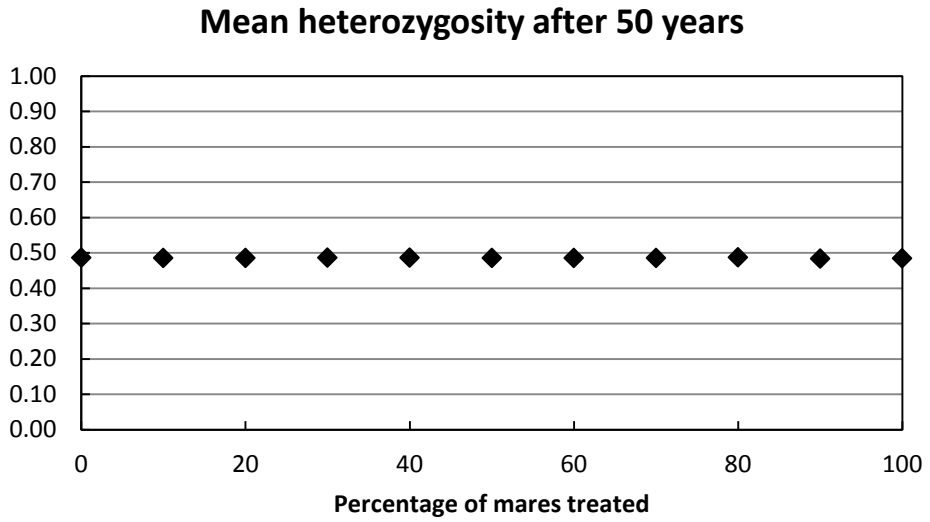


Figure 29. Initial population size = 1250, lambda = 1.20, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1447.5	1481.5	1392.3	1386.1	1362.7	1312.3	1309.2	1287.2	1182.8	1077.5	1116.7
Mean number of removals after 20 years	3615.2	3121.2	2802.8	2538.6	2179.6	2071.5	1859.4	1671.5	1576.1	1398.1	1103.3
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.48	0.49	0.48	0.48

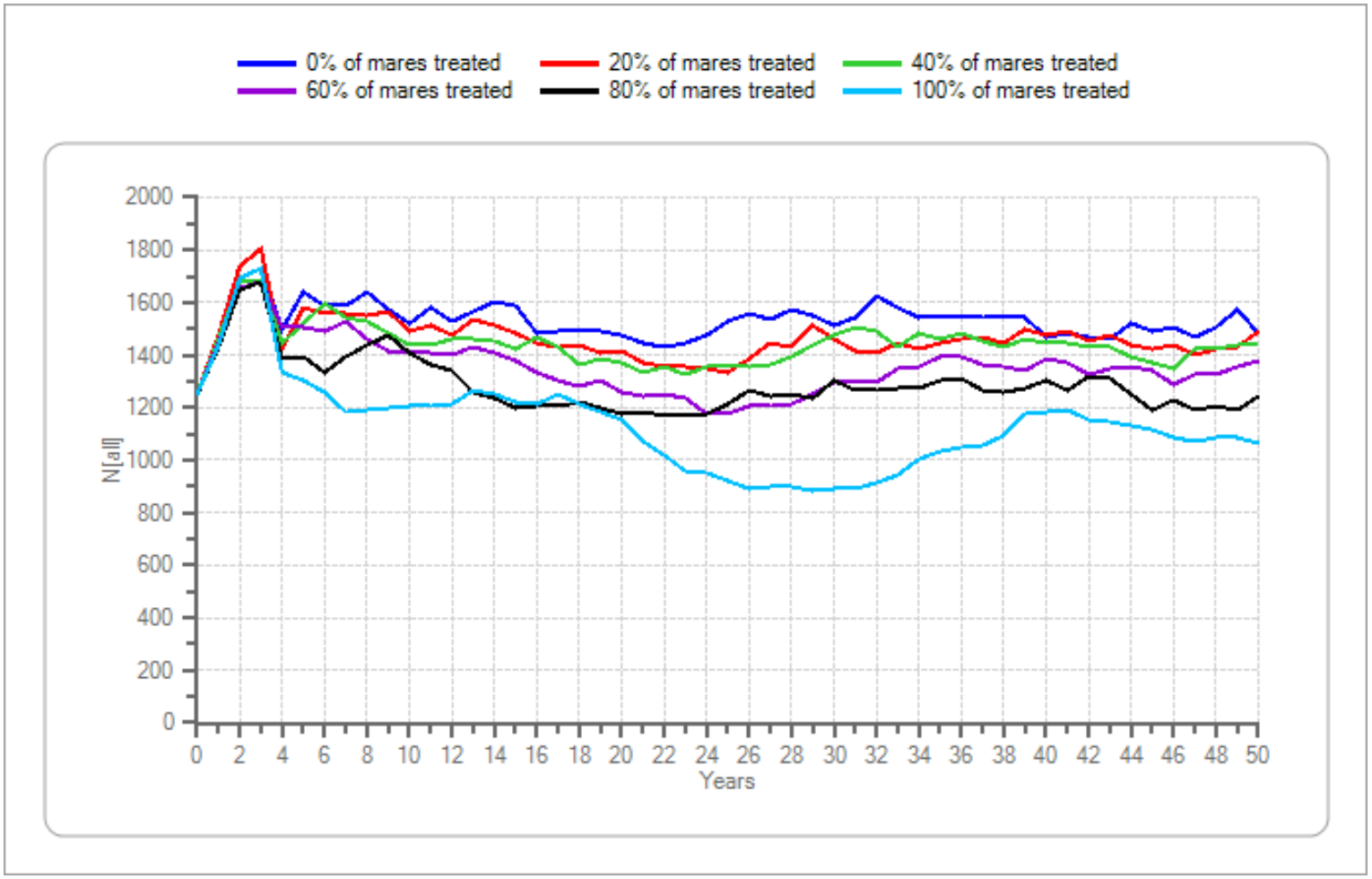
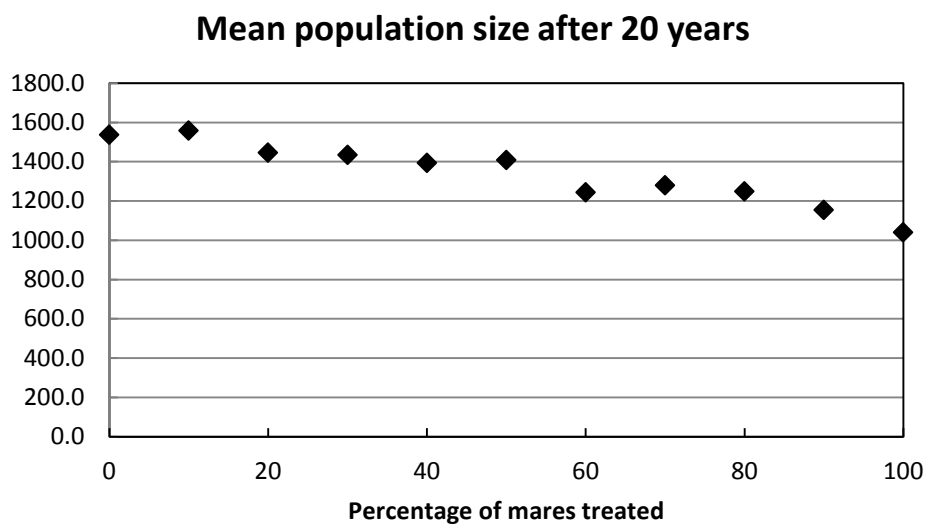
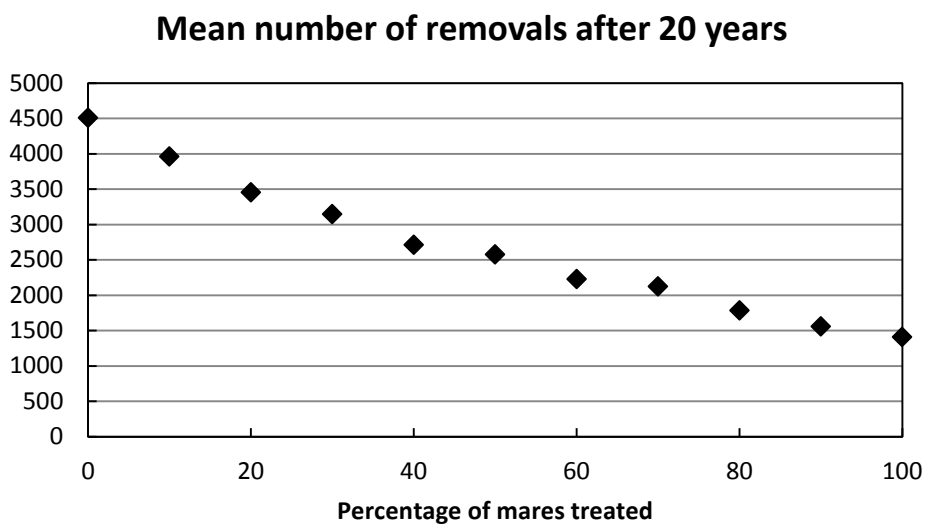
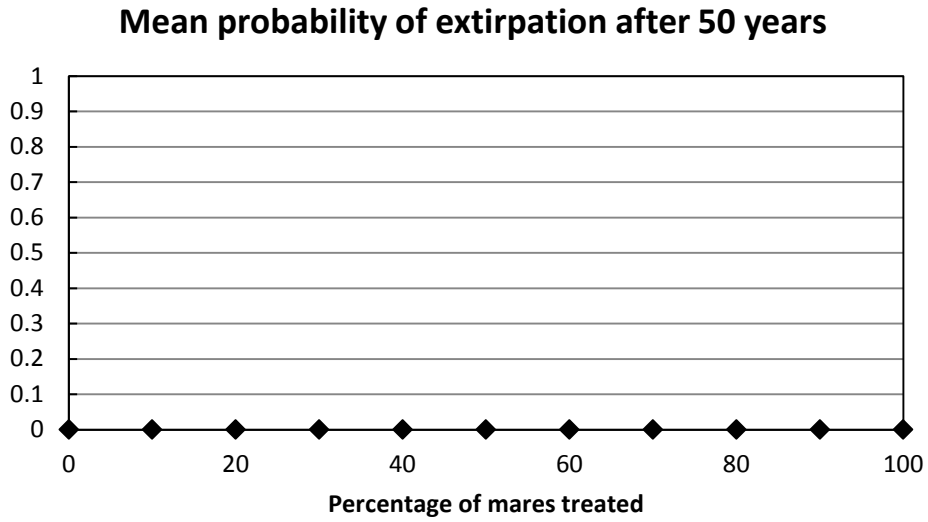


Figure 29. Initial population size = 1250, lambda = 1.20, genetic diversity = low.—Continued







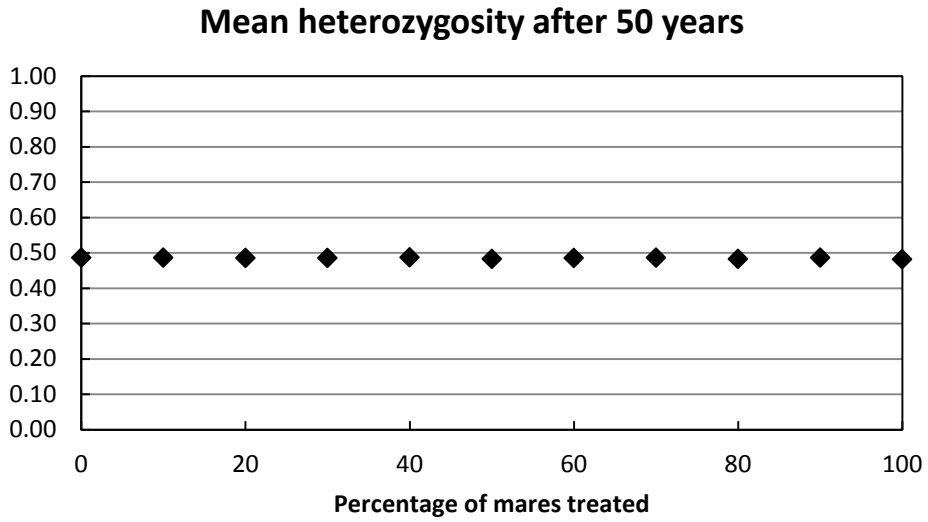


Figure 30. Initial population size = 1250, lambda = 1.25, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1536.3	1557.9	1445.1	1433.6	1394.1	1407.8	1243.9	1279.1	1248.4	1154.4	1039.9
Mean number of removals after 20 years	4509.1	3960.3	3453.9	3146.1	2711.4	2577.9	2226.4	2122.1	1782.3	1559.0	1409.3
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.48	0.49	0.48	0.49	0.49	0.48	0.49	0.48

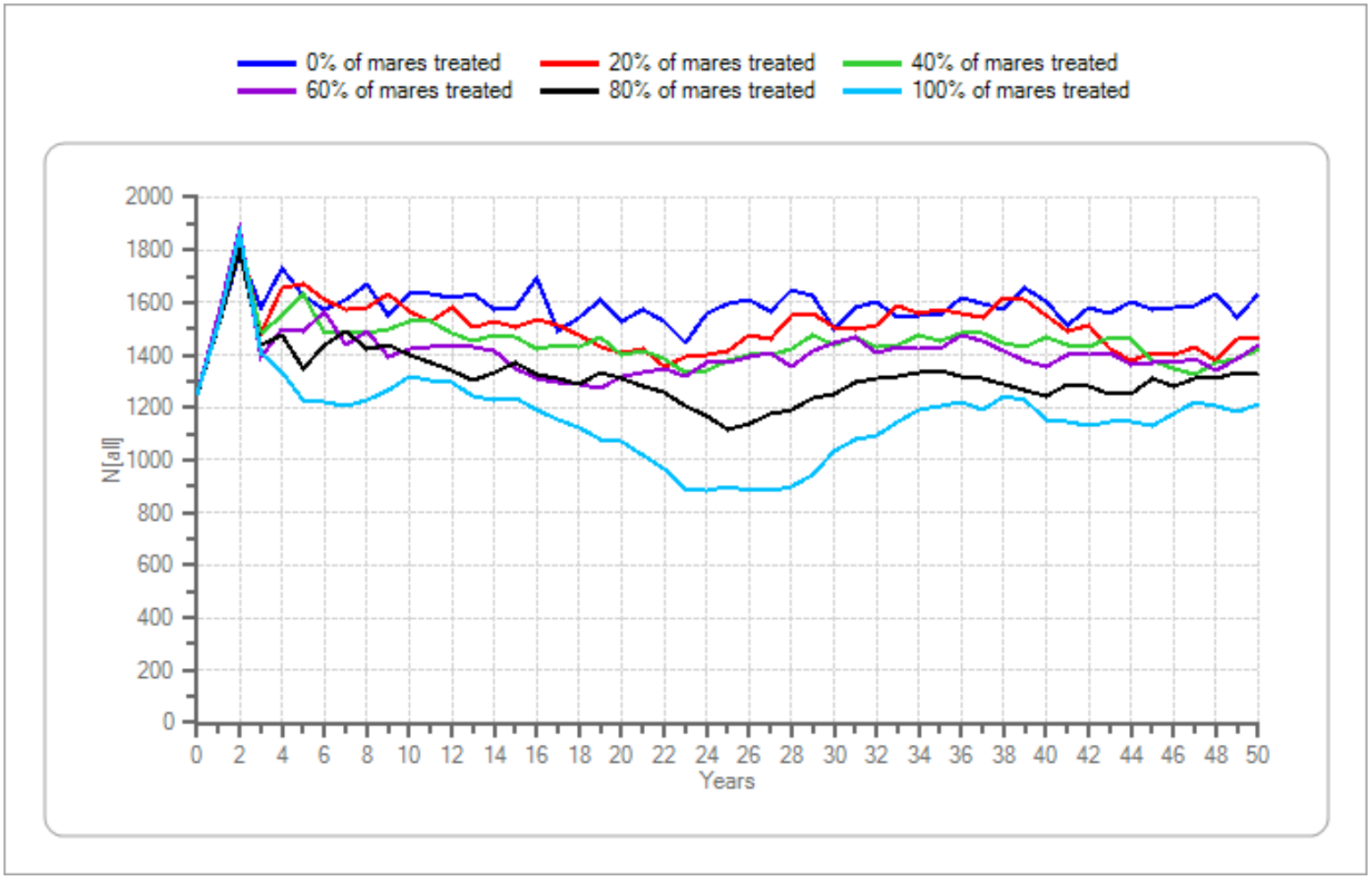
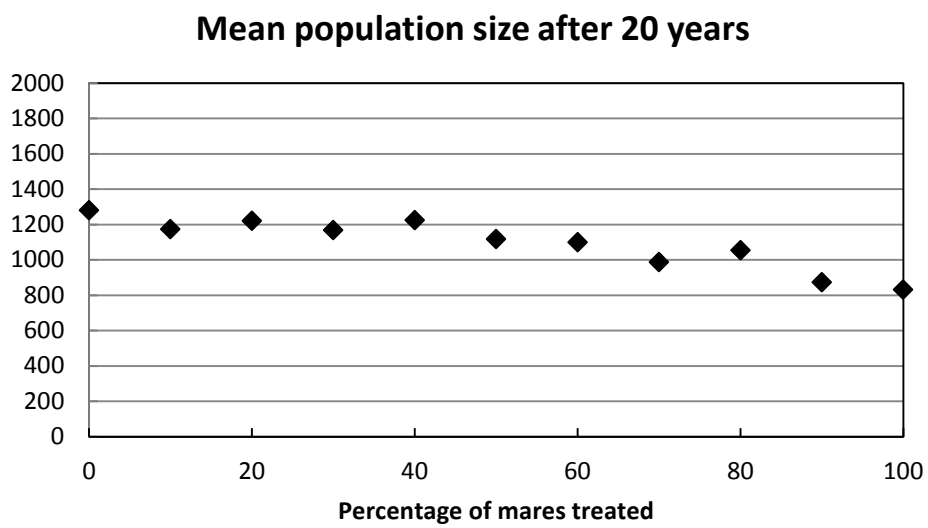
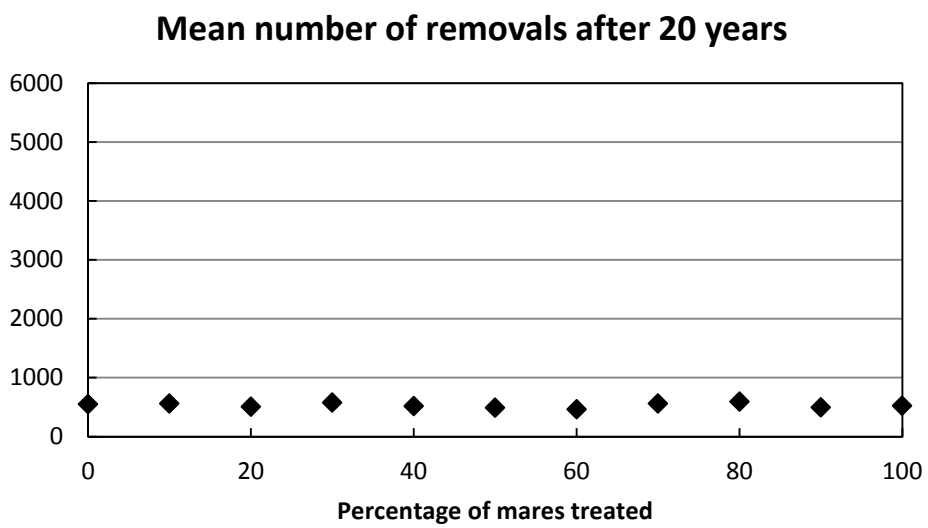
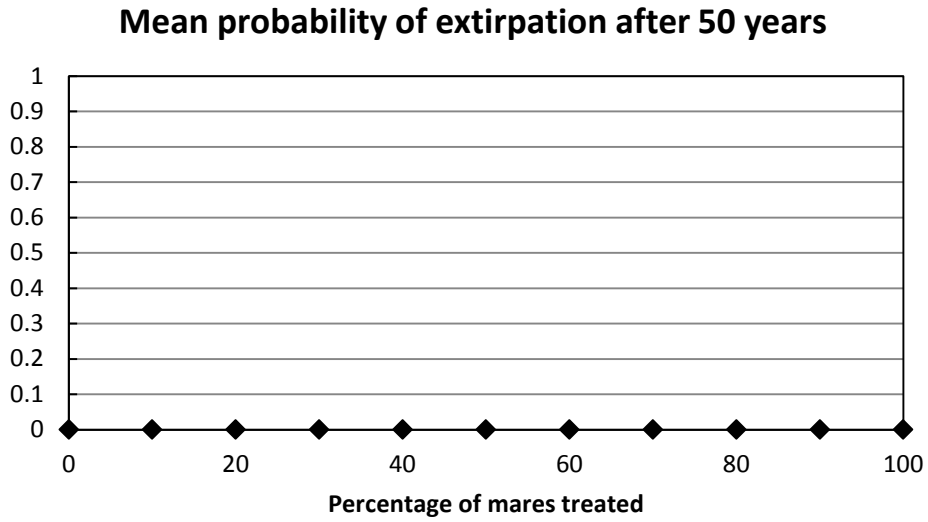


Figure 30. Initial population size = 1250, lambda = 1.25, genetic diversity = low.—Continued







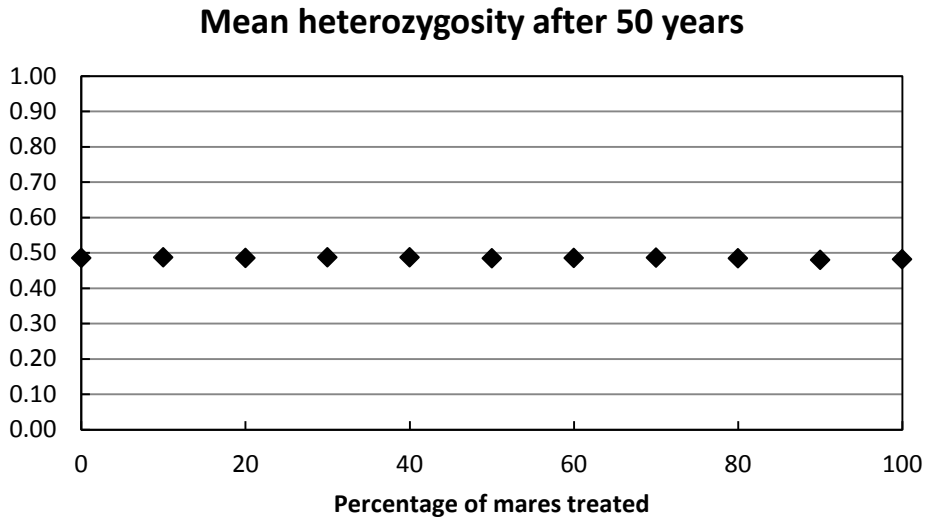


Figure 31. Initial population size = 1500, lambda = 1.05, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1279.4	1173.9	1220.8	1167.1	1224.9	1117.3	1098.7	987.4	1053.9	873.3	830.5
Mean number of removals after 20 years	547.92	557.55	504.35	573.75	514.62	487.4	459.94	558.95	591.28	496.54	524.2
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.49	0.49	0.48	0.49	0.49	0.48	0.48	0.48

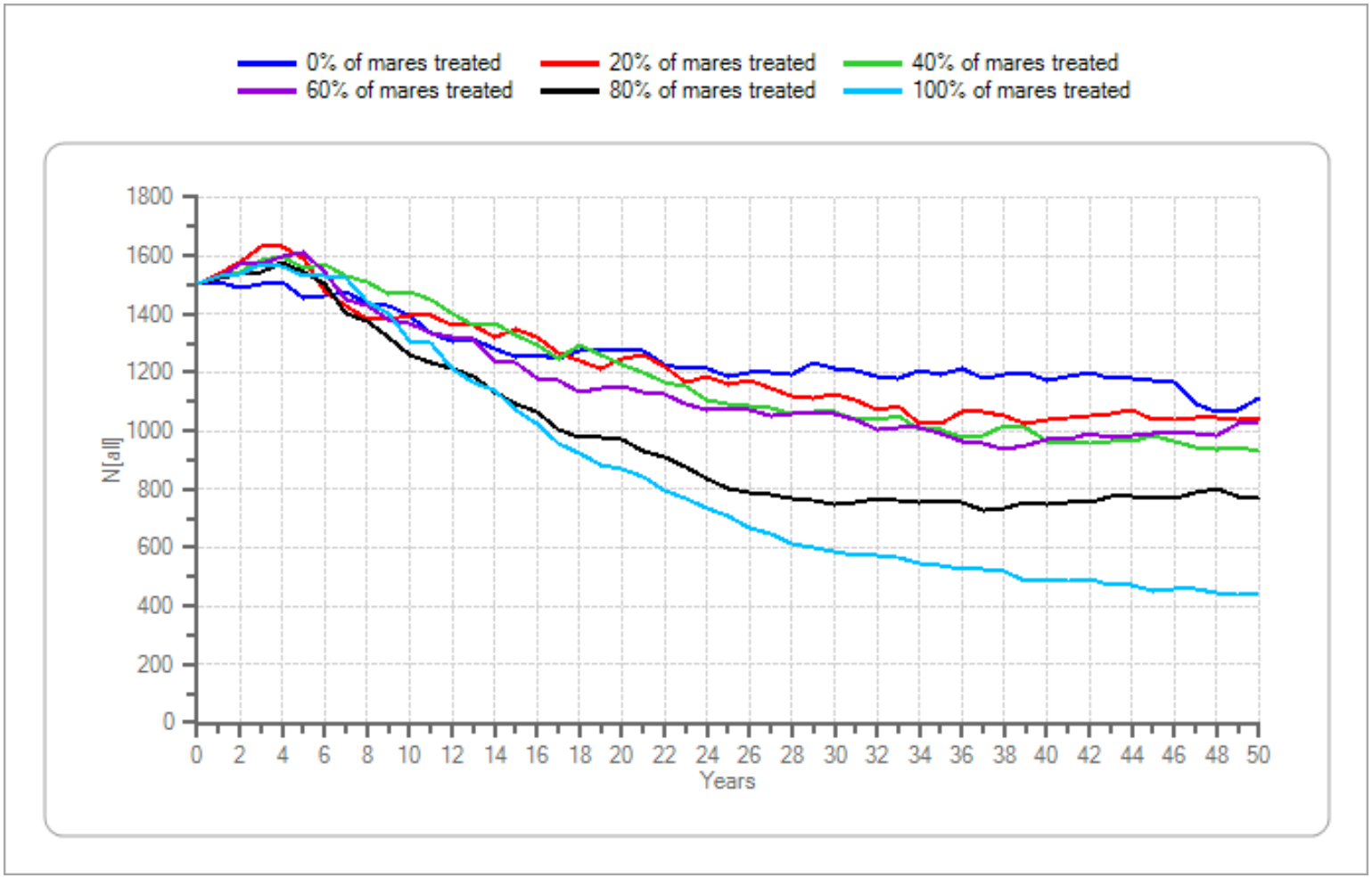
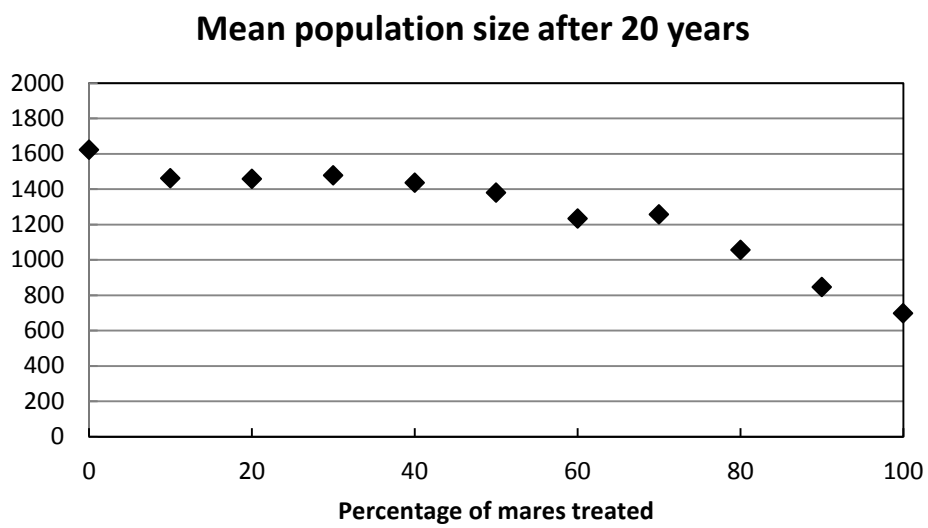
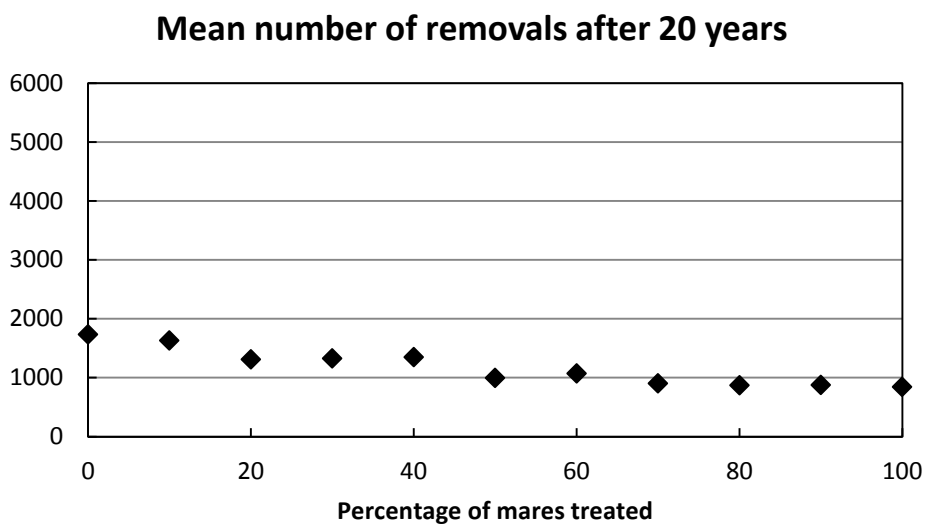
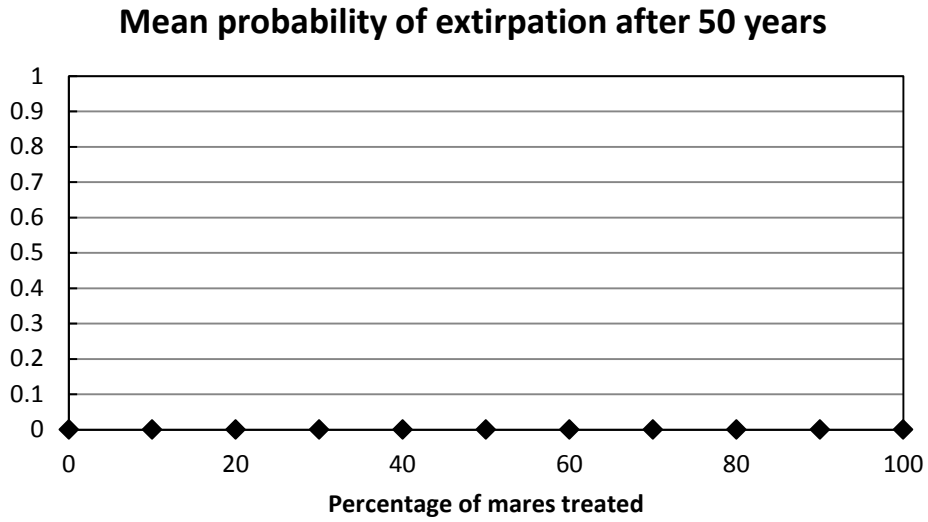


Figure 31. Initial population size = 1500, lambda = 1.05, genetic diversity = low.—Continued







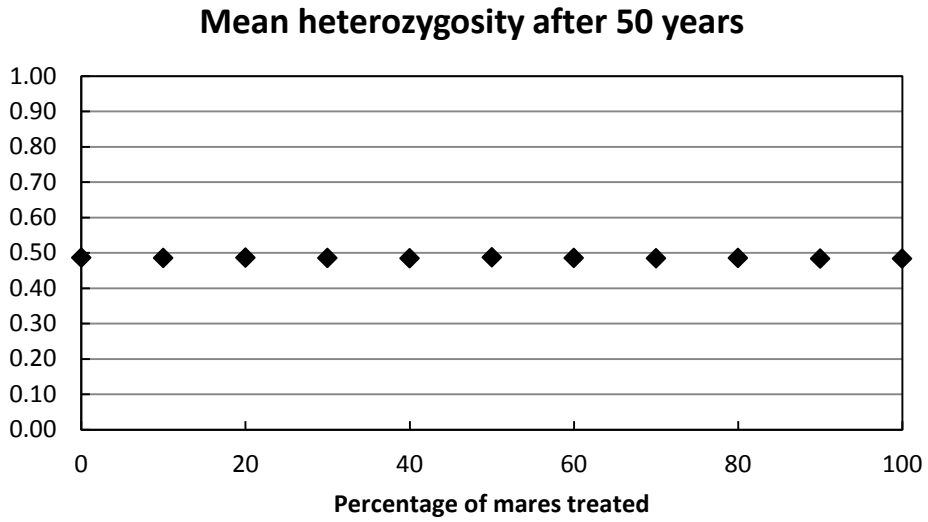


Figure 32. Initial population size = 1500, lambda = 1.10, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1622.8	1461.2	1457.5	1477.4	1435.9	1380.1	1232.4	1255.8	1054.9	846.0	697.3
Mean number of removals after 20 years	1735.0	1628.0	1308.3	1327.2	1348.6	995.9	1072.9	903.0	868.5	872.8	843.8
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.49	0.48	0.49	0.48	0.48	0.49	0.48	0.48

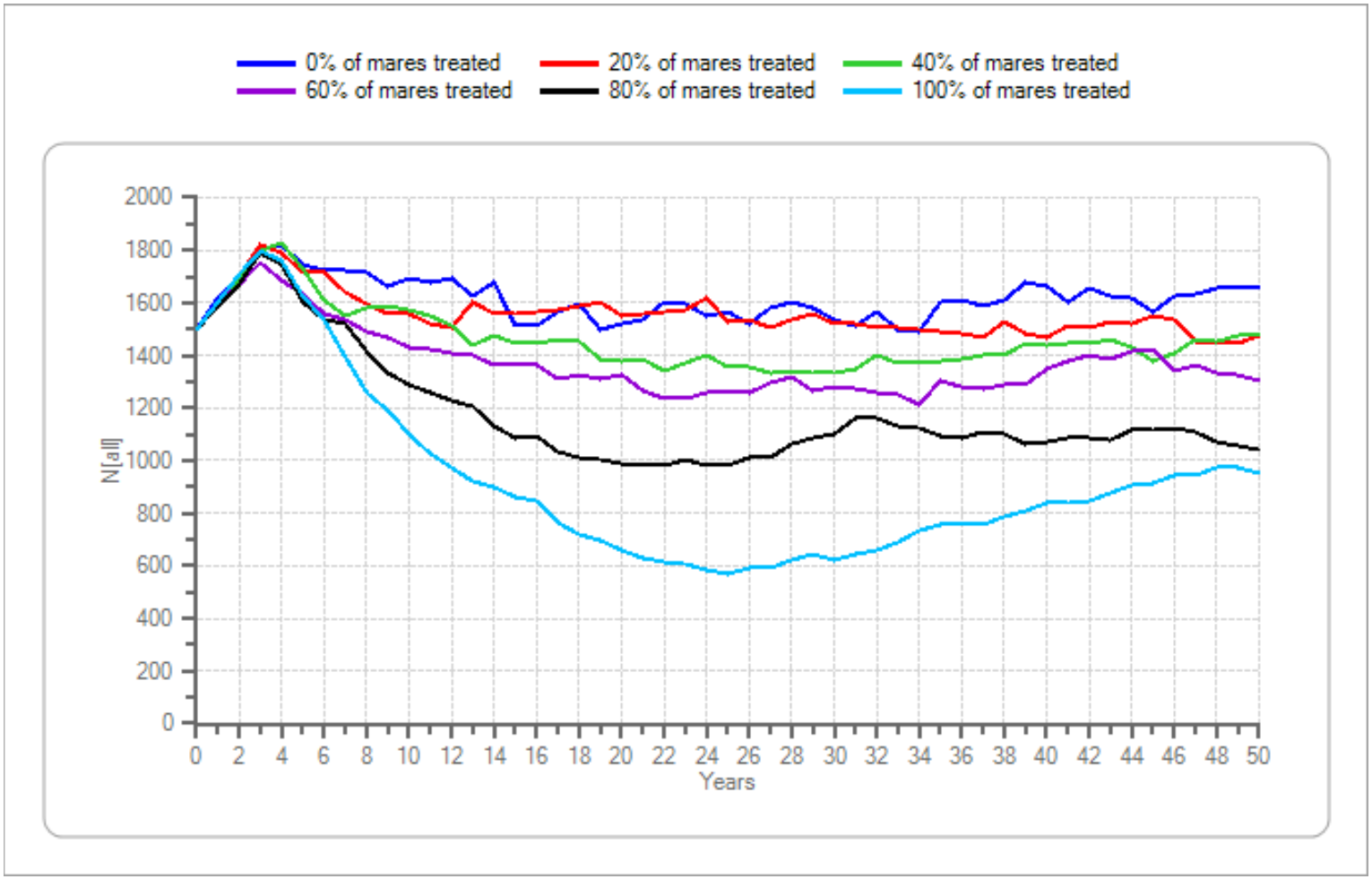
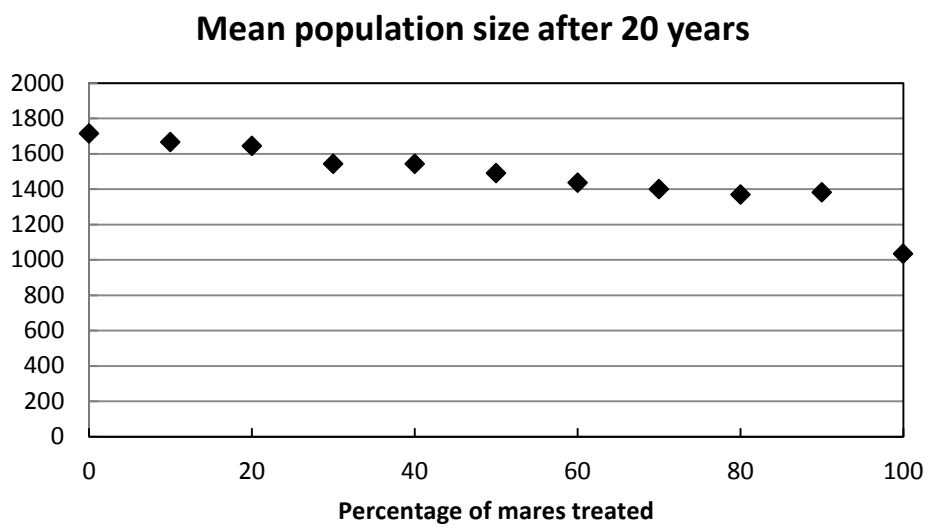
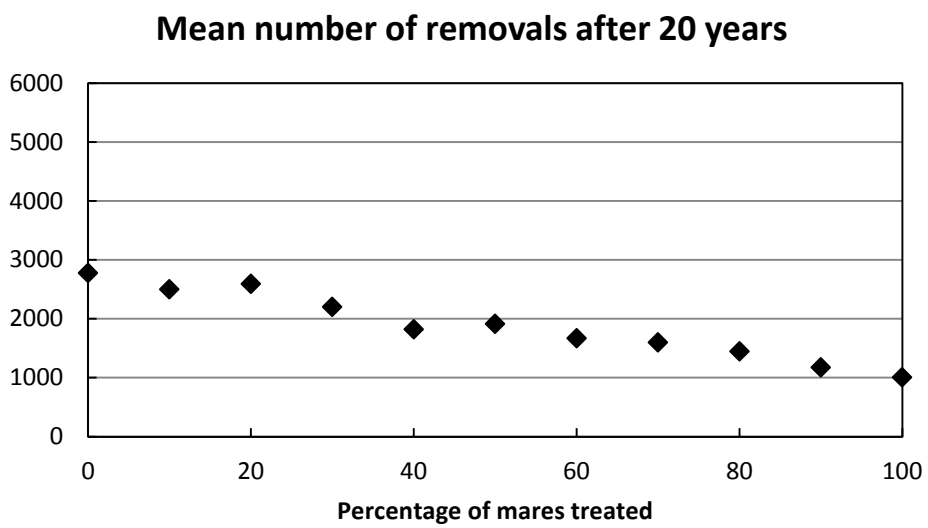
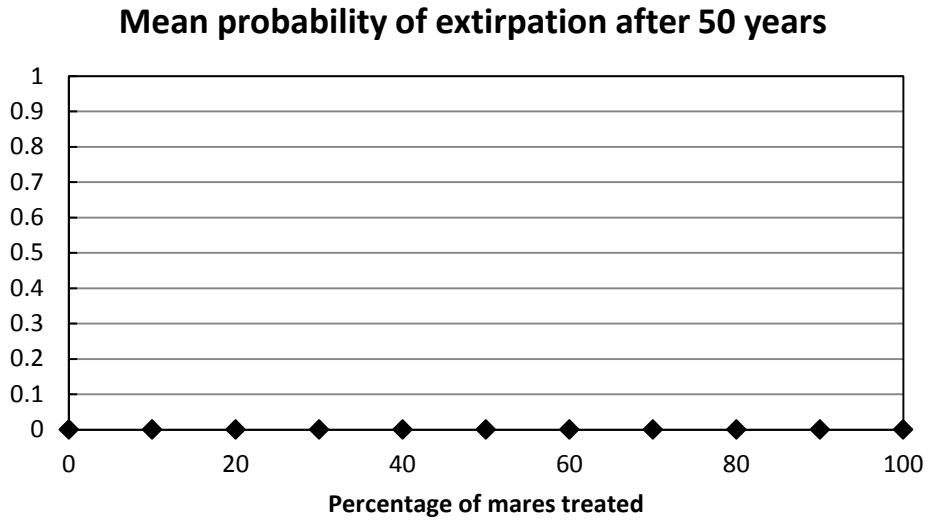


Figure 32. Initial population size = 1500, lambda = 1.10, genetic diversity = low.—Continued







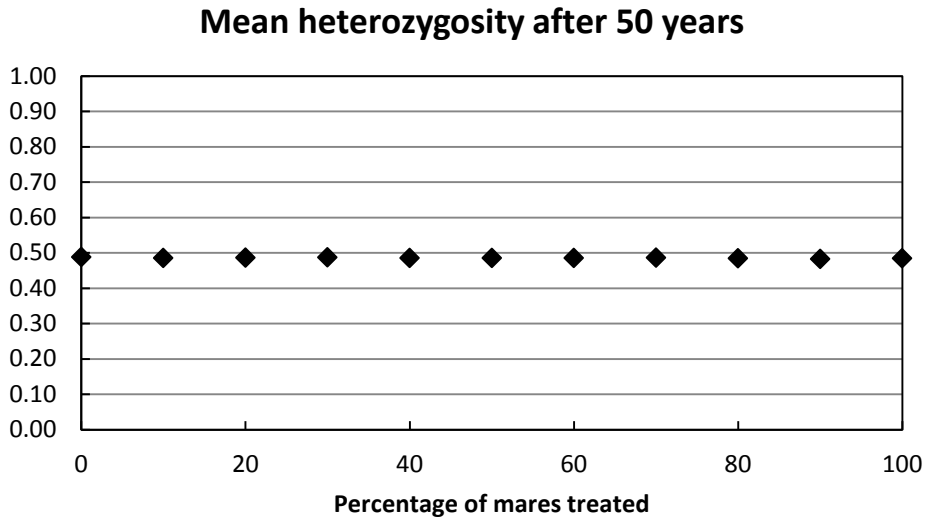


Figure 33. Initial population size = 1500, lambda = 1.15, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1714.8	1666.7	1643.6	1542.5	1542.7	1489.8	1436.2	1399.0	1368.2	1382.5	1033.5
Mean number of removals after 20 years	2777.7	2501.4	2591.7	2200.3	1822.5	1910.5	1669.9	1596.4	1444.0	1172.7	1004.9
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.48	0.48	0.48

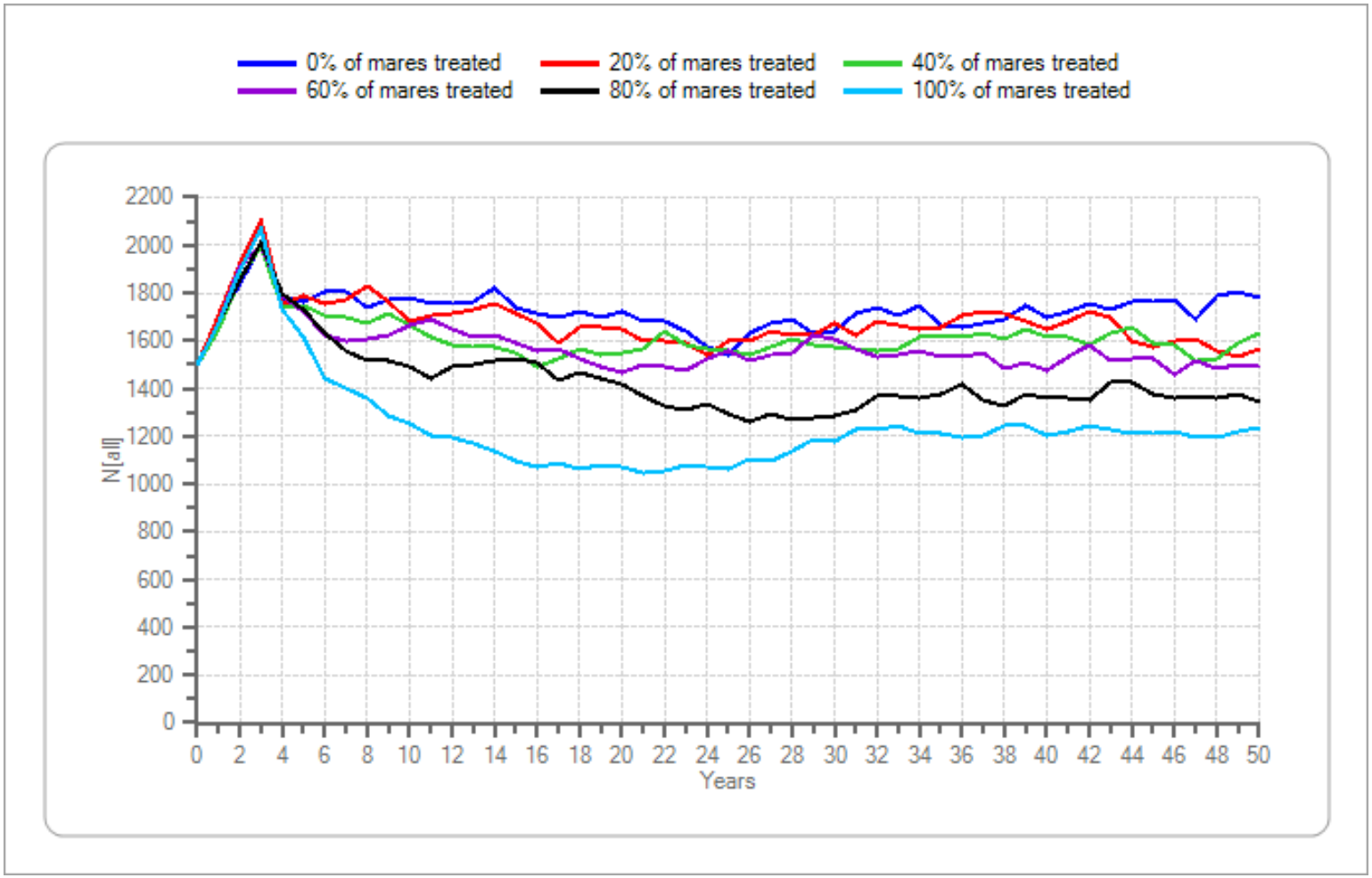
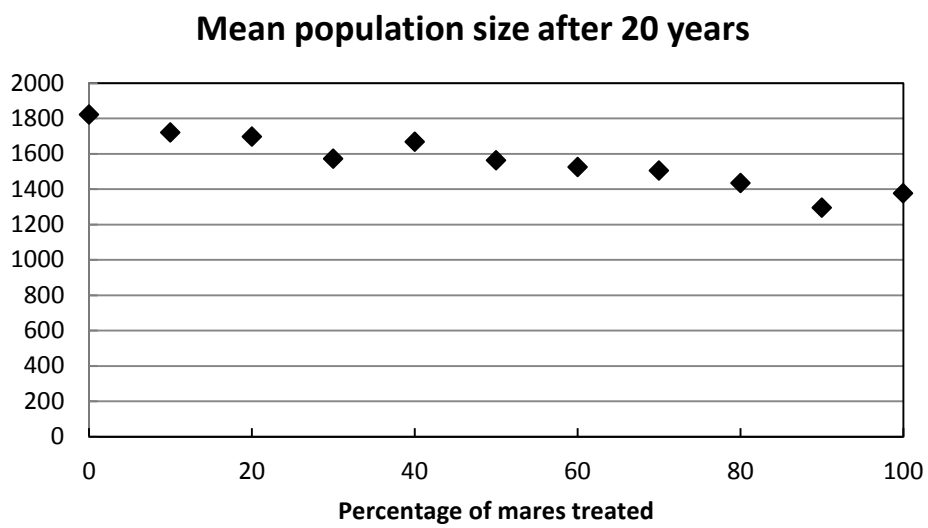
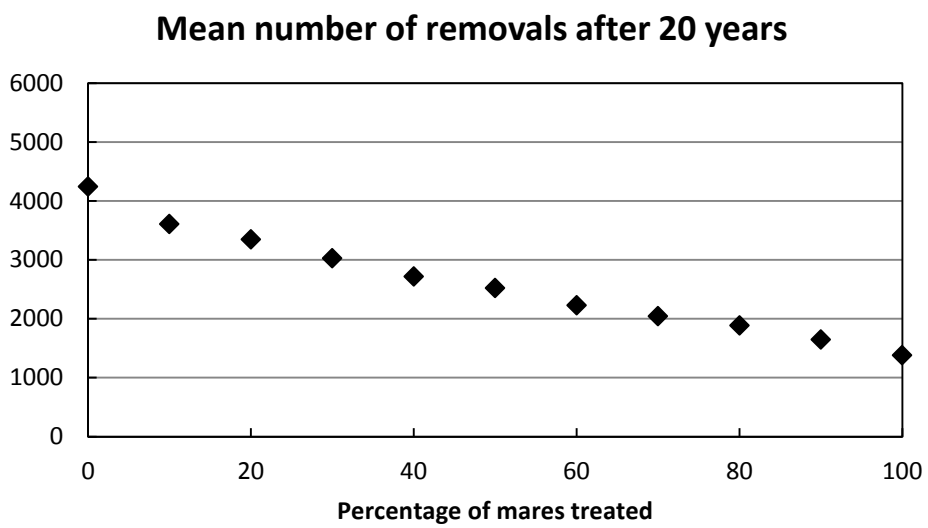
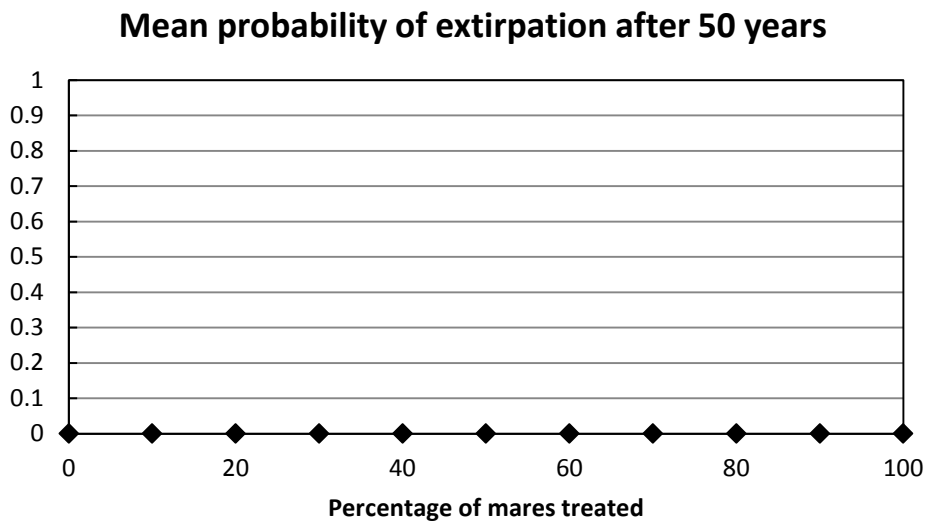


Figure 33. Initial population size = 1500, lambda = 1.15, genetic diversity = low.—Continued







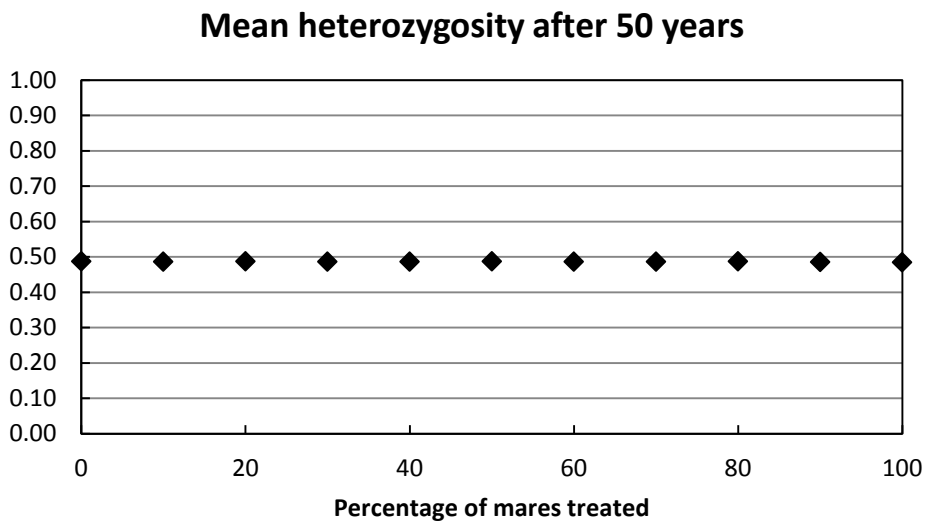


Figure 34. Initial population size = 1500, lambda = 1.20, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1821.8	1719.5	1697.0	1572.3	1667.0	1563.1	1523.7	1505.0	1433.3	1294.1	1376.3
Mean number of removals after 20 years	4241.3	3605.67	3348.38	3026.93	2716.34	2518.97	2225.75	2043.6	1886.32	1648.08	1381.6
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.48	0.48

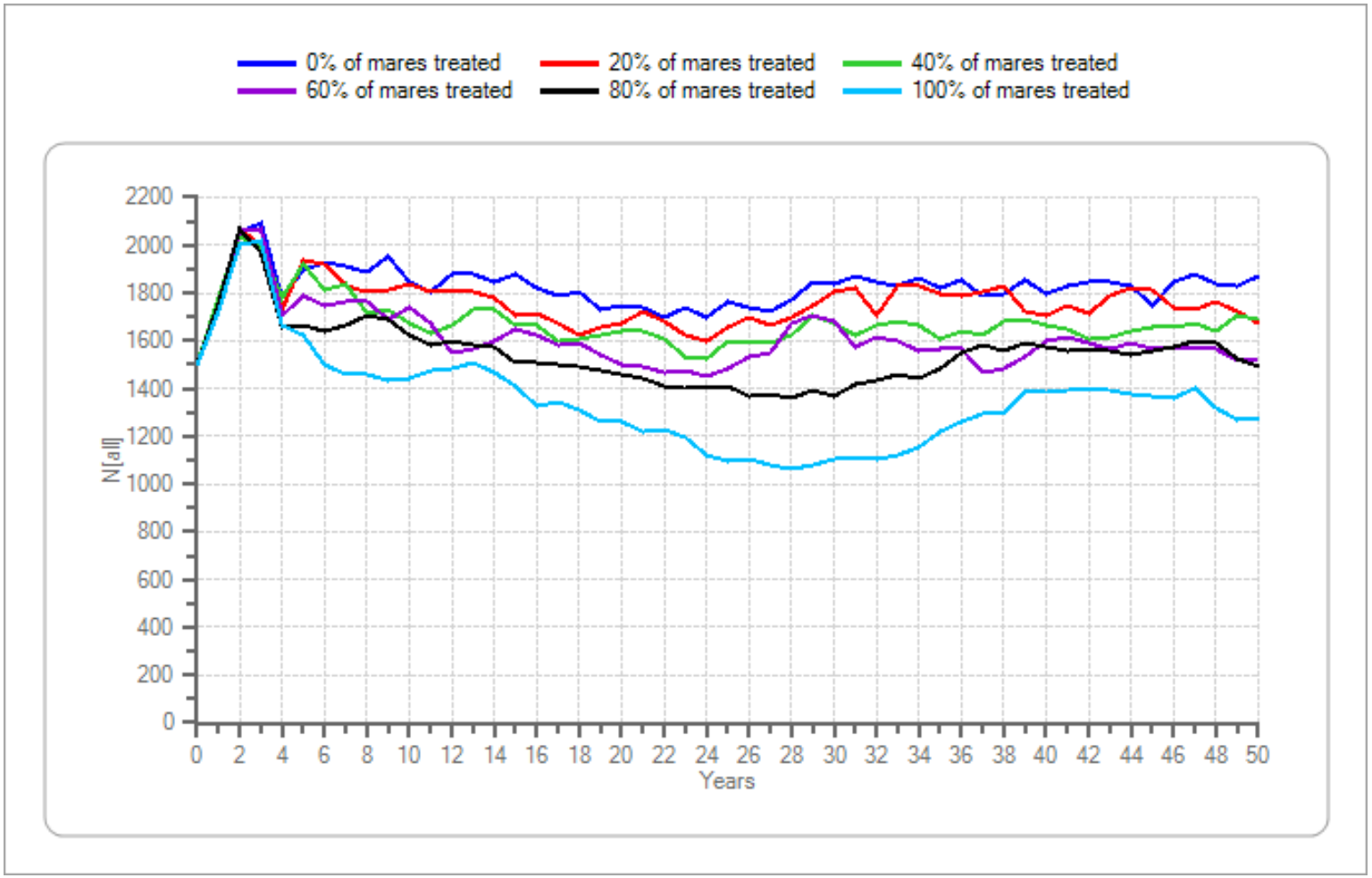
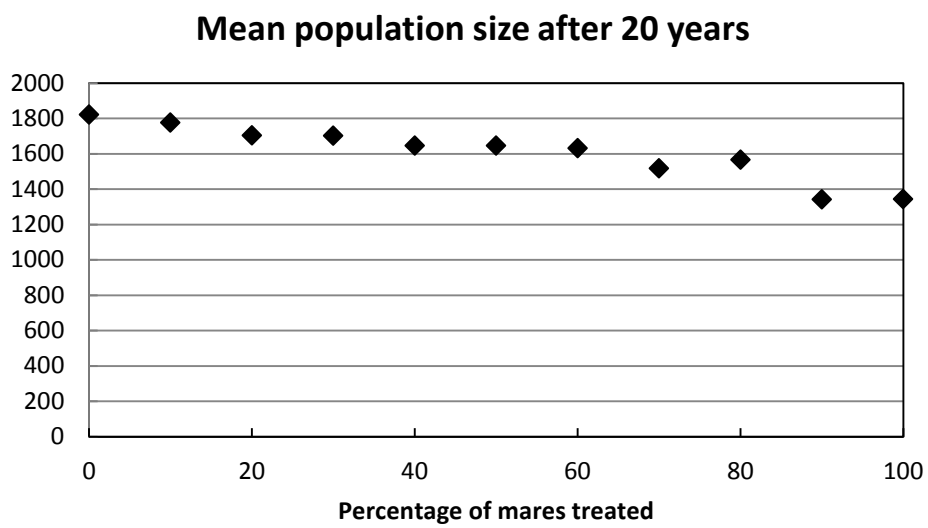
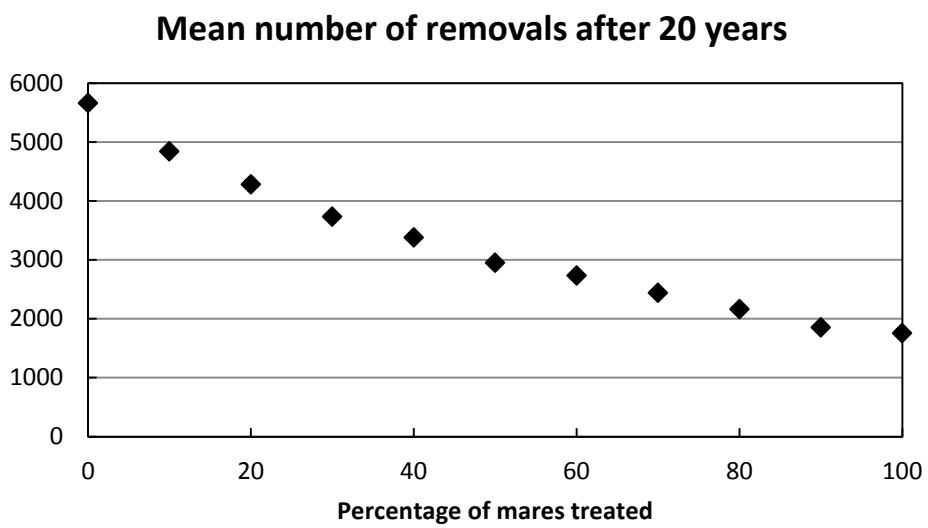
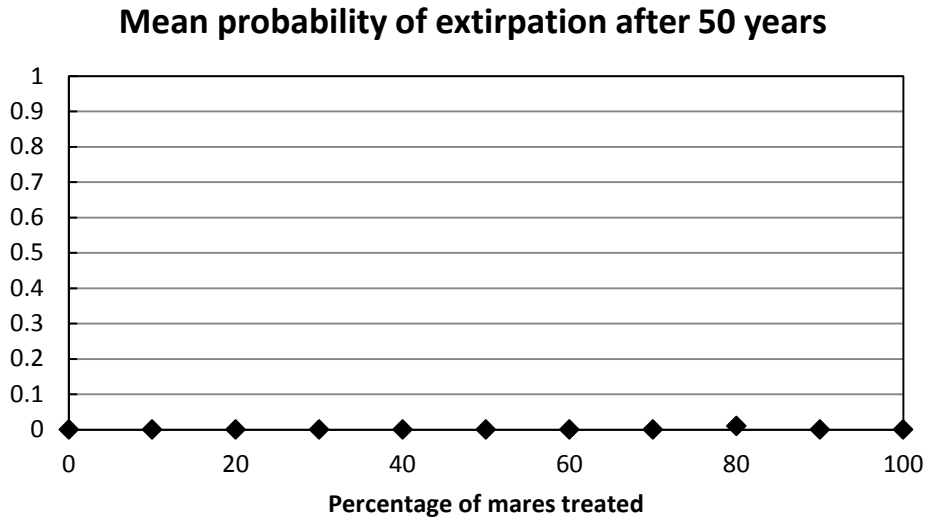


Figure 34. Initial population size = 1500, lambda = 1.20, genetic diversity = low.—Continued







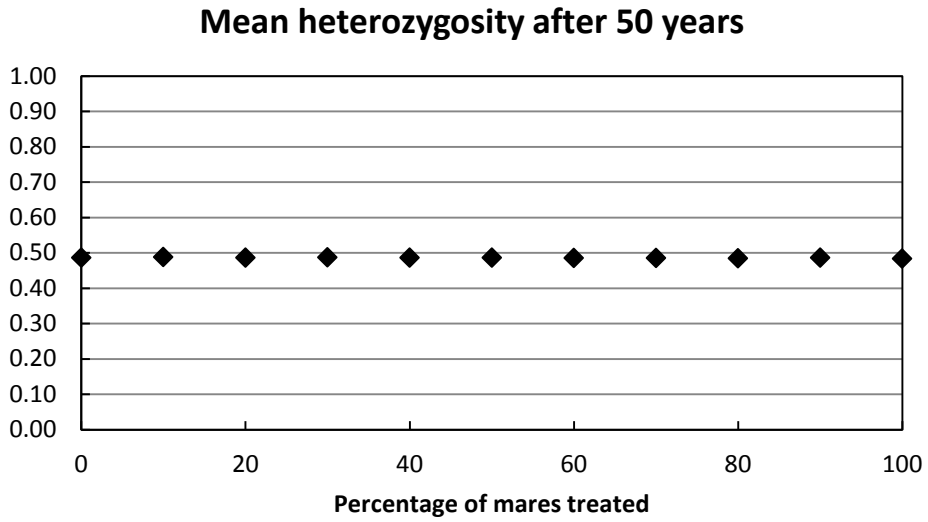


Figure 35. Initial population size = 1500, lambda = 1.25, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1821.3	1775.6	1704.2	1702.5	1645.8	1645.7	1631.5	1516.9	1566.6	1342.5	1343.4
Mean number of removals after 20 years	5662.1	4837.4	4282.2	3732.1	3380.2	2949.7	2731.2	2438.5	2164.2	1852.6	1756.3
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0.01	0	0
Mean heterozygosity after 50 years	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.49	0.48	0.49	0.48

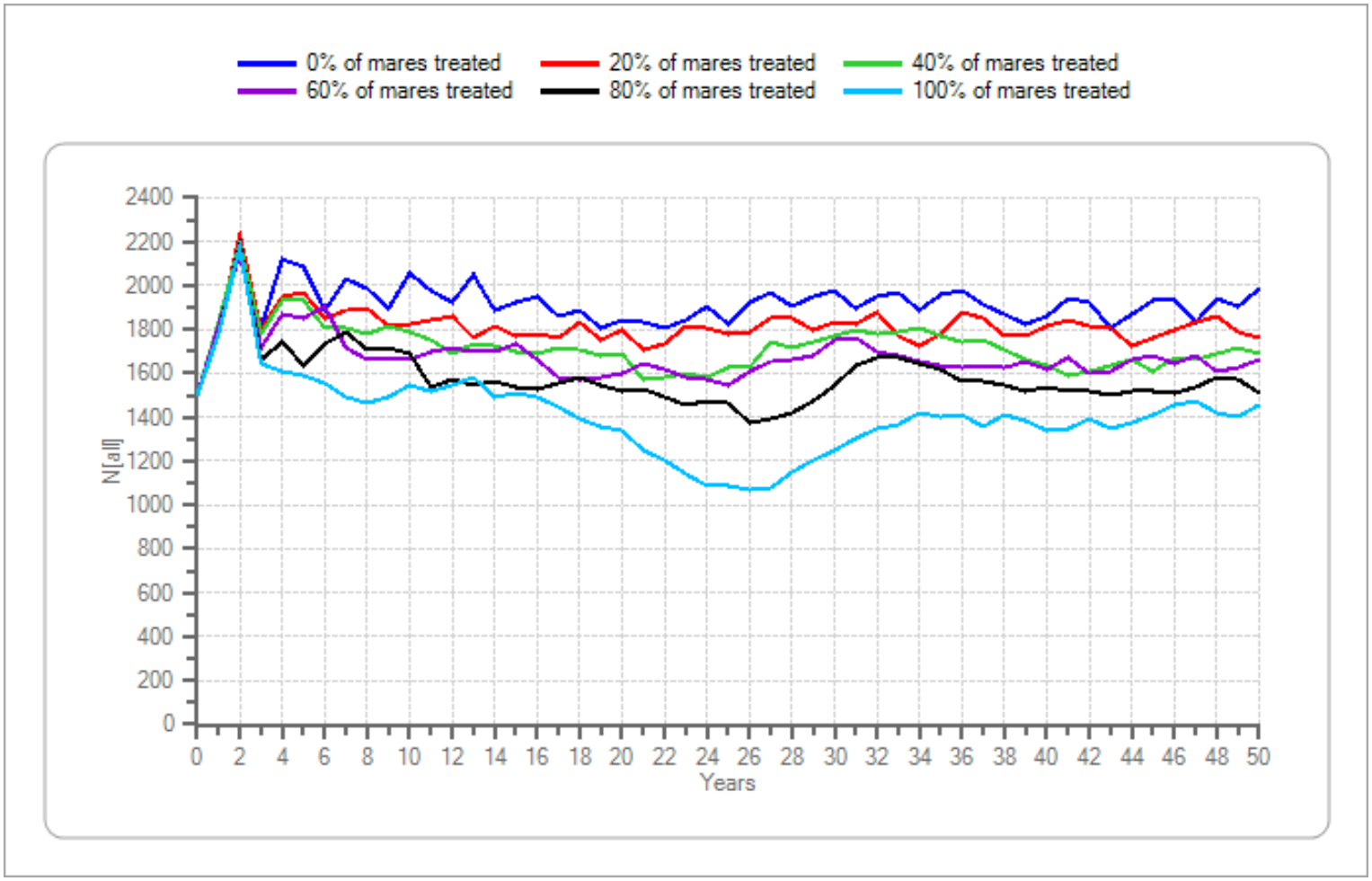
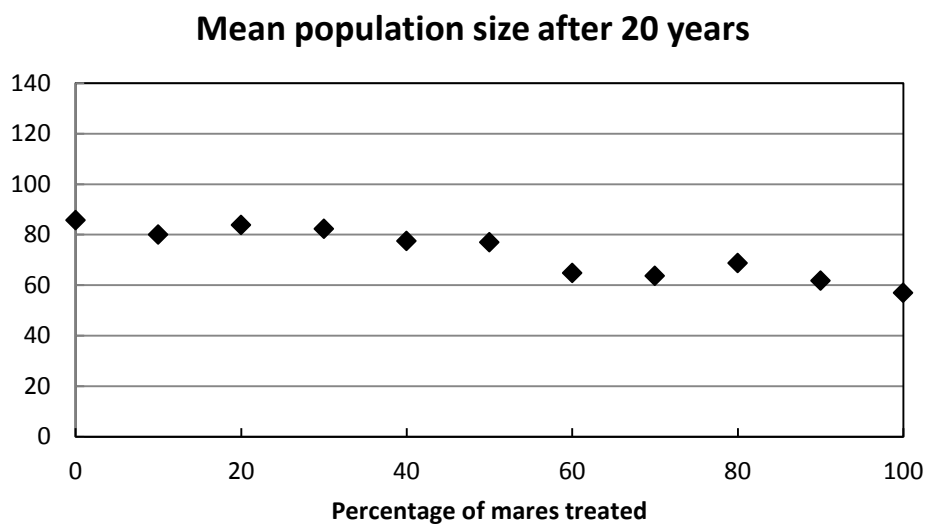
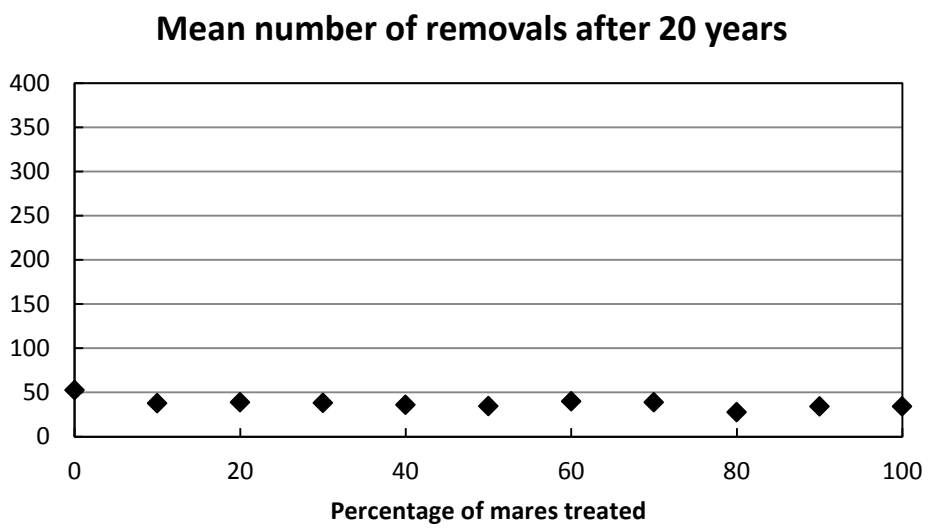
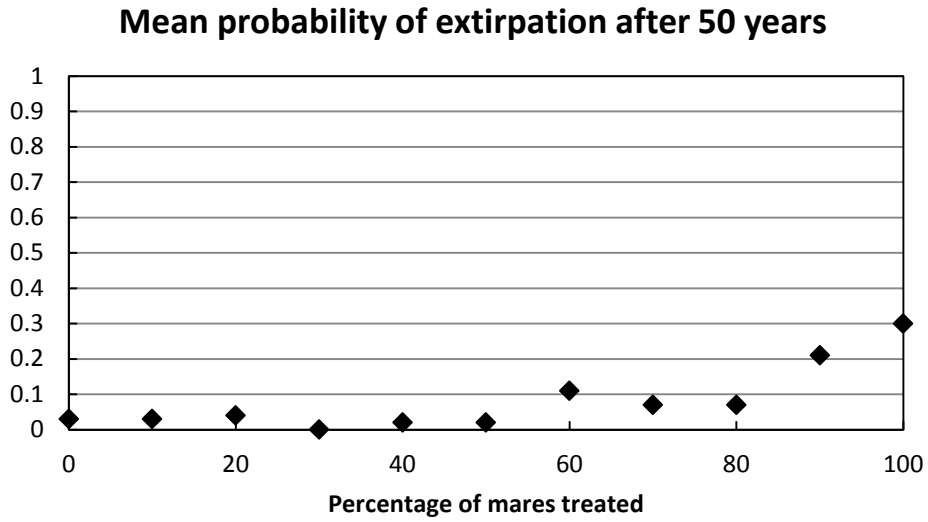


Figure 35. Initial population size = 1500, lambda = 1.25, genetic diversity = low.—Continued







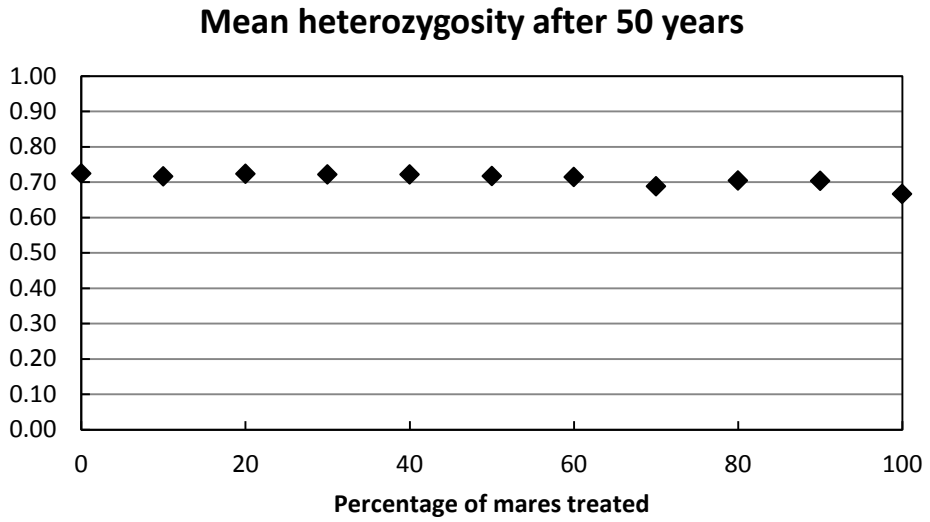


Figure 36. Initial population size = 100, lambda = 1.05, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	85.65	80.04	83.81	82.32	77.46	76.91	64.82	63.57	68.7	61.78	56.86
Mean number of removals after 20 years	52.51	37.59	38.83	38.02	36.03	34.27	39.99	38.8	27.57	34.2	34.23
Mean probability of extirpation after 50 years	0.03	0.03	0.04	0	0.02	0.02	0.11	0.07	0.07	0.21	0.3
Mean heterozygosity after 50 years	0.72	0.72	0.72	0.72	0.72	0.72	0.71	0.69	0.70	0.70	0.67

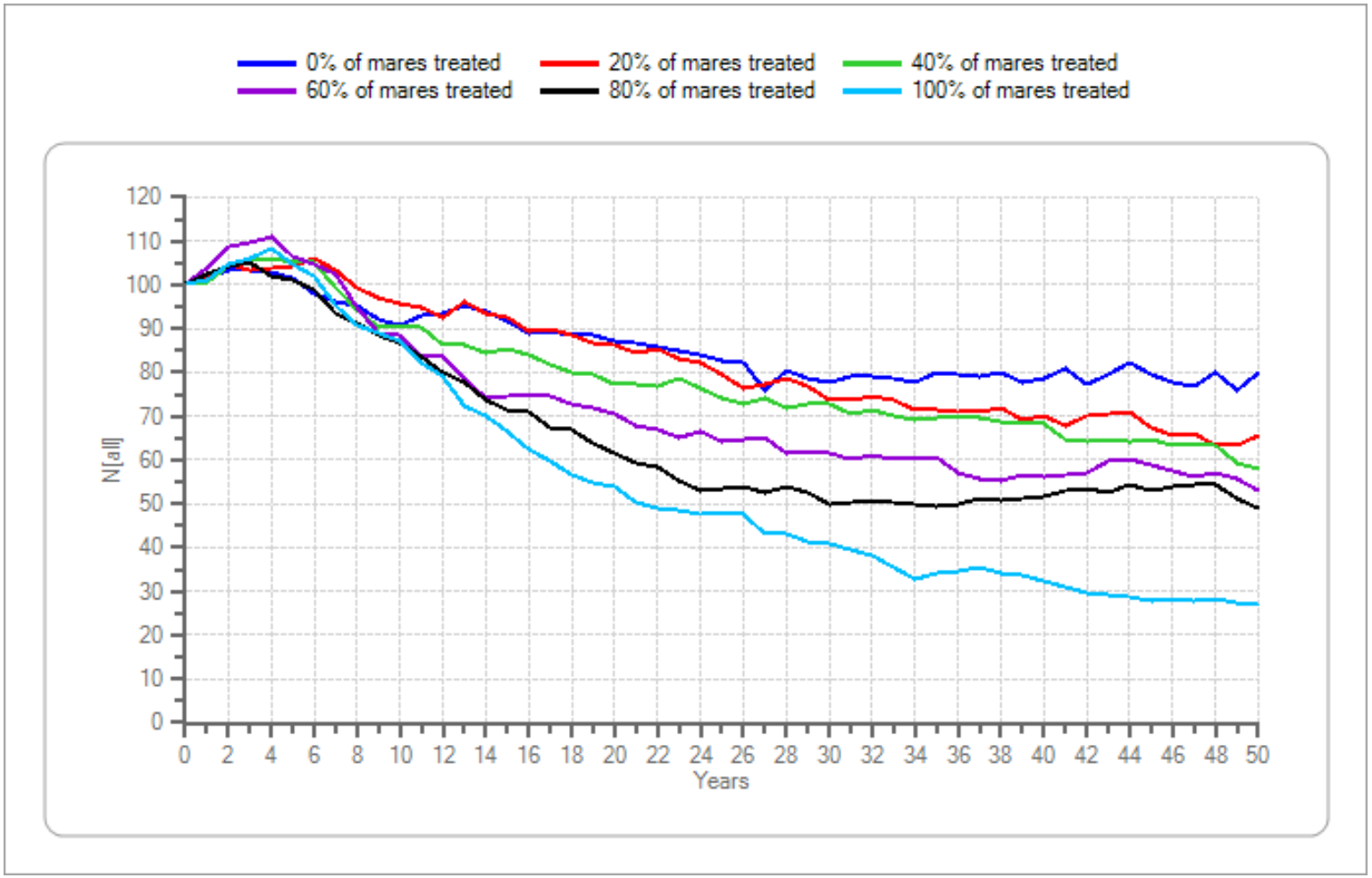
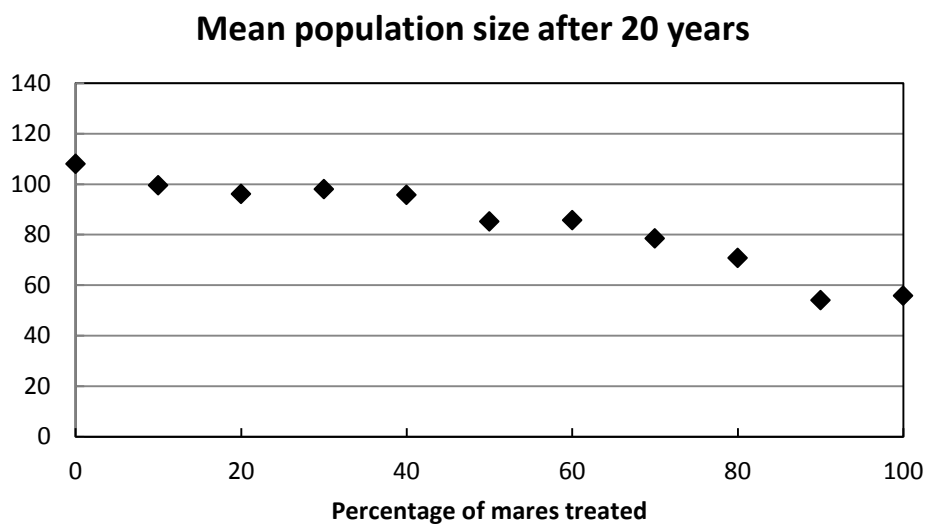
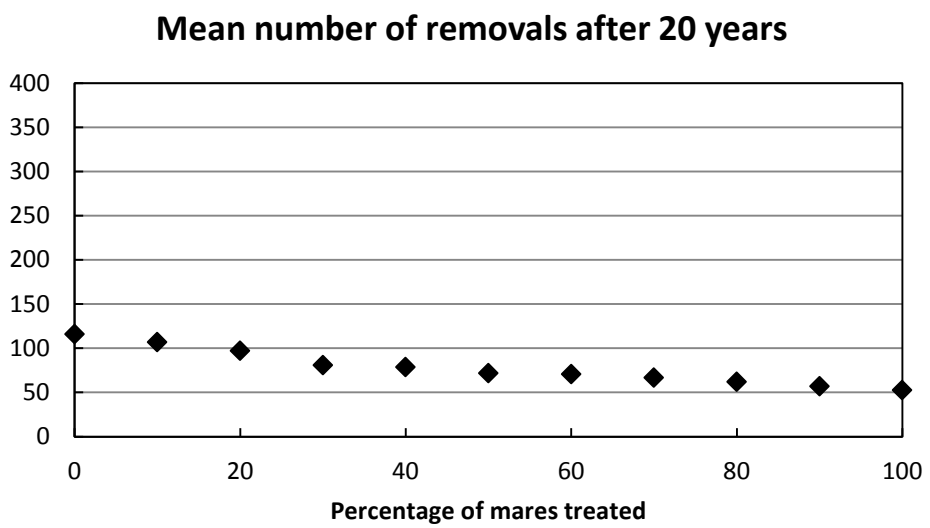
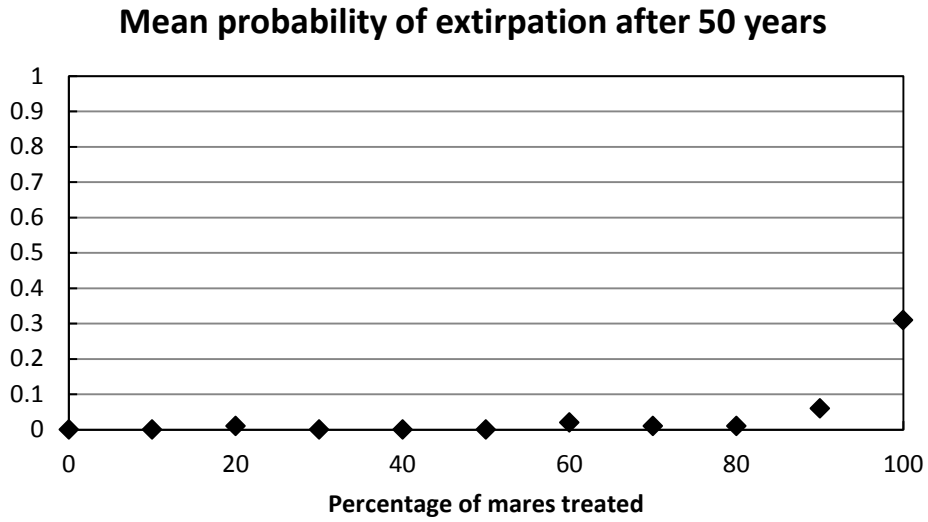


Figure 36. Initial population size = 100, lambda = 1.05, genetic diversity = high.—Continued







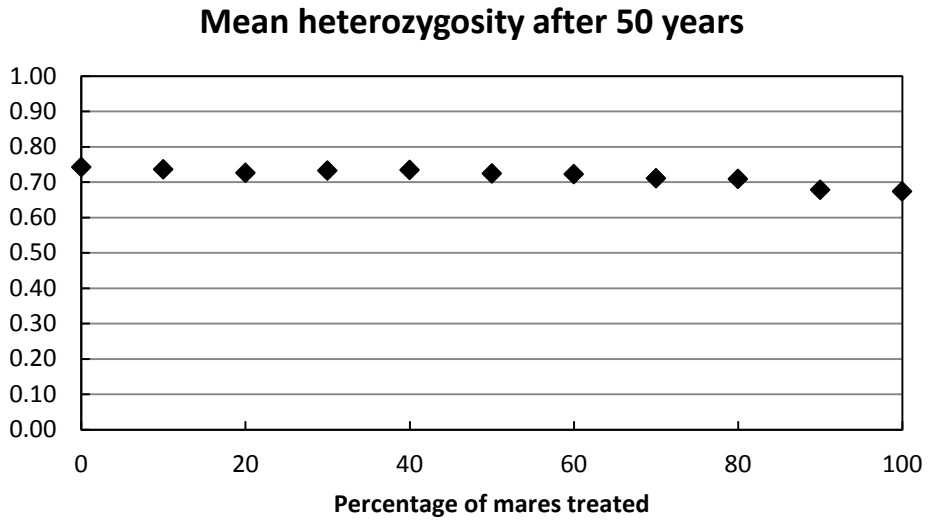


Figure 37. Initial population size = 100, lambda = 1.10, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	107.97	99.44	96.12	98.03	95.64	85.17	85.71	78.45	70.71	53.94	55.76
Mean number of removals after 20 years	115.94	106.96	96.94	80.92	78.45	71.85	70.62	66.8	62.01	56.75	52.5
Mean probability of extirpation after 50 years	0	0	0.01	0	0	0	0.02	0.01	0.01	0.06	0.31
Mean heterozygosity after 50 years	0.74	0.74	0.73	0.73	0.73	0.72	0.72	0.71	0.71	0.68	0.67

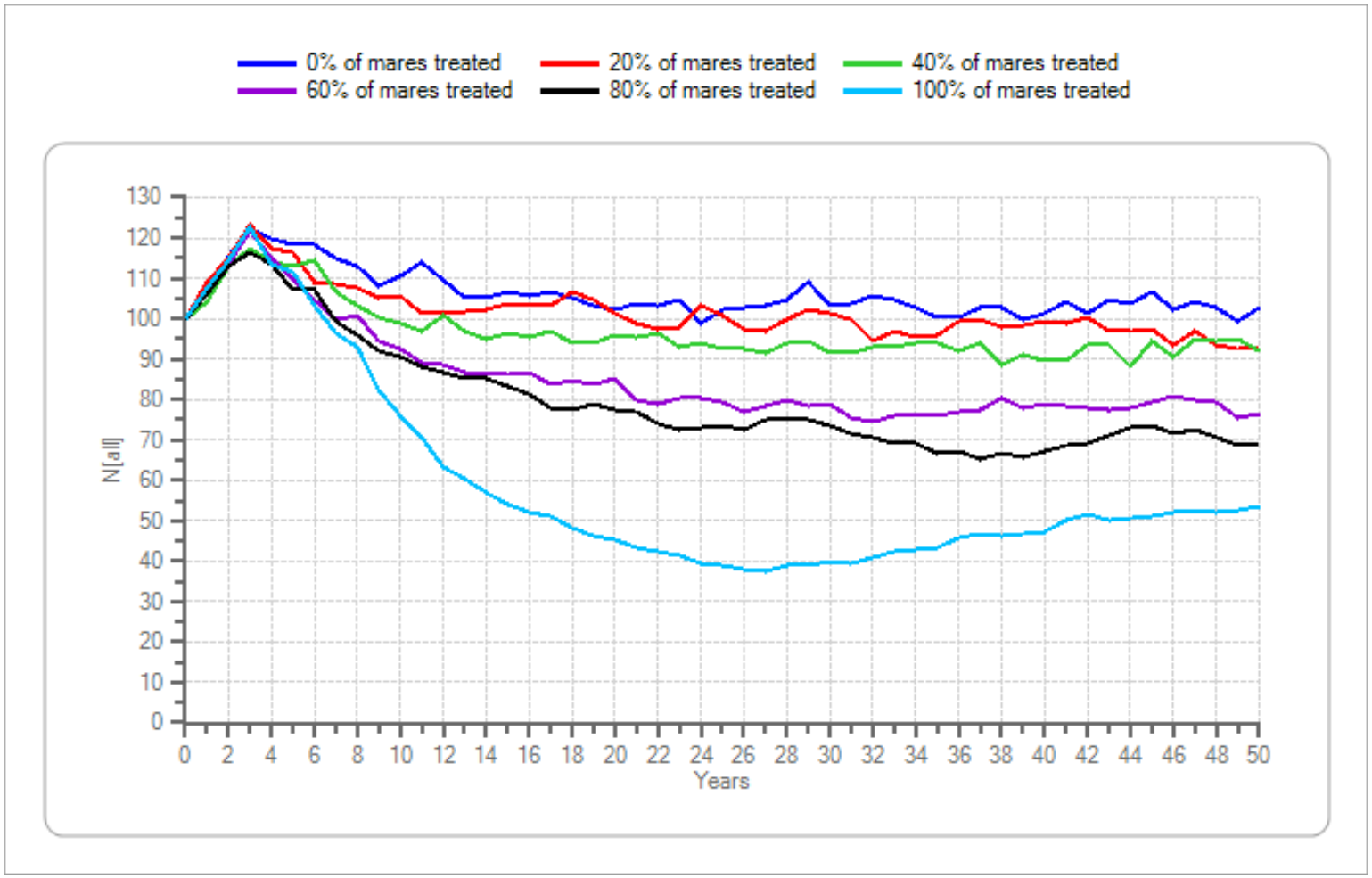


Figure 37. Initial population size = 100, lambda = 1.10, genetic diversity = high.—Continued

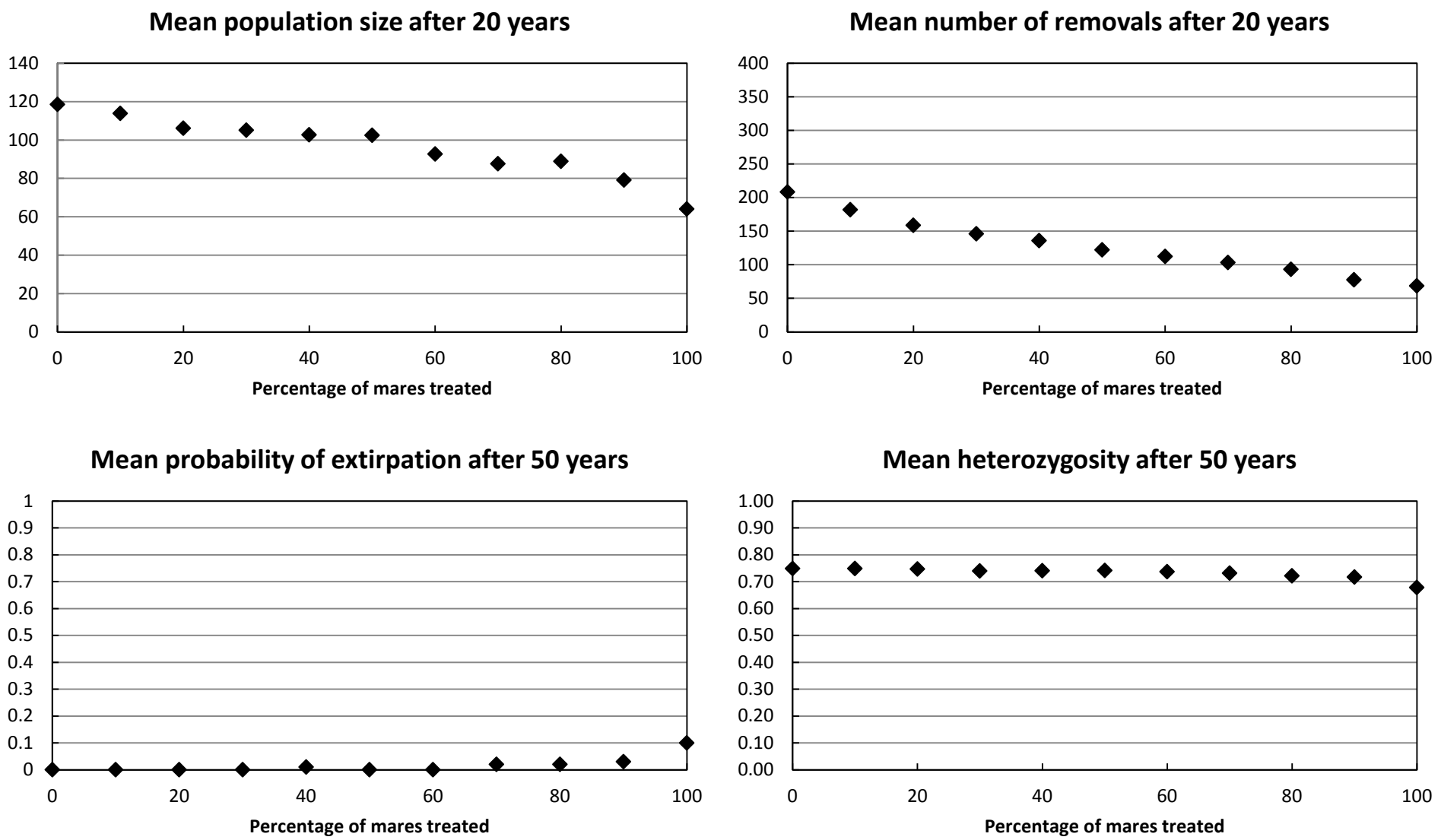


Figure 38. Initial population size = 100, lambda = 1.15, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	118.53	113.82	106.1	105.09	102.72	102.37	92.64	87.6	88.79	79.15	63.95
Mean number of removals after 20 years	208.4	181.74	158.65	145.93	135.73	121.88	112.21	103.11	93.06	77.44	68.55
Mean probability of extirpation after 50 years	0	0	0	0	0.01	0	0	0.02	0.02	0.03	0.1
Mean heterozygosity after 50 years	0.75	0.75	0.75	0.74	0.74	0.74	0.74	0.73	0.72	0.72	0.68

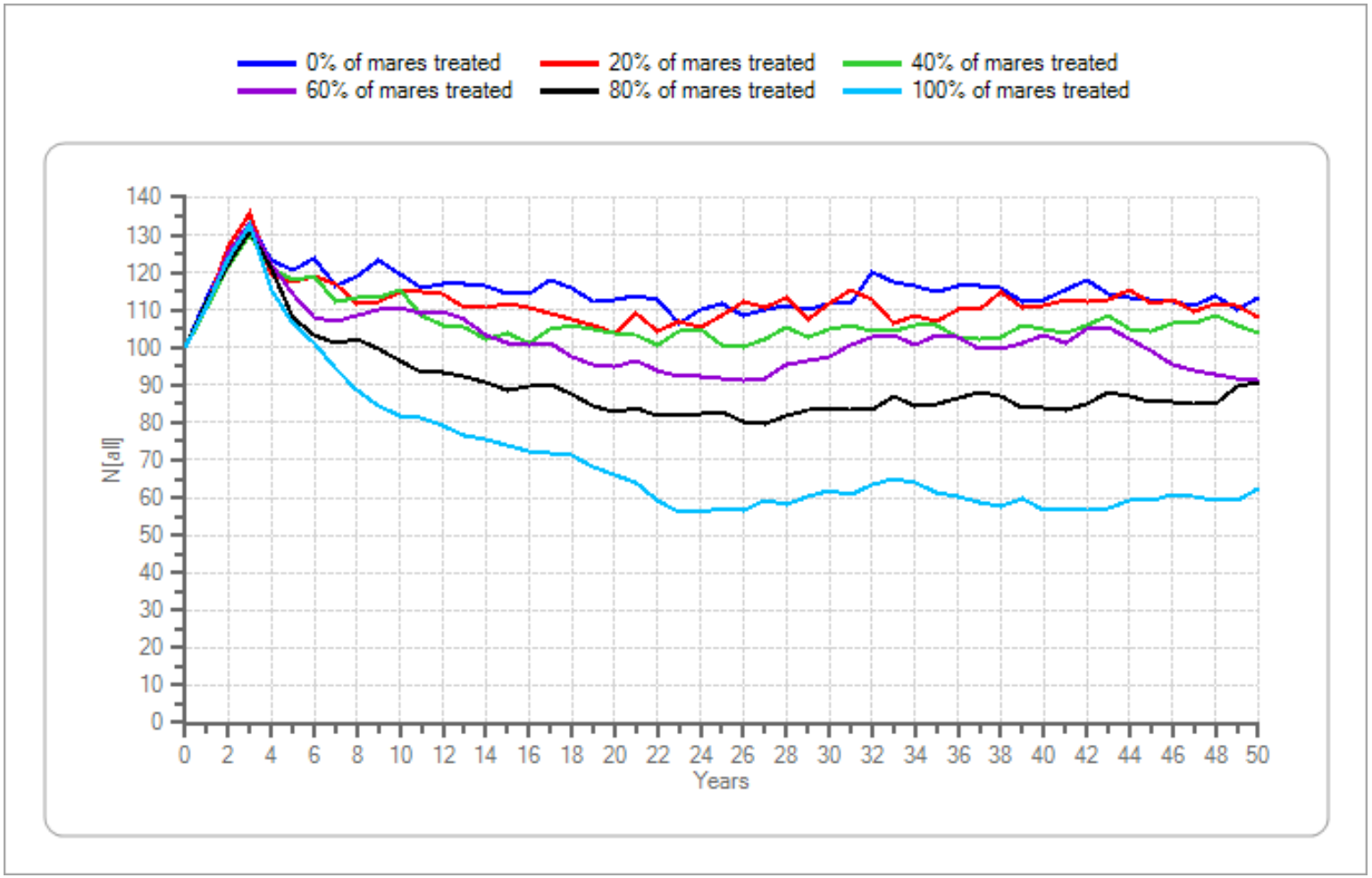


Figure 38. Initial population size = 100, lambda = 1.15, genetic diversity = high.—Continued

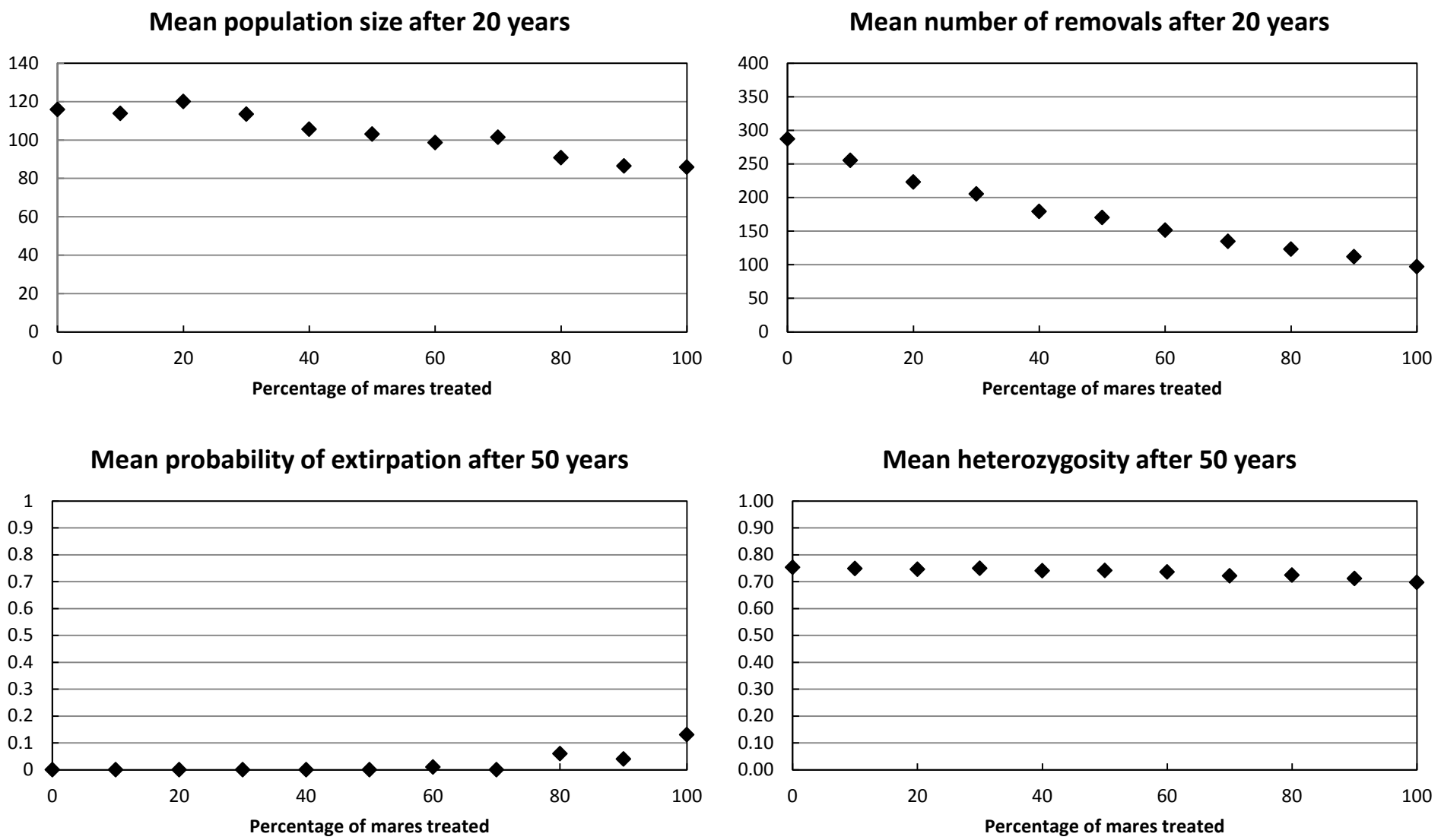


Figure 39. Initial population size = 100, lambda = 1.20, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	115.87	113.82	120.06	113.47	105.64	102.99	98.66	101.46	90.77	86.41	85.86
Mean number of removals after 20 years	287.24	255.15	222.93	205.42	179.11	170.34	151.32	134.9	123.26	112.03	97.01
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0.01	0	0.06	0.04	0.13
Mean heterozygosity after 50 years	0.75	0.75	0.75	0.75	0.74	0.74	0.74	0.72	0.72	0.71	0.70

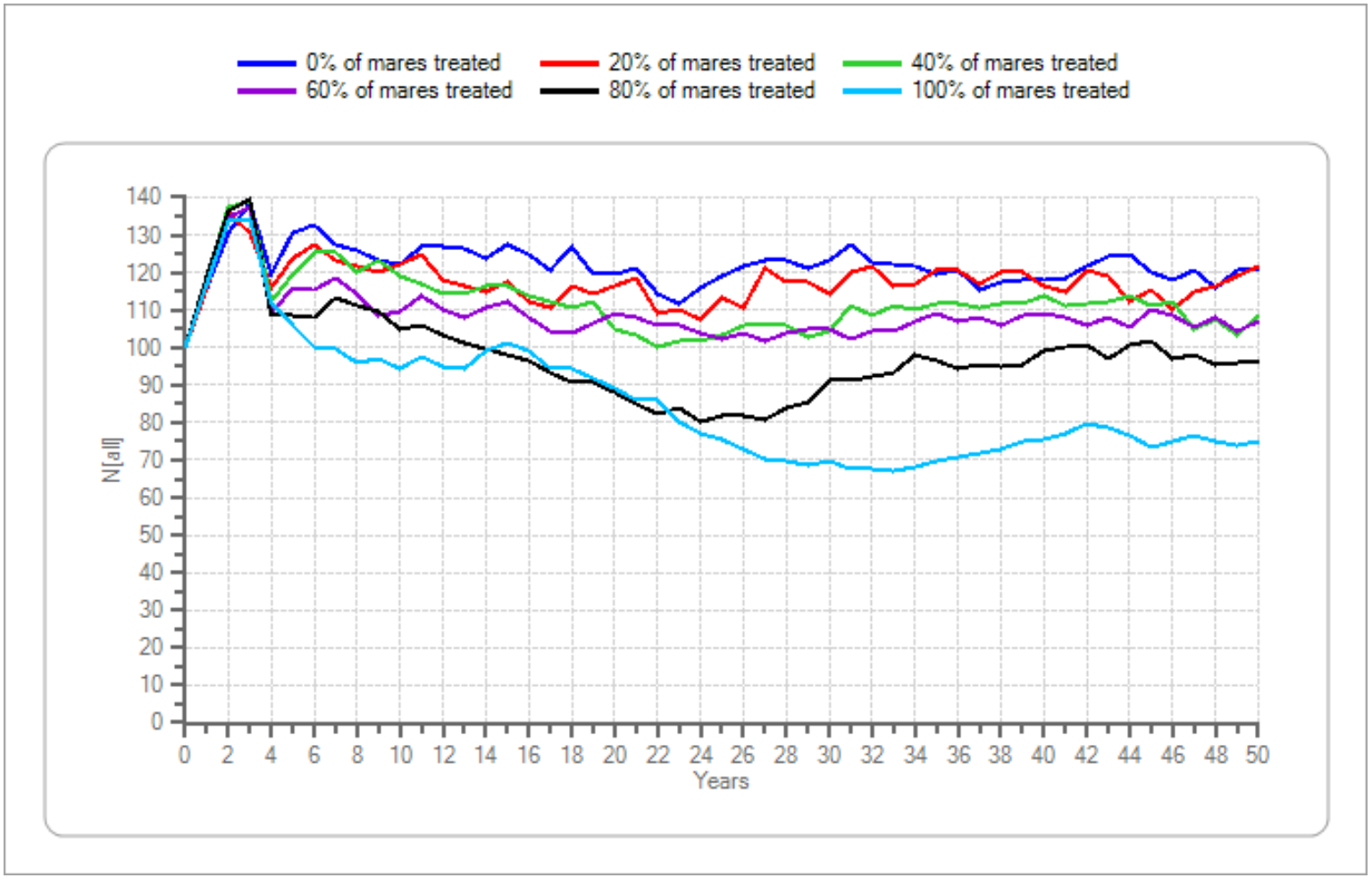


Figure 39. Initial population size = 100, lambda = 1.20, genetic diversity = high.—Continued

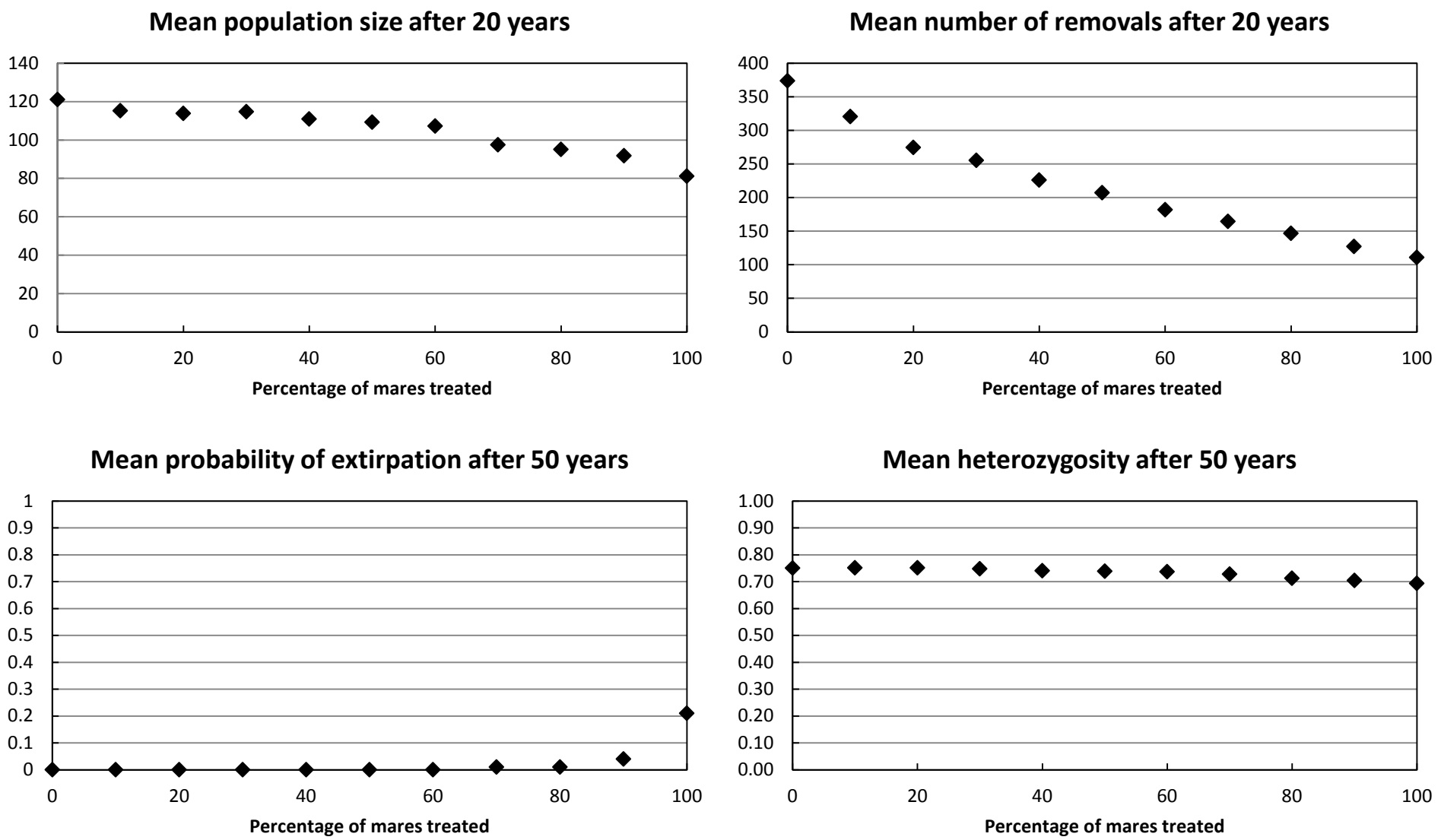


Figure 40. Initial population size = 100, lambda = 1.25, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	121.02	115.17	113.84	114.66	110.88	109.29	107.18	97.48	95.07	91.82	81.06
Mean number of removals after 20 years	373.76	320.53	274.59	255.43	225.87	207.24	181.97	164.56	146.74	127.02	110.7
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0.01	0.01	0.04	0.21
Mean heterozygosity after 50 years	0.75	0.75	0.75	0.75	0.74	0.74	0.74	0.73	0.71	0.70	0.69

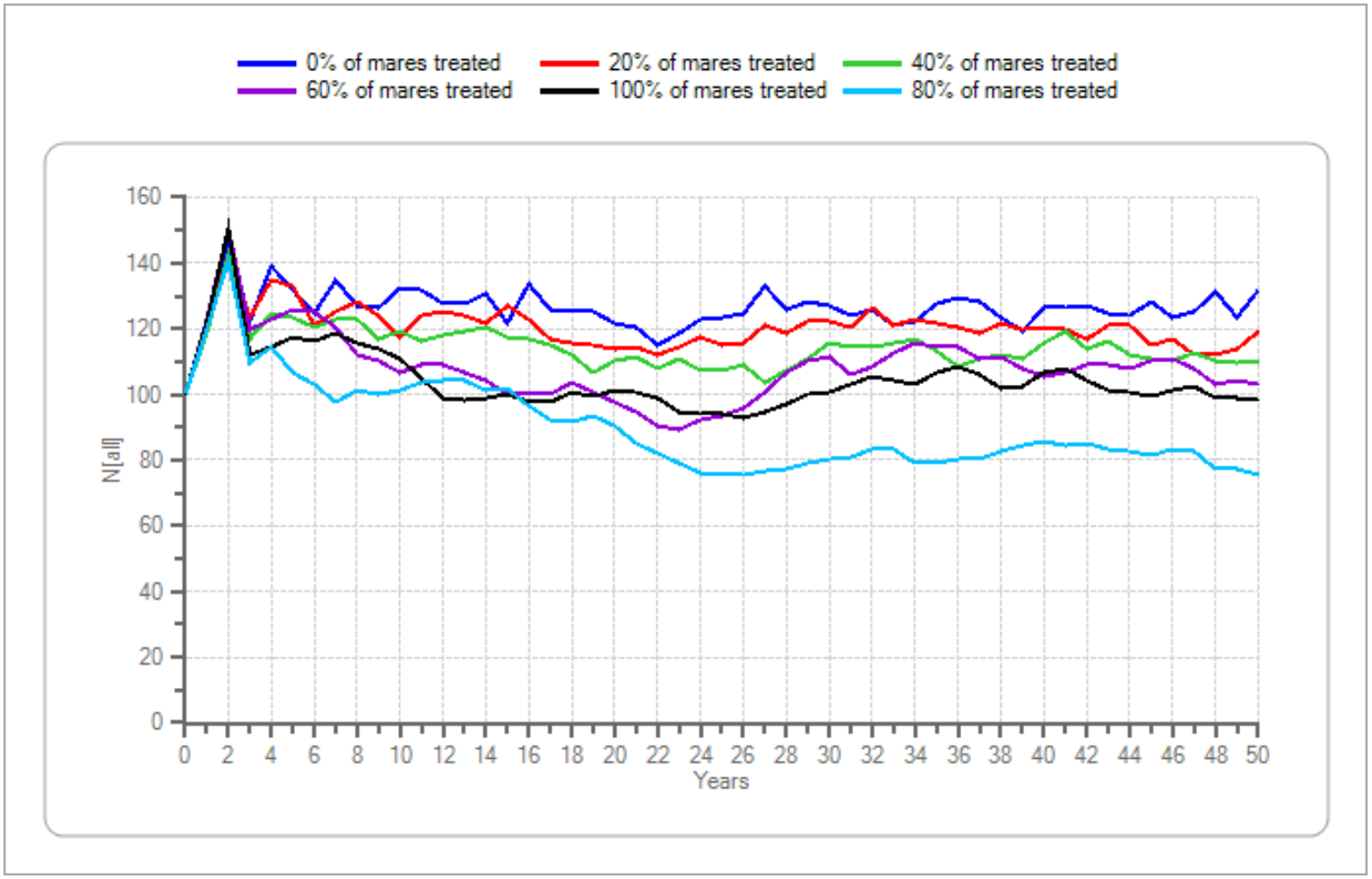
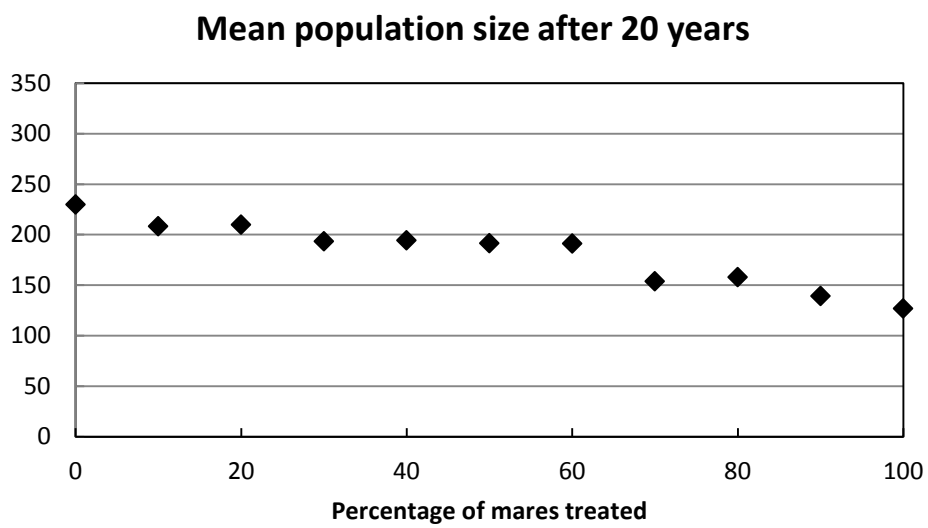
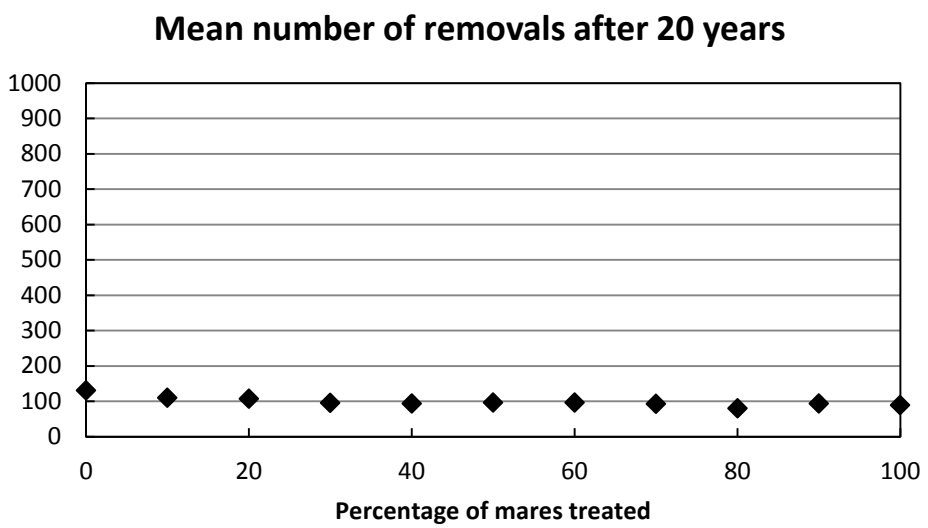
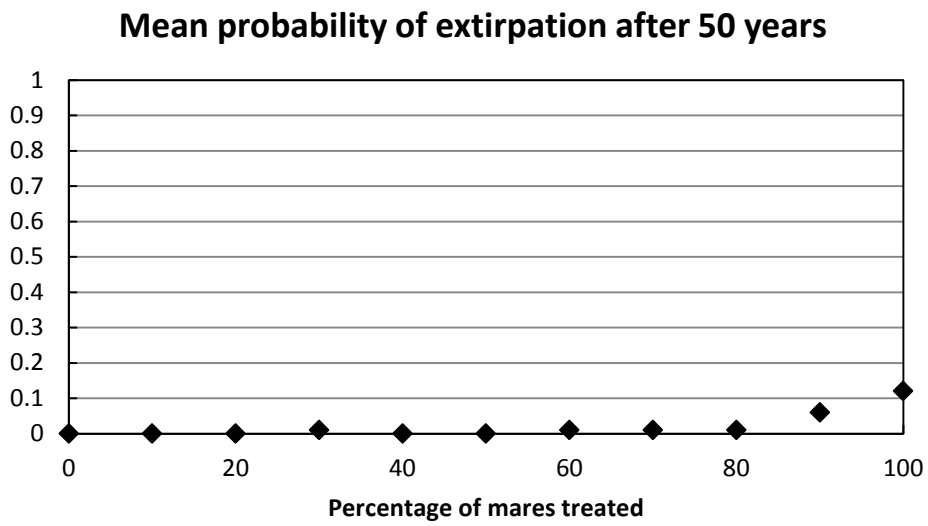


Figure 40. Initial population size = 100, lambda = 1.25, genetic diversity = high.—Continued







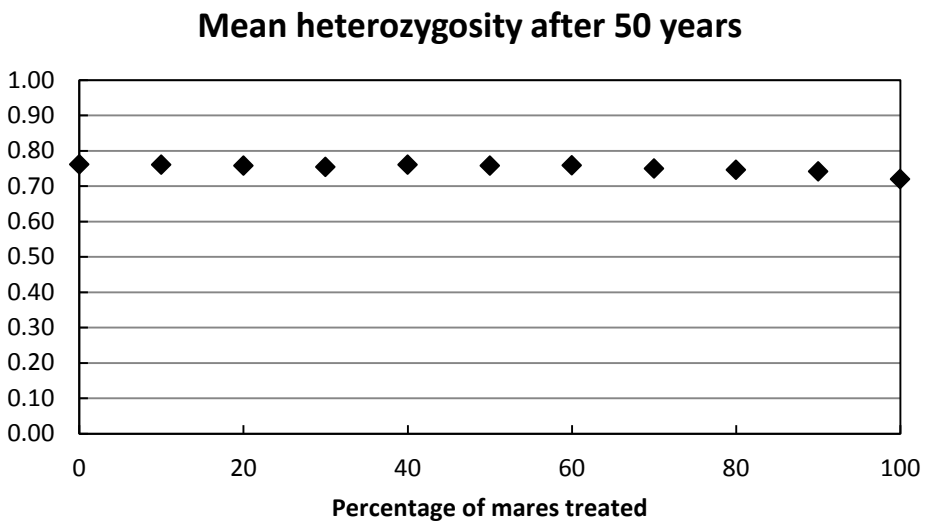


Figure 41. Initial population size = 250, lambda = 1.05, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	229.82	208.28	209.81	193.14	194.37	191.52	191.17	153.65	157.95	139.22	126.74
Mean number of removals after 20 years	130.33	109.21	106.87	95.15	93.21	95.93	96.1	91.98	79.41	93.08	89.13
Mean probability of extirpation after 50 years	0	0	0	0.01	0	0	0.01	0.01	0.01	0.06	0.12
Mean heterozygosity after 50 years	0.76	0.76	0.76	0.75	0.76	0.76	0.76	0.75	0.75	0.74	0.72

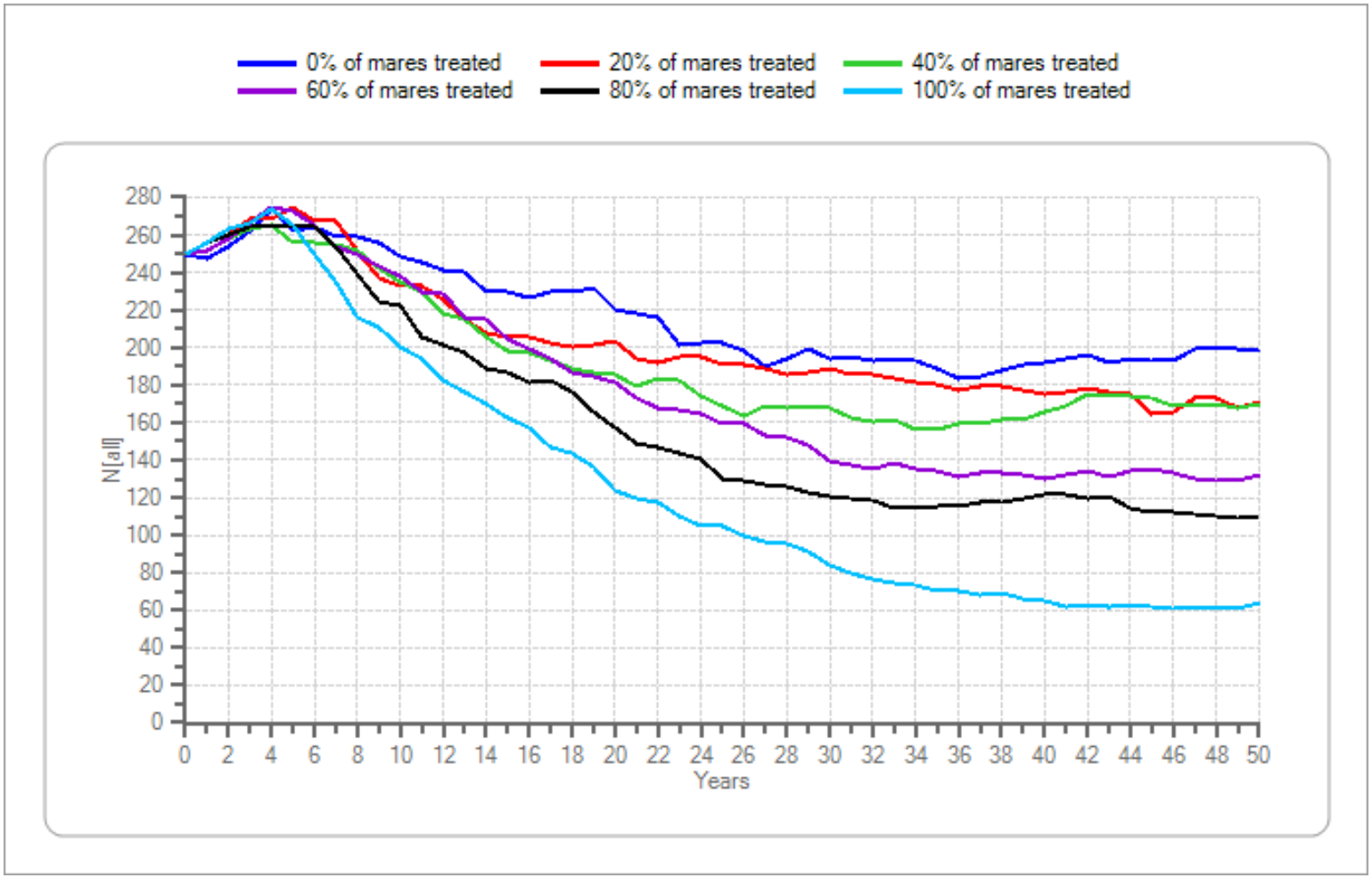
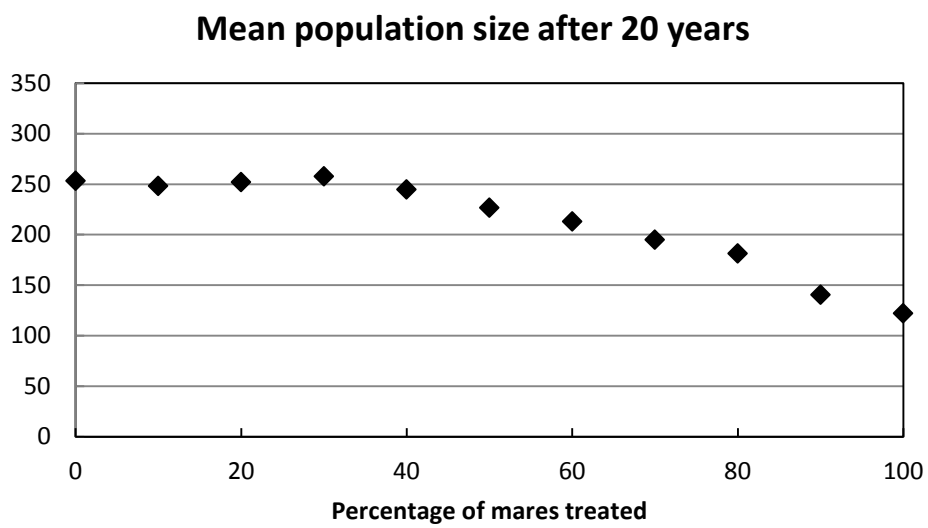
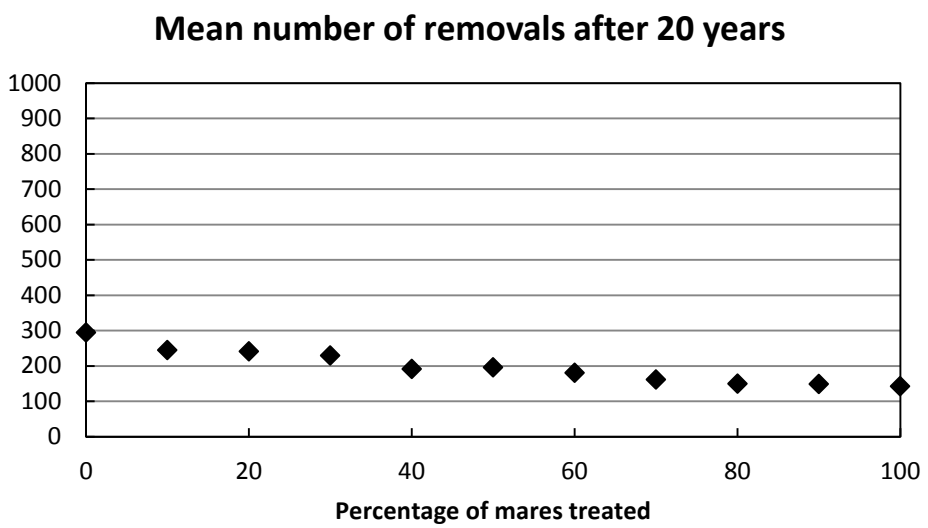
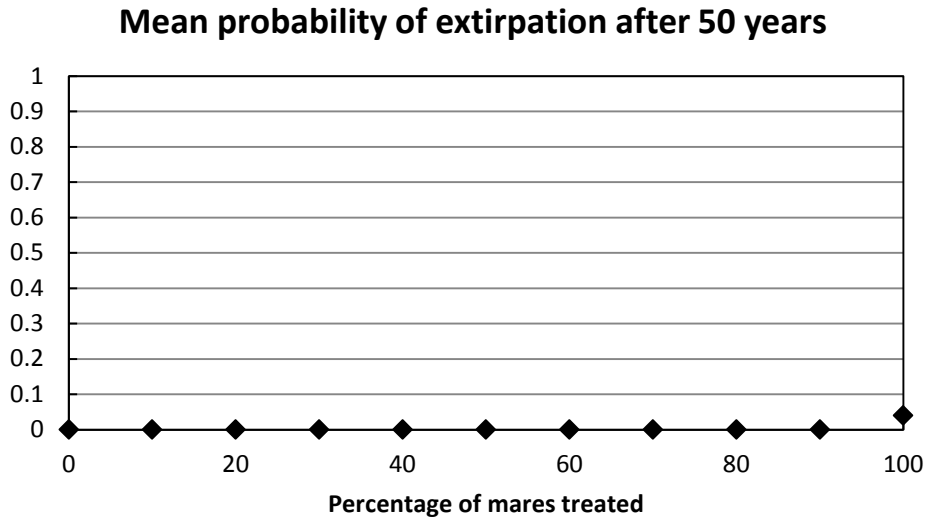


Figure 41. Initial population size = 250, lambda = 1.05, genetic diversity = high.—Continued







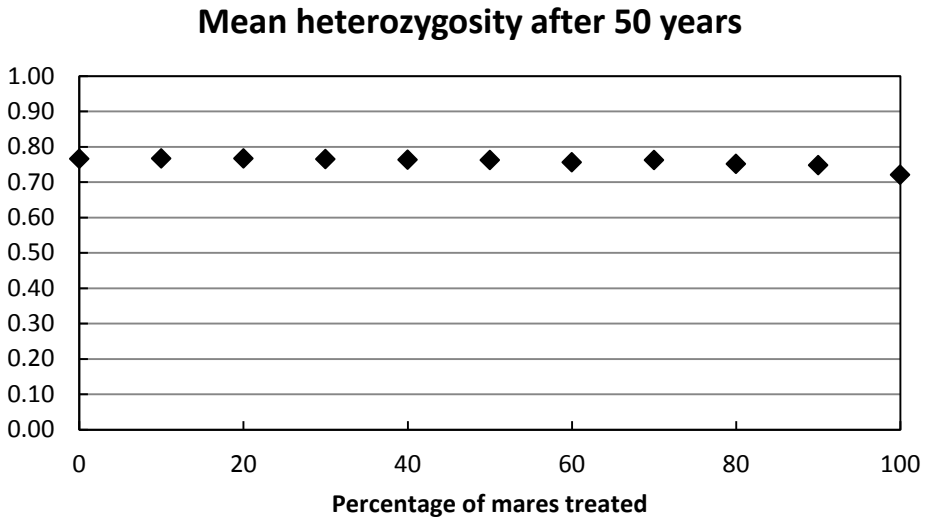


Figure 42. Initial population size = 250, lambda = 1.10, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	253.29	248.13	251.86	257.66	244.53	226.55	212.8	194.92	181.23	140.26	121.89
Mean number of removals after 20 years	294.42	244.72	240.7	229.15	190.87	195.41	180.34	161.04	149.36	148.76	141.8
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0.04
Mean heterozygosity after 50 years	0.77	0.77	0.77	0.77	0.76	0.76	0.76	0.76	0.75	0.75	0.72

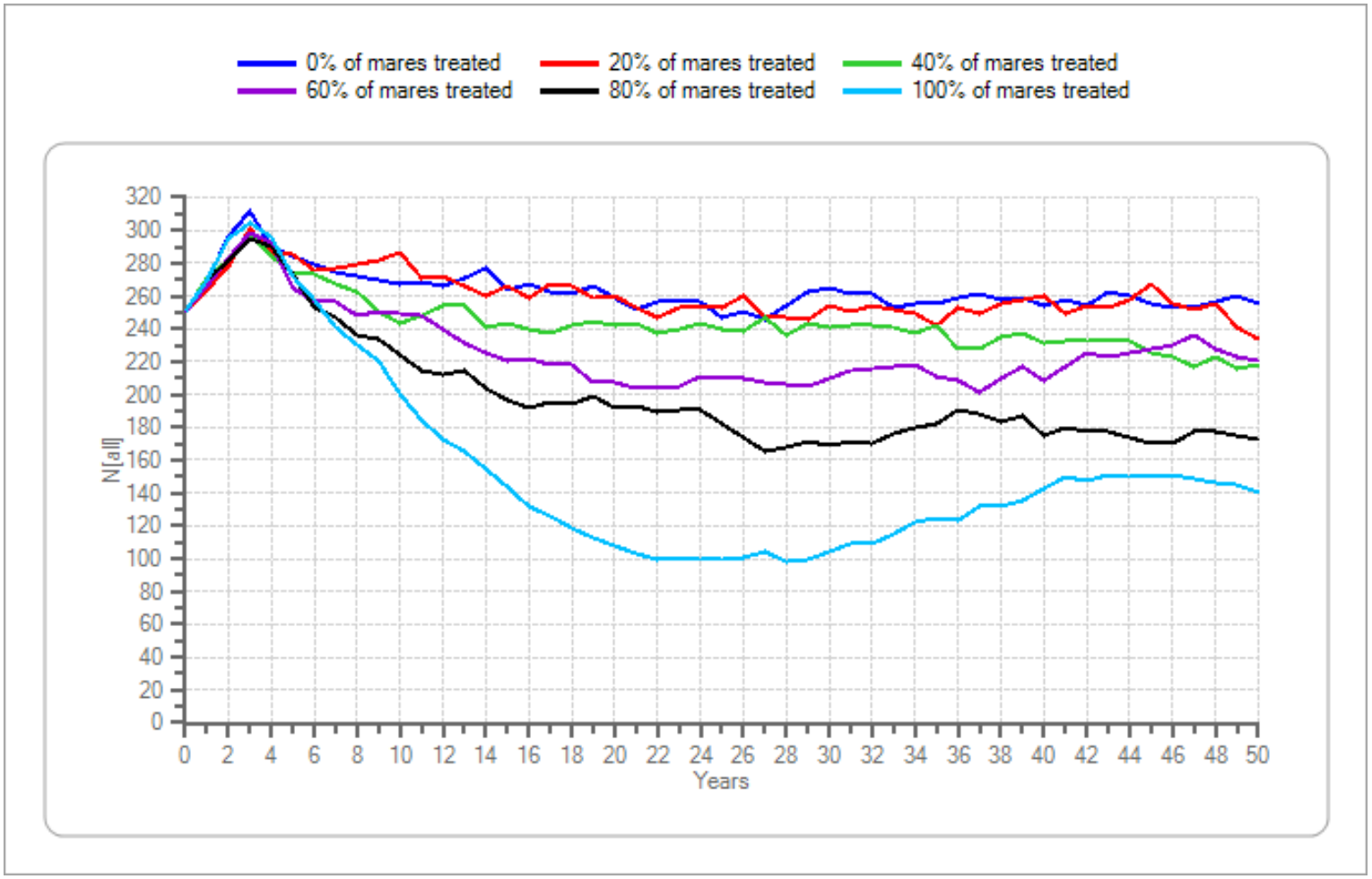
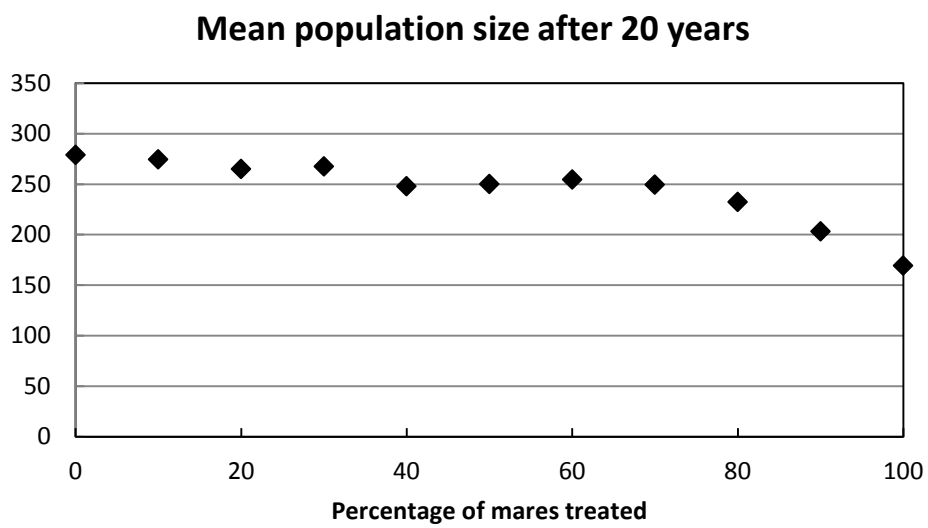
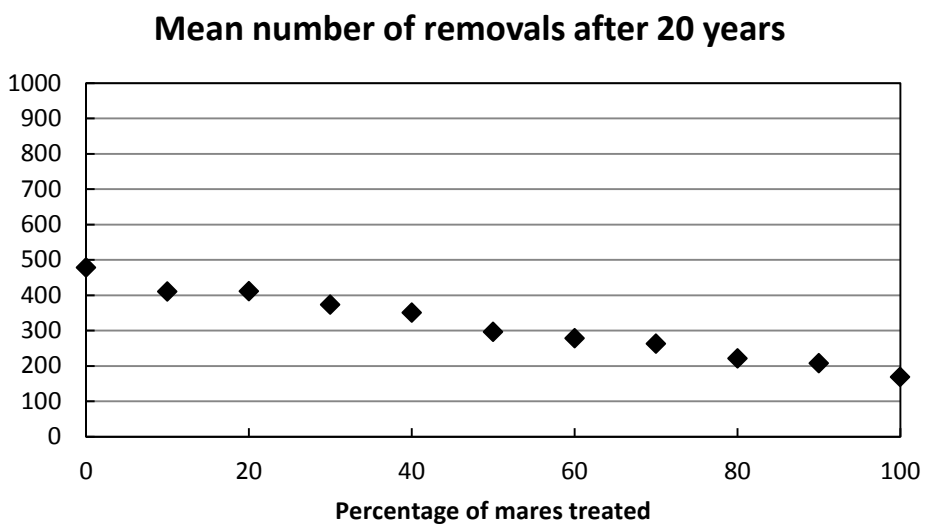
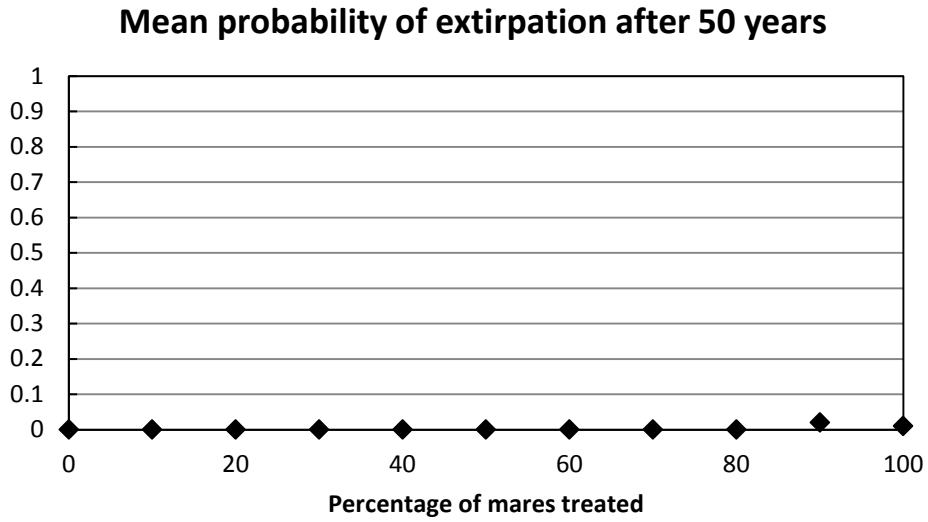


Figure 42. Initial population size = 250, lambda = 1.10, genetic diversity = high.—Continued







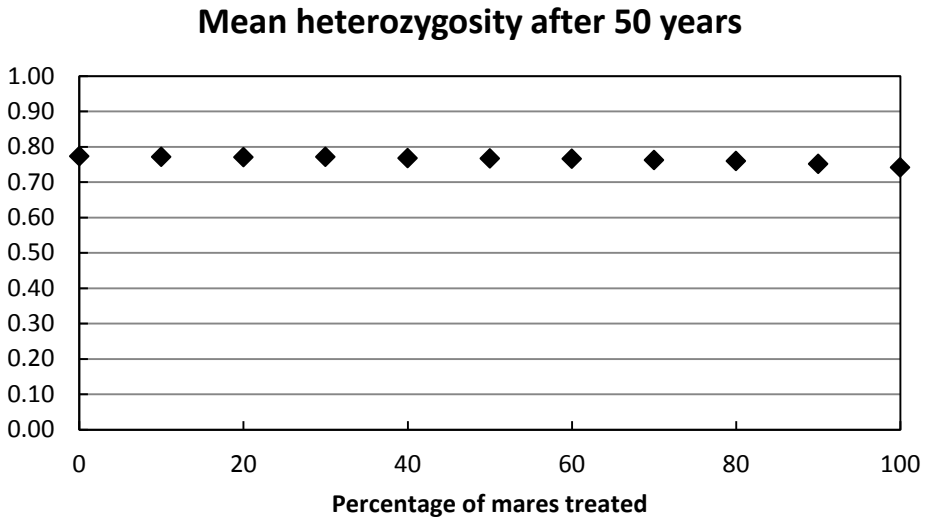


Figure 43. Initial population size = 250, lambda = 1.15, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	278.95	274.39	264.77	267.48	247.95	249.86	254.3	249.36	232.19	203.01	169.11
Mean number of removals after 20 years	478.13	410.55	411.23	373.12	350.21	295.77	278.33	262.32	220.73	207.34	168.69
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0.02	0.01
Mean heterozygosity after 50 years	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.76	0.76	0.75	0.74

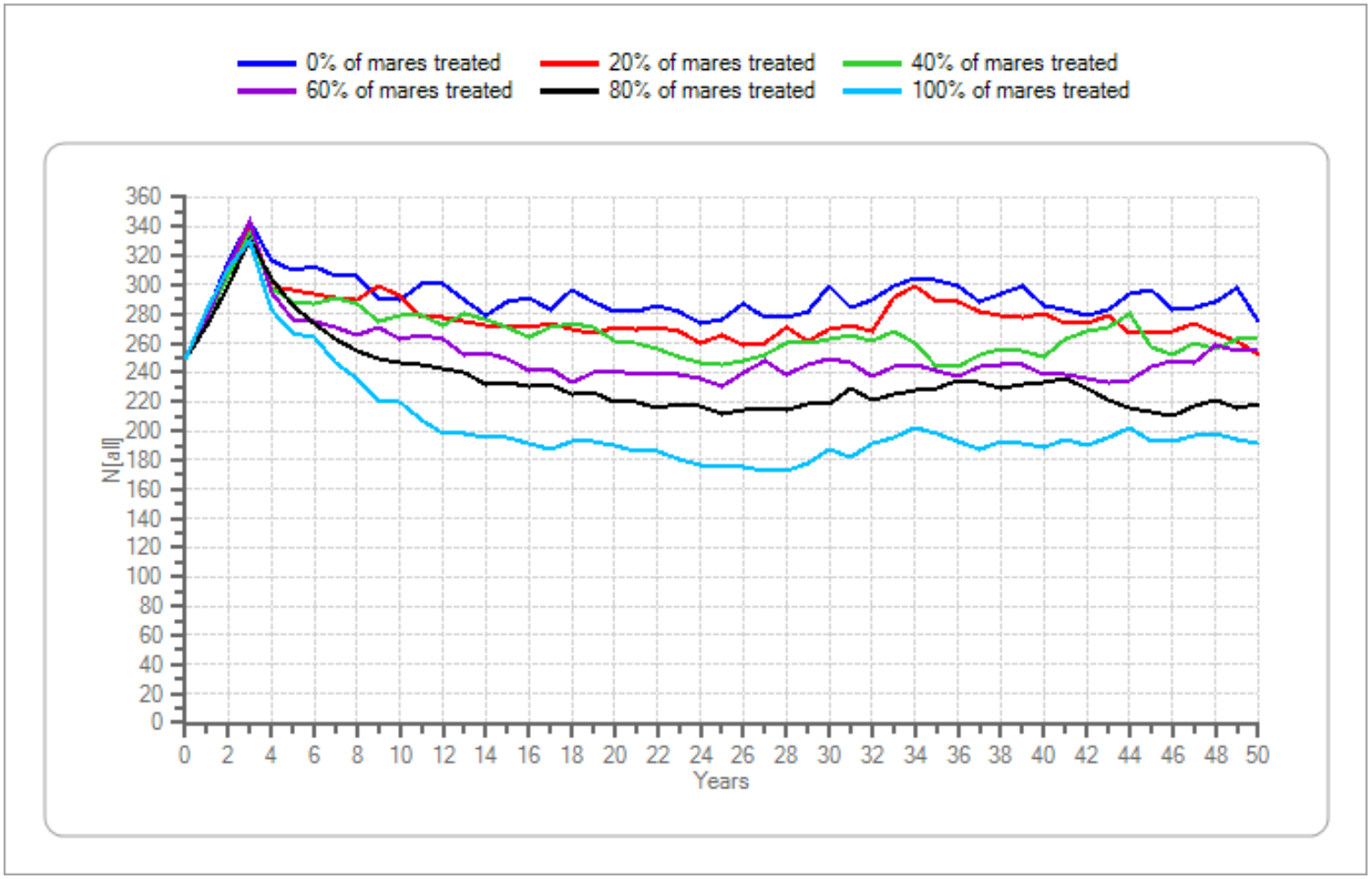
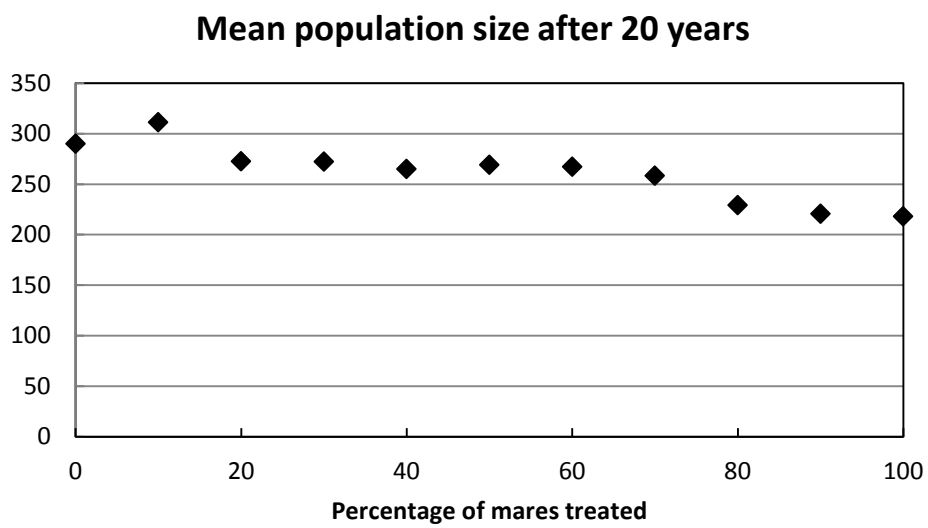
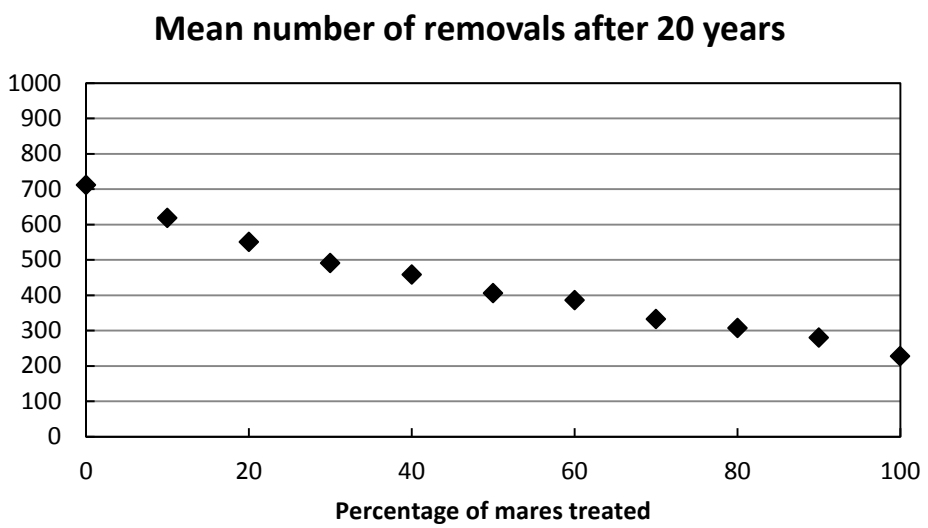
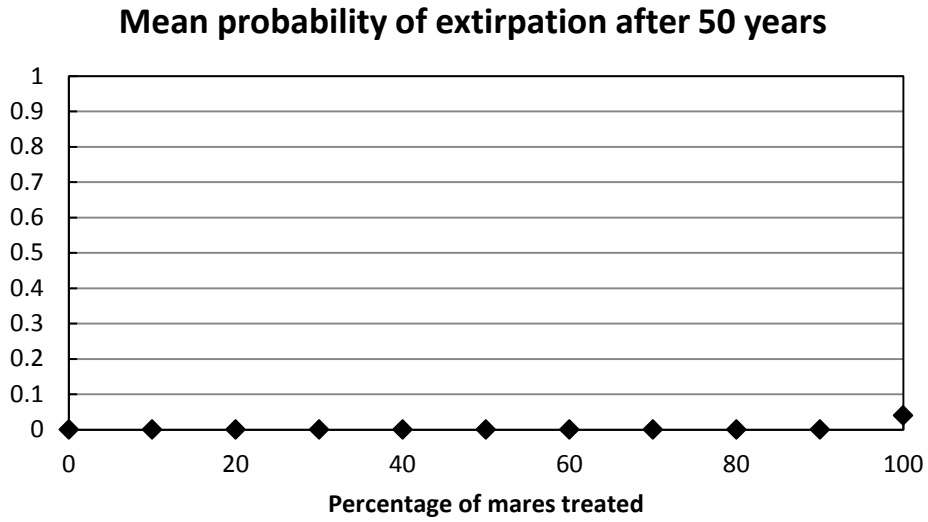


Figure 43. Initial population size = 250, lambda = 1.15, genetic diversity = high.—Continued







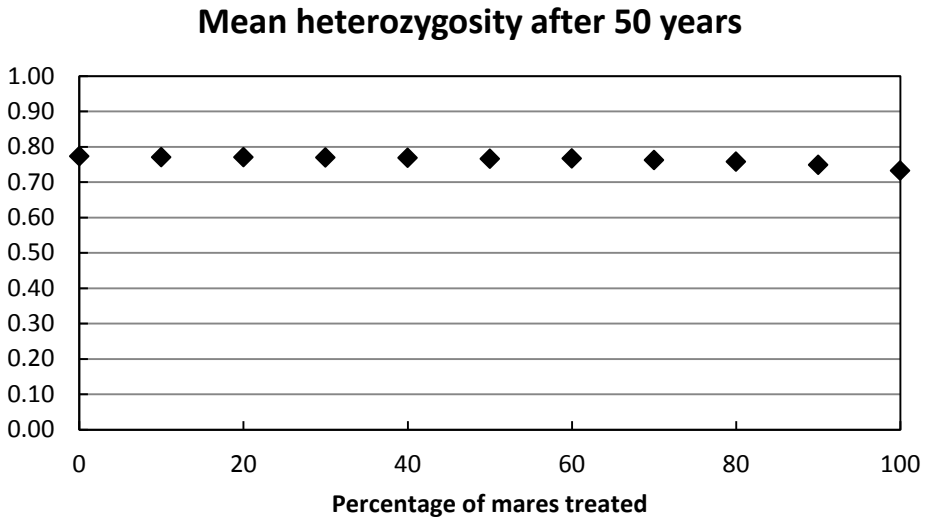


Figure 44. Initial population size = 250, lambda = 1.20, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	290.06	311.21	272.6	272.05	264.99	269.08	267.14	258.33	229.2	220.46	218.12
Mean number of removals after 20 years	711.9	618.06	550.39	491.13	458.39	405.16	385.49	331.92	307.21	279.49	227.5
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0.04
Mean heterozygosity after 50 years	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.76	0.76	0.75	0.73

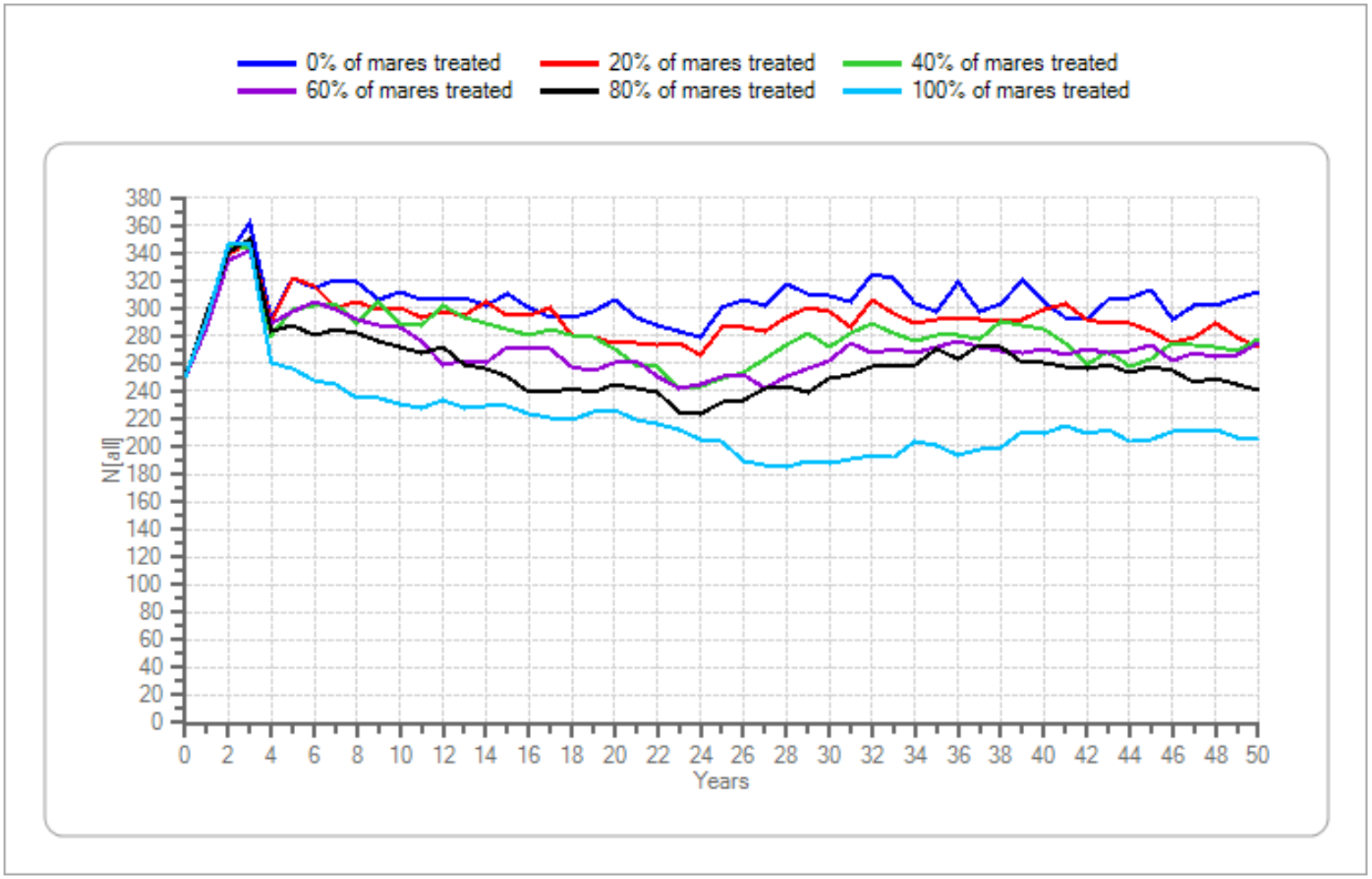
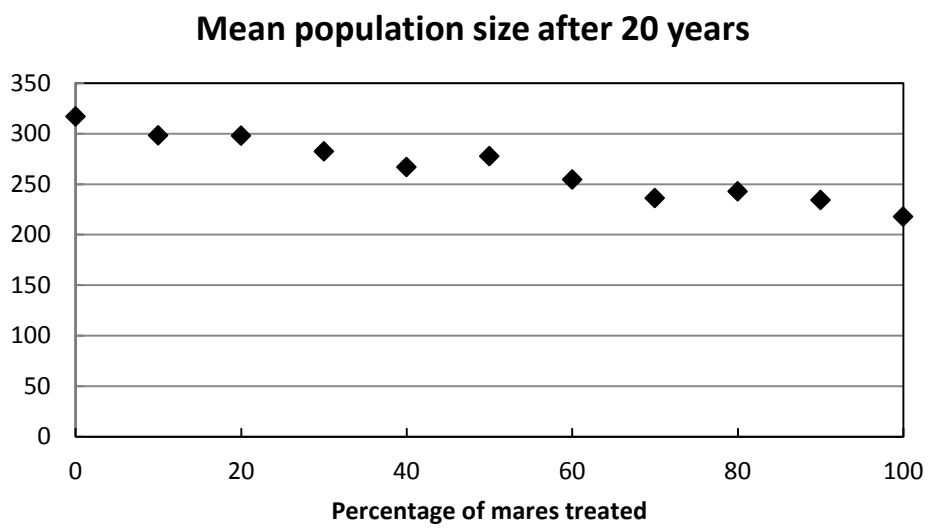
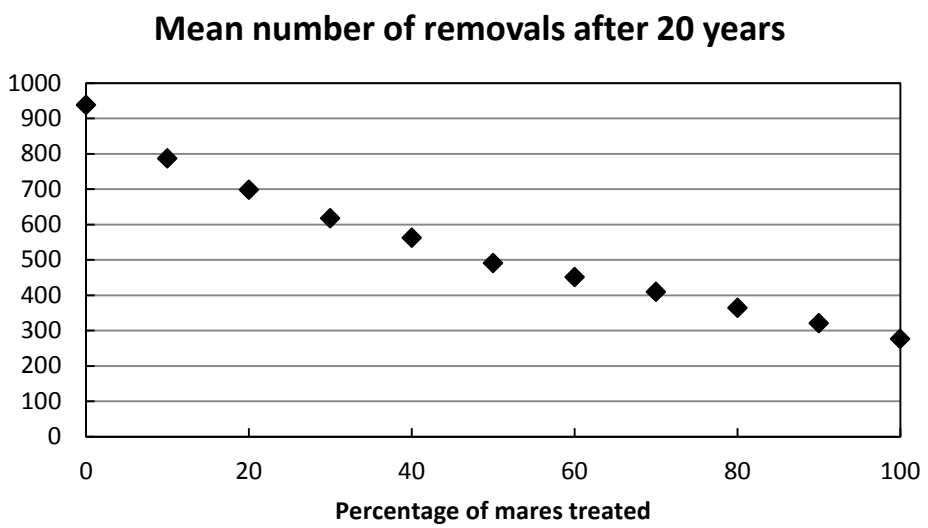
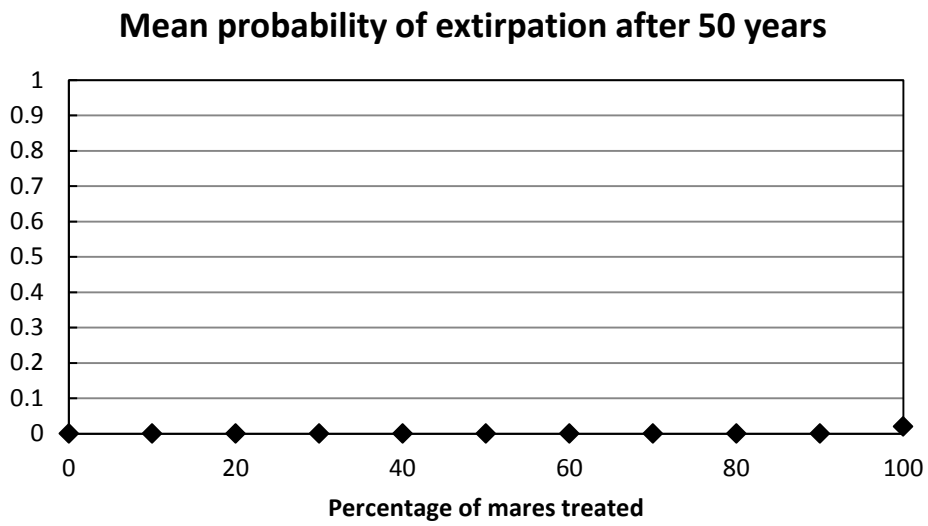


Figure 44. Initial population size = 250, lambda = 1.20, genetic diversity = high.—Continued







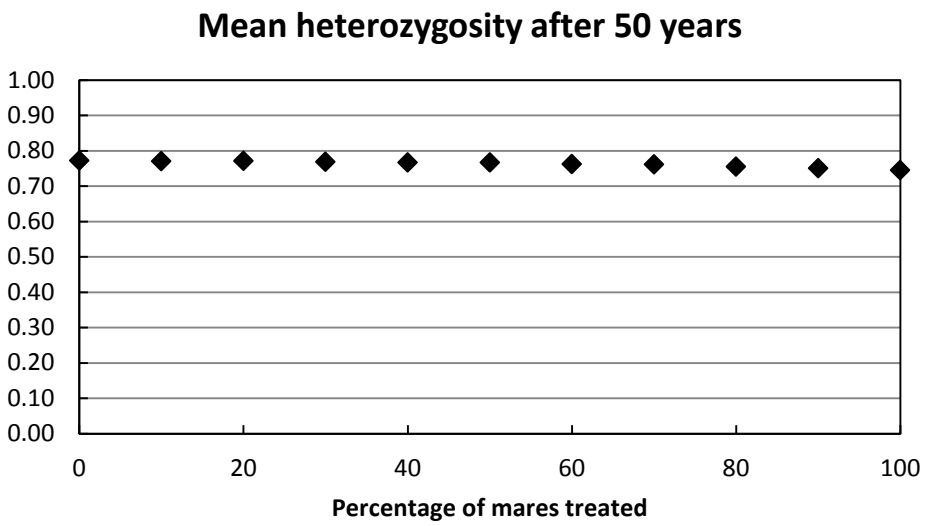


Figure 45. Initial population size = 250, lambda = 1.25, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	316.89	298.09	297.94	282.24	266.75	277.65	254.56	236.14	242.76	234.27	217.59
Mean number of removals after 20 years	937.55	786.93	697.69	617.57	562.54	490.47	450.66	409.16	363.89	320.86	276.24
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0.02
Mean heterozygosity after 50 years	0.77	0.77	0.77	0.77	0.77	0.77	0.76	0.76	0.76	0.75	0.75

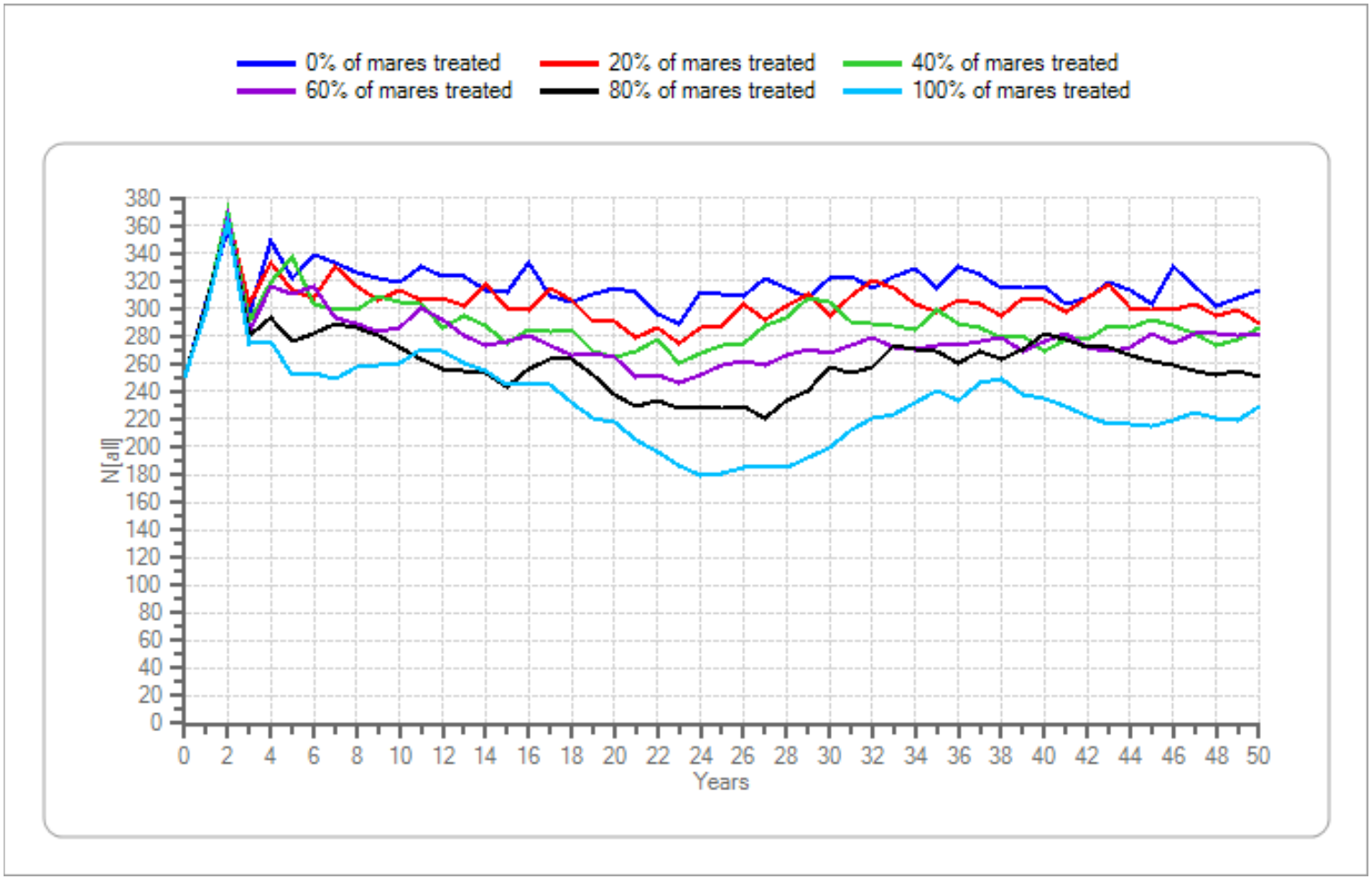
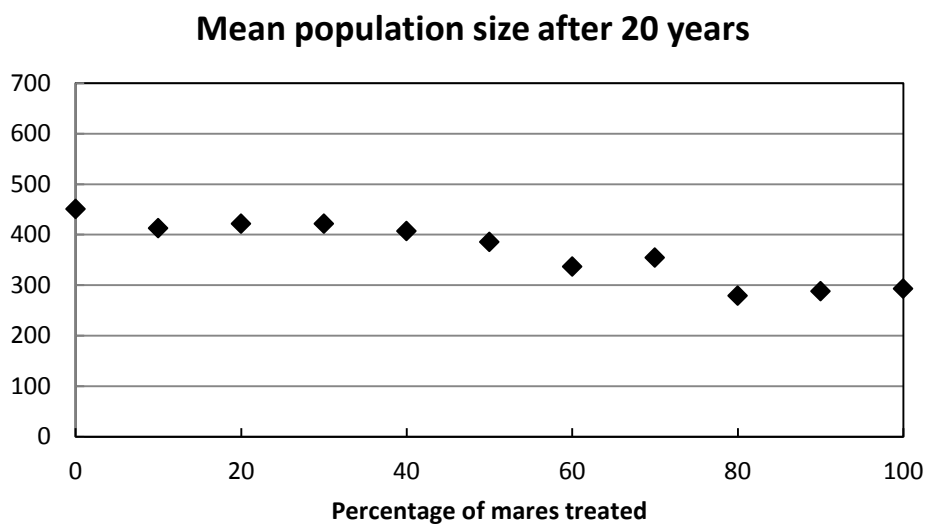
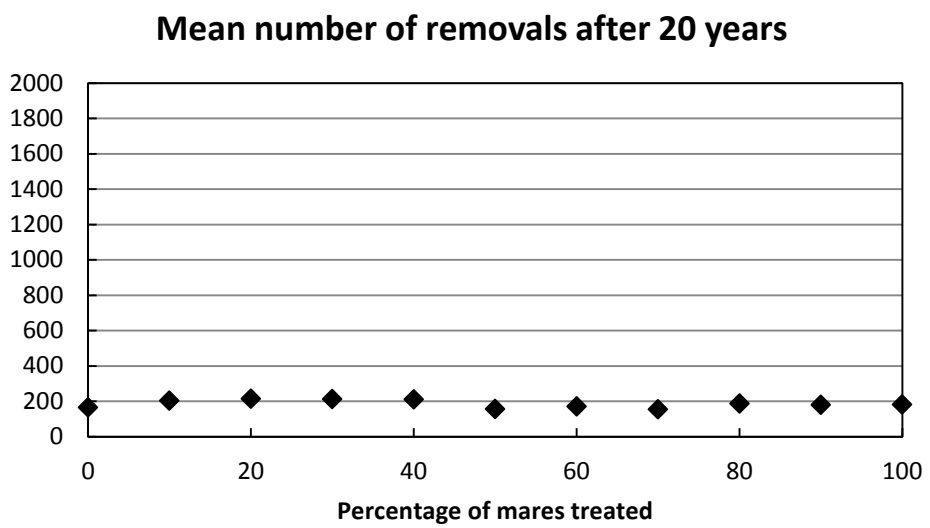
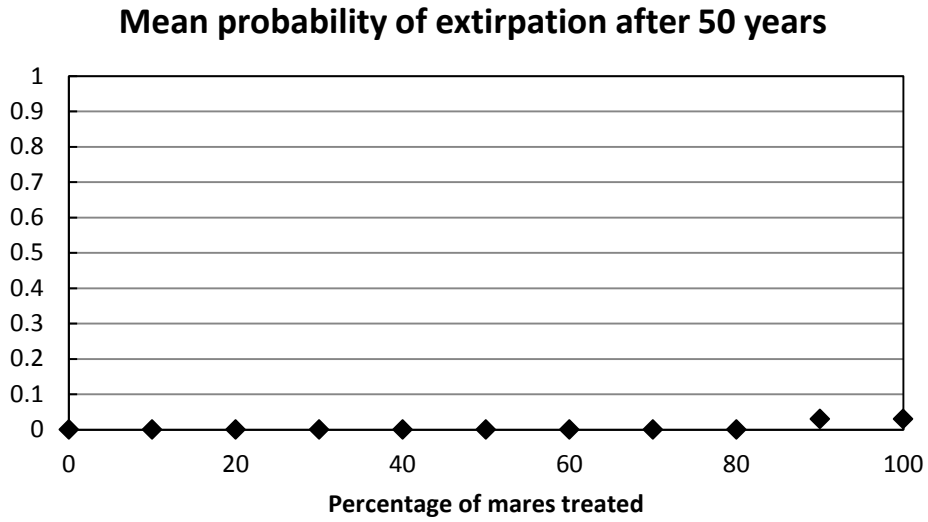


Figure 45. Initial population size = 250, lambda = 1.25, genetic diversity = high.—Continued







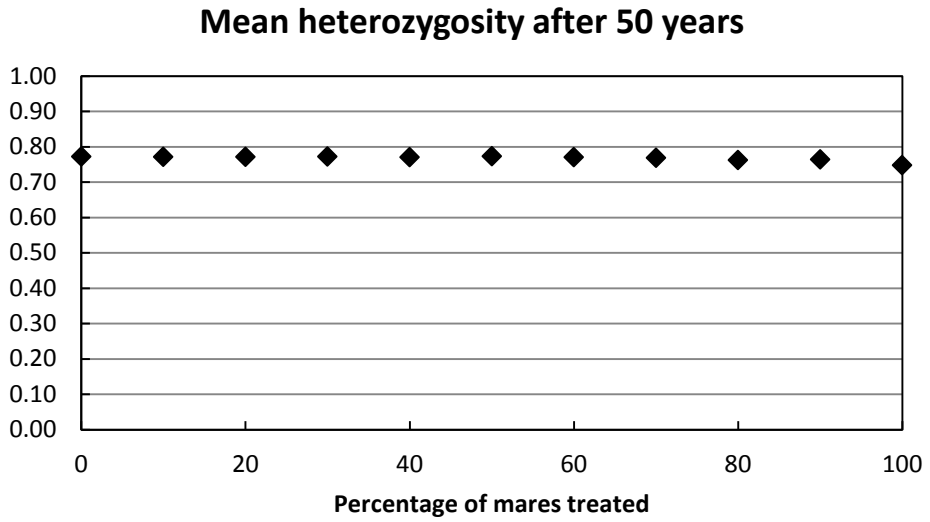


Figure 46. Initial population size = 500, lambda = 1.05, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	450.57	412.67	421.58	421.47	407.01	385.44	336.29	354.49	278.69	287.98	292.7
Mean number of removals after 20 years	165.39	203.66	214.47	212.76	209.18	155.55	170.07	154.49	186.23	178.59	180.8
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0.03	0.03
Mean heterozygosity after 50 years	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.76	0.76	0.75

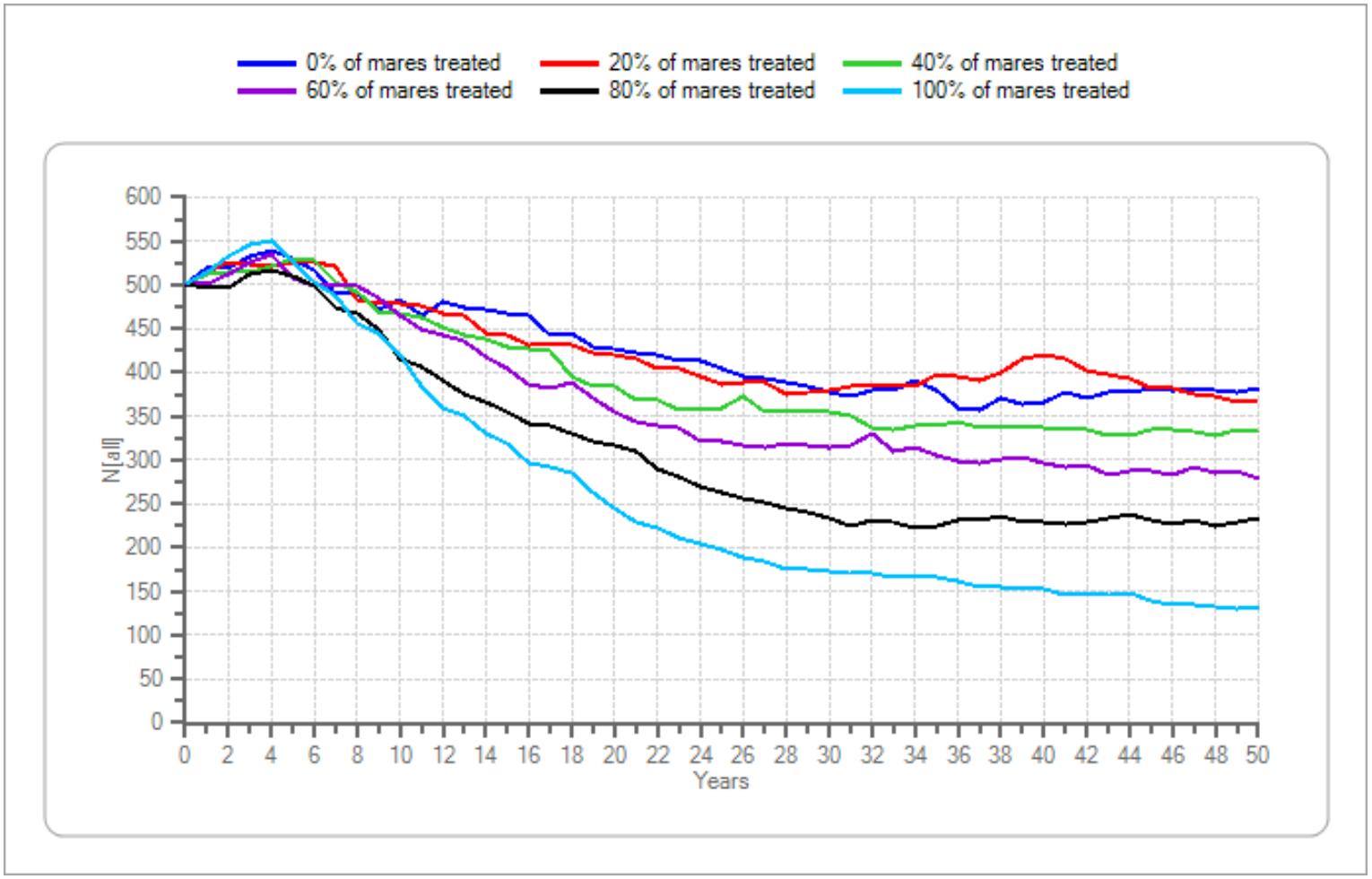
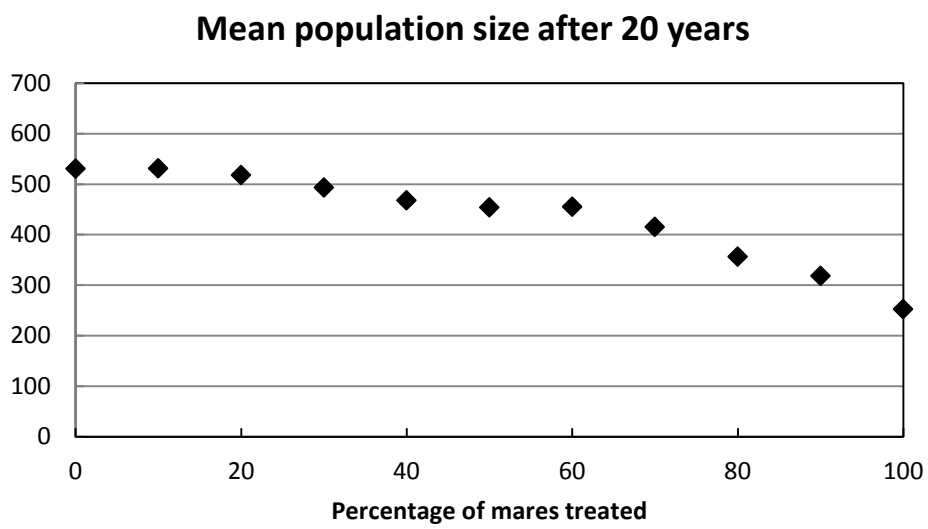
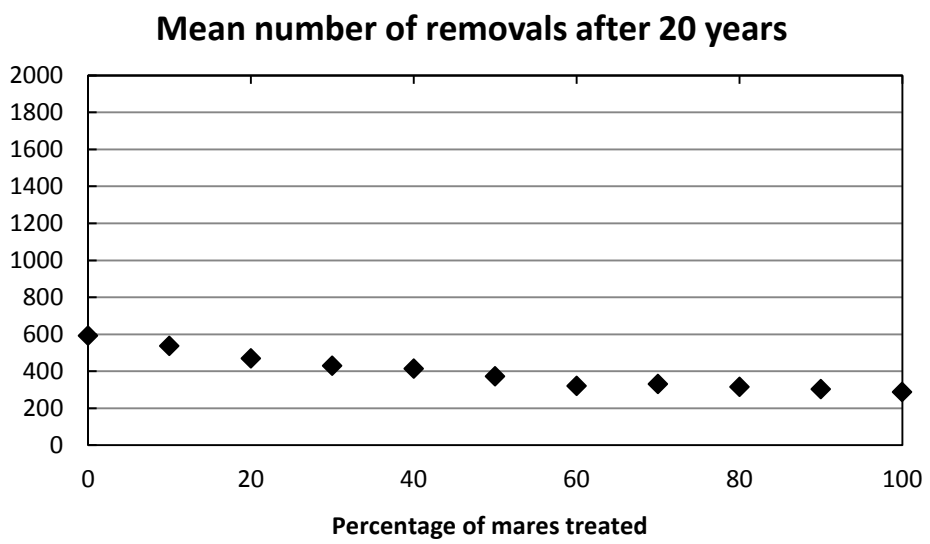
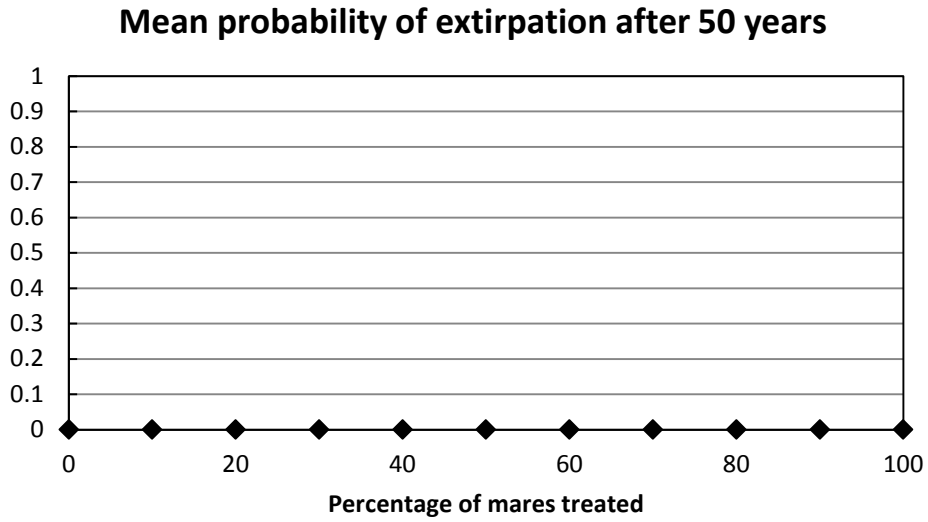


Figure 46. Initial population size = 500, lambda = 1.05, genetic diversity = high.—Continued







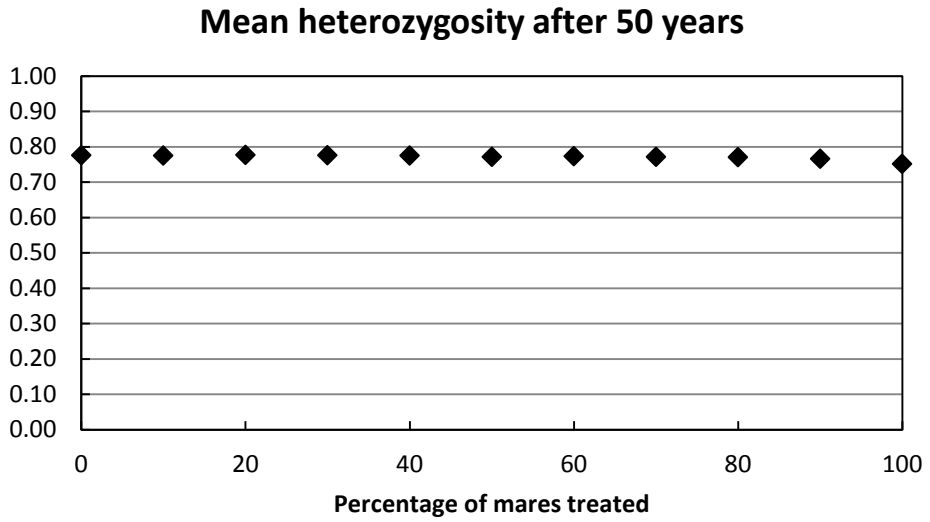


Figure 47. Initial population size = 500, lambda = 1.10, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	530.61	530.85	517.52	493.18	467.76	453.59	454.75	415.17	356.16	318.2	252.43
Mean number of removals after 20 years	591.77	536.39	469.19	428.35	413.44	371.39	319.96	329.77	315.36	302.16	286.23
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.77	0.77	0.77	0.77	0.77	0.77	0.75

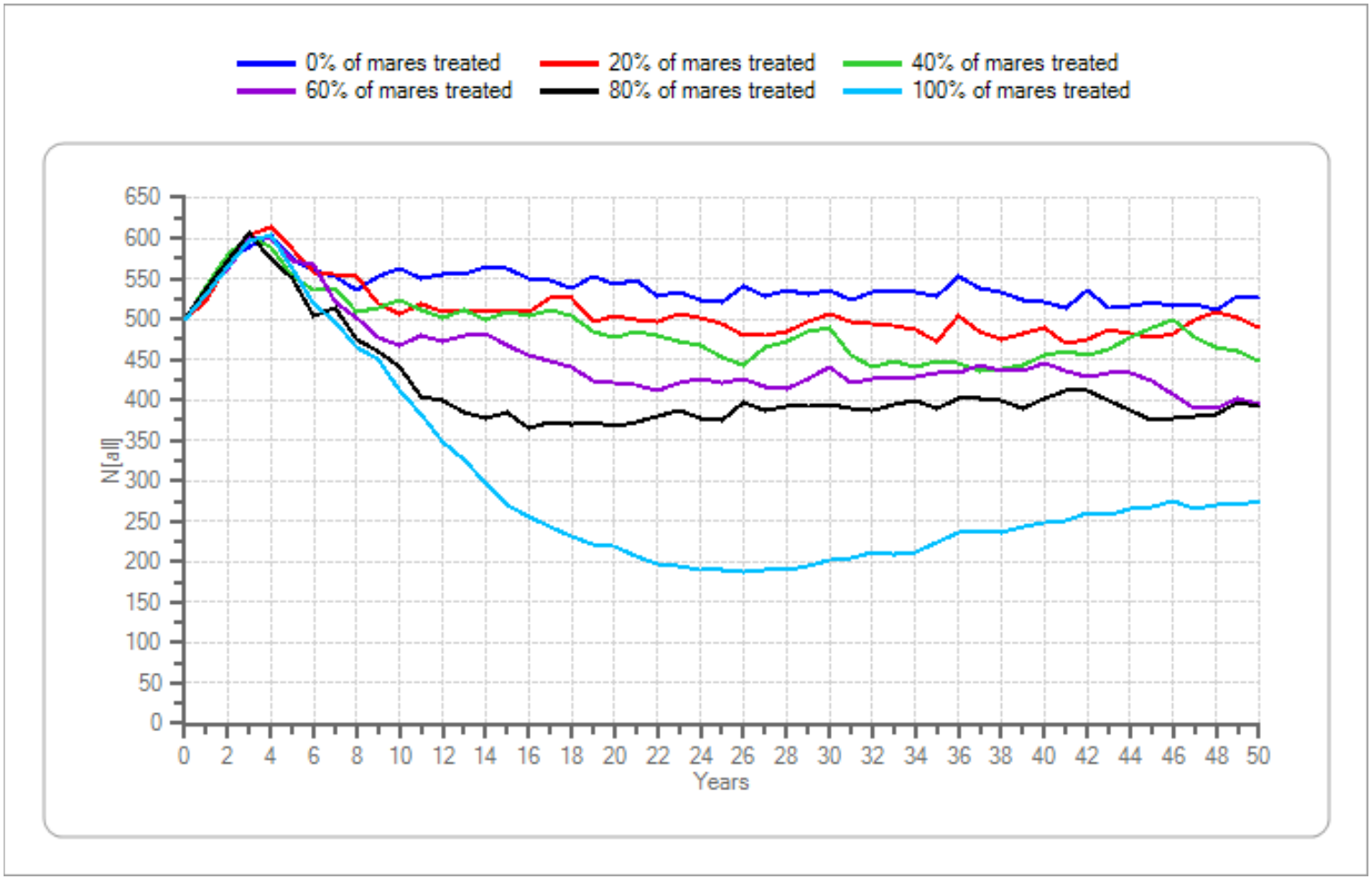
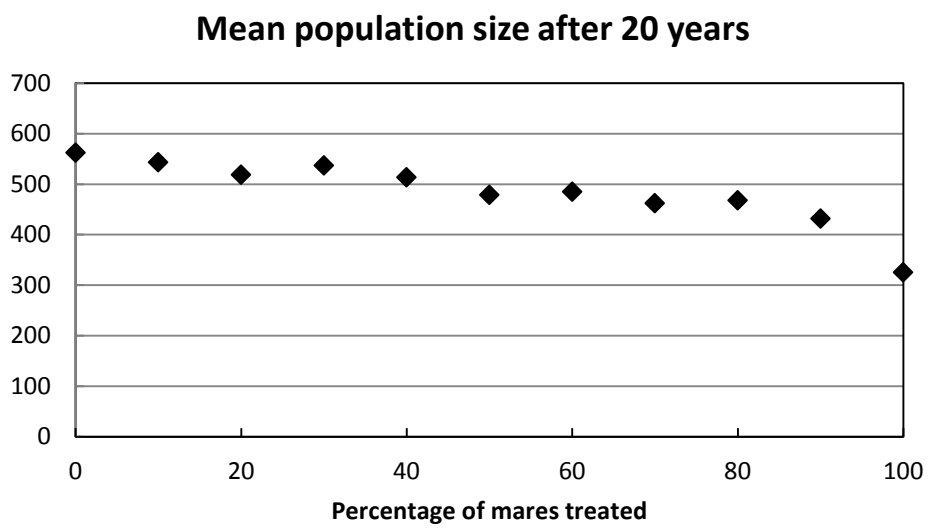
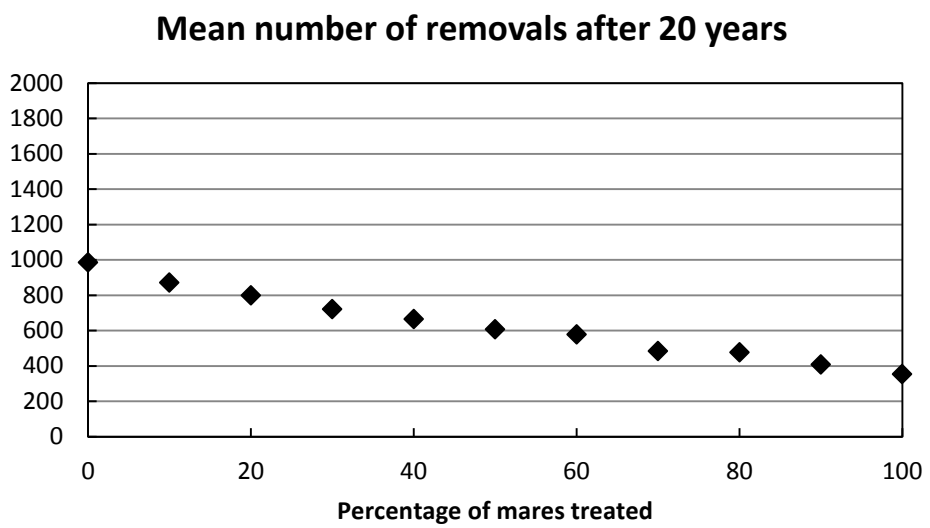
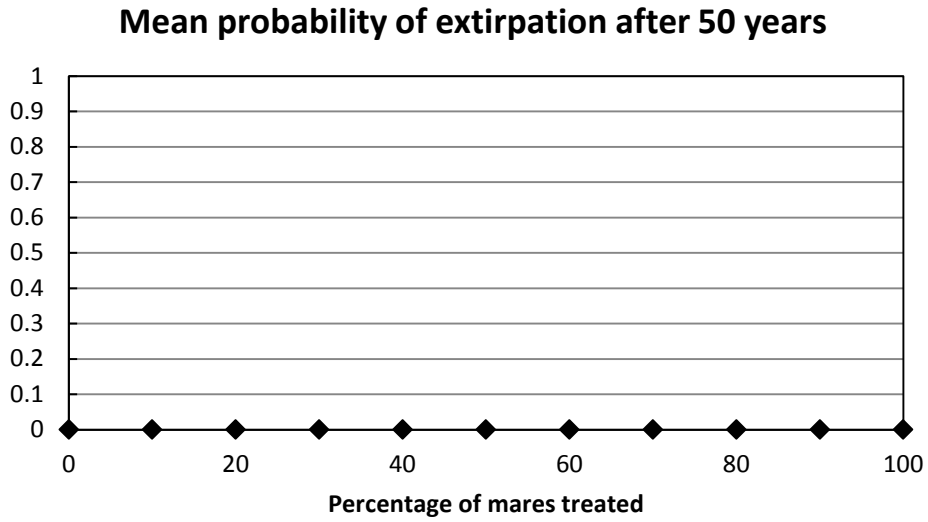


Figure 47. Initial population size = 500, lambda = 1.10, genetic diversity = high.—Continued







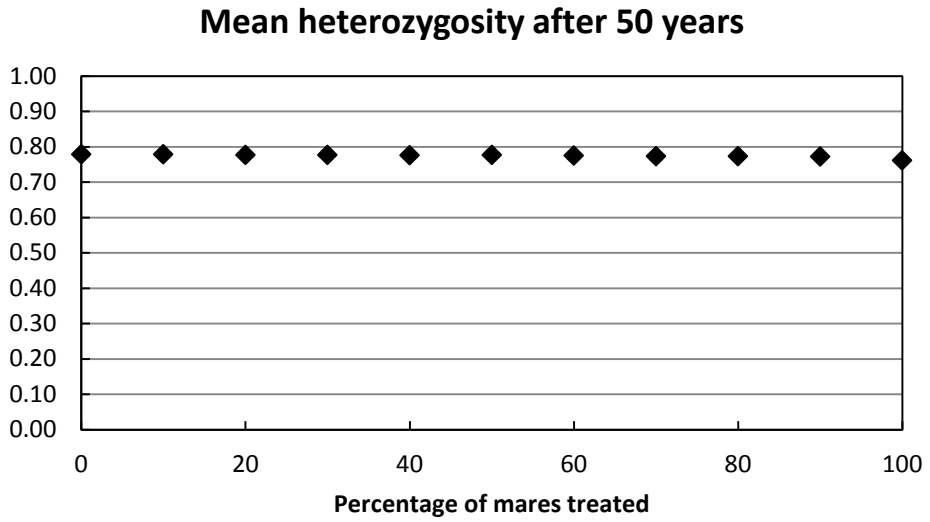


Figure 48. Initial population size = 500, lambda = 1.15, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	562.32	542.84	518.23	536.63	513.12	478.55	484.51	462.25	467.67	431.71	325.38
Mean number of removals after 20 years	985.18	870.87	799.25	721.09	665.02	606.24	577.73	482.76	475.37	406.87	352.26
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.77	0.77	0.77	0.77	0.76

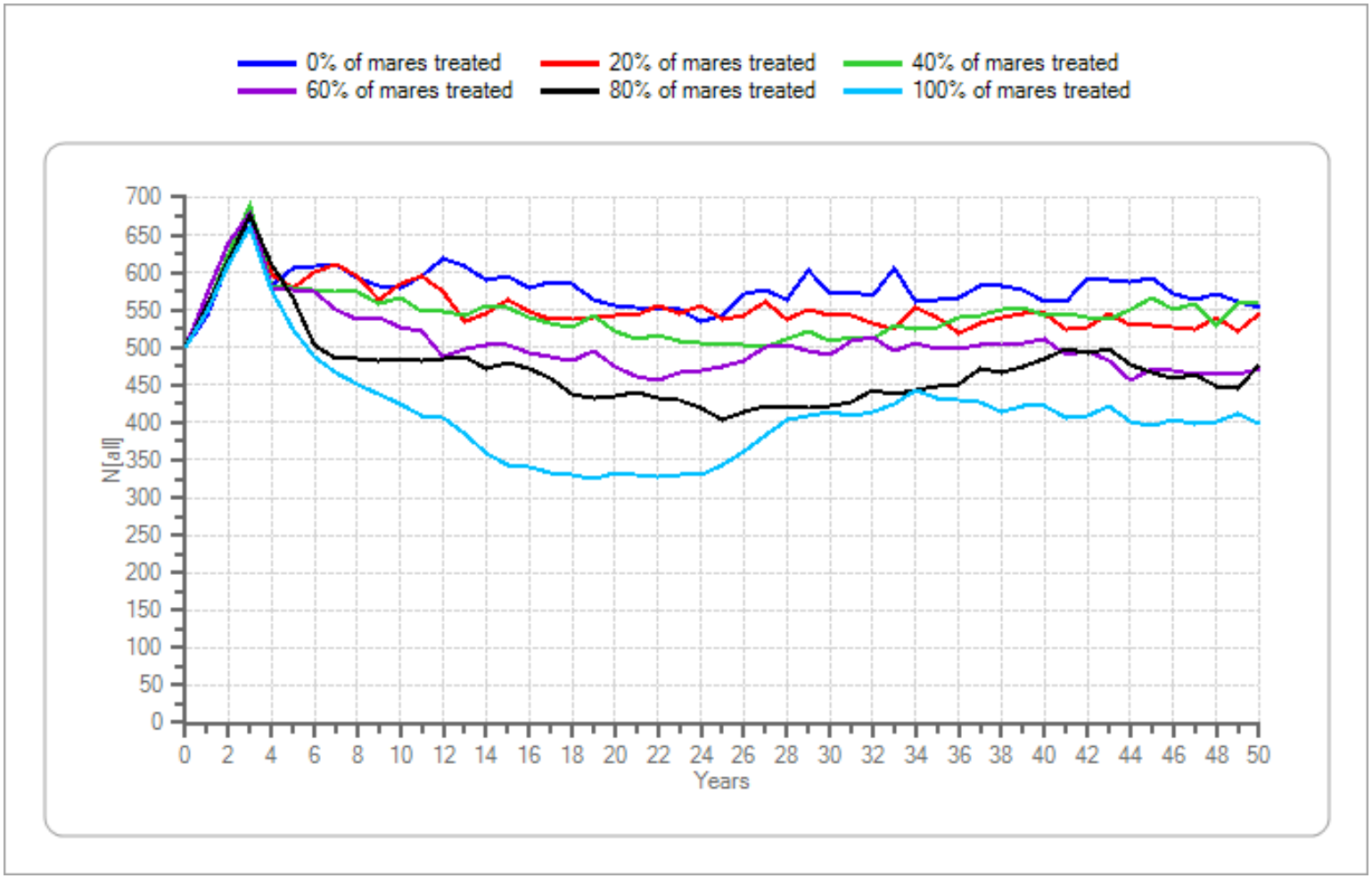
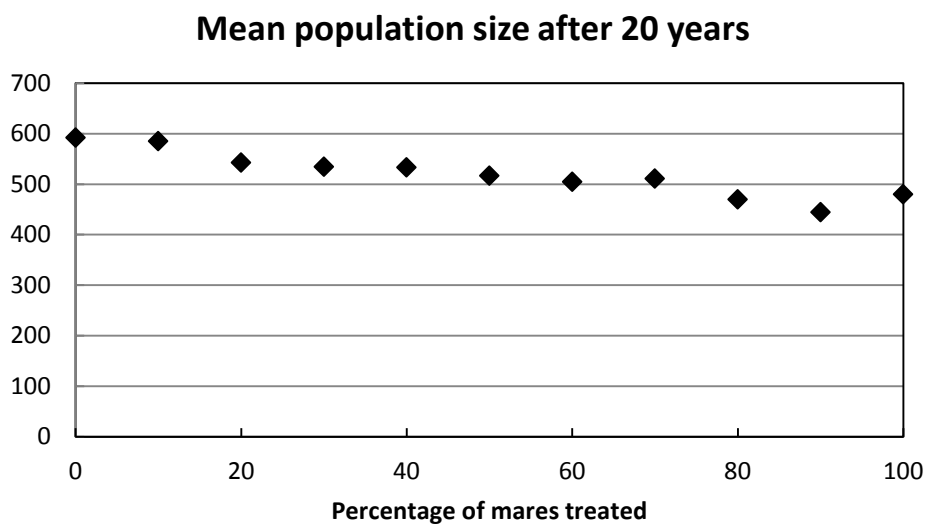
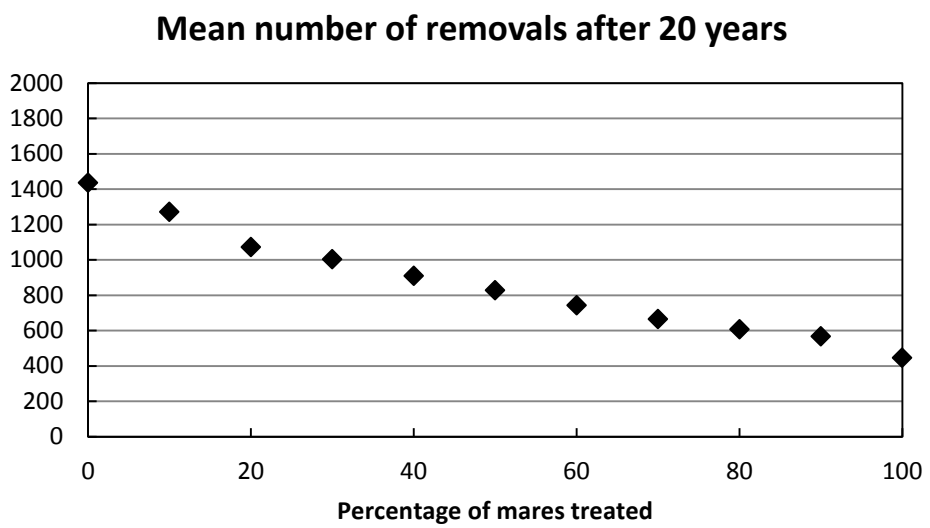
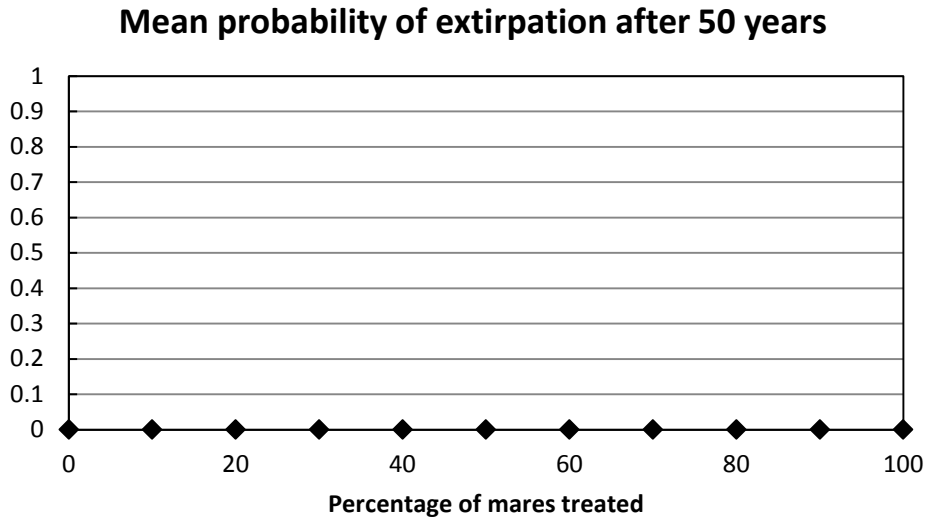


Figure 48. Initial population size = 500, lambda = 1.15, genetic diversity = high.—Continued







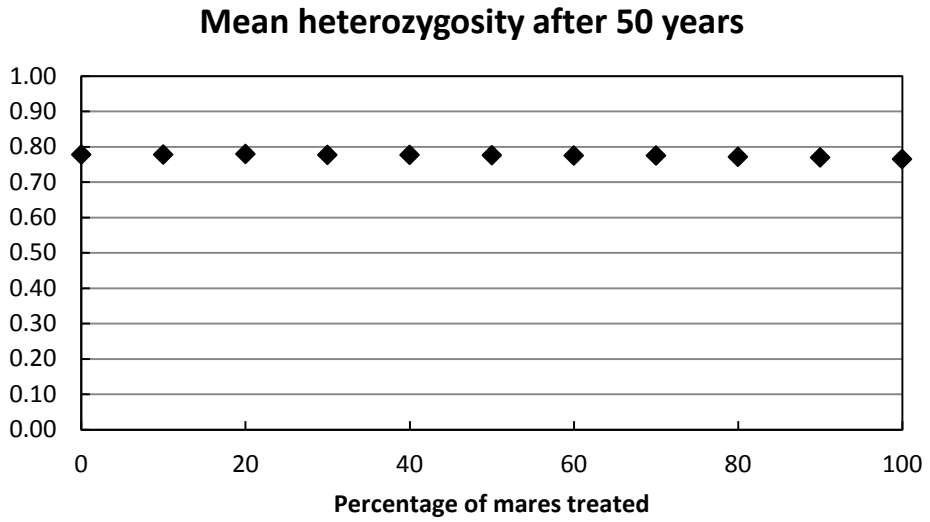


Figure 49. Initial population size = 500, lambda = 1.20, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	591.83	584.93	542.73	534.42	532.9	516.29	504.75	510.67	469.76	444.23	479.71
Mean number of removals after 20 years	1435.1	1271.0	1072.6	1003.7	908.9	826.8	741.6	663.7	606.5	567.2	445.4
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77	0.77	0.76

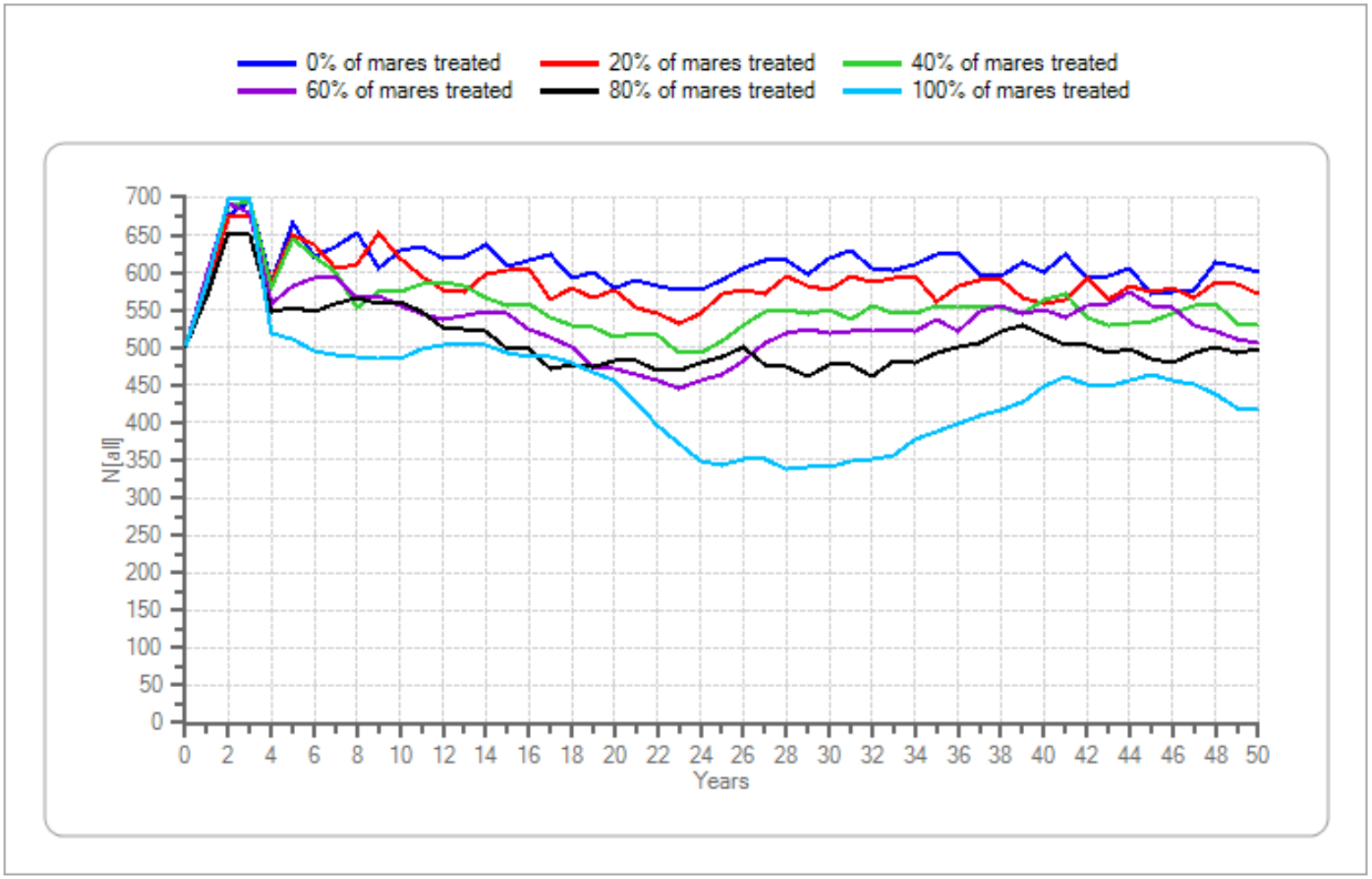
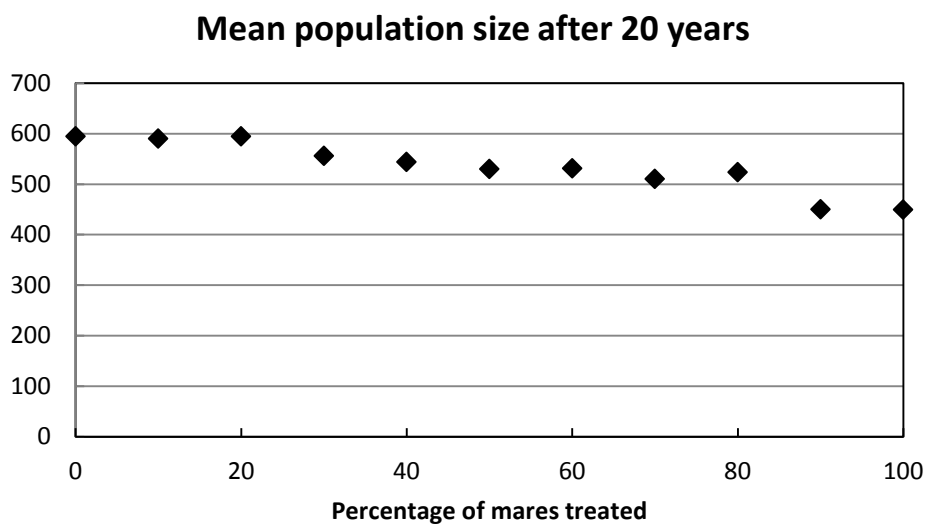
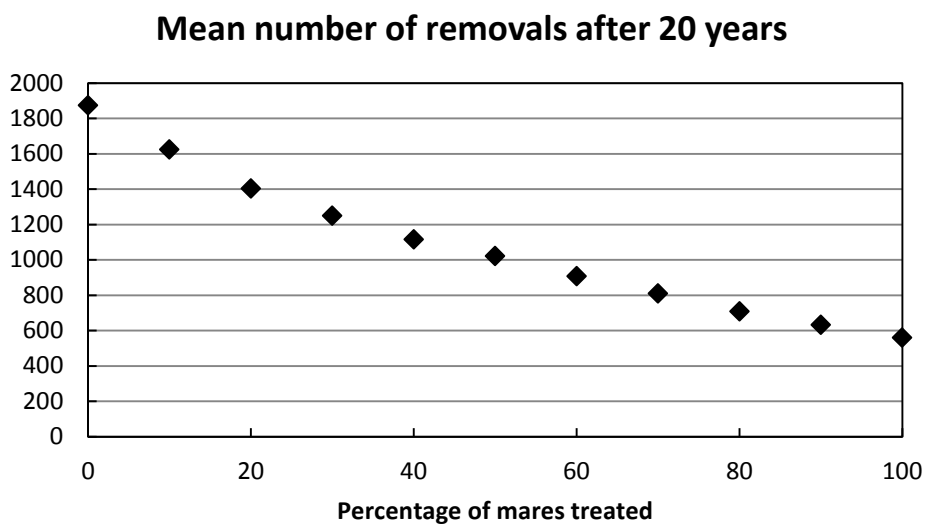
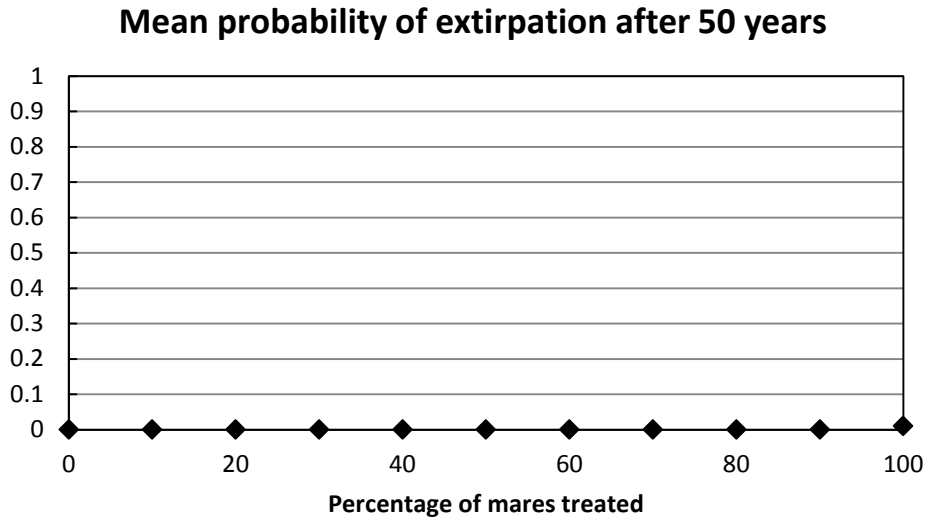


Figure 49. Initial population size = 500, lambda = 1.20, genetic diversity = high.—Continued







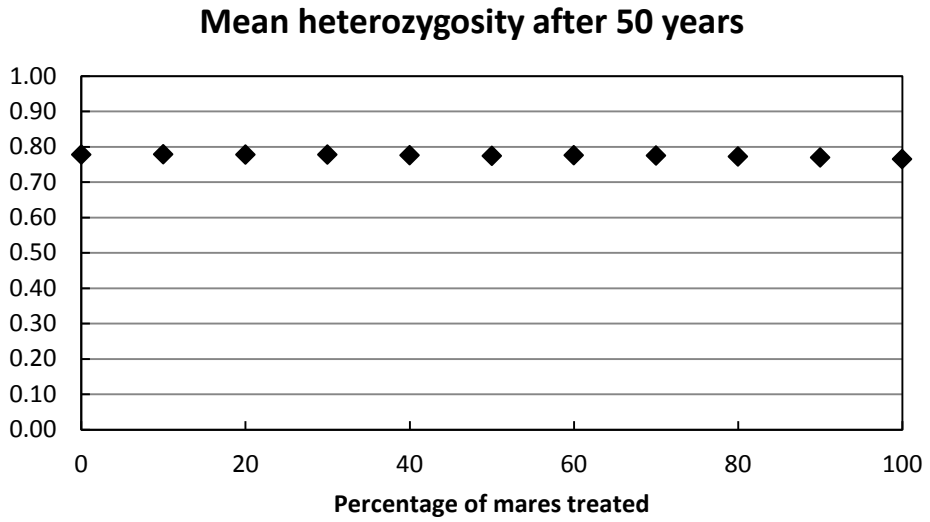


Figure 50. Initial population size = 500, lambda = 1.25, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	594.45	589.9	594.5	555.72	543.85	529.95	531.28	510.02	523.65	449.97	449.55
Mean number of removals after 20 years	1874.5	1624.6	1402.6	1249.2	1116.2	1021.7	907.0	809.4	707.9	631.4	559.9
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0.01
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.77	0.78	0.78	0.77	0.77	0.76

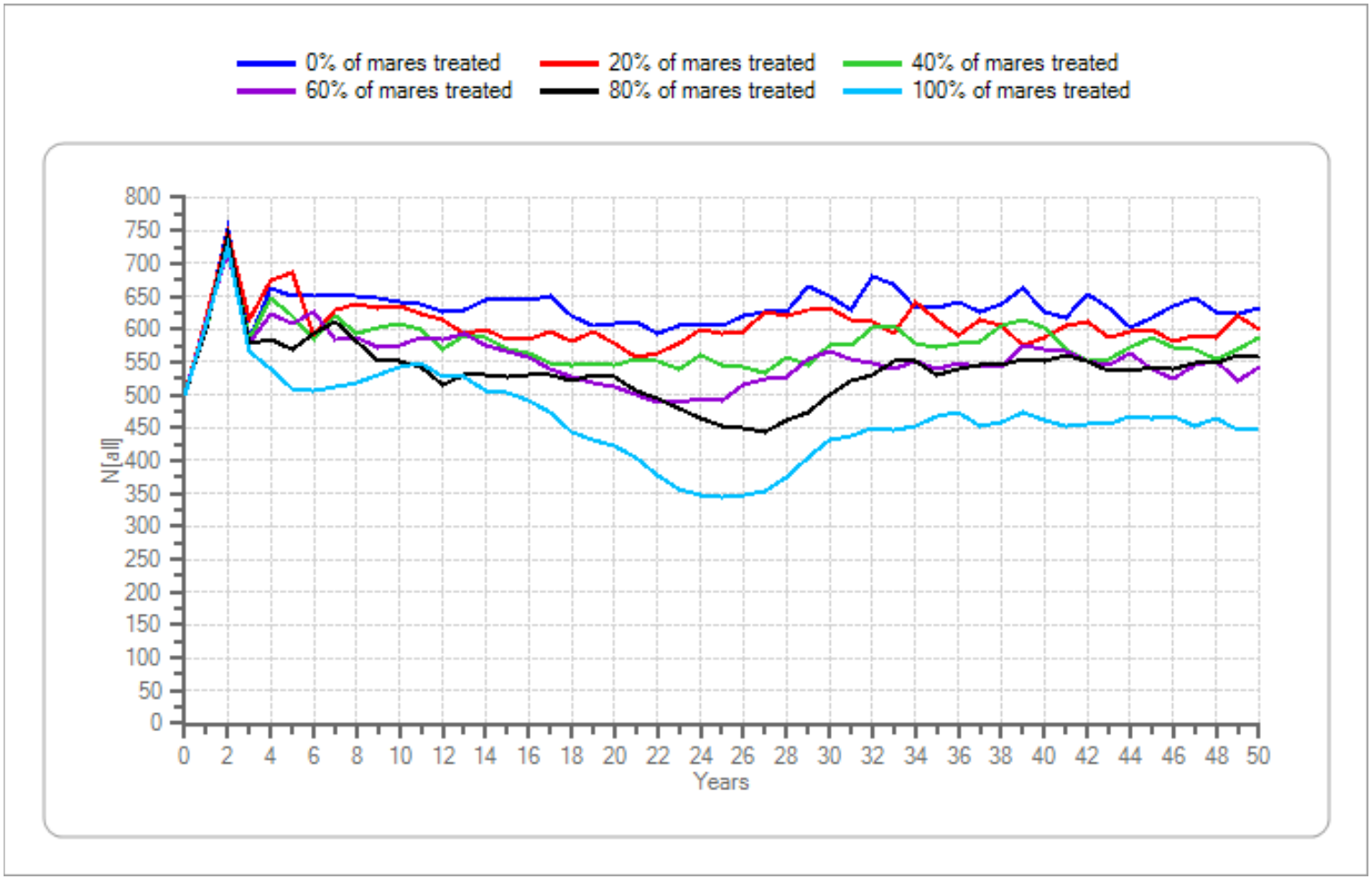
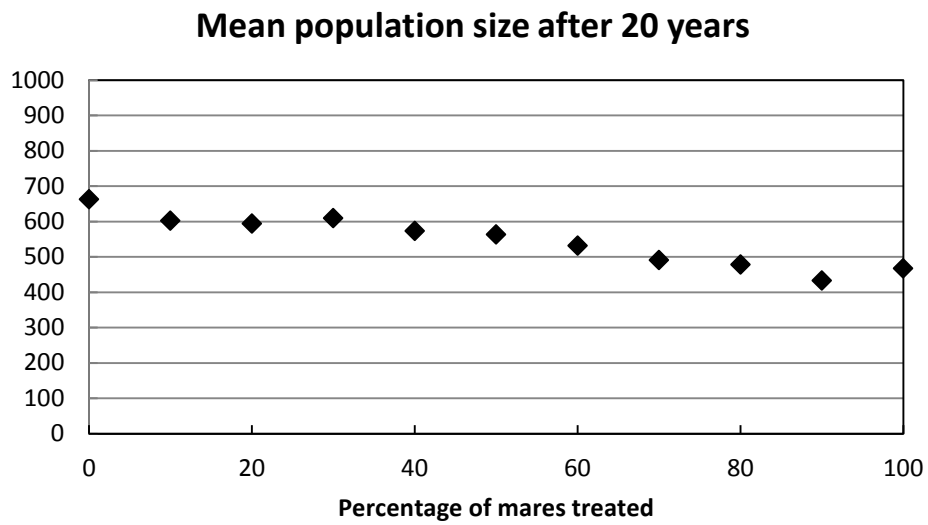
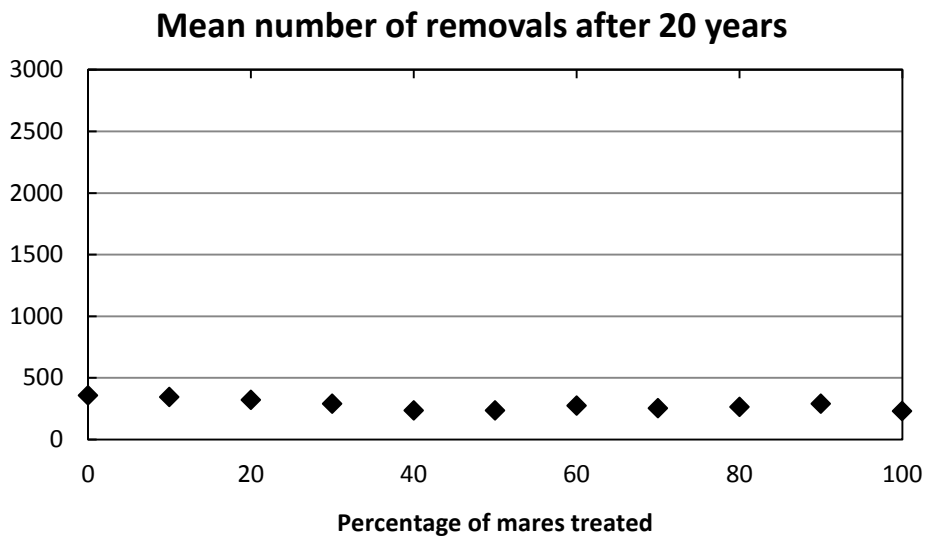
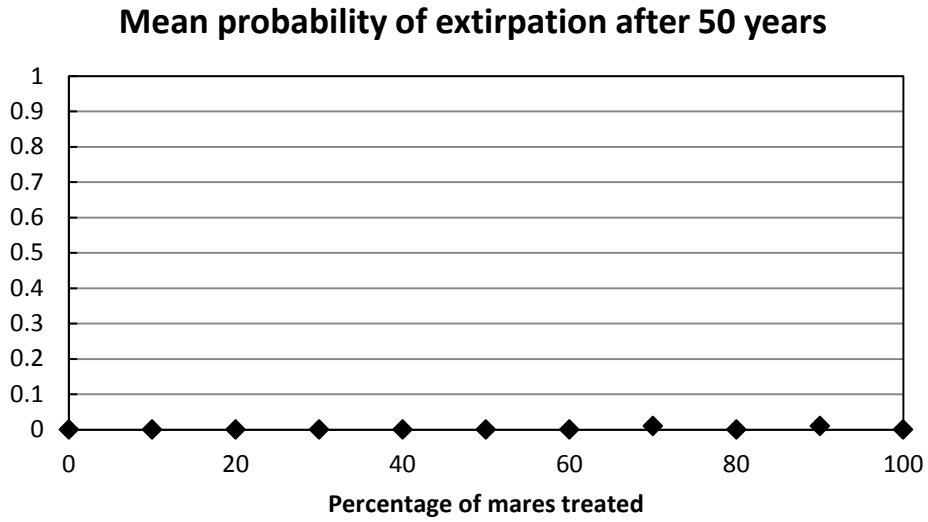


Figure 50. Initial population size = 500, lambda = 1.25, genetic diversity = high.—Continued







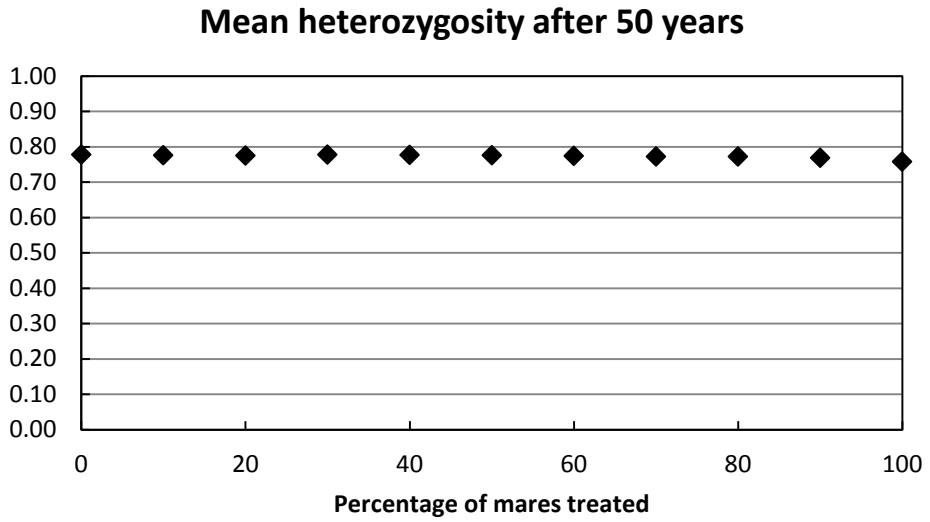


Figure 51. Initial population size = 750, lambda = 1.05, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	662.85	602.11	594.21	609.04	573.37	563.41	531.5	490.61	478.09	432.79	467.39
Mean number of removals after 20 years	357.45	343.82	320.45	290.07	234.09	234	273.83	253.99	263.92	289.45	228.35
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0.01	0	0.01	0
Mean heterozygosity after 50 years	0.78	0.78	0.77	0.78	0.78	0.78	0.77	0.77	0.77	0.77	0.76

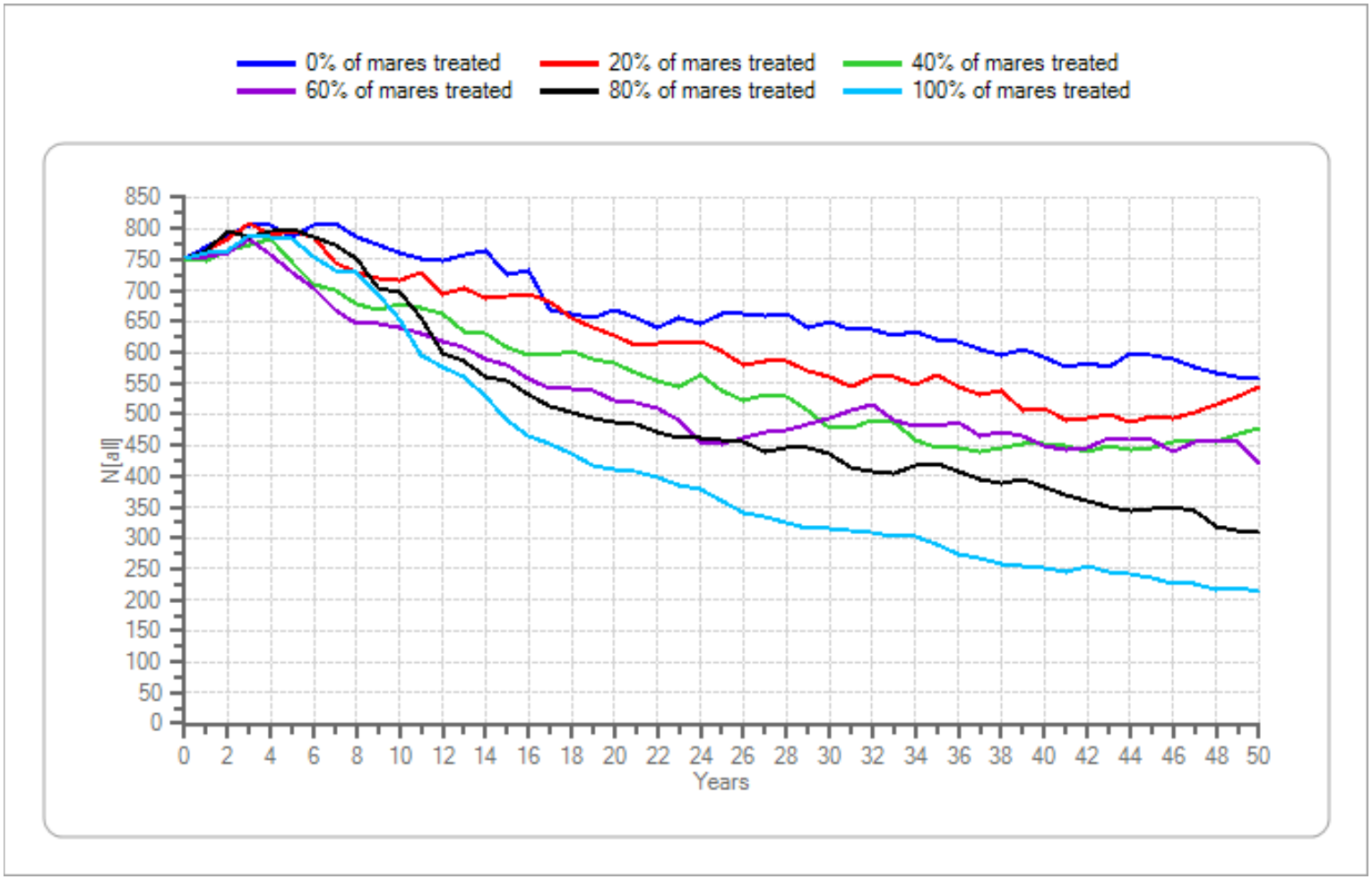
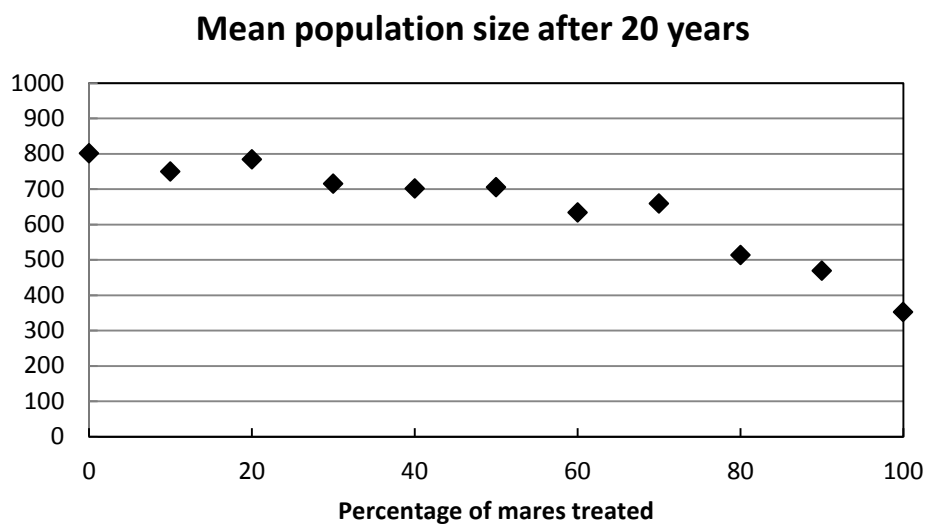
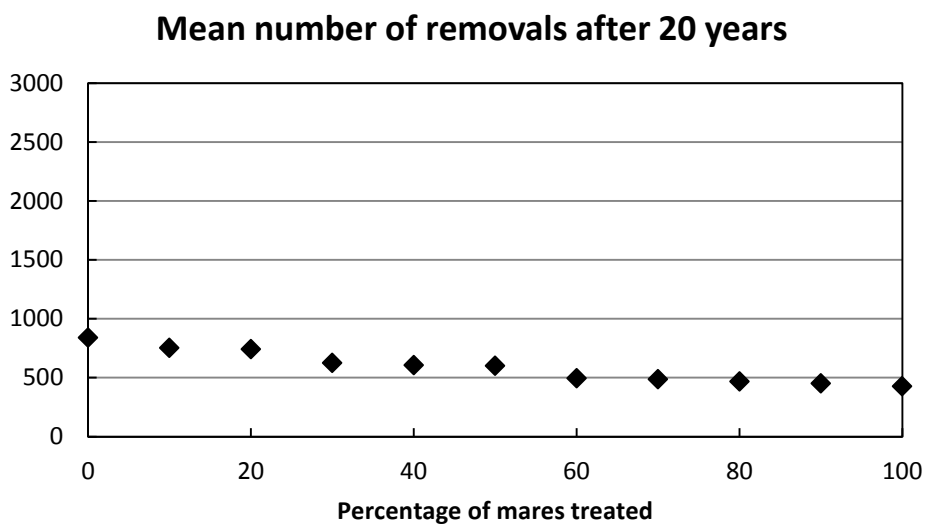
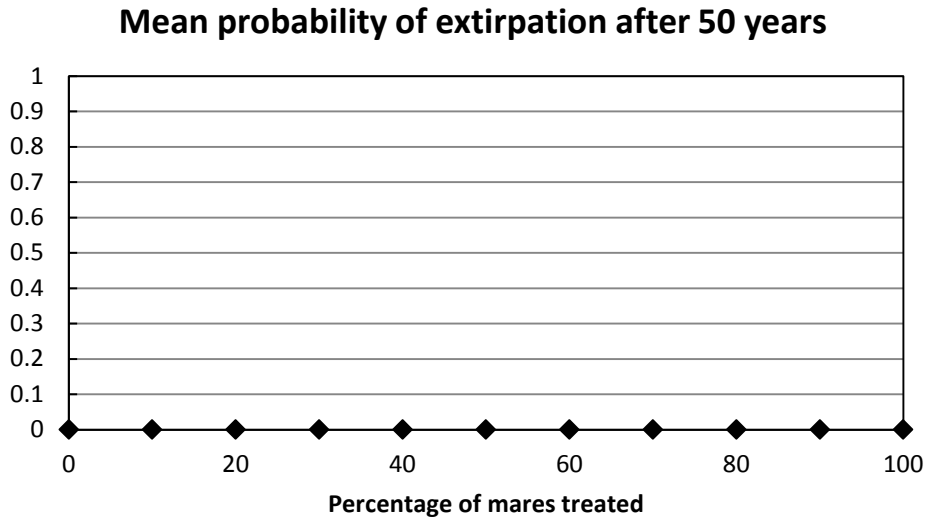


Figure 51. Initial population size = 750, lambda = 1.05, genetic diversity = high.—Continued







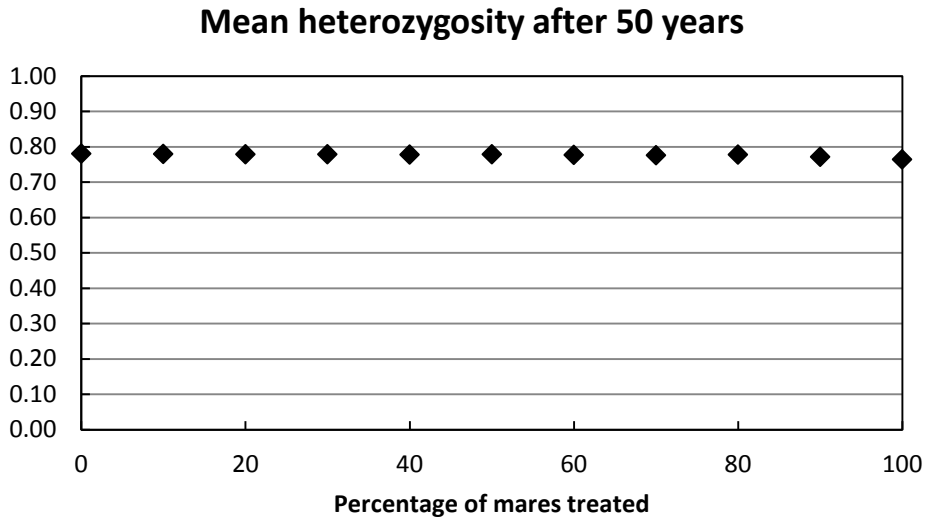


Figure 52. Initial population size = 750, lambda = 1.10, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	800.78	749.94	784.34	714.78	701.29	705.25	633.52	658.99	513.24	468.63	351.92
Mean number of removals after 20 years	838.9	753.64	742.14	625.1	605.75	599.52	493.98	486.5	466.16	451.41	427.27
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77	0.76

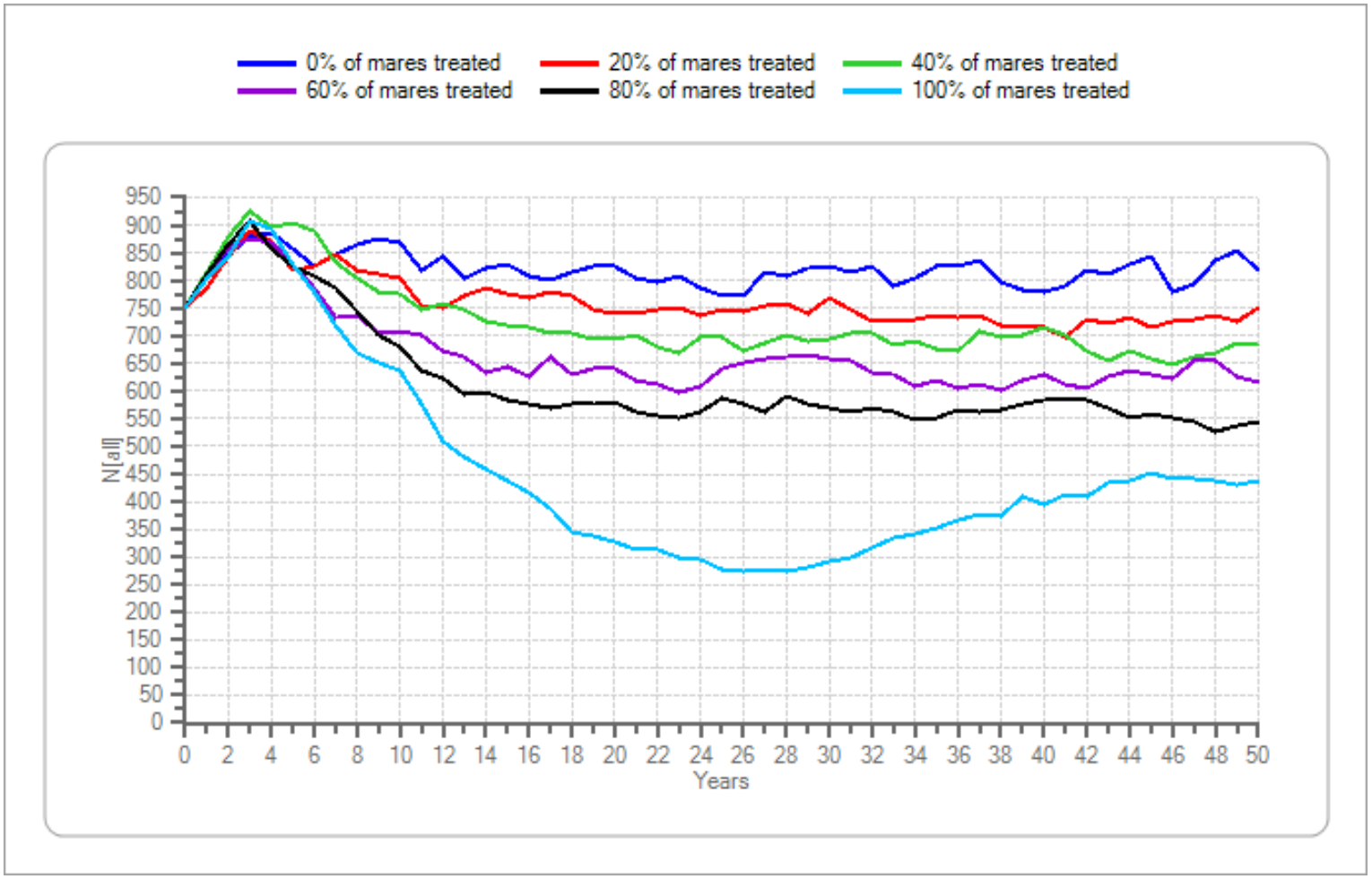
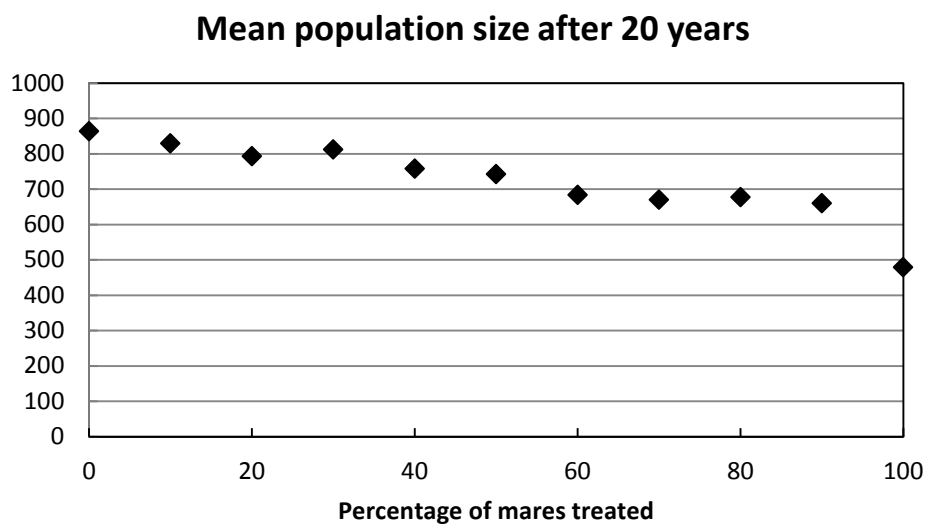
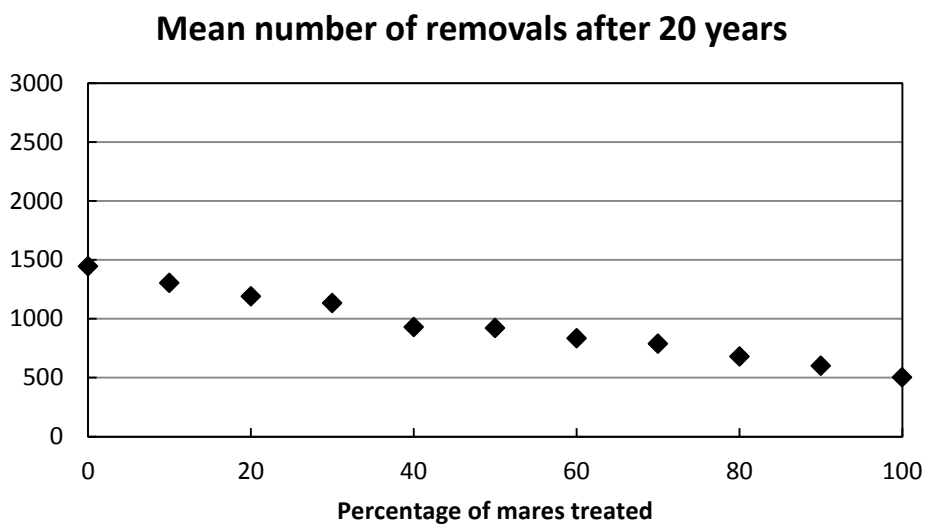
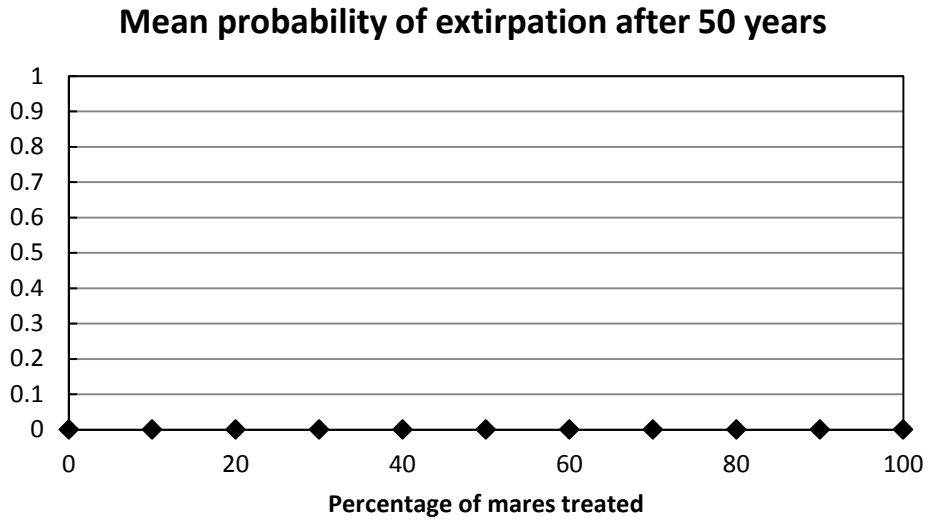


Figure 52. Initial population size = 750, lambda = 1.10, genetic diversity = high.—Continued







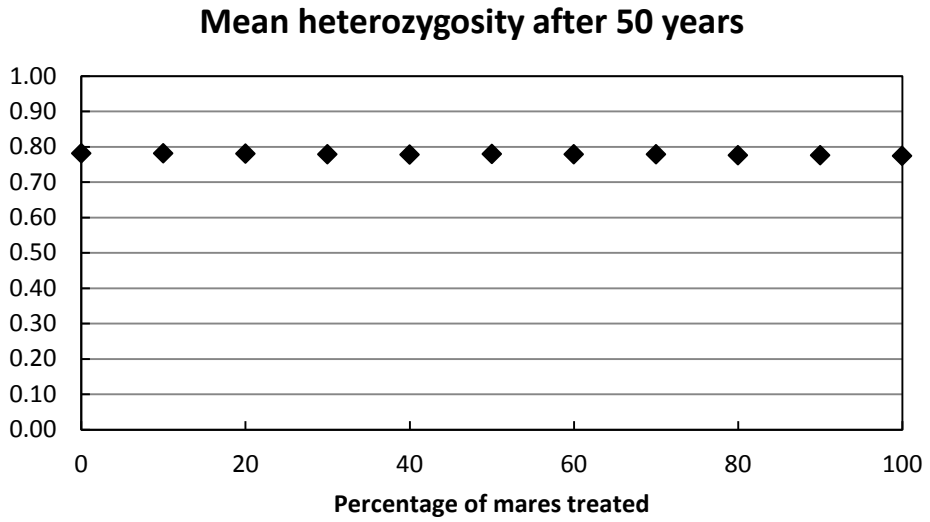


Figure 53. Initial population size = 750, lambda = 1.15, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	863.26	828.87	793.09	812.32	758.17	742.73	683.78	670.07	677.22	660.32	478.72
Mean number of removals after 20 years	1444.8	1304.1	1189.2	1132.7	929.9	920.5	834.1	788.1	679.1	599.5	501.4
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77

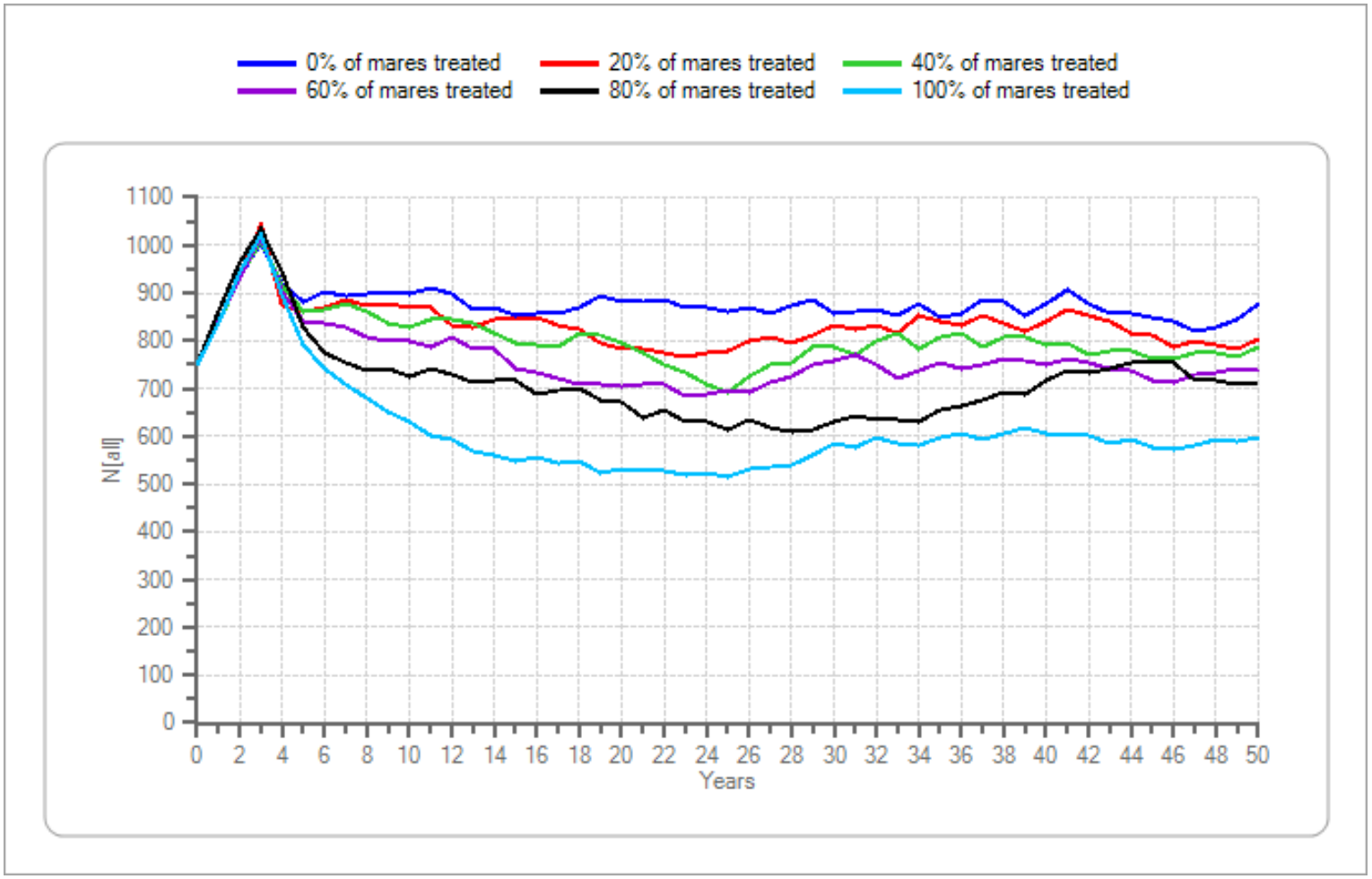


Figure 53. Initial population size = 750, lambda = 1.15, genetic diversity = high.—Continued

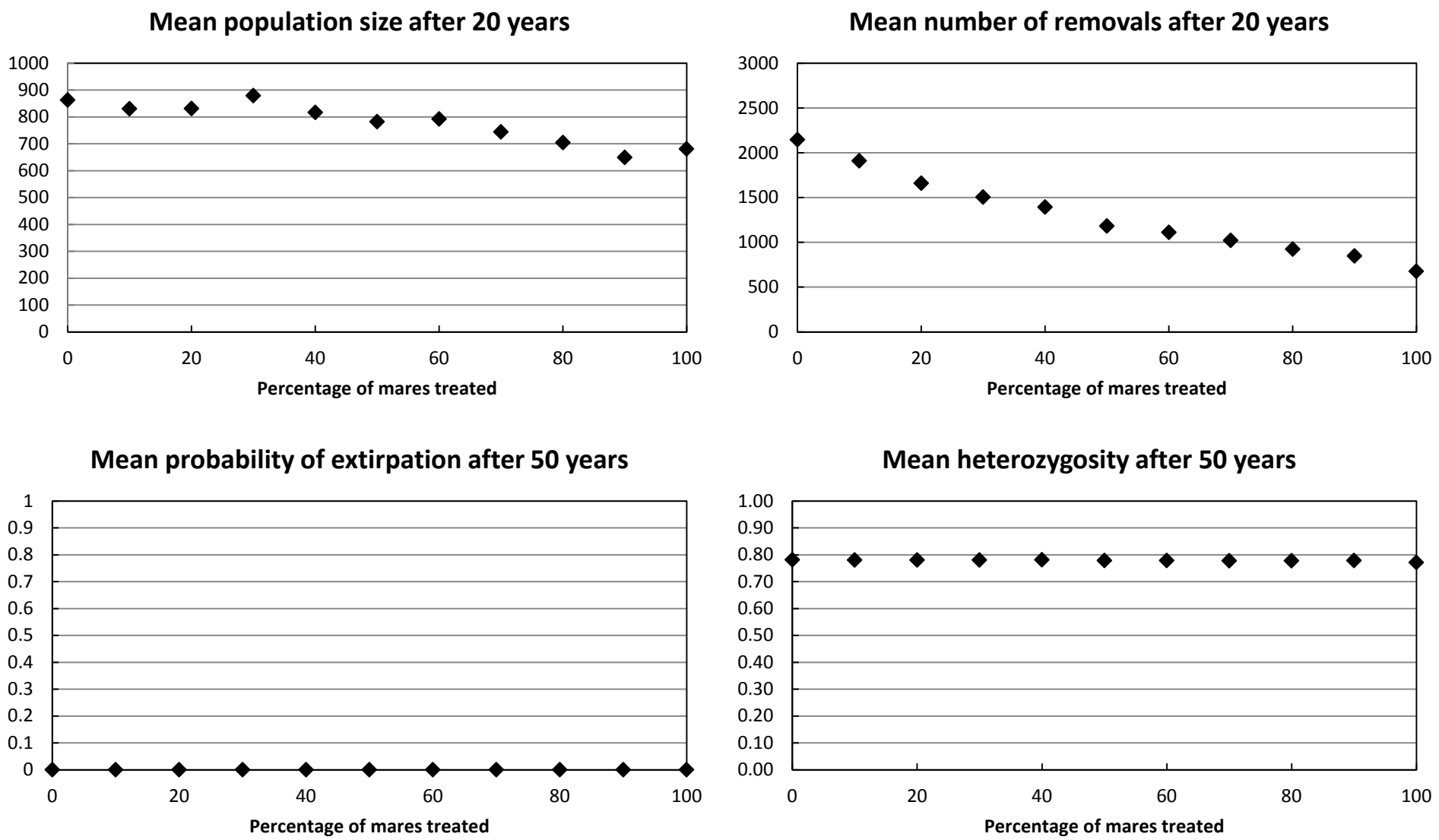


Figure 54. Initial population size = 750, lambda = 1.20, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	862.48	830.01	830.72	878.81	816.6	782.19	791.89	743.94	704.05	649.05	680.91
Mean number of removals after 20 years	2146.1	1909.2	1659.2	1506.0	1394.6	1181.1	1109.7	1021.6	923.1	847.1	675.6
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77

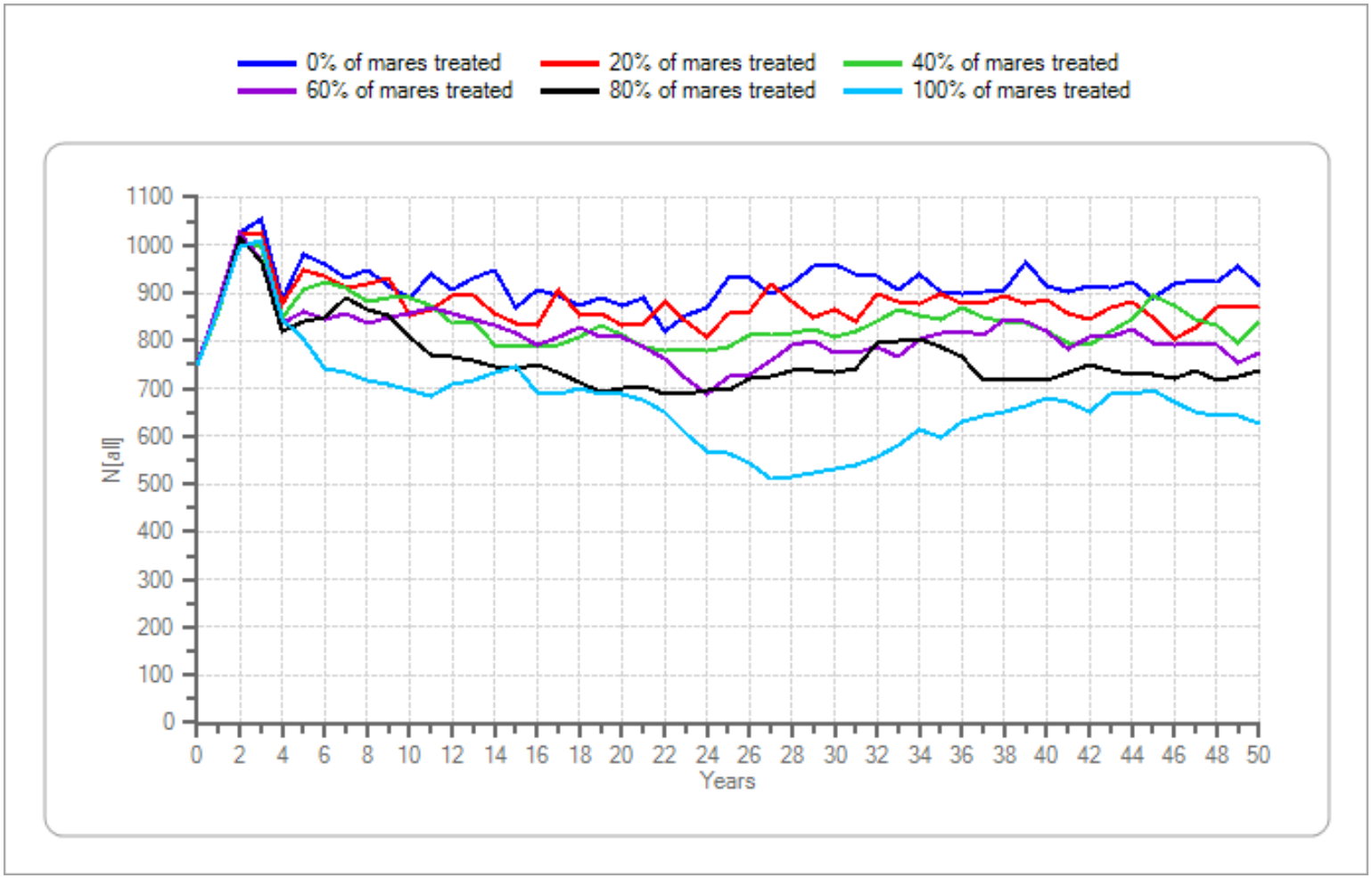


Figure 54. Initial population size = 750, lambda = 1.20, genetic diversity = high.—Continued

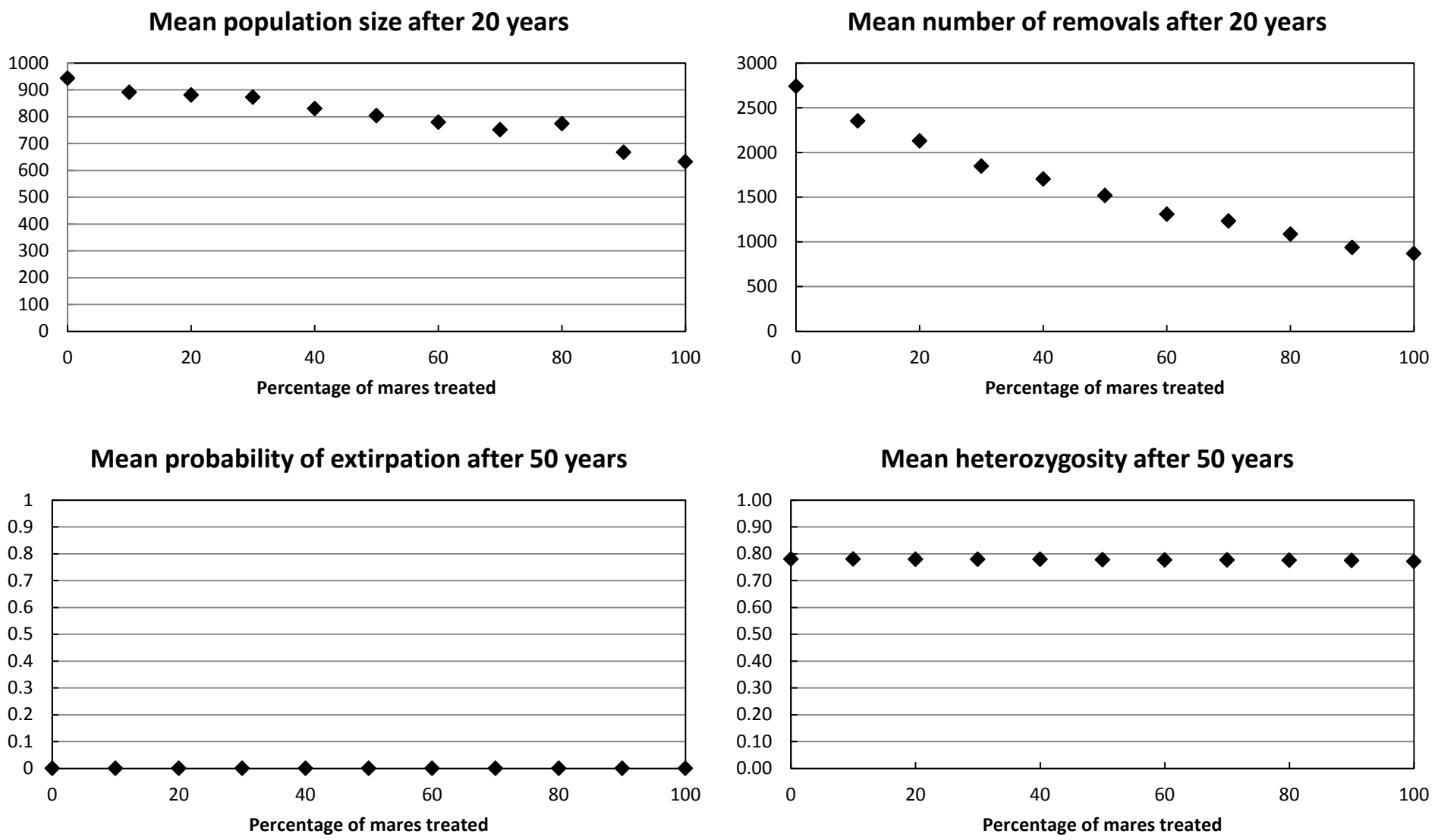


Figure 55. Initial population size = 750, lambda = 1.25, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	943.03	890.54	880.47	872.85	830.01	804.21	779.9	751.3	773.8	666.84	631.64
Mean number of removals after 20 years	2741.1	2352.9	2128.9	1845.6	1703.7	1518.4	1309.1	1234.3	1086.5	937.0	868.1
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77	0.77

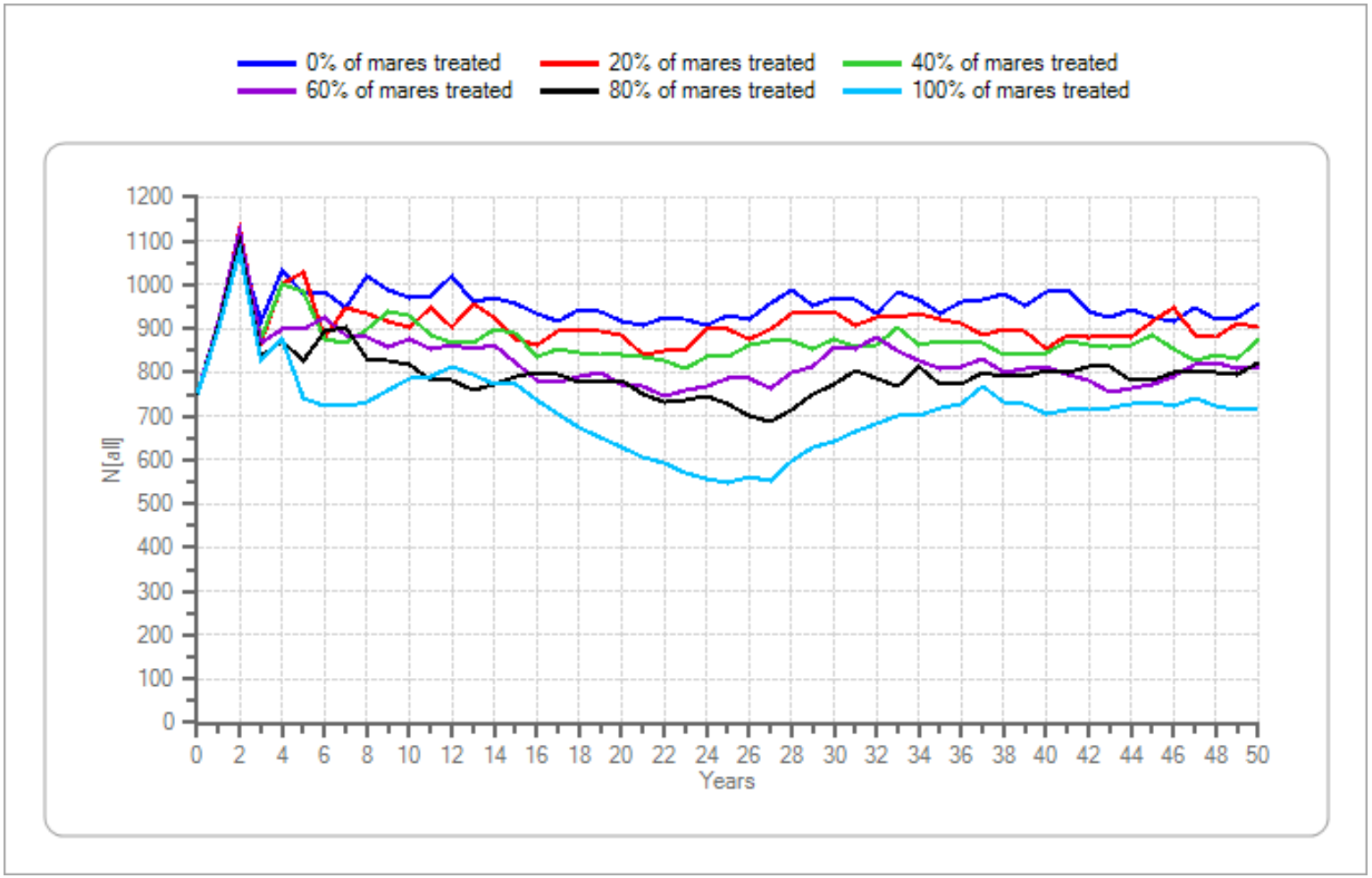
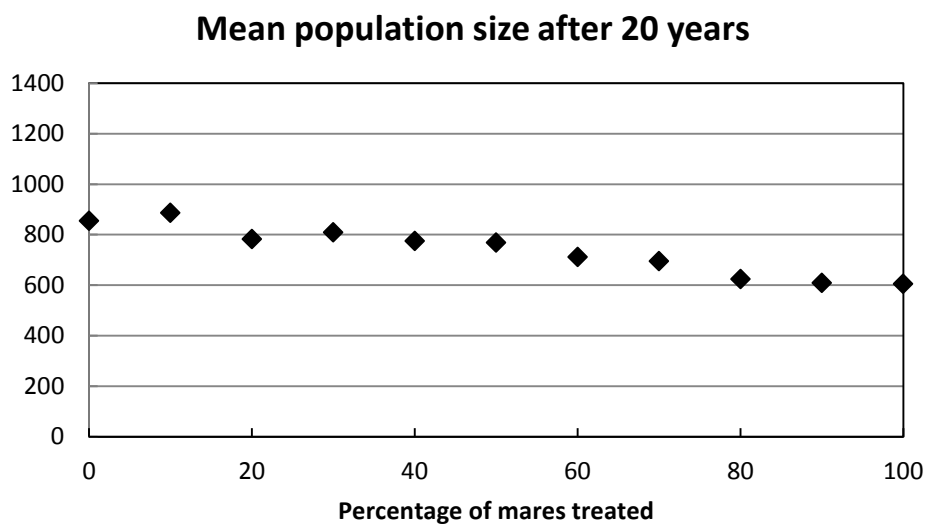
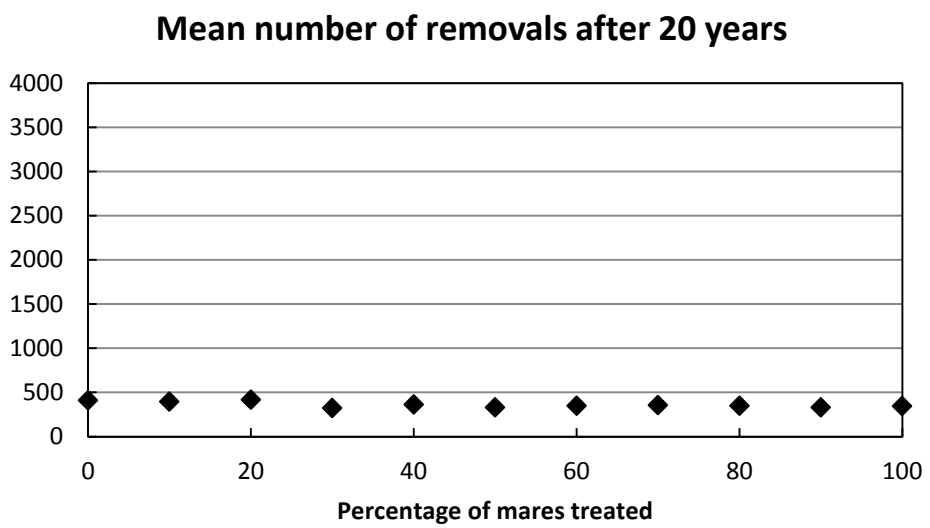
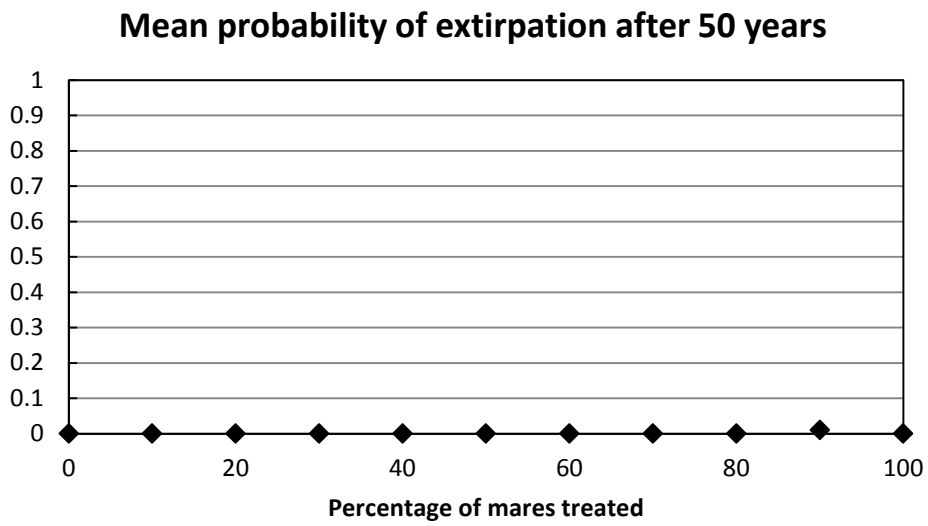


Figure 55. Initial population size = 750, lambda = 1.25, genetic diversity = high.—Continued







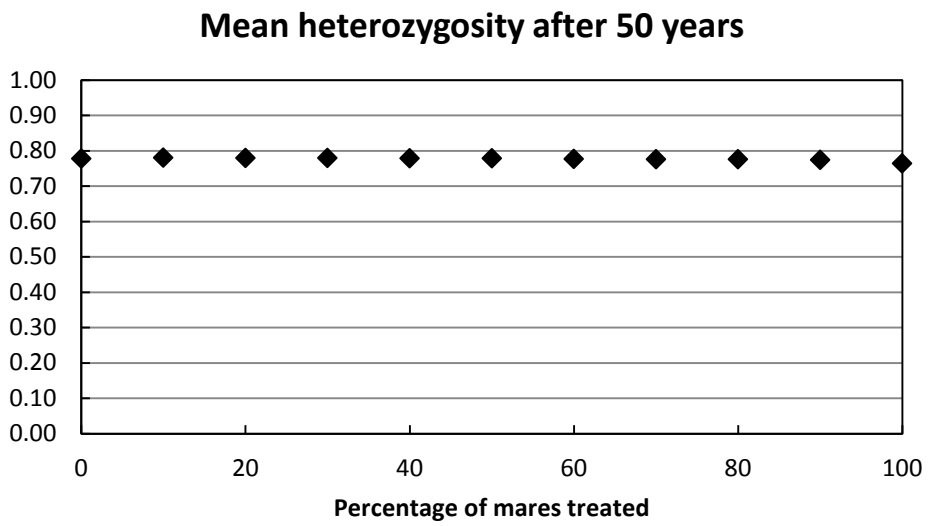


Figure 56. Initial population size = 1000, lambda = 1.05, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	854.42	886.33	782.46	808.7	774.68	767.58	711.38	694.91	623.41	608.7	604
Mean number of removals after 20 years	408.46	393.28	416.19	321.57	362.43	331.22	346.92	353.96	346.09	328.18	344.26
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0.01	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77	0.76

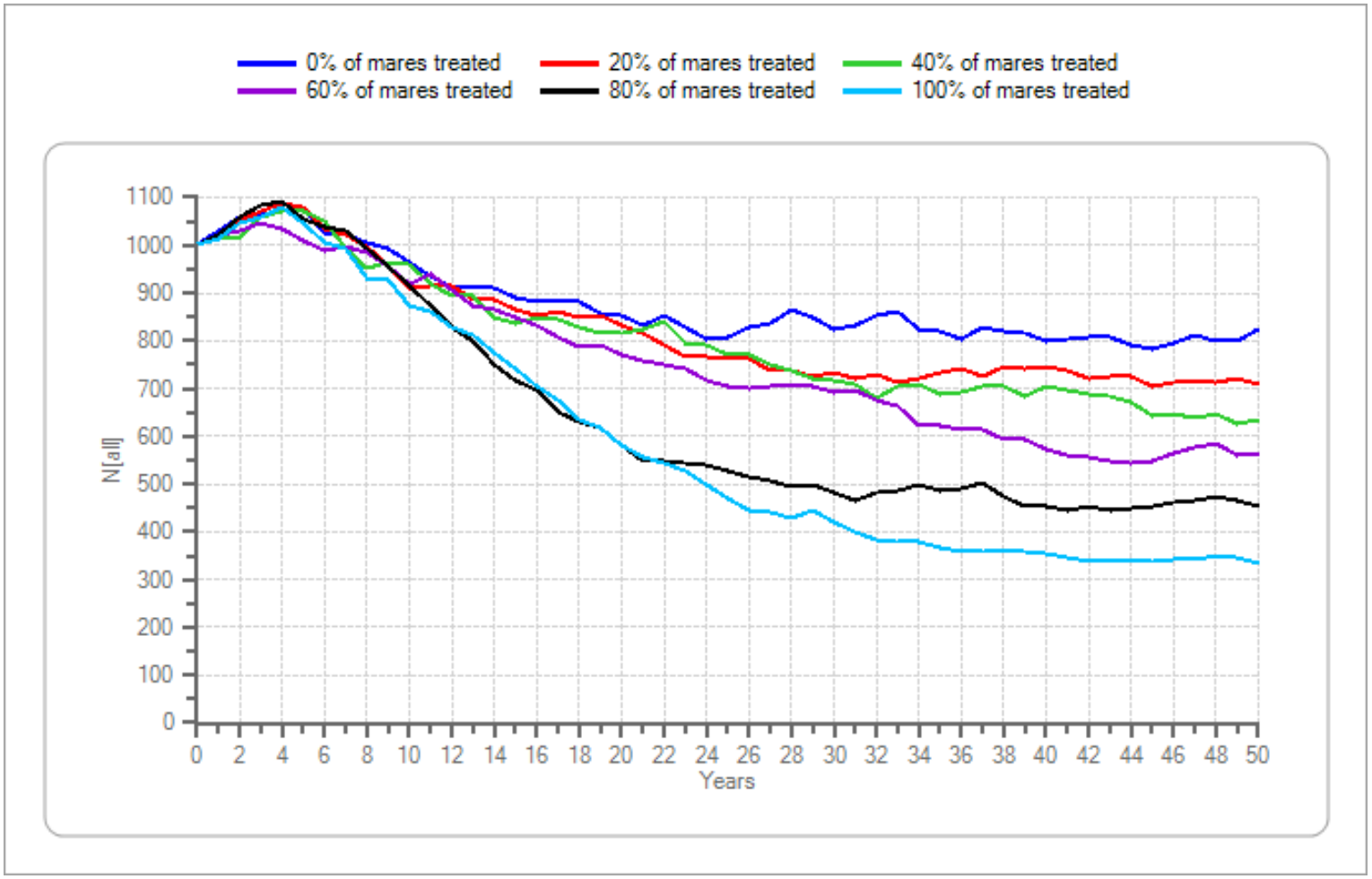
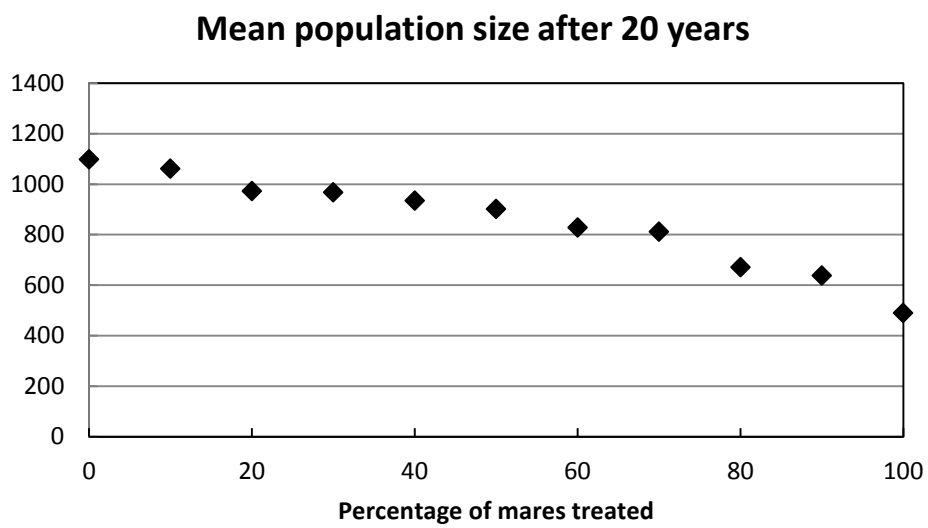
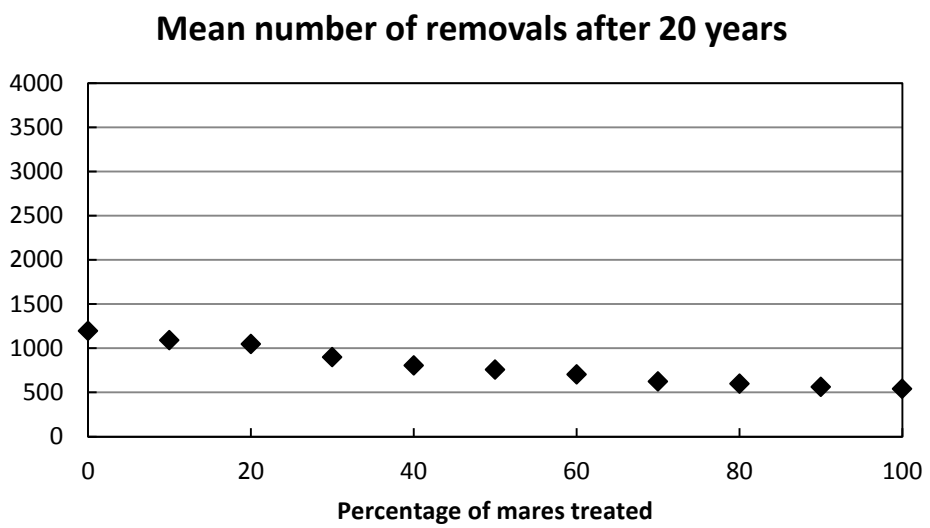
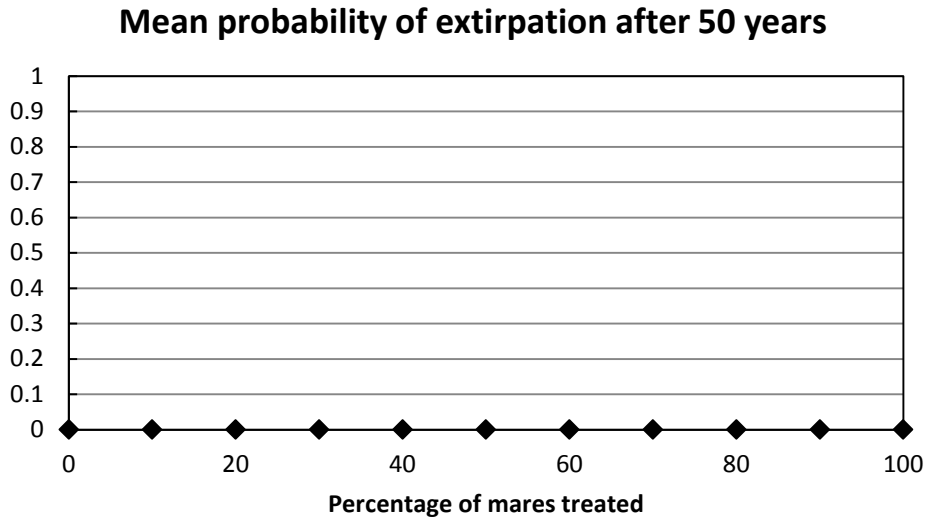


Figure 56. Initial population size = 1000, lambda = 1.05, genetic diversity = high.—Continued







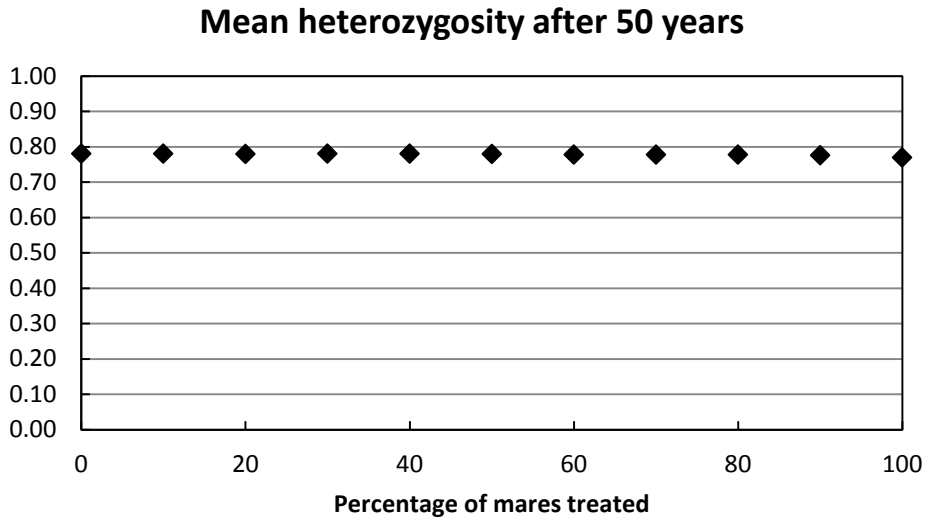


Figure 57. Initial population size = 1000, lambda = 1.10, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1098.09	1061.14	972.15	967.57	933.76	901.35	828.11	810.67	669.9	638.13	489.1
Mean number of removals after 20 years	1196.9	1091.1	1048.1	899.6	804.5	755.5	702.3	621.7	597.8	560.3	541.2
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77

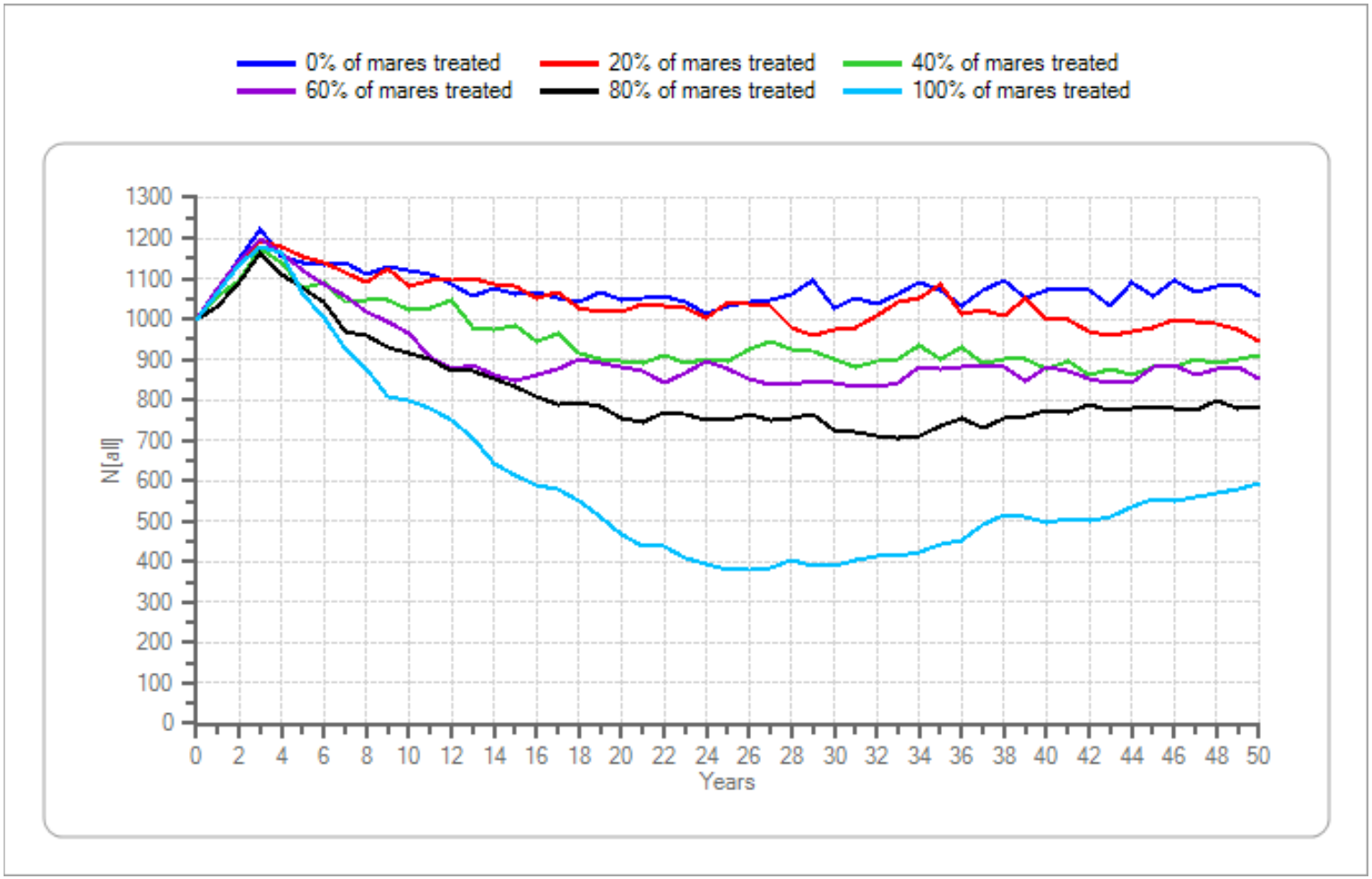
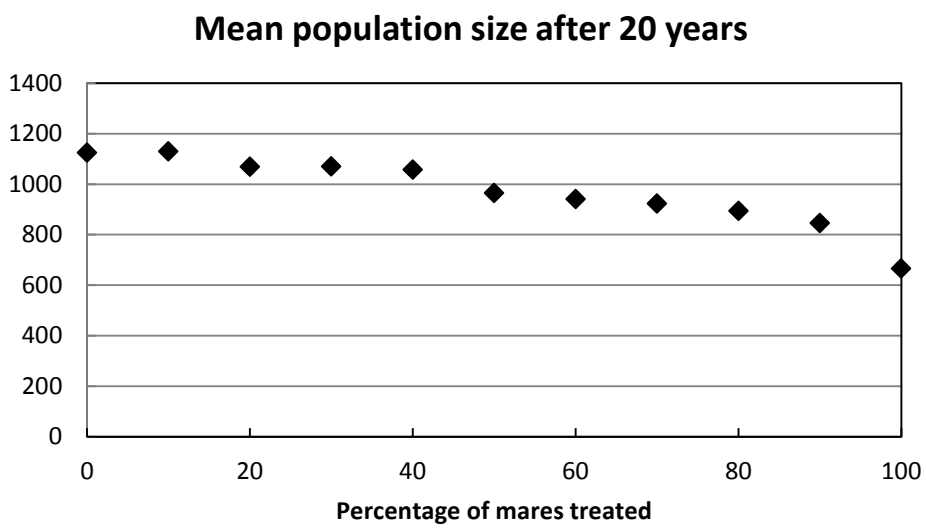
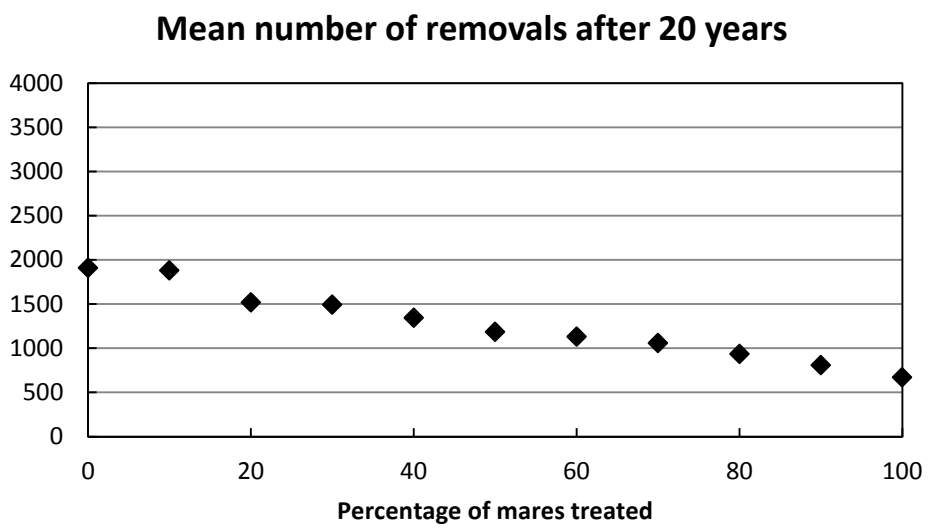
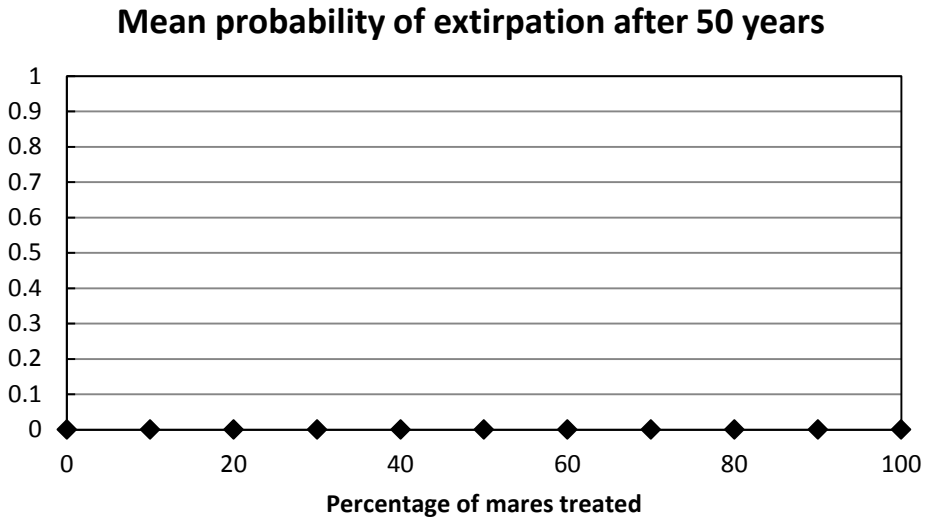


Figure 57. Initial population size = 1000, lambda = 1.10, genetic diversity = high.—Continued







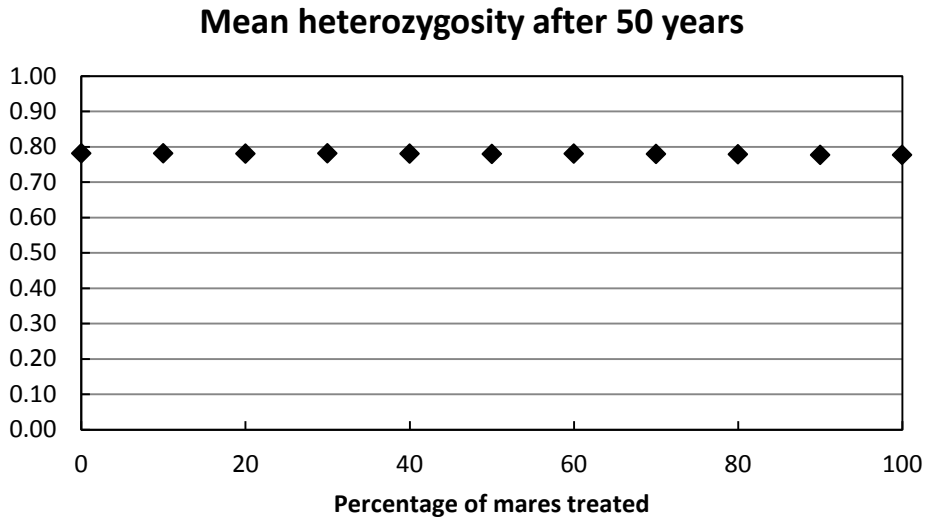


Figure 58. Initial population size = 1000, lambda = 1.15, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1124.15	1128.74	1069.1	1069.36	1056.52	964.86	940	922.21	893.25	845.4	665.68
Mean number of removals after 20 years	1907.4	1878.5	1517.7	1492.3	1343.0	1182.7	1130.2	1058.3	934.0	808.2	669.0
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

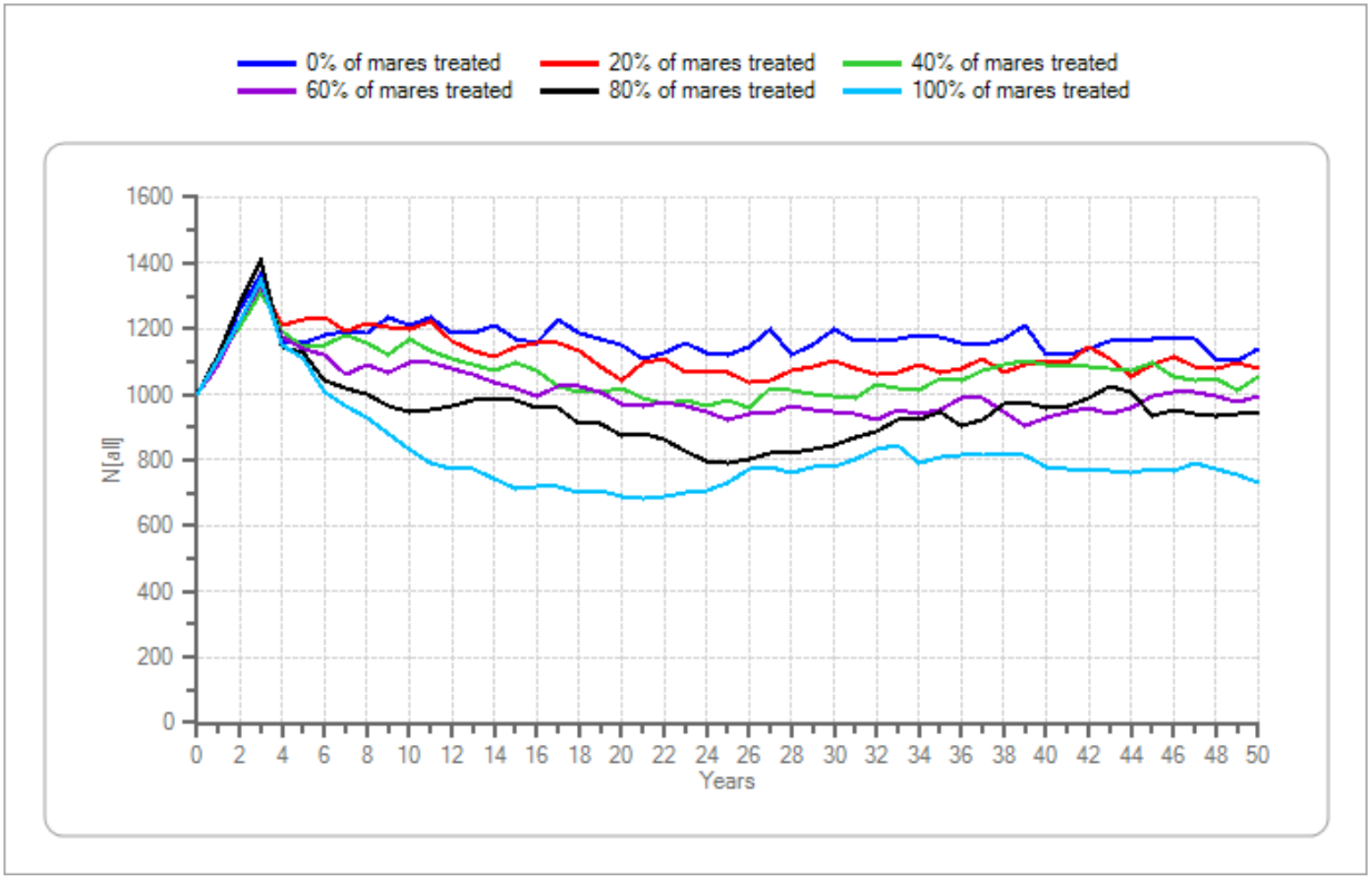
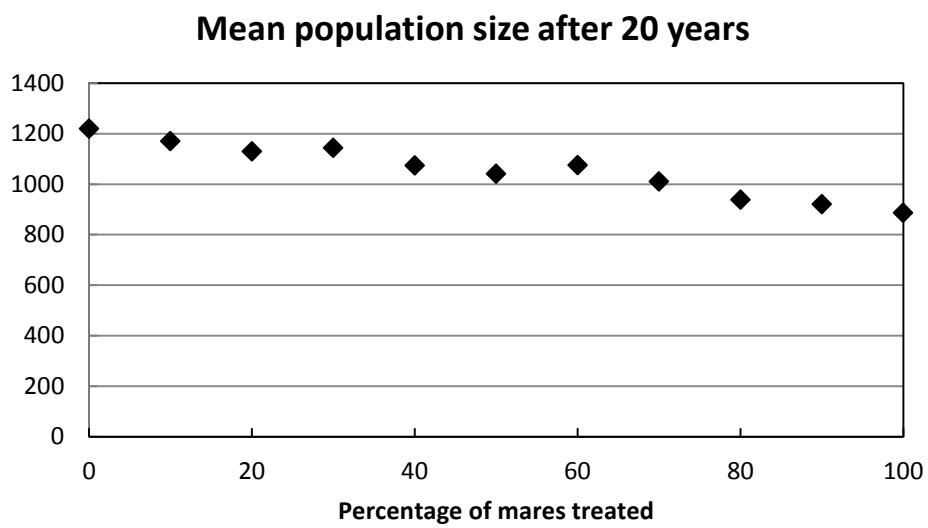
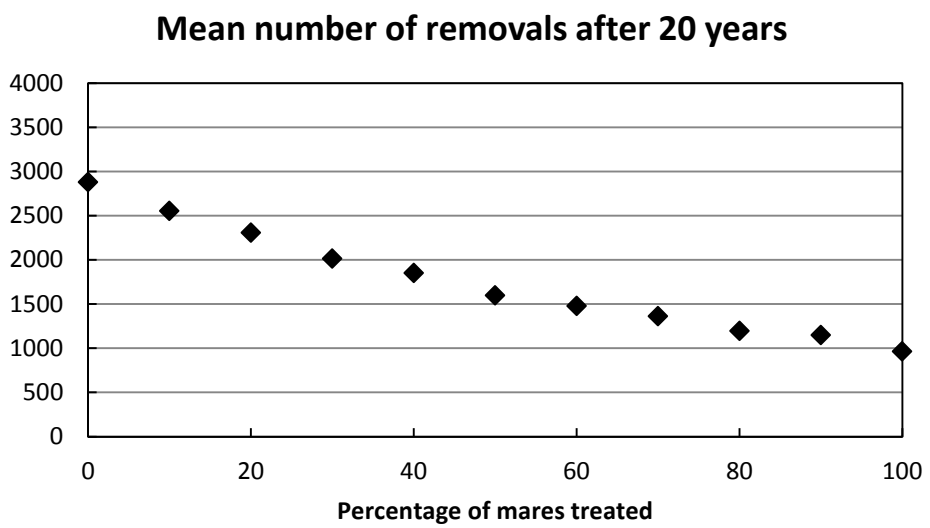
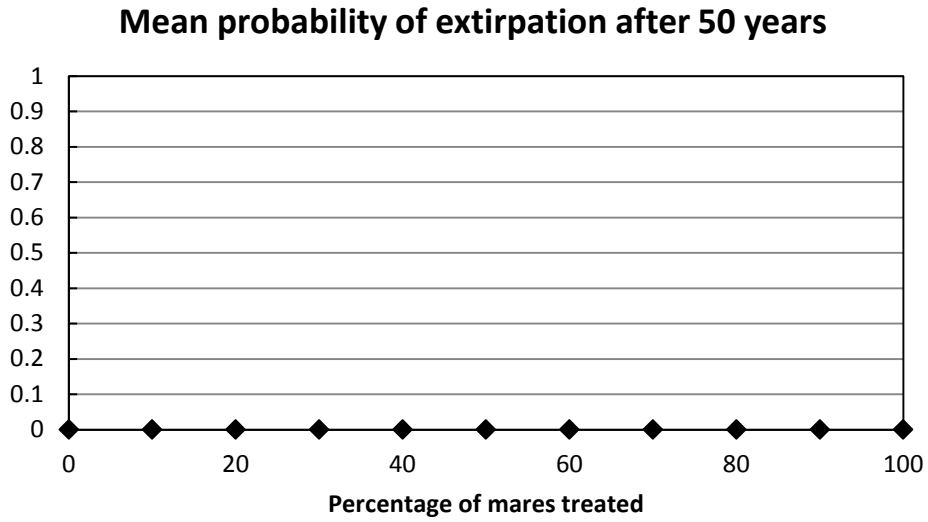


Figure 58. Initial population size = 1000, lambda = 1.15, genetic diversity = high.—Continued







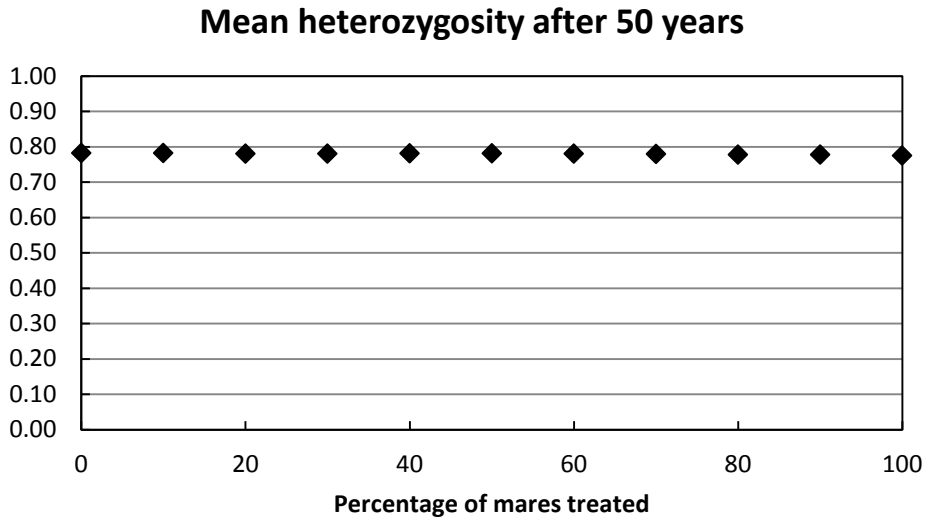


Figure 59. Initial population size = 1000, lambda = 1.20, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1219.7	1169.6	1129.0	1143.8	1073.6	1041.0	1075.2	1009.6	938.2	920.0	886.5
Mean number of removals after 20 years	2877.8	2551.9	2307.0	2013.1	1851.7	1597.8	1478.2	1362.8	1196.4	1149.4	963.0
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

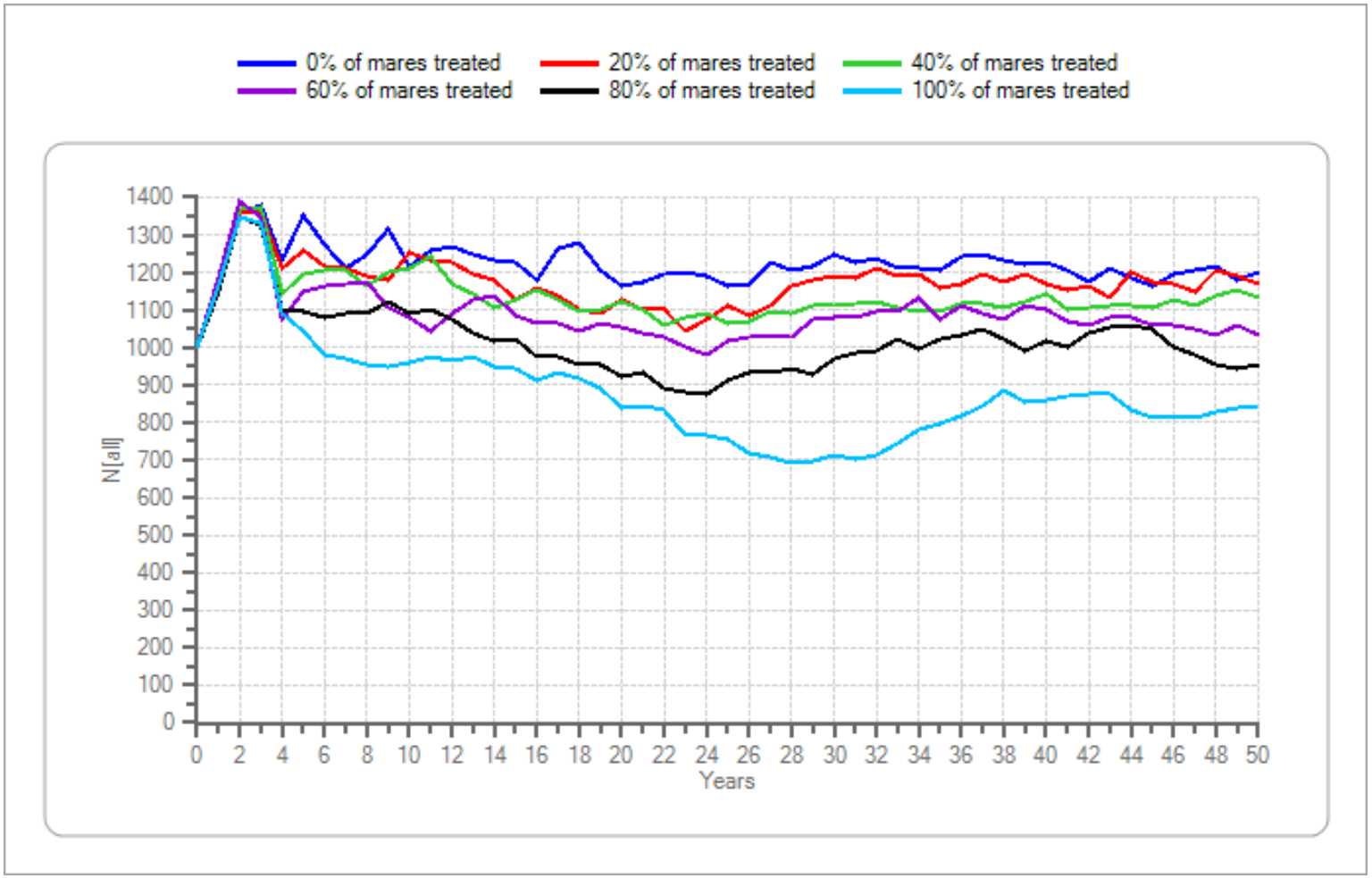
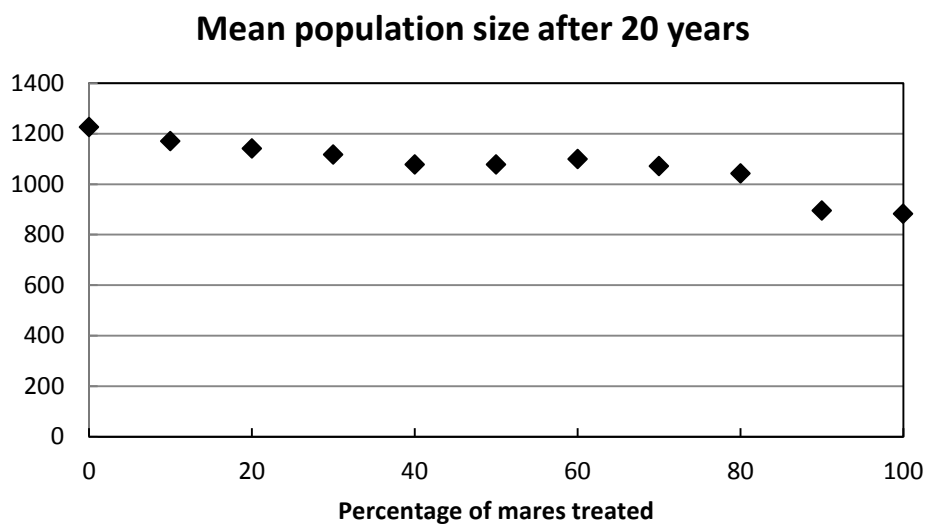
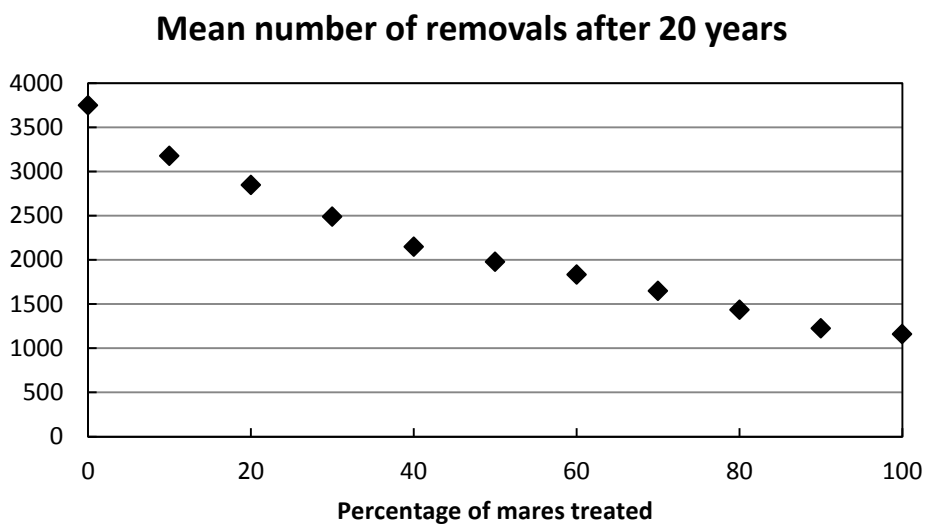
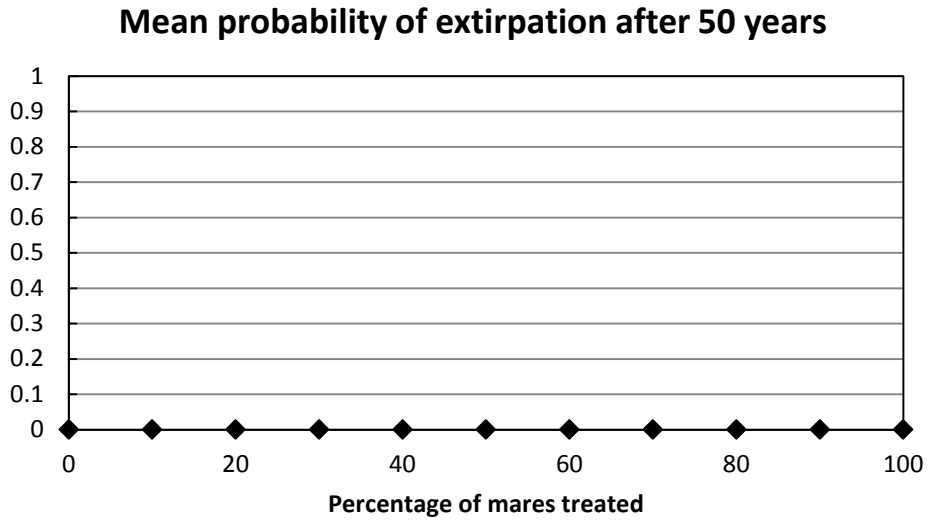


Figure 59. Initial population size = 1000, lambda = 1.20, genetic diversity = high.—Continued







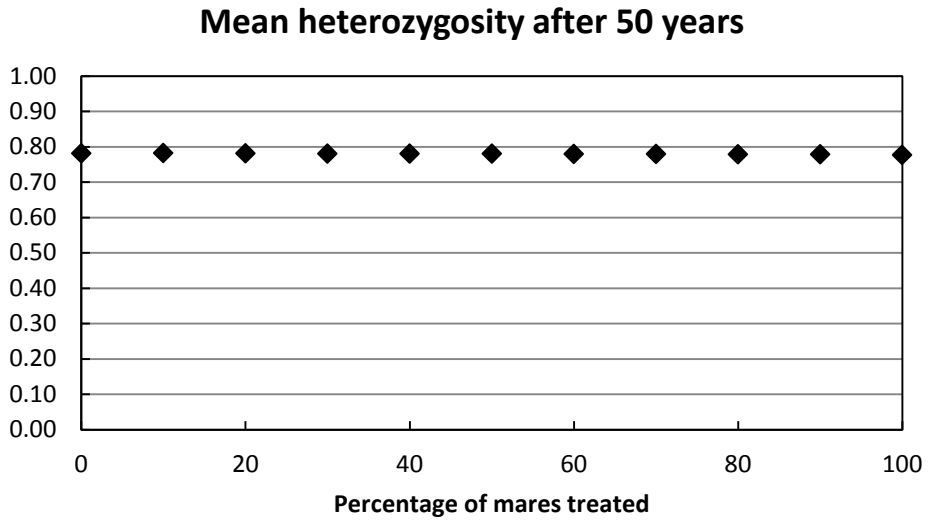


Figure 60. Initial population size = 1000, lambda = 1.25, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1225.4	1169.7	1140.7	1117.1	1077.6	1076.7	1098.6	1071.1	1041.7	894.4	882.7
Mean number of removals after 20 years	3749.6	3175.7	2844.7	2489.0	2147.9	1978.4	1831.9	1649.2	1432.6	1223.8	1159.1
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

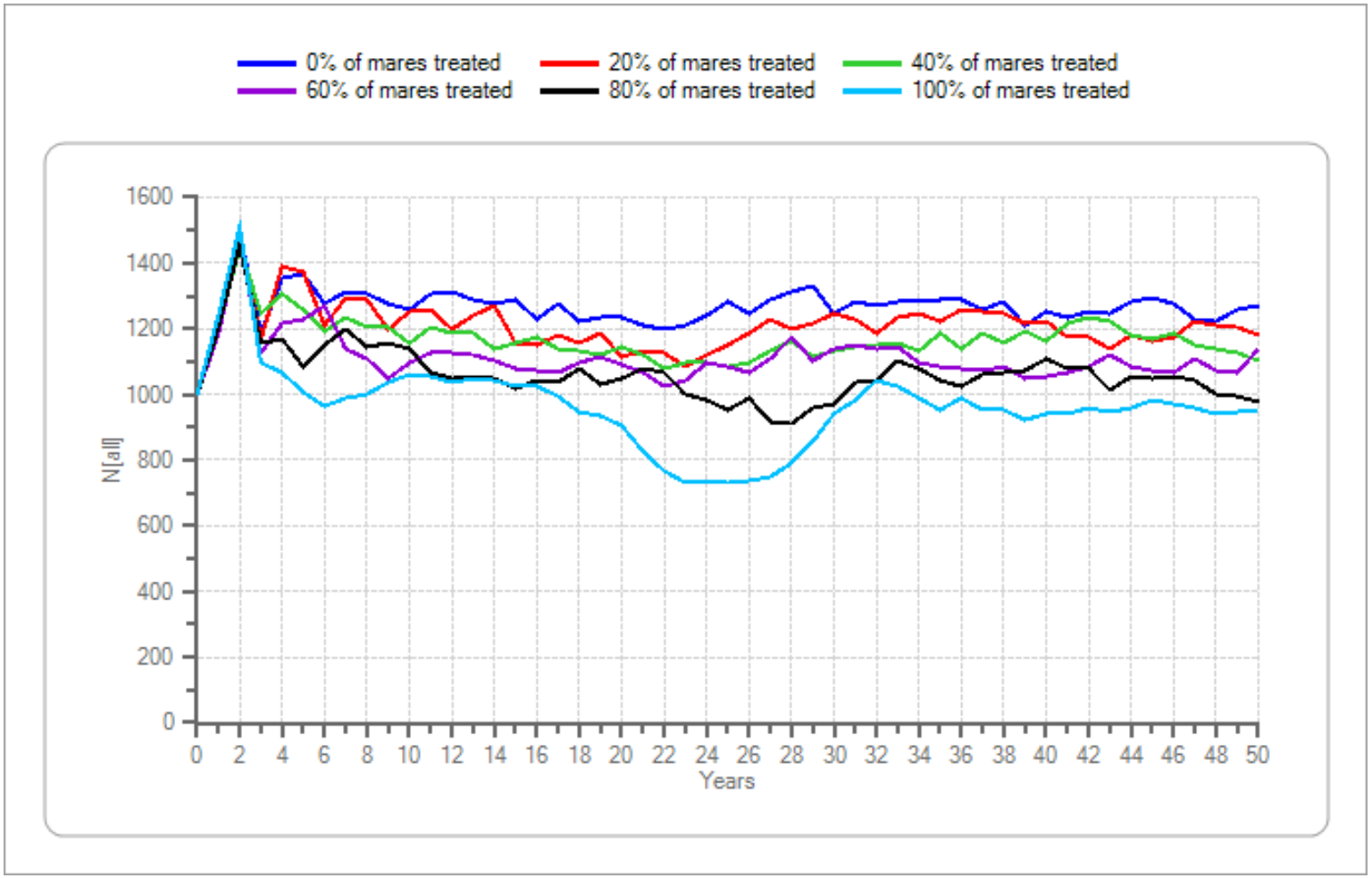
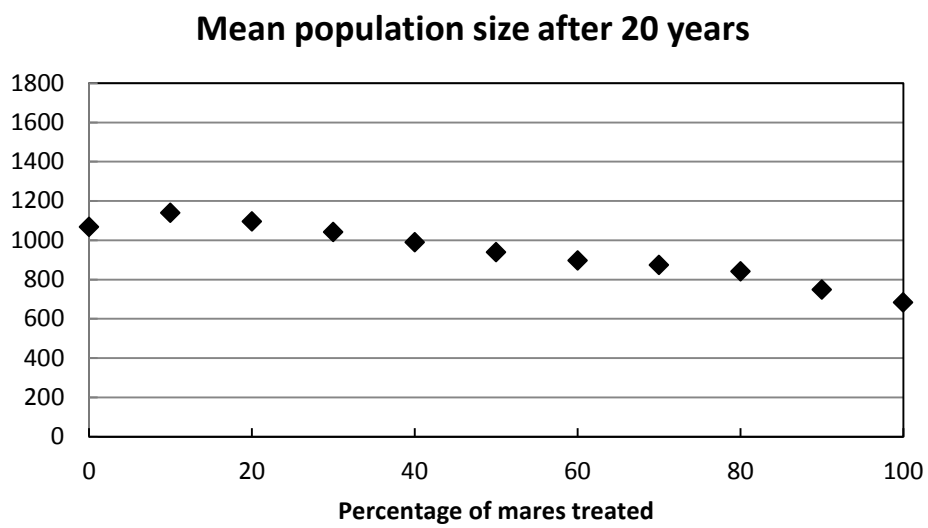
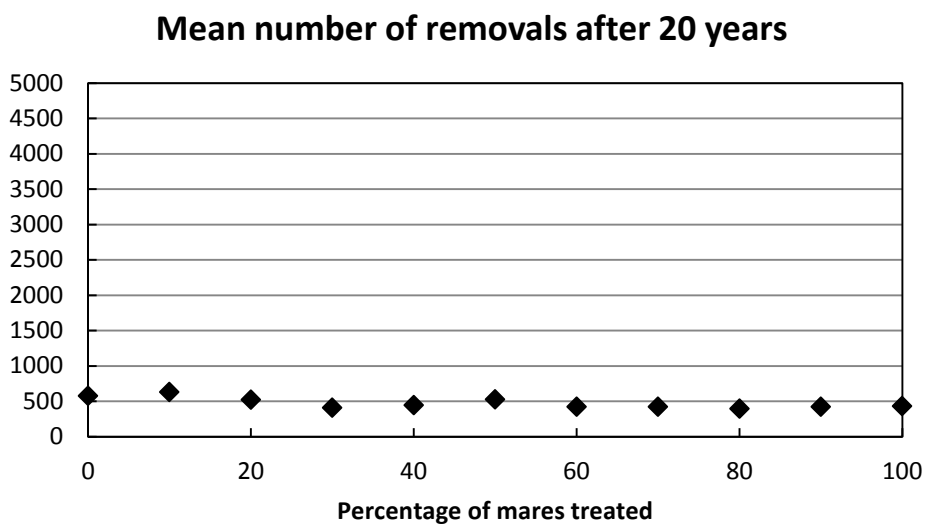
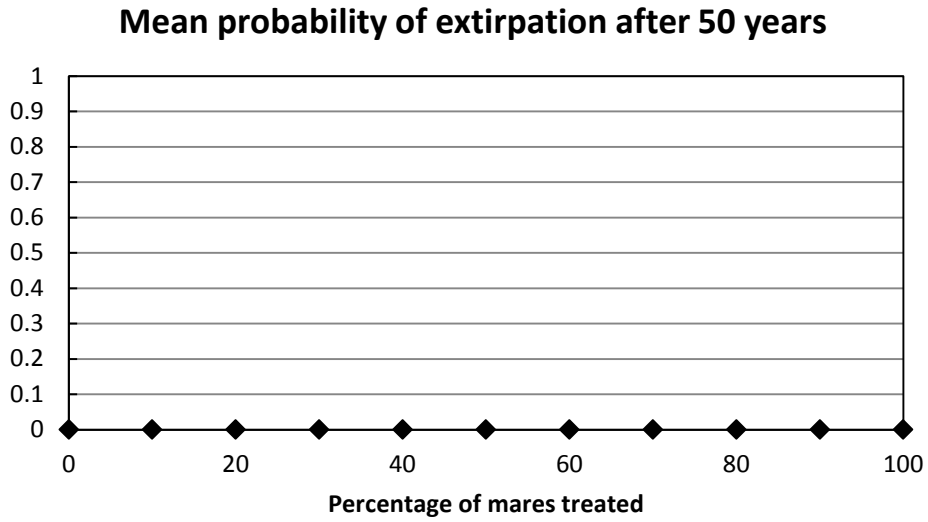


Figure 60. Initial population size = 1000, lambda = 1.25, genetic diversity = high.—Continued







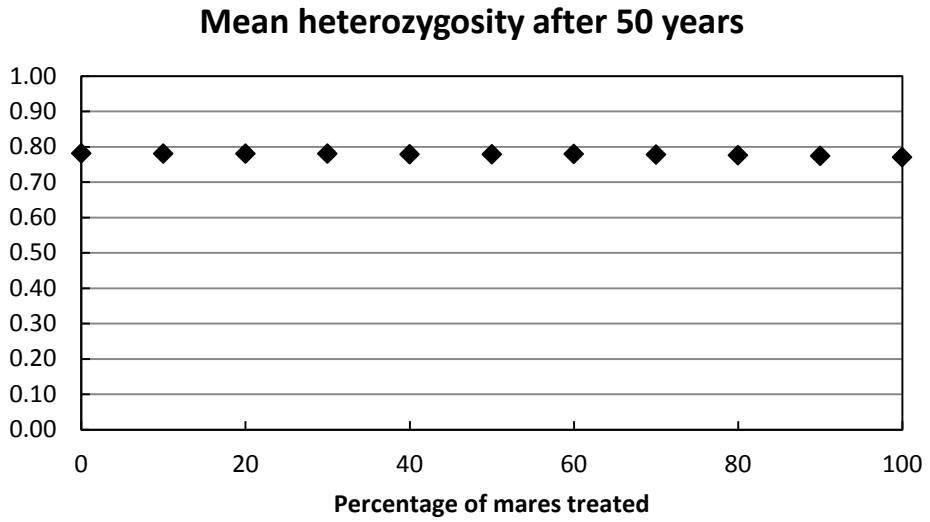


Figure 61. Initial population size = 1250, lambda = 1.05, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1066.7	1139.5	1095.1	1041.0	989.2	938.4	896.1	874.0	841.0	747.5	683.3
Mean number of removals after 20 years	575.5	629.3	519.5	406.3	442.6	524.2	422.0	420.3	393.0	422.2	428.9
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77	0.77

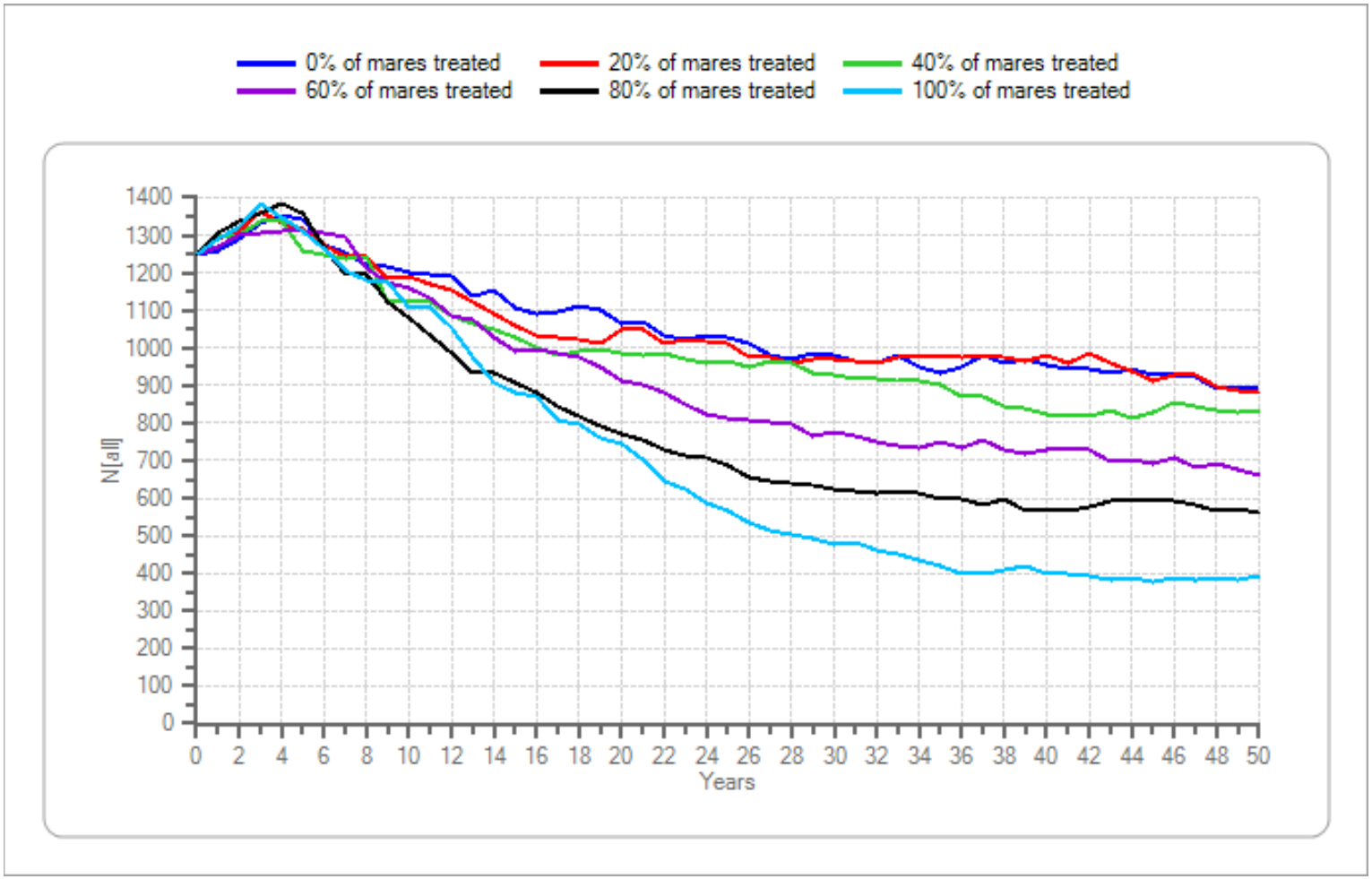
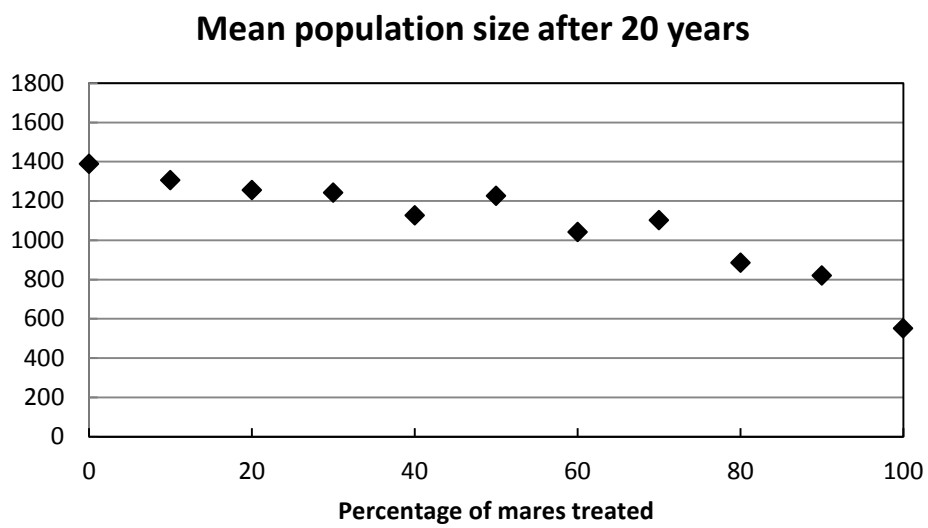
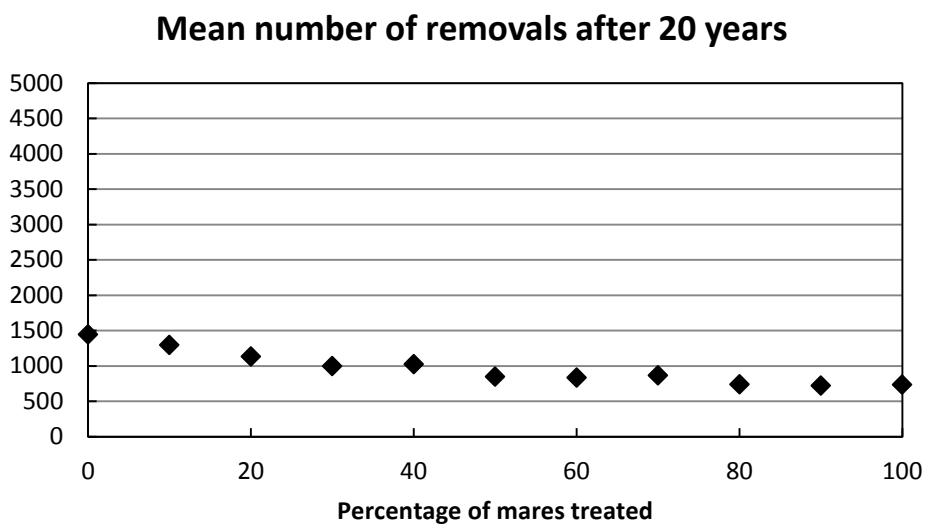
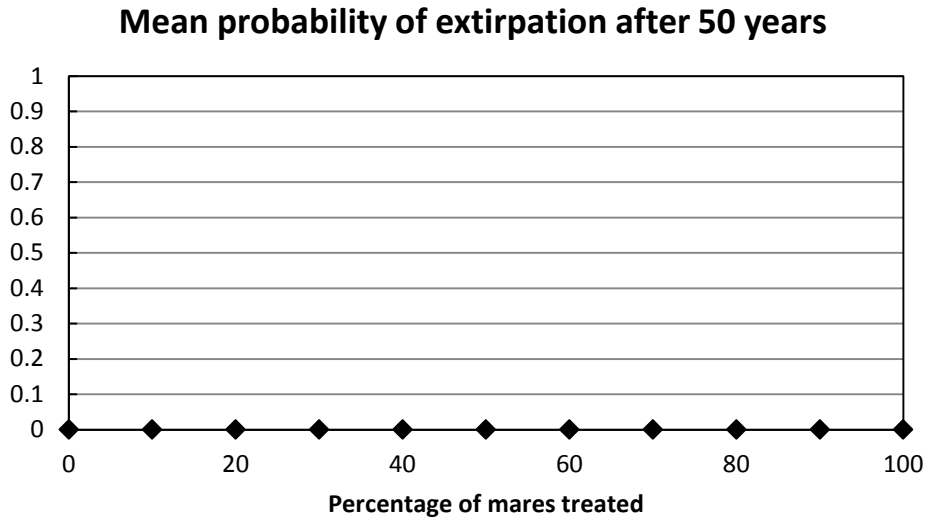


Figure 61. Initial population size = 1250, lambda = 1.05, genetic diversity = high.—Continued







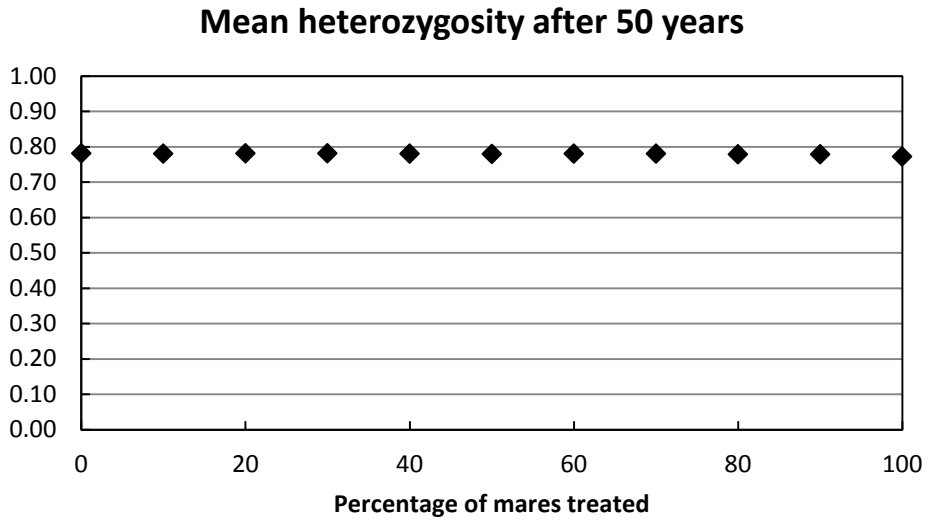


Figure 62. Initial population size = 1250, lambda = 1.10, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1388.7	1304.7	1254.2	1242.3	1126.8	1225.7	1041.3	1101.1	885.0	820.1	551.3
Mean number of removals after 20 years	1442.2	1296.3	1129.6	995.3	1024.7	845.1	834.5	866.0	738.2	721.3	735.6
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77

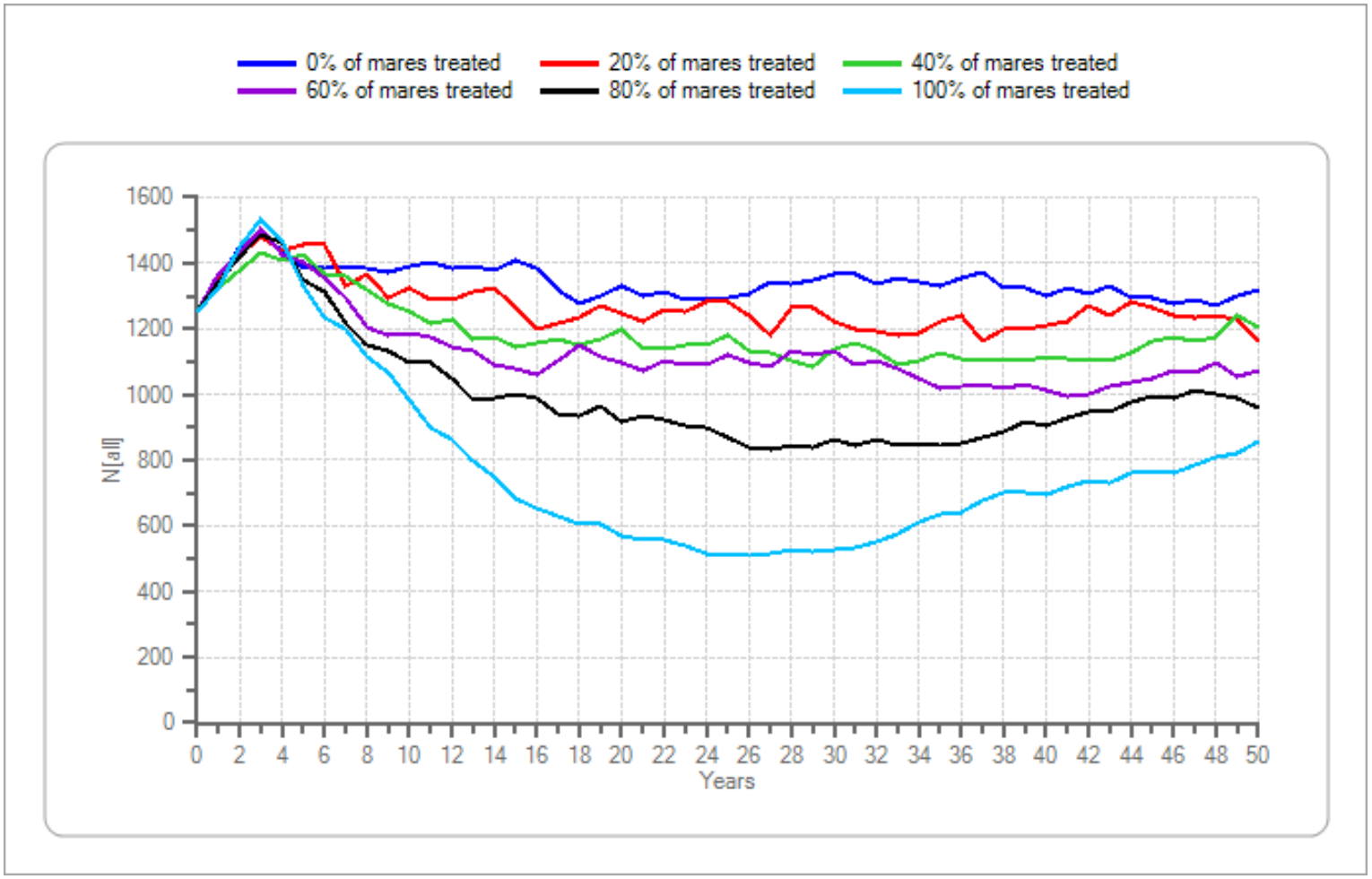
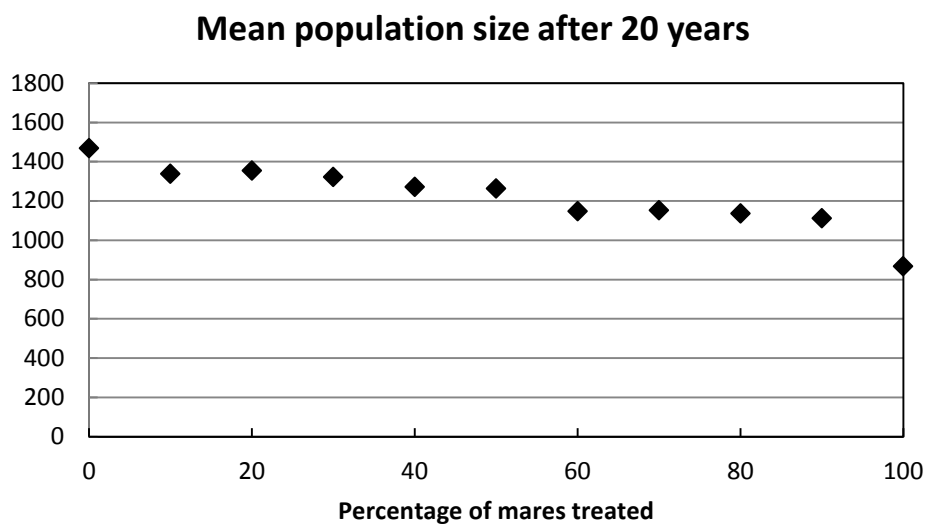
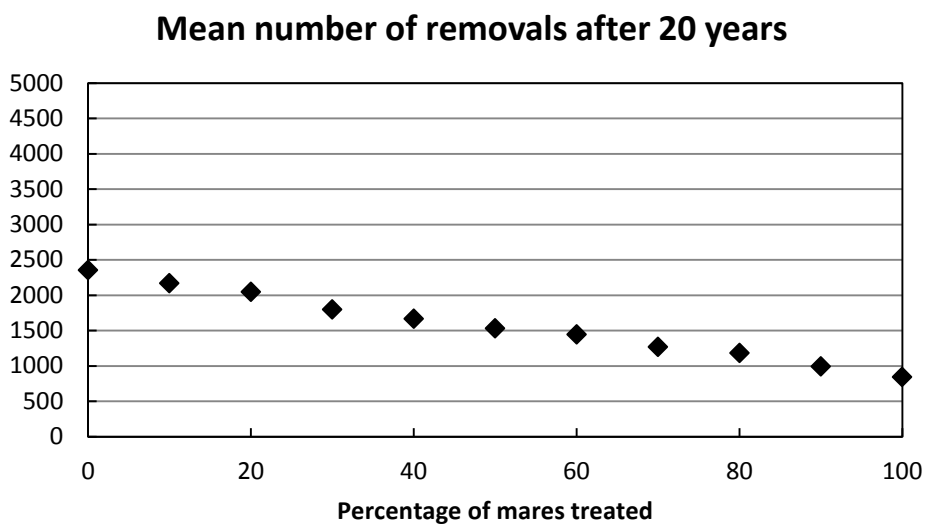
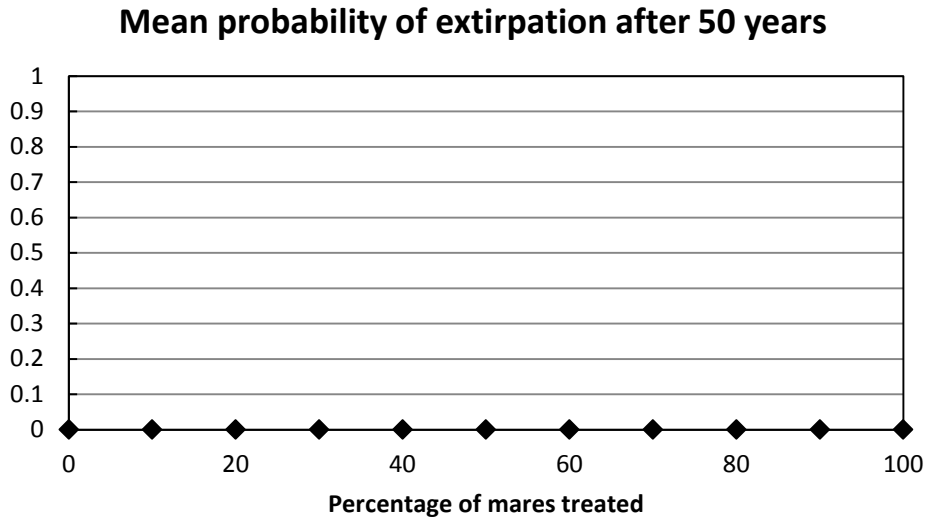


Figure 62. Initial population size = 1250, lambda = 1.10, genetic diversity = high.—Continued







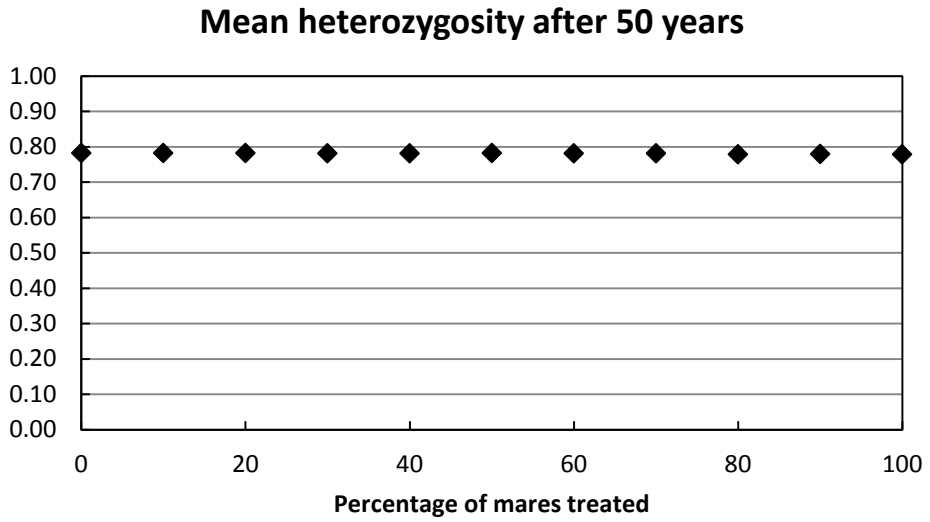


Figure 63. Initial population size = 1250, lambda = 1.15, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1468.9	1338.2	1354.4	1321.2	1271.8	1263.7	1147.8	1151.5	1135.1	1112.2	866.6
Mean number of removals after 20 years	2353.0	2166.1	2044.2	1797.3	1667.0	1529.1	1445.7	1267.1	1179.7	991.6	840.9
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

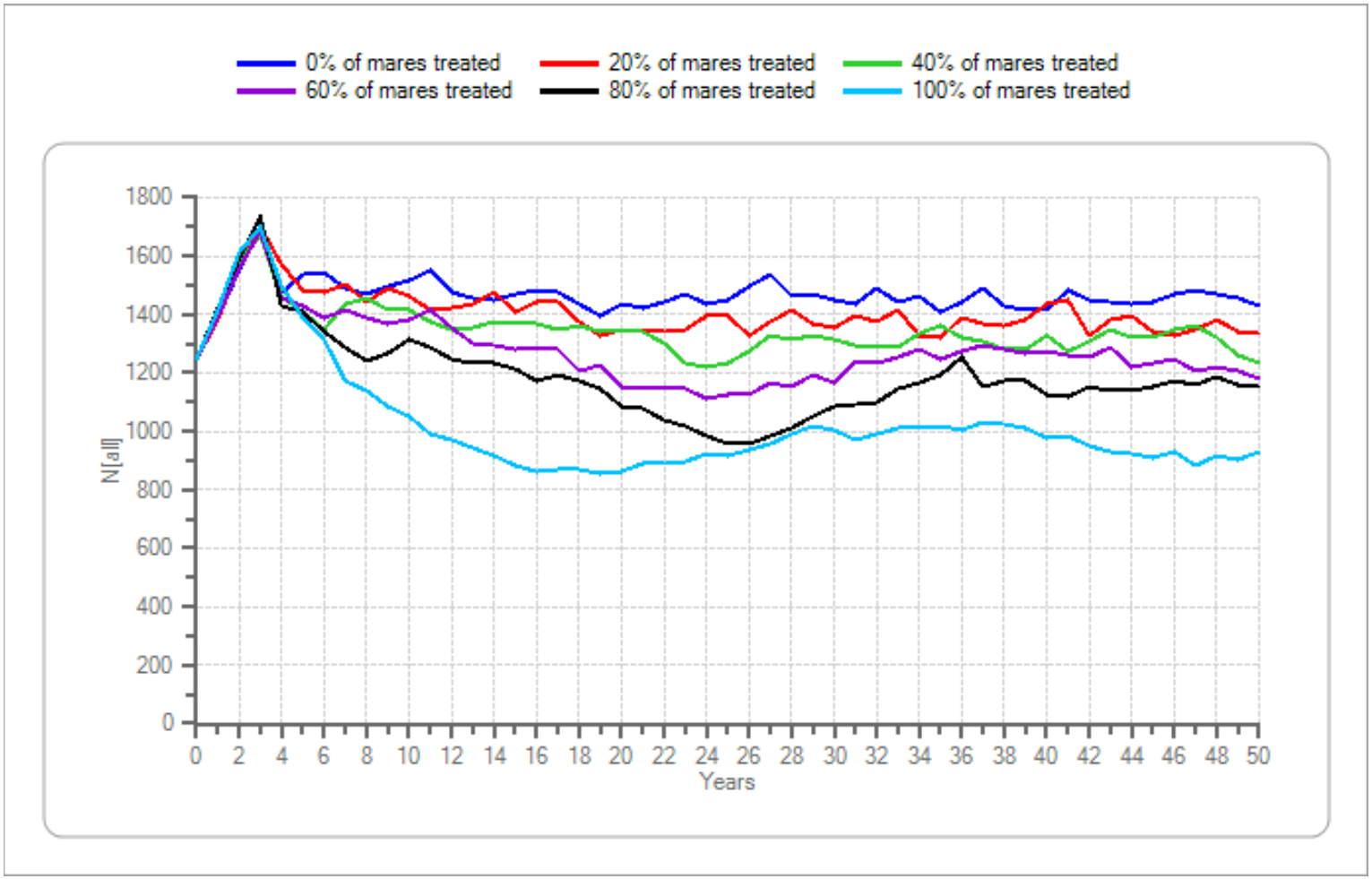
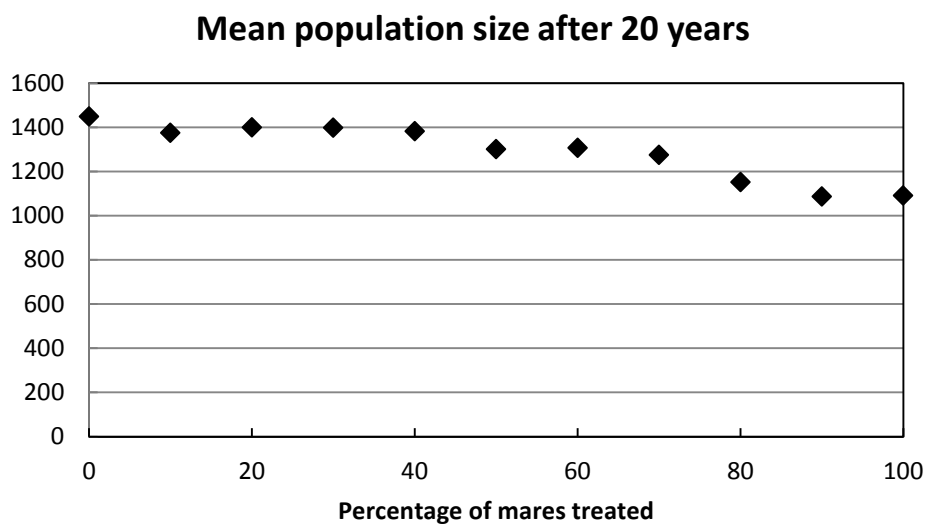
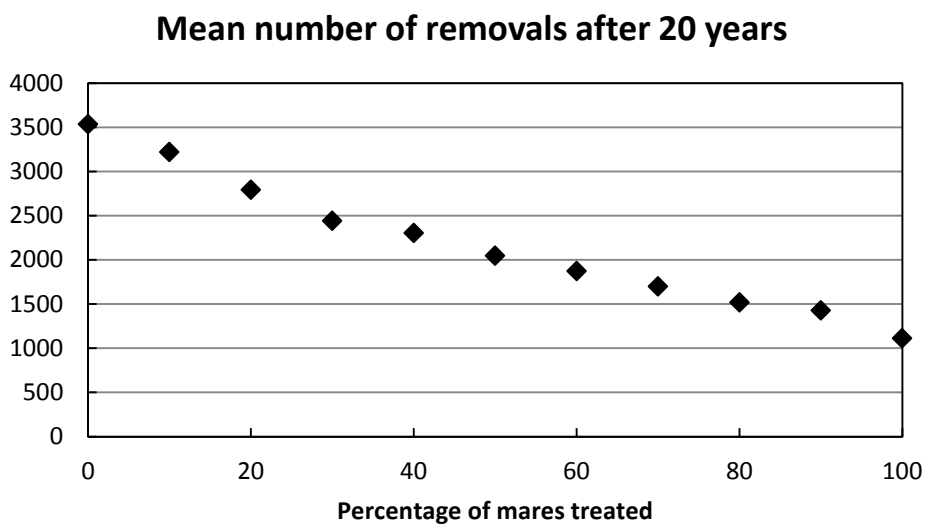
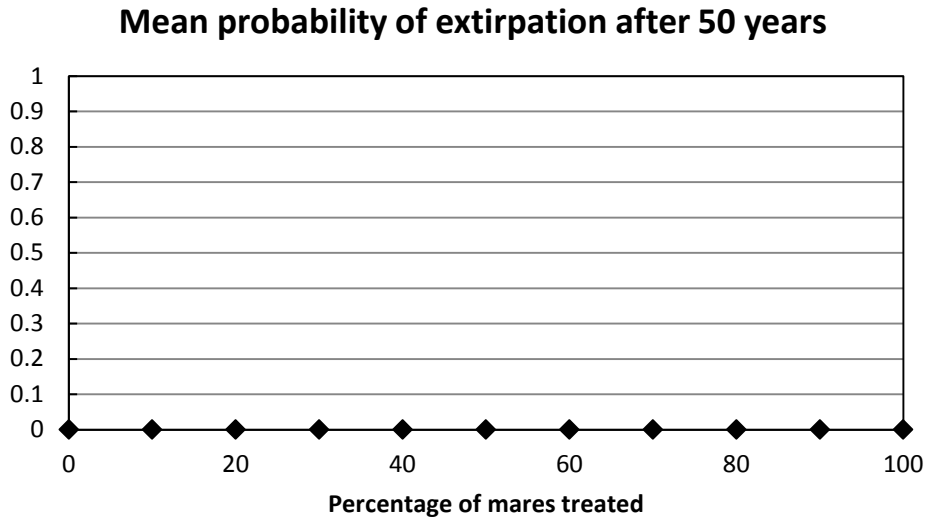


Figure 63. Initial population size = 1250, lambda = 1.15, genetic diversity = high.—Continued







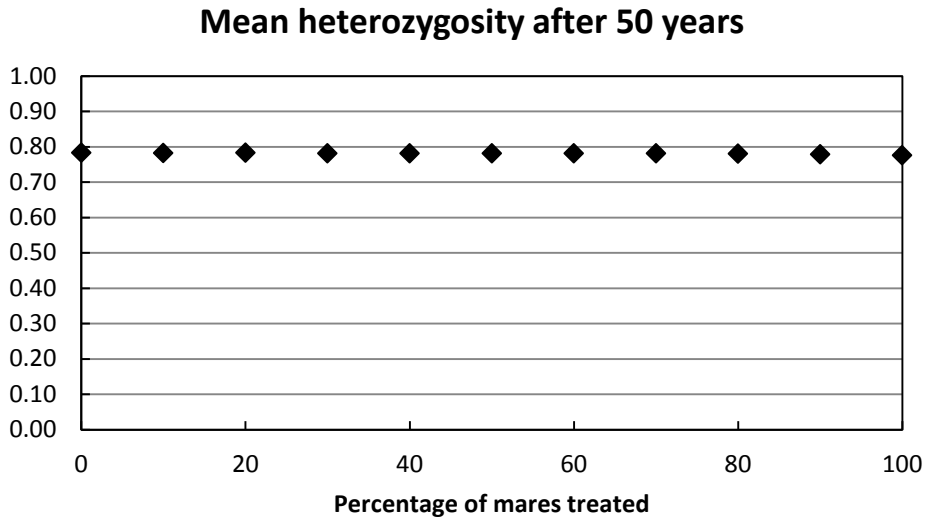


Figure 64. Initial population size = 1250, lambda = 1.20, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1449.1	1375.0	1398.9	1397.6	1381.6	1301.0	1306.8	1274.2	1152.3	1086.1	1090.1
Mean number of removals after 20 years	3536.0	3218.8	2791.6	2439.7	2302.0	2047.4	1874.1	1697.0	1517.3	1426.7	1110.3
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

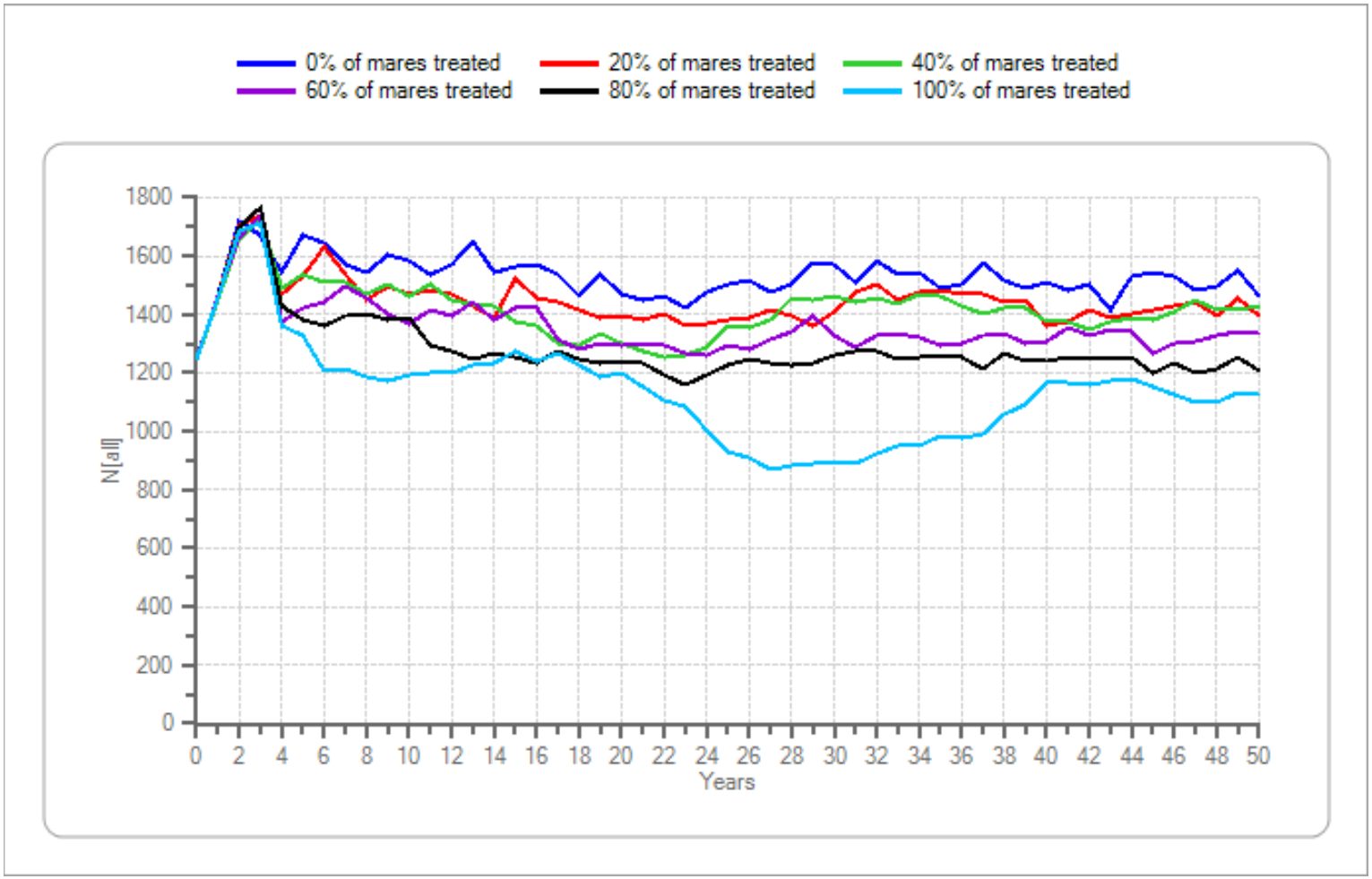
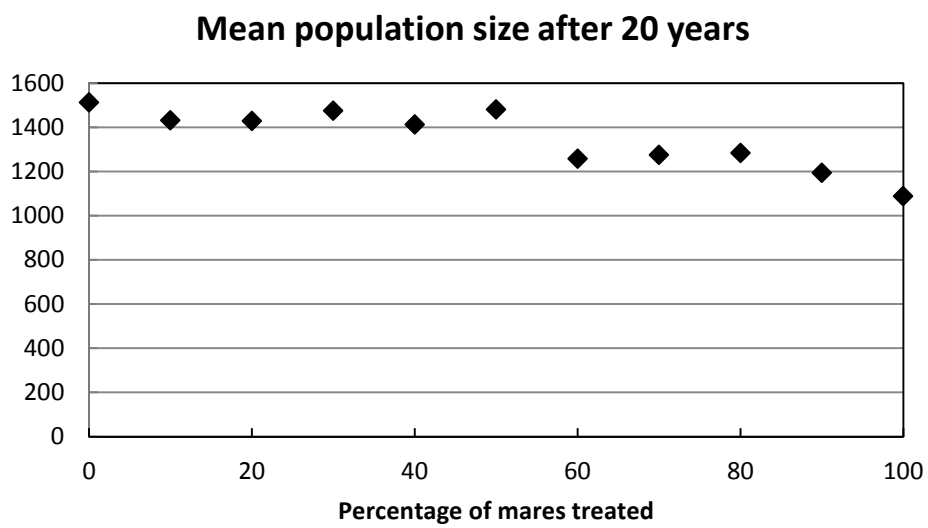
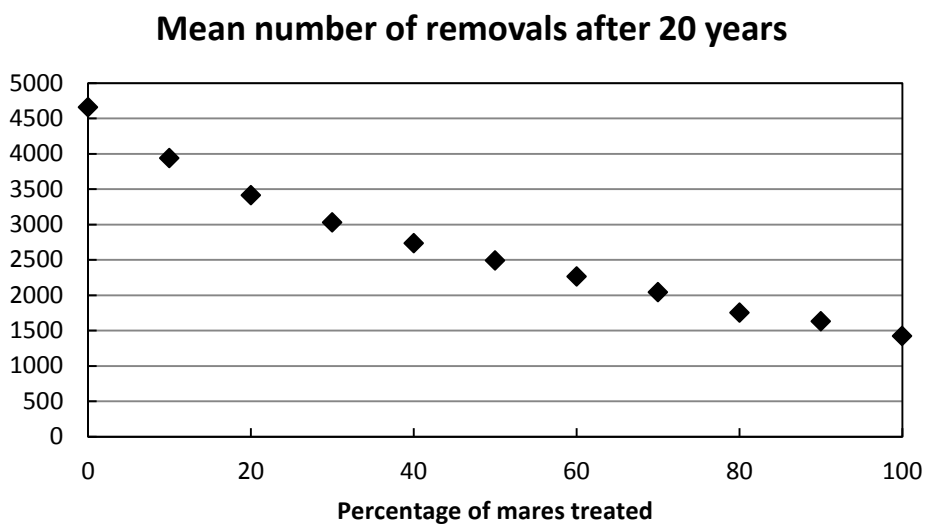
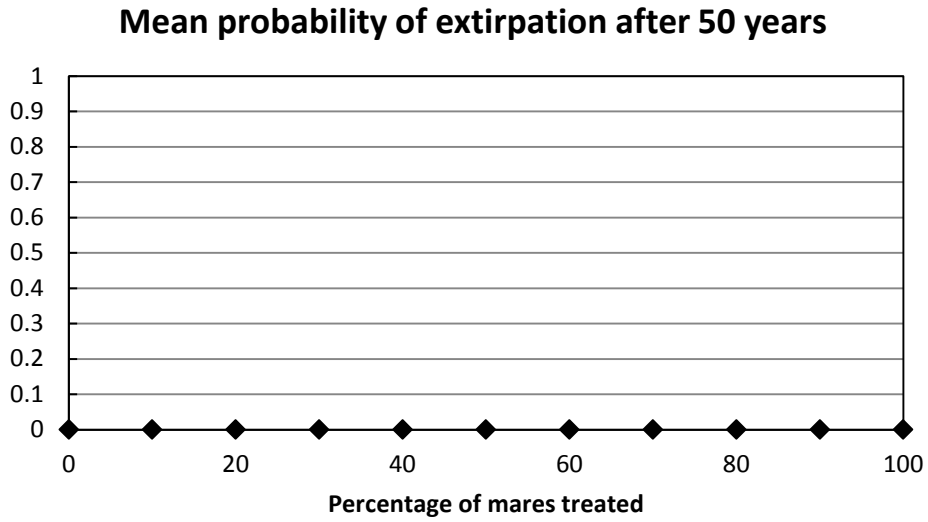


Figure 64. Initial population size = 1250, lambda = 1.20, genetic diversity = low.—Continued







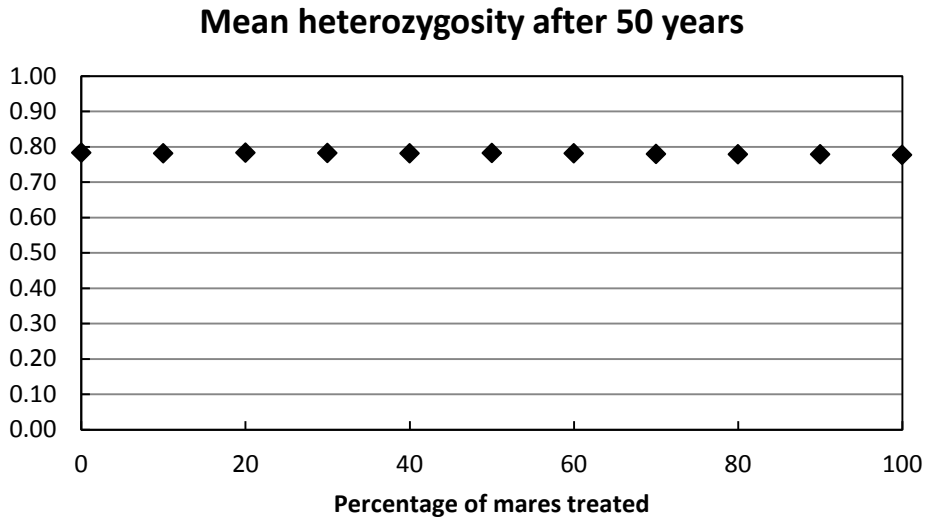


Figure 65. Initial population size = 1250, lambda = 1.25, genetic diversity = low.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1512.7	1430.9	1428.7	1474.7	1412.0	1480.7	1257.7	1274.9	1284.0	1193.1	1087.6
Mean number of removals after 20 years	4658.4	3937.6	3415.0	3030.1	2732.3	2491.5	2261.5	2041.8	1751.8	1629.4	1422.0
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

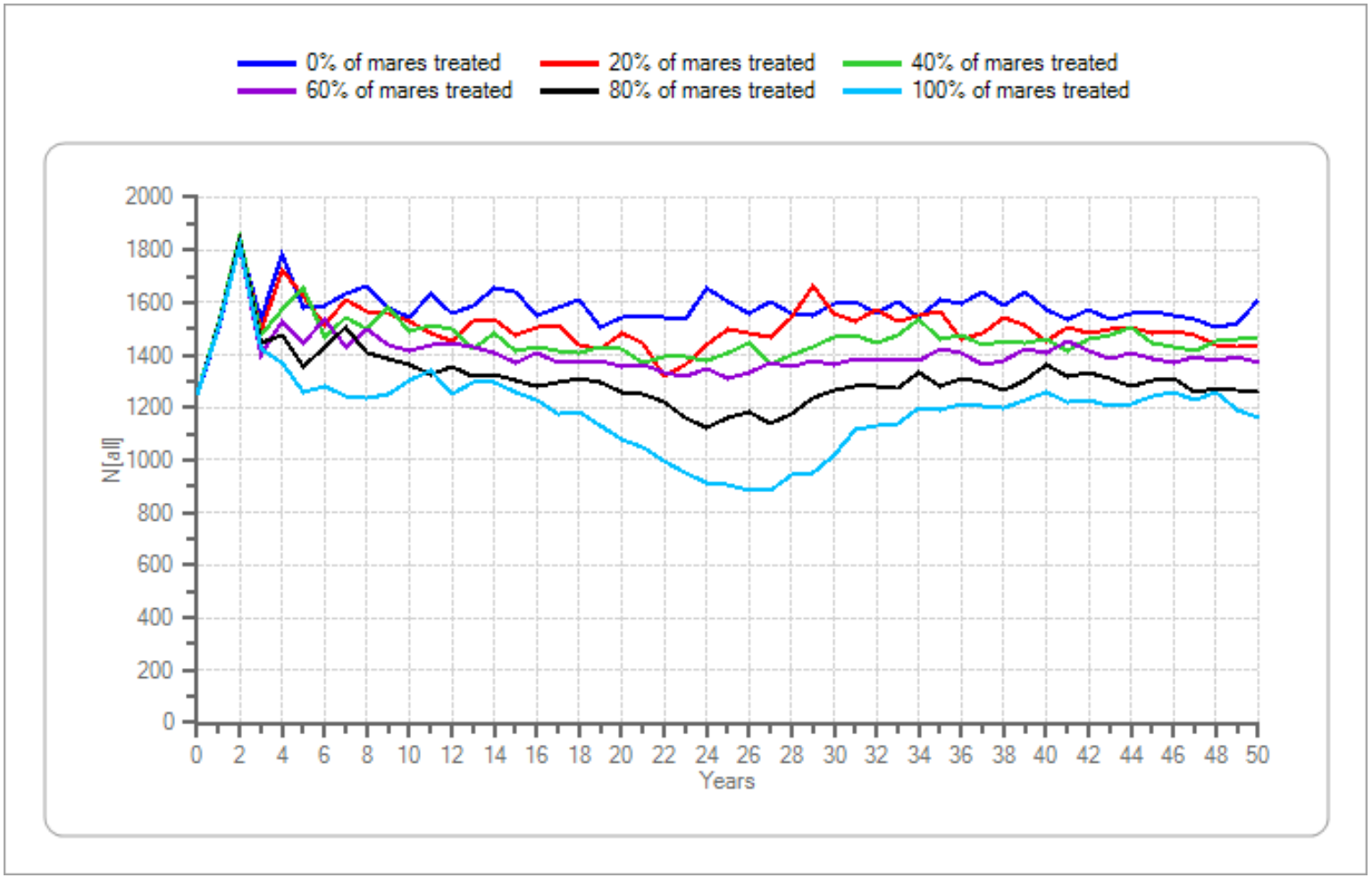
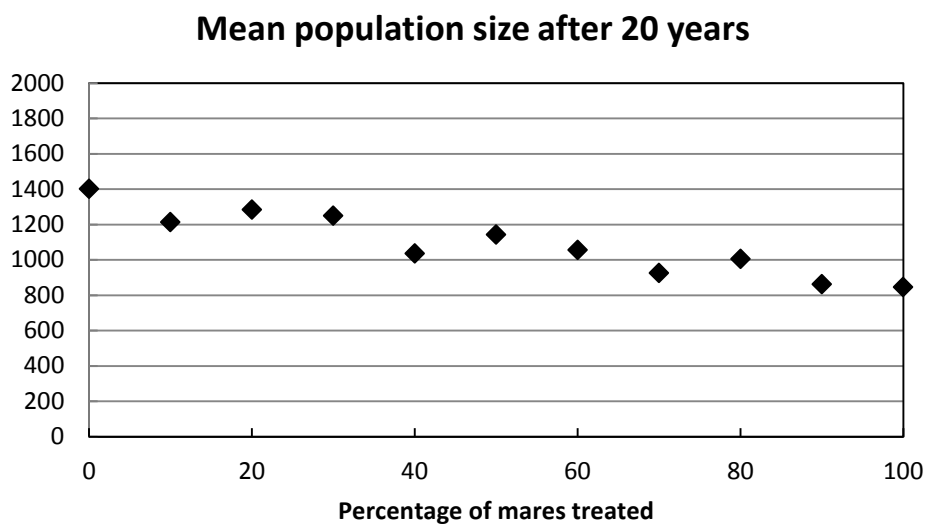
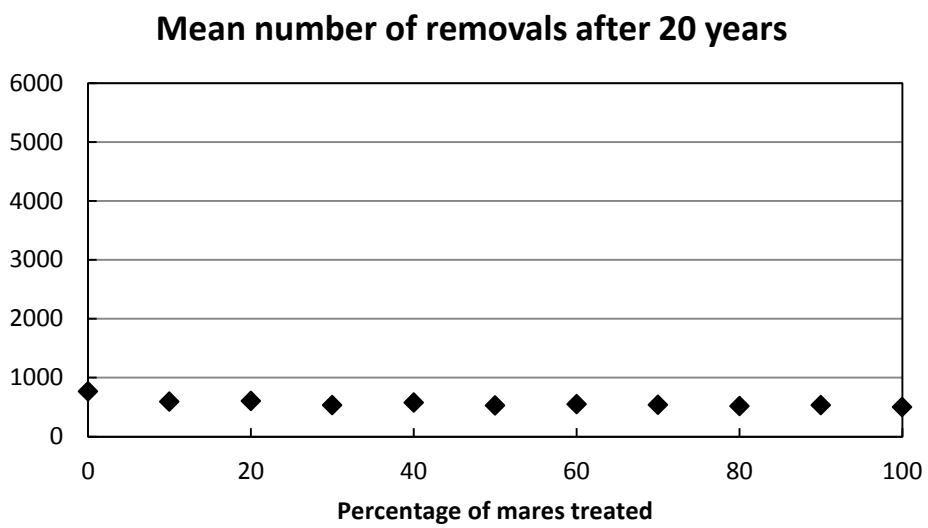
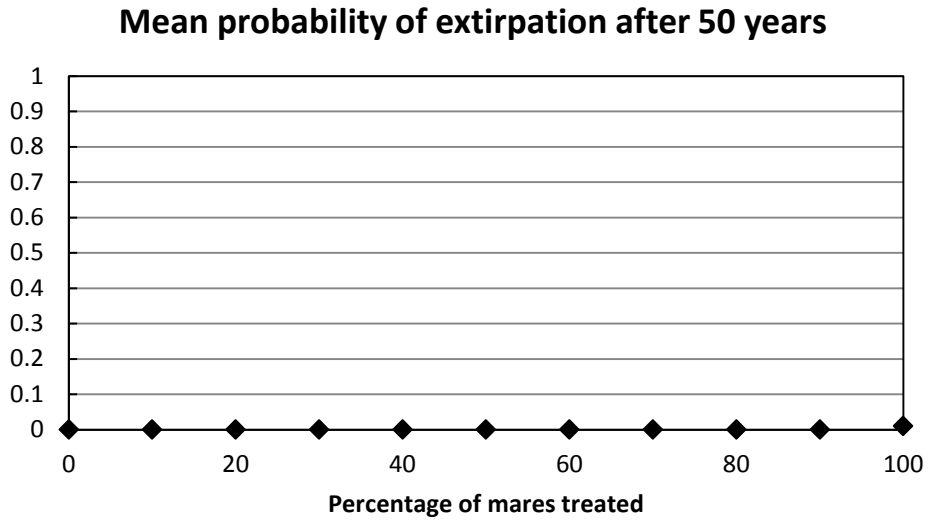


Figure 65. Initial population size = 1250, lambda = 1.25, genetic diversity = low.—Continued







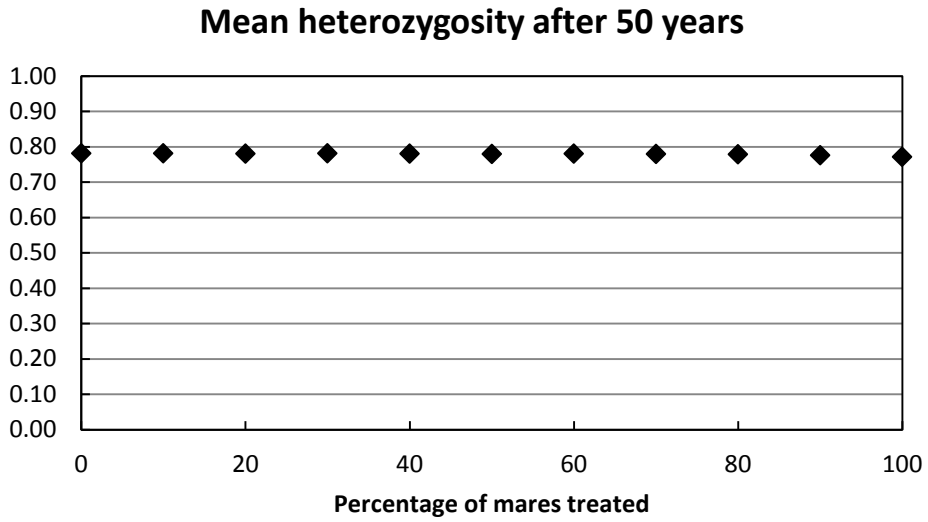


Figure 66. Initial population size = 1500, lambda = 1.05, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1401.3	1213.9	1283.2	1249.7	1035.4	1142.4	1056.3	925.6	1005.1	862.6	845.7
Mean number of removals after 20 years	764.8	589.6	604.0	530.4	578.3	527.0	547.6	539.9	516.7	534.7	497.8
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0.01
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77

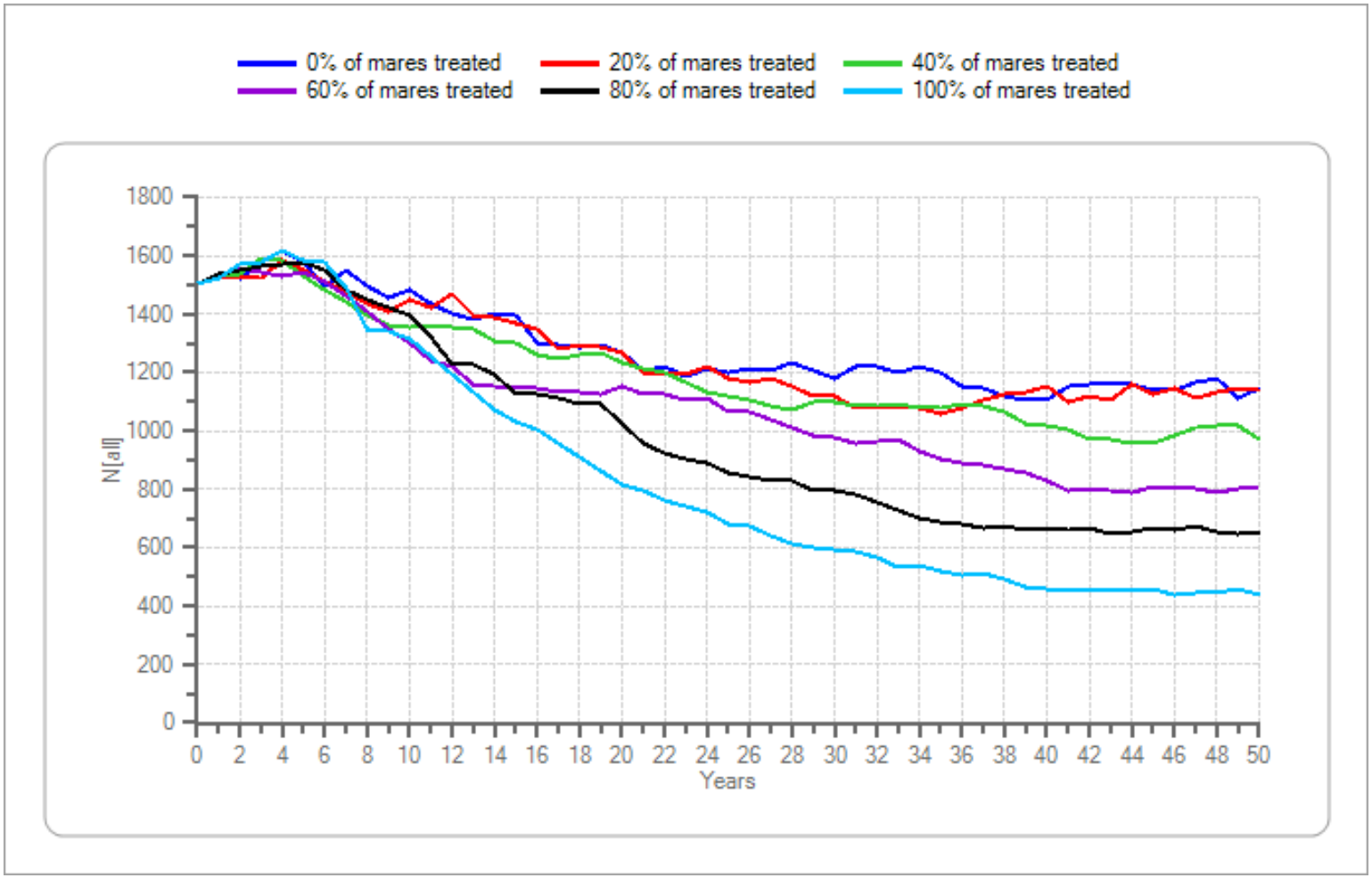
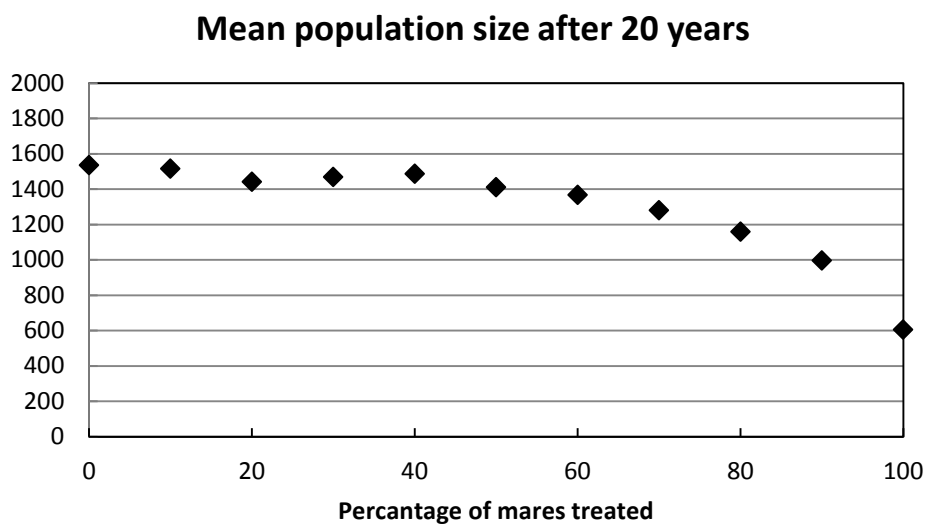
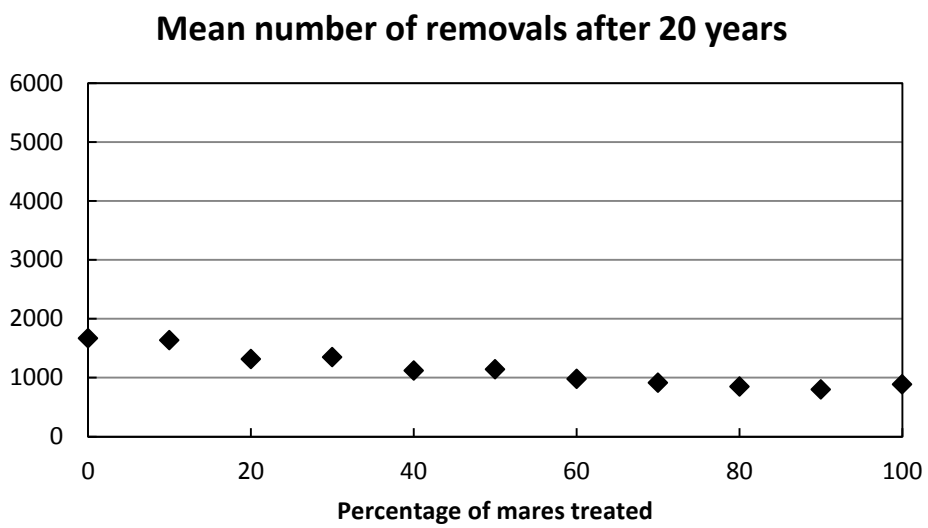
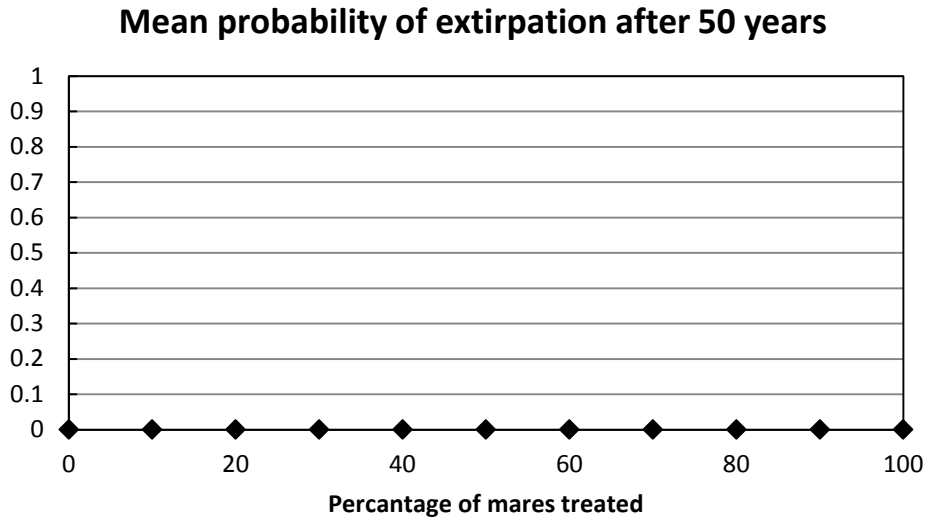


Figure 66. Initial population size = 1500, lambda = 1.05, genetic diversity = high.—Continued







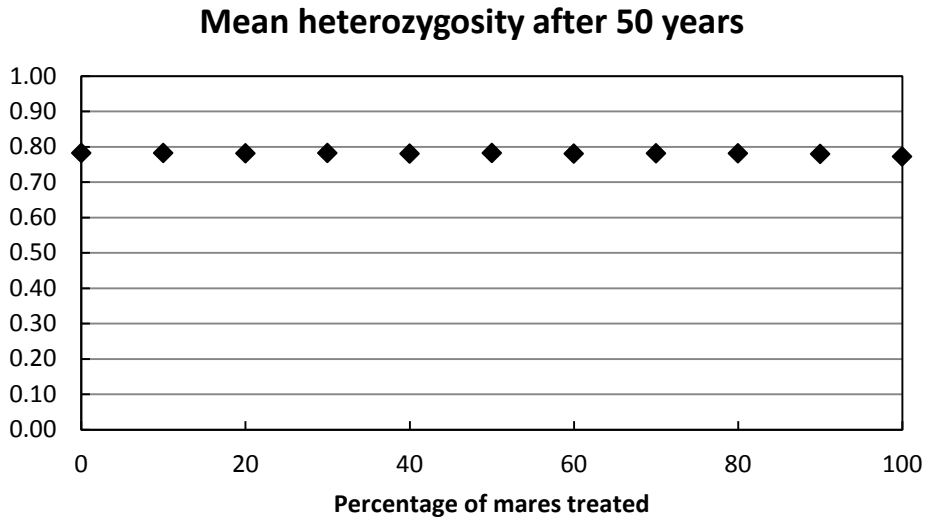


Figure 67. Initial population size = 1500, lambda = 1.10, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1534.7	1515.0	1441.5	1468.4	1487.3	1411.4	1367.4	1280.2	1158.3	996.3	604.3
Mean number of removals after 20 years	1669.4	1636.8	1316.9	1346.6	1118.7	1139.7	979.0	911.8	846.6	799.3	886.0
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.77

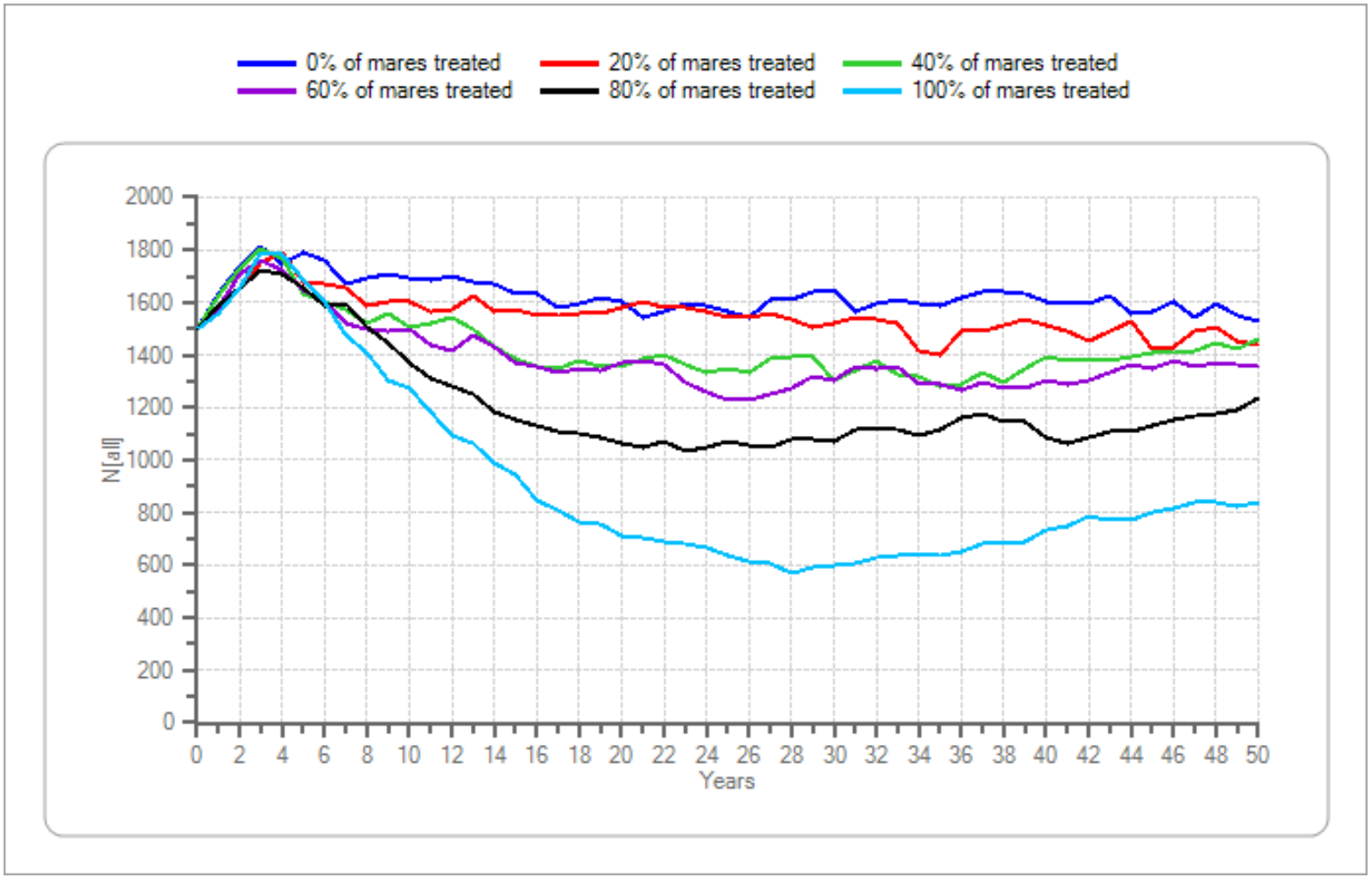
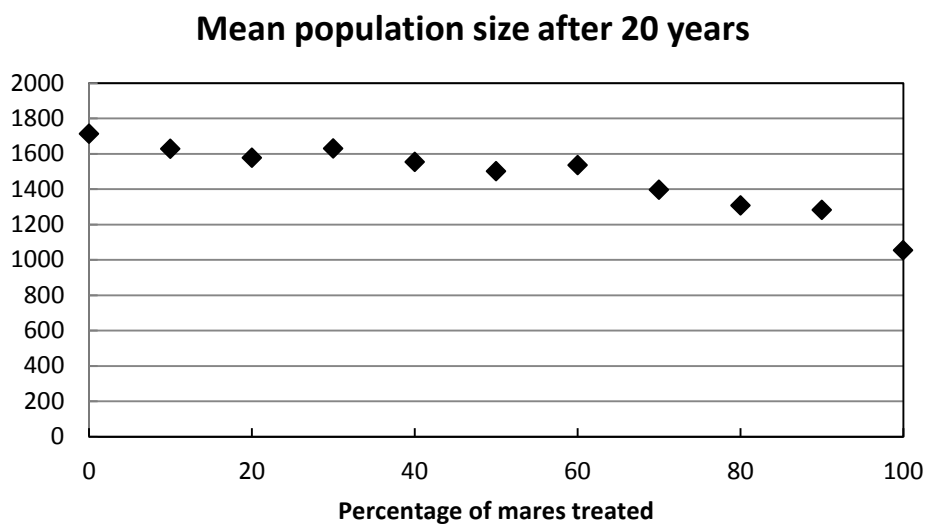
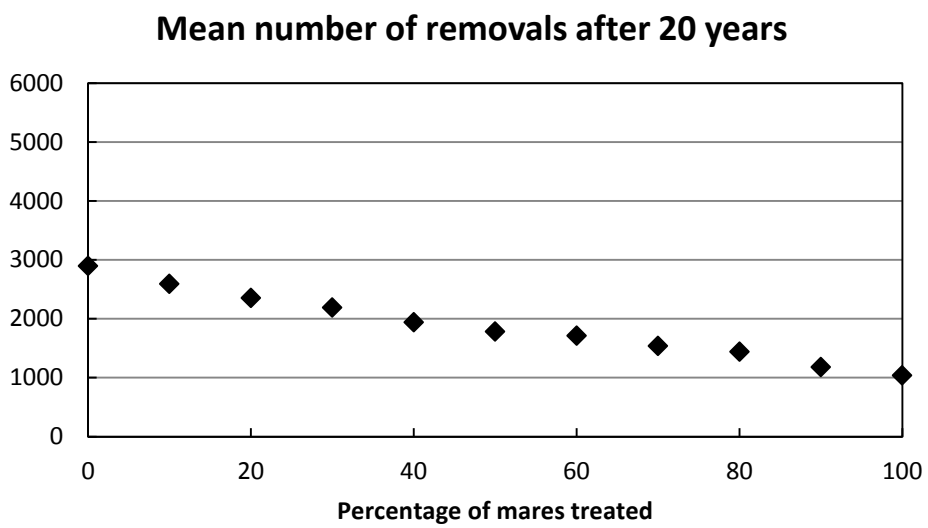
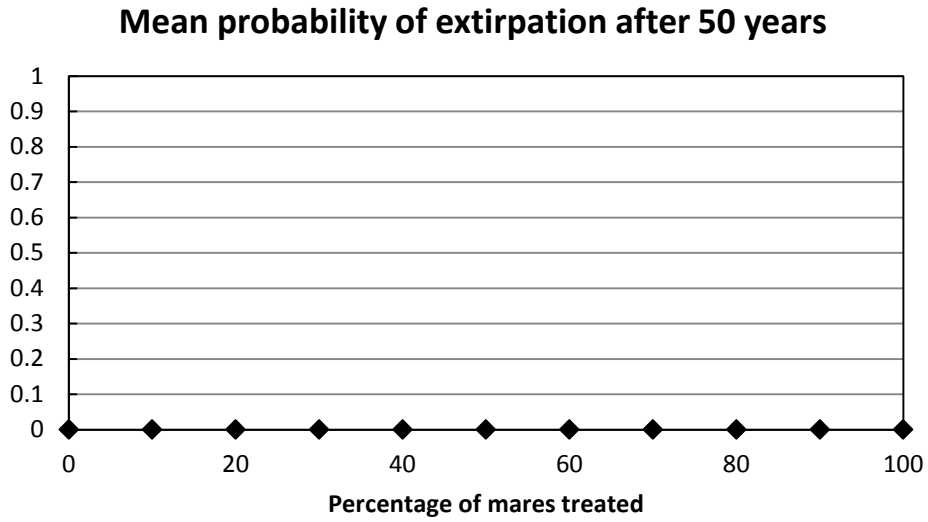


Figure 67. Initial population size = 1500, lambda = 1.10, genetic diversity = high.—Continued







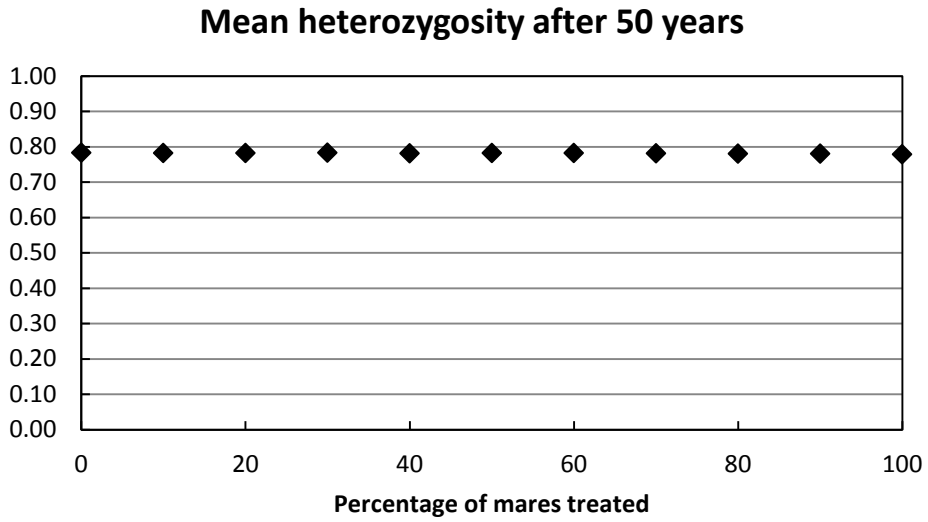


Figure 68. Initial population size = 1500, lambda = 1.15, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1712.0	1627.6	1576.6	1629.3	1553.6	1500.3	1535.1	1396.9	1306.5	1281.2	1053.2
Mean number of removals after 20 years	2896.0	2590.5	2349.9	2187.2	1938.7	1780.1	1713.2	1538.8	1441.8	1180.9	1038.4
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

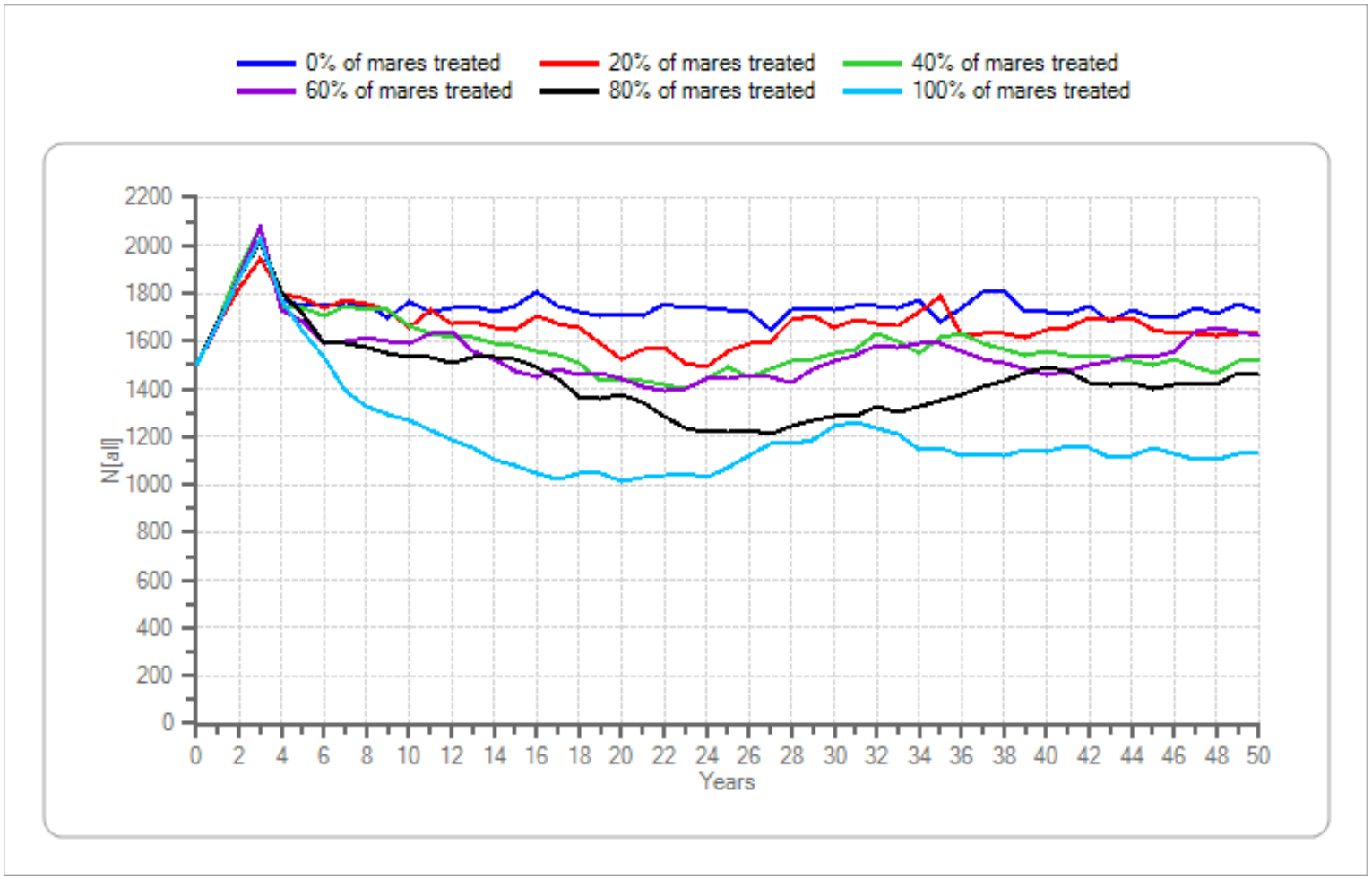
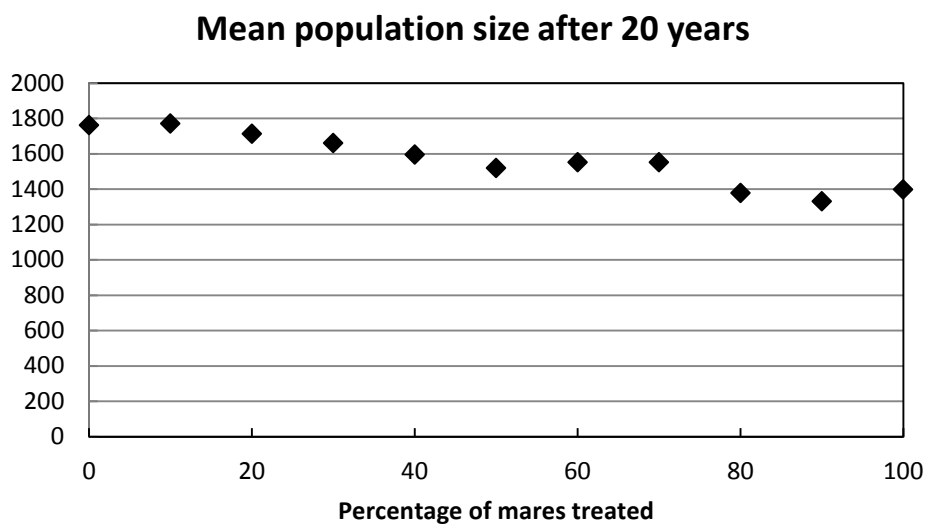
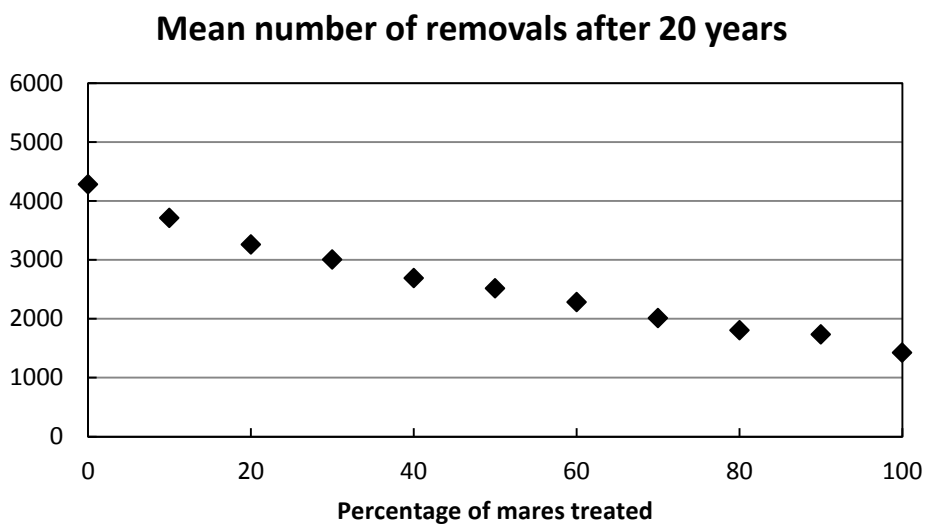
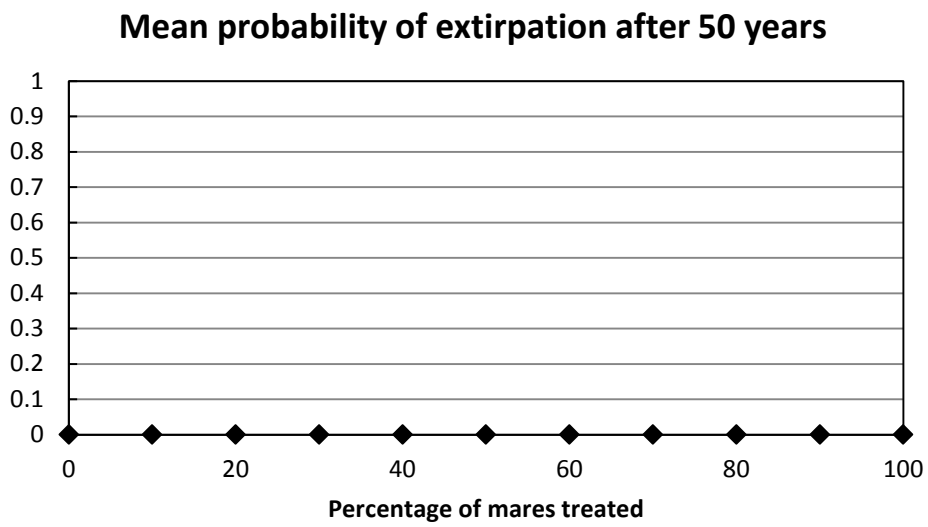


Figure 68. Initial population size = 1500, lambda = 1.15, genetic diversity = high.—Continued







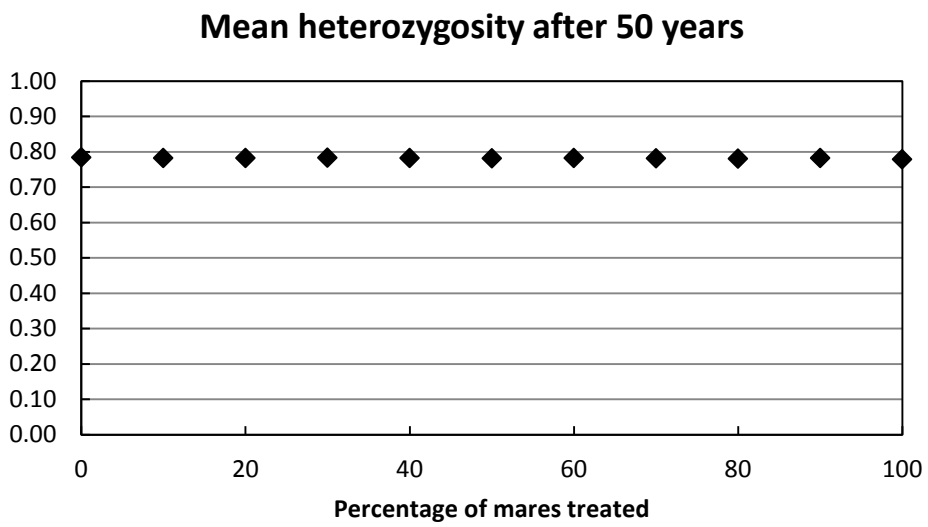


Figure 69. Initial population size = 1500, lambda = 1.20, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1761.4	1771.4	1712.8	1660.2	1595.1	1518.3	1551.7	1551.3	1378.4	1331.5	1397.5
Mean number of removals after 20 years	4280.8	3712.2	3260.2	3005.3	2689.8	2516.5	2281.8	2007.8	1802.1	1735.3	1423.0
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

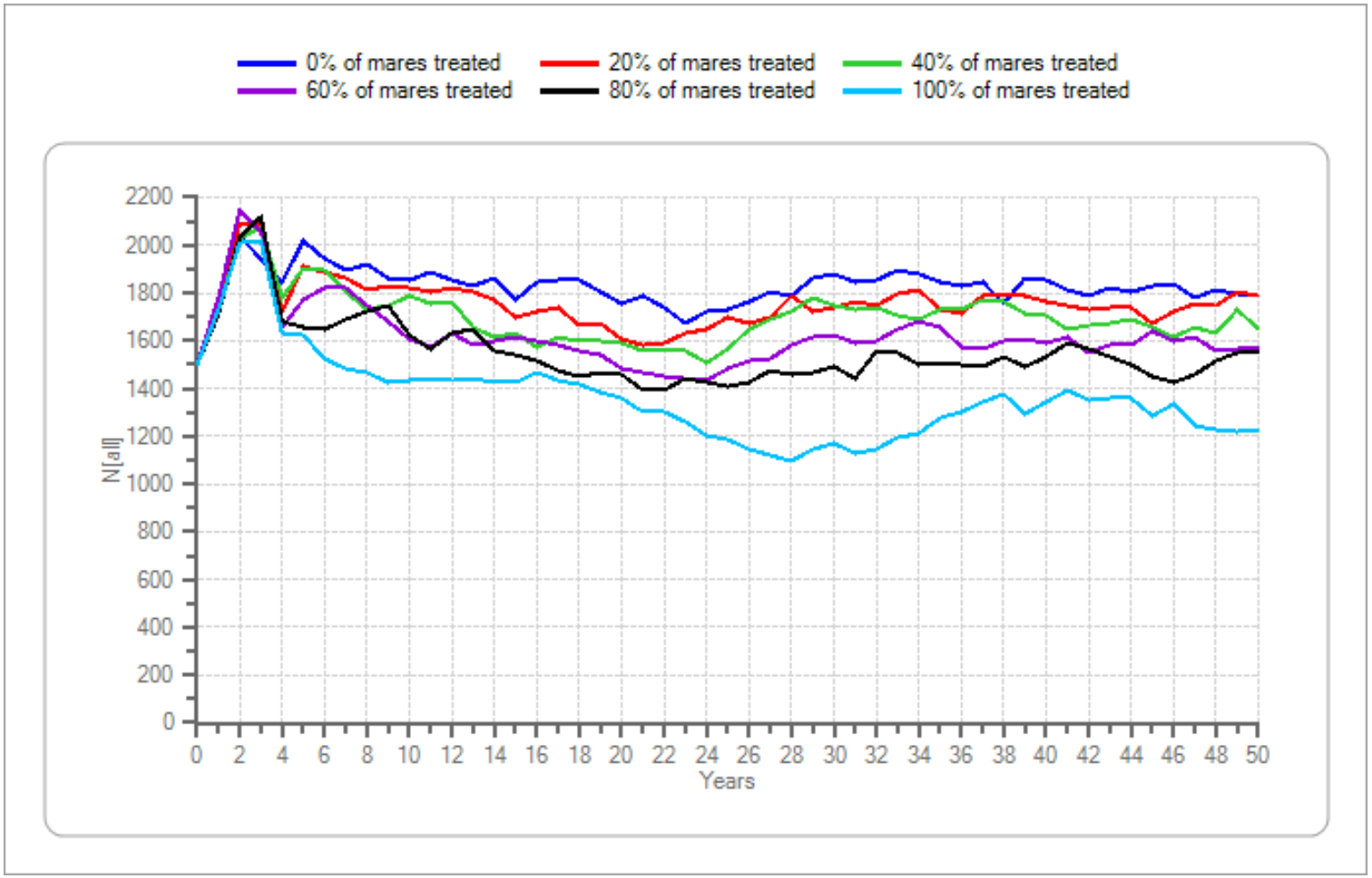
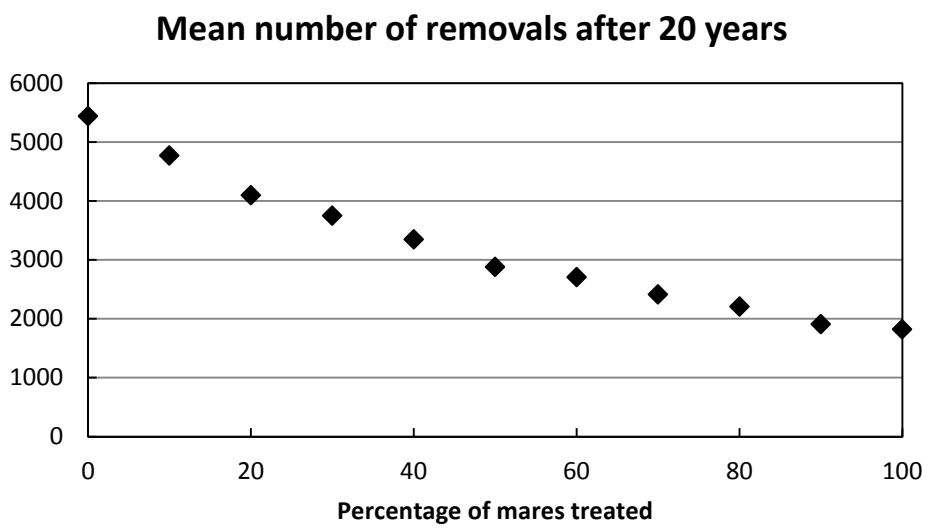
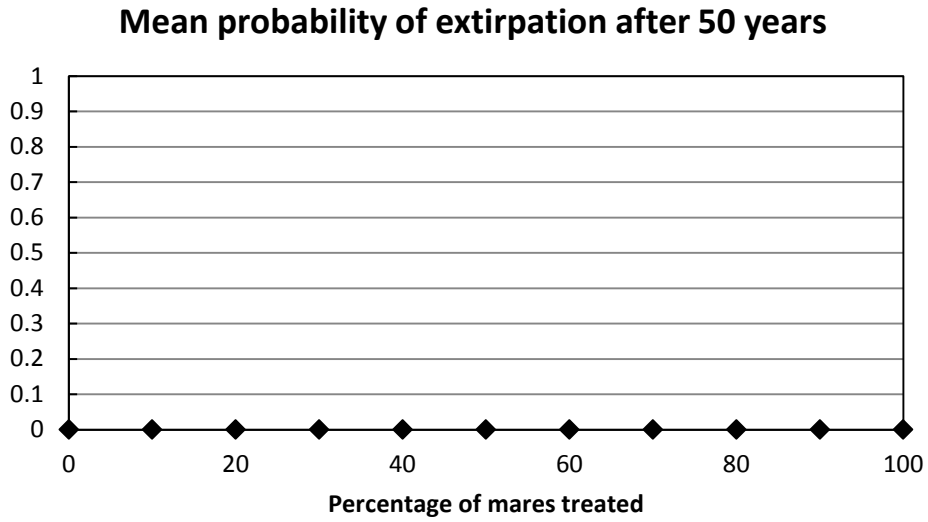


Figure 69. Initial population size = 1500, lambda = 1.20, genetic diversity = high.—Continued







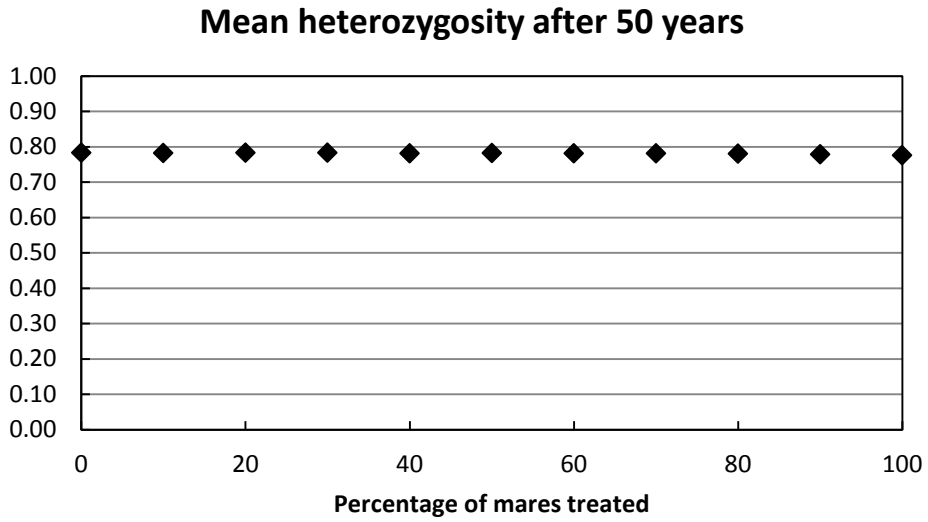


Figure 70. Initial population size = 1500, lambda = 1.25, genetic diversity = high.

Percentage of mares treated	0	10	20	30	40	50	60	70	80	90	100
Mean population size after 20 years	1902.9	1845.4	1761.8	1664.4	1620.0	1726.7	1585.1	1496.6	1543.0	1414.6	1267.5
Mean number of removals after 20 years	5436.7	4770.0	4094.4	3748.1	3344.8	2876.4	2706.6	2411.4	2203.6	1906.3	1821.2
Mean probability of extirpation after 50 years	0	0	0	0	0	0	0	0	0	0	0
Mean heterozygosity after 50 years	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78

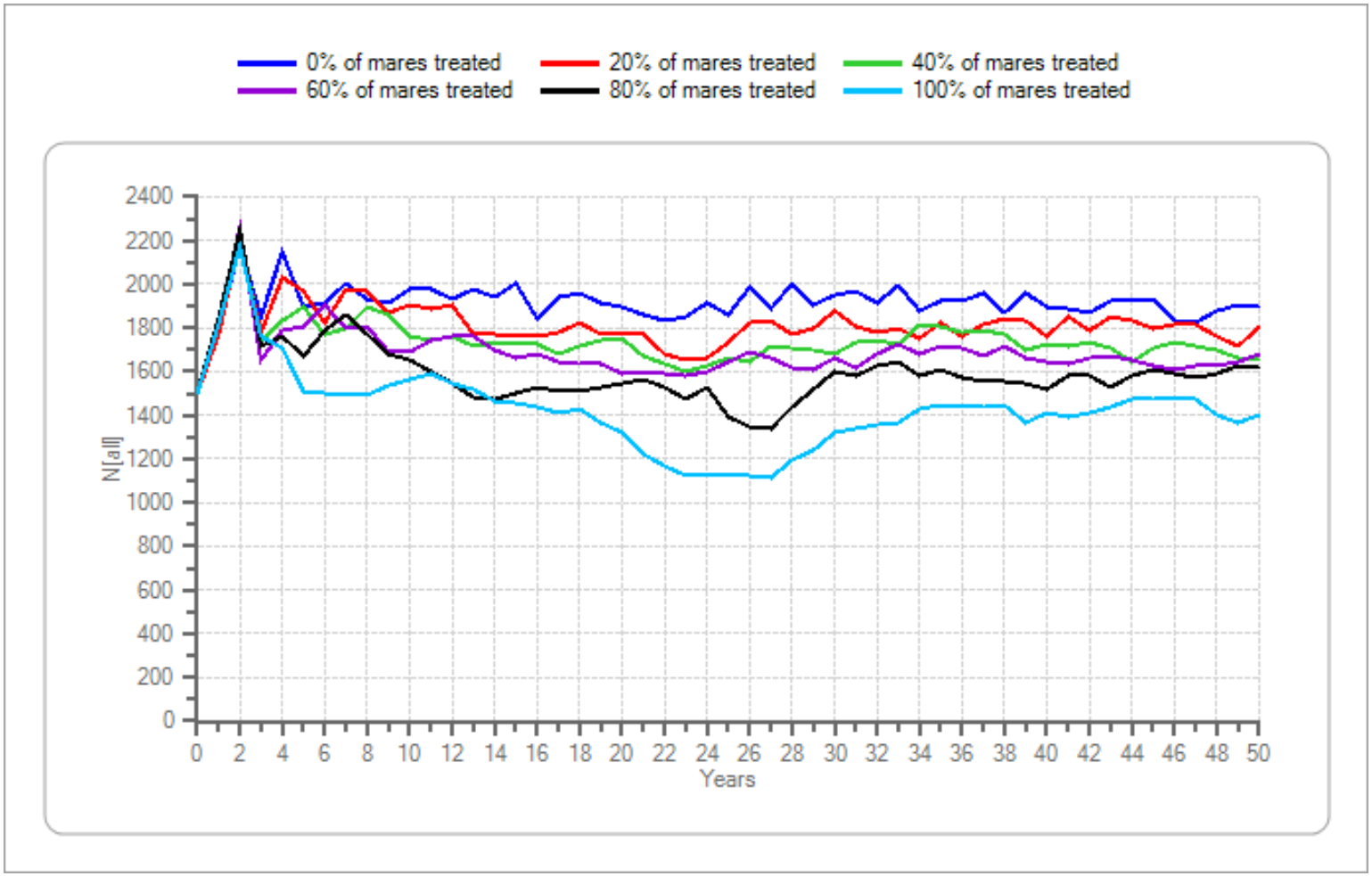


Figure 70. Initial population size = 1500, lambda = 1.25, genetic diversity = high.—Continued



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Injection-Site Reactions in Wild Horses (*Equus caballus*) Receiving an Immunocontraceptive Vaccine

Scientific Investigations Report 2009–5038

U.S. Department of the Interior
U.S. Geological Survey

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U.S. Geological Survey

U.S. Department of the Interior
KEN SALAZAR, Secretary

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Injection-Site Reactions in Wild Horses (*Equus caballus*) Receiving an Immunocontraceptive Vaccine

By James E. Roelle[1] and Jason I. Ransom[1]

Abstract

The U.S. Geological Survey and the Bureau of Land Management are conducting research on the efficacy of the immunocontraceptive agent porcine zona pellucida (PZP) in reducing fertility of wild horses (*Equus caballus*). As an antigen, PZP stimulates antibody production when injected into many mammalian species. These antibodies bind to the external surface of the ovum, preventing fertilization. By itself, PZP is only weakly immunogenic and is therefore delivered with an adjuvant, most commonly one of the Freund adjuvants, designed to further stimulate antibody production. Freund's complete adjuvant (FCA) in particular is known to be very effective, but may also be associated with undesirable side effects such as formation of abscesses at injection sites. Such reactions may be exacerbated when accompanied by the additional trauma of a remotely delivered dart. Because horses in our three study herds were individually identifiable by color markings and harem association, we were able to monitor mares for injection-site reactions (abscesses, nodules, swelling, and stiffness) following inoculation with PZP.

In 100 injections delivered by hand we observed a single nodule, two instances of swelling, and no other reactions. In two herds that received remotely delivered (dart) injections, the frequency of reactions was about 1 and 6 percent for abscesses, 25 percent for nodules (both herds), 11 and 33 percent for swelling, and 1 and 12 percent for stiffness. Abscesses were too infrequent to allow meaningful analysis of the relation to covariates, but for the other types of reactions we used logistic regression to examine the relation of occurrence to the delivery method (rifle or CO_2-powered blowgun), adjuvant (FCA, Freund's modified adjuvant, and Freund's incomplete adjuvant), dart trauma (normal or abnormal), and age of mare. Abnormal dart trauma included cases where the dart hit bone or the needle broke off. We found strong evidence (odds ratio = 5.023, $P = 0.001$) for a higher probability of occurrence of swelling when darts were delivered by blowgun. We found some evidence (odds ratio = 8.729, $P = 0.07$) that abnormal dart trauma led to a higher frequency of nodule formation. Nodules were the most common reactions observed and often persisted for a year or more, but in our observations they did not appear to change any animal's range of movement or locomotor patterns and in most cases did not appear to differ in magnitude from naturally occurring injuries or scars. We were unable to perform histological examinations of these nodules, but they may be similar to granulomas reported by other investigators following administration of Freund's adjuvant.

Introduction

The Wild Free-Roaming Horses and Burros Act of 1971 (P.L. 92–195, as amended) declared that "wild free-roaming horses and burros are living symbols of the historic and pioneer spirit of the West" and that these animals "shall be protected from capture, branding, harassment, or death." Responsibility for management of wild horses (*Equus caballus*) and burros (*Equus asinus*) on western lands was vested in the Bureau of Land Management (BLM) and the U.S. Department of Agriculture Forest Service (FS). BLM, our partner in the research described here, currently manages wild horses and burros on 199 Herd Management Areas (HMAs) encompassing over 13.9 million hectares. Given the protection afforded by the Act and largely unchecked by natural predators, wild horse populations can increase rapidly, often 15–25 percent per year (Eberhardt and others, 1982; Garrott and Taylor, 1990; Garrott and others, 1991) and can quickly exceed the capacity of the range to support them. Historically, BLM has managed excess animals by gathering them (every 3–5 years on most HMAs) and offering them for adoption or sale to good homes. More recently, BLM has sought to diversify its management program through the use of contraception to slow population growth.

In 2001, BLM and the U.S. Geological Survey (USGS), Fort Collins Science Center, initiated research to study the efficacy of the immunocontraceptive agent porcine zona pellucida (PZP) in limiting the fertility of mares in three horse herds in the Western United States: Little Book Cliffs Wild Horse Range (LBC) in Colorado, McCullough Peaks Herd Management Area (MCP) in Wyoming, and Pryor Mountain Wild Horse Range (PRY) in Montana and Wyoming. (See Kirkpatrick and Frank [2005] for a recent review of contraception in equids and other free-ranging animals.) PZP is produced from

[1]U.S. Geological Survey, Fort Collins, Colorado.

pig ovaries and acts as an antigen when injected into other species. PZP stimulates production of antibodies that bind to the zona pellucida, or external surface of the ovum, of the host species and interfere with fertilization (Asa, 2005). PZP by itself is only weakly antigenic and is usually delivered with an adjuvant to further stimulate the host immune system to produce antibodies. The adjuvant of choice for PZP applications in equids, at least for the first inoculation, has most often been Freund's complete adjuvant (FCA) (see, for example, Kirkpatrick and others, 1990; Turner and others, 1996, 1997, 2001). While very effective, FCA can have undesirable side effects, including the formation of abscesses and granulomas at the injection site, which appear to depend on the species, delivery method (hand injection or dart), injection location, and injection route (for example, intramuscular, subcutaneous) (Mahi-Brown and others, 1985; Broderson, 1989; Claassen and others, 1992; Leenaars and others, 1994, 1998; Lyda and others, 2005). In addition, accidental exposure to FCA can be hazardous to those administering the injections (Chapel and August, 1976).

In view of the potential for such adverse reactions, quantification of their occurrence is desirable. However, followup observations of injection sites is not often possible with free-ranging animals because individual animals are not easily identified unless they are marked (for example, with brands or ear tags) when treated (but see Naugle and others [2002] for an example involving individually recognizable white-tailed deer [*Odocoileus virginianus*]). In our three study herds, however, horses were individually recognizable through color markings and band (harem) associations, thus offering an opportunity to observe reactions at contraceptive injection sites. Here, we report the results of those observations; information on contraceptive efficacy will be presented elsewhere.

Study Areas

The Little Book Cliffs Wild Horse Range is located in Mesa County, Colorado, about 13 km northeast of Grand Junction and consists of 14,614 ha of sloping plateaus, sagebrush parks, steep-walled shale and sandstone cliffs, and four major canyon systems. A Wilderness Study Area (WSA) comprises 10,734 ha of the range and limits access by motorized vehicle. During our study, the herd was distributed across the entire range. Some horses traveled seasonally between the lower canyons and the higher elevations, and others did not move seasonally. Locating individual horses was often complicated by the predominance of Colorado piñon (*Pinus edulis*) and Utah juniper (*Juniperus osteosperma*) in association with the rough terrain. Once located, horses were generally approachable to within adequate observation distance using binoculars or spotting scopes; however, certain horses exhibited restless behavior when observers were present in wooded areas and would flee before detailed observation data could be collected.

The McCullough Peaks Herd Management Area is located in Park County, Wyoming, 32 km east of the town of Cody. It consists of 44,440 ha of predominantly flat, open sagebrush steppe, with rugged badlands along the western edge. A WSA occupies 9,943 ha of the range. There was no single seasonal congregation area for horses, though the majority of the population resided in the southern half of the range during our study. Horses on this portion of the range were generally quite visible due to the nature of the terrain and vegetation, but they were not approachable. Observation of injection sites was thus difficult, even with the aid of a spotting scope.

The Pryor Mountain Wild Horse Range is located 21 km north of Lovell, Wyoming, and lies within northern Bighorn County, Wyoming, and southeastern Carbon County, Montana. The range includes BLM land, portions of Custer National Forest and Bighorn Canyon National Recreation Area, and private lands, and consists of 16,046 ha of lowland desert, foothill slopes, forested montane slopes, steep canyons, and grassy plateaus. Three WSAs occupy the majority of PRY, limiting motorized access to the sparse road system. Vegetation types vary greatly from lower to higher elevations of the range, with lower elevations dominated by big sagebrush (*Artemisia tridentata*), mid-elevations dominated by curl-leaf mountain mahogany (*Cercocarpus ledifolius*), Utah juniper, and various grasses, and high elevations dominated by limber pine (*Pinus flexilis*), subalpine fir (*Abies lasiocarpa*), and alpine bluegrass (*Poa alpina*). Most horses moved from lower and mid-elevations in winter to high elevations in summer. Thus, horses were usually easy to observe in summer when they were in open subalpine and alpine meadows, but difficult to observe in spring and fall on forested montane slopes. Horses at PRY did not generally react to humans and, once located, could be closely approached for detailed observation.

Methods

We initiated treatments at each of the three study areas in concert with gathers scheduled by BLM (PRY in 2001, LBC in 2002, MCP in 2004). Ages and numbers of horses treated were in accordance with local plans developed by BLM (U.S. Department of the Interior, Bureau of Land Management, 2001, 2002, 2004). At LBC and MCP, mares of all ages ≥1 year were included. At PRY, however, we treated only mares 1–2 years old or >15 years old. At LBC and PRY, mares that were gathered received an initial inoculation (primer) by hand-held syringe or jabstick while restrained in a squeeze-chute, ungathered mares (sufficient to make up the total number of desired treatments) received the primer injection by remotely delivered dart, and all mares received a second inoculation (booster) by dart. At MCP, all inoculations were given in a squeeze-chute by hand-held syringe, jabstick, or hand-held trocar. All inoculations, regardless of delivery method, were given in the hip.

At LBC and PRY, primer injections consisted of 0.5 mL (100 μg) PZP and 0.5 mL (100 μg) FCA or Freund's modified adjuvant (FMA), and boosters consisted of 0.5 mL (100 μg)

PZP and 0.5 mL (100 μg) Freund's incomplete adjuvant (FIA). Adjuvants and PZP were provided by the Science and Conservation Center at ZooMontana in Billings. Darts were propelled from a Dan-Inject® (Dan-Inject of North America, Knoxville, Tenn.) CO_2-powered blowgun, a Dan-Inject® CO_2 rifle, or a Pneu-dart® (Pneu-Dart, Williamsport, Pa.) 193 rifle. The CO_2 delivery systems allowed user-defined power in pounds of pressure per square inch. The Pneu-dart rifle used .22 caliber CCI® (CCI, Lewiston, Idaho) green power-level charges that were individually weighed to ensure consistent delivery force. Abnormally light and abnormally heavy charges were discarded. Darts used in all delivery systems were 1-cc Pneu-dart darts with 3.8-cm barbless needles. These injections of liquid PZP at LBC and PRY were designed to provide 1 year of infertility, with annual boosters extending the period of contraception.

Treatment at MCP consisted of a primer injection (same composition as for the other two herds with FCA as the adjuvant) delivered by either hand-held syringe or jabstick and a second (but simultaneous) injection delivered by trocar and consisting of three pellets designed to release PZP in bolus fashion at 1, 3, and 12 months postinjection and provide 2 years of infertility. Pellets were developed at the Medical College of Ohio (now the College of Medicine, University of Toledo) and were formed using an organic solvent to mix polymer with PZP and the saponin adjuvant QA–21. The polymer was a mixture of polylactic acid and glycolic acid (both approved for use in humans by the Food and Drug Administration), with the ratio of the two components dependent on the desired release time. Subsequent evaporation of the solvent resulted in a polymer/PZP/adjuvant mixture viscous enough to be formed into pellets that were cut to the desired mass and loaded into a trocar for delivery (J. Turner, Jr., University of Toledo College of Medicine, written commun., 2005).

Liquid vaccine was provided as sterile components (PZP and adjuvant) that were emulsified in alcohol-sterilized syringes prior to application. For hand injections, the emulsion was loaded into alcohol-sterilized syringes fitted with single-use sterile needles. For remote delivery, the emulsion was loaded into darts as they were removed from the manufacturer's packaging, and a small dab of petroleum jelly was placed on the dart tip to prevent leakage of the vaccine. Pellets were dipped in alcohol, coated with a thin film of petroleum jelly, and loaded into sterile needles at the place of manufacture. Each needle tip was also closed with a small dab of petroleum jelly to prevent the pellets from slipping out of the needle during handling. For all hand injections, hip hair was shaved and the area washed with alcohol prior to immunization (J. Turner, Jr., written commun., 2008).

Mares treated at gathers were held in corrals for 28–48 hours and observed for any reactions to injections, then released back to the range. Following remotely delivered treatments, we immediately recovered the dart (if possible) and recorded information on its condition and performance. We then attempted to observe the animal for 30 minutes, which was sometimes not possible for those that fled. We also attempted, with varying degrees of success, to observe each injection site once per week for the first 3–4 weeks or until any reaction disappeared. Additional observations were recorded opportunistically for up to 6 years. Observation distances ranged from 1 m to 500 m, with various optical devices (binoculars, spotting scopes) employed at longer distances. We tracked multiple injections to the same individual by carefully noting the location of each injection on the mare and specifically referencing each new injection site to any observed reactions from previous injections. We also took photographs of reactions opportunistically to aid in referencing specific injection locations.

We coded injection-site reactions in four categories: abscess (an open sore, usually with drainage), nodule (a well-defined circular lump of unknown histology), swelling (a raised area of tissue, but not sufficiently well defined to be considered a nodule), or stiffness (any abnormal range of movement in the limb where the injection was delivered). These categories were not mutually exclusive with respect to either a single observation or a single injection. Swelling and stiffness, for example, were sometimes recorded for the same observation, and multiple reactions were sometimes recorded for a single injection site (for example, swelling might develop into a nodule). We also looked for muscle tremors, behavioral depression, or other systemic reactions, none of which was ever observed.

Because of the opportunistic nature of many of our observations, we could not calculate frequency of occurrence of injection-site reactions directly from the raw data. For example, if an injection site was observed on days 0 and 180, and no reaction was seen, it would be inappropriate to conclude that no reaction occurred because a reaction could have developed and disappeared in the time between the two observations. As an alternative, we divided all of our observations into a series of intervals representing elapsed time after the injection and calculated the frequencies with which the four types of reactions were observed in each interval. Multiple observations of an injection site in a given mare in a period were counted as a single observation in determining sample size, and the reaction was coded as present if it was recorded for any observation during the period. For this analysis, as well as the analysis of covariates (see below), we excluded all injections that were given by hand because much of the concern about possible injection-site reactions in horses is related to darting.

The fact that abscesses occurred infrequently, coupled with the fact that four of the eight observed abscesses occurred in a single horse, precluded meaningful analysis of the relation of abscess occurrence to covariates. For the remaining types of reactions (nodules, swelling, and stiffness), we used logistic regression (PROC LOGISTIC in SAS/STAT®; SAS Institute, Cary, N.C.) to examine the relation between occurrence and four covariates: adjuvant (FCA, FMA, or FIA), delivery method, dart trauma, and age of the mare. For delivery method, we used two classes: rifle or blowgun. We pooled deliveries from the Dan-Inject® CO_2 rifle with those from the Pneu-Dart®

193 rifle because both were scoped delivery guns with longer range, even though the CO_2 rifle allowed user-defined power. In addition, the sample size for treatments delivered with the CO_2 rifle was small (n = 8). For dart trauma, we divided the injections into two broad classes: those where the result may have caused additional trauma at the injection site (bone plug or needle broke off, which we labeled as abnormal) and all others (normal). We did not include the distance from which the dart was fired in this analysis because (1) there were some cases in which distance was not recorded (which would reduce our sample size) and (2) distance tended to be highly correlated with method. Probabilities reported from these regressions are based on the Wald chi-square test statistic.

As noted above, there were many injection sites that were not observed early enough or often enough to be certain whether the various reactions occurred. Therefore, we had to establish criteria for including an injection in the analysis of possible covariates, and those criteria differed by reaction type. Swelling and stiffness, for example, tended to appear earlier and disappear more quickly than nodules. After careful examination of the data, we arrived at the following criteria: for nodules, at least one observation between days 7 and 90 postinjection, inclusive; and for swelling and stiffness, at least one observation between days 0 and 7 postinjection, inclusive. Note that, for these analyses, we are concerned only with whether or not a reaction occurred, not with how long it persisted. In all of these analyses, we pooled data from LBC and PRY in order to maximize sample size.

Results

Thirty-six mares treated at MCP received 72 injections by hand-held syringe, jabstick, or trocar. Because reactions from these hand injections were very rare (see section on Hand Injections, below), and because of the difficulty of observing injection sites at the distances involved, we made only limited followup observations at MCP after the horses were released back to the range. For LBC and PRY combined, 90 mares received a total of 314 injections in 2001–2007. Five mares representing eight injections were not observed postimmunization, leaving us with 714 individual observations of 133 injection sites on 29 mares at LBC and 726 observations of 173 injection sites on 56 mares at PRY (85 total mares, 306 total injections, 1,440 total observations). The majority of injections at LBC and PRY were remotely delivered by dart; 28 were delivered by hand.

Raw Counts of Reactions

For the three areas combined, eight injections resulted in abscesses (table 1). Four of the five abscesses at LBC occurred on a single mare. Six of the eight abscesses were first observed within 39 days of the injection, but we cannot be more definitive about when they formed. One abscess appeared at least 24 days after the injection. Six of the eight abscesses were resolved by the time our formal observations ended in the fall of 2007. One of the two unresolved abscesses at PRY was from a recent injection (July 7, 2007). This abscess had resolved by the time she was first observed in 2008. The other was an abscess that was first observed on day 329 postinjection (we do not know when it formed). The mare subsequently developed a large wound in the same location, which prevented further observation of the injection site. A veterinary examination in September 2007 concluded that the muscle tissue was not infected but was inconclusive in determining whether the wound resulted from the PZP injection, dart penetration, or some natural cause. When observed in April 2008, the large wound had resolved with scar tissue evident, but no further external sign of the abscess was present. The mean diameter for all abscesses (± SE) was 3.3 ± 0.37 cm (n = 9 observations of 8 abscesses, range = 1–5 cm). Figure 1 illustrates a typical abscess.

Nodules were observed more often than abscesses and more often remained unresolved at the end of our observations (table 1). Nodules formed as early as the day of the injection, and 51 were first observed within 90 days postinjection; the remaining 11 may have appeared in this period also, but we cannot confirm this from our observations. Seventeen nodules appeared at least 8 days postinjection, and one nodule appeared at least 48 days postinjection. While nodules often persisted for long periods (26 were observed at least 1 year postinjection), persistence did not preclude eventual disappearance (for example, one disappeared after at least 1,337 days and another after at least 1,114 days). Mean nodule size was 2.8 ± 0.10 cm (n = 194 observations of 62 nodules, range = 1–12 cm). Figure 2 illustrates two typical nodules.

Swelling was the second most common reaction (after nodules; table 1) and usually appeared soon after the injection; 28 of the instances that we observed were recorded on the day of the injection or the following day. At least 26 of the instances of swelling disappeared within 30 days postinjection, although one was still present at 62 days. The two instances of unresolved swelling represent sites that were last observed (in 2007) 28 and 33 days postinjection. Mean swelling diameter at LBC and PRY was 4.6 ± 0.35 cm (n = 64 observations of 49 cases of swelling, range = 1–12 cm). The two instances of swelling at MCP (from hand injections) were both 2 cm in diameter.

Stiffness resulted from 1 injection at LBC and 14 at PRY. Stiffness usually developed shortly after the injection; in 11 of the 15 cases it was observed in the first 3 days postinjection. In most cases, stiffness lasted a relatively short time; in 11 of 15 cases it disappeared within 24 days postinjection. This may also be the case for the remaining four injections, but our observations were not sufficiently frequent to narrow the time of disappearance.

Multiple reactions were recorded for only 10 of 1,440 (0.7 percent) individual observations. A combination of swelling and stiffness accounted for eight of these observations, with the other two involving stiffness and a nodule,

Table 1. Raw counts of injection-site reactions observed. Includes reactions from both hand and dart injections.

[— = not applicable]

	LBC		MCP	PRY	
	Hand injection	Dart injection	Hand injection	Hand injection	Dart injection
Abscesses observed	0	5	0	0	3
Abscesses resolved[1]	—	5	—	—	1
Nodules observed	1	17	0	0	44
Nodules resolved[1]	1	12	—	—	24
Swelling observed	0	8	2	0	41
Swelling resolved[1]	—	8	2	—	39
Stiffness observed	0	1	0	0	14
Stiffness resolved[1]	—	1	—	—	14

[1] Resolved indicates that the reaction had disappeared by the end of our observations in fall 2007.

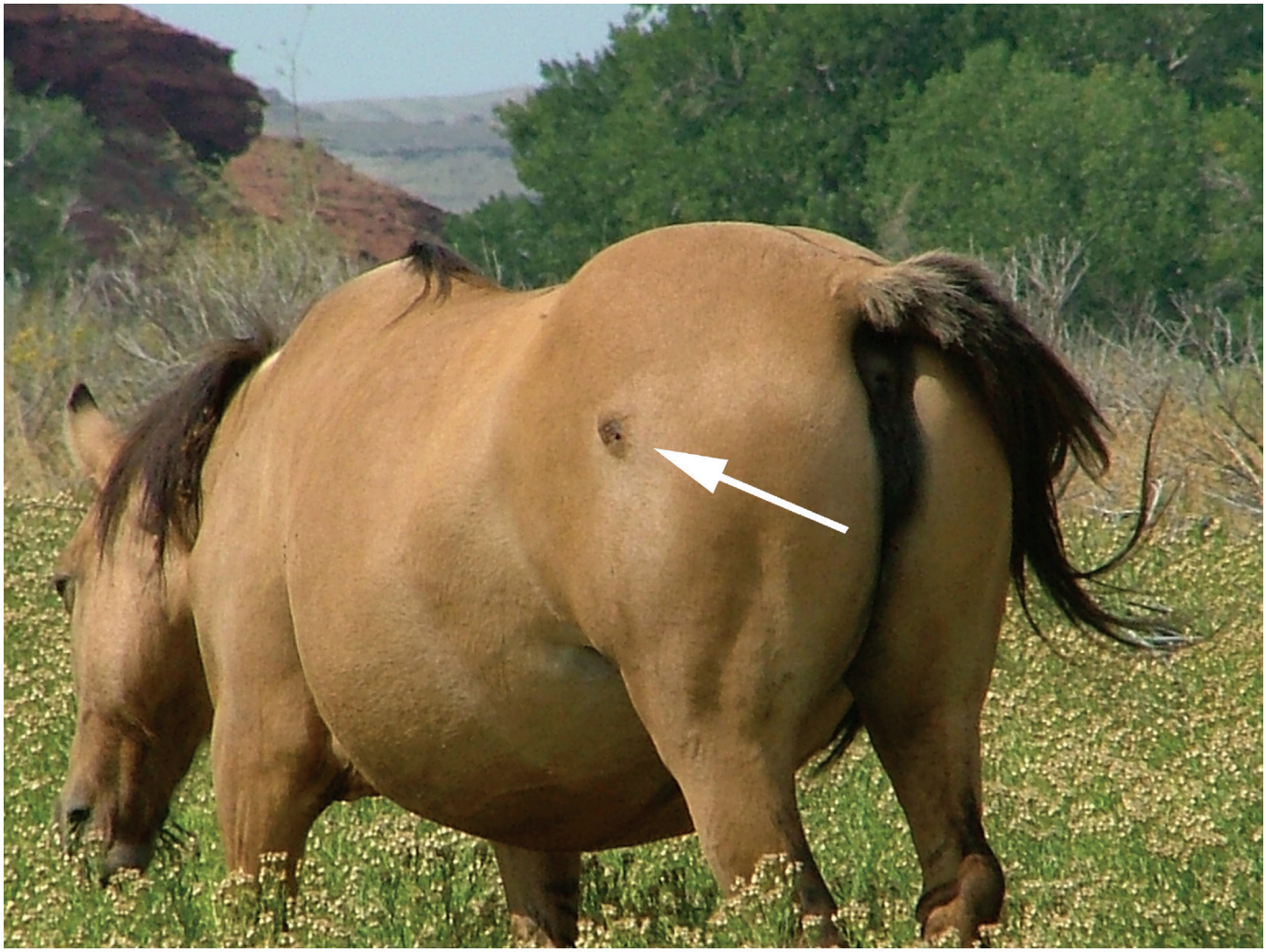


Figure 1. Abscess observed on Pryor Mountain horse #8901, 32 days after a booster injection delivered by CO_2 blowgun.

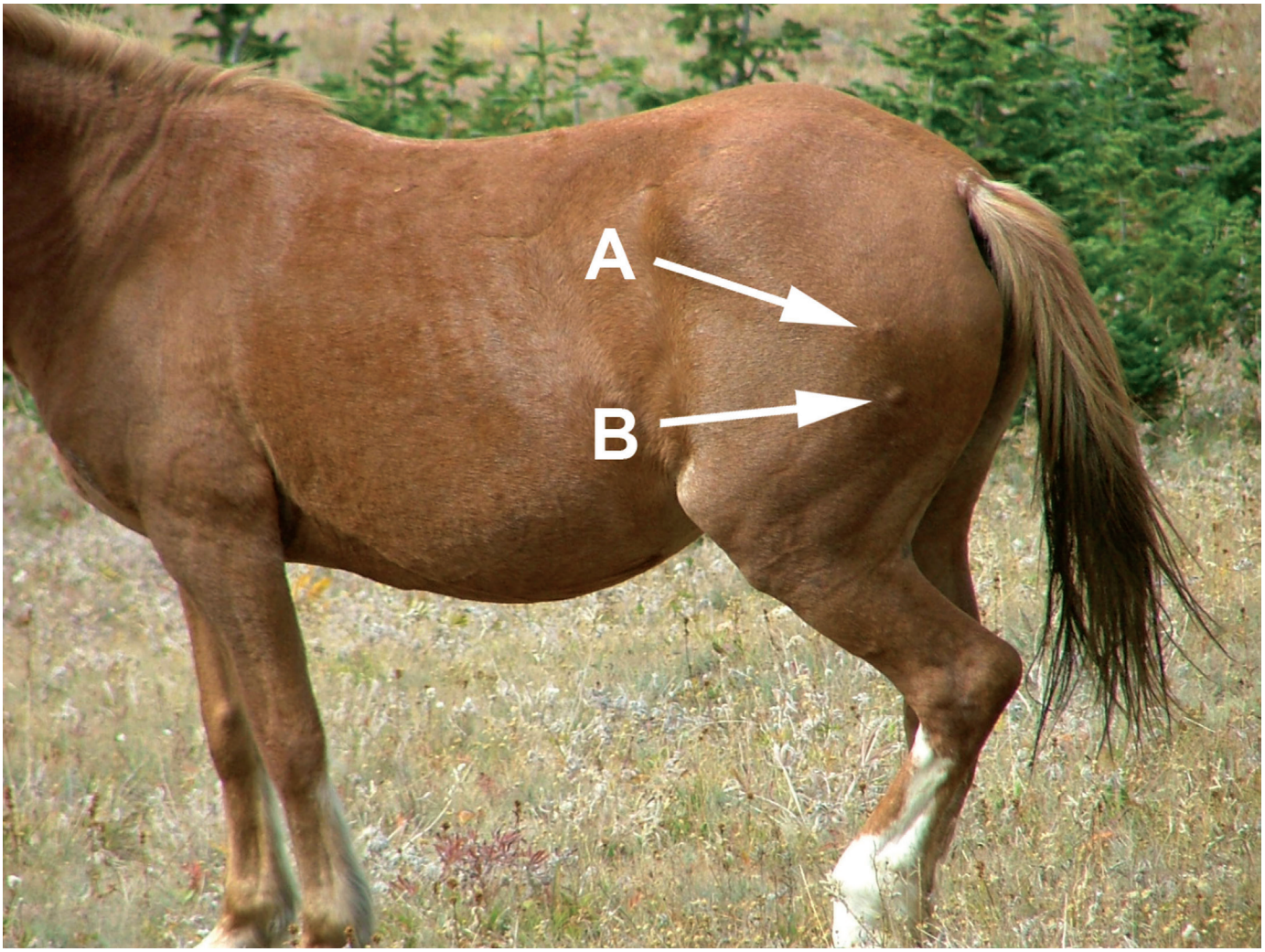


Figure 2. Two nodules observed on Pryor Mountain horse #9105, 2 years (*A*) and 56 days (*B*) after booster injections delivered by CO_2 blowgun.

and swelling and a nodule. In terms of injections, 35 (which includes the 10 mentioned above) of 306 (11.4 percent) resulted in multiple reactions, the most common combination being swelling and a nodule.

Hand Injections

The 72 injections by hand-held syringe, jabstick, or trocar at MCP resulted in two instances of swelling and no other reactions. In both cases, the swelling had disappeared by the time the animals were released back to the range. An additional 28 hand injections were given at LBC and PRY, resulting in only a single nodule, which formed on the day of the injection and persisted for about 150 days.

Dart Injections

Dart Performance

At LBC, 7 of 117 darts (6.0 percent) missed the animal completely and were not recovered. Four darts properly struck the target but were not recovered due to delayed ejection, rapid animal movement, thick vegetation, or some combination of these factors. Thus, 11 darts at LBC (9.4 percent of all darts fired) were not recovered. Similar data for PRY are incomplete and therefore are not reported here; however, due to the relatively short range of most shots at PRY, we believe that the unrecovered dart rate was likely lower than that at LBC.

The seven darts that missed the animal completely had no opportunity to discharge the drug properly and are thus not included in our assessment of dart performance (table 2). Based on the fact that the four other unrecovered darts struck the target properly, we assumed that they also functioned properly. Thus, for LBC and PRY combined, 278 injections were delivered remotely by dart from 2002 to 2007, and in 253 cases (91.0 percent) we judged that the injection was delivered properly (table 2). In 19 cases (6.8 percent), the dart either failed to discharge (the plunger was not depressed, two cases), partially discharged (the plunger was only partially depressed, three cases), split (the plastic portion split longitudinally, two cases; fig. 3), or came apart (the dart separated at the junction of the plastic and metal, 12 cases; fig. 3). In an additional six cases, the needle either broke off (three cases; fig. 3) or the dart hit bone (as evidenced by bone tissue in the recovered needle, three cases). When darts hit bone or came apart, the needles nevertheless fell free of the horse. In the few instances where the needle broke off, we do not know its fate. However, those three cases resulted in two nodules, both of which disappeared over time. The three injections where the dart hit bone cannot be considered a malfunction of the dart itself; excluding these three cases, 253 of 275 darts (92.0 percent) functioned properly.

Table 2. Performance of darts used in delivering the immunocontraceptive vaccine.

Dart performance	Number of darts		% of total
	LBC	PRY	
Bone plug	0	3	1.1
Came apart	5	7	4.3
Did not discharge	1	1	0.7
Functioned	102	151	91.0
Needle broke off	2	1	1.1
Partially discharged	0	3	1.1
Split	0	2	0.7
Total	110	168	100.0

Frequency of Reactions

Abscesses were relatively rare, resulting from at most 5–6 percent of the injections by dart (table 3). Most abscesses disappeared within 90 days of darting, though one persisted at PRY for more than 181 days. Nodules were both more frequent and more persistent than abscesses. Approximately 25 percent

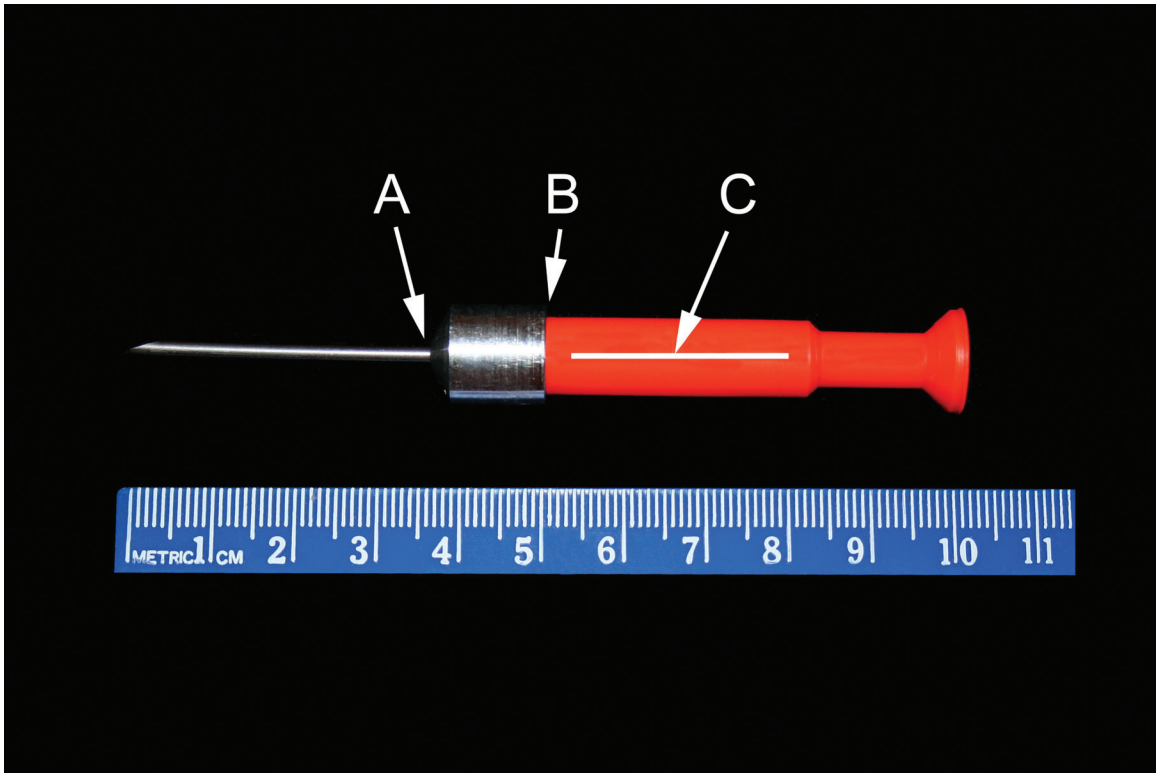


Figure 3. Example of a dart used for all injections. A is the point at which needles broke off, B is the point at which the metal separated from the plastic, and C illustrates the position of a typical longitudinal split in the cylinder.

of darts at both study areas resulted in nodules, and some nodules persisted into the fifth year. Most, however, eventually disappeared. Swelling resulted from about 11 percent of injections by dart at LBC and about 33 percent at PRY. At both study areas, swelling rarely persisted for more than 30 days and never for more than 90 days. Stiffness was recorded for 1.4 percent of the observations at LBC and 11.7 percent at PRY. In all cases, stiffness disappeared within the first 30 days.

Relation of Reactions to Covariates

For nodules, the criterion of at least one observation from day 7 through day 90 postinjection resulted in 185 of the 278 injections delivered by dart being selected for the analysis, including 50 (of the 61 known; see table 1) cases where a nodule occurred (27.0 percent) and 135 cases (73.0 percent) where none was observed (table 4). There perhaps was weak evidence that abnormal dart trauma (bone plug or broken needle) resulted in a higher occurrence rate for nodules, but the sample size for abnormal dart trauma was very small ($n = 4$). There was no evidence for a relation between nodule occurrence and either adjuvant formulation, delivery method, or age of mare.

The criterion of at least one observation from day 0 through day 7 posttreatment resulted in selection of 151 injections for analysis of swelling, including 43 (of the 49 possible) injections where swelling was known to have occurred (28.5 percent; see table 1), and an additional 108 (71.5 percent) injections for which swelling was not recorded (table 5). There was no evidence for a relation between swelling and either dart trauma, adjuvant, or age of mare, but strong evidence that swelling was more frequently observed when darts were delivered by blowgun than when they were delivered by rifle. Most (91.7 percent) of the 157 injections delivered by blowgun were at PRY.

Because the same criterion was used to select injections for analysis of both swelling and stiffness, the same 151 observations were chosen for both types of reactions. In the case of stiffness, this criterion resulted in the selection of 14 (of the 15 possible) injections known to have caused stiffness (9.3 percent; see table 1) and an additional 137 injections (90.7 percent) for which stiffness was not recorded (table 6). There was no apparent relation between stiffness and any of the covariates, though there were again few instances of abnormal dart trauma.

Table 3. Percentage of injection sites exhibiting abscesses, nodules, swelling, or stiffness as a function of time after injection. Only injections delivered by dart are included. Multiple observations of an injection site in a period were counted as a single observation in determining sample size (*n*). The reaction was coded as present if it was recorded for any observation during the time period.

[— = not applicable]

HMA	Time after injection	*n*	% Abscess	% Nodule	% Swelling	% Stiffness
LBC	0–30 days	73	5.5	12.3	11.0	1.4
	31–90 days	40	2.5	25.0	0.0	0.0
	91–180 days	16	0.0	18.8	0.0	0.0
	181–365 days	62	0.0	12.9	0.0	0.0
	Year 2	80	0.0	7.5	0.0	0.0
	Year 3	62	0.0	6.5	0.0	0.0
	Year 4	49	0.0	6.1	0.0	0.0
	Year 5	27	0.0	0.0	0.0	0.0
	Year 6	—	—	—	—	—
PRY	0–30 days	120	0.8	18.3	32.5	11.7
	31–90 days	75	1.3	25.3	2.7	0.0
	91–180 days	17	0.0	23.5	0.0	0.0
	181–365 days	66	1.5	22.7	0.0	0.0
	Year 2	76	0.0	19.7	0.0	0.0
	Year 3	21	0.0	28.6	0.0	0.0
	Year 4	21	0.0	19.0	0.0	0.0
	Year 5	38	0.0	5.3	0.0	0.0
	Year 6	11	0.0	0.0	0.0	0.0

Table 4. Relation between occurrence of nodules and the covariates dart trauma, adjuvant, delivery method, and age of mare (n = 185 injections).

	Frequency distribution[1]	
	Nodule occurrence	
Covariate	No	Yes
Dart trauma		
Abnormal	1 (25.0)[2]	3 (75.0)
Normal	134 (74.0)	47 (26.0)
Adjuvant		
FCA	24 (70.6)	10 (29.4)
FIA	100 (74.1)	35 (25.9)
FMA	11 (68.7)	5 (31.3)
Delivery method		
Blowgun	86 (70.5)	36 (29.5)
Rifle	49 (77.8)	14 (22.2)

	Logistic analysis			
		Odds ratio		
Covariate	*P*	Point Estimate	95% Wald Confidence Limits	
Dart trauma				
Abnormal vs. normal	0.07	8.729	0.874	87.235
Adjuvant				
FIA vs. FCA	0.64	0.820	0.313	2.148
FMA vs. FCA	0.87	0.998	0.254	3.922
Delivery method				
Blowgun vs. rifle	0.54	1.297	0.568	2.957
Age of mare	0.68	1.015	0.944	1.092

[1] Age of mare was analyzed as a continuous variable and thus is not included in this portion of the table.

[2] Numbers in parentheses = percentage of row total.

Table 5. Relation between occurrence of swelling and the covariates dart trauma, adjuvant, delivery method, and age of mare (n = 151 injections).

	Frequency distribution[1]	
	Swelling occurrence	
Covariate	No	Yes
Dart trauma		
Abnormal	3 (60.0)[2]	2 (40.0)
Normal	105 (71.9)	41 (28.1)
Adjuvant		
FCA	16 (66.7)	8 (33.3)
FIA	89 (74.2)	31 (25.8)
FMA	3 (42.9)	4 (57.1)
Delivery method		
Blowgun	55 (61.1)	35 (38.9)
Rifle	53 (86.9)	8 (13.1)

	Logistic analysis			
		Odds ratio		
Covariate	*P*	Point Estimate	95% Wald Confidence Limits	
Dart trauma				
Abnormal vs. normal	0.55	1.856	0.243	14.167
Adjuvant				
FIA vs. FCA	0.53	1.301	0.449	3.768
FMA vs. FCA	0.21	3.232	0.529	19.741
Delivery method				
Blowgun vs. rifle	0.001	5.023	1.921	13.136
Age of mare	0.17	0.949	0.882	1.022

[1] Age of mare was analyzed as a continuous variable and thus is not included in this portion of the table.

[2] Numbers in parentheses = percentage of row total.

Table 6. Relation between occurrence of stiffness and the covariates dart trauma, adjuvant, delivery method, and age of mare (n = 151 injections).

	Frequency distribution[1]	
	Stiffness occurrence	
Covariate	No	Yes
Dart trauma		
Abnormal	4 (80.0)[2]	1 (20.0)
Normal	133 (91.1)	13 (8.9)
Adjuvant		
FCA	19 (79.2)	5 (20.8)
FIA	112 (93.3)	8 (6.7)
FMA	6 (85.7)	1 (14.3)
Delivery method		
Blowgun	78 (86.7)	11 (13.3)
Rifle	59 (96.7)	2 (3.3)

	Logistic analysis			
		Odds ratio		
Covariate	*P*	Point Estimate	95% Wald Confidence Limits	
Dart trauma				
Abnormal vs. normal	0.46	2.604	0.209	32.444
Adjuvant				
FIA vs. FCA	0.24	0.263	0.066	1.042
FMA vs. FCA	0.80	0.384	0.034	4.336
Delivery method				
Blowgun vs. rifle	0.21	2.861	0.562	14.572
Age of mare	0.20	1.073	0.964	1.196

[1] Age of mare was analyzed as a continuous variable and thus is not included in this portion of the table.

[2] Numbers in parentheses = percentage of row total.

Discussion

Darting

Excluding the three injections that resulted in bone plugs, the overall failure rate of darts was 8 percent, but failures were not distributed evenly through time. In 2002–2003, 20 of 112 darts (17.9 percent) failed to function properly. Following changes in manufacturing, the failure rate in 2004–2007 dropped to 2 of 163 (1.2 percent). This was an important improvement in the dart manufacturing process given the time and effort expended to find and dart individual horses. The small number of darts lost on the range presumably represent little biological hazard should they be found because impact with the ground or other natural objects probably triggered the release mechanism in the dart and discharged the contents. The very small amounts of PZP and adjuvant discharged also presumably do not pose an environmental contamination threat. The physical hazards of darts, as well as pieces of darts that were unrecoverable (broken needles or needle/cap separations), could pose minor danger if found by visitors simply due to the sharp nature and aging metal present. However, given the intensity of search effort by the darters at the time of operations, it is unlikely that an unrecovered dart would be discovered, even if another person passed by the exact location in these remote areas.

Abscesses

Our results for injections delivered by hand-held syringe or jabstick are consistent with the scant information on abscesses reported in the literature for equids. We observed no abscesses in 72 hand injections of 36 mares at MCP, and no abscesses (but a single nodule) in 28 hand injections at LBC and PRY combined. Turner and others (1997) hand-injected 60 mares in the gluteus muscle with PZP and FCA, and held them in corrals for 30 days awaiting a booster injection. No abscesses were observed in this 30-day period, and the authors further concluded that abscesses were unlikely to have occurred in an additional group of mares that were treated with a single injection of PZP and FCA and released within 72 hours posttreatment. In a similar experiment, Turner and others (2001) hand-injected 95 mares in the gluteus muscle with PZP and FCA and an additional 60 mares in the same location with PZP, FCA, and a carbomer adjuvant. These 155 mares were held in corrals for 13–17 days awaiting a booster treatment, and no abscesses were observed during this period. Further observations of some of these mares after release showed no injection-site marks, leading the authors to conclude that abscesses had not occurred. Lyda and others (2005) treated 7 mares with PZP plus FCA and 8 mares with PZP plus FMA; all 15 mares received a booster injection of PZP plus FIA 27 days later. All injections were delivered by hand in the hip or gluteal muscles. The 30 total injections in their study resulted in one abscess (3.3 percent of injections) following a booster with FIA in a mare initially treated with FMA.

Thus, it appears that abscesses are rare in horses for injections of PZP and any of the formulations of Freund's adjuvant where the injections are delivered by hand in the hip region. The location of the injection may be important, however. Lyda and others (2005) cited a personal communication from I.K. Liu indicating that abscesses can occur more frequently in horses receiving injections of PZP and FCA in the neck.

Information on abscesses resulting from injections delivered remotely to horses is similarly scarce. Kirkpatrick and others (1990) darted mares at Assateague Island National Seashore with PZP and FCA (n = 26 mares), followed by PZP and FIA boosters (n = 26, injection 2; n = 18, injection 3). Following the third injection (with FIA), they observed three abscesses, or a frequency of 4.3 percent (in 70 injections). Following several additional years of treatment at Assateague, the number of abscesses was reported as either two (Turner and Kirkpatrick, 2002) or three (Lyda and others, 2005) in a total of 381 injections (0.5 or 0.8 percent). Interestingly, both of these later publications attributed only one of the abscesses to an injection containing FIA. In any case, the frequency of occurrence of abscesses was low.

Our results for frequency of occurrence of abscesses in mares darted at PRY (0.8 percent; table 3) were very similar to those reported above, but somewhat higher (5.5 percent) at LBC. The higher rate at LBC is mostly a result of four abscesses occurring in a single mare that was clearly prone to reaction. In addition, however, our approach to calculating frequency of occurrence of reactions, necessitated by the fact that we were unable to observe injection sites on a regular schedule, is strictly valid only if the observations can be considered a random sample of all of the injection sites present in an interval. This is probably not the case, because field personnel were instructed to monitor injection sites where there was a known reaction as closely as possible until the reaction was resolved, and then record observations only opportunistically. In fact, the mean number of observations for injection sites that showed a reaction (5.10) was slightly higher than the mean number of observations for injection sites that did not show a reaction (4.53; generalized linear model with Poisson distribution, P = 0.03). Thus, it is likely that our results for frequency of occurrence of all reaction types are biased somewhat high.

Kirkpatrick and others (1990) also reported that the three abscesses they observed formed within about 48 hours of the injection and healed completely within 14 days. The single abscess observed in the study by Lyda and others (2005) appeared 1 month after a booster inoculation; time until healing was not reported. While our observations on the timing of appearance and disappearance of abscesses are not as precise, we do know that one abscess became visible at least 24 days posttreatment and that six of the eight abscesses observed were present on day 15. Additionally, one abscess was present at day 77 postinjection, and another at day 329. Thus, it appears that abscesses can form as much as a month posttreatment and can occasionally persist for much longer than has been reported previously.

Other Reactions

We are unaware of any published information on occurrence of other injection-site reactions in equids treated with PZP. This is curious, particularly for nodules, which in our study were fairly common and sometimes persisted for long periods. It is unclear whether reactions similar to what we termed nodules simply did not occur in other studies, or perhaps were considered too inconsequential to report. Similar reactions have occurred in other species, however. Turner and others (1992, p. 155), working on white-tailed deer (*Odocoileus virginianus*), reported that "a raised area of about 20 mm in diameter developed in the final injection site on 3 does." These reactions disappeared in 8–10 days in two cases and after 5 weeks in the third. Patton and others (2005, p. 160) also mention that the Freund's adjuvants often cause formation of a small (marble size) granuloma at the injection site, but they do not provide any examples.

Covariates

We were unable to detect a relation between any of the reactions we observed and the adjuvant used. Coupled with the fact that reactions are rare for injections delivered by hand, this suggests that most reactions are probably the result of trauma associated with dart delivery, or a combination of dart trauma and adjuvant. With that observation in mind, we were somewhat surprised to find a higher probability of swelling when darts were delivered by blowgun, which was used at closer ranges and allowed for variable power. The average distance from which darts were fired by blowgun was 11.8 ± 0.24 m, whereas for darts fired by rifle it was 31.8 ± 1.03 m, and the majority of the shots fired by blowgun (144 of 157, or 91.7 percent) were at PRY, where horses are generally more approachable than at LBC. Greater approachability at PRY probably led to both more frequent use of the blowgun and greater ability to detect swelling.

Conclusion

PZP is a promising management tool for limiting population growth in wild horses; however, as with any management tool, side effects, practicality of use, and physiological and biological implications must also be considered. PZP has been clinically tested for safety and some potential physiological side effects in both domestic and wild equids (Liu, 1989; Kirkpatrick and others, 1992, 1995, 1996; Kirkpatrick and Turner, 2002;), but there is less information available regarding safety in field applications. Currently, the most time-efficient method for applying PZP is by hand-delivered injection of 2-year pellets when horses are gathered. We observed only two instances of swelling from this formulation. Use of remotely delivered, 1-year PZP is generally limited to populations where individual animals can be accurately identified and repeatedly approached. This formulation produced injection-site reactions of varying intensity, though none of the observed reactions appeared debilitating to the animals. The longer term nodules observed did not appear to change any animal's range of movement or locomotor patterns and in most cases did not appear to differ in magnitude from naturally occurring injuries or scars. We were unable to perform histological examinations of these nodules, but they may be similar to granulomas reported by other investigators following administration of Freund's adjuvant. Further analyses should be considered to determine whether they pose any long-term physiological threat to the animal. Injection-site reactions represent only one of the potential effects of PZP on horses. Other factors, such as treatment efficacy, long-term ovarian function, individual and social behavior, and seasonality of parturition by post-treated mares are also important considerations for the assessment of PZP as a management tool.

Acknowledgments

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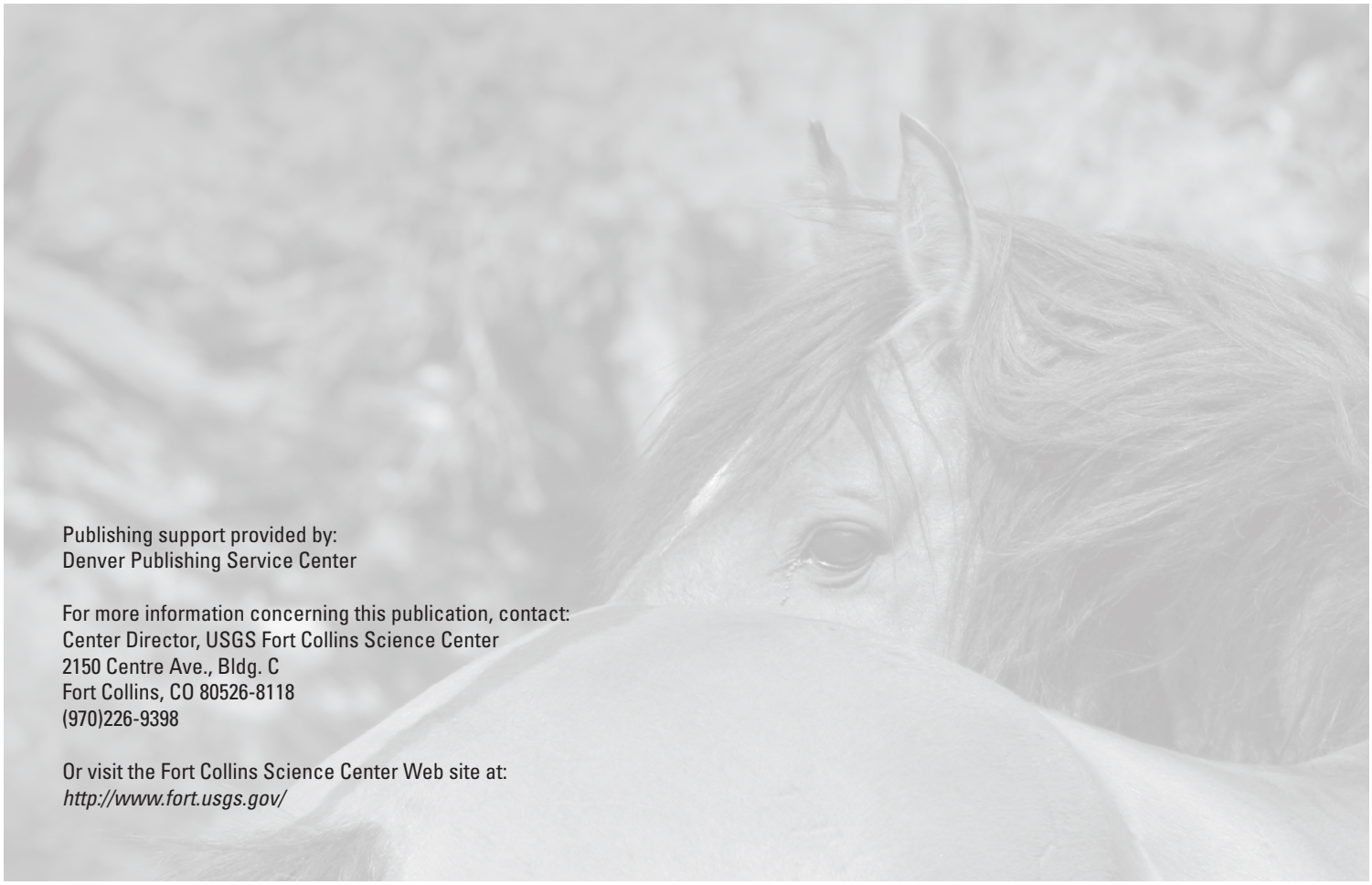
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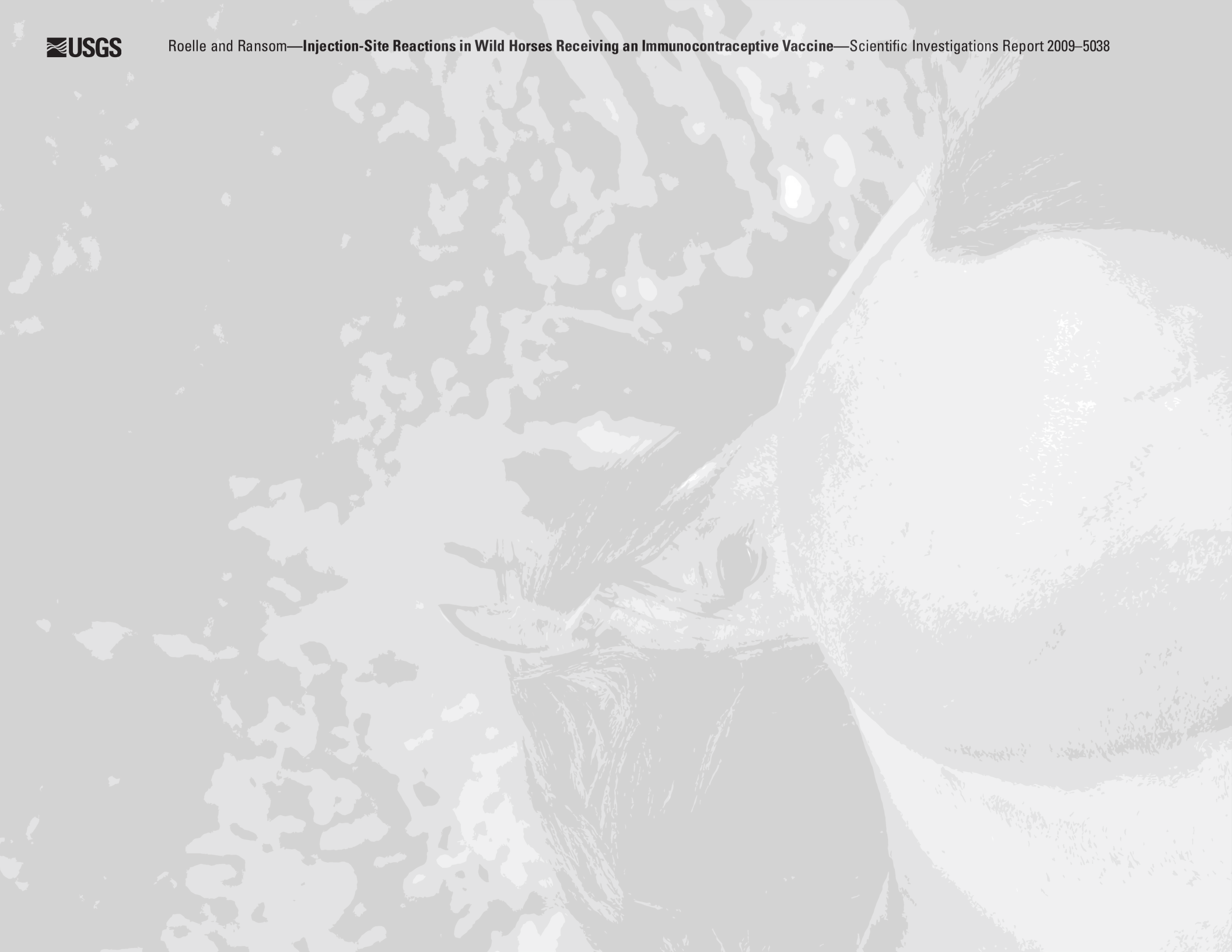
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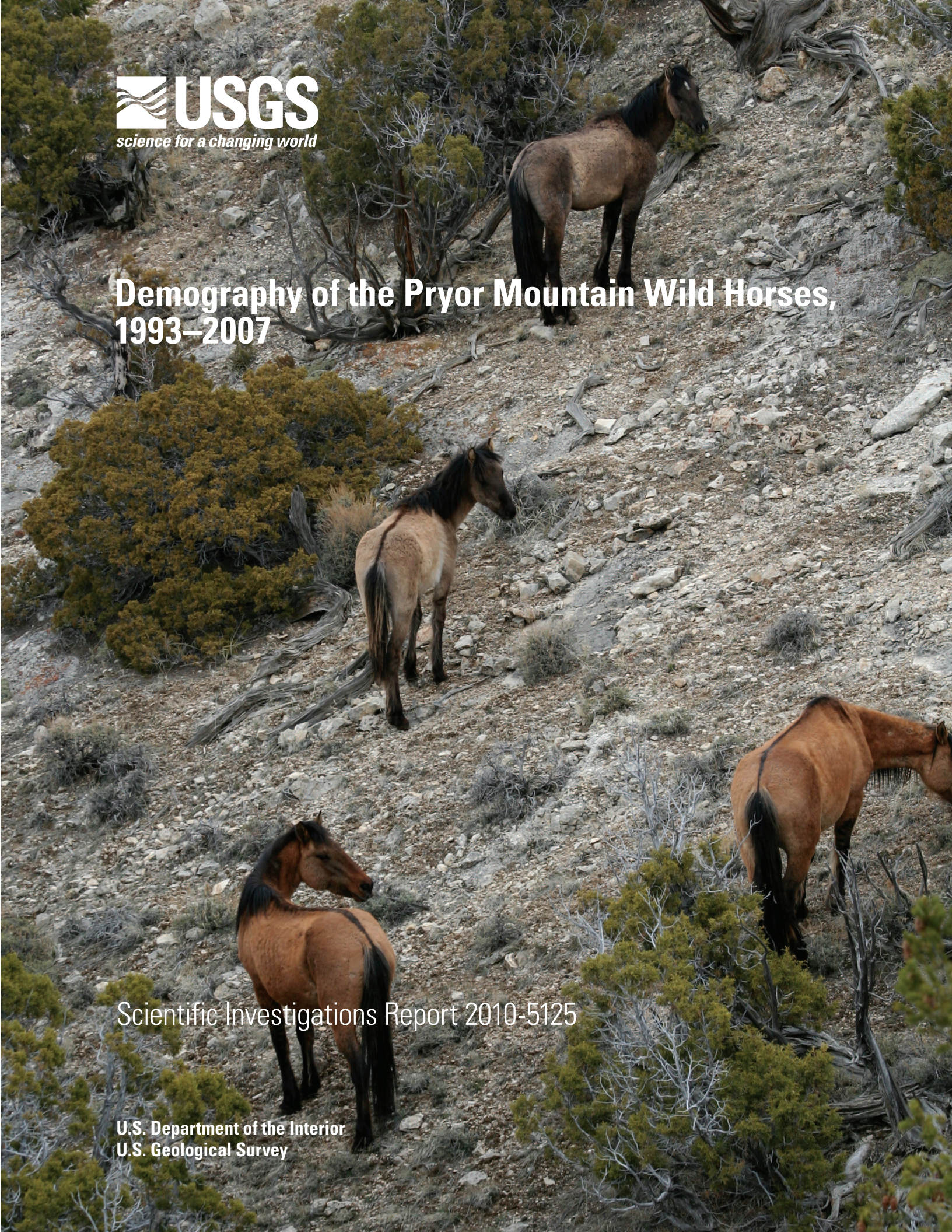


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Conversion Factors

Inch/Pound to SI

Multiply	By	To obtain
	Length	
inch (in.)	2.54	centimeter (cm)
foot (ft)	0.3048	meter (m)
mile (mi)	1.609	kilometer (km)
yard (yd)	0.9144	meter (m)
	Area	
acre	0.4047	hectare (ha)
square mile (mi^2)	259.0	hectare (ha)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

$$°F=(1.8×°C)+32$$

Acronyms Used in Report

AML	Appropriate Management Level
APHIS	Animal and Plant Health Inspection Service
BLM	Bureau of Land Management
FS	Forest Service
HMA	Herd Management Area
NCDC	National Climatic Data Center
NPS	National Park Service
PMWHR	Pryor Mountain Wild Horse Range
RAWS	Remote Automated Weather Station
USGS	U.S. Geological Survey

Demography of the Pryor Mountain Wild Horses, 1993–2007

By James E. Roelle,[1] Francis J. Singer,[1,2] Linda C. Zeigenfuss,[1] Jason I. Ransom,[1] Linda Coates-Markle,[3] and Kathryn A. Schoenecker[1]

Abstract

Wild horses (*Equus caballus*) at Pryor Mountain were studied by direct observation from 1993 through 2007. All horses present were individually identifiable on the basis of coat coloration, head and leg markings, gender, and band associations. Of the 609 horses either present prior to foaling in 1993 or born since, ages were precisely known for 491 (observed as a foal). Ages for 52 horses were estimated through tooth eruption and wear patterns, and for the remaining 66 horses through body size, morphology, and anecdotal evidence concerning when they were present on the range. At varying intensities, never less than 30 days per year, all horses were inventoried and their band associations noted. Foals were paired with dams based on observations of attachment during the early days and weeks of life. Year of death was determined by identification of the carcass where possible. In the absence of finding a carcass, an animal that was not observed for 2 years was considered to have died in the year that it went missing. Animals that were removed from the herd and mares that were part of a contraception study were excluded from calculations of survival and foaling rates, respectively, as appropriate.

The average prefoaling population over the 15 years of the study was 148.8 animals (range = 120–187), and the annual foal crop averaged 32.1 (range = 23–40). Large removals (19–60 animals) in four years helped maintain the herd at this level; apparent growth rate (calculated as though removals had not occurred) was 9.6 percent annually ($\lambda = 1.096$, range = 0.977–1.220). This annual growth rate is relatively low compared to that for many western horse herds, at least in part because of a decline in foal survival. Sex ratio of the foal crop varied widely among years, but pooled across years did not differ from 50:50. Sex ratio in the herd changed mostly as a result of removals. The average age of both males and females in the herd increased during the course of the study. Annual survival of males did not differ from that of females, nor did gender affect annual survival of foals. Pooled across years, ages, and sexes, the annual survival rate was 0.899. Annual foal survival rate was 0.697 and declined through time, with a tendency toward recovery in 2005–2007. Foal survival was higher in larger bands, but did not differ between foals born to primiparous and multiparous mares. A few 2-year-old mares produced foals; foaling rate (excluding contracepted mares and foals they produced) increased through age 10, remained high through age 15, and declined thereafter. Overall foaling rate for mares ≥3 years of age was 0.576 foals per mare, with no apparent trend during the period of our study. Foaling rate in years following gathers was somewhat lower than in other years. There was a positive relation between foaling rate and band size. Primiparous mares were somewhat less likely to foal in the following year than were multiparous mares. Most stallions that acquired a harem did so at age 5 or 6, and the average age of harem stallions increased during our study. Most harems had 1–3 mares ≥2 years of age, but harem size varied with age of the stallion, increasing through about age 11 and declining thereafter. About 6 percent of bands had a satellite stallion (≥5 years of age), but the mean number of mares did not differ between single- and multistallion bands. Most stallions left their natal band at age 2 or 3, but 17 percent remained with their natal band until age 4 or 5.

Foal survival rate was positively related to precipitation, suggesting a possible link to forage production and availability mediated through mare fitness. There also was evidence for density-dependent population regulation, as both population growth rate and survival rate were negatively correlated with population size from the previous year. These and other factors were not sufficient to stabilize the population during our period of study, however, as evidenced by the necessity for large removals in several years.

Introduction

The Pryor Mountain Wild Horse Range was created in 1968 by order of the Secretary, U.S. Department of the Interior, who directed that wild horses (*Equus caballus*) be managed in "a balanced program which considers all public values and without impairment of the productivity of the land" (Federal Register, Document 68-11056, Sept. 11, 1969;

[1] U.S. Geological Survey, Fort Collins, Colorado.

[2] Deceased.

[3] Bureau of Land Management, Wheat Ridge, Colorado.

Bureau of Land Management, 2006, p. 5). The Bureau of Land Management (BLM) was designated as the lead agency for management of wild horses, though the range also contains lands administered by the U.S. Forest Service (FS; Custer National Forest) and the National Park Service (NPS; Bighorn Canyon National Recreation Area). The Wild Free-Roaming Horses and Burros Act of 1971 (P.L. 92-195) further directed the FS and BLM to manage wild horses at Pryor Mountain and wild horses and burros (*Equus asinus*) wherever they occur "in a manner that is designed to achieve a thriving natural ecological balance on the public lands."

While laudable as general management goals, the conditions implied by phrases such as "a balanced program which considers all public values without impairment of the productivity of the land" and "a thriving natural ecological balance" have proven difficult to achieve for many wild horse herds. With the protection afforded by the Act and a dearth of natural predators, many wild horse herds can increase at annual rates of 15–25 percent (Eberhardt and others, 1982; Garrott and Taylor, 1990; Garrott and others, 1991b; Singer and others, 2000), thus threatening to exceed the carrying capacity of their ranges. BLM has addressed this problem largely through a gather and removal program in which excess horses are removed from the range and offered to the general public for adoption or sale to good homes. While hugely successful by some measures (BLM has adopted out more than 220,000 horses since 1971; *http://www.blm.gov/wo/st/en/prog/wild_horse_and_burro/wh_b_information_center/Fact_Sheet.html*), supply has exceeded demand, leaving nearly 32,000 horses in BLM corrals and long-term holding pastures. This approach is not embraced by all members of the public. Wild horse advocacy groups often favor a policy of minimum disturbance and intervention, arguing that there are too few, rather than too many, wild horses and that the gather and removal process is detrimental to horse health and well-being. On the other hand, ranchers and conservationists often see wild horses as competing with domestic cattle (*Bos taurus*) and native wildlife for forage, and would thus prefer that BLM reduce the size of many horse herds.

Given the contentious nature of the issues involved, BLM has a continuing need for the best scientific information available on the ecology and demography of wild horse herds. Since creation of the range in 1968, information on wild horses has been gathered nearly continuously by trained investigators and volunteers, making the Pryor Mountain horses perhaps the most studied herd in the Western United States. Furthermore, all of the horses at Pryor Mountain are individually identifiable (unique color markings), and many are of known (observed as a foal) or closely estimated (tooth eruption and wear patterns) age, making the resulting data dependable. The first study of horses at Pryor Mountain was initiated in 1970 by J.D. Feist, who focused largely on behavior (Feist, 1971), but also gathered some information on demography of the population (Feist and McCullough, 1975). Shortly thereafter, Hall (1972) performed an assessment of the herd and possible management alternatives. Perkins and others (1979) studied population dynamics, age distribution, and band composition in the Pryor Mountain herd from 1974 through 1978. Garrott and Taylor (1990), using information gathered by BLM personnel, reported on many aspects of herd demography from 1972 through 1986. F.J. Singer and several colleagues began studies at Pryor Mountain in the early 1990s, broadening their scope to include vegetation dynamics and conservation genetics as well as demography (Singer and Schoenecker, 2000). Prior to his untimely death in 2005, Singer also participated in work at Pryor Mountain on contraception and population estimation.

Here we report on the demographics of the Pryor Mountain wild horse herd from 1993 to 2007. The results contained herein represent a more detailed analysis of the data presented by Singer and others (2000) and an extension covering the next decade. While specific to the Pryor Mountain herd, and thus not necessarily representative of the other 180 Herd Management Areas (HMAs) overseen by BLM, we hope that our results will provide wild horse managers with useful insights as they continue to meet the challenges of managing wild horse herds in the Western United States.

Study Area

During the period of our study, horses actually used an area larger than the Pryor Mountain Wild Horse Range as originally designated. The northwestern and northern boundaries of our study area are not well defined, as there are no natural or manmade barriers to horse movement in that direction. As shown in figure 1, however, the study area encompasses about 22,300 hectares (ha). The following description of the area largely follows Singer and others (2000) and Ricketts (2004).

The study area lies along the Montana-Wyoming border at approximately latitude 45°4'N and longitude 108°19'W, 76 kilometers (km) south of Billings, Mont., and 14 km north of Lovell, Wyo. (fig. 1). The area is topographically diverse, dominated by narrow canyons and steep cliff faces. Elevations range approximately from 1,175 to 2,670 meters (m), with corresponding variation in climate and vegetation. Temperatures range from 40 ºC in the summer to -34 ºC in the winter. Mean annual precipitation ranges from 15 centimeters (cm) at lower elevations to nearly 70 cm at higher elevations, with much of the annual total falling in April–June. Midelevation ridges and plateaus often remain snow free or accumulate <10 cm of snow as a result of winds and southern and western exposures. Higher elevations often have winter snow accumulations of 1 m or more. In water years 1993–1998 (water year 1993, for example, runs from October 1992 through September 1993), mean annual precipitation at Lovell, Wyo. (*http://www.wrcc.dri.edu/summary/climsmwy.html*), was 17.2 cm, essentially identical to the 30-year average (Bureau of Land Management and others, 2008). From 1999 to 2004, however, severe drought conditions prevailed (mean annual precipitation = 12.3 cm). Prolonged drought can decrease plant health

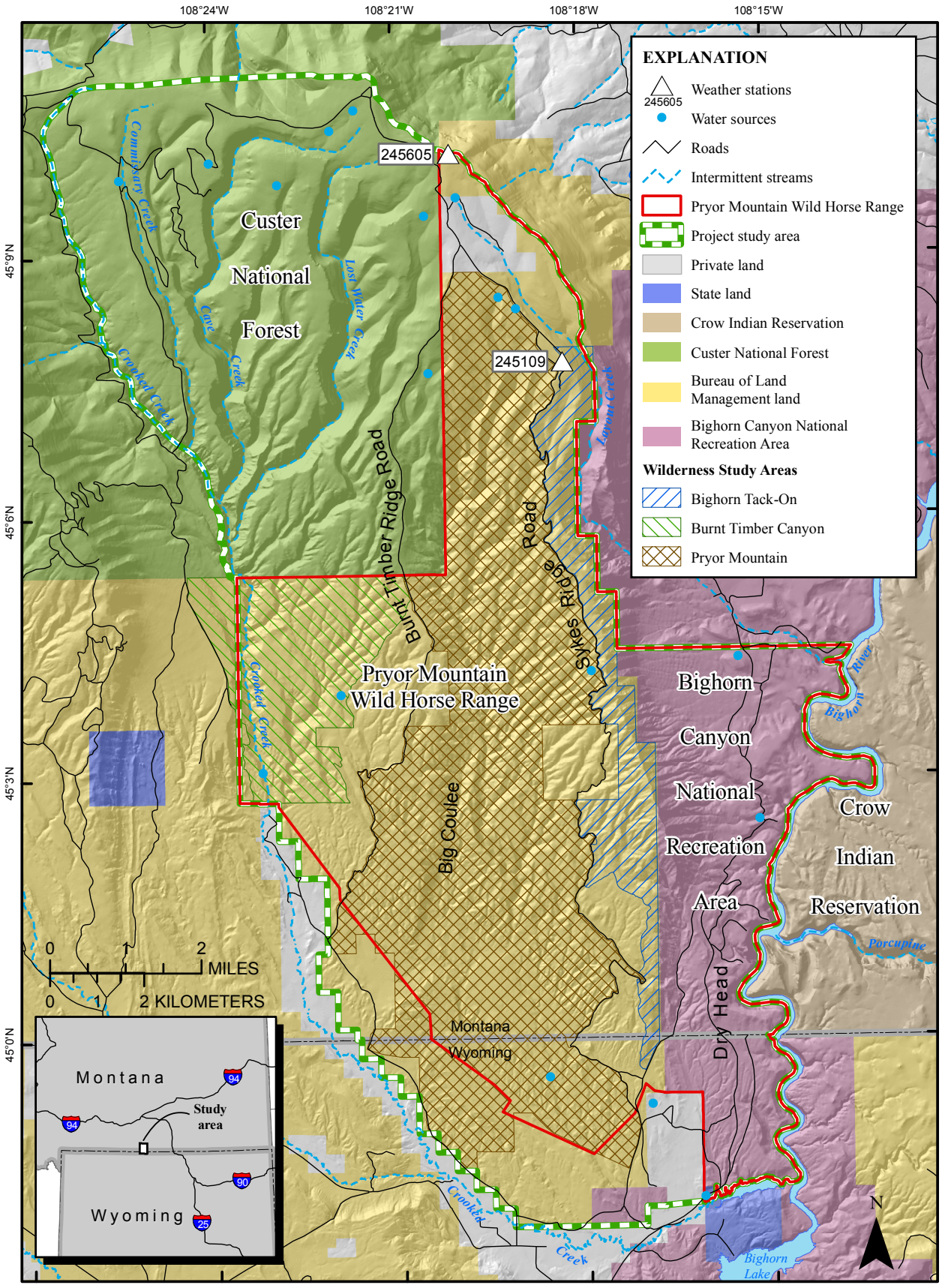


Figure 1. Location of the Pryor Mountain Wild Horse Range and study area.

and vigor and forage production. Temporary relief from the drought occurred in 2005 (annual precipitation = 21.2 cm) and 2007 (annual precipitation = 14.3 cm for 11 months, data missing for June) At least in 2005, greater precipitation probably resulted in improved forage production (Bureau of Land Management, 2006).

Vegetation in the study area changes dramatically with elevation. At the highest elevations, subalpine meadows (Idaho fescue, *Festuca idahoensis*; alpine timothy, *Phleum alpinum*; alpine bluegrass, *Poa alpina*; Ross sedge, *Carex rossii*; silky lupine, *Lupinus sericeus*) are interspersed with stands of subalpine fir (*Abies lasiocarpa*) and Engelmann spruce (*Picea engelmannii*). At somewhat lower elevations, Douglas fir (*Pseudotsuga menziesii*) occurs with an understory of snowberry (*Symphoricarpos* spp.). Still lower is a shrub community dominated by big sagebrush (*Artemisia tridentata*), rubber rabbitbrush (*Ericameria nauseosus*), shrubby cinquefoil (*Dasiphora fruticosa*), Utah juniper (*Juniperus osteosperma*), and curl-leaf mountain mahogany (*Cercocarpus ledifolius*) and a variety of grasses and forbs such as bluebunch wheatgrass (*Pseudoroegnaria spicata*), threeawn (*Aristida purpurea*), needle-and-thread (*Hesperostipa comata*), and blue grama (*Bouteloua gracilis*). For a more complete description of vegetation communities on the Pryor Mountain Wild Horse Range, see Ricketts (2004).

Herd Behavior and Management

The social structure of the Pryor Mountain wild horse population consists of bands that include a harem stallion, occasionally along with a satellite stallion, plus adult mares and yearlings and foals of both sexes. In general, we use the term band to refer to all members of the group and harem to refer to mares ≥2 years of age. Males older than yearlings that have not acquired a band usually are found in bachelor groups. Distribution and movements of the bands largely are restricted by topography to the west (Crooked Creek drainage) and east (Bighorn Canyon), and fences of varying integrity to the north and south (fig. 1). The wild horses occur in three more or less discrete subpopulations (Burnt Timber Ridge, Sykes Ridge, and Dryhead). These areas are separated by Big Coulee (a deep canyon) and a long, nearly vertical cliff face. However, the horses are able to move between these areas at high elevations and below Big Coulee, as well as occasionally by way of trails on the cliff face. Varying numbers of horses also use U.S. Forest Service land to the north and west of the original horse range boundary. Interchange of horses between subpopulations occurs regularly, but not with high frequency. The horses move with the seasons, spending winters at low and middle elevations where snow is less deep and summering at higher elevations. Distribution is restricted by available water in summer, when the horses tend to congregate near water sources. Water has less effect on distribution in winter, when the horses use snow to meet their water needs.

In the past, the herd has been managed consistent with national selective removal policy for the BLM Wild Horse and Burro Program, removing mostly young animals and conserving the core breeding component of the population. In general, BLM defines Appropriate Management Level (AML) as the number of adult wild horses (6 months and older) determined through the agency planning process to be consistent with the objective of achieving and maintaining a thriving natural ecological balance and multiple-use relation (Bureau of Land Management, 2006). Initially, the AML for the Pryor Mountain herd was set at 115–127, but was revised to 85–105 horses in 1992. However, based on genetic research (Cothran and Singer, 2000; Gross, 2000a,b), from 1996 to 2006 BLM attempted to manage the herd at an average of 140 adult horses to help conserve genetic diversity (Bureau of Land Management, 2006). More recently, BLM has determined that management at an AML of 90–120 adult horses is necessary to protect and improve rangeland health (Bureau of Land Management, 2009).

The BLM has managed the size of the Pryor Mountain herd primarily through a gather, removal, and adoption program. In 1994, wranglers on horseback attempted to capture the entire herd by moving horses into corral traps. In 1997 and 2001, a helicopter was used to move most of the family bands (individual bachelors typically were not gathered) into corral traps; approximately 125 animals were gathered in each of these years. Smaller gathers aimed at removing excess bachelor stallions and yearling females were conducted in 2003 and 2006. The 2003 gather involved a helicopter to move groups containing target horses into a corral trap. The 2006 gather was conducted using bait trapping to lure the horses into a trap, after which they were transported by vehicle to a holding corral.

Since 2001, some mares in the Pryor Mountain herd have received fertility control treatments using the contraceptive porcine zona pellucida [PZP; see Kirkpatrick and Frank (2005) for a recent review of contraception in equids and other free-ranging animals] as part of a cooperative research project between BLM, the U.S. Geological Survey (USGS), and the U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS). In 2001, six mares (age 1–2) that had not yet reached breeding status were treated with PZP by hand injection during the gather. A booster inoculation was administered to three of these females in August 2002; the remainder had either died or could not be located to deliver the booster. Fertility control was subsequently applied to younger mares (age 1–2, 2002–2004) and older mares (>14 years, 2002–2004; >10 years after 2004) that had already contributed to the genetics of the population. After 2001, all inoculations (both primers and boosters) were delivered remotely by darting. Because of the timing of booster inoculations relative to the breeding season, the first contraceptive effects were expected in 2004. From 2004 through 2007, 20.0, 29.0, 10.8, and 30.8 percent of the mares (≥3 years old) in the herd were presumptively contracepted.

BLM also has attempted to manage the herd to adjust the sex ratio in favor of males (Bureau of Land Management, 2006) and occasionally has introduced a limited number of horses (both mares and stallions) from outside herds in order to supplement the herd genetically. Introduction of outside horses was discontinued in 1994.

Methods

Field Data Collection

All data reported in this study were gathered through direct observations of the herd by trained BLM and USGS employees and volunteers. From 1995 to 1997, efforts were focused on the peak foaling season, May 1 to June 30. From 1998 to 2007, daily observations were made during the peak foaling and breeding period, May 1 to August 30. This effort totaled approximately 100 hours of observation per week during 1995–1997 and 100–150 hours per week during 1998–2007. Semi-weekly to weekly observations were made March–April and September–November. Fewer observations were made during midwinter, and they were limited to accessible areas of the range, which varied from year to year. Observations were made from the ground during systematic surveys along roads and trails that traverse the study area. Ground observations were augmented with two helicopter flights each fall or winter, 1995–2001, and one flight in late 2006, as well as 20–25 days of horseback surveys throughout each year, 1995–2005.

Greater than 90 percent of the bands were located at least twice per week during the May–July period, and all bands usually were located at least once per week from April to mid-May and August to mid-October. Reduced effort in the remainder of the year resulted in less than one observation per week per band. Observers collaborated during the peak observation season to develop a weekly list of band composition and foal presence.

Wild horses were approached within 100–250 m and could be observed for periods of 2–6 hours with no apparent effect on their behaviors. All horses were identified individually based on color, markings, gender, and band associations since 1993. All stallions, mares, yearlings, and foals observed in bands, bachelor groups, or alone were recorded. Lead harem stallions were identified based on behavior as described in Feist and McCullough (1975), Oom and Santos-Reis (1986), and Berger (1986). Interchanges between bands by mares and stallions were recorded, as were returns to the original band.

Each horse in the population was assigned a unique number during data compilation. Of the 609 animals in the population from 1993 to 2007, horses whose birth year was known from foal records included 480 of the 481 foals born during 1993–2007 and 11 horses born prior to 1993. Ages of an additional 52 animals were estimated by a veterinarian based on tooth eruption and wear patterns (American Association of Equine Practitioners, 2002). Ages of the remaining 66 animals were based on body size and morphology and anecdotal evidence of years the animals were on the range. Death year was determined by identification of the carcass of the deceased individual or, if an individual was not observed for two consecutive years, the animal was considered to have died in the year it went missing. Number of foals produced was determined by observation as described above. It is possible that some foals were born and died without being seen; however, given the intensity of the observations, we believe that this was rare. Identification of an individual's dam was based on observations of attachment of foal to mare during the early days and weeks of the foal's life.

Analysis

We conducted all of our analyses on the basis of biological years, which we defined as just prior to foaling in one year to the same time the next year. Thus, the biological year 1993 encompasses the period from spring 1993 to spring 1994. Because we defined the biological year in this way, because we made no attempt to pinpoint when during a year a horse was born or died, and because we have the benefit of hindsight (other reported data were often projections), our data will in some cases not match exactly those presented elsewhere (for example, various Environmental Assessments prepared by BLM).

We calculated annual population growth using the expression $\lambda_t = N_t / (N_{t-1} - R_{t-1})$, where λ_t is the growth multiplier from year $t-1$ to year t, N is the population size, and R_{t-1} is the number of animals removed in the period $t-1$ to t. In this formulation, λ represents the apparent growth rate of the population had horses not been removed (Garrott and Taylor, 1990).

Survival rates were calculated as the number of animals present at the end of a year divided by the number present at the beginning of that year. Animals that were removed from the herd were excluded from survival calculations in the year they were removed. Foaling rates were calculated as the number of foals born (regardless of how long they lived) divided by the number of mares present. Mares that were presumptively contracepted and foals that they produced were excluded from calculations of foaling rate.

Band composition has been recorded on "horse lists" regularly (usually weekly) since 1996. These are lists of all the harem stallions present and all the members of their bands, plus lists of bachelor males and animals in transit between bands. To derive information on harem stallions and band composition, we used two of these lists for each year from 1996 through 2007, one from the breeding season (usually early June) and one from fall (September–November, usually the last list available for the calendar year). For each harem stallion present, we calculated the number of mares ≥2 years of age in the band for each of the two time periods and then

took the maximum of those two values as the number of mares held in the harem in that year. We calculated total band size in an analogous manner, and we used these same lists to derive information on bachelors and satellite stallions, which we defined as ≥5 years of age. We defined a satellite stallion in this way in an effort to distinguish true multistallion bands from cases in which a male simply remained with his natal band longer than usual.

We looked for evidence of density dependence in foaling rate, herd survival, foal survival, and population growth rate in the Pryor Mountain wild horse population using stepwise multiple regression with the current year's postfoaling population and the previous year's postfoaling population as independent variables. For these analyses, we used data from Garrott and Taylor (1990) for 1976–1986 (foaling rate, herd survival, and population growth rate), as well as data from our study for 1993–2007. We excluded data from the winter of 1977–1978, during which 51 percent of the population died as a result of heavy snow and ice accumulations (Garrott and Taylor, 1990), from the analyses of density dependence in herd survival and growth rate. We also excluded data from 2004, when 96 percent of the foal crop was lost, from the analysis of foal survival. Foal losses in 2004 are suspected to have been the result of predation by mountain lions (*Puma concolor*). We believe that these exclusions are justified because the biological processes operating in these years were clearly different from those operating in other years.

It would be desirable to have consistent, long-term data on annual forage production for comparison with demographic parameters, but we are unaware of such data for the study area. As a possible surrogate, we examined weather records from a number of stations in the area, reasoning that forage production in this semiarid environment likely is related to precipitation, even though large elevation differences across the study area make it unlikely that any single station will be representative of the entire area. In terms of location, the best available data are from a Remote Automated Weather Station (RAWS; Wild Horse Montana, NWS ID #245605, elevation 2,675 m), which was operated in the study area from October 1991 to July 1997 (*http://raws.dri.edu/wraws/emtF.html*) (fig. 1). Unfortunately, overlap between this period of record and the period of our study is minimal. A National Climatic Data Center Station (NCDC #484770, elevation 1,170 m) at Lovell, Wyo., has a much longer period of record (1948–present; *http://www.wrcc.dri.edu/summary/climsmwy.html*), but its lower elevation caused us to question its relevance to higher elevations in the study area. For the 7 years of data in common, however, there was a strong correlation between the two stations for both total annual precipitation ($R^2 = 0.921$, $P = 0.0006$) and the minimum of the monthly average minimum temperatures ($R^2 = 0.763$, $P = 0.01$). We therefore used data from the Lovell station and stepwise multiple linear regression to analyze relations between weather and herd survival rate, foal survival rate, and foaling rate (mares ≥3 years of age). Data on annual herd survival and foaling rate were available from both our study and the work of Garrott and Taylor (1990), but data on annual foal survival were available only from our study. For the reasons cited above, we again excluded 2004 from the analysis of foal survival.

Other investigators (Heitschmidt and others, 2005; Smart, 2005) showed that spring precipitation (April–June) is related to annual forage production in the Great Plains. We therefore divided precipitation data into spring (April–June), summer (July–September), and winter (October–March). For each year, we also calculated the mean of the monthly average minimum temperatures (December–February) and the mean of the monthly average maximum temperatures (April–September) as indices of winter cold and summer heat.

For herd survival, we used the covariates winter precipitation in the previous year, spring precipitation in the current year, and summer precipitation in the current year (hypothesizing that all might have a positive relation with forage production in the current year), winter precipitation in the current year (potential negative effect of snow accumulation on forage availability in the current year), and average monthly minimum temperature in the current year (potential negative effect on ability to meet energy demands). For foal survival, we used the same set of covariates and added average monthly maximum temperature (possible effect of heat stress on more susceptible foals). For foaling rate, we used the covariates summer precipitation in the previous year (potential positive relation with forage production during early pregnancy), winter precipitation in the previous year (potential negative effect of snow accumulation on forage availability), average monthly minimum temperature (potential negative effect on ability to meet energy demands), and spring precipitation in the current year (possible positive relation with forage production during late pregnancy).

All data were entered into Microsoft Excel® spreadsheets (Microsoft Corporation, Redmond, Wash.). Analyses were conducted in SAS® v. 9.1 (SAS Institute, Cary, N.C.) using standard statistical techniques as noted in the Results section.

Results

Population Size and Growth

Prior to foaling in spring 1993, there were 128 horses ≥1 year of age in the study area. From 1993 to 2007, 481 horses were born; thus, our study encompasses 609 individual animals. During the period of study, the prefoaling population averaged 148.8 animals (range = 120–187), the foal crop (number born) averaged 32.1 (range = 23–40), and the postfoaling population averaged 180.9 animals (range = 149–227) (table 1). As we calculated it, the postfoaling population represents the maximum number of animals that could have been present at any time during the year, as it assumes no mortality of foals or adults during the foaling period. The number of deaths per year apparently increased during 1999–2006, while the foal crop remained relatively constant throughout

Table 1. Size and growth rate of the Pryor Mountain wild horse (*Equus caballus*) herd, 1993–2007.

[Growth rate (λ) is calculated based on the postfoaling population. —, not applicable; SE, standard error of the mean.]

Year	Prefoaling population	Foals born	Postfoaling population	Deaths	Removals	λ
1993	128	23	151	1	1	—
1994	149	34	183	3	60	1.220
1995	120	29	149	7	0	1.211
1996	142	32	174	7	2	1.168
1997	165	35	200	15	46	1.163
1998	139	23	162	7	1	1.052
1999	154	38	192	27	0	1.193
2000	165	37	202	15	0	1.052
2001	187	40	227	31	46	1.124
2002	150	32	182	28	0	1.006
2003	154	30	184	32	7	1.011
2004	145	28	173	38	0	0.977
2005	135	36	171	19	0	0.988
2006	152	30	182	16	19	1.064
2007	147	34	181	9	0	1.110
Total	2,232	481	2,713	255	182	—
Mean	148.8	32.1	180.9	17.0	12.1	1.096
SE	4.18	1.30	5.14	3.02	5.36	0.0228

the period of study. The mean annual apparent growth rate, calculated as though removals had not occurred, was 9.6 percent (λ = 1.096, range = 0.977–1.220), or 10.5 percent if the year (2004) of heavy foal loss is excluded. Although the growth rate rebounded somewhat in 2006 and 2007, there was a significant (R^2 = 0.514, P = 0.004) overall decline during the period of study as determined by linear least squares regression (fig. 2).

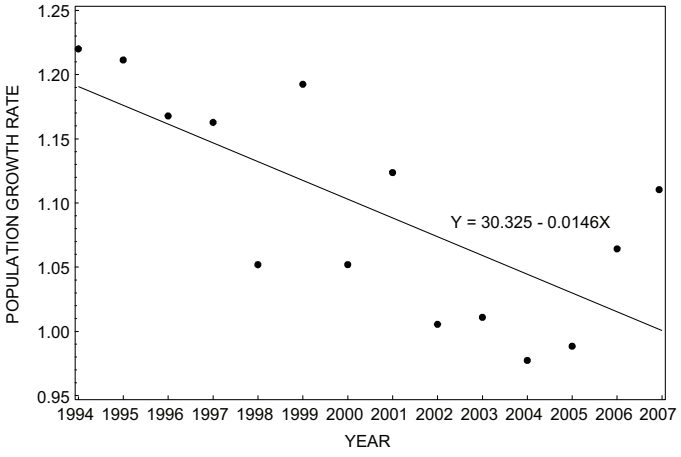


Figure 2. Trend in growth rate of the Pryor Mountain wild horse (*Equus caballus*) population, 1993–2007.

Removals

Major removals of wild horses occurred in 1994, 1997, 2001, and 2006, with smaller numbers of horses removed in four other years (table 2). For the entire period, adults (≥2 years of age) were removed most often (62.6 percent; n = 182), followed by yearlings (28.6 percent) and foals (8.8 percent). Across all age classes, slightly more males (56.6 percent; n = 182) were removed than females.

Sex and Age Structure

During the period 1993–2007, 477 foals of known sex were born; four foals disappeared before sex could be ascertained. Slightly more males (243) were born than females (234), but this difference was not statistically significant (χ^2 = 0.170, P = 0.68). On an annual basis, however, the proportion of males in the foal crop varied widely (range = 34.8–71.9 percent) (fig. 3). The proportion of males produced also varied by age of mare, declining from age 3 through about age 10, and then increasing in older mares (fig. 4).

The sex ratio of animals ≥1 year of age varied only moderately during the period of study, ranging from a high of 55.2 percent males in 1997 to a low of 44.2 percent males in 2000 and 2007 (fig. 5). The largest interannual changes in sex ratio occurred as a result of removals in 1994 (27 males, 33 females

Table 2. Removals of wild horses (*Equus caballus*) from the Pryor Mountain herd, 1993–2007.

	Foals		Yearlings		Adults		
Year	Males	Females	Males	Females	Males	Females	Total
1993	0	0	0	0	1	0	1
1994	8	6	7	7	12	20	60
1996	0	0	0	0	2	0	2
1997	2	0	11	1	20	12	46
1998	0	0	0	0	1	0	1
2001	0	0	10	6	8	22	46
2003	0	0	1	0	6	0	7
2006	0	0	4	5	10	0	19
Total	10	6	33	19	60	54	182

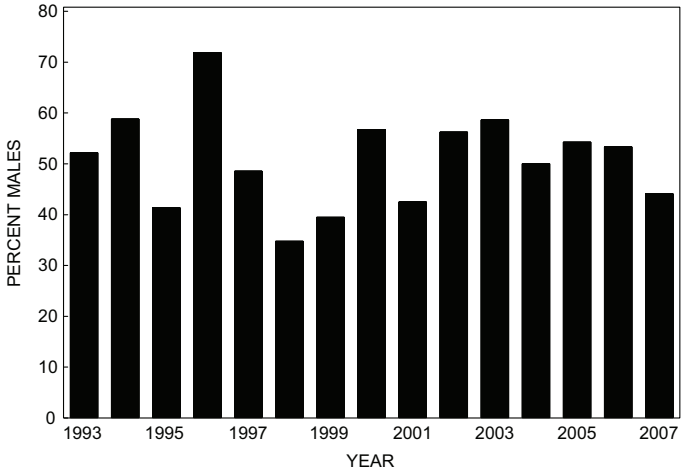


Figure 3. Sex ratio of wild horse (*Equus caballus*) foals born in the Pryor Mountain herd, 1993–2007.

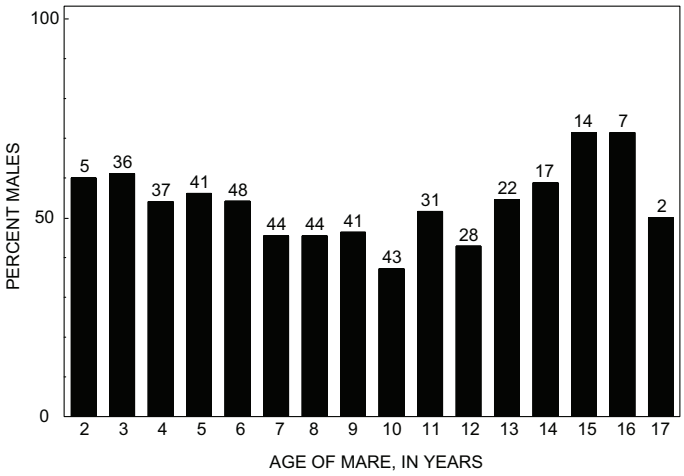


Figure 4. Variation in the sex ratio of wild horse (*Equus caballus*) foals by age of dam in the Pryor Mountain herd, 1993–2007. Values above the bars are number of foals born.

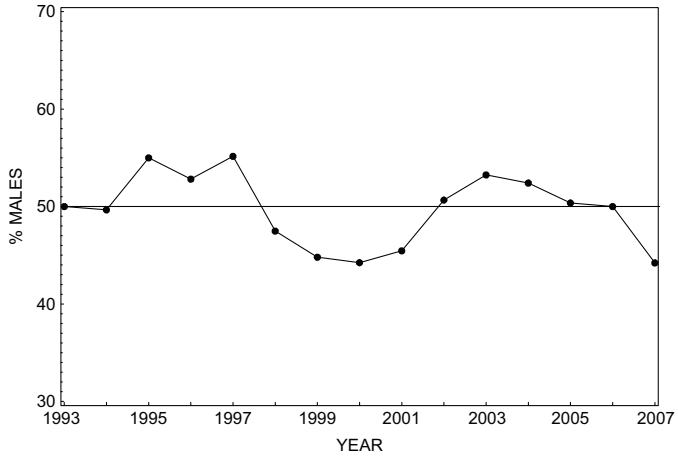


Figure 5. Annual sex ratio of adult (≥1 year old) wild horses (*Equus caballus*) in the Pryor Mountain herd, 1993–2007.

removed), 1997 (33 males, 13 females), 2001 (18 males, 28 females), and 2006 (14 males, 5 females).

The age structure of the Pryor Mountain wild horse herd, on the other hand, changed markedly between 1993 and 2007 (fig. 6*A*,*B*). In 1993, males in the herd averaged 4.4 years of age and females averaged 4.2 years. In 2007, the comparable values were 8.1 and 6.8 years, respectively. Both of these trends are statistically significant as judged by linear least squares regression (males, $P < 0.0001$, $R^2 = 0.911$; females, $P < 0.0001$, $R^2 = 0.832$).

Survival

Pooled across years, ages, and sexes ($n = 2{,}531$), the annual survival rate of horses on the study area was 0.899. Excluding four foals that disappeared before sex could be ascertained, survival of males (0.896, $n = 1{,}246$) did not differ from that of females (0.905, $n = 1{,}281$) ($\chi^2 = 0.486$,

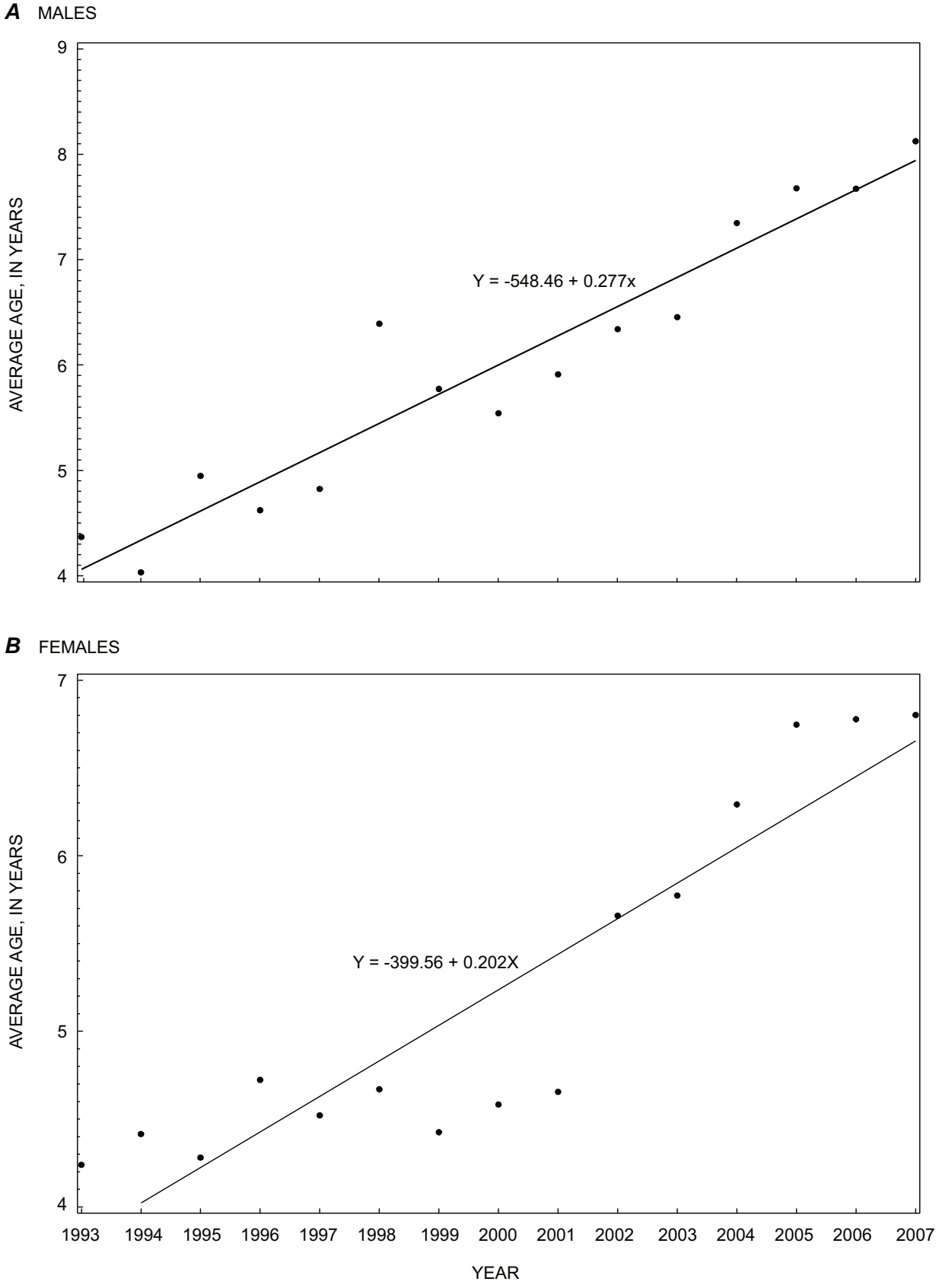


Figure 6. Average age of (*A*) male and (*B*) female wild horses (*Equus caballus*) in the Pryor Mountain herd, 1993–2007.

$P = 0.486$), nor was the difference between the sexes significant for foals ($\chi^2 = 2.500$, $P = 0.114$, $n = 461$). Data from both sexes were therefore pooled for calculations of survival by year.

Survival by year varied from a high of 0.993 in 1993 to a low of 0.780 in 2004 (table 3), when 27 of 28 foals were lost, with predation by mountain lions suspected as the cause (Bureau of Land Management, 2006). For the period of our study, there was a trend toward decreasing foal survival (fig. 7; $P = 0.020$, $R^2 = 0.353$), but there was no trend in survival of animals ≥1 year old ($P = 0.282$, $R^2 = 0.088$). Foal survival increased in the most recent three years of our study, however.

More than one half of the horses that died from 1993 to 2007 were foals, and annual foal survival pooled across years was 0.697 (table 4). Survival increased gradually from age 0 to age 3, remained high through age 15, and then declined gradually. Horses ≥18 years of age were pooled for this analysis because of small sample sizes in these age classes. The oldest animal recorded during the period of study was a male that died at age 26 in 2005. Nine horses lived to be 20 years of age or older, seven of which were males. Four horses (3 males, 1 female) age 20 and 21 were present on the range at the end of our study.

As judged by logistic regression, there was a positive relation between the probability of a foal surviving and the mean (average of summer and fall in a given year) number of members in the band ($n = 393$ foals in 1996–2007; Wald $\chi^2 = 14.964$, odds ratio = 1.244 with 95 percent CI = 1.114–1.390, $P = 0.0001$). Thus, for example, the regression yields a predicted foal survival rate of 0.457 (95 percent CI = 0.351–0.567) for a band size of two, and a predicted survival rate of 0.757 (95 percent CI = 0.688–0.815) for a band size of eight.

We also examined survival of foals in relation to parity of their dams—primiparous (those giving birth to their first foal) compared to multiparous (those that had given birth one or more times). For this analysis, we excluded all mares born before 1993 (since parity could not be determined with certainty) and any foals they produced. We also excluded mares that were presumptively contracepted or were present postcontraception (hereafter referred to as treated and posttreated) and foals that they produced. For the remaining subset of foals, there was no difference in survival based on parity of the dam ($n = 189$, $\chi^2 = 0.957$, $P = 0.328$).

Table 3. Annual survival rates of Pryor Mountain wild horses (*Equus caballus*), 1993–2007.

[Horses that were removed were excluded from survival calculations in the year of their removal.]

Year	n	Deaths	Survival
1993	150	1	0.993
1994	123	3	0.976
1995	149	7	0.953
1996	172	7	0.959
1997	154	15	0.903
1998	161	7	0.957
1999	192	27	0.859
2000	202	15	0.926
2001	181	31	0.829
2002	182	28	0.846
2003	177	32	0.819
2004	173	38	0.780
2005	171	19	0.889
2006	163	16	0.902
2007	181	9	0.950
Total	2,531	255	0.899

Foaling Rates

For ease of comparison with other studies, we calculated foaling rates of mares ≥2 years of age, ≥3 years of age, and ≥4 years of age. Treated and posttreated mares were excluded from these calculations, as were foals born to unknown dams. Pooled across years, productivity was 0.501 foals/mare (range = 0.254–0.705) for mares ≥2 years of age, 0.576 foals/mare (range = 0.300–0.795) for mares ≥3 years of age, and 0.597 foals/mare (range = 0.311–0.795) for mares ≥4 years of age (table 5). Regression analysis showed a slight increase in foaling rate of mares over time (for mares ≥3 years of age, $P = 0.088$), but variability was high ($R^2 = 0.208$).

From 1993 to 2007, 464 of the 481 foals produced in the study area were born to 118 known or highly probable dams (table 6), an average of 3.93 foals per mare. Many of the dams represented in table 6 are still producing foals; thus, the data represent only a single point in time. Twenty-seven dams that each produced seven or more foals accounted for a total of 237 foals, or 51.1 percent of the production by known dams. Of the 54 dams that have been monitored since birth (that is, those born in 1993 or later) and have not been contracepted, 6 (11.1 percent) first produced a foal at age 2, 29 (53.7 percent) at age 3, 14 (25.9 percent) at age 4, 3 (5.6 percent) at age 5, and 2 (3.7 percent) at age 7. The average age at first foaling for these dams was 3.4 years. Age-specific fecundity was 0.048 foals per mare for 2-year-old mares, increased nearly monotonically through age 10, remained high through age 15, and dropped rapidly thereafter (table 7). The maximum age at which any mare gave birth during the study period was 17 years, although that mare was presumptively contracepted and thus is not represented in table 7. A single set of twin foals (both females) was born during the period of our study, to a 6-year-old mare in 1994.

We used logistic regression to examine the probability of foaling in years following a gather as compared to other years for three age groups of mares: 2 years old, 3 years old, and ≥4 years old. Foal production (yes or no) was the dependent variable and year was the independent variable, and we used a specific hypothesis test (or contrast) to compare the mean probabilities of foaling. For this analysis we considered

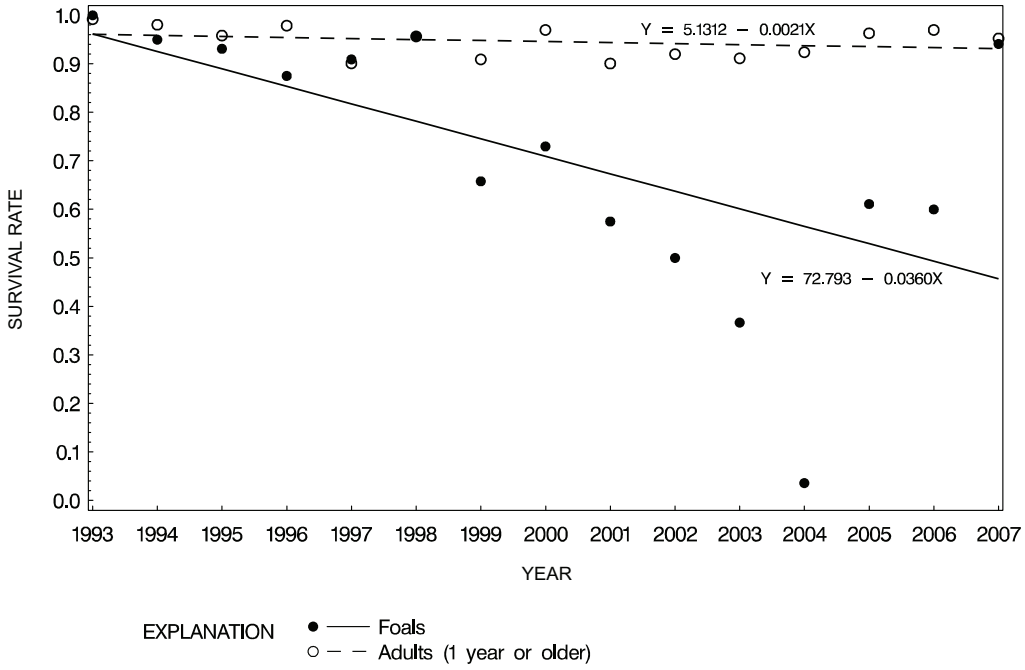


Figure 7. Trend in annual survival of Pryor Mountain wild horses (*Equus caballus*) for adults and foals, 1993–2007.

Table 4. Age-specific survival rates of Pryor Mountain wild horses (*Equus caballus*), 1993–2007.

[Horses that were removed were excluded from survival calculations in the year of their removal.]

Age	Males			Females			Total		
	n	Deaths	Survival	*n*	Deaths	Survival	*n*	Deaths	Survival
0	233	77	0.670	228	60	0.737	[1]465	141	0.697
1	115	9	0.922	137	12	0.912	252	21	0.917
2	91	5	0.945	108	9	0.917	199	14	0.930
3	77	3	0.961	89	2	0.978	166	5	0.970
4	72	1	0.986	84	5	0.941	156	6	0.962
5	70	1	0.986	87	4	0.954	157	5	0.968
6	67	4	0.940	76	3	0.961	143	7	0.951
7	60	2	0.967	73	0	1.000	133	2	0.985
8	60	1	0.983	69	0	1.000	129	1	0.992
9	56	1	0.982	65	1	0.985	121	2	0.984
10	53	0	1.000	57	4	0.930	110	4	0.964
11	49	3	0.939	48	3	0.938	97	6	0.938
12	38	2	0.947	40	5	0.875	78	7	0.910
13	34	1	0.971	32	4	0.875	66	5	0.924
14	33	1	0.970	27	1	0.963	60	2	0.967
15	30	1	0.967	22	2	0.909	52	3	0.942
16	30	1	0.967	18	4	0.778	48	5	0.896
17	26	4	0.846	9	2	0.778	35	6	0.829
≥18	52	12	0.769	12	1	0.917	64	13	0.797
Total	1,246	129	0.896	1,281	122	0.905	[1]2,531	255	0.899

[1] Includes four foals that disappeared before sex could be ascertained.

Table 5. Annual foaling rates of Pryor Mountain wild horse (*Equus caballus*) mares, 1993–2007.

[Treated and posttreated mares in 2004–2007 and foals that they produced were excluded, as were foals born to unknown dams.]

Year	Mares ≥ 2 years old			Mares ≥ 3 years old			Mares ≥ 4 years old		
	Foals	Mares	Foals/mare	Foals	Mares	Foals/mare	Foals	Mares	Foals/mare
1993	15	59	0.254	15	50	0.300	14	45	0.311
1994	32	64	0.500	32	59	0.542	30	50	0.600
1995	23	46	0.500	23	42	0.548	20	38	0.526
1996	32	51	0.627	30	44	0.682	27	40	0.675
1997	34	66	0.515	33	50	0.660	29	43	0.674
1998	23	55	0.418	23	48	0.479	22	43	0.512
1999	38	70	0.543	37	52	0.712	34	46	0.739
2000	37	76	0.487	36	61	0.590	27	44	0.614
2001	40	88	0.455	40	75	0.533	34	60	0.567
2002	32	63	0.508	32	57	0.561	28	53	0.528
2003	30	65	0.462	29	56	0.518	29	50	0.580
2004	28	52	0.538	28	48	0.583	28	48	0.583
2005	31	44	0.705	31	39	0.795	31	39	0.795
2006	23	42	0.548	23	42	0.548	23	37	0.622
2007	21	35	0.600	21	29	0.724	21	29	0.724
Total	439	876	0.501	433	752	0.576	397	665	0.597

Table 6. Total foals born to Pryor Mountain wild horse (*Equus caballus*) mares, 1993–2007.

[Includes only foals born to known dams. Many of the mares represented are still producing foals.]

Foals / Mare	Number of mares	Total foals	Percent of total foals
1	43	43	9.3
2	12	24	5.2
3	10	30	6.5
4	5	20	4.3
5	16	80	17.2
6	5	30	6.5
7	9	63	13.6
8	6	48	10.3
9	4	36	7.8
10	2	20	4.3
11	4	44	9.5
12	1	12	2.6
14	1	14	3.0
Total	118	464	100

only the gathers conducted in 1994, 1997, and 2001 in which most of the family bands were gathered; in 2003, only groups containing animals targeted for removal were gathered, and in 2006 a subset of the herd was captured by bait trapping. For mares 2 or 3 years old, the data were too sparse to allow calculation of the maximum likelihood estimate in the logistic regression (for example, in some years no mares in these age groups produced foals). For mares ≥4 years old the probability of foaling was lower in years following a gather than in other years ($P = 0.03$). The absolute value of this difference was small, however. The mean predicted foaling rate in years following a gather was 0.522 ($n = 3$ years, 134 mares) as opposed to 0.622 ($n = 12$ years, 531 mares) in other years.

We also used logistic regression to examine the probability of a mare foaling as a function of number of members in the band in summer and found a positive relation ($n = 577$ mare-years, Wald $\chi^2 = 24.122$, odds ratio = 1.254 with 95 percent CI = 1.146–1.372, $P < 0.0001$). As an example, the regression yields a predicted probability of foaling of 0.427 (95 percent CI = 0.347–0.511) with a band size of two, as compared to a probability of foaling of 0.743 (95 percent CI = 0.682–0.797) with a band size of eight.

Table 7. Age-specific fecundity of Pryor Mountain wild horse (*Equus caballus*) mares, 1993–2007.

[Treated and posttreated mares in 2004–2007 and foals that they produced were excluded, as were foals born to unknown dams. —, not applicable.]

Age	Number of foals	Number of mares	Foals/mare
0	0	234	0.000
1	0	156	0.000
2	6	124	0.048
3	36	87	0.414
4	34	81	0.420
5	37	72	0.514
6	43	69	0.623
7	41	68	0.603
8	44	70	0.629
9	42	69	0.609
10	43	59	0.729
11	31	48	0.646
12	28	40	0.700
13	22	30	0.733
14	14	23	0.609
15	14	20	0.700
16	4	9	0.444
17	0	4	0.000
≥18	0	3	0.000
Total	439	1,266	—

In addition, we compared the probability of foaling in the next year for primiparous and multiparous mares. Primiparous females were less likely to foal ($\chi^2 = 27.29$, $P < 0.0001$) in the year following their first foal (proportion foaling = 0.26, n = 43) than were multiparous females (proportion foaling = 0.72, n = 110). On the other hand, multiparous females were somewhat more likely to foal ($\chi^2 = 2.60$, $P = 0.107$) if they foaled in the previous year (proportion foaling = 0.72, n = 110) than if they did not foal in the previous year (proportion foaling = 0.61, n = 76).

Harem Stallions and Band Composition

During the 12 years (1996–2007) for which information was available, the number of bands varied from 29 to 38 (table 8), with an increasing trend through time (least squares regression, $P = 0.012$, $R^2 = 0.48$). There was no relation, however, between number of bands and band size (least squares regression, $P = 0.88$); that is, band size did not decline significantly as the number of bands increased. Bands were held by 64 different stallions during this period, the youngest being age 3 and the oldest being age 23 (fig. 8). The vast majority of bands, however, were held by stallions aged 5–17 (fig. 8; n = 399). A single stallion gained his first harem at age 3, but 18 of the 29 harem stallions (62.1 percent) for which we have records since birth (born in 1993 or later) acquired their first harem at age 5 or 6 (fig. 9). As might be expected from the increasing age of all males in the herd (fig. 6A), the average age of harem stallions also increased through time (table 8) (least squares regression, $P < 0.0001$, $R^2 = 0.82$).

Harems with as many as six breeding-age females (≥2 years old) were recorded occasionally, but more than 86 percent of the harems contained 1–3 breeding-age females (fig. 10; n = 399). Harem size varied by age of stallion, increasing from age 3 to about age 11 and declining thereafter (fig. 11; n = 399). There were four somewhat anomalous cases in which a male was listed as a harem stallion but the harem size was zero (fig. 10). In two of these cases there were no harem members at all when the observations were recorded. These represent harems in flux, with one or more mares sometimes with the stallion and sometimes not. In the other two cases the only band members were the harem stallion and a yearling female, and we counted only females ≥2 years old in our calculations of harem size.

Of 399 band-year combinations for which we have observations, only 25 (6.3 percent) involved satellite stallions, which we defined as a male ≥5 years old that was associated with a harem stallion. Three pairs of stallions that lived in the same band for 6, 6, and 5 years accounted for 17 of these 25 associations. The remaining eight associations lasted only 1 or 2 years. In only one case were three stallions involved, and that association was maintained for a year or less. Two of the long-term associations (≥5 years) involved stallions that were the same age (7 through 12 years of age) or 1 year apart (harem stallion 5 through 9 years of age; satellite stallion 6 through 10 years of age). The third long-term association included a harem stallion 17 through 22 years of age and a satellite stallion 7 through 12 years of age. The mean number of mares (≥2 years old) did not differ between single- and multistallion bands (2.2 mares in both cases), although 5 of the 25 multistallion bands had 4–6 mares.

Of the 180 male foals born from 1993 through 2003 (those born after 2003 would have been only 3 years old or younger at the end of our study), 124 died or were removed before reaching age 4 and thus had virtually no chance of becoming a harem stallion. An additional 17 died or were removed at age 4 or 5 and thus had a much reduced chance of becoming a harem stallion. Three died at age 6, and two were removed at age 7 without having held a harem. Of the original 180 male foals born, 29 became harem stallions. Of the remaining five animals, two were still bachelors at the end of our study (ages 4 and 6 in 2007), two briefly held harems (at ages 4 and 8) but reverted to bachelor status, and one was a satellite stallion in his natal band (at age 5).

Table 8. Number of wild horse (*Equus caballus*) bands, mean age of harem stallions, mean harem size (mares ≥2 years old), and mean band size (all members) in the Pryor Mountain herd, 1996–2007.

[—, not applicable.]

Year	Number of bands	Mean age of harem stallions	Mean harem size	Mean band size
1996	29	8.76	1.90	5.14
1997	31	9.68	2.06	5.23
1998	31	10.26	1.87	4.90
1999	31	10.71	2.68	5.74
2000	31	11.10	2.68	5.87
2001	38	10.71	2.39	5.24
2002	34	11.06	1.94	4.59
2003	36	10.89	2.06	4.53
2004	34	11.21	2.06	4.03
2005	35	12.11	2.11	4.20
2006	35	13.00	2.20	4.66
2007	34	11.97	2.38	5.18
Total	399	—	—	—

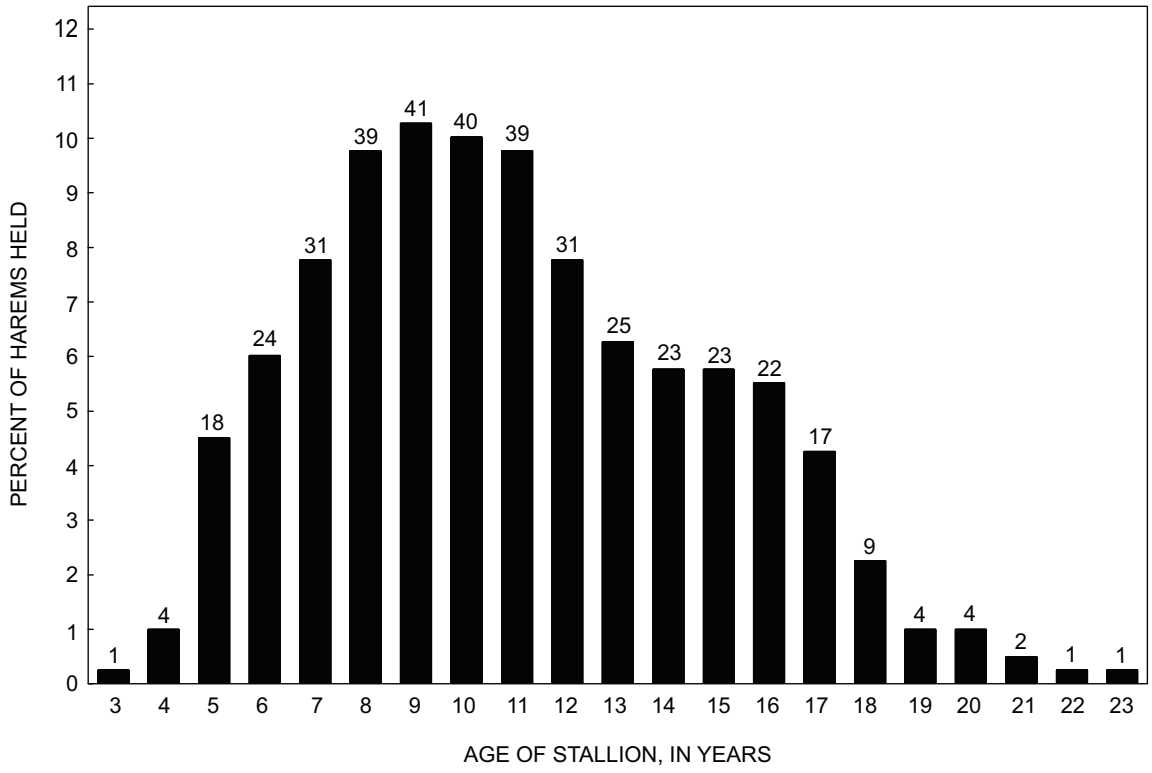


Figure 8. Age of wild horse (*Equus caballus*) stallions holding harems in the Pryor Mountain herd, 1996–2007. Values above the bars are number of harems.

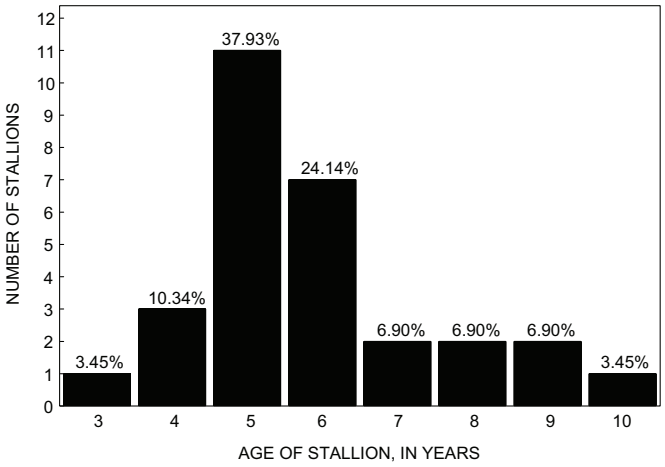


Figure 9. Age at which wild horse (*Equus caballus*) stallions first held a harem in the Pryor Mountain herd, 1996–2007. Values above the bars are percent of the total stallions represented on the chart.

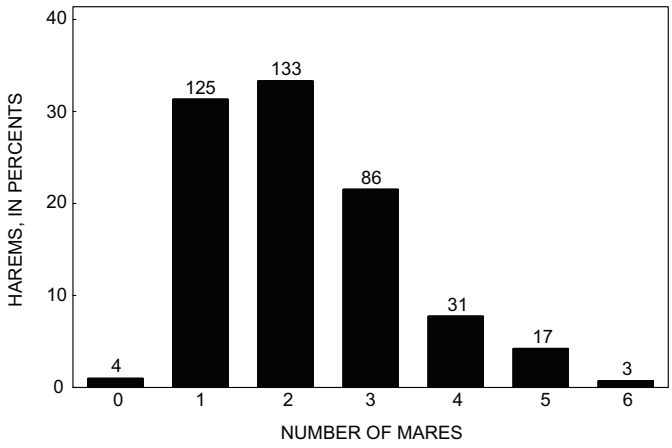


Figure 10. Frequency distribution of wild horse (*Equus caballus*) harem size in the Pryor Mountain herd, 1996–2007. Values above the bars are number of harems.

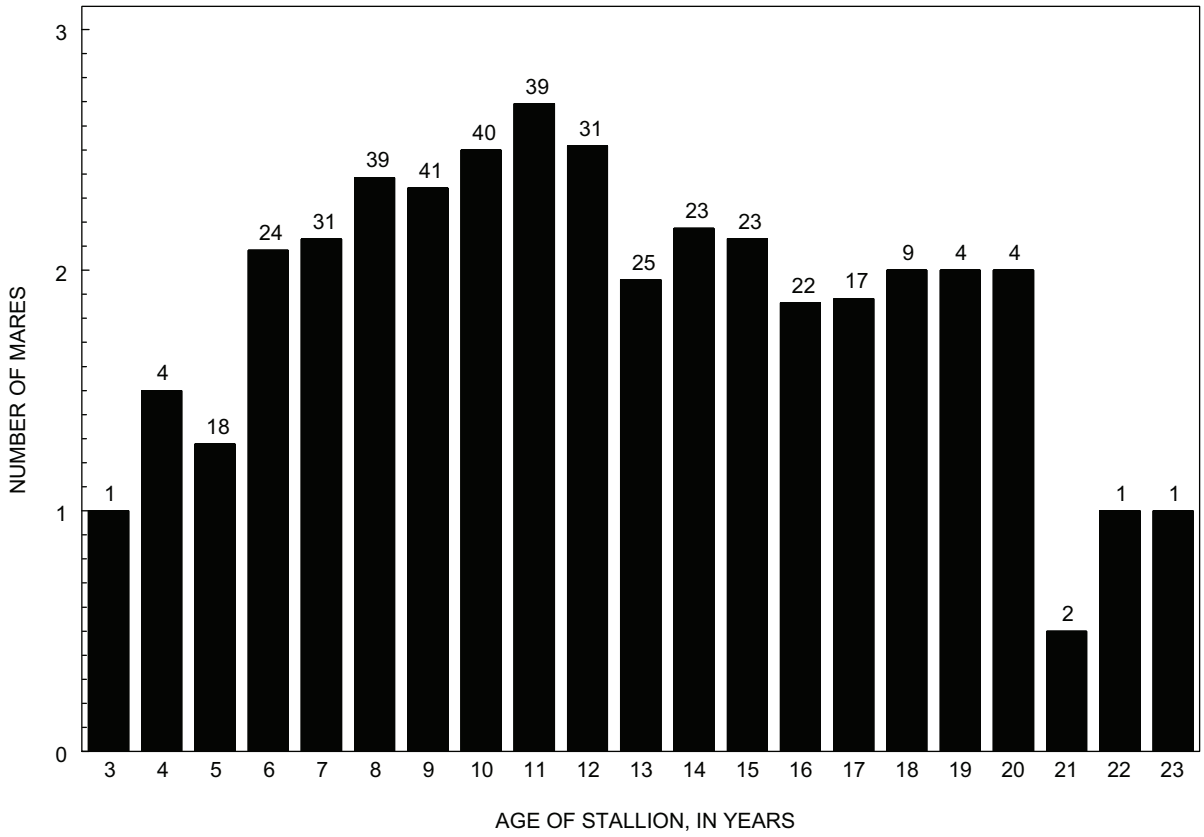


Figure 11. Mean number of wild horse (*Equus caballus*) mares held relative to age of harem stallions in the Pryor Mountain herd, 1996–2007. Values above the bars are number of harems.

Bachelors

The number of bachelor males in a wild horse herd changes through the course of a year, as young males are expelled from or leave bands, middle-aged males (formerly bachelors) gain bands, and older males gain and lose bands. In the Pryor Mountain herd, the number of bachelor males in summer (early to mid-June) varied between 16 and 36 during the period of study (fig. 12). The largest interannual change occurred between 1997 and 1998, in part because of the removal of 18 bachelors in fall 1997. In total, 40 bachelors were removed from the herd from 1996 through 2007. Pooled across years, 188 of 328 bachelors (57.3 percent) were 2 through 5 years of age (fig. 13).

To examine the question of when young stallions leave their natal bands and become bachelors, we used a subset of our data that included all males born in 1996 or later (thus ensuring that we had complete information on their band associations since birth). Of the 59 males that were born in 1996 or later and reached bachelor status (many died or were removed before becoming bachelors, others were born too late in the study period to have an opportunity to become bachelors), 4 (6.8 percent) became bachelors at age 1 (though one subsequently returned to his natal band), 32 (54.2 percent) at age 2, 13 (22.0 percent) at age 3, 7 (11.9 percent) at age 4, and 3 (5.1 percent) at age 5.

Population Regulation

Density Dependence

Regression analyses showed no relation between the foaling rate of mares ≥3 years of age and population size in either the current year or the previous year ($P \geq 0.143$). Foaling rate of 2-year-old mares may have declined somewhat with larger population in the previous year ($n = 13$ years, $P = 0.090$), but variability was high ($R^2 = 0.239$). Other young age classes (3–5 years) did not show similar relations, nor did combinations of these age classes.

Overall herd survival was related negatively to the previous year's postfoaling population (fig. 14A) ($n = 23$ years, $P = 0.006$), but there was a great deal of variability ($R^2 = 0.305$). Foal survival, however, was unrelated to population size ($n = 13$ years, $P > 0.15$). There also was a relation between population growth rate and the previous year's postfoaling population (fig. 14B) ($n = 23$ years, $P = 0.002$, $R^2 = 0.382$). This is perhaps not surprising, because survival rate is an important component of population growth rate.

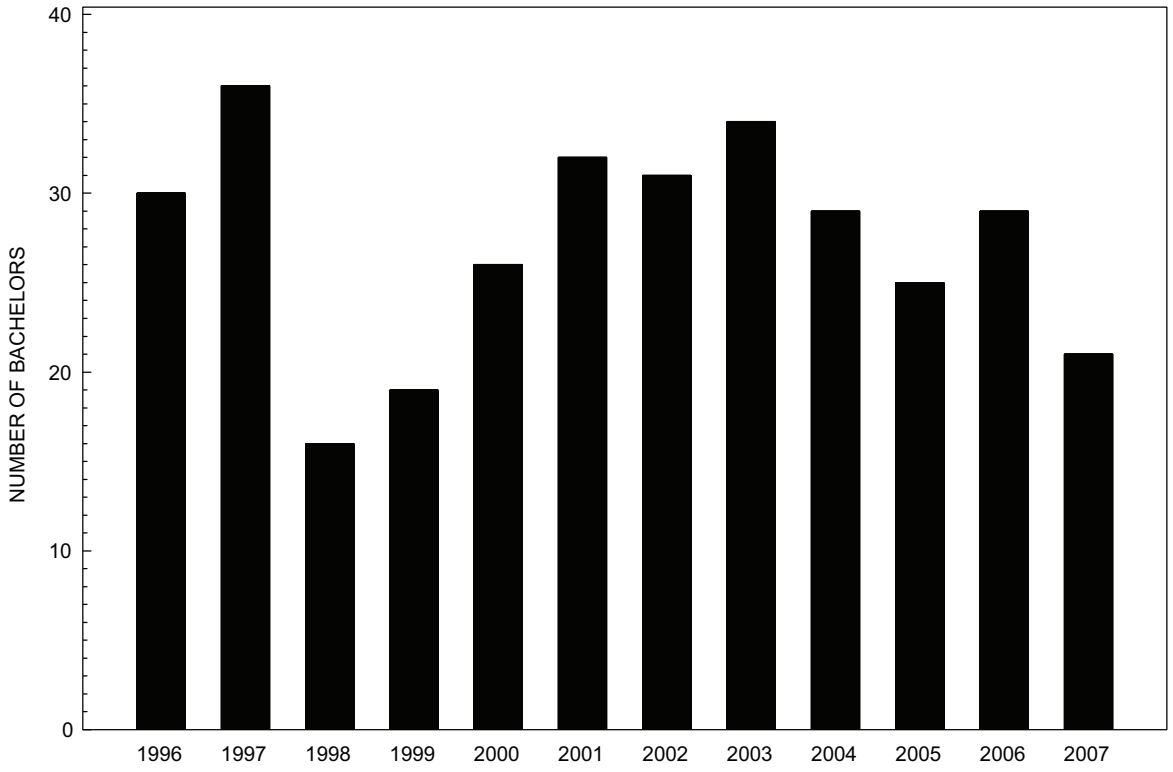


Figure 12. Number of bachelor wild horses (*Equus caballus*) in the Pryor Mountain herd, 1996–2007.

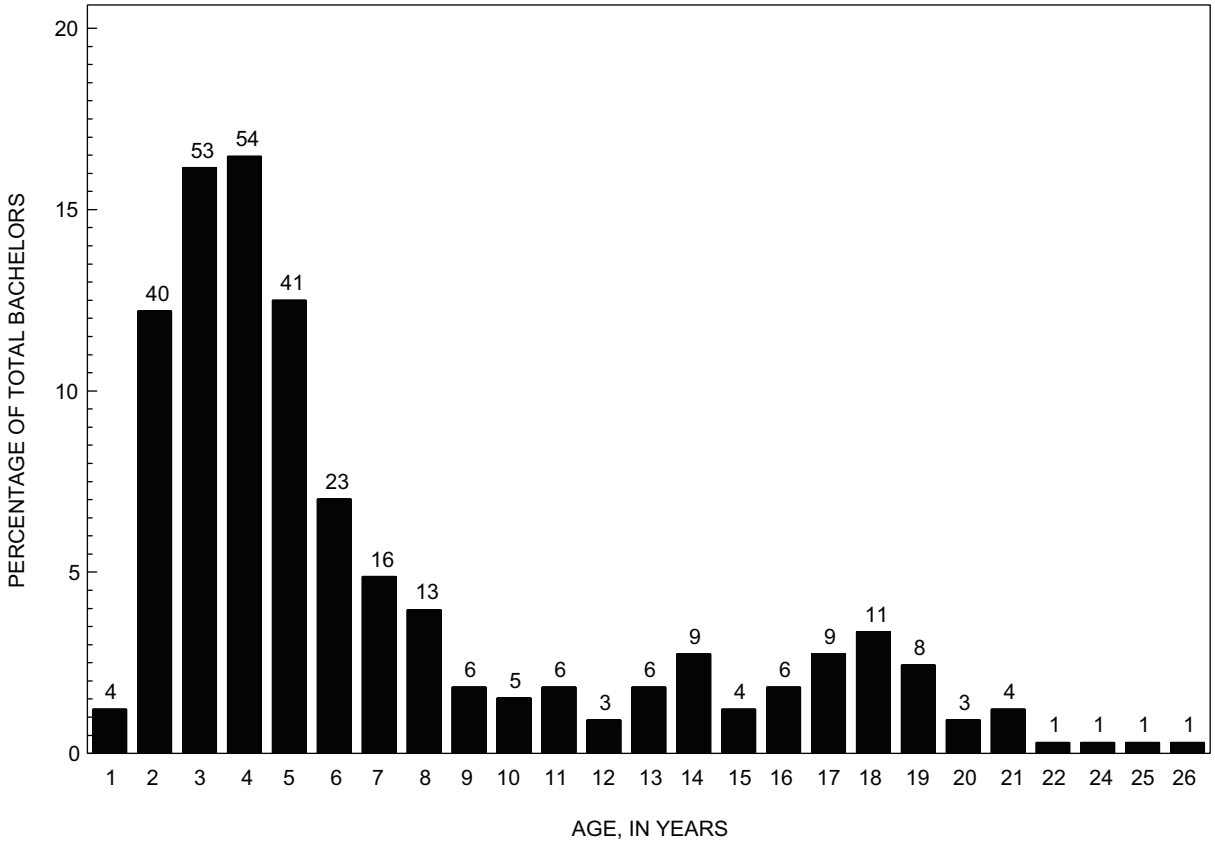


Figure 13. Age distribution of bachelor wild horses (*Equus caballus*) in the Pryor Mountain herd, 1996–2007. Values above the bars are number of bachelors.

Weather

None of the covariates that we used entered the stepwise multiple regression model for herd survival ($P > 0.15$; table 9). For foal survival, both summer and winter precipitation in the current year met the criterion for entry in the model ($P < 0.15$), and both were related positively to foal survival. In the case of winter precipitation in the current year, the positive relation was contradictory to our hypothesis. In combination, these parameters accounted for about 81 percent of the variability in foal survival. In terms of mare foaling rate, only the mean of the monthly average minimum temperature in the previous winter entered the model, and the relation accounted for only about 15 percent of the variability.

Discussion

Population Size and Growth

From 1993 to 2007, the prefoaling (adult) population averaged about 149 animals, only slightly more than the target population of ≈140 adults established on the basis of genetic considerations (Bureau of Land Management, 2006). Removals and a declining survival rate for foals appear to have been the primary factors keeping the population at this level. Contraception also may have played a role if it prevented the birth of any foals. However, it seems likely that the effect of the contraceptive program on population growth was small, given that relatively few mares were treated and that those mares were not in the most productive age classes.

The subject of population growth in wild horse populations has been controversial since early reports (for example, Cook, 1975) of rates as high as 30 percent annually. Other investigators challenged the accuracy of such estimates, and several used simulation modeling in combination with the scant data then available for wild horses and extrapolation of data from other ungulates to show that growth rates of 20–30 percent seemed highly unlikely given apparently reasonable assumptions about model parameters (Conley, 1979; Wolfe, 1980). Frei and others (1979) suggested that the apparently inflated growth rates might be an artifact of improved aerial census techniques and greater experience of observers.

As additional data were accumulated, however, it became apparent that initial assumptions regarding population parameters, particularly survival rates and age of mares at first reproduction, were probably conservative for many wild horse herds (Wolfe, 1986). Eberhardt and others (1982) calculated rates of increase of 20 and 22 percent for two herds

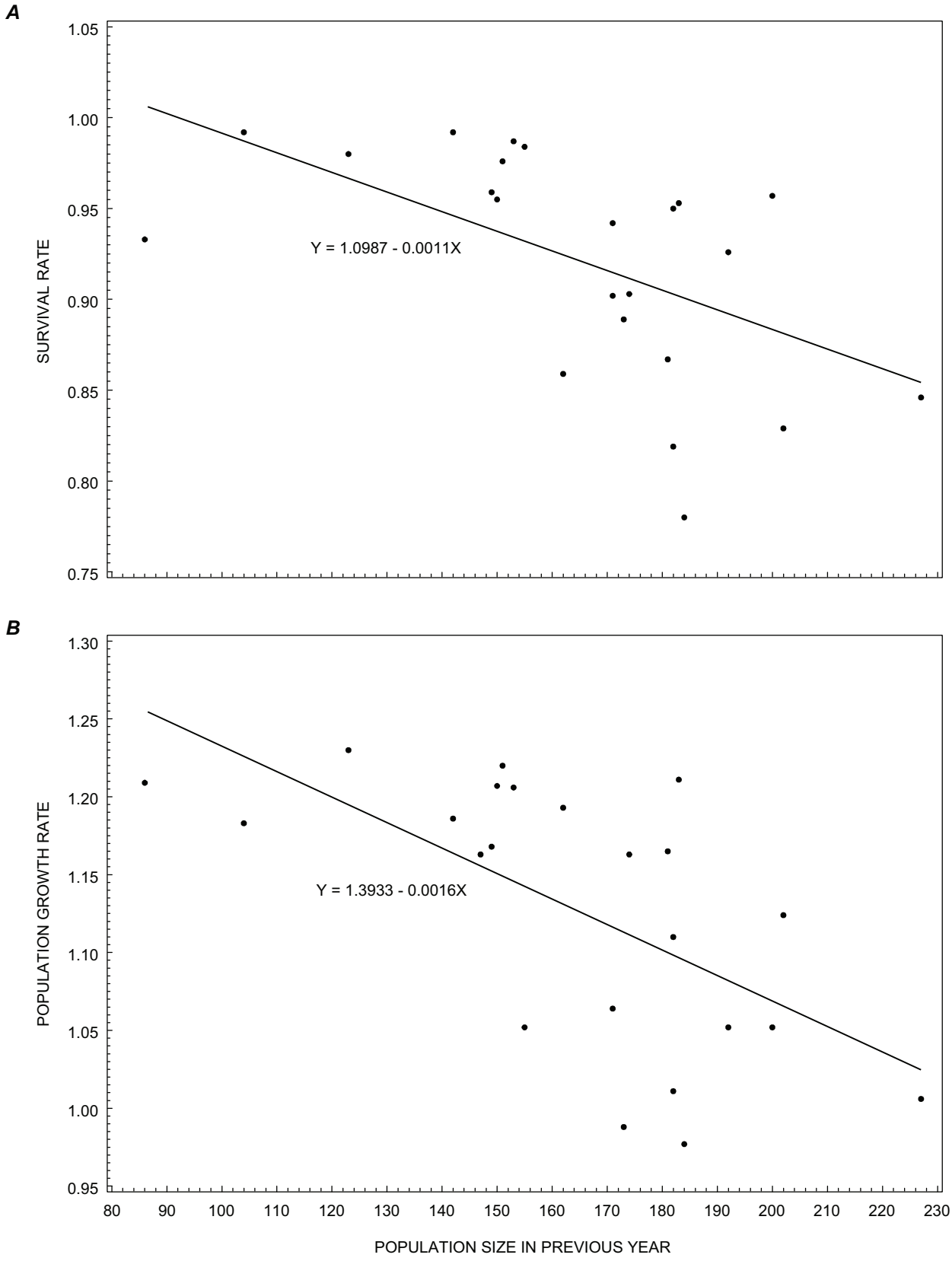


Figure 14. Relation of (*A*) survival and (*B*) population growth rate to the previous year's population for wild horses (*Equus caballus*) in the Pryor Mountain herd. Includes data from this study and from Garrott and Taylor (1990), excepting the abnormally severe winter of 1977–1978.

Table 9. Results of stepwise multiple regressions between wild horse (*Equus caballus*) herd survival, foal survival, and foaling rate (mares ≥3 years of age) and various weather parameters for the Pryor Mountain herd.

[Includes data from Garrott and Taylor (1990) and this study for herd survival and mare foaling rate, but only from this study for foal survival. *n*, sample size; SE, standard error of the estimate; R^2, coefficient of determination; *P*, probability; —, no variables entered the model; SUMP, summer precipitation in the current year; WINP, winter precipitation in the current year; LMNT, average monthly minimum temperature (December–February) in the previous year.]

Population parameter	*n* (years)	Covariate	Parameter estimate	SE	Partial R^2	Model R^2	*P*
Herd survival	19	—	—	—	—	—	—
Foal survival	10	SUMP	0.164	0.041	0.71	0.71	0.002
		WINP	0.117	0.059	0.10	0.81	0.090
Foaling rate	19	LMNT	0.008	0.005	0.15	0.15	0.105

in Oregon and, by comparing pre- and postremoval populations with known removals, showed that the aerial counts used to derive the growth rates must have been reasonably accurate. Berger (1986, p. 76), based on a 5-year study of horses in the Granite Range of Nevada, reported an average annual growth rate of 31 percent. [Note, however, that Berger (1986, p. 77) also reported an exponential growth rate of 0.188, which is equivalent to a finite growth rate of only 20.7 percent annually.] Wolfe (1986), using data from an unspecified number of BLM herds (>40,000 animals), calculated an annual growth rate of 14 percent for the period 1981–1983 and 15 percent for 1983–1985. Garrott and others (1991b) reported an unweighted mean growth rate of 21 percent (range = 15–27 percent) for 12 western herds. High rates of population growth are not limited to western horse populations. Franke Stevens (1990), for example, reported that the horse population at Rachel Carson National Estuary, made up of four small islands off the coast of North Carolina, increased from 44 animals in 1984 to 68 animals in 1986, from which we calculated an annual growth rate of 24.3 percent.

Reported growth rates of wild horse herds have not been universally high, however. Keiper and Houpt (1984), for example, stated that the horse population on Assateague Island National Seashore grew from 42 to 80 animals during the period 1975 to 1982, which represents an average annual growth rate of 9.6 percent. Goodloe and others (2000) found an average annual growth rate of 4.3 percent (range = 3.0–6.8 percent) for horses on Cumberland Island, Georgia, from 1986 to 1990 and suggested that the low rates likely were the result of density-dependent response to limited forage. Greger and Romney (1999), studying a horse population at the Nevada Test Site (now the Nevada Wild Horse Range), found an average growth rate of -7.0 percent, which they attributed to very low foal survival as a result of predation by mountain lions.

The first multiyear study of horses at Pryor Mountain was that of Perkins and others (1979). While they did not calculate population growth rates, they presented information sufficient to do so for two years. Based on information in their table 1, we calculated growth rates of 7.5 and 16.0 percent for 1976 and 1977, respectively, for the postfoaling population. Garrott and Taylor (1990) reported growth rates at Pryor Mountain for 1977–1986 with a mean of 11.9 percent, but 17.8 percent if a year with heavy winter mortality (51 percent of the population died) was excluded. Singer and others (2000) found a mean growth rate of 17.6 percent at Pryor Mountain from 1992–1997.

Our mean annual growth rate (9.6 percent; 10.5 percent if the year of heavy foal loss is excluded) for the period 1993–2007 is thus toward the lower end of the range reported for the Pryor Mountain and other herds. The declining trend in growth rate through time appears to be a result of decreasing survival of foals, though both foal survival (2005–2007) and growth rate (2006–2007) increased in recent years. Contraception also may have played a small role in reducing the average growth rate, though it is difficult to separate the effects of multiple factors.

Sex and Age Ratios

Studies at Pryor Mountain and elsewhere have found foal sex ratios favoring both males and females, with the pattern sometimes switching for the same herd in different time periods. At Pryor Mountain, for example, Feist and McCullough (1975) reported that 54.3 percent of 35 foals were males, Singer and others (2000) found that 58.3 percent of "surviving" foals were males (sample size not given), but Perkins and others (1979) found 48.5 percent males among 103 foals. Similarly, Keiper (1979) reported 52.6 percent males among 190 foals on Assateague Island, but Keiper and Houpt (1984) found 46.6 percent males in 103 foals born at the same location. In many of these studies with relatively small sample sizes, of course, the results did not differ significantly from parity, which also is true of most studies with larger samples, including ours. Speelman and others (1944), for example, found 49.4 percent males in 568 foals born to domestic mares raised under range conditions in Montana. Garrott (1991), who

studied the sex ratio of more than 10,000 foals removed from 74 BLM herds, found no significant difference from parity in 65 of those herds; the foal sex ratio favored females in seven herds and males in two herds. Interestingly, one exception to this pattern is found in the study by Garrott and Taylor (1990) at Pryor Mountain, who reported a sex ratio of 39.7 percent males ($P < 0.001$) in a sample of 330 foals from 1972 to 1986. Garrott and Taylor offered no explanation for this result, but they did comment that other data from their study might suggest low forage availability. Other investigators have hypothesized that females in poor condition (as through low forage availability) should produce fewer male offspring (Trivers and Willard, 1973).

The foal sex ratio did not differ from parity during the course of our study, and there was no overall difference in survival between the sexes. Thus, apart from minor variations resulting from each foal crop entering the population, consistent departures from a 50:50 sex ratio are the result of removals. This is evidenced by the fact that the largest interannual changes in sex ratio occurred following the larger removals. The drop in the proportion of males in the herd in 2007 was because of the removal of 14 males and 5 females in 2006, but also because natural mortality in 2006 consisted of 13 males and only 3 females. The increasing age of the herd also is mostly explained by removals; for all animals removed during the period of study, the average age was 3.2 years for females and 2.7 years for males. This is in keeping with BLM's management strategy of retaining harem stallions and core breeding-age mares (6–10 years) on the range (Bureau of Land Management, 2006). Low survival of foals in 2003 (36.7 percent) and 2004 (3.6 percent) also reduced the proportion of young animals in the herd and thus increased the average age. To the extent that it prevented any mares from foaling, the contraceptive program also would have contributed to increasing age of the herd, but the magnitude of any such effect is unknown.

Survival

The most striking feature of the survival data from our study is the general decline in foal survival in 1993–2004, followed by an apparent increase in the next 3 years. It would be tempting to attribute at least the declining part of this pattern to increased observational effort and hence increased detection of neonatal loss. We do not believe this to be the case, however, because there was no trend in the number of foals produced through time (linear least squares regression, $R^2 = 0.06$, $P = 0.38$), and because all animals present in the herd in 1993 and all foals born since then have been accounted for. Predation by mountain lions is suspected as the cause of low foal survival in 2004 and perhaps in 2003, though only four foals were confirmed to have been taken by mountain lions (Bureau of Land Management, 2006). Three mountain lions were removed from the area by hunters in winter 2004–2005 (Bureau of Land Management, 2006), which might in part explain the increase in foal survival observed in 2005–2007. It also seems possible that predation began on a smaller scale much earlier than 2003, but we know of no evidence to support this hypothesis. Predation by mountain lions has been shown to be an important factor in foal mortality in at least one herd (Turner and Morrison, 2001) and has been implicated in others (Salter, 1979; Greger and Romney, 1999). Wolves (*Canis lupus*), when present, are also suspected of preying on wild horses (Salter, 1979; Oom and Santos-Reis, 1986). Another possible contributor to the observed pattern in foal survival is drought. The drought began in 1999, the same year as the first real drop in foal survival, and was somewhat ameliorated in 2005 and 2007, at which time foal survival increased. The results of our regression analyses between precipitation and foal survival would seem to support this explanation. On the other hand, the initial response to reduced forage availability would likely be a decline in body condition of mares and foals, and such a decline was not observed during the early years of the drought (LC-M).

Overall, survival rates from our study for 1993–2007 are similar to those reported by Perkins and others (1979), but lower than those reported by Garrott and Taylor (1990) and Singer and others (2000) for the same study area (table 10). The foal survival rate reported by Garrott and Taylor, however, probably is an overestimate because it covers the period from midsummer to midsummer; neonatal mortality, which often constitutes the majority of foal mortality (Boyd, 1979; Keiper, 1979; Berger, 1986; Monard and others, 1997; Goodloe and others, 2000), likely was missed. Age of the herd also may account for some of the difference between our survival rate and that calculated by Garrott and Taylor (1990). At the end of their study in 1986, about 22 percent of the herd was ≥11 years of age, whereas the comparable number for 2007 was 39 percent. Perkins and others (1979) and Singer and others (2000) did not provide sufficiently detailed age data to make similar comparisons. Survival rates from our study were also moderate compared to those reported previously for other horse herds in North America (table 10).

Relations between foal survival and band size have not often been reported in the literature on wild horses, and the few available results are equivocal. We found that foals from larger bands tended to survive better than those from smaller bands. Working on Camargue horses in France, Feh (1999) found that there was less foal mortality in bands with an alliance of two stallions (3.2 percent), than in single-stallion bands (22.0 percent). Multiple stallions often are associated with larger bands. Cameron and others (2003), on the other hand, found that among Kaimanawa horses in New Zealand more foals died in multistallion bands (survival = 80 percent, $n = 30$) than in single-stallion bands (survival = 94 percent, $n = 50$), but the difference was not quite significant ($P = 0.056$). Similarly, evidence for a relation between mare age or parity and foal survival is equivocal. We found no relation between foal survival and parity of the mare. Keiper and Houpt (1984) reached the same conclusion, but only 10 foals died in the course of their study. In contrast, Cameron and

Table 10. Survival rates reported for North American wild horse (*Equus caballus*) herds.

[Data are arranged in order of increasing foal survival. Sample size (*n*), when available, is given in parentheses. NA, data not available; PMWHR, Pryor Mountain Wild Horse Range.]

Source and location	Survival rate (*n*)				Comments
	Foals	Yearlings	Adults	Herd	
Greger and Romney (1999), NV	0.12	NA	NA	NA	Foal survival is a mean for 8 years and is a maximum estimate because some neonatal mortality probably went undetected.
Turner and Morrison (2001), CA and NV	0.32	0.88	0.92	NA	All data represent means for 10 years.
Goodloe and others (2000), GA	0.600 (105)	NA	0.923 (263)	NA	Adult rate is for mares only, probably ≥3 years old.
This study, (PMWHR)	0.697 (465)	0.917 (252)	0.949 (1,814)	0.899 (2,531)	
Perkins and others (1979), PMWHR	0.75	NA	NA	0.903 (579)	Foal survival is a mean for 4 years. Herd survival is a weighted mean calculated from Table 1, Perkins and others (1979).
Boyd (1979), WY	0.824 (51)	NA	NA	NA	Data for 1978 only.
Singer and others (2000), PMWHR	0.87	NA	NA	Males, 0.965 Females, 0.975	Not clear that data reported for the herd actually includes all animals.
Siniff and others (1986), Pine Nut, NV	0.875 (40)	NA	0.972 (72)	NA	Adult rate is a weighted mean for 2 years representing only mares, probably ≥2 years old
Siniff and others (1986), Pah Rah, NV	0.877 (73)	NA	0.975 (81)	NA	Adult rate is a weighted mean for 2 years representing only mares, probably ≥2 years old
Keiper and Houpt (1984), MD and VA	0.883 (86)	NA	NA	NA	Foal rate is a weighted mean for 8 years.
Pellegrini (1971), NV	0.905 (21)	NA	NA	NA	Data are for 1969. However, in 1968 no foals were observed, and in 1970 only one foal was observed.
Berger (1986), NV	0.917 (120)	0.969 (64)	0.951 (329)	0.945 (513)	Herd rate calculated from data on the three age groups.
Garrott and Taylor (1990), PMWHR	0.968 (249)	0.977 (173)	0.962 (865)	0.965 (1,287)	Excludes data from a severe winter. Adult and herd rates calculated from Table 2, Garrott and Taylor (1990). Foal rate measured from midsummer to midsummer and may be an overestimate.

others (2000) found significant differences in mortality of foals born to mares 3–5 years old (18 percent mortality), 6–8 years old (3 percent), and ≥9 years old (9 percent).

Foaling Rates

Foaling rates of mares at Pryor Mountain most often have been low to moderate when compared to those reported for other North American herds (table 11). In fact, the rate of 0.432 foals/mare for mares ≥4 years of age calculated from Feist and McCullough (1975) is among the lowest for any herd, although their study was primarily behavioral and lasted only 1 year. In addition, they attempted to classify mares aged 1, 2–3, and ≥4 years without the benefit of examining tooth eruption and wear, and they admit that some classification errors probably occurred. If there was a tendency to misclassify 3-year-old mares as being ≥4 years old, the resulting foaling rate would be biased low. Garrott and Taylor (1990), however, reported similarly low rates, and mares in their study were aged by dental characteristics. As noted above, Garrott and Taylor (1990) reported on foals present in midsummer; some neonatal loss likely was missed, which would tend to bias foaling rates low. Perkins and others (1979) did not report age-specific foaling rates of mares, but for the three years of their study for which data are complete (1975–1977), the foal crop was 15.1 percent of the prefoaling population (weighted average, $n = 411$ animals ≥1 year of age). The comparable value during our study was 21.6 percent (table 1, $n = 2{,}232$ animals). These values, of course, are affected by the sex and age structure of the herd. Similarly, Singer and others (2000) did not report age-specific foaling rates in sufficient detail to be included in table 11. They did, however, show rates of 0.107 foals/mare for mares of age 2, 0.54 for age 3, 0.67 for ages 4–11, and 0.46 for ages ≥12 in the period 1992–1997. Comparable values from our study (1993–2007) were 0.048, 0.414, 0.588, and 0.636, respectively. Thus, it appears that foaling rates at Pryor Mountain have varied considerably through time, with those from our study being intermediate.

Several investigators have suggested that forage availability can affect the condition of feral mares and thus foaling rates (Green and Green, 1977: Nelson, 1978; Berger, 1986; Siniff and others, 1986; Garrott and Taylor, 1990). Hall (1972) described range conditions at Pryor Mountain as being poor to extremely poor through much of the area. Feist and McCullough (1975) reported a low foaling rate in 1971, as did Garrott and Taylor (1990) in the first 3 years (1976–1978) of their study. Furthermore, Garrott and Taylor (1990) suggested that low foaling rates likely were a result of poor forage, also noting that the foaling rate increased markedly in 1979 following loss of 51 percent of the herd in the severe winter of 1977–1978. Singer and others (2000) reported somewhat higher foaling rates than were observed in the earlier studies and also noted that former managers who visited Pryor Mountain in 1997 remarked about the overall improvement in "plant condition." Again, our values for foaling rate appear to be intermediate. It also may be relevant that mean annual precipitation in the biological years covered by the study of Singer and others (17.55 cm; 1992–1997, all years included regardless of missing data) was greater than that for the remainder of our study (13.79 cm; 1998–2007).

In addition to its effects on foaling rates of adult animals, forage availability may affect age of first reproduction by mares. Feist and McCullough (1975), Perkins and others (1979), and Garrott and Taylor (1990) all worked at times when forage production may have been poor at Pryor Mountain and all found that mares first foaled at age 3. Singer and others (2000), on the other hand, at a time when forage production may have been somewhat better, found that 10.7 percent of 2-year-old mares produced foals; the comparable value from our study was 4.8 percent. First foaling at age 2 (Salter and Hudson, 1982; Berger, 1986; Lucas and others, 1991) and age 3 (Boyd, 1979; Keiper and Houpt, 1984; Goodloe and others, 2000) has been reported for other herds, with the rate for 2-year-olds varying both among herds and within herds in different years (Garrott and others, 1991a). In at least two studies, low foaling rates in young mares have been attributed to high rates of fetal and neonatal loss (Lucas and others, 1991; Linklater and others, 2004).

Other factors that have been shown or hypothesized to affect foaling rate include gathers, band stability, and parturition history of the mare. In the case of gathers, we found a small but statistically significant effect, with foaling rate being lower in years following gathers. It must be remembered, however, that we did not design an experiment specifically to test a hypothesis about the effect of gathers. In particular, we had to assume that the majority of mares were affected by the gather (either actually moved into a trap or significantly disturbed by helicopter activity) in each of the three years that we considered to be gather years. Given the nature of the gathers in those three years, we believe that such an assumption is reasonable. Other evidence for an effect of gathers on foaling rate is equivocal. Hansen and Mosley (2000) studied two herds, one in Idaho and one in Wyoming, and found no difference in foaling rate between mares ungathered, gathered and released, or gathered and adopted. The ungathered samples were small, however, consisting of only 14 mares in each herd. Ashley and Holcombe (2001), on the other hand, found that in one of the two years of their study, ungathered mares foaled at a higher rate than did those that were gathered and released or gathered and removed. They found no evidence for an effect of a gather in the other year.

Evidence for an effect of band stability on mare foaling rate is more consistent. Berger (1983) found that mares in bands with resident stallions produced foals at a higher rate than those in bands taken over by a new stallion. Kaseda and others (1995), working on Misaki horses in Japan, reported that mares in "stable consort relationships" had higher reproductive success than those that changed stallions. Similarly, Goodloe and others (2000) found that mares that changed bands had a lower probability of foaling than those that did

Table 11. Foaling rates reported for North American wild horse (*Equus caballus*) herds.

[To the extent possible, data are arranged in order of increasing production by mares ≥3 years old. Sample size (*n*), when available, is given in parentheses. NA, data not available; PMWHR, Pryor Mountain Wild Horse Range.]

Source and location	Foals/mare (*n*)			Comments
	Mares ≥2 years old	Mares ≥3 years old	Mares ≥4 years old	
Siniff and others (1986), Pine Nut, NV	0.385 (104)	NA	NA	Combined data for 2 years. Age of mares inferred from other information.
Feist and McCullough (1975), PMWHR	NA	NA	0.432 (81)	Some animals may have been aged incorrectly. Includes two foals born to 3-year-old mares.
Greger and Romney (1999), NV	NA	0.360	NA	Unweighted mean for 5 years.
Garrott and Taylor (1990), PMWHR	NA	0.488 (600)	NA	Calculated from Table 3, Garrott and Taylor (1990).
Wolfe and others (1989), NV	0.432 (206)	NA	NA	Calculated from Table 5, Wolf and others (1989), for lactating mares.
Boyd (1979), WY, 1979	NA	0.526	0.545	Sample sizes not given.
Turner and others (1992), CA and NV	NA	0.537	NA	Unweighted mean for 6 years.
Keiper and Houpt (1984), MD	NA	0.571	NA	Unweighted mean for 8 years.
Nelson, (1978), NM	NA	NA	0.553 (38)	
This study, PMWHR	0.501 (876)	0.576 (752)	0.597 (665)	
Keiper (1979), MD	NA	0.614	NA	Unweighted mean for 5 years.
Salter and Hudson (1982), Alberta	0.521 (71)	0.621 (58)	NA	Calculated from Table I, Salter and Hudson (1982).
Seal and Plotka (1983), OR	NA	0.626 (99)	NA	Foaling rate based on lactating mares from Table 4, Ashley and Holcombe (2001).
Ashley and Holcombe (2001), Granite Range, NV, 1994	NA	NA	0.714 (28)	Calculated only for ungathered horses.
Ashley and Holcombe (2001), Granite Range, NV, 1997	NA	NA	0.741 (54)	Calculated only for ungathered horses. Age of mares inferred from other information.
Wolfe and others (1989), WY	0.535 (172)	NA	NA	Calculated from Table 5, Wolfe and others (1989), for lactating mares.
Garrott and others (1991a), NV	0.584 (901)	NA	NA	Foaling rate based on lactating mares gathered in June. Calculated from Table 1, Garrott and others (1991a).
Keiper (1979), VA	NA	0.744	NA	Unweighted mean for 4 years.
Ashley and Holcombe (2001), Garfield Flat, NV, 1997	NA	NA	0.824 (17)	Calculated only for ungathered horses. Age of mares inferred from other information.
Boyd (1979), WY, 1978	NA	0.781	0.863	Sample sizes not given.
Siniff and others (1986), Pah Rah, NV	0.670 (109)	NA	NA	Combined data for 2 years. Age of mares inferred from other information.

not. Data on mare interchanges are available from the Pryor Mountain herd, but have not yet been analyzed.

The question of whether parturition history of the mare affects foaling rate has been of interest since early investigators reported what they perceived to be a tendency to foal in alternate years (Green and Green, 1977; Nelson, 1978). This question has been addressed in a number of ways and results have varied. Seal and Plotka (1983) found that 68.4 percent (n = 76) of pregnant mares also had a foal at their side or were lactating, and Wolfe and others (1989) found no difference in pregnancy rates for lactating and nonlactating mares in six different populations. Neither of these studies, however, addressed the question of whether lactating and nonlactating mares are equally successful in bringing their pregnancies to term. On Assateague Island, 10.2 percent of 49 mares followed for 4 years foaled in alternate years, whereas 55.1 percent foaled in either 3 or 4 years (Keiper, 1979). Similarly, Keiper and Houpt (1984) could not see any tendency to foal in alternate years among 14 mares followed for 8 years. At Pryor Mountain, in both our study and that of Garrott and Taylor (1990), primiparous mares were less likely to foal in the next year than were multiparous mares. Cameron and Linklater (2000), on the other hand, found that the probability of foaling in a given year was reduced significantly if the mare had a foal in the previous year. Thus, while not universal, it appears that foaling in one year can sometimes reduce the probability of foaling in the subsequent year, particularly in primiparous mares.

Harem Stallions and Band Composition

Comparisons among band or harem sizes reported by various investigators are hampered by inconsistencies in defining what constitutes a harem member, or failure to do so at all. We defined harem members as mares ≥2 years of age, and found that the mean harem size was 2.2 mares with a range of 1.9 to 2.7 over 12 years; the number of bands ranged from 29 to 38. In other studies at Pryor Mountain, Feist and McCullough (1975) reported only average band size (5.0 including all members, not just mares), but they found 44 bands and 106 females ≥2 years of age, so the average harem size was likely somewhere near 2.4 mares. Note that the total population during the single summer of their study was about 225 animals, which probably explains the somewhat larger number of bands. Perkins and others (1979), working on a population that ranged from 95 to 168 animals (1974–1978), found 19–27 bands with a mean size of 4.9 (total animals), but did not report harem size in terms of number of mares. During a period (1992–1997) when the mean population was 161 animals, Singer and others (2000) reported an increase in the number of bands (18 to 31) and a decline in the number of mares (age unspecified) per harem from four to two. They attributed these trends to an increasing proportion of males in the population, death or waning vigor of a number of older males, and removal of members from the bands of several stallions aged 10–15 years. Investigators working on other herds have found similar or perhaps slightly larger average harem sizes. Mean harem size on Cumberland Island was 1.8 mares (Goodloe and others, 2000); the authors did not specify the age of mares included, but only mares ≥3 years old produced foals. Greger and Romney (1999) reported an average harem size of 2.4, which included all females ≥1 year of age, excluding daughters. Berger (1986, p. 130) found an average harem size of 3.1 mares with a range of 2.73–3.67 across years; age of mares was not specified but, because 2-year-old females reproduced, mares ≥2 years old may have been included. In Misaki horses (Japan), the annual average harem size ranged from 1.8 to 5.3 mares ≥2 years old; one stallion in one year had a harem of nine mares, and 55.0 percent of 60 harems (14 harem stallions) observed from 1979 to 1994 had four or more mares (Kaseda and Khalil, 1996).

In our study, 62.1 percent of harem stallions first acquired a harem at age 5 or 6. Other investigators have found comparable results: Berger (1986, p. 142), age 5–6; Kaseda and Khalil (1996), age 4–6; Asa (1999), age 5; Singer and others (2000), mean age = 6 years. In the last two years of our study, the mean age of harem stallions was 13 and 12 years, which is remarkably similar to the mean of 12.8 years reported at Pryor Mountain by Perkins and others (1979). Hall (1972), however, reported a mean age of 7.8 years, which is more similar to the data from the early years of our study. Age structure of removals is undoubtedly an important determinant of such results.

During our study, 6.3 percent of bands had multiple stallions. Feist and McCullough (1975) did not mention whether any of the 44 bands present during their study had multiple stallions. Perkins and others (1979) found only two multistallion bands in the 19–27 bands present during the 4 years of their study; if the mean number of bands present was, say, 23 per year, multistallion bands would have made up about 2.2 percent of the total. These percentages are lower than those reported for other herds: Goodloe and others (2000), 10.7 percent, n = 177; Nelson (1978), 11.8 percent, n = 17; Berger (1986, p. 129), 12 percent, sample size not given; Salter and Hudson (1982), 17.4 percent, n = 23; Asa (1999), 28 percent and 33 percent in 2 years, n = 18 and 9; Miller (1979), 23–45 percent for 3 years, n = 12–18; Bowling and Touchberry (1990), 39.1 percent, n = 69; Franke Stevens (1990), 50 percent, n = 12; Green and Green (1977), 45 percent and 54 percent in 2 years, n = 53 and 67, respectively. Kirkpatrick and Turner (1986) suggested that differences in proportions of bands with multiple stallions may be related to population density and sex ratio, with a greater ratio of males to females tending to lead to multistallion bands, and noted that some of the reported proportions may be high as a result of inaccurate aging of the animals based on body size [for example, Green and Green (1977)].

Bachelors

We have information on the total number of bachelors, but not on individual groups, which makes comparisons with other studies at Pryor Mountain difficult. Hall (1972) reported that the average size of bachelor groups was three, with individuals ranging in age from 1 to 5 years, and that older stallions usually lived alone after losing their harems. Feist and McCullough (1975) found 23 bachelor groups with a mean of 1.8 animals per group. Perkins and others (1979) found six bachelor groups, all with 2–3 individuals ranging in age from 3 to 8 years, as well as six older stallions that lived alone. With allowances for varying population size, our results do not appear to be greatly different from those found at other times at Pryor Mountain.

Most evidence suggests that males leave or are expelled from their natal band when they reach sexual maturity, which usually occurs at age 3 and occasionally at age 2 (Angle and others, 1979; Kirkpatrick and Turner, 1986). Our data show a somewhat different pattern. A few males left their natal band at age 1, most (54.2 percent) left at age 2, but 17 percent remained with their natal band until age 4 or 5. Of the three individuals that remained in their natal bands until age 5, two subsequently acquired harems and one was still a bachelor (6 years old) at the end of our study. Interestingly, one of these stallions took over his natal band, at which point his dam left the band for another. It seems possible that instances of stallions remaining with their natal band until age 4 or 5 account for some of the reports in other studies of a high proportion of multistallion bands.

Population Regulation

Several investigators have suggested a relation between forage availability and foaling rate, but few studies have been long enough and had sufficient variation in population size to allow detection of density-dependent effects. Berger (1986, p. 97) found little evidence for density dependence in his data from the Granite Range and suggested that the confounding effects of weather were responsible. He did, however, find some evidence for a decline in foaling rate with increasing population size in data from Keiper and Houpt (1984). Singer and others (2000) found that population growth rate at Pryor Mountain declined with increasing population size, but this result was clearly dominated by data from a single year [Singer and others (2000), fig. 5]. They did not find evidence for a density-dependent effect on either foaling rate or survival rate. Jenkins (2000) examined data from the Granite Range and Pryor Mountain herds, both having individually identified animals, as well as five additional herds where population size was estimated using aerial surveys. In all seven cases, he found that population growth rate decreased with increasing population size, but only one was statistically significant ($P \leq 0.05$). However, his meta-analysis of the entire data set indicated that the probability of the same result through chance alone was only 0.001.

Our results are similar to those of both Singer and others (2000) and Jenkins (2000) in that we also found that population growth rate declined with increasing population size, albeit population size from the preceding year, which suggests that lag effects may be important. Furthermore, we found similar evidence for a negative relation between overall herd survival and population size, and perhaps some weaker evidence that the foaling rate of 2-year-old mares is affected by population size. We did not, however, find evidence for a density-dependent effect on foal survival, perhaps in part because Garrott and Taylor (1990), whose study encompassed a range of population sizes, did not provide annual foal survival rates.

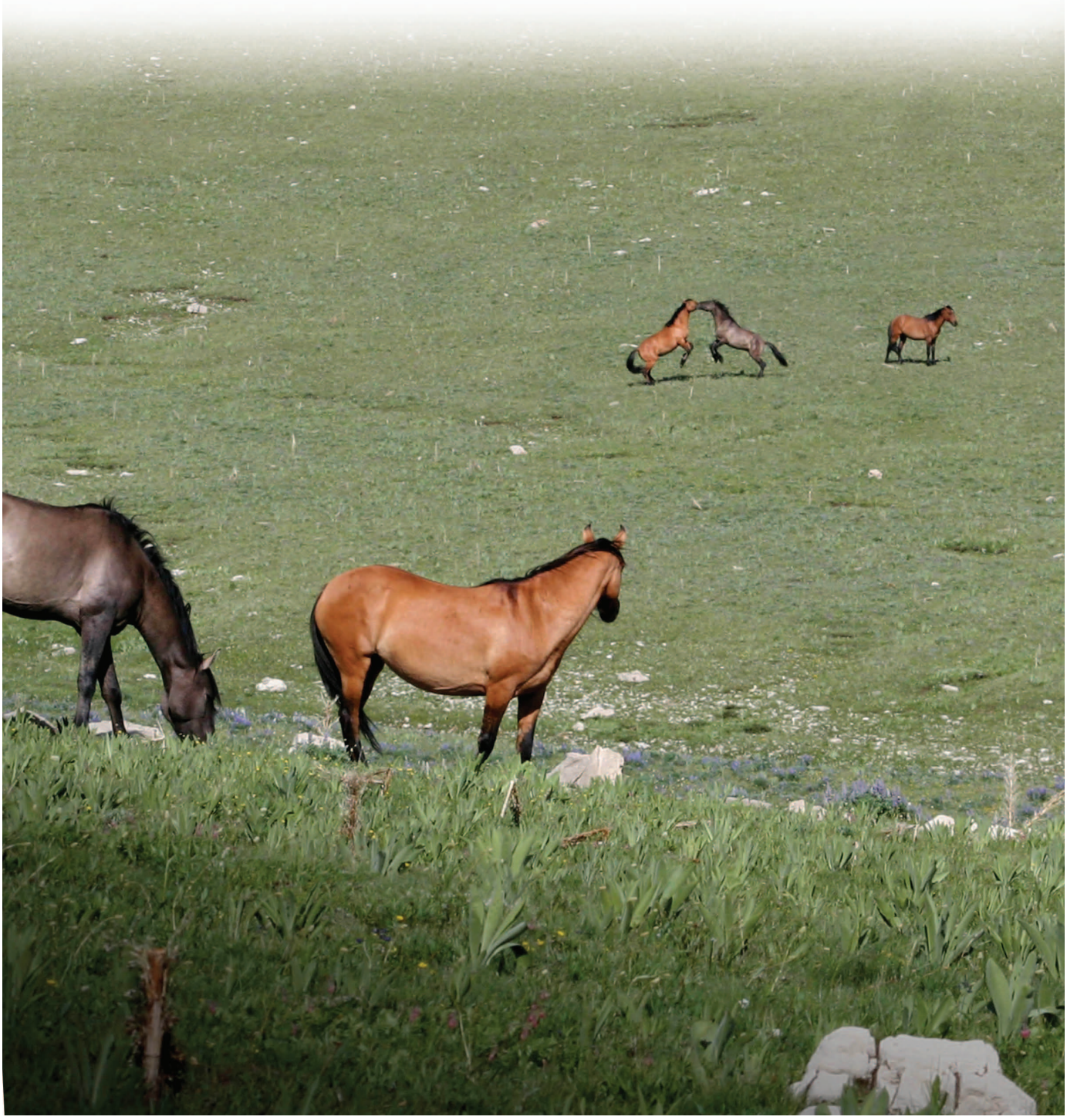
We used population size as a surrogate for population density in these analyses, as did all of the authors cited in this section. In doing so, we made the implicit assumption that the area used by horses did not change significantly through the course of the study. In the case of the Pryor Mountain herd, however, horses probably made increasing seasonal use of Forest Service lands north and west of the original wild horse range. It seems likely that any density-dependent effects would have been more pronounced had this increase in use of Forest Service land not occurred.

In all cases where we found evidence for density dependence, variability was high, suggesting that other factors also may be important in regulating the Pryor Mountain wild horse herd. Assuming that the cause of mortality was correctly identified, mountain lion predation may have had a dramatic effect on foal survival in at least one year. In the latter years of our study, contraception also may have had some effect on foaling rate, though it seems likely that this effect was small given that relatively few mares were treated and that they were not in the most productive age classes. One of the most important regulatory mechanisms appears to be precipitation, which we found to be strongly related to foal survival. Both density dependence and precipitation effects are consistent with an interpretation of forage availability per individual as an important parameter. All of these factors combined, however, were not sufficient to stabilize the population during our period of study, as evidenced by the necessity for large removals in several years.

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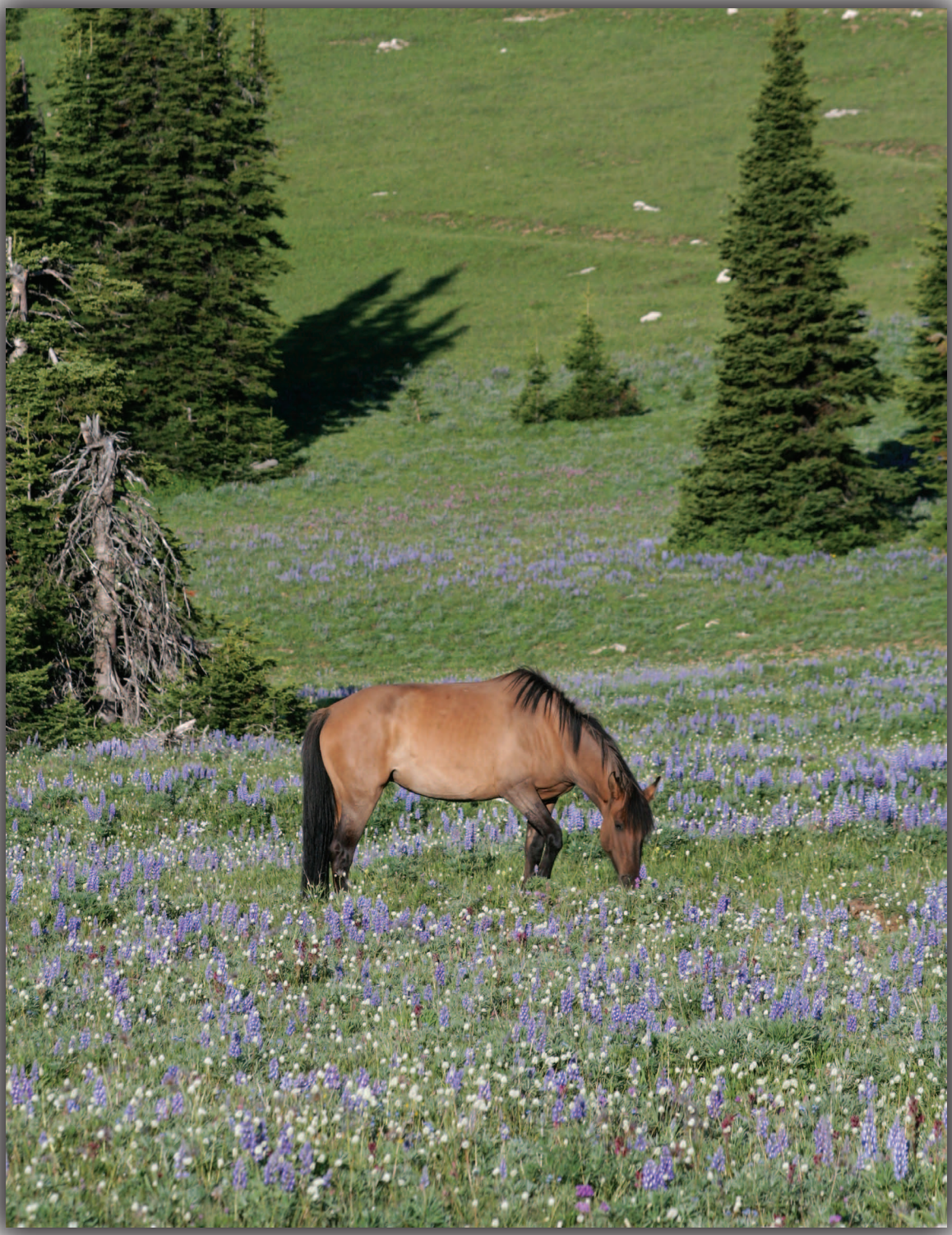
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Original Article

Efficacy of SpayVac® as a Contraceptive in Feral Horses

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ABSTRACT We tested the efficacy of 2 formulations of the immunocontraceptive SpayVac®, which packages the immunogen porcine zona pellucida (PZP) and an adjuvant in multilamellar liposomes, as a contraceptive in captive feral horses (*Equus caballus*) for 3 consecutive breeding seasons (Pauls Valley, OK, USA; 2012–2014) following a single inoculation. Annual fertility rates in control adult female horses ($n = 30$ each yr) were 100%, 96.7%, and 100%. In the nonaqueous treatment group, fertility was 16.7% in the first year ($n = 30$) and 75.9% in the second year ($n = 29$), at which point we dropped the group from the study. Fertility rates in the aqueous group were 13.3%, 46.7%, and 43.3% ($n = 30$ each yr). Fifteen of the females in the aqueous group were infertile in all 3 years. Across 11 sampling dates postvaccination, mean PZP antibody titers in serum were 33.7–91.9% greater in nonpregnant females than pregnant females for the aqueous treatment group and 7.8–82.8% greater for the nonaqueous group. However, the 15 consistently infertile females did not necessarily have the greatest antibody titers. Reactions at the injection site occurred in 29.8% of the 84 females that received an injection other than saline solution, but there was no evidence that the reactions were painful or affected mobility. The nonaqueous formulation produced more local reactions than did the aqueous, but presence of PZP did not increase the frequency of reactions above that seen with liposomes + adjuvant. Uterine edema was not found at frequencies greater than would be expected in untreated females. Additional research to explore relationships between vaccine dose, adjuvant, and efficacy is warranted. Published 2017. This article is a U.S. Government work and is in the public domain in the USA.

KEY WORDS *Equus caballus*, feral horse, fertility control, immunocontraception, porcine zona pellucida, SpayVac®, wildlife contraception.

Feral horse (*Equus caballus*) populations managed by the Bureau of Land Management (BLM) in the western United States often grow rapidly and, in the absence of intervention by BLM, would frequently exceed the carrying capacity of their ranges (Eberhardt et al. 1982, Garrott et al. 1991*b*, Roelle et al. 2010). The BLM has most often controlled herd size through a gather (round up) and removal process, offering excess horses to the public for adoption. Over time, the supply of horses has exceeded adoption demand, and BLM has moved thousands of animals to long-term holding pastures where they are cared for as long as they live at considerable expense to American taxpayers. In 2007, for example, the cost of holding animals off the range was approximately US$21 million, or about 67% of the budget for BLM's Wild Horse and Burro Program (U.S. Government Accountability Office 2008:9).

The BLM has long been interested in fertility control as a means of regulating feral horse reproduction and partial solution to the problem of escalating off-range holding costs. The most promising fertility control agents to date are based on porcine zona pellucida (PZP) glycoproteins extracted from pig (*Sus scrofa*) ovaries. When injected into mammalian species such as the horse, these proteins induce production of antibodies that bind to the surface of the ovum and block sperm penetration and fertilization (Liu et al. 1989). Possible effects on ovarian function have been noted as well (Kirkpatrick et al. 1992, Bechert et al. 2013). The most widely used form of PZP available at the present time is an emulsion registered as ZonaStat-H by the U.S. Environmental Protection Agency for use in horses and burros (*E. asinus*). Unfortunately, ZonaStat-H has 2 important drawbacks relative to use in BLM herds. First, a booster inoculation is required, ideally 3–4 weeks after the primer is administered (Liu et al. 1989). Because darting is rarely feasible on western rangelands, most BLM herds must be gathered in order to provide access to the horses for inoculation. Female horses would need to be held in captivity long enough to administer a booster 3–4 weeks later. Second, ZonaStat-H has a reported duration of efficacy of only 7–10 months, after which annual booster inoculations are required to maintain infertility (Liu et al. 1989, Kirkpatrick et al. 1990, Lyda et al. 2005). Some progress in overcoming

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these drawbacks has been made by substituting time-release pellets, delivered at the same time as the primer injection, for the booster at 3–4 weeks and first annual booster. This formulation, sometimes referred to as PZP-22, has a reported efficacy of 22 months (Turner et al. 2007), but this is still less than ideal from the BLM's perspective.

A third formulation of PZP known as SpayVac®, produced by ImmunoVaccine Technologies Inc. (IVT; Halifax, NS, Canada) using a proprietary liposome technology, has been reported as having long-term, single-dose efficacy in several species, including grey seals (*Halichoerus grypus*; Brown et al. 1997*a*), fallow deer (*Dama dama*; Fraker et al. 2002), and most recently feral horses (Killian et al. 2008, Fraker and Brown 2011). Killian et al. (2008), for example, reported contraceptive efficacy of 100%, 83%, 83%, and 83% in 4 years following inoculation of 12 feral horses in Nevada, USA, with SpayVac. This result sparked renewed interest within the BLM to conduct further investigations on the efficacy of SpayVac in feral horses.

The 3 main objectives of our study were to 1) quantify the efficacy of SpayVac in preventing pregnancy and foaling over a maximum of 5 breeding seasons postinjection when females are exposed to breeding males in a pasture setting; 2) determine the relationship between PZP serum antibody titers and fertility; and 3) assess the frequency and severity of injection-related and general side effects, if any, following intramuscular injection by hand. In addition, for the 4 years of their study, Killian et al. (2008) reported that 70–100% of females treated with SpayVac showed some degree of uterine edema. Although we do not view uterine edema as a serious contraindication, others have indicated concern, so a fourth objective was to estimate the frequency and degree of uterine edema in females treated with SpayVac. Finally, Bechert et al. (2013) reported that 93% of females that they treated with SpayVac ceased estrous cycling within 3–4 months after vaccination. Our fifth objective was therefore to determine what proportion of our SpayVac-treated females stopped cycling.

STUDY AREA

We conducted our captive breeding trial at BLM's wild horse and burro adoption facility near Pauls Valley, Oklahoma, USA. Adult female horses resided year-round in 3 pastures of approximately 12 ha each, and were accompanied by breeding males from approximately the first week in June (2011) or the first week in May (2012 and 2013) to the first week in October (all years). During the remainder of the year, space limitations forced us to house the males in a separate facility in Nebraska, USA. We transported males to and from their winter quarters by trailer, moving them back to Oklahoma in the spring 2–3 weeks before beginning the breeding trial for that year. We immunized the females and drew blood samples in a processing facility consisting of a series of alleys and pens and a padded hydraulic squeeze chute located 100–300 m from the pastures.

Horses fed on pasture bermudagrass (*Cynodon* sp.) *ad libitum* and alfalfa hay provided at a rate of approximately 6.4 kg/animal/day. FlaxLic® protein/fat supplement (New Generation Feeds, Belle Fourche, SD, USA) and Ranch House trace mineralized salt blocks (United Salt Corporation, Houston, TX, USA) were also provided *ad libitum*. Bureau of Land Management personnel annually immunized horses against eastern and western equine encephalomyelitis, equine influenza type A_2, the EHV-1 and EHV-4 strains of equine herpes virus, and tetanus (Fluvac Innovator® 5; Zoetis, Florham Park, NJ, USA); West Nile virus (Vetera® WNV; Boehringer Ingelheim Vetmedica, St. Joseph, MO, USA); *Streptococcus equi* (Strepvax® II; Boehringer Ingelheim Vetmedica); and rabies (Imrab® 3; Merial Limited, Duluth, GA, USA). Additionally, BLM personnel dewormed horses at least annually (more frequently for specific susceptible individuals) using Ivermax™ Equine Oral Liquid (RXV Products, Westlake, TX, USA).

METHODS

Vaccine Preparation

We conducted all work on this study under the auspices of a protocol (#2011-06) approved by the Animal Care and Use Committee at the Fort Collins Science Center, U.S. Geological Survey, Fort Collins, Colorado, USA. We chose 2 formulations of SpayVac (an aqueous emulsion and a nonaqueous form) for testing based on a recommendation from a companion study on safety and mechanism of action conducted at Oregon State University, Corvallis, Oregon, USA (Bechert et al. 2013). Personnel at IVT prepared the vaccines as described by Brown et al. (1997*b*). Briefly, they obtained frozen pig ovaries from SiouxPreme Packing Co. (Sioux Center, IA, USA) and isolated PZP by grinding thawed ovaries and passing the resulting mixture through a series of nylon mesh screens with successively decreasing pore sizes to recover oocytes. They then homogenized the oocytes to release PZP, mixed the PZP at a rate of 200 μg/dose with phosphate buffered saline (pH = 7.4), and added 0.2 g lecithin and 0.02 g cholesterol (Lipoid, Newark, NJ, USA) per dose to form multilamellar liposomes. One half of the PZP-liposome mixture was added to Modified Freund's Adjuvant (MFA; Calbiochem, San Diego, CA, USA; 1:1 v/v) to form a water-in-oil emulsion (the aqueous vaccine; 1 mL/dose). The second half of the PZP-liposome mixture was lyophilized and then reconstituted with MFA to form the nonaqueous vaccine (0.5 mL/dose). Vehicle controls were produced in the same manner but without the PZP antigen. Saline controls consisted of 1 mL of phosphate-buffered saline. Technicians at IVT loaded all vaccines and control doses into individual syringes and delivered them to us frozen on dry ice.

Treatments and Blood Sampling

We randomly selected 90 females—ages 4 ($n = 68$), 5 ($n = 18$), 6 ($n = 2$), or 7 ($n = 2$)—from 120 females that were present at the Pauls Valley facility when we initiated our study. All females were thus at or near prime breeding age (Garrott et al. 1991*a*, Roelle et al. 2010), and all were open (nonpregnant) at the time of enrollment by virtue of separation from males during the previous breeding season. Age was either known precisely (i.e., removed from the range as a foal or born in captivity) or estimated by tooth eruption

and wear (American Association of Equine Practitioners 2002). Bureau of Land Management personnel uniquely marked each female on the left hip with a 4-digit freeze mark in numerals 7.6 cm high. We assigned each female to 1 of 3 experimental groups (aqueous vaccine, nonaqueous vaccine, and control) in a stratified-random fashion such that each group was equally represented in each of the 3 pastures. We further subdivided the 30 females in the control group into aqueous (4 F/pasture), nonaqueous (4 F/pasture), and saline (2 F/pasture) groups. After allowing all materials to thaw for approximately 30 min at room temperature, we delivered vaccines and sham doses on 30 March 2011 by hand injection in the left rump while each female was restrained in a padded hydraulic squeeze chute.

At the time of vaccination and on 7 additional occasions in 2011 (3 May, 7 Jun, 6 Jul, 2 Aug, 7 Sep, 5 Oct, and 14 Dec), we drew 10 mL of blood from each female in serum separator vacutainers by jugular venipuncture for PZP titer assays. We also drew blood samples on 7 March 2012, 9–10 January 2013, 6 March 2013, and 13–14 November 2013. Following collection, we allowed the blood samples to clot for approximately 2 hr at room temperature. We then centrifuged the samples at 1,300 G for approximately 12 min and removed 2 aliquots of approximately 2 mL of serum by pipette. We placed each aliquot in a 10-mL polypropylene transport vial labeled with the date and horse identification number, froze the samples overnight in a household freezer, and shipped them on dry ice to Fort Collins, Colorado, where they were stored at −80° C until being shipped on dry ice by overnight courier for titer assays.

To corroborate our foaling observations (see below), we drew an additional 10 mL of blood from each female in a plain red-top vacutainer on 12 December 2011, 9–10 January 2013, and 13–14 November 2013. We separated serum from these samples as described above and shipped the frozen samples to the Endocrinology Lab, Animal Health Diagnostic Center, Cornell University, Ithaca, New York, USA, for pregnancy testing. Personnel at the lab assayed the samples for pregnant mare serum gonadotropin and estrone sulfate and characterized each female as open, pregnant, or inconclusive.

Female Horse Health and Condition

During each blood draw through 7 March 2012, we observed females carefully for general health and palpated each injection site to ascertain whether a reaction had occurred. We characterized each site as follows: none = no reaction apparent; swelling = a diffuse raised area, perhaps irregular in shape, may be firm, but does not appear or feel hard; lump = a distinct, roughly circular nodule that appears or feels hard; abscess = a draining sore or pocket of fluid or pus. We characterized the severity of each reaction on a scale from 0 to 3, using the same scoring system both in palpation of the injection sites and pasture observations (see below): 0 = no reaction observable or palpable; 1 = reaction (swelling, lump, or abscess) present, but no pain or lameness noticeable; 2 = reaction present, mild to moderate lameness or response to palpation; 3 = reaction present, severe lameness or strong response to palpation. On 30 March 2011 and 7 March 2012, we also estimated body condition of our females using the system of Henneke et al. (1983). We assigned each female a score from 1 to 9 following observation in the chute and palpation of fat and soft tissue of the neck, back, ribs, and rump.

Serum Assays

Technicians at IVT used an enzyme-linked immunosorbent assay to determine PZP antibody titers in horse serum. They coated 96-well polystyrene microtiter plates (Bio-Rad, Hercules, CA, USA) with PZP antigen (prepared in-house, 1 μg/mL) and incubated them overnight at 4° C. They then washed the plates with a Tris wash buffer (Tris-buffered saline/Tween-20) and blocked them with 3% gelatin (Bio-Rad) for 30 min at 37° C. Following another wash, they added a standard horse serum sample and unknown horse sera samples to the wells and incubated the plates at 4° C overnight. They diluted unknown serum samples and a positive reference serum sample to 1/25,000 and added each sample to the plates in triplicate. They then washed the plates, added a secondary antibody, Protein G (Calbiochem) conjugated to alkaline phosphatase enzyme, to each well at a dilution of 1/1,000, and incubated the plates for 1 hr at 37° C. Following another wash, they incubated the plates for 1 hr at 37° C with a substrate solution containing 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland) at a concentration of 1 mg/mL. They then used a microtiter plate reader (ASYS Hitech GmbH, Eugendorf, Austria) to measure the optical density or absorbance of each well at a wavelength of 405 nanometers. They calculated titers of the unknown serum samples as the average absorbance of the triplicate unknown samples expressed as a percentage of the average absorbance of the triplicate positive reference sample. The positive reference sample was from a female previously vaccinated (in a separate study) with SpayVac and determined to have a strong, PZP-specific antibody response. The positive reference sample was considered to have a titer of 100%.

Male Horses

We randomly selected 12 adult males, 6–9 years of age at the start of the study, from a group gathered at the Adobe Town/Salt Wells Creek herd management complex in Wyoming, USA, in October 2010. We gelded and dropped 1 of these males from the study because of his aggression. We allowed 2 months following inoculation for anti-PZP antibody titers to build and introduced 3 randomly selected males into each pasture with the females on 7 June 2011. We anticipated that the pastures would be large enough for each male to acquire a stable harem from the 30 females present and coexist with minimal aggression, but this turned out not to be the case. Fighting was not uncommon, and in 2 of the pastures breeding activity was dominated by a single male. On 6 July 2011, we randomly assigned a single male to each pasture and developed a random rotation such that pastures received a new male approximately every 2 weeks, and all males had a rest period of ≥2 weeks following each session in a pasture. Following the first rotation, we eliminated another male because he was never observed breeding a female. In 2012

and 2013, we used a new randomly assigned order each year and each pasture session lasted approximately 3 weeks. Two males died of causes unrelated to the study, one due to impaction and rupture of the large colon and the other due to a spinal injury, during the winter of 2012–2013, leaving only 8 in the rotation in 2013.

Pasture Observations

A technician who was blind to the treatment status of individual females observed each pasture $\geq$2 hr/day and $\geq$3 days/week to record information on injection-related (2011 only) or other health issues, estrous and breeding behavior, and foaling. We tried several vantage points or modes of transportation (e.g., from the fence surrounding the pasture, from a truck in the pasture, mounted on a domestic horse) for making these observations, ultimately settling on a golf car as the best platform; using the car, we were able to move within a few meters of the females while evoking little reaction other than curiosity. We recorded the nature and severity of reactions to the injections using the system described above. We characterized estrous and breeding behavior on a scale from 0 to 5 as follows: 0 = no evidence of estrous or breeding behavior observed; 1 = warm = squatting, winking, or urination observed, but female is not completely receptive to the male; 2 = hot = squatting, winking, or urination observed and female is receptive to the male; 3 = mounting observed; 4 = penetration observed; and 5 = ejaculation (flagging of the male's tail) observed. The main purpose of these observations was simply to ensure that females were being bred. We associated foals with individual dams based on nursing by the foal that was readily accepted by the female and protective behavior by the female, especially during the first few days and weeks of the foal's life. Female–foal pairings were observed $\geq$3 times/week from birth through the end of the breeding season.

Uterine Edema

On 9–10 January 2013 and 13–14 November 2013, we examined each female in the study per rectum using a Universal Medical Systems 900 (Bedford Hills, NY, USA) portable ultrasound and a 5-MHz linear transducer. Uterine edema was characterized as none, slight, moderate, or extensive. During these examinations, we also recorded information on follicular development, stage of estrous cycle, and pregnancy. All examinations were performed while females were restrained in a squeeze chute; none required sedation.

Cyclicity

On 3 June, 10 June, 17 June, 23 June, and 1 July 2014, we drew 10-mL samples of blood in a plain red-top vacutainer from each female treated with the aqueous formulation of SpayVac. We extracted serum as previously described and shipped it to the Endocrinology Lab, Animal Health Diagnostic Center, Cornell University for progesterone analyses. Females with $<$0.5 ng/mL progesterone in all 5 samples were considered to be not cycling.

Analyses

We had the following information available for assessing drug efficacy: foal observations (2012 and 2013), palpation and ultrasound (2013 and 2014), and hormonal pregnancy tests (all 3 yr). In the hormonal pregnancy tests, there were 20 cases where the results were inconclusive; in 18 of those 20 cases, no foal was observed. It thus seemed important to include those cases in our analyses, by using the other 2 criteria, to obtain the best estimate of drug efficacy. We therefore performed all of our analyses on a composite outcome measure in which a female was judged to be fertile if any of the 3 criteria (ultrasound, hormone test, foal observation) was positive. Use of this composite measure allowed us to include females whose hormone assays were inconclusive, but likely had only a small effect on our overall results, because results from the various measures of fertility were in complete agreement in 220 (92.1%) of 239 cases. We also performed duplicate analyses (data not shown) using only the results of hormonal pregnancy testing (because data were available for all 3 yr); in no case did the results differ substantively from those presented here for the composite measure of fertility.

We compared the binomial composite measure of fertility across the 2 treatment groups (aqueous and nonaqueous) with logistic regression when we were interested in estimating treatment group differences after adjusting for effects of years since treatment, body condition, and pasture assignment. We did not include female age in our models, but rather controlled for the effects of age through randomization and restricting enrollment to females 4–7 years of age. We used Akaike's Information Criterion (AIC; Burnham and Anderson 2002) to select those models better supported by the data. Because control females were fertile in 89 of 90 possible instances, the logistic model estimates were limited to comparisons of aqueous and nonaqueous groups. We also compared fertility in the control group with each of the treated groups without any additional covariates using Fisher's exact tests for the homogeneity of proportions fertile in each year. Comparisons of 2 treatment groups with Fisher's exact test provided a corresponding 95% confidence interval on the odds ratio. In addition, we compared injection-site reactions across the control and treatment groups, and by formulation (aqueous vs. nonaqueous, treated and control females combined) and ingredient (liposomes plus adjuvant with PZP or without PZP, treated and control females combined) with logistic regression and Fisher's exact test for homogeneity of proportions.

RESULTS

There was the potential for females in the control group to be fertile in 90 cases (30 F $\times$ 3 yr) during the course of our study. Using the composite measure, fertility occurred in 89 cases, indicating that males were able to cover females adequately with the rotation schedules that we used. Thus, differences in pregnancy rate or composite fertility rate between control and treatment groups can likely be attributed to the treatment, rather than to a failure of the males to breed.

The aqueous formulation of SpayVac was effective in 2012 as evidenced by a fertility rate of only 0.133 foals/female as compared with 1.00 foals/female in the control group (odds ratio 95% CI = [29.8, ∞], $P < 0.001$; Table 1). Fertility in

Table 1. Fertility rates in treated and control adult female feral horses enrolled in a captive breeding trial of the immunocontraceptive SpayVac at Pauls Valley, Oklahoma, USA, 2012–2014.

	Aqueous[a]			Nonaqueous[a]			Control	
Year	Fertility rate[b]	*n*	*P*[c]	Fertility rate[b]	*n*	*P*[c]	Fertility rate[b]	*n*
2012	0.133	30	<0.001	0.167	30	<0.001	1.00	30
2013	0.467	30	<0.001	0.759	29	0.026	0.967	30
2014	0.433	30	<0.001	Withdrawn			1.00	30

[a] Aqueous and nonaqueous refer to 2 formulations of SpayVac.
[b] Fertility rate is expressed as proportion of females. Fertility was determined through hormone assay (pregnant mare serum gonadotropin and estrone sulfate), rectal palpation and ultrasound, and direct observation of foals. A female was considered fertile if any of the 3 measures was positive.
[c] *P*-value is the probability that the fertility rate is the same in the treatment and control groups as determined by Fisher's exact test.

the aqueous group increased in 2013, but remained different from rates in the control group (odds ratio 95% CI = [4.1, 1,424.5], $P < 0.001$), which also occurred in 2014 (odds ratio 95% CI = [7.7, ∞], $P < 0.001$). Fertility rate in the nonaqueous group differed statistically from that of the control group in 2012 (odds ratio 95% CI = [24.5, ∞], $P < 0.001$) and 2013 (odds ratio 95% CI = [1.0, 428.9], $P = 0.03$). In 2013, however, the difference was quite small biologically (nonaqueous group = 0.76 foals/F, control = 0.97 foals/F), and we judged that the BLM would not find the small reduction in foaling due to contraception to be of interest. Having no reason to suspect that drug performance would improve in subsequent years and not wanting to contribute foals unnecessarily to the off-range population of BLM horses, we removed the nonaqueous group from the experiment at the end of the second foaling season. The fact that few treated animals were fertile in the first year indicates that the time we allowed between vaccination and introduction of the males (~2 months) was sufficient for contraceptive efficacy to develop.

We used logistic regression to model probability of foaling in the aqueous and nonaqueous treatment groups in 2012 and 2013. Our initial model contained the potential explanatory variables treatment, year, and a treatment by year interaction. Of these, only year was significant ($z = 2.675$, $P = 0.007$), consistent with the fact that the fertility rate increased in both groups in 2013. Addition of body condition at the time of treatment did not improve the model ($\Delta AIC = 0.96$).

Mean recorded PZP antibody titers reached a maximum more quickly for the nonaqueous formulation, but also declined more quickly and were consistently lower than means for the aqueous formulation after 6 July 2011 (Fig. 1). A similar pattern was apparent in the number of females that exhibited their maximum recorded titer on each sampling date (Table 2). Greater than half of the 30 females treated with the nonaqueous formulation exhibited their maximum recorded titer on 3 May 2011, and 29 of the 30 did so by 6 July 2011. In contrast, individual females treated with the aqueous formulation exhibited their maximum titers over a longer period of time. Across 11 sampling dates postvaccination, mean PZP titers were 33.7–91.9% greater in nonpregnant females than in pregnant females in the aqueous treatment group (Fig. 2A) and 7.8–82.8% greater in the nonaqueous group (Fig. 2B). The relationship between antibody titer and fertility in individual females was less clear (Table 3). Female number 609, for example, had a maximum titer of 60.11%, was not one of the females that ceased cycling postvaccination, and did not become pregnant in any of the 3 years of our study, whereas several females with greater maximum titers did become pregnant.

None of the 6 females that received saline control inoculations exhibited any reaction at the injection site (Table 4). Of the 84 animals that received either SpayVac or the liposome+adjuvant control doses, 25 (29.8%) exhibited a reaction of some kind (swelling, lump, or abscess). Abscesses were present in 5 (6.0%) of the 84 during the year in which we monitored the injection sites; all 5 occurred in animals that received either the nonaqueous vaccine or the nonaqueous control injection. Abscesses appeared as early as 2 months postinoculation and as late as 5 months; some healed only to reappear at a later date. On female 3425, for example, an abscess was first recorded on 7 September 2011, was recorded again on 5 October, had disappeared by 14 December, and reappeared by 7 March 2012. In no case,

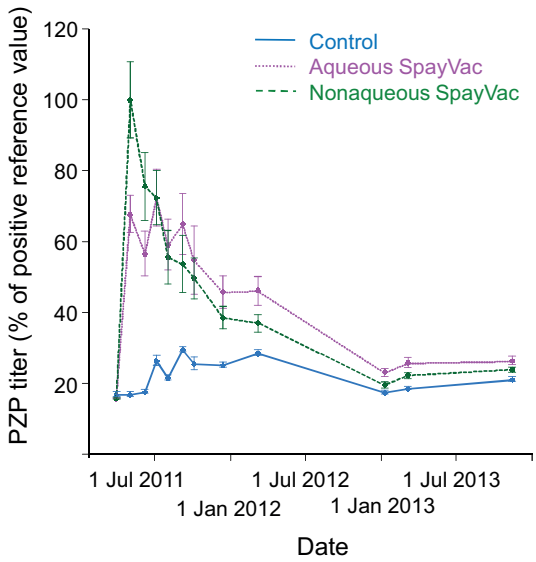


Figure 1. Mean porcine zona pellucida antibody titers, expressed as % of a positive reference, in adult female feral horses enrolled in a captive breeding trial of the immunocontraceptive SpayVac at Pauls Valley, Oklahoma, USA, 2012–2014. Aqueous and nonaqueous refer to 2 different formulations of SpayVac. Vertical bars represent ±1 standard error.

Table 2. Number of adult female feral horses for which maximum antibody titers were recorded on each sampling date in a captive breeding trial of the immunocontraceptive SpayVac at Pauls Valley, Oklahoma, USA, 2012–2014. Females were treated with either aqueous ($n = 30$) or nonaqueous ($n = 30$) SpayVac on 30 March 2011.

Sampling date	Aqueous SpayVac	Nonaqueous SpayVac
3 May 2011	8	17
7 Jun 2011	6	4
6 Jul 2011	7	8
2 Aug 2011	2	0
7 Sep 2011	5	0
5 Oct 2011	1	1
14 Dec 2011	1	0

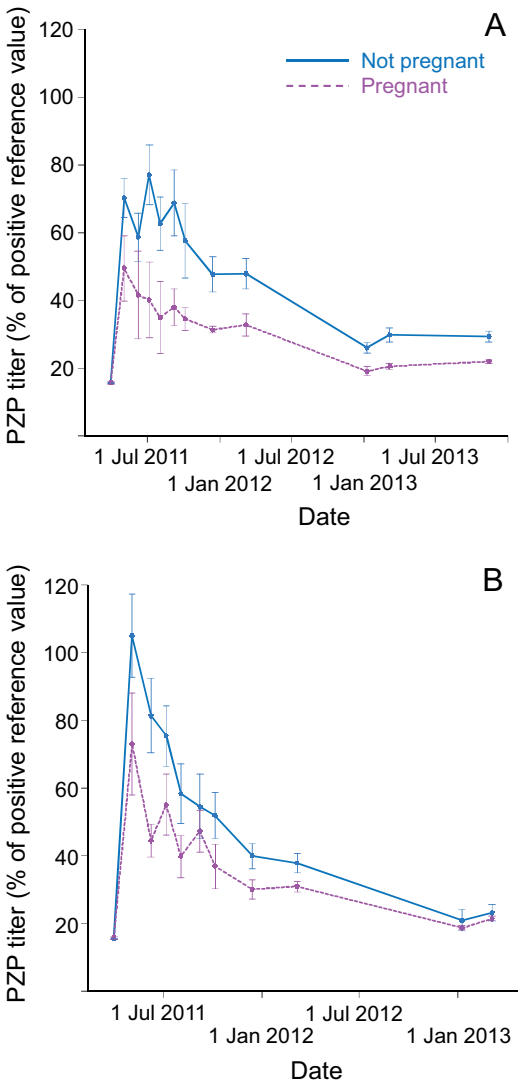


Figure 2. Mean porcine zona pellucida antibody titers, expressed as % of a positive reference, in pregnant and nonpregnant adult female feral horses enrolled in a captive breeding trial of the immunocontraceptive SpayVac at Pauls Valley, Oklahoma, USA, 2012–2014. Aqueous (A) and nonaqueous (B) refer to 2 different formulations of SpayVac. Vertical bars represent ±1 standard error.

however, did we record a severity score >1 (reaction present, but no pain or lameness noticeable).

Neither Fisher's exact test ($P = 0.28$) nor logistic regression analysis (deviance = 2.648, df = 2, $P = 0.27$) revealed an effect of treatment (control, aqueous vaccine, or nonaqueous vaccine) on the probability of a female exhibiting a reaction at the injection site. However, combining control and treated animals by formulation (aqueous or nonaqueous) in a logistic regression model revealed a greater ($z = 2.183$, $P = 0.03$) incidence of injection-site reactions in animals receiving the nonaqueous formulation (41.5%) than in those receiving the aqueous formulation (19.0%). Interestingly, adding a variable for presence or absence of PZP did not improve the model ($\Delta AIC = -1.52$); that is, reactions were as likely to occur in animals receiving liposomes+adjuvant as in animals that received the vaccine with antigen. Addition of body condition also failed to improve the model ($\Delta AIC = -1.91$).

On 9 and 10 January 2013, we rectally palpated 59 treated females (1 F in the nonaqueous treatment group died of torsion of the small intestine unrelated to the study in Feb 2012). Of those, 25 were open and only 1, treated with the aqueous vaccine, exhibited slight uterine edema. On 13 and 14 November 2013, we rectally palpated 30 animals treated with the aqueous vaccine. Of the 17 females that were open, 2, 2, and 1 showed slight, moderate, and extensive uterine edema, respectively.

Of the 30 females treated with aqueous SpayVac, 5 (numbers 461, 3763, 3904, 4305, and 4330) had progesterone levels consistently <0.5 ng/mL and were thus judged to be not cycling. None of the 5 was fertile in any of the first 3 years of the study, but they were not the females with the highest maximum antibody titers (Table 3).

DISCUSSION

A single-injection contraceptive having multiyear efficacy in horses would be of great utility to the BLM and other agencies that manage feral horses. In the only other trial of SpayVac in horses where pregnancy or foaling was an endpoint, Killian et al. (2008) reported pregnancy rates in 12 treated adult females of 0% in the first year after vaccination and 17% in the next 3 years, as compared with pregnancy rates of 75%, 75%, 88%, and 100% in 8 control females. In addition, although breeding was not a part of their study, Bechert et al. (2013) found that 93% of 14 adult females treated with SpayVac ceased estrous cycling within 3–4 months of vaccination. Thus, both of these earlier studies offered hope that SpayVac might provide the single-injection, multiyear efficacy desired by the BLM for management of wild horses. Although we received all vaccines frozen, the nonaqueous vaccine was of particular interest because the lyophilized material could be stored unfrozen and easily reconstituted with adjuvant in the field, rather than having to create an emulsion as with the aqueous version.

We were unable to attain contraceptive efficacy as great as that reported by Killian et al. (2008). Individual variation in response to the vaccine may account for some of the difference between our results and those of Killian et al.

Table 3. Number of pregnancies and maximum recorded porcine zona pellucida (PZP) antibody titer, expressed as percent of a positive reference, in adult female feral horses treated with the aqueous formulation of the immunocontraceptive SpayVac in a captive breeding trial at Pauls Valley, Oklahoma, USA, 2012–2014. Females are listed in order of increasing maximum PZP antibody titer.

Female ID number	Max. PZP titer[a]	No. of pregnancies[b]	Female ID number	Max. PZP titer[a]	No. of pregnancies[b]
7802	31.19	3	4305	85.00	0
3908	37.94	2	3904	85.40	0
1688	39.66	3	8816	86.38	2
500	41.90	2	4257	92.69	0
4320	44.31	2	504	96.88	0
9186	46.33	1	3763	99.12	0
9655	51.33	2	3724	107.74	0
6105	56.23	2	4301	111.07	0
609	60.11	0	4330	111.39	0
8789	60.38	2	4253	134.79	1
3901	69.62	3	4133	135.26	0
394	70.60	1	3795	137.72	0
4124	72.98	3	3815	176.06	0
461	73.98	0	1029	196.89	0
8667	78.12	2	4319	297.25	0

[a] PZP antibody titer is expressed as a percentage of a positive reference, represented by a female vaccinated in a previous study and judged to have a strong, PZP-specific antibody response. The positive reference sample was considered to have a titer of 100%.
[b] Pregnancy was determined through hormone assay (pregnant mare serum gonadotropin and estrone sulfate), rectal palpation, and direct observation of foals. A female was considered pregnant if any of the 3 measures was positive.

(2008); other factors that may be important include dose, adjuvant, and injection site. Henderson et al. (1988) showed that dose (amount of PZP administered) can affect both estrous cyclicity and duration of infertility. In the study by Killian et al. (2008), each dose contained 400 μg of PZP, whereas our vaccines and those used by Bechert et al. (2013) contained only 200 μg of PZP/dose. This change was first instituted by the manufacturer in the vaccine provided to Bechert et al. (2013) based on the fact that a primer dose of 65 μg of PZP emulsified with Freund's Complete Adjuvant (FCA) followed by a booster dose of 65 μg of PZP emulsified with Freund's Incomplete Adjuvant was sufficient to contracept horses on Assateague Island, Virginia, USA (Kirkpatrick et al. 1990, 1995). The manufacturer thus expected that 200 μg of PZP in the liposome formulation used in SpayVac would be more than sufficient for contraception. The fact that 93% of females treated by Bechert et al. (2013) stopped cycling seemed to support that expectation.

Our choice of adjuvant was influenced by several factors. First, we were uncertain that AdjuVac™, which was used by Killian et al. (2008), would be readily available in the future. The various versions of Freund's adjuvants, on the other hand, are available from several suppliers. Second, MFA is thought to be less likely to cause injection-site reactions than FCA. Lyda et al. (2005) showed that antibody titers in adult females treated with MFA+PZP were greater than those in females treated with FCA+PZP, although this finding was not statistically significant. Finally, Bechert et al. (2013) achieved what appeared to be positive results with SpayVac formulated with MFA. All of these factors influenced our choice of MFA as the adjuvant.

Table 4. Percent of treated and control adult female feral horses exhibiting an injection-site reaction in a captive breeding trial of the immunocontraceptive SpayVac at Pauls Valley, Oklahoma, USA, 2012–2014.

		Females exhibiting a reaction	
Treatment	*n*	No.	%
Saline control	6	0	0
Aqueous control	12	1	8.3
Nonaqueous control	12	5	41.7
Aqueous SpayVac	30	7	23.3
Nonaqueous SpayVac	30	12	40.0

In both previous studies of SpayVac in horses, injections were given in the neck (Killian et al. 2008, Bechert et al. 2013). Although no data were presented, several authors suggested that reactions to injections given in the rump were less common and perhaps less severe than to injections given in the neck, which influenced our decision on injection site (Lyda et al. 2005, Kirkpatrick et al. 2011, Bechert et al. 2013). Additional work to clarify the relationship between injection location and reactions may be warranted.

Antibody titers in response to vaccination with PZP have been shown to vary considerably within a species even when dose and adjuvant are the same (Kirkpatrick et al. 2011). Differences in response may to some extent depend on an animal's physiological state, but we saw no effect of body condition on probability of foaling. This is perhaps not surprising in that body condition ranged only from 4.0 to 6.0 ($\bar{x} = 4.9$ for all 90 females) when vaccinations were given in March of 2011, and improved substantially by March of 2012 ($\bar{x} = 6.6$, range = 5.0–8.0).

Comparisons between antibody titers measured in our study and those reported by Killian et al. (2008) and Bechert et al. (2013) are difficult because of differences in measurement scale and units in which titers were quantified. In general, however, it appears that, for unknown reasons, high titers were sustained longer in those 2 previous studies than in ours. Other studies have shown a correlation between antibody titers and contraception in females, with antibody

titers 50–60% of a positive reference serum being sufficient to render 90–95% of adult females infertile (Liu et al. 1989, 2005; Turner et al. 1997). However, none of these studies were >1 year in duration. Our data suggest that a similar threshold may exist for multiple-year efficacy in horses treated with SpayVac. Only a single foal was produced (female 4253) in 3 years following inoculation in females having a maximum titer greater than approximately 90% of the positive reference; we believe that datum may be an error in foal assignment to a dam, because the female tested inconclusive for pregnancy by hormone analysis and negative for pregnancy by palpation in that year. Despite our careful efforts to associate foals with dams, an occasional error is possible because many of our females, particularly those with bay coloration, were very similar in appearance. Titers reported as a percentage of a positive reference compare serum taken from a successfully contracepted female (the positive reference) with sera from other treated and control females. The lower values reported here, for example in the control females, were probably below the limit of detection for the assay and likely reflect background issues, which are not uncommon when testing for polyclonal antibodies. As such, most values below approximately 30% of the reference serum likely do not represent true immune responses, which may explain the fluctuations in control values through time.

Our study and that of Bechert et al. (2013) clearly showed that reactions at the injection site were fairly common even with MFA as adjuvant and, contrary to a previous report (Roelle and Ransom 2009), even when injections are delivered by hand rather than by dart. Both studies reported that reactions can resolve only to reappear at a later time, and both studies provided some evidence that reactions at the injection site are attributable to the adjuvant rather than the immunogen. Increased rates of injection-site reactions in these 2 studies are most likely the result of more frequent and closer examination, both visually and by palpation. Interestingly, however, even such close scrutiny does not detect all reactions. In our study, necropsy of female 740, which died of torsion of the small intestine, revealed a sterile abscess that had gone undetected in 7 examinations (visual and by palpation) over a period of 7 months. It thus seems highly likely that studies based on field observation alone under report injection-site reactions. Bechert et al. (2013) found injection-site reactions to be more frequent for the aqueous formulation of SpayVac (5/7 = 71.4%) than for the nonaqueous formulation (3/7 = 42.9%), whereas the reverse was true in our study. Reasons for this difference are unclear, but the small sample sizes ($n = 7$ F/group) in the study by Bechert et al. (2013) may be a contributing factor. Despite the fact that injection-site reactions were fairly common in our study, perhaps the most striking and important observation was that none of the reactions appeared to be painful or caused any noticeable change in mobility or gait.

Unlike Killian et al. (2008), we found uterine edema in only a small proportion of open females in January of 2013, likely because most of the females were in anestrus. In November of 2013, however, we found uterine edema in a proportion of females similar to the expected normal frequency (~25–30%) suggested by Killian et al. (2008) assuming a 21-day estrous cycle and 5–7 days of the cycle actually in estrus. Our November examinations should be roughly comparable to those done by Killian et al. (2008) in mid to late October, so the reasons that they found much higher rates of uterine edema remain unclear. We reiterate, however, that we view the frequencies of uterine edema observed here as normal and not a contraindication to the use of SpayVac.

Bechert et al. (2013) found that 93% of females treated with SpayVac ceased cycling 3–4 months after vaccination. Unfortunately, they were unable to follow the females to see whether normal ovarian function was restored over time. We found that 5 of 30 females treated with the aqueous formulation of SpayVac were not cycling >3 years after vaccination, which may suggest that loss of ovarian function is permanent when it occurs. As would be expected, none of these 5 females was fertile in the first 3 years of our study. However, an additional 10 females that appeared to be cycling were also infertile in the first 3 years of our study, which suggests that some mechanism other than disruption of ovarian function is responsible for infertility. One possibility is that antibody titers, even though they declined significantly following vaccination, were still high enough to prevent sperm binding to ova in these 10 females.

MANAGEMENT IMPLICATIONS

We did not find SpayVac to be as efficacious as other investigators have. However, both modeling (Ballou et al. 2008) and field studies (Kirkpatrick and Turner 2008) have shown that significant reductions in fertility can be achieved with <100% contraceptive efficacy. If even the modest results that we observed could be duplicated on a large scale, which would require registration of SpayVac by the U.S. Environmental Protection Agency, the BLM and other agencies charged with managing feral horses would have a more effective fertility control tool than has heretofore been available, especially given the fact that no booster inoculation is required with SpayVac. Concerns about reactions at the injection site and uterine edema appear to be minimal and should not deter BLM or others from using SpayVac.

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Behavioural ecology of island feral horses

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Summary

In a horse population on Shackleford Banks, an island off the American east coast, major changes in social organisation have occurred. Instead of typically living in harems occupying overlapping home ranges, it was found that some harems defended territories and some horses did not even live in fixed membership groups. The existence of these social variations appeared to be correlated with the unique habitat and geographical features of the island. Territories, whose boundaries ran the width of the island, only occurred where the island was narrow, the visibility unrestricted and the essential vegetation zones ran along the island's long axis. Only here were the difficulties in defending reduced and feeding and male mate-guarding opportunities increased. Where the sand dunes were high and forest dense temporary assemblages occurred and non-territorial harems occupied the patchy semi-open area with the batchelor males.

Once territories were defended many aspects of individual social relationships became altered. Territorial stallions were less likely to influence the daily activity patterns of their groups and more likely to groom their dominant females and round-up their subordinate females than were their non-territorial counterparts. In addition, interpersonal distances were likely to be greater among horses living in territorial, as opposed to non-territorial, harems.

Introduction

ISLAND biotas differ in many ways from their mainland counterparts. Perhaps the most salient features of islands are that they possess fewer species than nearby mainland areas and that, in general, large near-shore islands have more species than smaller more distant islands. But the differences in behaviour and ecology of insular populations are just as striking. These populations often show more dimorphism (Schoener 1967; Wallace 1978), forage in more foliage levels (Karr 1971) and display reduced stereotype in seed selection (Pulliam 1973) than their mainland counterparts. Even changes in social organisation have been observed. For example, the yellow faced grassquit *(Tiaris olivacea),* which is gregarious in Costa Rica, is territorial on Jamaica (Pulliam *et al* 1972). Typically, these effects are attributed to insular isolation, small size and topographic simplicity (Preston 1962; MacArthur and Wilson 1967; Lack 1976). But the way in which these factors actually alter island fauna appear to be complex and are, as yet, incompletely understood.

The work reported here was undertaken to examine whether islands affect the social behaviour of feral horses and, if so, what attributes of islands are responsible for these changes. This entailed comparing the social behaviour and patterns of social organisations of feral horses inhabiting a number of different mainland and island habitats. Apart from the data gathered during the last 5 years on the ponies of Shackleford Island, the other data were derived from the published studies of Welsh (1973) on Sable Island, Berger (1977) in the Grand Canyon and Feist in the Pryor Mountains (Feist and McCullough 1976).

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Social organisation of wild equids

A brief look at the social organisation of the living equids — the zebras, asses and horses — suggests that phylogenetic constraints will only minimally influence feral horse social organisation. Wild equids show a remarkable diversity of social organisation (Table 1). Basically, 2 categories of social organisation exist; that involving permanent groups and that involving temporary assemblages. Typically the asiatic wild horse *(Equus prezwalski)* (Mohr 1971; Groves 1974) and the plains *(E burchelli)* and mountain *(E zebra)* zebras (Klingel 1974) live in small permanent groups that are composed of females, their offspring, juveniles and a dominant stallion. Adult males not possessing harems live either alone or in permanent bachelor groups. Both kinds of groups inhabit large overlapping home ranges. For plains zebra in the Serengeti, home ranges vary in size from 600 km^2 in the dry season to 300 km^2 in the wet season (Klingel 1974). Some zebra populations undertake seasonal migrations in search of suitable food and water. The Serengeti populations, for example, migrate up to 150 km to their dry season foraging ranges. The few anecdotal accounts of the ranging behaviour of the Asiatic wild horse suggest that harems have well-defined home ranges centred around water holes during the spring foaling period and summer dry season but that they wander more widely during the winter when snow and melt water are widely available (Mohr 1971).

In the case of wild asses and Grevy's zebra the pattern of social organisation is very different. According to Klingel (1977), in both the African *(E africanus)* and the Asiatic *(E hemionus)* wild asses, there are no permanent associations other than small mother-offspring groups. Nevertheless, temporary assemblages consisting of only males, only females or of both sexes do occur. During the day these small groups and solitary individuals may even coalesce into large feeding herds but by evening these herds break up. During the breeding season, however, a large proportion of the adult males establish territories. These territories are large, averaging 23 km^2, and are only defended against other males when a female in oestrus actually enters the territory.

Klingel's observations show that the social organisation of Grevy's zebra *(E grevyi)* is basically similar to that of the wild asses (Klingel 1974). Permanent associations among adults are rare and most of the adult males restrict their activity to territories that are only defended in the presence of females in oestrus. These territories appear to be smaller than those of the asses (6 km^2) and they appear to be maintained throughout the year except during the dryest years.

Why there should be 2 major types of equid social organisa-

TABLE 1: Social groups exhibited by different equid species

	Permanent groups			Temporary groups	Solitary territorial males
	Harems	Bachelor groups	Solitary males	All males; all females; mixed sexes	
Wild horses	5-6	?	?	No	No
Plains zebra	4-7	2-3	?	No	No
Mountain zebra	6-7	4-8	?	No	No
Grevy's zebra	No	No	—	2-8	Yes
Wild asses	No	No	—	2-6	Yes

Numbers reflect range of group sizes, otherwise presence or absence is indicated

tion is not obvious. What appears to be common to all equids is that in drier habitats and in areas where resources are distributed in a more patchy fashion, systems of temporary assemblages and solitary territorial males occur more frequently than systems involving harems. This relationship probably arises largely because low quality vegetation distributed in small widely scattered patches makes it uneconomical for females to form permanent fixed membership groups by intensifying intraspecific competition. Without female associations to defend, males instead defend large areas containing the resources females require. Only larger more evenly distributed patches of higher quality vegetation allow females to feed in permanent membership groups, thus fostering a harem social system.

Social organisation of mainland feral horse populations

In general, the social organisation of mainland feral horses is similar to that of the Asiatic wild horse and the plains and mountain zebras, with one exception; there appear to be no long distance seasonal migrations. However, as with the non-migrating plains zebra of Ngorongoro Crater, feral horse populations are constrained either by geological features, such as the Grand Canyon, or man-made barriers. Thus it is difficult to surmise whether feral horses would migrate if the opportunity were available.

Some details of the population characteristics and social organisation of 2 mainland populations are shown in Table 2. At the time of the surveys both populations were composed of a variety of permanent groups. Typically, most dominant stallions possessed small harems which consisted of females, their young and juveniles. The average size of these harems was about 5 horses although they ranged in size from 2 to 20. There were also bachelor groups consisting only of adult males and during the breeding season these tried to establish their own harems by stealing females from neighbouring harems. In the Pryor Mountains the average size of these bachelor groups was 1.8 horses. Occasionally solitary males were found which were thought to be older males that had lost their harems. The stability of the groups in these populations seemed pretty high; only 7.6 per cent of the Pryor Mountain adult females changed bands in a year while none changed groups during a 6-month period in the Grand Canyon. In these 2 mainland populations, groups possessed home ranges of about 20 to 25 km^2 but these often overlapped with those of neighbours.

Social organisation of island feral horses

The social organisation of feral horses on islands is more variable than that on mainlands. Sable Island, which lies about 100 miles east of Nova Scotia, is inhabited by a population of feral horses which probably originated in the early 1700s. The population, which fluctuated between 150 and 300 horses, was dispersed along the island in about 50 groups. Some were typical harems which contained a dominant stallion, females, offspring, juveniles and occasionally a subordinate male. Others were bachelor groups or even groups whose composition continuously changed. Although this type of group is characteristic of asses and Grevy's zebra, it is not generally described for horses. In addition, there were a few solitary males in the population. The sizes of these groups were slightly larger than those of the mainland populations (Table 2). Furthermore, on Sable Island, which is approximately 40 km long and 1.3 km wide, the population density was greater than that of the mainland populations, while the average size of the overlapping home range was less. The age structure, however, was not too different from that of the Pryor Mountain population.

Shackleford Banks, a barrier island off the coast of North Carolina, is inhabited by approximately 100 horses whose ancestors were probably shipwrecked on the outer banks in 1565 (Anon 1975). Certain facets of the social organisation of this

TABLE 2: Population characteristics and social patterns of 4 feral horse populations

	Pryor Mountains (1970)	Grand Canyon (1974)	Sable Island (1970)	Shackleford Island (1974)
Population characteristics				
Population size	225	78	240	104
Population density	2.0/km^2	0.2/km^2	6.3/km^2	11.0/km^2
Age structure				
Adults	58%	?	64%	61%
Juveniles	28%	?	21%	21%
Infants	13%	?	15%	19%
Activity areas				
Group ranges	25 km^2	20 km^2	<7 km^2	6 km^2
Group territories	No	No	No	3 km^2
Social patterns				
Group types ($\bar{N}$)				
Harems	5.0	4.5	6.0	12.3
Bachelors	1.8	?	<2.0	2.6
Solitary males	Yes	Yes	Yes	Yes
Temporary assemblages	No	No	Yes	Yes
Band stability				
Adult female group changes	7.6%	0.0%	?	10.8%

population were found to be very different from those of the other studies. As in other horse populations there were stallion dominated harems, bachelor groups, solitary males and even temporary assemblages containing adults of both sexes. But the population on Shackleford was unique in that two-thirds of the harems maintain well-defined, non-overlapping territories. Furthermore, these territories were permanent. During the course of this study the boundaries remained remarkably constant, shifting not more than 15 to 20 m. Even when harems were stampeded together and driven far from their territories, all the horses returned to their previous territories after human harassment had ceased.

Territorial boundaries ran the width of the island from the ocean to the back sound. Generally, these boundaries coincided with subtle geographic features such as a row of low dunes, a tidal inlet or a patch of fresh water marsh. Although large dung piles occasionally lay along the boundaries, equally large dung piles were situated throughout the territory. But even though these boundaries were not conspicuously marked, they were rarely crossed. Two reasons may account for this. First, stallions are very solicitous of females, especially during the breeding season, and when females approach the boundary stallions herd them towards the centre of the territory. Secondly, neighbouring stallions and bachelor males rarely cross these boundaries because the resident stallion defends his territory vigorously. Typically, encounters stop after a ritualised sequence of defaecations and dung sniffing. But often these encounters escalate into physical contests in which the horses kick and chase each other. During the period of this study resident stallions won all encounters. The costs of such vigorous territorial defence, however, were presumably quite high because resident stallions often received deep cuts around the mouth and jaw.

The feral horse populations on Sable and Shackleford Islands had similar age structures, population densities and ranging areas (Table 2). Shackleford had significantly larger harems and smaller ranges than some of the other populations but these characteristics might just as likely be the consequences of territoriality as its cause. Furthermore, band stability, as measured by the percentage of adult female movements, was not much lower than that found in the Pryor Mountain population.

Advantages of territoriality

Why then did the population on Shackleford Island exhibit such a different pattern of social organisation? There appears to be an absolute correspondence between particular habitats and the types of groups that inhabit them and it is therefore proposed that a number of features concerning the topography and habitat structure of Shackleford were responsible for the pattern of social organisation. Shackleford Banks is a long, narrow island approximately 17 km long and 1 km wide. The eastern half of the island is virtually flat and is composed of 3 major vegetation zones, each running parallel with the long axis of the island. Running inwards from the ocean (Fig 1) there is a sandy beach about 10 m wide followed by a narrow zone of low dunes that are predominantly covered with sea oats *(Uniola paniculata)*. The dunes are rarely higher than 5 m but they do provide the centre of the island with some protection from ocean waves and salt spray. There is an extremely wide flat swale that is dominated by salt-meadow cordgrass *(Spartina patens)*, the rush *(Juncus rosemerianus)* and other grasses and sedges. The distribution of species is often patchy and in places the ground cover is fairly sparse. In this zone there are both permanent and temporary pools of fresh and brackish water. Finally, there is a salt marsh bordering the sound in which saltwater cordgrass *(Spartina alterniflora)* and glasswort *(Salicornia virginica)* are dominant. It was only on this flat, eastern half of the island that territorial harems were found.

The western end of the island has a strikingly different profile. There are virtually no flat open areas (Fig 2). Along the beach there are tall dunes reaching 10 to 15 m in height. These dunes stretch across the width of the island and eventually slope into dense maritime forest. Occasionally, there are small flat areas among the dunes and these are covered by typical swale vegetation. Intruding into the forest from the rear are pockets of fresh marsh which support a rich and varied flora. In this topographically complex area horses were often found alone, in bachelor groups or in temporary assemblages. At this end of the island animals of both sexes mingled freely but when groups formed their composition continuously changed.

Between the eastern and western end of the island there is a small flat area which is punctuated by 2 small stands of



Fig 1. Cross-section of the eastern end of Shackleford Island

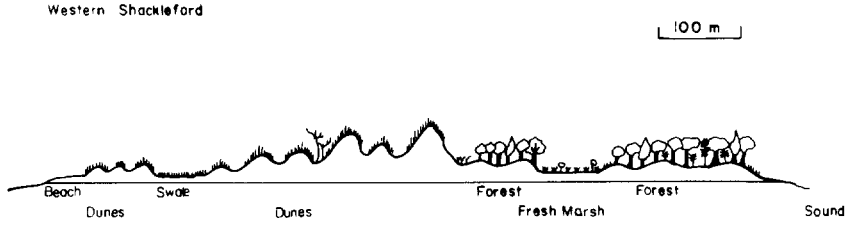


Fig 2. Cross-section of the western end of Shackleford Island

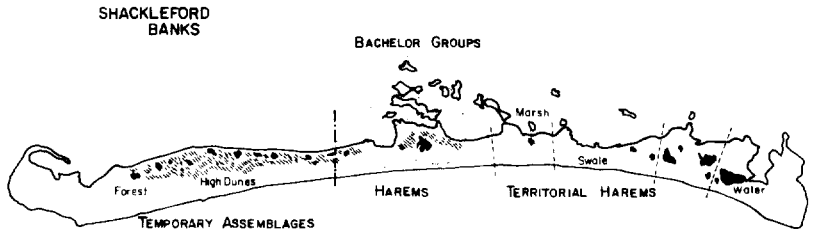


Fig 3. Map of Shackleford Island showing the location of the major environmental features and the location of the territorial harems, non-territorial harems which overlap with the bachelor groups, and the location of the temporary assemblages

maritime forest (Fig 3). In the centre of this area were situated several small groups of bachelor males and on the fringes of the forest were located 2 harem groups. The ranges of the harems were not exclusive and they did overlap almost completely with the ranges of the bachelor males. As a result they were not considered to be true territorial harems. The location of the various habitat areas and the arrangement of the types of groups inhabiting them is depicted in Fig 3. Note the fairly even distribution of fresh or brackish water along the back side of the island.

It is suggested that territoriality would exist in all horse populations if the costs associated with maintaining it were offset by large enough benefits. Only on the eastern end of Shackleford, however, did it appear that the benefits of exclusive access to a variety of seasonal food types and of increased control over females in oestrus exceeded the costs of excluding competitors. This favourable benefit/cost relationship might have arisen because:

(1) The island is narrow;
(2) the habitat is open and visibility is excellent;
(3) each of the 3 essential feeding zones runs lengthwise along the island;
(4) water is fairly evenly distributed along the length of the island.

Two facts suggested that foraging considerations were important and that having territories might provide horses with increased feeding advantages. First, the horses spent an average of 75 per cent of their time foraging. Fig 4 shows that there were daily and seasonal effects but time spent feeding made up a considerable part of a horse's daily time budget. Secondly, despite this large proportion of time spent feeding, the overall condition and health of the animals remained low (Fig 5). Ribs could be seen through the coat year round and most deaths occurred just before the foaling season when the nutrient and energy content of the sparse forage was lowest.

On Shackleford the abundance of forage types varies seasonally and the grazing behaviour of the horses reflected this changing availability. Typically, horses spent most of their time feeding on the saltmarsh and the swale during the summer but during the winter they switched their activity patterns and concentrated feeding on the swale and on the dunes. These seasonal feeding patterns are shown in Fig 6 for horses living in territorial harems but the pattern also applied to horses living in non-territorial harems and bachelor groups. Apparently horses needed to forage in all 3 vegetation zones in order to survive.

Since home ranges traversing the island from ocean to sound would secure access to each of the 3 major zones, why did some horses establish territories and maintain exclusive access to this forage? This was done possibly to derive 2 additional feeding benefits. First, home ranges had large areas of overlap (Fig 3) and this might have temporarily prevented individuals in a group from obtaining a particular resource at a particular time. Obviously establishing a territory, even if more costly in terms of time and energy expenditure, would alleviate this problem. Secondly, exclusive use of an area might have enabled individuals to use the forage more efficiently. By regularly rotating grazing patches in a particular zone, each horse could continuously harvest the highly nutritious new vegetative growth. Preliminary analyses showed that territorial harems seemed to alternate consistently among regenerating patches. Although there was much variation, the time from abandoning a patch to recommencing feeding in that patch was longer within territories (10 to 14 days) than in areas of overlapping home ranges (less than 7 days). As a result, the grass was longer in territorial as opposed to non-territorial areas. Even if difficult at

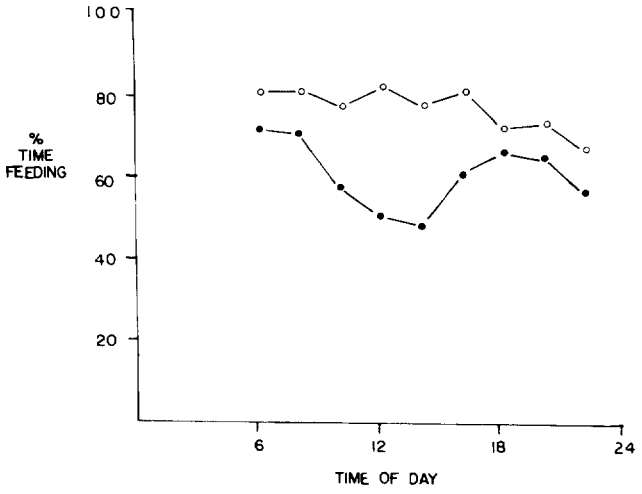


Fig 4. Seasonal changes in the daily pattern of time spent foraging. ●——● *summer;* o——o *winter*



Fig 5. A Shackleford pony grazing on submerged saltwater cordgrass (Spartina alterniflora) *and drinking sea water. This individual typifies the poor body condition of horses as the new vegetation begins to grow in March*

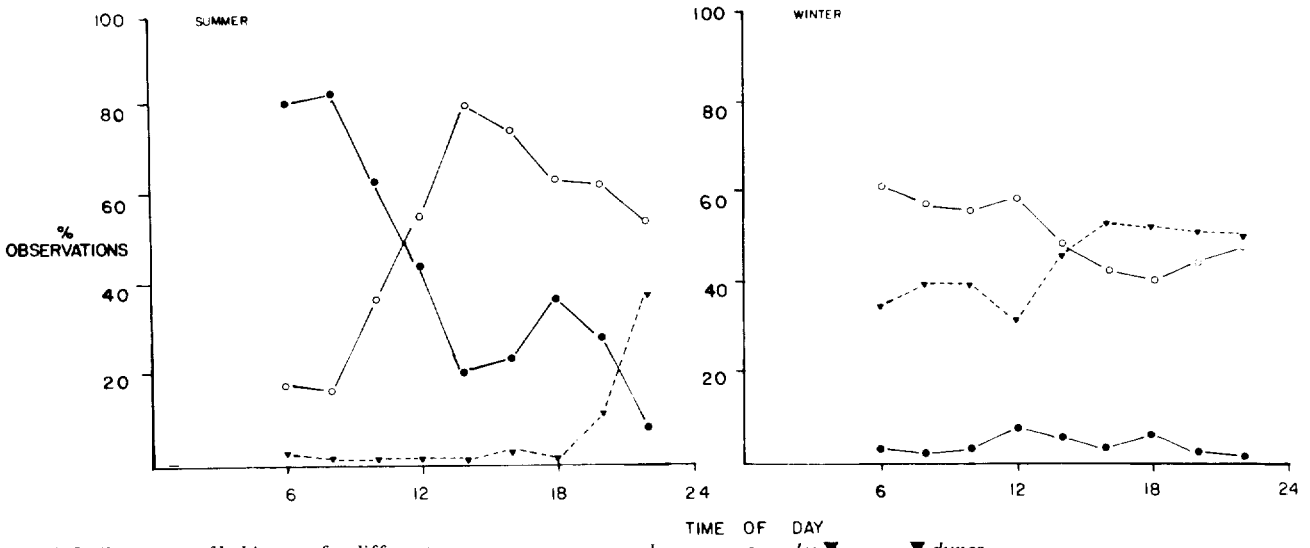


Fig 6. Daily pattern of habitat use for different seasons. ●——● marsh; o——o swale; ▼– – –▼ dunes

present to explain why individuals did not cheat and crop the forage prematurely, it would appear that individuals in groups that had exclusive access to resources were deriving increased feeding benefits.

Because stallions performed all the territorial defence, it was possible that they could increase the number of females in their harems and increase the likelihood that they actually fathered their offspring by maintaining a territory. Two observations suggested that the reproductive success of territorial males may indeed be higher than that of non-territorial males. First, it was found that male-male contacts were more likely to occur further away from the harem when a male had a territory than when he did not. Thus, the risk of a female being stolen by a neighbouring stallion was lower for territorial stallions than for non-territorial stallions. Secondly, territorial harems consisted of more adult females than non-territorial harems and, more importantly, territorial harems tended to increase in size, whereas non-territorial harems either did so more slowly or decreased in size.

Costs of territoriality

On Shackleford, guaranteed access to fresh water and to the 3 essential vegetation zones seemed to provide all horses, regardless of sex, with some additional feeding benefits. Also, males appeared to derive additional mating benefits. But what about the costs associated with this unique occurrence of equid territorial behaviour? During the study, it was not possible to estimate the costs associated with territoriality but normally the costs of excluding other individuals from an area were high (Gill and Wolf 1975; Carpenter and MacMillan 1976; Rubenstein 1981). On the eastern end of Shackleford Banks, however, these costs may in fact have been quite low because first, visibility was high and stallions could monitor their boundaries with a minimum of lost time and energy and secondly, half the perimeter, on average, of a territory running the width of such a narrow island was bounded by water and thus devoid of potential competitors. Therefore, even if the additional costs to ungulates of being territorial were usually high, the habitat structure on the eastern end of Shackleford kept them low, making "affordable" the small feeding and mating advantages associated with territorial behaviour.

Circumstantial evidence lends support to this hypothesis. In the mainland populations water was not evenly distributed about the habitats and as a result bands shared watering holes and had overlapping home ranges. The same situation seemed to exist on Sable Island (Welsh 1973). Even on Shackleford, where water was less uniformly distributed, the visibility poorer and the island wider, territoriality did not occur. In the transition zone where there was only one large centrally located pool, forests and accessible islands almost touching the sound side of the island, both harems and bachelor groups maintained overlapping ranges. Although in this area groups sometimes drank simultaneously from this pool, one group usually waited in the forest until the drinking group had left. Furthermore, Hoffman (1980, personal communication) noted that on an island less than 2 miles from Shackleford, with similar vegetation but with different spatial arrangements and with centrally located water holes, the horses lived in harems that did not possess territories. So it appeared that the dispersion of forage and water played an important role in determining whether harems will have overlapping home ranges or territories.

Consequences of territoriality

Once territories were formed subsequent changes in other aspects of social behaviour were possible. For example, the reduced likelihood of interference from neighbours probably enabled a stallion to control his harem and prevent raiding males from stealing his females, with less effort. Therefore, it was not surprising that during and immediately before the breeding season the individual distance among members of territorial harems was significantly larger than among members of harems that did not have exclusive home ranges (Fig 7).

Territorial stallions were also less likely than non-territorial stallions to direct or influence many daily activities such as choosing feeding areas, determining the duration of feeding episodes, leading local or short distance movements and intervening in intragroup conflicts. Only when initiating and directing long distance movements and when herding widely dispersed females did territorial and non-territorial stallions behave in a similar fashion.

Dominance relationships within a harem were also affected by the territorial nature of the group. Although harem stallions dominated all their females, females differed in their ability to dominate each other. By recording how often females threatened each other (eg, headshakes, ear lay backs, neck extensions, rump turns and kicks) it was possible to rank

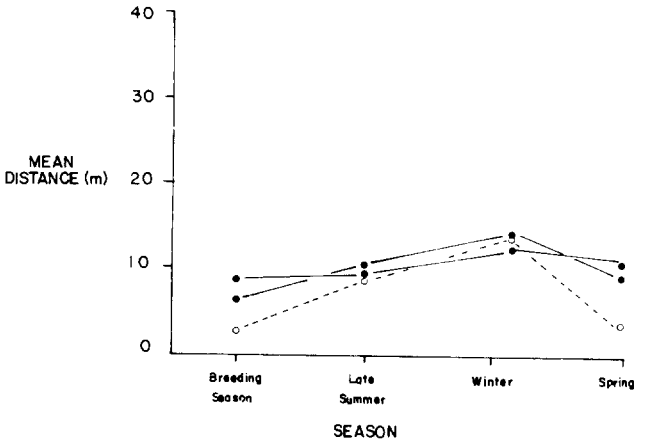


Fig 7. Seasonal changes in the mean distance among group members. ●——● territorial groups; o- - -o non-territorial groups. Differences significant during breeding season (t_{25} = 2.81; $P < 0.01$) and spring (t_{31} = 3.02; $P < 0.005$)

females. Both territorial and non-territorial harems exhibited nearly linear female dominance hierarchies. Typically, adult females ranked above yearlings and yearlings ranked above 2 year olds, regardless of harem type (Fig 8). But territorial and non-territorial stallions threatened their females differently, especially during round-ups. Territorial males tended to round-up lower ranking females disproportionately more than they did higher ranking females, whereas non-territorial males tended to round-up females more equally, regardless of rank (Table 3).

Territoriality also seemed to affect the grooming relationship between stallions and their females. Among horses living in territorial harems a correlation was found between the extent to

TABLE 3: Number of times territorial and non-territorial stallions rounded-up high and low ranking females in their harems

		Female rank	
		Upper 50%	Lower 50%
Type of stallion	Territorial harem	12	71
	Non-territorial harem	9	12

Statistical test: chi = 12.5; significance level P-squared <0.001.

which females were groomed by the stallion and their rank. The male groomed higher ranking females more than lower ranking females. Among horses living in non-territorial harems, however, the stallion appeared to groom all his adult females equally (Fig 8).

From these few examples it appeared that the relationship between a stallion and his females depended on whether he had established a territory. With respect to "bonding" behaviours, as measured in terms of interpersonal distance, and grooming and round-up behaviour, territorial males responded differently to females depending on their relative rank than did non-territorial males. It seemed likely that such differences were the consequences of the different risks of mating failure suffered by each type of stallion.

The linear arrangement of territories also had consequences for inbreeding and kin relations. Adult and, to a lesser extent, juvenile females moved only short distances when they left a band, whereas juvenile males usually moved longer distances to the western end of the island or to the bachelor groups in the

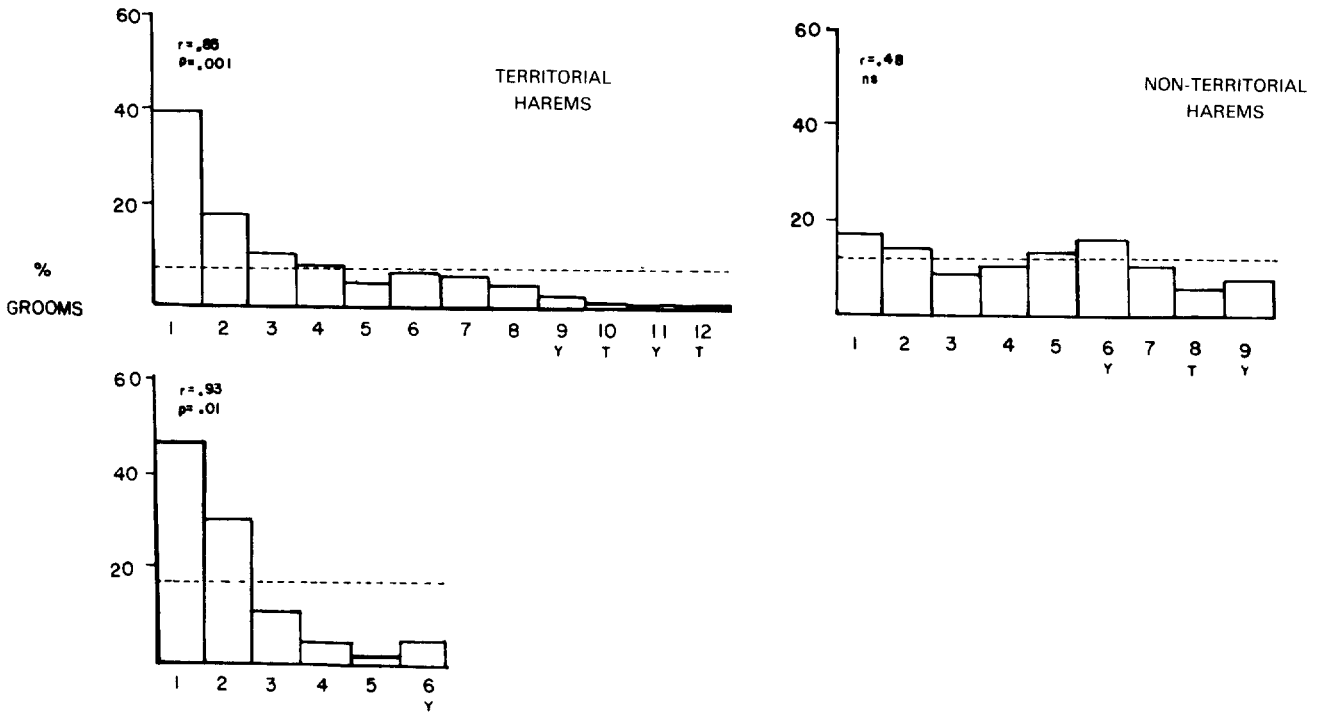


Fig 8. Relationship between a female's rank and the frequency of male grooming contacts. Dashed line shows the expected level if all females were groomed equally frequently by the male. The correlation coefficient (r) and level of significance (P) reveal the magnitude and significance of the relationship. ns Not statistically significant at $P < 0.05$. Y = Yearling, T = Two-year-old

transition areas. Some never seemed to leave their natal territory. However, larger samples are needed before the ramifications of these different sex-related dispersal patterns on group dynamics can be completely unravelled.

Conclusions

These comparisons reveal that horses have a typical pattern of social organisation but that variations can arise under unusual environmental conditions. On Shackleford Banks the types of groups that formed, relationships between groups and the social cohesion within groups were largely controlled by the habitat structure of the island, which in turn was influenced by the size and shape of the island. But does Shackleford Bank represent a topographically and vegetationally unique situation? Or are there other areas that might provide novel arrangements of habitats or unusual environmental pressures? It would not be surprising to find that most islands, because of their peculiar shapes, limited floral diversity, ruggedness, fluctuating fresh water levels and extreme climates, are areas that are most likely to provide habitats that must be adapted to in novel ways. The spectacular variety of endemic bird species on islands certainly suggests that forceful selection pressures are operating.

But even on mainlands, a species' range is comprised of a variety of environments. And as 2 recent and continuing studies of mainland horse populations demonstrated, some of these differences also influence equid sociality. In the New Forest where the adult sex ratio was highly skewed and females greatly outnumbered males, males appeared to possess harems and exclusive non-overlapping home ranges (Pollock 1980, personal communication). But unlike the Shackleford horses, a proportion of females appeared to live apart from harems. Some lived in harems only during the breeding season, leaving the group during the winter as the forage abundance and quality declined. Others never attached themselves to harems, wandering among harems regardless of season. In a Camargue horse population another social variant occurs. In a population consisting of 42 adult horses, 36 lived in a cohesive large group comprising many harems. Duncan and Vigne (1979) were able to demonstrate that individuals in larger groups were parasitised by fewer biting flies. They argued that the debilitating effects on condition associated with blood loss were a major factor in affecting this social arrangement.

These studies on feral horse populations demonstrate that a species such as the horse may exhibit a "typical" social system under "normal" conditions. However, the social system is likely to alter when changes occur in ecological factors, such as habitat structure, the distribution of forage and water and the diversity and quality of the vegetation, as well as demographic factors such as size, sex ratio and age structure. In addition, because islands differ quite markedly from the ideal or normal environment, populations living on them will most likely exhibit novel and exciting social variations.

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Résumé

Dans la population équine de l'île de Shacklesford Banks au large de la côte est est USA, des changements majeurs de l'organisation sociale sont apparus. Au lieu de vivre classiquement en harems occupant des territoires se "chevauchant", on s'est rendu compte que certains harems défendaient leur territoire et même que certains chevaux n'appartenaient même pas à des groupes fixes. L'existence de ces variations sociales semble être due à l'habitat et aux caractéristiques géographiques uniques de l'île. Les territoires, dont les limites couraient le long de l'île, n'existaient que là où celle-ci est étroite, la visibilité non obstruée et où les principales zones de végétation courent le long de l'axe principal de l'île. Ce n'est qu'à ces endroits que les difficultés de défense étaient réduites et les posibilités de se nourrir et d'assurer la garde pour les mâles étaient supérieures. Dans les zones de hautes dunes de sable et de forêt épaisse, des assemblages provisoires ont été observés et des harems sans territoire fixe occupaient les zones semi-ouvertes accidentées avec des mâles célibataires.

Une fois les territoires défendus, de nombreux aspects des relations sociales individuelles changent. Les étalons à territoire ont moins tendance à influencer les activités quotidiennes de leurs groupes et plus à s'occuper de leurs femelles dominantes et à regrouper leurs femelles subordonnées que les étalons sans territoire. De plus, les distances individuelles sont généralement plus grandes entre animaux vivant dans des harems territoriaux par opposition à des harems non territoriaux.

Zusammenfassung

Bei einer Pferdepopulation auf Shacklesford Banks, einer Insel vor der amerikanischen Ostküste, haben sich größere soziale Organisationsveränderungen ereignet. Anstatt in den typischen Harems in sich überschneidenden Heimatgebieten zu leben, wurde festgestellt, daß einige Harems ihre Territorien verteidigten und einige Pferde noch nicht einmal in festen Gruppen lebten. Das Bestehen dieser sozialen Variationen schien mit dem einzigartigen Lebensraum und den geographischen Besonderheiten der Insel in Zusammenhang zu stehen. Territorien, deren Grenzen über die ganze Breite der Insel verliefen, gab es nur an den Stellen, wo die Insel schmal und die Sicht unbegrenzt war und wo die Vegetationszonen entlang der Längsachse der Insel verliefen. Nur hier waren die Schwierigkeiten der Verteidigung reduziert und die Ernährung sowie die Möglichkeit der männlichen Partnerbewachung besser. Wo die Sanddünen hoch und die Wälder dicht waren, fanden zeitweise Zusammenschlüsse statt, und nicht-territoriale Harems bewohnten zusammen mit den weibchenlosen Männchen die uneinheitliche, halboffene Landschaft.
Mit dem Beginn der Territoriumsverteidigung veränderten sich auch zahlreiche Aspekte der individuellen sozialen Verhältnisse. Territoriale Hengste neigten weniger dazu, das tägliche Aktivitätsmuster ihrer Gruppe zu beeinflussen, und tendierten eher dazu, ihre dominanten Weibchen zu versorgen und zu pflegen und ihre untergeordneten Weibchen zusammenzutreiben als ihre nicht-territorialen Gegenstücke. Bei territorialen Harems wurden auch größere Abstände zwischen den einzelnen Tieren beobachtet als bei nicht-territorialen.

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ABSTRACTS

Digestive system and diseases

Mesodiverticular bands as a cause of small intestinal strangulation and volvulus in the horse

FEEEMAN, D. E., KOCH, D. B. and BOLES, C. L. (1979) *J. Am. vet. med. Ass.* **175**, 1089-1094.

MECKEL'S diverticulum and persistent vitello-umbilical bands are already documented as causes of intestinal obstruction in horses and cattle respectively. A third and distinct developmental anomaly, described as a mesodiverticular band, was responsible for bowel strangulation and secondary volvulus in the 3 cases reported in this paper. The band derives from a persistent vitelline artery and extends from one side of the mesentery to the antimesenteric surface of the bowel, usually along the jejunum as in these animals. The resultant double fold of mesentery thus forms a pouch into which intestines may migrate and become incarcerated. An interesting pathological feature in these cases was strangulation of the bowel, not within the pouch itself but through a tear in the jejunal mesentery close to the hernial opening, which is thought to develop as a result of increased tension caused by incarceration. The presence of ramifications of the vitelline artery may render the anomalous mesenteric band stronger than the normal jejunal mesentery. Fixation of bowel within the pouch also led to volvulus as an additional complication. Mesodiverticular bands may be present without causing pathological changes. A further case is reported in which 2 such bands were incidental findings at post mortem examination.

H. PEARSON

Neoplasia

Treatment of bovine and equine ocular squamous cell carcinoma by radiofrequency hyperthermia

GRIER, R. L., BREWER, W. G., PAUL, S. R. and THEILEN, G. H. (1980) *J. Am. vet. med. Ass.* **177**, 55-61.

THE treatment of ocular squamous cell carcinomas in cattle and horses using radiofrequency (RF) hyperthermia is described. The efficacy of the treatment in destroying tumours is ascribed to the apparent greater sensitivity of malignant cells to non-coagulating temperatures of between 41°C and 45°C. The tissue hyperthermia was created by passing a RF (2MHz) electric current for approximately 30 secs between 2 electrodes placed on the tumour surface or within the tumour mass. Most of the animals were treated under topical anaesthesia and sedation, without resort to general anaesthesia. Total treatment time ranged from 30 secs to 15 mins depending on the size and number of tumours. Forty-five ocular squamous cell carcinomas in 17 cattle and 8 horses were treated. In cattle 88 per cent of tumours receiving a single treatment regressed completely while there was 67 per cent complete regression in those tumours which received 2 or more treatments. In horses there was 75 per cent complete regression in the 8 tumours treated. The conclusion is that hyperthermia is an effective and easily applied treatment for ocular squamous cell carcinoma in cattle and horses when the tumour mass is small, less than 5cm diameter, and there is no deep eyelid or conjunctival penetration.

G. WYN-JONES

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Contraceptive efficacy of priming and boosting doses of controlled-release PZP in wild horses

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Abstract

Context. At some sites, wild horse populations have been effectively and non-invasively regulated using remote darting with porcine zona pellucida (PZP) immunocontraceptive vaccines. However, this model has not been widely applied to wild horse herds in western USA, many of which are difficult to access because they roam large areas and are wary of people. Single-treatment, multi-year contraceptive vaccines would significantly broaden the scope for successful contraceptive management.

Aims. The aims of the present field studies were to (1) test the contraceptive effectiveness and longevity of primers incorporating PZP–adjuvant emulsions plus PZP and adjuvant in controlled-release pellets ('PZP-22'); and (2) compare the contraceptive effectiveness and duration of PZP–adjuvant emulsion-only boosters with those of PZP-22 boosters when administered by hand or remote darting to PZP-22-primed mares.

Methods. Wild horses in herd management areas in Colorado and Utah, USA, were rounded up in 2008 and in Utah again in 2012. Previously untreated females received a single hand-injection of PZP–emulsion plus controlled-release PZP pellets ('PZP-22' vaccine) and were then released. In Autumn 2010, 50 Colorado females treated in 2008 were booster-darted with either the PZP-22 vaccine or the PZP emulsion alone. In Utah, 57 previously treated females rounded up in 2012 received hand-injections of either the PZP emulsion–pellet vaccine or the PZP emulsion alone. Maternity was established through field observation of nursing and other close associations.

Key results. Effectiveness of initial controlled-release PZP treatments over 2 years was low relative to previous trials. However, boosters delivered by dart or by hand to PZP-22-primed mares yielded high levels of contraceptive effectiveness for 3 years, with no consistent difference between simple PZP-emulsion boosters and boosters incorporating controlled-release pellets.

Conclusions. Priming mares with PZP-22 extends the efficacy of a subsequent PZP booster to at least 3 consecutive years.

Implications. Regulation of wild horse populations may be achieved with existing contraceptive agents by developing models and management plans that account for the increased longevity of repeated contraceptive treatments.

Additional keywords: feral horse, immunocontraception, porcine zona pellucida, remote delivery.

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Introduction

The 1971 *Wild Free Roaming Horse and Burro Act* (P.L. 92-195) assigned primary responsibility for managing wild horses and burros occupying public lands to the US Bureau of Land Management (BLM), with secondary responsibilities to the US Forest Service (USFS) for managing the species on USFS lands. In 1978, prompted by growth of the herds subsequent to passage of the *Act*, Congress authorised the BLM and USFS to gather, remove and make available for adoption by the public wild horses and burros deemed excess relative to appropriate management levels set by public land use planning processes (*Public Lands Improvement Act*, P.L. 95-514).

Through the fiscal year (FY) 2015, the BLM had adopted out more than 239 000 wild horses and burros to the public (BLM 2016*a*). However, the rate at which horses have been adopted has failed to keep up with the number of horses removed from the range, especially since FY2001, resulting in increases in wild horse numbers in captive-holding facilities and on the range. As of March 2015, more than 45 000 formerly free-roaming wild horses were being maintained in short- and long-term holding facilities at a budgeted cost of US$62 million for FY 2016 (BLM 2016*b*). In addition, the 67 027 wild horses and burros estimated to be living on the range as of 1 March 2016 significantly exceeds BLM estimates of a system-wide

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appropriate management level (AML, the number of horses and burros defined by the BLM as the number that can thrive in balance with other public-land resources and uses) of 26 715 (BLM 2016*b*). While BLM estimates of AML can be challenged, it is clear that the system is unsustainable as currently being operated.

Both BLM documents and outside reviews have repeatedly identified on-the-range fertility control as a plausible option for reducing the rate of growth of wild-horse herds, thereby reducing pressure on the system of removal and adoption as well as the range itself. In response, BLM has been funding research into practical, safe, and effective wild-horse contraception since the 1970s (Turner and Kirkpatrick 1982; Plotka *et al*. 1988, 1992; Turner *et al*. 1997, 2007; National Research Council 2013). Several potentially suitable immunocontraceptive agents have emerged from BLM-funded research, including porcine zona pellucida (PZP) and GnRH vaccines (Killian *et al*. 2008; National Research Council 2013). Porcine zona pellucida has the deepest research history, having been previously tested and applied for several years at Assateague Island National Seashore, MD, and elsewhere (Kirkpatrick *et al*. 1990; Kirkpatrick and Turner 2002, 2003, 2008; Ransom *et al*. 2011; National Research Council 2013).

In a minority of BLM herd management areas (HMAs), wild horses are approachable enough so that contraceptive vaccines can be delivered directly by dart (Ransom *et al*. 2011; Naugle and Grams 2013). However, in others, horses are too wary to approach closely enough for darting, and are thinly dispersed over wide areas (Naugle and Grams 2013). In these areas, either gathers or trapping must be used to make horses accessible for treatment. Where accessing horses for treatment is challenging, the availability of a multi-year, single-treatment contraceptive vaccine would be enormously helpful in effectively and substantially reducing foaling rates and, consequently, rates of population growth.

Evidence for multi-year effectiveness of contraceptive vaccines is mixed. Killian *et al*. (2008) showed that single doses of SpayVac® (Immunovaccine Technologies, Halifax, NS, Canada) and GonaCon™ (USDA/NWRC, Fort Collins, CO, USA) significantly reduced fertility in a small sample of captive horses for up to 4 years. Gray *et al*. (2010) showed that GonaCon-B™ and a formulation of PZP with Adjuvac™ (USDA/NWRC, Fort Collins, CO, USA) reduced fertility in free-roaming Nevada mares for 3 years, although efficacy was modest for both vaccines (reducing fertility from control levels by 25–59%). Turner *et al*. (2001) showed that a single dose of native PZP plus PZP prepared in controlled-release microspheres produced a 1-year reduction in fertility comparable to that of two doses of native PZP administered 3 weeks apart. However, the microspheres tended to clump in the syringe and proved difficult to handle under field conditions (Turner *et al*. 2002). In a subsequent field trial at Clan Alpine HMA, NV, a single injection of 65 µg PZP emulsified in Freund's complete adjuvant (FCA) plus 410 µg PZP and 875 µg QA-21 adjuvant in controlled-release pellets reduced fertility in treated mares by 91%, 73%, and 38% from control levels in Years 1–3 after treatment (Turner *et al*. 2007).

On the basis of the results of the Turner *et al*. (2007) trial and supporting data on captive and non-captive mares (Turner *et al*. 2002; Liu *et al*. 2005), BLM initiated wider use of the PZP emulsion plus PZP/QA-21 pellet vaccine (dubbed 'PZP-22,' based on antibody titer concentrations displayed in Liu *et al*. 2005; National Research Council 2013), treating between 500 and 1000 mares annually between 2007 and 2013. However, the Clan Alpine study was not replicated, and information on the efficacy of BLM administrations at both the individual and population levels was anecdotal. Also lacking are any studies of the effectiveness and longevity of PZP boosters administered to mares primed with PZP-22.

As a part of a larger study to further evaluate the individual- and population-level effectiveness of PZP-22, and to establish the effectiveness and longevity of PZP boosters administered to mares that had previously been primed with PZP-22, we conducted field studies in two BLM herd management areas.

Materials and methods

Study areas

Sand Wash Basin Herd Management Area, Colorado

The Sand Wash Basin (SWB) HMA comprises ~638 km^2 located ~72 km west of Craig, CO, USA. Elevation ranges from 1900 to 2660 m. SWB receives 18–30 cm of annual precipitation, and the climate is typical of the cold deserts of the Rocky Mountain Region, with warm summers and very cold winters. Vegetation types within the HMA include sagebrush (*Artemesia spp*), bunchgrasses, saltbush (*Atriplex* spp.), and pinyon–juniper woodlands. Horses, livestock and wildlife at SWB rely for water on a combination of developed wells, undeveloped springs and seeps and artificial reservoirs.

According to BLM aerial-survey estimates, there were ~463 wild horses on the SWB HMA and 50 wild horses off HMA. Our initial ground inventory after the BLM gather in October 2008 indicated the presence of 194 horses (K. Grams and A. Rutberg, unpubl. data). The appropriate management level at SWB is 163–362 (BLM 2017).

Cedar Mountains Herd Management Area

The Cedar Mountains (CM) HMA comprises 727 km^2 and is located ~80 km west of Salt Lake City, UT, USA. Approximately 425 km^2 within the HMA is part of a congressionally designated candidate wilderness area; vehicles and permanent structures are not permitted within the designated wilderness area. Elevations vary from 1400 to 2460 m. Annual rainfall in the lower elevations averages 20–25 cm, and 25–38 cm of rainfall is received in the upper elevations. Desert shrubs and bunchgrasses are common in the lowlands, with sagebrush and juniper woodlands dominating higher elevations. Water is seasonally scarce, and is concentrated in dry summer months at cattle troughs and a few natural springs.

Our ground-based population inventory indicated that there were at least 608 horses on CM when the study began in 2008, with additional horses on adjacent military lands that had not yet been documented. Initial estimates by BLM based on their aerial survey indicated that 191 remained after the December 2008 BLM gather, although subsequent ground-based population inventories indicated that there were at least 265 horses present post-gather. The appropriate management level for CM is set by the BLM at 190–390 (BLM 2017).

Horse identification

Beginning in 2008, information was documented in the field and then entered into a database. This working document included sex, colour, face and leg markings, unique descriptions, mane and tail description, year of birth and the subpopulation in which the horses lived. Each horse was assigned an ID number to establish individual identity. Horses, including bachelors, were grouped by band in the areas in which they were most recently found. The horse list was updated weekly to reflect any changes or additions and was used in the field to help identify horses. Horses were individually recorded in the Wild Horse Identification Management System (WHIMS, US Geological Survey, Fort Collins, CO, USA). Information from the horse list was placed into this database along with individual pictures of each horse. A photo catalogue allowed for viewing each horse, with the pertinent information to that horse.

Horses that were identified during the study but were not observed within the HMA were excluded from the analysis.

PZP treatments

Wild-horse mares were gathered by the BLM and restrained in squeeze chutes for PZP treatments at Sand Wash Basin on 23 October 2008, and at Cedar Mountains on 17 December 2008 and 25–28 February 2012. Porcine zona pellucida treatments were also administered remotely by dart at SWB from 11 September to 17 November 2010. Thus, boosters were administered at SWB 2 years after the initial treatment, and at CM 3.2 years after the initial treatment.

Hand-injections

In October 2008 and December 2008 respectively, 62 mares (2+ years old) at SWB and 70 mares (2+ years old) at CM were gathered and treated with hand-injections of PZP-22, consisting of one priming injection of 100 µg PZP in 0.5-mL phosphate buffer solution (PBS) emulsified in 0.5 mL of modified FCA (mFCA, Calbiochem, LaJolla, CA, USA) (following Kirkpatrick *et al*. 1990), and a second injection of three heat-extruded lactide–glycolide polymer pellets (polymers from Lactel/Durect, Pelham, AL, USA) containing a total of 450 µg of PZP and 900 µg of QA-21 (Antigenics, Lexington, MA, USA), a saponin adjuvant, engineered to release at 1, 3 and 12 months (Turner *et al*. 2008). The 1- and 3-month pellets each contained 100 µg of PZP and 200 µg QA-21, and the 12-month pellet contained 250 µg PZP and 500 µg of QA-21. Both injections were delivered intra-muscularly into the hip, the PZP–mFCA emulsion with a hand-held syringe fitted with an 18-gauge needle, and the pellets with a trocar fitted with a 14-gauge needle. In the squeeze chute, the horses were also freeze-branded on the hip with a marking that identified them as PZP-treated mares. In February 2012, 85 previously untreated mares at CM were similarly treated and released. Of these mares, 23 were 2 years old, and 62 were ≥3 years old.

Booster trials

To examine the relative effectiveness of the PZP-22 booster when hand-injected, 58 mares that had been treated at CM in 2008 and recaptured for treatment in February 2012 received a hand-injection of either PZP-22 (with Freund's incomplete adjuvant (FIA, Calbiochem, LaJolla, CA, USA) substituted for mFCA, 'PZP-22–FIA'; $n = 39$) or the PZP–FIA booster alone ($n = 19$).

A parallel test of the PZP-22 booster when delivered remotely, by dart, was conducted at SWB. From 11 September to 17 November 2010, 34 mares received PZP-22–FIA delivered in a Pneu-dart® (Williamsport, PA, USA) dart designed with a rod within the 1.5-inch needle, to simultaneously inject both the emulsion and the dart (see Walter *et al*. 2012 for a similar device). Another 16 mares received only the PZP–FIA emulsion by dart (Kirkpatrick *et al*. 1990). Two dart projectors were utilised for short and longer distances. The Dan-Inject CO2 pistol (Børkop. Denmark) projected darts at a distance of 10–18 m. The Pneu-Dart X-Caliber Gauged CO2 projector rifle projected darts at a distance of 17–45 m. Depending on the tractability of the animal, the Dan-Inject was primarily used for close range.

Observations

Observational data were collected beginning in 2008, focusing on the months from June to October in SWB and April through to December at CM. Observations were conducted from the ground, usually by one but sometimes by two observers. Depending on distance, observations were made through binoculars or 20–60 × 80 spotting scope.

For each band encountered, observers identified all known horses present, described any new horses in the band that had not previously been described, tabulated the age–sex composition of the group, and, using maps and field notes, noted the location of the band. Mares were matched with foals within 2 weeks of birth, as indicated by persistent proximity and nursing. Within limits of visibility and accessibility, the observer also estimated and recorded body condition scores on a 1–5 scale (after Rudman and Keiper 1991) and noted any apparent injuries, lameness, abscesses and other injection-site reactions, or other signs of ill health, for each band member, as well as any reproductive activity or mortalities that occurred.

Data analysis

Foaling data inferred from observations of foal–mare associations and behaviour were compiled by mare age (when age data were available) and PZP treatment status. The relationship between age and foaling rates was examined using the linear regression function of IBM SPSS®-22, using study site, age and age squared as independent variables (modified from Ransom *et al*. 2011). Foaling rates were compared among treatment groups and between treatment groups and untreated mares by year after treatment, using Pearson's chi-square tests or, if sample sizes were too small, by two-tailed Fisher's exact tests. Sham controls were not used, because Turner *et al*. (1997) had previously demonstrated that fertility rates among sham controls and untreated controls were virtually identical.

The proportions of untreated and pretreatment mares of known age that foaled in a given year were calculated for each site. Foaling data comprised 170 foals born to mares of known age (2–17 years old) in 311 mare-foaling opportunities at SWB between 2008 and 2014, and 193 foals born to mares of known age (2–24 years old) in 376 mare-foaling opportunities at CM

between 2008 and 2015. Because fewer records existed for older mares, ages were consolidated into groups of at least 10 mares in each age category, assigning the median age to each group. Sample size per age group ranged from 12 to 154.

Because of uniformly low foaling rates among untreated 2-year-old females (see below), all comparisons between treatment groups and untreated controls were performed using mares 3 years old and older at the time of foaling. The criterion for rejecting null hypotheses was set at $\alpha = 0.05$.

Results

Effects of age on likelihood of foaling

In both study sites, 2-year-old females were less likely to foal, then older females (Fig. 1). Site, as an independent variable, was not a significant predictor of foaling rate ($P = 0.824$), and was eliminated from the analysis. Because the form of the relationship between age and fertility appeared to differ at both sites, each site was analysed separately. At CM, the probability of foaling followed a fairly strong parabolic relationship with age ($R^2 = 0.817$, $F_{2,5} = 11.14$, $P = 0.014$; Fig. 1*a*). However, at SWB, the age–foaling relationship was flat for mares older than 2 years old relative to CM, and the parabolic relationship was not significant ($R^2 = 0.580$, $F_{2,5} = 3.46$, $P = 0.114$; Fig. 1*b*).

Initial treatments with PZP emulsion and controlled-release pellets

Sample sizes for observation of foaling in treatment groups showed only slight attrition during the study, whereas samples of untreated controls observed for foaling generally grew from year to year as new individuals in the population were identified or were recruited as foals born into the herd during the study aged into the 3+ years age group (Table 1).

In all three priming treatments (CM 2008 and 2012 and SWB in 2008), foaling rates were statistically indistinguishable between treated and untreated mares during the foaling period immediately after treatment, defined as Year 0 (reflecting pretreatment pregnancy rates; Fig. 2). Significantly lower foaling rates were found among mares ≥3 years old in treated mares in Year 1 at CM in the 2008 ($\chi_1^2 = 29.04$, $P < 0.0001$; Fig. 2*a*) and 2012 ($\chi_1^2 = 24.99$, $P < 0.0001$; Fig. 2*b*) treatments, but not in the 2008 SWB treatments ($\chi_1^2 = 3.4$, $P = 0.065$; Fig. 2*c*), relative to untreated controls. In Year 2, foaling rates in treated mares were significantly lower than those in untreated mares for both the 2008 and 2012 CM treatments ($\chi_1^2 = 5.94$, $P = 0.015$; $\chi_1^2 = 5.57$, $P = 0.018$) and the SWB treatments ($\chi_1^2 = 6.2$, $P = 0.013$). Summed over the two post-treatment years, foaling

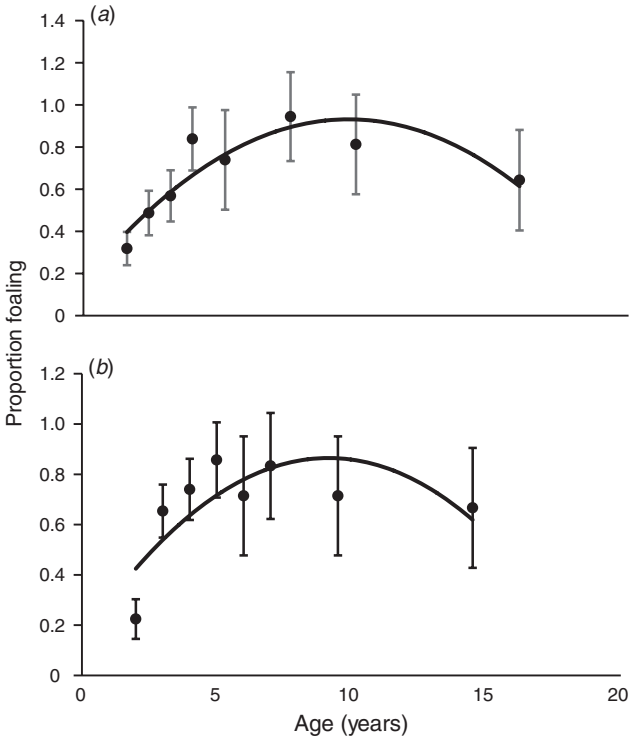


Fig. 1. Proportion of untreated and pretreated mares foaling by age. $N > 10$ for each age category. Error bars represent 95% CI. (*a*) Cedar Mountains, UT, USA, 2008–15 ($Y = -0.005X^2 + 0.126X + 0.165$, $R^2 = 0.817$, $F = 11.14$, d.f. = 2,5, $P = 0.014$). (*b*) Sand Wash Basin, CO, USA, 2008–14 ($Y = -0.009X^2 + 0.157X + 0.144$, $R^2 = 0.580$, $F = 3.46$, d.f. = 2,5, $P = 0.114$).

Table 1. Number of mares 3+ years old for which foaling data were obtained in each treatment group at Cedar Mountains (CM) Herd Management Area, Utah, USA, and Sand Wash Basin (SWB) Herd Management Area, Colorado, USA

At CM, 39 mares received PZP-22–FIA boosters and 19 received PZP–FIA boosters. At SWB, 34 mares received PZP-22–FIA boosters, and 16 received PZP–FIA boosters

Year	Cedar Mountain HMA			Sand Wash Basin HMA		
	PZP-22 primer	PZP boosters (total)	Untreated control	PZP-22 primer	PZP booster (total)	Untreated control
2008	70 hand-treated	–	–	62 hand-treated	–	–
2009	69	–	61	62	–	15
2010	69	–	63	61	50 dart-treated	18
2011	68	–	73	61	50	22
2012	85[A] hand-treated	58 hand-treated	38	–	50	52
2013	83	57	44	–	49	68
2014	80	56	56	–	28[B]	107
2015	–	55	94	–	–	–

[A]Twenty-three (23) mares that were 2 years old at the time of treatment were not included in Year 0 (2012) foaling-rate calculations.

[B]Twenty-one (21) mares were lost to the study because they received additional PZP treatments in 2013. Of the 28 remaining in the study, 17 had received PZP-22 boosters and 11 had received PZP emulsion only.

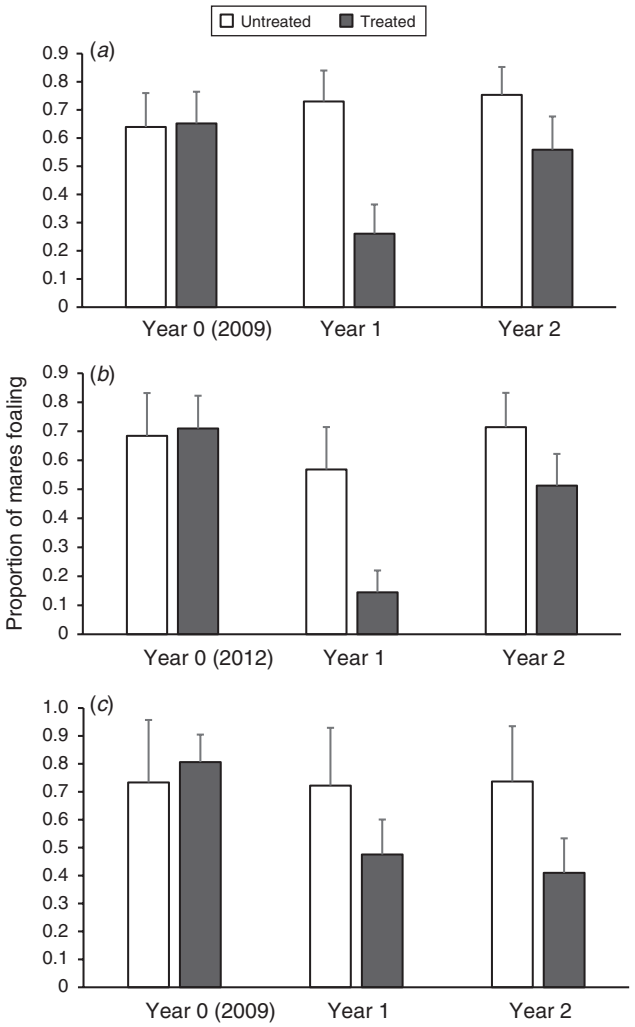


Fig. 2. Foaling rates in mares ≥3 years old. Error bars represent 95% CI. (*a*) Cedar Mountains herd-management area (HMA), UT, USA, following December 2008 administration of porcine zona pellucida (PZP)-22 primers by hand. Year 0 = 2009. (*b*) Cedar Mountains HMA, UT, USA, following February 2012 administration of PZP-22 primers by hand. Year 0 = 2012. Twenty-three PZP-22-treated females that were 2 years old in 2012 were excluded from the Year 0 analysis. (*c*) Sand Wash Basin HMA, CO, USA, following October 2008 administration of PZP-22 primers by hand. Year 0 = 2009.

rates among treated mares were reduced by 39% at SWB in 2008, by 45% at CM in 2008, and by 50% at CM in 2012.

No injection-site reactions were observed in mares after priming via hand-injection.

PZP booster treatments

At both SWB and CM, PZP boosters were associated with significant reductions in foaling rates among mares ≥3 years old every year for 3 years after treatment (Fig. 3).

At SWB, foaling rates in mares boosted with PZP by remote darting averaged 25.2% over 3 years (range 22.0–32.1%) versus 74.9% (65.4–79.4%) in untreated mares (Fig. 3*b*), i.e. foaling rates in booster-treated mares were reduced by 66% relative to those of untreated mares. In each year, foaling rates were significantly lower in treated mares than in untreated mares (Year 1, $\chi_1^2 = 19.46$, $P < 0.0001$; Year 2, $\chi_1^2 = 34.94$, $P < 0.0001$; Year 3, $\chi_1^2 = 20.00$, $P < 0.0001$). Mares darted with PZP-22–FIA showed lower foaling rates (averaging 17.8% across 3 years) than did mares darted with PZP–FIA emulsions alone (averaging 39.5% across 3 years), but there were no significant differences in foaling rates between the two treatment groups in any year.

Injection-site reactions were seen in 32 of 47 darted SWB horses (68.1%) observed in the weeks following darting. Of these reactions, 5–8 were draining abscesses (uncertainty owing to observation quality), of which at least five had resolved by the last time the horse was observed in the 2010 field season (15–52 days after darting). The remainder were swellings and granulomas averaging 2.75 cm (s.d. = 0.87 cm) in diameter. The two treatment types did not differ from one another in either the frequency of swellings and granulomas or in the frequency of draining abscesses.

At CM, hand-injected PZP boosters were also highly effective, with foaling rates of treated mares across 3 years averaging 14.9% (ranging from 7.0% to 20.0%) versus 67.5% (range 56.8–71.4%) for untreated females ≥3 years old, i.e. foaling rates in booster-treated mares were reduced by 78% relative to those of untreated mares (Fig. 3*a*). Treated mares had significantly lower foaling rates than did untreated controls in all years (Year 1, $\chi_1^2 = 30.09$, $P < 0.0001$; Year 2, $\chi_1^2 = 30.38$, $P < 0.0001$; Year 3, $\chi_1^2 = 35.03$, $P < 0.0001$). At CM, no consistent differences in efficacy emerged between the two treatment groups, and there was no significant difference in foaling rates between the PZP-22–FIA and the PZP–FIA-only group in any treatment year. No injection-site reactions were observed in hand-boosted mares.

Discussion

Mares hand-injected with PZP-22 primers generally demonstrated lower foaling rates than did untreated control mares. However, the effectiveness and longevity of PZP-22 were much lower at both CM and SWB than was reported in Turner *et al*. (2007) for wild horses in Clan Alpine. Several differences in manufacturing and protocol may account entirely or in part for the differences.

(1) *Treatment month of administration.* The 1-, 3- and 12-month pellet-release times were engineered to optimise effectiveness of vaccines delivered December to February. The highest 2-year efficacy of primers in the present study occurred when vaccinations were delivered in February (CM 2012), followed by vaccinations delivered in December (CM 2008), with those delivered in October (SWB 2008) being least effective. However, even with optimal administration timing, the efficacy levels observed in these trials following Year 1 did not match those observed at Clan Alpine, suggesting that timing alone is not a sufficient explanation for the differences.

(2) *Differences in timing of antigen release*. Subsequent *in vitro* testing of the heat-extruded controlled-release pellets of batches used in this trial was undertaken to ascertain whether changes in pellet-release characteristics originally described in Turner *et al*. (2007) and Turner *et al*. (2008)

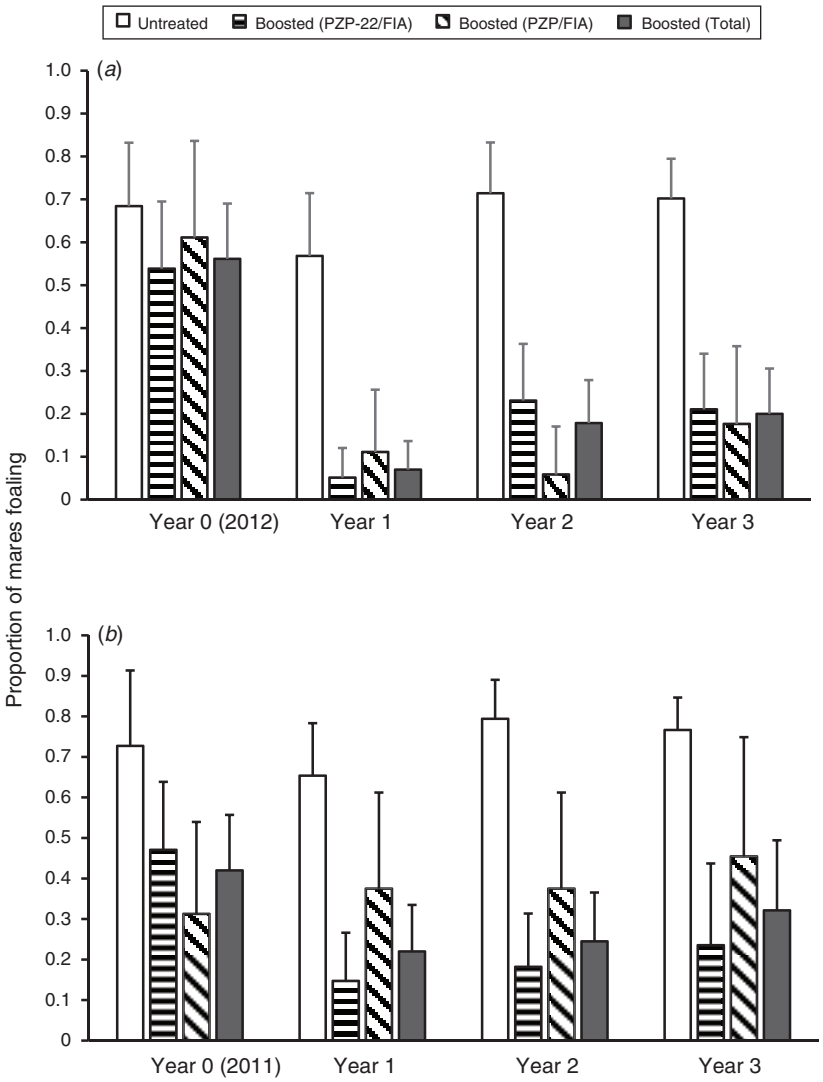


Fig. 3. Foaling rates in mares ≥3 years old. Error bars represent 95% CI. (*a*) Cedar Mountains herd-management area (HMA), UT, USA, following February 2012 administration of PZP-22–FIA or PZP–/FIA boosters by hand. Year 0 = 2012. (*b*) Sand Wash Basin HMA, CO, USA, following Autumn 2010 administration of PZP-22–FIA or PZP–FIA boosters by dart. Year 0 = 2011. Note that the Year 0 (2011) foaling rates for treated mares are the same as those for Year 2 in Fig. 2*c*.

contributed to suboptimal PZP-22 performance. Comparison of release characteristics of pellets produced before and after 2007 revealed that post-2007 pellets of the 12-month delay type released prematurely and more gradually than was expected (J. W. Turner, unpubl. data). Possible contribution of polymer-batch and pellet-manufacture variations to this circumstance are under investigation.

(3) *Method of injection.* PZP-22 at Clan Alpine was delivered by jab stick, and both the emulsion and pellet components were delivered together at the same injection site, whereas emulsion and pellets were delivered by two separate injections in all hand-injection protocols of PZP-22 followed in the present study. Although being an implausible explanation for the differences, this hypothesis also receives weak support from the improved PZP-22 efficacy relative to the PZP–FIA emulsion associated with dart delivery in booster trials (described below).

(4) *Difference in adjuvant.* PZP vaccine prepared before 2005 utilised FCA, and, thereafter, mFCA was used. Similar anti-PZP titer responses to primer-dose PZP treatment with these two adjuvants have been reported (Lyda *et al*. 2005). However, contraceptive efficacy was not determined, and it is possible that the antibody type and quality generated by these two adjuvants differed sufficiently to influence the immune memory generated by pellet-based boosters (M. Wooten, pers. comm.). In light of the limited future for FCA (related to tissue reactions and false-positive TB tests) and the promise of alternative multi-target adjuvants (e.g. Lu *et al*. 2015), comparison of mFCA with such novel adjuvants to address the above issue may be warranted.

Although the initial PZP-22 treatments tested in the present study showed disappointing effectiveness, a single PZP booster (either the PZP–FIA emulsion or PZP-22–FIA) effectively reduced fertility across three consecutive years. Whether delivered by dart, as at SWB in 2010, or by hand, as at CM in 2012, PZP boosters reduced foaling rates in treated mares by roughly 65–72% relative to untreated control mares over the 3 years. We do not know, because the studies had to be ended 3 years after boosting, whether some contraceptive effectiveness might persist into a 4th year.

Most previous research on PZP in western wild horses has presented data only on the effectiveness and longevity of the initial treatment (Turner *et al.* 1997, 2001, 2007). Working on wild horses at Little Book Cliffs Wild Horse Range, CO, USA, and the Pryor Mountain Wild Horse Range, MT, USA, Ransom *et al*. (2011) examined the relationship between the number of consecutive years of treatment (up to 4 years) with PZP emulsion boosters and the interval between the last booster and the first foaling. This interval ranged from 7 to 18 months, with some trend towards longer intervals as the number of consecutive years of treatment increased, although the sample size for 3 and 4 consecutive years of treatment was very small and the trend was not significant. In their study of reversibility of PZP treatments of wild horses at Assateague Island National Seashore, MD, USA, Kirkpatrick and Turner (2002) showed that most mares receiving two initial injections and up to one subsequent annual booster returned to fertility within 1 year, whereas mares receiving three or four consecutive years of treatment experienced delays of 3–4 years in return to foaling. Although our measures of the longevity of vaccine effectiveness differ from those used in other studies, namely annual foaling rates of treated mares relative to those of controls, rather than interval between last booster and first foaling, as used by Ransom *et al*. (2011) and Kirkpatrick and Turner (2002), our results appear to parallel those for Assateague mares receiving three or more consecutive years of treatment. Thus, even if the initial effectiveness of PZP-22 was low relative to previous studies, the combination of a PZP-22 primer and a single PZP booster appears to have yielded effectiveness comparable to three or more years of consecutive treatments (i.e. 4+ injections) of Assateague mares initially treated with the PZP–mFCA emulsion primer.

Our study failed to show that the addition of the controlled-release pellets to the PZP emulsion increased the efficacy and longevity of the booster. At CM, addition of pellets in hand-administration did not increase the efficacy or longevity of the PZP emulsion over the 3 years of the study. Dart delivery of the booster was associated with a doubling of efficacy across all 3 years at SWB, but because the sample is small, this test warrants replication.

Conclusions

Long-term management of wild horse populations with a non-invasive reversible contraceptive will require repeated treatments over the life of the wild mare. Thus, we believe that the research emphasis placed on the effectiveness and longevity of the primer vaccination may be misguided. The data presented here, as well as in Kirkpatrick and Turner (2008), suggest that population models should be constructed that allow for increased longevity of vaccine effectiveness with successive boosters. In practice, the initial application of contraceptives to a wild horse herd may produce only a modest reduction in population foaling rates, but sustained contraceptive applications over time could result in a much greater reduction in population foaling rates, even with current technology.

At Assateague, the initial management step was to treat every mare with a single primer of PZP–FCA emulsion (Kirkpatrick and Turner 2008). Although the primer alone was only somewhat effective as a contraceptive, subsequent boosting yielded increased effectiveness, and, ultimately, a reduction in herd numbers. This lesson needs to be applied in Western herds.

Also encouraging is the demonstration of management flexibility in PZP-22 application. First, the data suggest that the interval between initial and booster treatments, i.e. 2 years at SWB, and 3.2 years at CM, does not obviously influence effectiveness or longevity of the booster over the range of times tested. Second, the SWB data indicate that PZP-22–FIA can be effectively delivered remotely, at least matching the effectiveness of the PZP–FIA emulsion. However, until technical improvements and additional tests show that the controlled-release pellets significantly increase booster efficacy, managers can employ the much less costly PZP–FIA emulsion as a booster following administration of the PZP-22 primer.

Continuing to improve vaccine longevity is essential. The more individual mares are darted, the more difficult they become to re-treat (Kirkpatrick and Turner 2008; Naugle and Grams 2013). As the controlled-released pellets are refined, re-darting can be performed less frequently, making contraceptive control more cost effective.

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PASSAGE OF ZONA ANTIBODIES VIA PLACENTA AND MILK FOLLOWING ACTIVE IMMUNIZATION OF FEMALE MICE WITH PORCINE ZONAE PELLUCIDAE

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Female mice heteroimmunized with heat-solubilized porcine zonae produced a significant antibody response to this immunogen as measured by radioimmunoassay (RIA) but their fertility was unaffected and normal litters were delivered. Monitoring for anti-zonae pellucidae activity in the sera of offspring from such immunized mothers by RIA indicated that the zonae antibodies were transferred from mother to young via the placenta and milk. Such transferred antibodies cross-reacted with and bound to the zonae of female offspring as demonstrated by immunofluorescent techniques. These data are significant to the future consideration of use of specific zona antigens for development of a contraceptive vaccine.

INTRODUCTION

Immunocontraception is described as the prevention of fertility by immunologic methods. The high level of specificity exemplified in the antigen–antibody binding reaction as well as the neutralizing potential of antibody molecules make the immunologic inhibition of fertility an attractive approach to contraception. Recently, the zona pellucida has gained much attention as a potential immunocontraceptive target antigen (Sacco, 1981). The zona contains one or more tissue specific antigens, antibodies directed against such antigens totally inhibit fertility and, the pig and human share a similar zona specific antigen(s) (Sacco, 1981; Takai et al., 1981; Shivers and Dunbar, 1977; Sacco, 1977). This latter observation has identified the pig as a potential source of zona antigen material in attempts to develop a contraceptive vaccine with possible human application.

The consideration of an immunocontraceptive approach, as applicable to the human female and which utilizes porcine zonae, would likely ultimately involve active immunization using a purified porcine zona macromolecule as immunogen. Experiments describing the inhibition of fertility following active immunization of females with zonae pellucidae, although few in number to date, have been encouraging (Shivers et al., 1981; Gwatkin and Williams, 1978; Gwatkin et al., 1977). Also, the inhibition of in vitro fertilization of human eggs by antibodies to pig zona pellucida has been recently reported (Trounson et al., 1980). However, other studies (Sacco et al., 1981a,b) have also vividly demonstrated that the degree of immunologic similarity between zonae used as immunogen and zonae of the female immunized is of critical importance for the resulting cross-reacting zona antiserum to exert its contraceptive effect in the injected female. For

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example, although heteroantibodies produced against either mouse or pig zonae do cross-react with the other species' zona and are contraceptive in their respective homologous system (Sacco, 1981; Sacco et al., 1981a, b; Jones, 1980), mouse antibodies to pig zonae do not inhibit mouse fertility in vivo (Sacco et al., 1981a). Likely, although components present in mouse and pig zonae do possess some antigenic similarities as evidenced by the cross-reaction of their respective anti-zonae sera, mouse and pig zonae are not sufficiently similar for zona antibodies to exert a contraceptive effect in respect to heterologous zonae (Sacco et al., 1981a, b). In the present study we have taken advantage of the observation that while mice heteroimmunized with porcine zonae produce a potent immune response to this antigen, mouse fertility is unaffected by such antibodies and viable litters are produced. We demonstrate here, for the first time to our knowledge, the transfer of zona antibodies from an immunized mother to her offspring. While antibody transfer from mother to young is not unique (Collins et al., 1980; Castro, 1971; Cramet et al., 1974; Barnes and Holliday, 1970; Desay and Creger, 1963), with the recent detection of zona pellucida antigen(s) in primordial follicles of the human (Bousquet et al., 1981), this observation regarding zona antibodies is of considerable importance in respect to the possible use of the zona in an immunocontraceptive approach to fertility regulation.

MATERIALS AND METHODS

Immunogen preparation

Porcine solubilized isolated zonae pellucidae (SIZP) were prepared as detailed elsewhere (Sacco et al., 1981a). Briefly, the procedure consisted of homogenizing pig ovaries in Tris buffer (0.05 M Tris/0.15 M NaCl/0.02% NaN_3/0.004 M Na_2EDTA, pH 8.0) using a commercial meat grinder and isolating oocytes by sieving the homogenate on nylon screens (Tetko, Inc.) of decreasing pore size (500–100 μm). The washed oocytes were resuspended in a measured volume of buffer (approx. 100 ml), a measured aliquot of this suspension withdrawn, and its oocyte content counted using a stereomicroscope. The total number of oocytes present in the suspension was then calculated. Following the determination of oocyte concentration, zonae were then solubilized by heating the oocyte suspension in a 73°C water bath for 20 min. Intact oocytes were removed by centrifugation at 27 000 $\times g$ for 15 min and the solubilized zonae material (SIZP) contained in the supernatant concentrated by the stepwise addition of ammonium sulfate to a final 70% saturation. The resulting zonae precipitate was dissolved in and dialyzed against 0.1 M phosphate-buffered saline (pH 7.3). The volume of the dialyzate was measured and the number of SIZP per unit of volume calculated. Aliquots of this preparation were used as SIZP immunogen as indicated below. Antisera raised against such heat-solubilized zonae material has been previously shown to be specific for zonae pellucidae (Gwatkin et al., 1980; Gwatkin and Williams, 1978).

Immunizations and antisera collections

Groups of Sch:ARS(CF1)f female mice (10 mice/group) were each heteroimmunized, using the methodology of Vaitukaitis et al. (1971) of multiple intradermal injections, with quantities equivalent to 1000, 2000, 4000 or 50 000 porcine SIZP per mouse, respectively, as detailed elsewhere (Sacco et al., 1981a). Blood samples for antisera

(approx. 150 μl/bleeding) were collected from adult mice via the orbital sinus using 75 mm capillary tubes. For newborn and young (approx. 22 days old) mice, blood was obtained by decapitation immediately followed by collection into a capillary tube. Following centrifugation, the antiserum was stored frozen at −20°C. Immunization and bleeding schedules are indicated in Figs. 1, 2, 4 and 5. Female mice immunized with adjuvants only or unimmunized mice served as controls.

Antibody detection

(a) Radioimmunoassay (RIA). The double antibody RIA method used to detect and monitor mouse antibodies to porcine SIZP has been previously described (Sacco et al., 1981c). SIZP radiolabeled with $Na^{125}I$ by the chloramine-T procedure was used as the labeled antigen ([^{125}I]SIZP). For use in the RIA, activity in the tube immediately following the peak tube was adjusted by dilution with PBS containing 0.5% BSA to 25 000 dpm/100 μl (equal to approx. 0.1 zona). Sera for antibody evaluation were diluted serially in PBS containing 1% normal mouse serum. The precipitating second antibody was a rabbit anti-mouse gamma-globulin (1:30 dilution). Triplicate tubes devoid of anti-zona activity were included in each assay to estimate nonspecifically bound (NSB) radioactivity. For the assay itself, 200 μl of various dilutions of mouse anti-zona serum were incubated with 100 μl [^{125}I]SIZP and 500 μl PBS–BSA for 4 h at 37°C, following which 200 μl of second antibody was added. After an overnight incubation at 4°C, 3 ml of cold PBS were added to each of the tubes, the tubes centrifuged (1100 × *g*, 35 min), the supernatants discarded and the precipitates drained and counted in a Searle Model 1185 gamma counter. The NSB tubes and the radioactivity precipitated in the antibody-containing tubes were calculated as the percentage of total counts added to the assay system.

(b) Indirect immunofluorescence. The indirect immunofluorescent antibody technique was used to detect the presence of mouse anti-porcine SIZP serum on the zonae of superovulated mouse eggs. Eggs were obtained from the female offspring (age = 49–59 days) of mothers actively immunized with porcine SIZP by flushing the reproductive tracts of hormonally primed animals. Established procedures for inducing superovulation and removal of cumulus cells with hyaluronidase (300 μl/ml PBS, Sigma Type 1) were used. Rabbit anti-mouse IgG serum labeled with fluorescein isothiocyanate (Cappel Laboratories) was employed as the second antibody. Methodology used was patterned after previously reported procedures (Sacco and Shivers, 1978). Eggs obtained from offspring of adjuvant only injected mothers served as controls and were processed and viewed simultaneously with the experimental eggs.

Matings

Immunized and control mice were allowed to undergo spontaneous matings with males of proven fertility and the presence of a copulatory plug was considered as evidence of a successful mating. Mice injected with adjuvants alone and/or unimmunized mice served as the controls. For experiments involving the transfer of litters between an immunized mother and a control mother, matings were initiated so pregnant immunized and control females would deliver at approximately the same time. Litters were switched immediately after delivery using a gloved hand and out of view of the mothers. In studies which

involved monitoring antibody levels over a period of days in such transferred offspring only those mothers having large litters (10–12 offspring) were selected since a portion of the litter had to be killed at intervals over this experimental period for serum collection.

For studies involving nonsuckled newborn mice, pregnant females, just prior to delivery, were placed in cages having a wire screen floor. During delivery, the newborn mice dropped through the screen and thus had no contact with the mother. All mice were weaned at approximately 21 days.

RESULTS

Immune response to porcine SIZP

The individual immune responses for four mice injected with 1000, 2000, or 4000 porcine SIZP and as monitored by RIA using a 1×10^{-4} antiserum dilution are presented in Fig. 1. Complete mouse immune profiles produced in response to this antigen for mice in all the immunized groups have been presented in detail and discussed elsewhere (Sacco et al., 1981a). For this study, these particular females were selected because they were representative of mothers whose litters had been monitored for anti-SIZP activity. Although demonstrating considerable individual biologic variation, Fig. 1 indicates that each of the four mice, regardless of SIZP dosage injected, produced a significant immune response to this antigen. The percentages of [^{125}I]SIZP bound by a 1×10^{-4} antibody dilution ranged from approximately 26–42% during their respective periods of pregnancy (Fig. 1).

Transfer of SIZP antibodies to offspring

Fig. 2 presents the antiserum titration profiles of a single bleeding from each of these mothers (first bleeding following delivery and designated by arrow in Fig. 1) and three consecutive bleedings from a representative female offspring from each. All offspring (including males) possessed significant antibody activity directed against porcine SIZP when initially tested at 26–36 days of age and which gradually decreased to barely detectable levels at 106–116 days of age. No antibody activity was detected in control immunized animals or their offspring. This anti-SIZP activity present in the female offspring from immunized mothers cross-reacted and bound to native intact mouse zonae as indicated in Fig. 3. Superovulated mouse eggs obtained from such female offspring at 49–59 days of age demonstrated moderate fluorescence associated with their zonae when tested by the indirect immunofluorescent assay. Furthermore, anti-SIZP activity could also be demonstrated by immunofluorescence procedures when these sera were reacted against zonae obtained from either control immunized mice or their offspring.

Antibodies to porcine SIZP were present in offspring immediately following birth as indicated in Fig. 4A. Sera, from newborn mice from a mother immunized with 50 000

Fig. 2. Antibody titration profiles of a single bleeding from mothers (M, ▪) and three consecutive bleedings of a single representative female offspring (●) of each as assayed over a period of days (d). A, mother immunized with 1000 SIZP; B, 2000 SIZP; C, 4000 SIZP; D, 4000 SIZP. The bleeding titered in this figure for each mother is designated by arrow in Fig. 1.

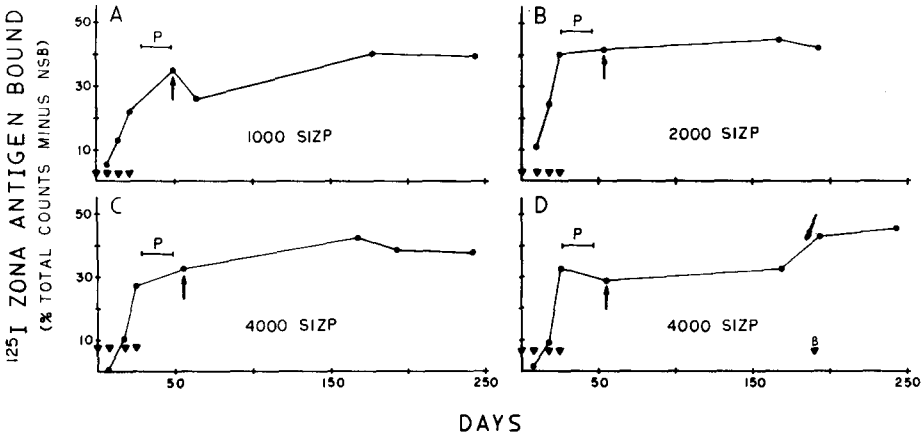


Fig. 1. Female mouse immune response to injection with various dosages of heat-solubilized isolated porcine zonae pellucidae (SIZP) as monitored by radioimmunoassay (RIA) using a 1×10^{-4} serum dilution. Female immunized with: A, 1000 SIZP; B, 2000 SIZP; C, 4000 SIZP; D, 4000 SIZP. ▼, injections in initial series of immunizations. $\overset{B}{\blacktriangledown}$, booster immunization following initial injection series. P, time period that mouse was pregnant. Arrow represents first bleeding assayed following delivery of litter.

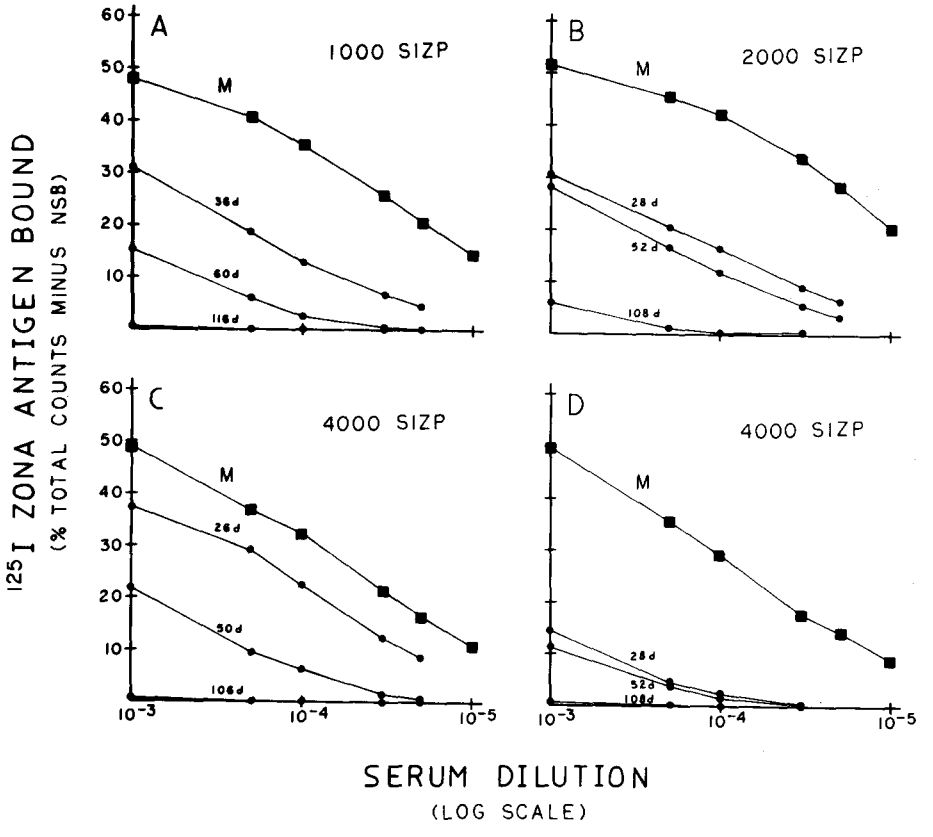


Fig. 2. See opposite page for legend.

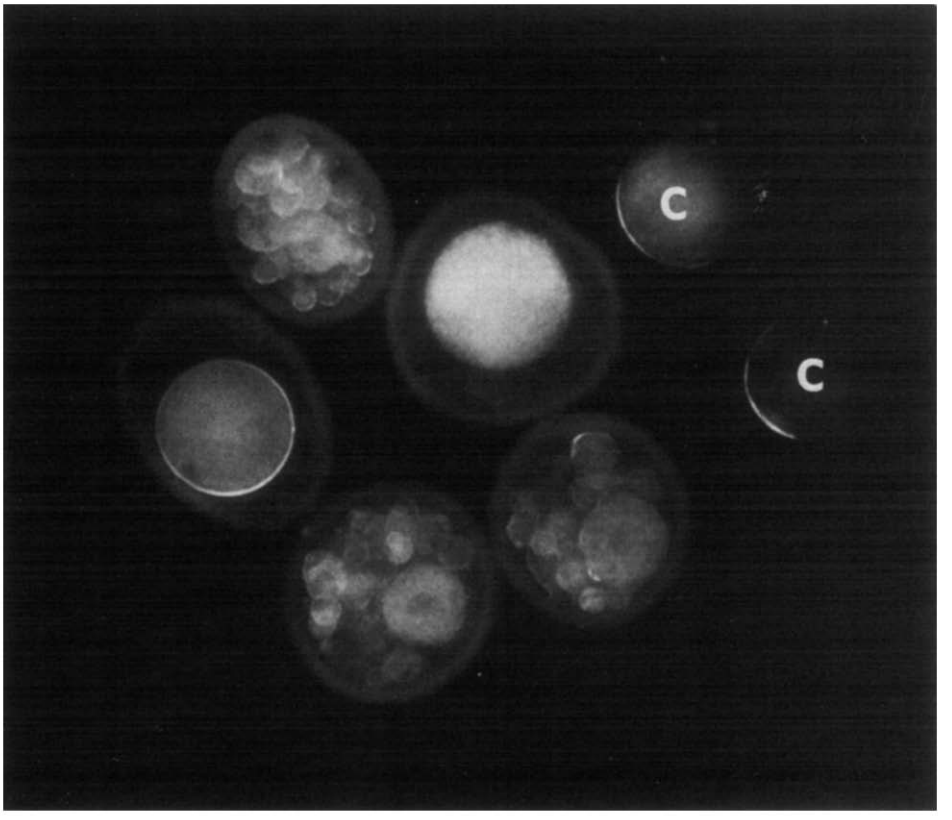


Fig. 3. Superovulated zona-encased mouse eggs obtained from a representative female offspring and tested for antibodies bound to zonae pellucidae by the indirect immunofluorescent assay. The mother of this particular female offspring was immunized with 4000 solubilized porcine zonae. Eggs were obtained and tested when the age of this female offspring was 49 days. Note moderate fluorescence associated with zonae pellucidae indicating the in vivo cross-reaction of antibodies to porcine zonae with mouse zonae in female offspring. No fluorescence is associated with zonae (C) of offspring obtained from control immunized mother. Fluorescence by vitellus is attributed to nonspecific autofluorescence.

SIZP and which were killed immediately after delivery and prior to nursing, precipitated 30.25 ± 1.23% ($\bar{x}$ ± SD) [^{125}I]SIZP at a 1×10^{-3} dilution. The mother's serum at time of delivery precipitated 35% [^{125}I]SIZP at a comparable serum dilution. However, nursing also contributed to antibody presence in offspring as suggested in Fig. 4B. Another mother immunized with 50 000 SIZP produced a litter of 12 offspring at 46 days following her initial immunization. During the period of time that included this pregnancy (which extended from 27–46 days following the initial immunization) the percentage of [^{125}I]SIZP bound by a 1×10^{-3} dilution of the mother's serum was increasing from 43% (at 21 days) to 58% (at 54 days). The percentage of [^{125}I]SIZP precipitated by a 1×10^{-3} serum dilution from offspring ($n = 6$) killed immediately after delivery and prior

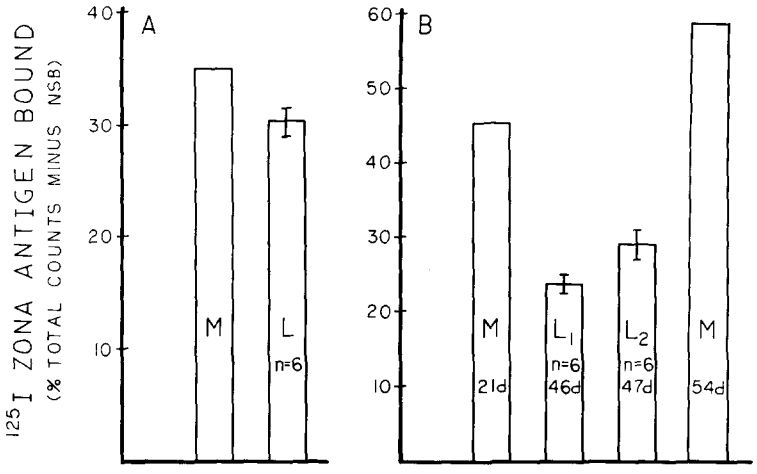


Fig. 4. Percentage of ^{125}I-labeled zona antigen bound by sera (1×10^{-3} dilution) from immunized mothers (M, 50 000 SIZP/female) and their litters (L). A. Comparison of percentages of zona antigen bound by serum from a mother and her newborn offspring ($n = 6$) which were tested immediately after their birth and prior to nursing. B. Comparisons of percentages of zona antigen bound by sera from a mother obtained at 21 and 54 days (d) following immunization and her litter ($n = 12$) which was delivered at 46 days following mother's initial immunization. L_1, offspring ($n = 6$) killed immediately following birth and prior to nursing. L_2, offspring ($n = 6$) allowed to nurse during 16 h exposure to mother. Vertical lines represent ± 1 S.D.

to nursing was 23.84 ± 1.35 ($\bar{x}$ ± SD) and was significantly lower (Student's t-test, $P < 0.001$) than the percentage of [^{125}I]SIZP bound by sera from the remaining offspring ($n = 6$) killed approximately 16 h following delivery and which were allowed to nurse (29.01 ± 1.89 [^{125}I]SIZP bound).

The passage of antibodies directed against porcine SIZP to offspring via mothers' colostrum and milk was confirmed by litter transfer experiments. Litters were switched between immunized mothers (50 000 SIZP) and control mothers immediately after delivery and prior to nursing as illustrated in Fig. 5. The percentage of [^{125}I]SIZP bound by a 1×10^{-3} serum dilution from offspring of the control mother and killed immediately after delivery was insignificant (Fig. 5A, day 1). However, when the remaining offspring, immediately following their birth, were switched to and allowed to nurse from the immunized mother and their sera tested 15 days later, significant anti-SIZP activity was present (56.62% [^{125}I]SIZP bound at a 1×10^{-3} dilution) in their sera. These titers slowly decreased over a period of time (15–65 days) as indicated in Fig. 5A. The anti-SIZP activity in sera of offspring from the immunized mother was initially high immediately following their birth and prior to nursing (40.4% binding on day 1; Fig. 5B). Following the transfer of the remaining litter mates immediately after their birth and prior to nursing to the control mother, the anti-SIZP activity in their sera gradually decreased over the period ranging from 1 to 65 days (Fig. 5B). During this particular experiment the titer of the immunized mother was increasing from a percent binding level

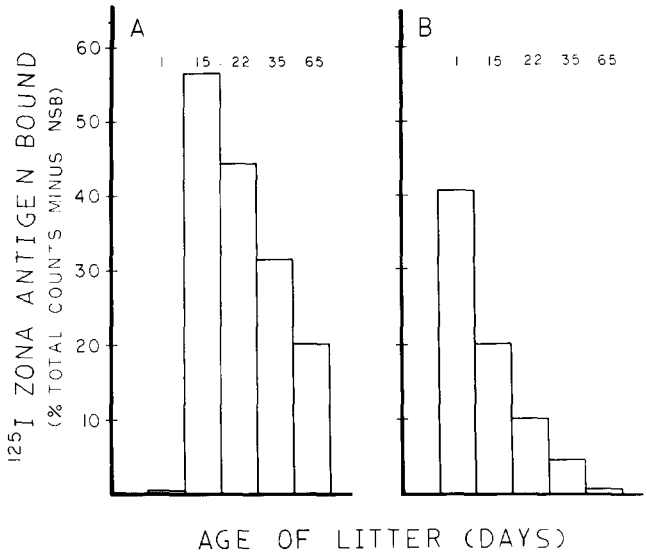


Fig. 5. Percentages of ^{125}I-labeled zona antigen bound by sera (1×10^{-3} dilution) of offspring from a control mother and offspring from immunized mother. Immediately after delivery, the offspring from the control mother were transferred to immunized mother and the offspring from immunized mother were transferred to control mother. A. Data from litter born from the control mother and switched to the SIZP-immunized mother immediately after birth. B. Data from litter born from the SIZP immunized mother and switched to the control mother immediately after birth. $n = 3$ for day 1 values; $n = 2$ for all other days. See text for details.

of 49% on day 1, the day of the delivery, to 58% at 65 days following delivery. No anti-SIZP activity was detected in serum from the control mother.

DISCUSSION

Zonae pellucidae represent a potent heteroimmunogen as demonstrated both in the present investigation by the high anti-SIZP titers achieved using RIA (Fig. 1) and by others using the more conventional biologic zona antibody detection assays (Shivers et al., 1981; Tsunoda et al., 1981; Tsunoda and Sugie, 1979; Mahi and Yanagimachi, 1979; Sacco and Shivers, 1978; Gwatkin et al., 1977). Zona antibodies exert their contraceptive effect by inhibiting sperm attachment to and passage through the zona (Shivers et al., 1972). Recently, it has been suggested that this inhibition is achieved by a generalized zona antibody lattice formation occurring on the zona surface rather than a specific antigen–antibody reaction directed against sperm receptors (Ahuja and Tzargos, 1981; Aitken and Richardson, 1981). The present study has demonstrated that antibodies to SIZP produced by the mother are passively transferred via the placenta and milk to her offspring (Figs. 2–5). Furthermore, such antibodies retain their biologic activity within the offspring, as indicated not only by their ability to precipitate [^{125}I]SIZP in the RIA but, more importantly, by their binding in vivo to the zonae of female offspring (Fig. 3). Similar in vivo antibody binding to the zonae of actively immunized mothers has been

previously shown (Shivers et al., 1981; Gwatkin et al., 1977).

In investigations where active immunization of females with zona material produces a contraceptive effect during periods of high anti-zona levels, fertility of the immunized female gradually returns once zona antibody titers decline to low levels (Shivers et al., 1981; Gwatkin and Williams, 1978; Gwatkin et al., 1977). Our current findings strongly indicate that even in those situations where pregnancy is prevented due to high zona antibody levels, once titers decrease and a pregnancy ensues, any residual zona antibodies from the immunized pregnant mother will be transferred to the offspring with their subsequent binding to zonae in the females. All present data indicate that zona antibodies produce no physiologic effect other than the inhibition of fertility, a fact substantiated both by the demonstrated tissue specificity of zonae antibodies (Subramanian et al., 1981; Gerrity et al., 1981; Palm et al., 1979) and by the return of normal fertility and the production of normal pregnancies and offspring in zonae immunized females (Shivers et al., 1981; Gwatkin and Williams, 1978; Gwatkin et al., 1977). Nonetheless, to evaluate fully the immunocontraceptive approach to fertility regulation, based upon a zona pellucida target antigen, detailed evaluation of possible effects such zonae antibodies may have in fetal, newborn and prepuberal females must now also be considered.

ACKNOWLEDGMENTS

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BREEDING AND GENETICS

Direct and Correlated Response to Divergent Selection for Serum Immunoglobulin M and G Levels in Chickens

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ABSTRACT Two pairs of chicken lines were divergently selected for serum immunoglobulin M and G levels at 10 wk of age to examine the effect of selection on direct and correlated traits. After three generations of selection, the lines were tested for their ability of antibody production against SRBC, a T cell dependent antigen. At 14 and 17 wk of age, the lines were immunized intravenously with 1 mL of 5% SRBC solution. Blood samples were collected at 7 and 14 d of post primary immunization (PPI) and post secondary immunization (PSI). Selection was effective ($P < 0.01$) in changing the serum immunoglobulin M levels. The G levels were changed in a downward direction initially in the high line. The differences between the high immunoglobulin M (HIM) and low immunoglobulin M (LIM) lines as well as high immunoglobulin G (HIG) and low immunoglobulin G (LIG) lines became larger during the course of selection. Selection differentials varied considerably during the selection, but the selection response was almost constant. Realized heritability was relatively high, ranging from 0.58 to 0.66 and from 0.40 to 0.60 for the IgM and IgG selected lines, respectively. Total antibody titers to SRBC differed significantly ($P < 0.01$) between the HIM and LIM lines at 7 and 14 d PPI, and 7 d PSI. The HIM line also produced significantly higher mercaptoethanol resistant antibody titers to SRBC than the LIM line at 7 and 14 d PPI and PSI. The LIG line had significantly higher total antibody titers to SRBC than the HIG line at both 7 and 14 d of PPI and PSI. From these results, it is suggested that selection of chickens on the basis of serum immunoglobulin isotypes may change antibody producing cells as well as other immunocompetent cells that modulate the immune response of the selected lines.

(*Key words*: chicken, selection, immunoglobulin M, immunoglobulin G, immune response)

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INTRODUCTION

The production of poultry continues to be plagued with disease problems, although vaccination programs have dramatically reduced the incidence of diseases. Vaccination programs alone cannot cope adequately with infectious diseases. A combination of vaccination and genetic resistance is essential to maximize the protection from diseases. Genetic resistance of chickens can be improved through selection for immunocompetence (Gavora and Spencer, 1983; van der Zijpp, 1983a; Warner *et al*., 1987). Consequently, several lines of chickens have been developed for humoral or cell mediated immune responses. Developing lines against specific antigens is the most popular and frequent approach of selection to improve the immune response and to increase disease resistance. In mice, Biozzi *et al.* (1979, 1984) reported that differences in antibody production within lines selected for high (H) or Low (L) antibody production to SRBC were associated with antigen handling and antigen presentation of macrophages and the multiplication rate of B lymphocytes. Antibody production against SRBC has also been used in selection experiments in chickens (Siegel and Gross, 1980; van der Zijpp, 1983b; Martin *et al*., 1990; Pinard *et al*., 1992). Siegel and Gross (1980) found that the high antibody production line was more resistant to parasites and viruses than the low antibody production line. Selection for antibody response to glutamic acid-alaline-tyrosine (GAT) and also for antibodies against *Salmonella pullorum* has also been reported (Pevzner *et al*., 1981a,b). Pitcovski *et al.* (1987) selected for high and low early antibody response to Newcastle disease virus vaccine and *Escherichia coli*, and after four generations of

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Abbreviation Key: GAT = glutamic acid-alaline-tyrosine; GVHR = graft-vs-host reaction; HIG = high immunoglobulin G; HIM = high immunoglobulin M; LIG = low immunoglobulin G; LIM = low immunoglobulin M; LPS = lipopolysaccharide; MER = mercaptoethanol-resistant; PPI = post primary immunization; PSI = post secondary immunization; SD = selection differentials; SR = selection response; SP = *Salmonella pullorum*; SRID = single radial immunodiffusion.

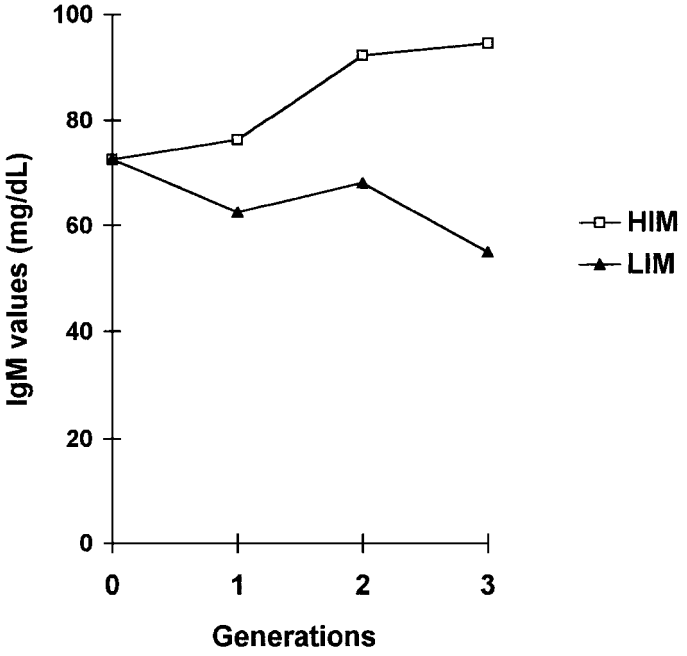


FIGURE 1. Effect of divergent selection for a serum IgM level over three successive generations in chickens. The serum IgM level changed considerably over the course of selection. A significant difference ($P < 0.01$) was found between the high (HIM) and low (LIM) lgm lines during the course of selection.

selection they found that the average antibody production of the high responder line exceeded that of the low responder line. Selection on the basis of multitrait immunocompetence, as well as high and low donor competence of splenomegaly was also conducted (Okada and Mikami, 1974; Cheng *et al*., 1991; Kean *et al*., 1994).

On the contrary, improved disease resistance may also be achieved without challenging animals with disease agents. Selection of chickens on the basis of serum immunoglobulin isotypes, which represent the response to a wide variety of unknown antigens, may be a useful tool in this regard. However, considerably less attention has been paid towards this technology thus far. Tamaki (1981) determined that chicken lines selected for a high serum IgG level were more susceptible to Marek's disease than their low IgG counterparts. In another experiment, Okada and Yamamoto (1987) demonstrated that the high IgG line was associated with higher incidence and mortality to Marek's disease, high antibody responses to SRBC, and lipopolysaccharides (LPS). However, Krieg *et al.* (1978) reported that low IgG levels were associated with mortality to myeloblastosis virus. Therefore, the purpose of the present selection program was to develop divergent chicken lines for serum IgM and IgG levels and to determine the effects of selection on direct and correlated traits.

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MATERIALS AND METHODS

Chickens

The base population for this divergent selection experiment consisted of crosses of six lines. The lines were HA, LA, HG, LG, C, and GSP. Lines HA, LA, HG, and LG were established from the N strain of Hokkaido University, Sapporo, Japan, by four generations of selection for high and low competences of splenomegaly in graft-vs-host reaction (GVHR). The details about these lines have previously described (Okada and Yamamoto, 1987). Briefly, lines HA, LA, HG, and LG were developed from the N strain of Hokkaido University, Sapporo, by selection for high and low competences of splenomegaly index in GVHR in 1969. Line C was a randombred control line and established from crossbreds of White Leghorns and White Rocks maintained at the authors' laboratory for 35 yr. The GSP line (Somes, 1988) was established by pedigree breeding from the Fayoumi breed at the Nippon Institute for Biological Science, Kobuchizawa, Yamanashi, Japan in 1971. To construct the base population, firstly the lines were crossed to produce GHA (GSP♂ × HA♀), GHG (GSP♂ × HG♀), CLA (C♂ × LA♀), and CLG (C♂ × LG♀) populations. Then these GHA and CLG, and GHG and CLA were crossed reciprocally to established the base population.

From this combined gene pool, progeny with highest IgM and those with highest IgG, from 10 sires and 20 dams, became the parents of HIM and HIG lines.

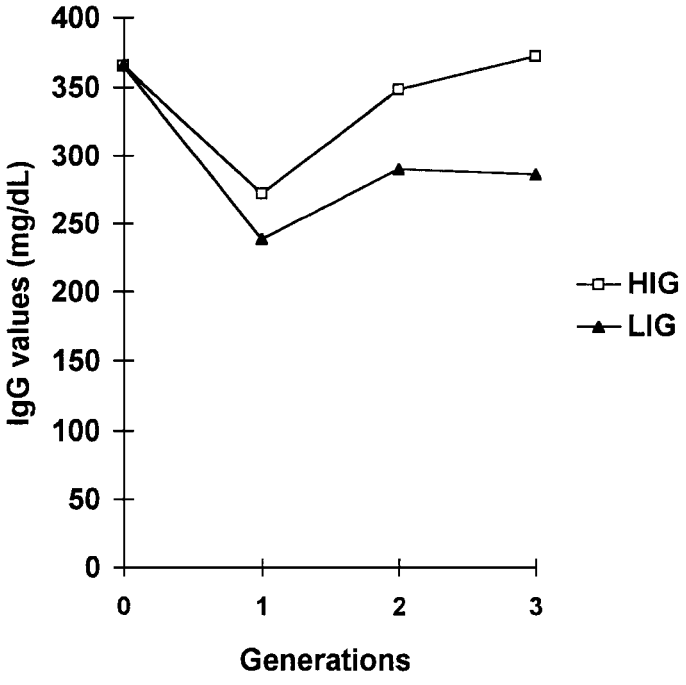


FIGURE 2. Effect of divergent selection for a serum IgG level over three successive generations in chickens. The serum IgG level changed gradually over the course of selection. A significant difference ($P < 0.01$) was found between the high (HIG) and low (LIG) lgG lines as the selection progress.

TABLE 1. Least squares means and standard error for IgM values in three successive generations of selection

			Generations				
Line[1]	Sex	n[2]	First	n	Second	n	Third
HIM	Male	33	66.86 ± 9.94	55	81.51 ± 11.79	44	90.42 ± 14.42
	Female	48	85.65 ± 9.85**	40	103.02 ± 11.99**	52	97.97 ± 13.95
	Male and female	81	76.25 ± 11.06[A]	95	92.26 ± 15.92[A]	96	94.51 ± 14.59[A]
LIM	Male	35	52.95 ± 8.48	59	67.98 ± 11.19	55	55.15 ± 11.56
	Female	50	72.05 ± 11.56**	35	68.01 ± 13.87	54	54.35 ± 12.75
	Male and female	85	62.50 ± 11.42[B]	94	67.99 ± 12.18[B]	109	54.84 ± 11.98[B]

[A,B]Means of combined sex within the column with no common superscript differ significantly ($P < 0.01$).

[1]HIM = high IgM; LIM = low IgM.

[2]No. of chickens used in each generation of selection.

**Sex means within the lines differ significantly ($P < 0.01$).

Similarly, progeny with lowest IgM and IgG, respectively, from the same 10 sires and 20 dams, became the parents of LIM and LIG lines. Thereafter, individual phenotypic selection was carried out within each closed line for serum IgM and IgG levels at 10 wk of age. In each generation, chickens with highest serum IgM and IgG as well as chickens with lowest IgM and IgG were chosen as breeders for the next generation. Chicks from all the lines were hatched in a single hatch and reared together under the same environmental condition. Simultaneously, at hatching, all the chicks were wing-banded and vaccinated for Marek's disease. Also, fowl pox and Newcastle disease vaccines were administered per schedule. Birds of all the lines were provided *ad libitum* access to water and a commercial diet. After three generations of selection, birds of all lines were tested for their ability to produce antibody against SRBC antigen.

Quantification of IgM and IgG

The IgM and IgG were quantified by a single radial immunodiffusion (SRID) technique (Mancini *et al*., 1965). At 10 wk of age, blood samples were collected from the ulnar vein and separated by centrifugation at $1,700 \times g$ for 10 min. Four percent special noble agar[2] was dissolved in PBS (8 g sodium chloride, 0.2 g potassium chloride, 1.15 g disodium phosphate, 0.2 g monopotassium phosphate in 1 L of distilled water, pH 7.5) containing 0.02% sodium azide (NaN_3) at 62 C to prepare the agar solution. Goat anti-chicken IgM (μ chain specific[3]) or goat anti-chicken IgG (heavy and light chain specific[3]) was incorporated in the amount of 0.01 mL in 1.90 mL of PBS at 62 C. Finally, 3.5 mL of well mixed agar antiserum solution was used to make the gel for one agar diffusion plate. The serum sample, 0.003 mL, was applied in duplicate consecutively into the wells (1 mm of diameter) of the agar plate. The same amount of chicken immunoglobulin reference serum[3] was also applied to the same plates. The plates were incubated at 37 C for 24 and 8 h for IgM and IgG, respectively. After completion of the incubation period, the diameter of precipitation circle was measured by a slide caliper and the average concentration (milligrams per deciliter) of IgM and IgG was determined by comparison of the average diameter of the ring of precipitation in the test sample with diameters obtained using varying concentrations of purified IgM and IgG.

Administration of Antigen

At 14 and 17 wk of age, 30 chickens from the respective lines were injected intravenously with 1 mL of SRBC antigen. Sheep red blood cell, a T cell dependent antigen, was collected in Alsever's solution (2.05% dextrose, 0.42% sodium chloride, 0.8% sodium citrate). Cells were washed three times and were diluted to 5% in PBS. Blood samples were collected from the ulnar vein at 7 and 14 d post primary immunization (PPI) and post secondary immunization (PSI), respectively.

Agglutination Assay

Total and mercaptoethanol-resistant (MER) antibody titers were measured by agglutination test as described by van der Zijpp and Leenstra, (1980). The agglutination titer was expressed as the $\log_2$ of the reciprocal of the highest dilution where agglutination occurred.

Selection Differentials, Selection Response, and Realized Heritability

Selection differentials (*S*D) was measured by subtracting the means of tested population from the selected population of the high and low lines. Selection response (*S*R) was the difference of the tested populations of two consecutive generations. Realized heritability was calculated dividing the selection response by selection differentials. Therefore, realized heritability $h^2 = SR/SD$.

Statistical Analysis

Data were analyzed using the General Linear Models (GLM) procedure of SAS® software (SAS Institute, 1985). Treatment means were separated by Duncan's multiple range test.

TABLE 2. Least squares means and standard error for IgG values in three successive generations of selection

			Generations				
Line[1]	Sex	n[2]	First	n	Second	n	Third
HIG	Male	27	262.42 ± 34.59	53	335.18 ± 32.21	46	368.16 ± 36.81
	Female	34	279.42 ± 34.18**	59	359.65 ± 28.87	43	375.36 ± 43.04
	Male and female	61	271.67 ± 35.07[A]	112	348.07 ± 32.74[A]	89	371.64 ± 39.88[A]
LIG	Male	36	231.50 ± 28.79	48	297.28 ± 32.42	59	285.95 ± 36.81
	Female	36	245.24 ± 24.72	63	283.22 ± 31.60	41	283.85 ± 34.76
	Male and female	72	238.37 ± 27.52[B]	111	289.30 ± 32.57[B]	100	285.09 ± 34.10[B]

[A,B]Means of combined sex within the column with no common superscript differ significantly ($P < 0.01$).

[1]HIG = High IgG; LIG = low IgG.

[2]No. of chickens used in each generation of selection.

**Sex means within the lines differ significantly ($P < 0.01$).

RESULTS

Serum IgM and IgG levels at 10 wk of age changed considerably over the course of selection. Figures 1 and 2 demonstrate the changes in serum immunoglobulin M and G levels for HIM and LIM lines and HIG and LIG lines, respectively, after three generations of selection. The divergence of the selected lines became gradually larger with each successive generation of selection. Tables 1 and 2 shows the means of IgM and IgG values in sexes and lines over generations. A significant difference ($P < 0.01$) was observed between the sexes of HIM line in the first and second generations of selection. In the LIM line, the sex difference was observed only in the first generation. Even after a single generation of selection, a significant ($P < 0.01$) line difference was observed and followed in the subsequent generations. By contrast, in the IgG selected lines, the sex difference was significant ($P < 0.01$) only in the HIG line at the first generation of selection. The HIG line had significantly ($P < 0.01$) higher IgG levels over the course of selection than the LIG line.

Selection differentials, SR, and h^2 in the respective generations are presented in Tables 3 and 4. In the present study, no control population was used; therefore, the SR was measured by the difference between the high and low lines. Although the SD varied substantially over the course of selection, the SR was almost constant. Realized heritabilities were relatively high, with a mean value of 0.61 and 0.52, respectively, for M and G selected lines.

TABLE 3. Selection differential, selection response, and realized heritability on the basis of difference between the HIM and LIM lines[1]

Generation	Selection differential	Selection response	Realized heritability
1	23.47	13.75	0.58
2	17.70	10.52	0.59
3	15.55	10.40	0.66

[1]HIM = high IgM; LIM = low IgM.

Least squares means for total and MER antibody titers to SRBC in the HIM and LIM lines and HIG and LIG lines are presented in Tables 5 and 6, respectively. In the IgM selection, the females of the HIM line had significantly ($P < 0.01$) higher total antibody titers to SRBC than the males at 14 d of PPI and PSI than the males. Similarly, the females of the LIM showed significantly higher total and MER antibody titers to SRBC than the males at 14 d PSI, and at 7 and 14 d of PSI, respectively. The HIM line had significantly ($P < 0.05$) higher total antibody titers to SRBC than the LIM line except at 14 d PSI. The MER antibody titers to SRBC were also observed to be significantly higher in the HIM line than the LIM line on both the PPI and PSI. In the IgG selection, the females of the HIG line had significantly ($P < 0.01$) higher total and MER antibody titers than the males at 7 d PPI, and 14 d PPI, and at 7 and 14 d PSI, respectively. In the LIG line, total and MER antibody titers were significantly higher in the female than in the male at 7 d PPI, 7 and 14 d PSI, and 7 d PSI, respectively.

Total antibody titers to SRBC were significantly ($P < 0.01$) higher in the LIG line than in the HIG line at both 7 and 14 d of PPI and PSI; whereas, MER antibody titers were not significant between the two lines.

DISCUSSION

In this study, divergent selection for serum immunoglobulin M and G levels resulted in significant line differences during the course of selection. Successful selection for serum IgG levels as well as selection of chickens for antibody titers to SRBC has been demonstrated (Tamaki, 1981; Martin *et al.*, 1990; Dunnington *et al.*, 1996). The average h^2 for M and G selected lines were 0.61 and 0.52, respectively. Although comparable data were not available in the literature regarding selection of chickens for a serum IgM level, relatively high h^2 as found in this study indicates the usefulness of this kind of selection. Eide *et al.* (1991) reported h^2 of 0.19 for antibody response to diphtheria toxoid in goats.

TABLE 4. Selection differential, selection response, and realized heritability on the basis of difference between the HIG and LIG lines[1]

Generation	Selection differential	Selection response	Realized heritability
1	74.50	29.75	0.40
2	49.84	29.74	0.60
3	48.70	27.06	0.56

[1]HIG = high IgG; LIG = low IgG.

In chickens, h^2 for antibody response to *Escherichia coli* were 0.23 and 0.32 for high and low response lines, respectively (Leitner *et al*., 1992). Realized heritabilities for antibody response to SRBC varied from 0.17 to 0.52 (Siegel and Gross, 1980; van der Zijpp *et al*., 1983a; Martin *et al*., 1990). However, the upper value is in concordance with our study. The estimate of h^2, especially for IgG selected lines, varied over the course of selection. This divergence could have been due to the absence of a control population in this study.

In the present experiment, the female chickens generally had higher serum IgM and IgG levels as well as antibody titers than the males. This result was in agreement with the result of Leitner *et al*. (1992). Similar results were also reported in humans and mice (Ahmed *et al*., 1985; Astorquiza *et al*., 1987; Nelson and Steinberg, 1987; Grossman, 1989). From this result, it is suggested that hormones of the females may have modulated the immune response.

van der Zijpp (1983b) studied the total and MER antibody titers to SRBC of three lines of chickens with different genetic origins, and found that there were significant differences among them. In the other bidirectional experiments carried out on antibody response to SRBC and *Salmonella pullorum* (SP), the difference between the high and low responder lines was significant after three to five generations of selection (Siegel and Gross ,1980; Pevzner *et al*., 1981b). Genetic control of antibody response to SRBC, SP, and GAT has also been reported (Haddad *et al*., 1994). In our study, significant line differences were found for total and MER antibody titers to SRBC, which further demonstrates that genetic differences are major factors that influence the antibody production to the SRBC antigen. Okada and Yamamoto (1987) reported that a high IgG line had higher antibody response to SRBC. However, in the present experiment, the LIG line produced significantly higher antibody titers to SRBC than the HIG line (Table 6). This discrepancy may be due to the differences in the genetic background of the lines. Another possibility is that the LIG line may have more T lymphoproliferative cells than the HIG line, which react efficiently with SRBC, a T cell dependent antigen. The LIG line was also associated with higher degree of splenomegaly indices and lower level of antibody production to *Brucella abortus*, a T cell independent antigen, than the HIG line (Sarker *et al*., 1997, 1998). On the other hand, the HIM line had higher total and MER antibody titers than the LIM line at all points of PPI and PSI. The HIM line may have more B cells for multiplication and differentiation as well as T cells which react specifically to SRBC for antibody production. The HIM line was also associated with higher antibody production to *B. abortus* (Sarker *et al*., 1998), which also led to the possibility of better antigen processing and presentation of macrophages in this line as demonstrated by Biozzi *et al*. (1979, 1984) in mice.

From the results, it is postulated that selection of chickens for serum immunoglobulin isotypes may change other immunocompetent cells as well as antibody producing cells. However, further investigation of the T cell subsets and T cell receptor along with B cell number would be helpful to better understand the immune mechanism of this line. An effect of selection on IgM and IgG levels as well as correlated response to SRBC as found in this study may be helpful in understanding the immune mechanism and disease resistance in chickens.

TABLE 5. Least squares means and standard error for total and mercaptoethanol resistant (MER) antibody titers (IgG) to SRBC at 7 and 14 d post primary (PPI) and post secondary (PSI) immunizations in the chicken lines selected for IgM levels

		Day 7 PPI		Day 14 PPI		Day 7 PSI		Day 14 PSI	
Line[1]	Sex	Total	MER	Total	MER	Total	MER	Total	MER
HIM	Male	9.43 ± 0.16	5.40 ± 0.22	6.87 ± 0.27	4.73 ± 0.23	8.92 ± 0.23	6.33 ± 0.20	7.73 ± 0.23	5.53 ± 0.21
	Female	9.53 ± 0.16	5.93 ± 0.22	7.93 ± 0.28**	5.31 ± 0.22	9.14 ± 0.23	6.46 ± 0.21	8.30 ± 0.25**	5.76 ± 0.23
	Male and female	9.48 ± 0.12^{A}	5.66 ± 0.15^{a}	7.40 ± 0.19^{A}	5.02 ± 0.16^{A}	9.03 ± 0.16^{A}	6.39 ± 0.15^{A}	8.02 ± 0.17^{A}	5.65 ± 0.15^{A}
LIM	Male	8.40 ± 0.16	5.33 ± 0.22	6.26 ± 0.28	3.86 ± 0.23	8.20 ± 0.22	5.35 ± 0.21	7.42 ± 0.24	4.28 ± 0.22
	Female	8.20 ± 0.16	4.80 ± 0.22	6.86 ± 0.28	3.93 ± 0.23	8.61 ± 0.24	6.23 ± 0.21*	8.21 ± 0.24**	5.23 ± 0.23**
	Male and female	8.30 ± 0.12^{B}	5.06 ± 0.16^{b}	6.56 ± 0.19^{B}	3.90 ± 0.16^{B}	8.40 ± 0.16^{B}	5.79 ± 0.15^{B}	7.82 ± 0.17^{A}	4.75 ± 0.16^{B}

^{a,b}Means of combined sex within the column with no common superscript differ significantly ($P < 0.05$).

^{A,B}Means of combined sex within the column with no common superscript differ significantly ($P < 0.01$).

[1]HIM = high IgM; LIM = low IgM.

*Sex means within lines differ significantly ($P < 0.05$).

**Sex means within lines differ significantly ($P < 0.01$).

TABLE 6. Least squares means and standard error for total and mercaptoethanol resistant (MER) antibody titers (IgG) to SRBC at 7 and 14 d post primary (PPI) and post secondary (PSI) immunizations in the chicken lines selected for IgG levels

		Day 7 PPI		Day 14 PPI		Day 7 PSI		Day 14 PSI	
Line[1]	Sex	Total	MER	Total	MER	Total	MER	Total	MER
HIG	Male	8.25 ± 0.20	3.93 ± 0.21	5.86 ± 0.27	4.66 ± 0.21	7.00 ± 0.25	5.84 ± 0.18	7.15 ± 0.30	4.53 ± 0.24
	Female	9.40 ± 0.19**	4.64 ± 0.22	6.50 ± 0.28	5.50 ± 0.22*	7.69 ± 0.25	6.23 ± 0.18**	7.15 ± 0.30	5.53 ± 0.24**
	Male and female	8.84 ± 0.13^{A}	4.28 ± 0.15^{A}	6.18 ± 0.20^{A}	5.08 ± 0.15^{A}	7.34 ± 0.17^{A}	6.03 ± 0.13^{A}	7.15 ± 0.21^{A}	5.03 ± 0.17^{A}
LIG	Male	8.78 ± 0.20	4.28 ± 0.22	6.92 ± 0.28	4.92 ± 0.22	8.00 ± 0.25	5.22 ± 0.22	7.07 ± 0.30	4.22 ± 0.29
	Female	9.92 ± 0.20**	4.21 ± 0.22	7.28 ± 0.28	4.64 ± 0.22	9.23 ± 0.25*	5.92 ± 0.18*	8.53 ± 0.30**	4.78 ± 0.23
	Male and female	9.35 ± 0.13^{B}	4.25 ± 0.16^{A}	7.10 ± 0.20^{B}	4.78 ± 0.15^{A}	8.72 ± 0.17^{B}	5.57 ± 0.14^{A}	7.80 ± 0.21^{B}	4.50 ± 0.18^{A}

^{A,B}Means of combined sex within the column with no common superscript differ significantly ($P < 0.01$).

[1]HIG = high IgG; LIG = low IgG.

*Sex means within the lines differ significantly ($P < 0.05$).

**Sex means within the lines differ significantly ($P < 0.01$).

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SAFETY DATA SHEET

ZONASTAT-H (PORCINE ZONA PELLUCIDA IMMUNOCONTRACEPTIVE VACCINE)

SECTION 1 – IDENTIFICATION

PRODUCT IDENTIFIER: Zonastat-H

OTHER MEANS OF IDENTIFICATION: Porcine Zona Pellucida Vaccine, Porcine Zona Pellucida Immunocontraceptive Vaccine, Native PZP

MANUFACTURER: Science and Conservation Center

RESPONSIBLE PARTY:

MAILING ADDRESS: 2100 South Shiloh Road, Billings, MT 59106

PHONE: 406-652-9718

HOURS: 8:00 AM TO 4:00 PM, MST Mon-Fri.

PRODUCT USE: Used to inhibit fertility in elephants, all members of Family Ursidae, all ungulates, including the Perissodactyla, Artiodactyla, excluding the Family Suidae, and pinnipeds.

RESTRICTIONS ON USE: None

SECTION 2 – HAZARD INFORMATION

PRIORITY IDENTIFIERS: ANIMAL REMEDY
KEEP OUT OF REACH OF CHILDREN
FOR ANIMAL TREATMENT ONLY

EMERGENCY OVERVIEW: Non-toxic; possible abscess after im injection; temporary reproductive inhibitor if injected intramuscular.

Adverse Effects: Adverse effects in target animals may include injection-site abscess, or minor swelling. Material cannot pass through the food chain, thus ingestion is not dangerous.

SECTION 3 – COMPOSITION INFORMATION ON INGREDIENTS

CHEMICAL NAME: Native Porcine Zonae Pellucidae

COMMON NAME AND SYNONYMS: Porcine Zona Pellucida Vaccine, Porcine Zona Pellucida Vaccine, Porcine Zona Pellucida Immunocontraceptive Vaccine, Zonastat-H, Native PZP

Table 1. Chemical Components of Porcine Zonae Pellucidae*

Component	ng/zona
Protein	35.5 ±1.8
Hexose	2.5±0.1
Sialic Acid	0.02±0.00
Uronic acide	0.34±0.03
Amino Sugars	3.01±0.13

Active Ingredients:

The 4 ZP glycoprotein family protein separation via two dimensional polyacrylamide gel electrophoresis (2D- PAGE) and 2D-PAGE immunoelectrophoresis revealed approximately 7% contamination with non-ZP materials. Heat solubilization of ZP yields a product that does not contain organ cross-reactive antigens.

The major PZP component is ZP3, which is a 55 kD glycoprotein comprising 71% of the purified PZP. This glycoprotein serves as the primary immunogenic antigen in the PZP vaccine. The species-specific antigen that generates fertilization-blocking antibodies is an 11 kD fragment of the 55 kD ZP3 protein.

Nominal Concentration in Weight Percent: 500 μg PZP/1.03 g vaccine = 0.052%. 100-550 μg PZP (equivalent of 5-20 × 103 zonae) per immunization (intramuscular).

Purpose of Ingredient: The ingredient serves as an antigen to generate anti-PZP antibodies, which bind to ovulated eggs and block sperm attachment to the egg, preventing fertilization.

Impurities of Toxicological Significance Associated with Active Ingredient No detectable toxicological impurities are present in the immunization-ready PZP preparation.

SECTION 4 – FIRST AID MEASURES

Inhalation: No known effects of inhalation

Eye: May cause irritation. Flush with water

Skin: This material cannot be absorbed through the skin, but flushing with water is recommended

Ingestion: Because of its protein nature this vaccine cannot be absorbed in the gastrointestinal tract.

Poisoning Symptoms: No information regarding the symptoms of poisoning is available for this product.

Workplace Facilities: Other than a reliable freezer, no special facilities are required for storage. In the field some form of storage with cold or freezer capabilities must be available.

Hygiene Practices: Avoid ingestion, inhalation and contact with skin and eyes. Do not eat, drink or smoke while using this product. Wash hands and exposed skin before eating, drinking or smoking and after work.

SECTION 5 – FIREFIGHTING MEASURES

Vaccine is not flammable.

SECTION 6 - ACCIDENTAL RELEASE MEASURES

Spill Response: Spilled or released vaccine, on the ground or in water, will quickly degrade upon exposure to the elements, because of its protein nature. Because vaccine is packaged in 0.5 cc volumes, no more than 0.5 cc of the vaccine can be released at any one time.

Procedure for Disposal: Dilute with water and flush to sewer. Empty containers can be decontaminated with water. Syringes and needles and used darts must be disposed of in accordance with state regulations for medical waste.

SECTION 7 – HANDLING AND STORAGE

Storage: Vaccine is stored frozen and kept frozen or cold in the field. It may be refrozen after thawing as long as it has been kept cold. Expiration is two years from date of acquiring the vaccine.

Handling: No special technical protective measures required. No special handling advice required beyond that given in ***required*** training.

Handling Practices: Avoid ingestion, inhalation and contact with skin and eyes. Do not eat, drink or smoke while using this product. Wash hands and exposed skin before eating or smoking and after work.

Approved Handlers: Acquisition of the vaccine and application to equine requires a three-day training program, conducted at the Science and Conservation Center.

SECTION 8 – EXPOSURE CONTROL/PERSONAL PROTECTION

Application in the workplace: Vaccine may be loaded into darts in the workplace or most commonly in the field.

Exposure Standards Outside the Workplace: There are no established exposure limits.

Personal Protection: The following procedures are meant for people coming into frequent and lengthy contact with this product. For occasional use, take precautions suitable for conditions under which the product is used.

Respiratory: It is safe to avoid using dust masks or respirator protection because of the nature of the product and handling procedures.

Eyes: Protective eyewear is not necessary when using this product. However, it is always prudent to use protective eyewear because of other conditions encountered in the field.

Skin: Because the product cannot be absorbed through the skin, no special precautions need be taken.

Hands: Rubber, latex or PVC gloves are not necessary, but are desirable if prolonged use of the product is occurring.

SECTION 9 – PHYSICAL AND CHEMICAL PROPERTIES

Appearance: Aqueous solution

Odor: None

Specific Gravity:

Melting Point: 0° C

Boiling Point:

pH: 7.0 -7.1

Flashpoint: Not Applicable

Solubility in Water: Soluble

Oxidizing Properties: Not applicable: this product is not an oxidizer

Corrosive Properties: Not applicable; this product is not corrosive

Vapor Pressure:

SECTION 10 – STABILITY & REACTIVITY

Reactivity: None

Chemical Stability of Substance: The product is stable for up to two years if stored frozen and kept cold in the field and for up to 48 hours in darts, if kept refrigerated

Conditions to Avoid: Store frozen, keep at 2-8o C in the field.

Incompatible Materials to Avoid: None known

Hazardous Decomposition Products: No products of decomposition, either in or out of fire exist.

Hazardous Polymerization: The product cannot polymerize

SECTION 11 – TOXICOLOGICAL INFORMATION

TOXICOLOGY – ACUTE

Exposure: Principal routes of exposure are by skin contact and accidental needle stick. As with any chemical product, contact with skin or ingestion in any form should be avoided by observing good work/medical practices. This material is not regarded as pathogenic in humans.

TOXICOLOGY – CHRONIC/LONG TERM EFFECTS

Fate of Product after Injection: Following injection, both components of the

vaccine follow the common immunological and metabolic path of vaccines detected by the humoral immune system. Briefly, both the PZP and the bacterial peptidoglycans are hydrolyzed in cells of the immune system. The breakdown products of this hydrolysis bear no resemblance to PZP or bacterial peptidoglycans, and are indistinguishable from other products of lysosomal hydrolyis. After hydrolysis, these metabolic products are excreted and eliminated from the body in forms (such as CO2, water, lactic acid, and urea) that are indistinguishable from other metabolic products. Likewise, the antibodies produced in response to ZonaStat-H injection are broken down into their component amino acids, and recycled into other body proteins or metabolized and excreted as urea, CO2, and water. PZP and the adjuvant antigens are not stored in body tissues in a stable or physiologically active form, thereby eliminating the possibility of continued exposure of the target animal to the vaccine components, or of non-target animals and humans of exposure to vaccine components from excreted products. 30% of the hexadecane component of the adjuvant mineral oil remained at the injection site after 10 months. The mineral oil is readily incorporated into lipids and appears as triglycerides, sterol esters, and free sterols in the liver, at 3 months in liver phospholipids, and were absent from the liver by 10 months. Metabolic products also appeared as triglycerides in depot fat, a small amount of which still remained after 10 months and are lost slowly over time through metabolism and elimination via respiratory CO2, and there was no bioaccumulation of mineral oil. Mannide monooleate is dissipated more quickly from the injection site than mineral oil, with the oleate component largely being incorporated into lipids, with subsequent elimination through respiratory CO2, and the mannide largely eliminated from the body in urine in the form of non-esterified sugars – 25% in the first 24 hr after administration.

SECTION 12 – ECOTOXICITY INFORMATION

Ecotoxicity Effects: Delivery of this product to the target animal is highly specific, by either hand injection or dart delivery, thus toxicity to birds, fish, algae, aquatic invertebrates, soil dwelling organisms or bees does not occur.

Environmental Fate: Once thawed the vaccine degrades rapidly by bacterial degradation and pH changes.

SECTION 13 – DISPOSAL CONSIDERATIONS

Product Disposal: Preferably dispose of the product by use. Otherwise dispose of the product at an approved landfill, or other approved facility in accordance with local, regional and national regulations.

Container Disposal: dispose of vaccine vials, needles, syringes and used darts in accordance with state, regional or federal regulations for medical waste disposal. All needles, syringes and used darts should be kept in a sharps container until proper disposal.

SECTION 14 – TRANSPORT INFORMATION

Not classified as dangerous by rail, sea or air transport regulations. Keep frozen during transportation.

SECTION 15 – REGULATORY INFORMATION

Registered as a Pesticide by the Environmental Protection Agency (EPA Registration No. 86833-1). Also authorized for Equids under FDA INAD # 8857-G0002 and for Captive Animals and Deer under FDA INAD 8841-G0003-004.

SECTION 16 – OTHER CONSIDERATIONS

NOTE: This product is a veterinary medicine and must therefore be used in accordance with the container/label/insert directions and as indicated in required training. A comprehensive package of toxicological and environmental data for the active ingredient of the product has been provided and is on file with the Environmental Protection Agency and has been inspected by expert toxicologists and environmental scientists before approval in February 2012.

This material safety data sheet summarizes our best knowledge of the health and safety hazard information of the product and how safely to handle and use the product in the workplace and field. Each user should read the MSDS and consider information in the context if how the product will be handled and applied, including in conjunction with other products.

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A polyanhydride-based implantable single dose vaccine platform for long-term immunity

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Traditionally, immune priming followed by an antigen-boost is utilized for vaccine-induced protective immunity and maintaining long-term immunity often requires repeated exposure to antigen. Here, a novel single dose vaccination platform designed with gonadotropin releasing hormone multiple antigenic peptide as a model antigen is shown. Initially a soluble component will prime the immune system followed by vaccine release from a biodegradable polyanhydride (PA) copolymer rod of 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(*p*-carboxyphenoxy) hexane (CPH) for immune boosting (Appendix A) [1]. A second PA-based vaccine depot is enclosed within an implant of biocompatible high molecular weight polyethylene, which limits PA degradation and antigen release to a single surface, significantly extending the release kinetics (Supplemental Table). Collagen is used to generate an immunodiffusion barrier [2] over the PA wherein antigen-antibody complex formation can occur for an immune feedback design (Fig. 1). Therefore, antigen release is impeded, theoretically, by high antibody titers. The cap of the implant is a hydrophilic poly (vinylidene fluoride) membrane with 0.65-μm pores that allows flow of proteins into and out of the implant, while excluding leukocytes. Thus, this device enables vaccine priming, boost and long term maintenance with a single administration of antigen.

Acknowledgments

Patent # 14/814,148 - *Vaccine Delivery Devices* is pending. This work was supported by an Iowa State University Bailey Award, the Nanovaccine Institute, and a grant from Michelson Prize & Grants, a program of the Michelson Found Animals Foundation, which is supported by the generous contributions of Dr. Gary Michelson and Alya Michelson. B.N. acknowledges the Vlasta Klima Balloun Faculty Chair.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2017.11.067.

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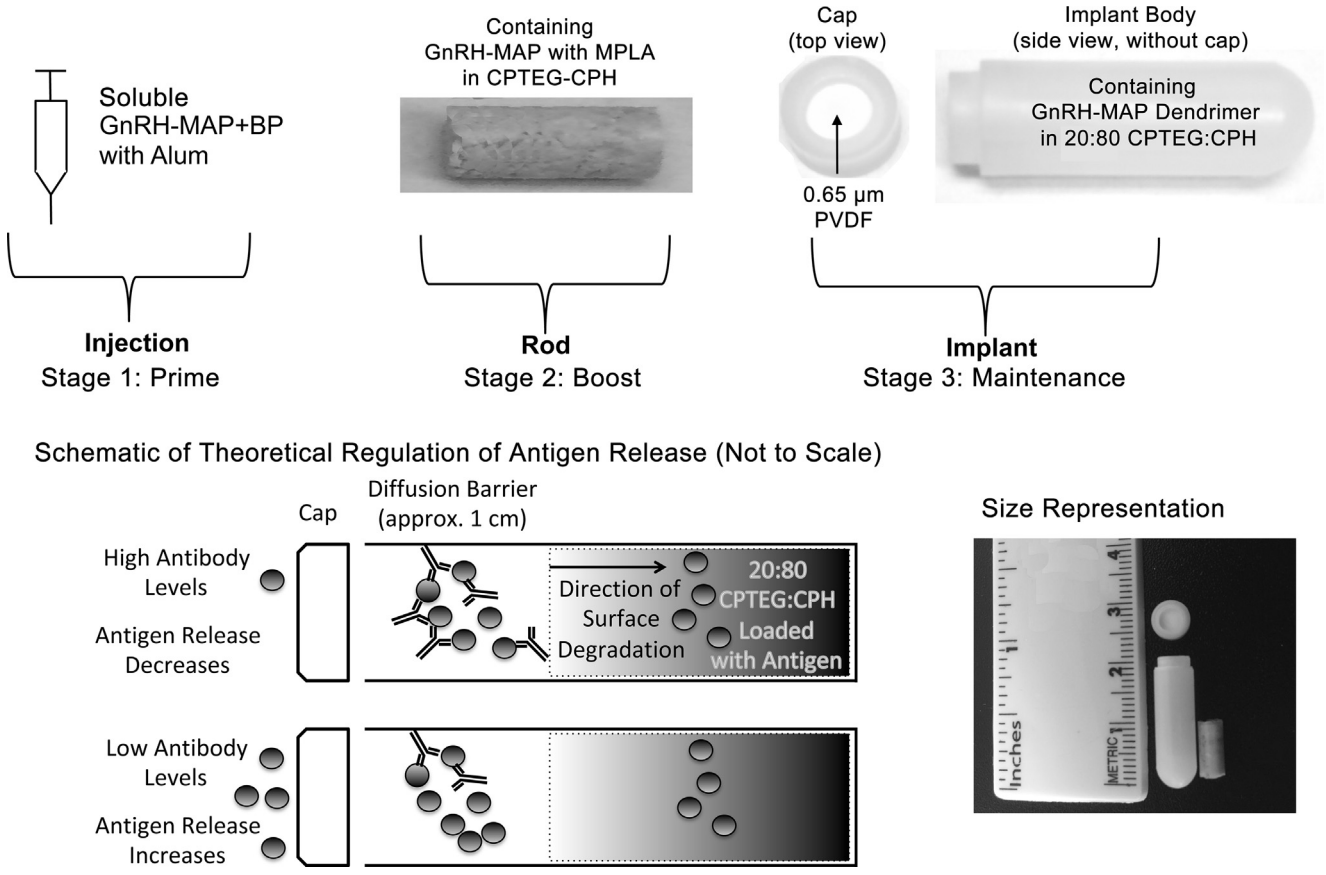


Fig. 1. Schematic of 3 stage single injection vaccine delivery device (top) with soluble priming dose, polyanhydride (PA) depot boosting dose, and implant for maintenance of immunity. The lower left schematic illustrates the theoretical passive immunoregulation of vaccine release from the implant. The lower right is an image of the implant with the PA depot that will be placed within.

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Short Communication

Reversibility of the effects of GnRH-vaccination used to suppress reproductive function in mares

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Summary

Reasons for performing study: Active immunisation against gonadotrophin-releasing hormone (GnRH) provides a reversible method for control of oestrous behaviour and fertility in mares. Previous reports failed to demonstrate the interval to resumption of cyclic ovarian activity after GnRH-vaccination.
Hypothesis: Administration of the GnRH-vaccine Improvac in a large group of mares of various ages will result in effective, reliably reversible suppression of ovarian activity within a 2 year period.
Methods: The mares, subdivided into 3 age categories, were vaccinated twice (with a 35 day interval) using 400 μg Improvac and monitored via blood samples until Day 720 after initial vaccination for serum progesterone concentration determination by radioimmune assay and anti-GnRH antibody titre by enzyme immunoassay. Samples were collected until individuals resumed cyclic ovarian activity.
Results: All mares showed suppression of cyclic ovarian activity by clinical examination and serum progesterone concentration (SPC) ≤1 nmol/l by Day 70 and 92.2% resumed cyclic activity by SPC at Day 720 with a mean interval = 417.8 days (s.d. ± 23.9; range 232–488 days, median 344 days). A significant age effect (P = 0.028) on the interval, but not on GnRH-antibody titre response, was observed between the youngest (≤4 years) and oldest (≥11 years) categories.
Conclusions: Immunising adult mares of all ages with Improvac resulted in a reversible suppression of cyclic ovarian activity in most mares. An age effect, with the youngest mares showing a longer interval to reversibility, was observed.

Keywords: horse; mare; GnRH-vaccination; anoestrus; reversibility; titres

Introduction

Suppressing reproductive function and associated oestrous behaviour in mares has been managed by several approaches. Pharmacological methods utilising progesterone or progestins and potentially reversible GnRH-agonist or -antagonist therapy and their associated shortcomings including costs, practicality and potential negative side effects are reported [1–3]. Surgical ovariectomy is generally considered to be undesirable, due to its associated risks and irreversible loss of breeding potential [4].

Various immunocontraceptive methods have been used to control fertility in mares. Although porcine zona pellucida (pZP) vaccine is effective, it neither prevents mares from cycling nor suppresses oestrus-related behaviours [5]. Gonadotrophin-releasing hormone (GnRH) vaccines utilise a modified form of GnRH hormone conjugated to a foreign protein administered to stimulate the production of anti-GnRH antibodies. These antibodies neutralise endogenous GnRH, preventing natural binding to receptors on the pituitary gonadotrophs, suppressing pituitary secretion of follicle stimulating hormone (FSH) and luteinising hormone (LH) [6–8]. Studies report successful but variable suppression of ovarian activity [6–13]. Age effects on response and of using serum progesterone concentration (SPC) to reliably monitor cyclic ovarian activity have been reported [8,12]. Short monitoring periods generally limit these reports. Reliable reversibility and the associated duration of anoestrus induced by vaccination have not been reported.

This study investigated the reversibility of anoestrus over a period of 2 years in a large group of mares of different ages after 2 treatments with the GnRH-vaccine Improvac[a].

Materials and methods

Experimental design

A previous controlled study reported on the efficacy, safety and interval to suppression of reproductive activity after vaccination of nonpregnant mares with Improvac[a] [12]. The current study was designed as a continuation to observe the reversibility of treatment. Mares (n = 51) aged between 3 and 17 years and either Thoroughbred or similar light-horse type, were subdivided into 3 age categories: Category 1 (≤4 years, n = 25), Category 2 (5–10 years, n = 16) and Category 3 (≥11 years, n = 10). The trial was approved by The University of Pretoria's Animal Use and Care Committee (V068/05).

GnRH immunisation

The mares were injected into the gluteal muscles with 2 ml Improvac[a] (400 μg GnRF-protein conjugate) approximately 3 months after the onset of the physiological breeding season in South Africa. All showed cyclic reproductive activity via transrectal palpation and ultrasound examination at the time of vaccination. One booster (2 ml) was administered 35 days after primary vaccination. By 70 days after primary vaccination, 100% of mares showed SPC reflecting anoestrus concentrations and ovarian activity was absent on clinical examination [12]. Resumption of ovarian activity was henceforth defined by a blood sample exceeding a baseline SPC prospectively set at ≤6 nmol/l, the reported upper threshold concentration for winter anoestrus [11].

Blood sampling

Blood samples were collected by jugular venipuncture from Day 232 after primary vaccination at 2-weekly intervals until resuming cyclic activity or failing that, until the end of the study on Day 720. The blood samples were collected in plain Vacutainer tubes[b] and left to clot. Serum was separated by centrifugation (4000 × ***g***, 10 min) on the same day before storage at -20°C until assayed for both SPC and anti-GnRH antibody titre.

Serum progesterone assay

Assays for SPC were conducted by means of radioimmune assay (RIA)[c]. Assay sensitivity was 0.06 nmol/l. The main cross-reactivity was with progesterone (100%), 5α-pregnane-3,20-dione (9.0%), 17α-hydroxyprogesterone (3.4%) and 5β-pregnane-3,20-dione (3.2%). The intra- and inter-assay coefficients of variation were 8.8, 3.6 and 2.7 and 9.7%, 3.9 and 3.9% for low, medium and high concentrations, respectively.

GnRH-antibody titre determination

The anti-GnRH titres were determined by enzyme immunoassay (EIA), using a modification of the method described by Elhay *et al*. [11]. Briefly, 96 wells MaxiSorp MTPs[d] were coated overnight (ON) at 4°C with 10 μg/ml GnRH peptide in sodium carbonate buffer at pH 9.6. The plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and then blocked ON at 4°C with 0.3% BSA in PBS. Plates were incubated with serial dilutions (dilution factors 200, 400, 800, 1600, 3200, 6400, 12,800) of test mare serum and standards for 1 h at 37°C. The plates were washed and bound antibody was detected by incubating plates with protein G-horseradish peroxidise (HRP)[e] for 1 h at 37°C. Subsequently, plates were washed and bound protein G-HRP was visualised with tetramethylbenzidine microwell peroxidase substrate[f]. Pooled samples of high titre (high standard) at Day 70 and low titre (low standard) at Day 35 after primary vaccination and samples of unvaccinated horses (negative control) served as reference points for the unknown samples on all plates analysed.

The plates were normalised by dividing all optical densities (ODs) by the maximal OD, thereby setting the high standard in 1:200 dilution to 1.0 for each plate. Subsequently, a negative titre threshold was defined as OD of the average of the negative controls plus 2 s.d. In this regard, titres were determined as the first OD exceeding the negative titre (negative control) threshold (starting from dilution 1:12,800 and decreasing). For method validation, titres of standards and positive controls were recorded for all 26 plates. The high and low standard titres were 3200 for all 26 plates and 800 for 18 plates and 400 for the remaining 8 plates, respectively. The negative control titres were 0 for all plates.

Data analysis

Data analysis was performed using Sigma Stat[g]. A Kruskal–Wallis one way analysis of variance (ANOVA) was performed on the ranked data to assess significance of age on the variables for general linear models. A Dunn's Multiple Comparison Test for means separation was also performed with Category 1 as control. Statistical significance was defined as $P<0.05$.

Results

Interval to resumption of cyclic activity

By Day 720, 47/51 (92.2%) mares had resumed cyclic activity with a mean interval of 417.8 days (s.d. ± 23.9; range 232–488 days, median 344 days). The remaining 4 mares (all Category 1) were still in anoestrus.

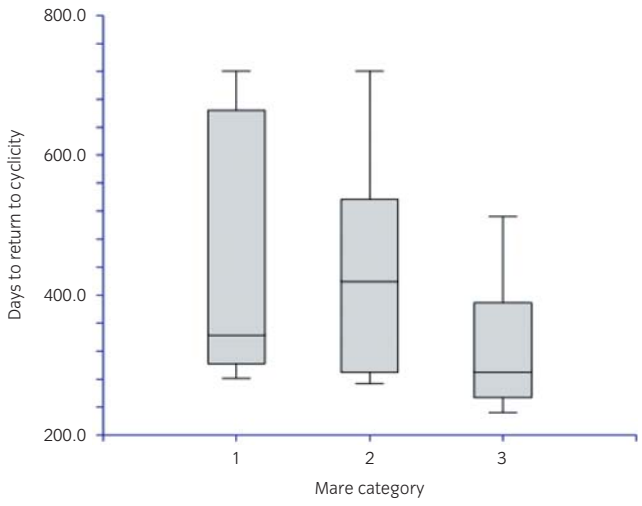


Fig 1: Box plot of days to return to cyclicity for different age categories of mares. Category 1 (≤4 years, n = 25), Category 2 (5–10 years, n = 16) and Category 3 (≥11 years, n = 10).

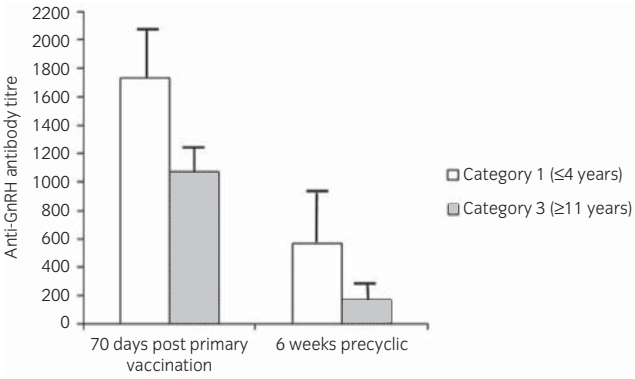


Fig 2: Mean (s.e.) gonadotrophin-releasing hormone (GnRH) antibody titres for selected mares in Categories 1 and 3 at 70 days post primary vaccination and 6 weeks prior to resumption of cyclic activity.

Effect of age on resumption of cyclic activity and GnRH-antibody titres

The median interval in Category 1 was significantly greater than in Category 3 mares ($P = 0.028$) (Fig 1). The mean anti-GnRH-antibody titres of 6 mares randomly selected from each of Categories 1 and 3 at 70 days post primary vaccination and at 6 weeks prior to resuming cyclic activity showed no significant difference (Fig 2) although mean titres were higher at both intervals for Category 1 mares.

Discussion

In previous GnRH-vaccine studies, animal numbers were either small or monitoring discontinued before most had reversed. Dalin *et al*. [8] treated 3 mares with GnRH-bovine serum albumin-conjugate vaccine, 2 responded with reversal at 13.5 and 15 months after primary vaccination, respectively. Imboden *et al*. [10], treating 9 mares with Improvac[a], reported down-regulation of ovarian activity in all mares, but no reversibility in 4 mares within a 2-year period. Elhay *et al*. [11] reported using Equity[h] in 24 mares. No treated mare exceeded anoestrus SPC threshold levels at 20 weeks and ovarian shutdown lasted 4–23 weeks in 10/16 mares. The other 6 mares remained inactive at the end of the 29–34 weeks observation period. Killian *et al*. [13] comparing contraceptive methods, treated 15/43 mares with a single-shot GnRH-vaccine (GonaCon). The rates of contraception in these mares decreased from 94–40% between years 1–4, but time to resumption of cycling was undetermined.

The resumption of cyclic ovarian activity was used to monitor the reversibility of anoestrus induced in all mares following Improvac[a] administration. By Day 720, all but 4 mares (all Category 1) had resumed ovarian activity with a median interval of 344 days. The effect of vaccination on subsequent reproductive performance in these mares was not observed. A significant effect of mare age on reversibility was found between the oldest and youngest categories. Stout and Colenbrander [2] similarly reported 2 treatments of GnRH-tandem-dimer vaccine suppressed testosterone secretion in young sexually-mature stallions whereas older stallions generally required further boosters. In this study, although anti-GnRH-antibody titres were not significantly different between youngest and oldest mares, the mean titres were higher for the youngest and may explain their later return to cyclic activity.

In conclusion, variable reversal of anoestrus was shown in 92.2% of mares within 2 years after vaccination with Improvac[a]. Older mares showed significantly earlier resumption of cyclic ovarian activity than the youngest mares.

Conflicts of interest

No conflicts of interest have been declared.

Source of funding

The Equine Research Centre of the University of Pretoria funded the study.

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[b]BD Vacutainer Systems, Plymouth, UK
[c]Coat-a-Count, Diagnostic Products Corp, Los Angeles, California, USA
[d]Nunc, Roskilde, Denmark
[e]Sigma-Aldrich, Steinheim, Germany
[f]KLP, Gaithersburg, USA
[g]Systat Software Inc., San Jose, California, USA
[h]Pfizer Animal Health, West Ryde, New South Wales, Australia

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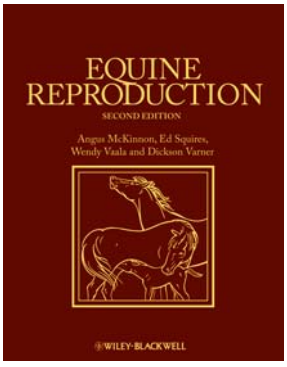
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WHITE-TAILED DEER ACTIVITY, CONTRACEPTION, AND ESTROUS CYCLING

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Gary Killian
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CONCURRENT SESSION #5 (UNGULATES/MONKEYS)

WHITE-TAILED DEER ACTIVITY, CONTRACEPTION, AND ESTROUS CYCLING

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Abstract: Reliable activity measures in free-ranging white-tailed deer could be useful for the assessment of estrous cycling and general activity changes that can occur after immunocontraceptive vaccine treatments. Electronic data-logging (Countcard™) devices attached to 9 white-tailed doe deer on neck collars were used to monitor their movement activities during 2 rutting seasons in 2.5 acre fenced enclosures. Direct daily behavioral observations on buck responses toward individual does were used to detect estrus and to validate changes in 24-hour activity counts as another indication of estrous cycling. When individual activity counts for the estrus days on the does were compared to diestrus counts as estimated by mean values over days 10 through 15 post-estrus, a significant high product moment correlation of 0.946 was obtained indicating similar and proportional changes in activity under the 2 behaviorally-detected conditions. As predicted, mean activity counts for the estrus days were significantly higher ($P<0.0039$) than the mean counts for the diestrus days. This indicated some potential utility for the devices as sensors for estrus detection. Even though the individual deer showed different activity baseline levels and a high degree of daily variation, estrus days were frequently characterized by minor peaks in the daily activity counts followed within a day or a day and a half by a sharp activity decline, an indication that the does were at this point receptive to mounting by bucks.

Pages 124-131 *in* C. D. Lee and S.E. Hygnstrom, eds. Thirteenth Great Plains Wildl. Damage Control Workshop Proc., Published by Kansas State University Agricultural Experiment Station and Cooperative Extension Service.

Key Words: deer activity, contraception, data-logger, estrous cycle, *Odecoileus virginianus*

Control of localized white-tailed deer (*Odecoileus virginianus*) populations using immunocontraceptive treatments is a potentially attractive wildlife management technique in terms of social acceptance, humaneness, and feasibility (Kirkpatrick and Turner 1991; Turner, Liu and Kirkpatrick 1992; Turner, Kirkpatrick and Liu 1996; Mc Shea, Wemmer and Stuwe 1993). In some areas of the Northeastern and Midwestern U. S., hunting and other management methods have failed to stem deer overpopulation levels. As a result, damage to fruit trees, vegetable gardens and ornamental plantings; automobile and ground aircraft traffic hazards; and the spreading of Lyme disease from ticks have been identified as deer-related problems. In newly sub-divided rural areas adjacent to urbanized cities, individual land owners are often opposed to hunting as a deer management method, and prefer to apply repellents or to alter habitats. Because these

methods may achieve only temporary or partial reduction of the deer-related problems, immunocontraception is viewed as an attractive possibility for a more permanent solution.

Although Porcine Zona Pellucida (PZP) treatment has been demonstrated to be a highly effective immunocontraceptive for preventing pregnancies in white-tailed deer, treated does also show extended estrous cycling beyond the normal rut season (Turner et al. 1992; Shumake and Wilhelm 1995) and 3 or 4 extra cycles are not uncommon. Gonadotropic releasing hormone (GnRH), on the other hand, will generally reduce reproductive behaviors in both sexes with extended treatment leading to regression and atrophy of the gonads (Kirkpatrick and Turner 1991). The overall effects of the absence of estrus in does on buck behavior have not been fully documented.

Bucks are normally attracted to powerful chemosignals generated in does at the time of estrus (Murphy et al. 1994) and their behavior toward individual does can be used to detect receptive animals. It may be also possible to detect the time of estrus in individual does by either a slight change in doe body temperature or movement activity as is the case with dairy goats and cows (Doherty et al. 1987; Redden et al. 1993). The objectives of the 1995-1996 study were: (1) to evaluate the effects of PZP treatment on buck pursuit/mounting/copulation, and (2) to determine whether neck-collar-mounted Countcards™ could be used to detect behavioral estrus in PZP-treated does. Objectives of the 1996-1997 study were: (1) to compare the effects of PZP treatment in 4 previously dosed does with 4 control does treated with vehicle only, and (2) to evaluate the neck-collar-mounted count card sensors on both PZP-treated and control doe deer. Detection of estrus in the white-tailed doe deer was accomplished by observing the behavior of bucks housed in enclosures with small doe herds at the Deer Research Facility during 2 rut seasons. Statistical correlations and comparisons between the movement activity counts for individual does during detected estrus and the counts for diestrus days were performed. This allowed an assessment of the Countcard™ data logger devices as a potential means of estrus detection.

STUDY AREA

Both studies were conducted at the Pennsylvania State University Deer Research Facility, University Park, PA. The facility consists of a 22 acre enclosure with high fencing for the captive management and propagation of white-tailed deer. The area is further divided into 9 paddocks or sub-enclosures, each separated by 8 ft (2.44 m) diamond mesh wire fencing, and topped with 3 strands of barb wire. A centrally-located runway building is used for handling, weighing, blood sampling, injecting, and examining the animals. The first study extended over 107 days from November 8, 1995 through February 22, 1996. The second study lasted 86 days from November 8, 1996 through February 3, 1997.

METHODS

Observation Procedures

To detect behavioral signs of estrus in each of the does for the 1995-96 rut, observations were made by trained staff twice per day from 0800 to 0900 EST and from 1600 to 1700 EST. In addition, reproductive behaviors were observed at other hours during the day by Deer Research Center staff. Individual does were identified by their ear tag colors and by numbers. For does with similar tag colors, a small patch of hair was shaved on unique areas of their bodies. The complete list of tag numbers, colors, and shave marks was made available to all observers. Animals were identifiable from a minimum of 20 yards from all locations within the 2.5 acre paddock.

For the 1996-97 rut, observation procedures were identical to those described for the previous year's rut. All animals were observed 3 times per day over 1-h intervals (0800 to 0900, 1130 to 1230, and 1600 to 1700 EST) throughout the 86 days of observation. Individual doe interactions with the bucks were again recorded on data sheets in the pen enclosure and periodically on videotape recordings for later comparisons of activity and behavioral signs of estrus in the does as extrapolated by buck interest.

Data Logger Devices

Countcard™ devices are credit card sized data logger units that can be preprogrammed to automatically sense and record daily movements. The devices are normally used to monitor automobile and truck highway traffic and are imbedded into the road surface. Operational detection is achieved by disturbances in the earth's magnetic field forces as the vehicles pass over the device. The Countcard™ units will, however, also register movement counts when they are moved themselves. Data generated in this manner are based on discrete episodes or bouts of movement activity. In other words, rapid movement of the devices without interruption will not register high activity counts, but repetitive stop-and-start movements are registered. This use of the devices has some advantage in terms of filtering some forms of rapid activity (e.g. scratching), but also has a disadvantage of not registering extended movements (e.g. long distance running) unless interrupted by pauses. A method for mounting the devices inside of plastic project boxes (Radio Shack®, Tandy Corp., Fort Worth, Tex.) and then onto neoprene deer collars (Advanced Telemetry Systems, Isanti, Minn.) was developed for attachment to individual does.

Determining Behavioral Estrus

For the 1995-96 rut, does were equipped with model NC-30X Countcard™ devices (Nu-Metrics, Uniontown, Penn.) and were previously treated with PZP vaccine in 1992, 1993, and 1994. They were not re-boosted with vaccine injections prior to the Fall 1995-96 rut. This initial deer herd size was 17, including 9 PZP-treated and 8 GnRH-treated does. Because no does in the GnRH group were instrumented with Countcard™ devices, their observed behaviors are not reported here. Of the PZP-treated does, 5 animals were monitored with devices (#0033, #0040, #0049, #228, and #327).

For each observation period, student observers entered the paddock, located the buck(s), and verified individual ear tags using binoculars. For the remainder of the observation period, the behaviors of 1 to 3 bucks and the responses of individual does were observed from the maximal distance that allowed animal identification. For each encounter in which the buck(s) showed an interest in a doe, rut behaviors were classified and recorded as one of eight categories as further described in Table 1.

Table 1. Eight categories of rutting behavior in white-tailed deer used to detect estrus in does.

Behavioral Category (Level)	Brief Description
No Interest 0	No male displays any interest in activities of a given doe. The doe is assumed to be in diestrus.
Following/Standing/Lying 1 within 8 yards	The buck visually orients in the direction of the doe and may exhibit urine sniffing and the Flehmen response. The buck and doe are observed together, either walking or bedding down, for a period of at least 15 minutes.
Short Pursuit 2	The buck follows the doe wherever she moves in the paddock, for a period of less than 2 minutes. While exhibiting this activity, the couple is frequently observed standing within 8 yards of one another.
Extended Pursuit 3	This is the same description as listed above for short pursuit, but the activity is continued over at least a 5 minute interval.
Aggressive Guarding/Pursuit 4	Highly aggressive behavior is shown by the buck toward any other male that approaches a doe in estrus. If the doe attempts to escape from this buck, the alpha male, he continually pursues her.
Female Stands for Male 5	This activity involves the buck licking the urogenital area of a receptive, estrous doe, with a mounting attempt. This is often accompanied with the buck resting his chin on the back of the doe. At this time, the alpha male aggressively defends the doe from pursuits of other bucks.
Mounting and Copulation 6	The doe allows mounting and copulation. Often, the doe will step out from under the buck on his first few mounting attempts. Actual mating lasts only 8-15 seconds and is therefore rarely observed.
Post Copulatory Posture 7	These postures exhibited by does typically follow a successful copulation, where ejaculation has occurred. The posture of the doe consists of a urination stance (legs spread, tail raised, and a hunched back). The buck may also urinate at this time.

All data were recorded manually and were documented on videotape. The recorded data sets were later analyzed to extrapolate the time periods in which each doe came into estrus. Repeated estrous cycling was a strong indication that the doe was not pregnant even though matings may have been repeatedly observed.

For the 1996-97 rut, student observers were employed again to make the 3, 1-h observations each day of the test. Inclement weather conditions as well as disturbances due to humans, dogs, and construction activities were noted each day in an attempt to account for potential aberrations in the doe activity. The same 8-category scale of buck interest toward individual does (Table 1) was used for detection of behavioral estrus in each instrumented animal. Countcard™ collars were installed on 8 deer. Five of the animals (i.e., #0001, #0040, #0049, #139, and #228) had been previously treated with the PZP vaccine but had not received booster vaccinations since 1994, and 3 of the animals (i.e., #661, #793, and #8108) had never received the PZP vaccine treatment. Animals were included as a part of a herd of 18 does penned together in a single enclosure with 1 to 3 bucks.

Data Analyses

Descriptive analysis involved tabulation of the number of estrous cycles observed in each doe treatment group. For the 1995-96 rut, final fawn birth data were tabulated by July 19, 1996, for comparisons of contraceptive efficacy among treatments. To evaluate the degree of proportional changes in movement activity counts for behaviorally-detected estrus versus diestrus days, correlation analyses were performed. Differences in the daily movement activity count means for the estrus versus diestrus conditions were compared with Student paired t-tests and Wilcoxon signed rank tests to assess the potential of using doe movement counts to serve as a supplemental method of estrus detection.

RESULTS AND DISCUSSION

1995-96

Data for the 1995-1996 rut were obtained from only 3 of the 5 Countcard™ devices on the collared does (#0033, #0049 and #228) when down-loaded manually or via computer through the serial port. Reasons for failure to obtain movement activity count data on does #0040 and #327 remain obscure. Reasons were probably not related to battery failure, but were most likely due to programming errors since the obtained outputs were in the form of counts per hour rather than counts for the previous 24-h period. Neither of these 2 does showed signs of estrus as detected by observation of buck behavior after November 23, 1995. Table 2 is a summary of the number of estrus cycles as detected by buck behavior directed toward the 3 does that yielded movement activity count data over the 107-day observation interval. Countcard™ movement activity data for selected observation days when estrus was detected by buck behavior are included along with the mean movement count values for days 10 through 15 post-estrus (diestrus). Daily changes in the movement counts, particularly for does #049 and #0033, indicated that a sharp increase or peak in activity did not necessarily correspond with an estrus day as detected by observers. With deer #228, there was an indication of a brief increase in movement activity counts followed by a sharp decrease. This would be a predicted pattern since the bucks normally show extended pursuit toward does in early estrus followed by a reduced movement of the does when they stand for the buck as he attempts to mount and copulate. In other words, when deer #228 was most receptive and allowed mounting, she also became relatively less active. The remaining 4 non-instrumented does showed up to 2 extra cycles with the last sign of estrus observed on February 11, 1996.

Of these 9 PZP-treated deer, 3 became pregnant and all had produced fawns by June 3, 1996. Deer #0033 produced a male and a female fawn on May 21, 1996 even though only the short pursuit category (level 2) of buck interest was observed and mounting attempts were never observed. Deer #327, also instrumented with a Countcard™, produced 3 female fawns but, unfortunately, no movement activity count data were obtained for this animal. The remaining doe, #16B, produced a single female fawn.

1996-97

During the 1996-1997 rut, count data were obtained for 3 control does (#0062, #661, #8108). There was again an indication of an increased movement activity count on the day that estrus signs were recorded by student observers as indicated in Table 2. Based on the 5-day means for the putative diestrus interval, there was an approximate 20 to 25 percent decrease in movement activity 10 days post-estrus.

Movement activity counts for does #661 and # 8108 reflected a high degree of variance within and between the untreated does. For example, whereas #661 showed extreme lapses of activity down to <1000 counts/day (i.e., days 2, 6, 7, 8, 11, 12, 14, 20

etc.), #8108 was more stable and never produced <4750 counts/day throughout the observation interval. At the time of this writing, the pregnancy rate for these animals as indicated by fawn births was unknown for this season, but no estrus signs for the 3 control does were recorded after the date of November 24, 1996 (i.e., Day 16).

PZP-treated animals that had not received booster immunocontraceptive vaccine for the past 3 years still continued to show repeated estrous cycling. There were 4 cycles detected for #0040, and all animals (i.e., #0001, #0040, #0049, #139, #228) showed estrus signs (as indicated by buck behavior) through the month of January, 1997 (i.e., Day 67). As with the previous year's rut, buck pursuit generally produced an increase in daily movement activity counts of does when estrus signs were first detected. The advanced categories of buck interest (e.g., mounting, aggressive guarding, copulation) were again sometimes correlated with generally below average daily activity counts. This pattern in doe activity at estrus was fairly consistent and repeatable within and between animals. For several animals (#0001, #0040, #139, and #228) pursuit responses by bucks produced higher daily counts followed within a day by lower counts when the does allowed buck mounting and copulation. The daily activity counts for deer #228 (Figure 1) exemplify the pattern of peaks followed by sharp declines that correspond with signs of estrus as recorded by the student observers.

Comparison of Movement Activity Counts and Estrus Detection

Assuming a nominal value of 25 days between estrus cycles for white-tailed deer, a 12-day post-estrus interval is a logical period of elapsed days for taking a 1-day sample of activity during diestrus. The prediction can be made that there should be consistently less movement activity (i.e., fewer proportional counts) on day 12 post-estrus when compared to the counts on the estrus-detected day. However, this was not confirmed by our data sets when a Pierson product moment correlation coefficient was calculated for the 9 collared animals. The r value was 0.3787 (not significant). However, both a Student paired t-test (T = 3.02, df = 8, P<0.0165) and a Wilcoxon signed rank test (P<0.004) on the data sets detected reliable differences between the 2-sample counts for each animal. Whereas the mean for diestrus days was 4718.0 Å 3304.3 and the mean for estrus days was 8224.5 Å 2665.7, the individual animal variation lowered the correlation value. With a low n of only 9 does, 1 or 2 animals with extremely low counts during diestrus can degrade the correlation coefficient value.

On the other hand, when a 5-day mean of the daily movement counts for days 10 through 15 post-estrus was used (instead of the 1-day count for 12 days post-estrus), a significant correlation was achieved ® = 0.946; T = 7.722, df = 7, P<0.00011). A paired t-test on the data set for estrus counts compared to the 5-day means for diestrus counts was significant (T = 6.187, df = 8, P<0.00026) as was a Wilcoxon signed rank test (P<0.0039). This 5-day-mean-count procedure tended to dampen the extremes for 2 does that showed disproportionately low counts during some days during the diestrus stage and thus improved the consistency of proportional activity changes. Both procedures were also influenced by 1 doe that had extremely low counts during both estrus and diestrus. Figure 2 shows a scatter plot of the correlated count measures using the mean-count procedure. Influences of other more subtle effects such as rain, snow, temperature, and human disturbance were also, at times, evident in the data set with some animals more sensitive than others to these disturbances. Despite these uncontrolled extraneous factors, a moderately consistent pattern of movement activity changes was detected in the data sets.

The consistent proportional decrease in movement activity counts for estrus days versus corresponding 5-day mean diestrus days ® = 0.946) implied that 89% of the count variation was accounted for by estrous cycling. If baselines for individual doe activity levels could be also factored into a detection equation algorithm, the activity data could become a useful additional means of detecting estrus in deer. A moderately high degree of predictive power for detection of estrus has been previously reported using body temperature and pedometer measurements in dairy cows (Redden et al. 1993). With captive white-tailed deer in small herd situations, a peak in activity followed by a sudden and sharp drop within 1 or 2 days of the peak may be a likely candidate for comparison with a 5-day mean estimate value for a potential diestrus interval. Variability between does and within the same animal was, however, a limiting factor in the predictive power and more experimental data will be needed to fully assess the potential of the devices. Studies will be continued in the Deer Research Facility pens and may be extended to field testing if results appear encouraging. Whether the Countcard™ devices could be useful for estrus detection in free-ranging field situations is unknown. However, the deer-movement effects of other management tools such as buffer-fencing, hunting to thin herds, or habitat modification probably could be monitored using these kinds of data logging devices. Devices are in fact available that would allow for deer movement activity monitoring with radio telemetry and remote down-loading of the data. These systems are, however, currently quite expensive (i.e., $2000 per doe vs. $250 per doe for Countcards™). This would either limit the number of does that could be monitored when working within a reasonable research budget or could easily become cost prohibitive when aircraft radio-tracking and other expenses are included.

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Figure 1. Daily activity counts for Deer #228 during the 1996-97 rut. Instances of buck interest are indicated for days 17, 41, and 67.

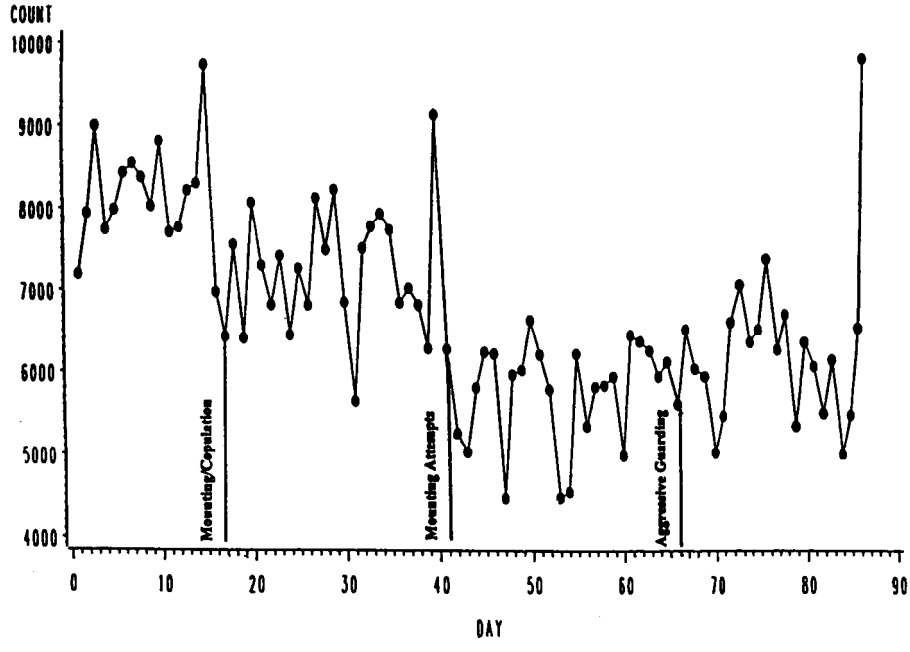
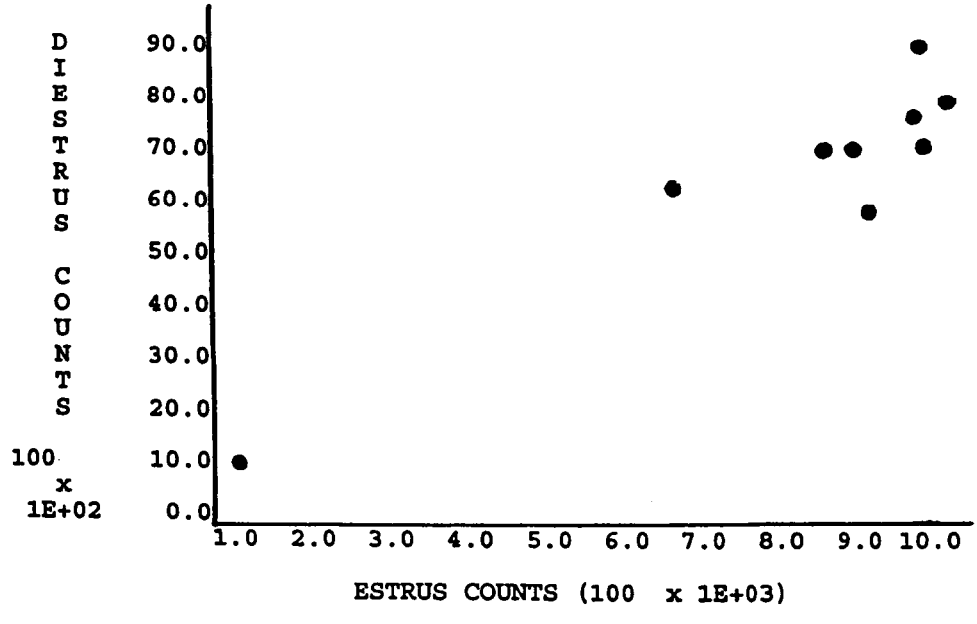


Figure 2. Scatter plot of movement activity counts on the day that estrus was detected through observation of buck behavior versus a mean count value for days 10 through 15 post-estrus for each of 9 white-tailed doe deer. The product moment correlation for these data (r = 0.946. P<0.000l) indicated proportionally decreased mean movement counts for diestrus compared to estrus counts. The decrease was also significant using a Wilcoxon signed rank test (P<.00039).



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Article Navigation

Immunization with Zona Pellucida Proteins Results in Abnormal Ovarian Follicular Differentiation and Inhibition of Gonadotropin-Induced Steroid Secretion

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Abstract

Changes in rabbit ovarian hormonal responses and cellular differentiation of ovarian follicles after immunization with porcine zona pellucida (ZP) have been examined. Steroid and peptide hormone levels were monitored after immunization to evaluate ovulation and pseudopregnancy cycles in immunized and control animals. All immunized rabbits developed serum antibodies to specific ZP antigens and failed to form functional corpora lutea in response to hCG administration, as evidenced by the absence of elevated serum progesterone concentrations. This is in contrast to control rabbits, which had elevated progesterone levels 8–9 days after hCG administration. Furthermore, all immunized animals showed greatly increased serum levels of FSH and LH compared to those of control animals. These effects on ovarian function were apparent within 20 weeks of the primary immunization. Follicular development was analyzed by light and electron microscopies.

The numbers of primary, secondary, and tertiary follicles in ovaries of immunized animals were markedly reduced within 7 weeks compared with control values. By 23 weeks, few if any growing follicles were present. Although numerous distinct clusters of cells with ultrastructural properties that resemble those of normal follicular cells were present in immunized animals, they contained no oocytes. These studies suggest that antibodies to ZP glycoprotein alter ovarian function by interfering with cells during the stage of follicle differentiation at which the ZP proteins are being synthesized and secreted. This system should provide an excellent model with which to study the early events associated with ovarian follicular cell differentiation and subsequent hormonal responsiveness.
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Issue Section: Articles

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MANAGING AFRICAN ELEPHANT POPULATIONS: ACT OR LET DIE?

Preface

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20. **Ian Whyte:** The feasibility of current options for the management of wild elephant populations

Report

Conclusions

Colophon

Preface

During the last century, the number of African elephant (Loxodonta africana) declined dramatically as a result of over-hunting, poaching for ivory and, more recently, the loss of habitat area due to encroachment of the human population. In some areas, however, the trend to declining numbers was reversed after the elephant was placed on Appendix 1 of the Convention for International Trade in Endangered Species (CITES) and a worldwide ban was imposed on the sale of ivory. Unfortunately, the resulting recovery in elephant numbers within both National and privately owned game parks poses a threat to the survival of many other species. Indeed, when elephant numbers exceed the carrying capacity of a game park, the gradual destruction of vegetation and habitat is catastrophic and, if left unchecked, is only arrested when the elephants themselves begin to die of starvation. Over the last 30 years, it became generally accepted that the best way to prevent this boom-and-bust scenario and, in addition keep epidemic diseases under control, was to maintain elephant numbers below the carrying capacity by culling. During the 1990s, however, popular opinion turned against culling as a means of controlling elephant populations and the focus shifted instead to translocating animals from areas of high to areas of low population density. Self-evidently, while relocation may be a useful way to control elephant numbers in relatively small parks, it is unrealistic to expect to relocate thousands of animals per year to keep the population under control in the large parks, for example, in northern Botswana.

Of course, the best method for controlling population growth depends critically on the size and rate of growth of that population and, in this respect, it has been proposed that contraception may offer an alternative to relocation or culling in relatively small parks with a limited number of elephants. However, while the first experiments to examine the efficacy of immune contraception in female elephants have offered promising results, and preliminary experiments to modulate the behaviour or fertility of bulls have also begun, there are as yet no agents proven to be reliable, effective in field conditions and without significant side effects. The aim of this workshop is to discuss the current policies for controlling elephant numbers and to assess the suitability of newer strategies being developed to address this problem. Given the increasing interest in pharmacological means of controlling population size, it is hoped that experiences with various compounds in other species may help to indicate the approaches most likely to be applicable to the African elephant. It is also hoped that this workshop will stimulate collaborative research projects to identify the best approach to population control in a given situation, and to develop the means to implement that plan safely and effectively. This information may come to provide a valuable source of support when the management decisions required to ensure the health and viability of a game park and its elephant population are complicated by politics.

It goes without saying that a workshop of this nature would have been extremely difficult to organise without financial support from The Prince Bernhard Nature Fund. I would also like to offer my sincere thanks to Mr. Paul Fentener van Vlissingen for his foresight in proposing this initiative and his interest in nature conservation in Africa.

Ben Colenbrander

Chairman Organising Committee



Induced abortion to control population increases in African elephants

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Morphological, histological and histochemical examinations, and hormone precursor incubations, of biopsies of placenta, gonad and fetal liver recovered post mortem from 59 pregnant elephants carrying conceptuses estimated, on the basis of fetal weight, to be between 20 days and 21 months of age, showed:

1. A zonary placenta encircles the equatorial region of the ovoid unicornuate conceptus. It is endotheliomonocytochorial in structure and consists of intensely folded or pleated lamellae. They contain maternal capillaries that are closely invested by a mononuclear trophoblast layer which is itself closely invested by fetal capillaries. The placental zone is attached to the endometrium only by a remarkably narrow fibrous pedicle through which all the maternal blood vessels pass to vascularise the maternal side of the fetomaternal interface. A haemophagous region forms at the lateral edges of the placental zone in which phenotypically specialised trophoblast cells phagocytose leaked maternal red blood cells.

2. Between 2 and 5 large, plum-like corpora lutea persist in one (usually) or both (occasionally) ovaries throughout pregnancy. These are present from the earliest stages of gestation and are appreciably bigger (3-5 cm diameter) than the biggest (1.5 cm diameter) follicle observed at any stage of the oestrous cycle or pregnancy. However, no form of chorionic gonadotrophin (CG) is detectable in elephant placenta.

3. Incubation of placenta and fetal gonad, alone and in combination, with radioactively labelled steroid hormone precursors showed that whereas the enlarged fetal gonads are capable of synthesising progesterone and other progestagens from cholesterol or pregnenelone, the placenta itself is unable to synthesise any progestagens and it does not possess any aromatising capacity to synthesise oestrogens.

This endocrinological inertia of the elephant placenta suggests the likelihood that the maintenance of pregnancy in this species is entirely dependent upon luteal sources

of progestagens. Hence, it would be reasonable to expect that, as in some large domestic animal species, uncomplicated abortion induced by luteolysis would follow the administration of a prostaglandin F analogue at any stage of pregnancy. If so, a single intramuscular injection of PGF analogue delivered by dart gun could perhaps constitute an efficacious method for controlling unwanted increases in maintained elephant populations. However, intramuscular administration of cloprostenol (Estrumate; Schering-Plough, Middlesex, U.K.) on each of 3 consecutive days to a young Indian elephant at 6 months of gestation caused a rapid but temporary decline in urinary pregnanediol concentrations and the pregnancy continued unscathed. Hence, further research is necessary before induced abortion could be considered as a means of preventing unwanted births in elephants.

Contraception of African elephant cows in two pivate conservancies using porcine zona pellucida vaccine, and the control of aggressive behaviour in elephant bulls with a GnRH vaccine

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Contraception of elephant cows with pZP vaccine

Previously, Fayrer-Hosken et al. (1999) demonstrated that the pig and African elephant share zona pellucida epitopes, and suggested that it should be possible to use porcine zona pellucida (pZP) proteins as the basis of a contraceptive vaccine for elephants. Two subsequent field trials performed in the Kruger National Park proved that pZP vaccine is an efficacious contraceptive treatment for female elephants (Fayrer-Hosken et al. 2001); further studies are continuing in two smaller populations of elephants in private game reserves.

Makalali Game Reserve

When the first pZP vaccination programme commenced in May 2000, the total elephant population in Makalali Game Reserve stood at 66, including 16 adult and seven 9-12 year-old cows. The vaccine was manufactured at The Science and Conservation Centre of ZooMontana, USA. The primary vaccination (600 µg) was administered in Freund's modified, and boosters (400 µg) in Freund's incomplete, adjuvant. Initially, 18 cows were immunised using a primary course of three injections administered at 3-week intervals via dropout darts shot from the ground. The darts were retrieved to confirm discharge of the vaccine, and behavioural patterns returned to pre-darting patterns within two days. During 2001, all of the immunized cows were 'boosted' with a single injection, while a further two cows were treated with a 3 injection primary course and added to the trial group. The following year, 3 new cows were given a single primary vaccination, and the rest were boosted. In 2003, all of the immunised cows were boosted; 17 were darted from a helicopter and the rest from the ground.

The effects of the contraception programme to date are shown in Table 1. In summary, no calves have been born to matings dating from after the commencement of vaccination. Bearing in mind that the average inter-calving period in the Lowveld is ± 4 years, contraception has therefore definitely been achieved in 5 cows (their calves are now 5-6 years old). In addition, four cows were immobilised and examined

for pregnancy using ultrasound in July 2003; none were pregnant, and blood progesterone concentrations suggested very little or no luteal activity. The next two years will provide a lot more information with respect to the efficacy of contraception. The safety of pZP vaccination during pregnancy was confirmed by the fact that 14 cows immunised during months 2-21 of gestation subsequently gave birth to normal healthy calves at term. One of the criticisms levelled at the use of pZP-immunocontraception, is that it may lead to an increased number of matings and presence of bulls. This has not been the case in Makalali. During 2002, only five cows were observed to be in heat, and two were mated. Their calves were 14 and 18 months and 3½ years (x 3) old at the time.

Table 1: Summary of the Makalali cows vaccinated with pZP during 2000-2003

Cow		Date and number of primary vaccinations	Age of calf at 1st vaccination	Pregnancy status at 1st vaccination	Birth of calf after vaccination	Age of calf on Sept 2003	PD July 2003 [progesterone] Heats in 2002
ID	Age						
Queeny	A	May 00 x 3	3 y	NP	None	6 y	-
Friendly	A	May 00 x 3	3 y	± at conception	04-02	17 m	-
Stripper	A	May 00 x 3	5 y	18 m	09-00	36 m	-
Waves	A	May 00 x 3	4 y	12 m	03-01	30 m	-
Plain Jane	A	May 00 x 3	20 m	NP	None	5 y	Heat 10-02
Smelly	A	May 00 x 3	5 y	15 m	12-00	33 m	Heat 02-02
Holey Ear	A	May 00 x 3	5 y	21 m	08-00	37 m	Heat 02-02
Toni	A	May 00 x 3	3 y	2 m	12-01	21 m	-
#2	A	May 00 x 3	No calf*	4 m	11-01	22 m	NP [0.41 nmol/l]
U-boat	A	May 00 x 3	4 y	13 m	02-01	31 m	-
Kwatile	A	May 00 x 3	2 y	NP	None	5 y	Mated 05/02
Anna	A	May 00 x 3	5 y	18m	09-00	36 m	NP [3.42 nmol/l]
Dracula	A	May 00 x 3	No calf*	17 m	10-00	35 m	-
Yvonne	A	May 00 x 3	17 m	NP	None	5 y	Mated 10-02
Markina	A	May 00 x 3	4 y	6 m	09-01	24 m	NP [1.24nmol/l]
Pokenhontas	A	May 00 x 3	3 y	NP	None	6 y	-
Tiny	9-12	May 00 x 3	None	NP	None	None	-
Knop-Kop	9-12	May 00 x 3	None	5 m	10-01	23 m	-
Cheeky	9-12	June 01 x 3	None	18 m	10-01	23 m	NP [0.62 nmol/l]
Madam M	9-12	June 01 x 3	None	8 m	08-02	25 m	-
Cindy	9-12	June 02 x 1	None	Just calved	None	27 m	-
Connie	9-12	June 02 x 1	None	6 m after calving	None	21 m	-
Enigma	9-12	June 02 x 1	3 y	20 m	08-02	13 m	-

A = Adult cows; *Calf probably lost after arrival at Makalali before project started

Mabula Game Reserve

Mabula Game Reserve has a herd of 9 elephants, including one sub-adult and 3 adult cows. The details of vaccination are provided in Table 2. As the project is only

18 months old, it is too early to assess the contraceptive effects of the vaccine.

Table 2: Elephant cows vaccinated at Mabula Game Reserve with pZP and ISA 50

Cow		Date and number of primary vaccinations	Age of calf at 1st vaccination	Pregnancy status at 1st vaccination	Birth of calf after vaccination	Age of calf on Sept 2003
ID	Age					
Nikita	A	May 02 x 3	5 m	NP*	None	22 m
Dabula	A	May 02 x 3	6 m	NP*		23 m
Bontle	A	Aug 02 x 1	2½ y	16 m	02-03	7 m
Bekile	9-12	May 02 x 1 Aug 02 x 1	None	?		-

*Likelihood - based on age of calf

Controlling aggressive behaviour in elephant bulls using a GnRH vaccine
Both captive and free-ranging elephant bulls can present serious management problems if they display aggressive behaviour, either in or out of musth. They can so endanger the lives of other animals and people, that trained elephants usually have to be removed from work programmes and restrained to an extent that may become a welfare issue. As there is currently no proven practical way of controlling musth, culling often constitutes the only solution to free-ranging problem bulls. However, both musth and aggressive behaviour appear to be related to high concentrations of testosterone, and a project is currently underway to test the effects of a GnRH vaccine on testosterone secretion and behaviour, with a view to developing the vaccine as a means to control musth and aggressive behaviour in elephant bulls.

The vaccine (GnRH-tandem-dimer conjugated to ovalbumin, Pepscan Systems, The Netherlands) will be administered with either the Montanide ISA 51 or Covaccine adjuvants (Oonk et al. 1998). Five elephant bulls will be injected with 2 mg GnRH plus adjuvant by darting, 3 times at 3-week intervals. In addition, two control bulls will be darted with adjuvant only. From a few weeks prior to the first vaccination until 4 months after the last injection, faecal samples will be collected weekly, and testosterone secretion monitored via an EIA for faecal epiandrosterone. Over the same period, sexual and aggressive behaviour will be monitored.

In fact, the first elephant bull has already vaccinated at Johannesburg Zoo. This bull has been exhibiting aggressive behaviour and temporal gland secretion for a considerable time. Four vaccinations using the ISA 51 adjuvant have been administered, and the faecal epiandrosterone profile following the first two vaccinations is shown in Figure 1. Although, during this period, no change in the faecal epiandrosterone profile was apparent, the elephant has displayed calmer episodes after each vaccination and has shown a reduction in temporal gland secretion. This effect was more marked after the 4th vaccination, while no side effects have been noted. Subsequently, a further one and three bulls have been vaccinated three times and once, respectively.

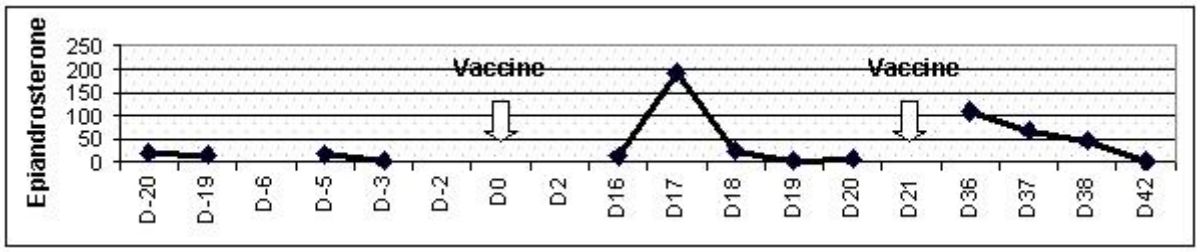


Fig 1: Faecal epiandrosterone concentrations (ng/g dry faeces) in a bull elephant before and after the first two vaccinations against GnRH

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Epididymal approaches to contraception

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The epididymis performs a variety of functions, notably transport, maturation, storage and protection of spermatozoa (Cooper 1999). It is the cause of some cases of infertility and provides an attractive target for male contraception. However, to know if epididymal contraception is relevant for elephants it must first be ascertained whether these animals do indeed have an epididymis. Short et al. (1967) stated that “The elephant does not have an anatomically distinct epididymis”, and some years later Jones et al. (1974) agreed that there was no regional demarcation of the paired genital duct of the male African elephant comparable to its differentiation in scrotal mammals. Subsequently, Jones (1980) showed that many aspects of sperm maturation do indeed occur in the elephant’s organ, thereby concurring with the earlier work of Glover (1973) that “signs of sperm maturation occur in the epididymis of testicond mammals” and that the cauda epididymidal sperm store is near the exterior.

Assuming that the elephant’s epididymal physiology is similar to that of scrotal mammals; namely, that the maturing spermatozoa are influenced by the nature of secretions in the luminal fluid bathing them, three basic approaches to the design of a contraceptive to act on this organ become apparent (Cooper 2002): (i) alteration of the time of contact of spermatozoa with secretions, via peritubular contractions and alteration of transit times; (ii) interference with the composition of luminal fluid by inhibiting synthesis, transport, function or availability of secretions and (iii) attack upon sperm-specific/sensitive pathways.

Studies in rats have examined the effects of local or systemic cholinergic, adrenergic and purinergic agonists and antagonists, and peptides that either increase or decrease sperm transport through the epididymis. Where examined, fertility was not always affected, but in no case was an entire population of treated rats infertile. Those animals that were, displayed only subfertility.

Theoretically, elimination of an epididymal secretion involved in the (epididymal) acquisition of the spermatozoon’s fertilising capacity should have a contraceptive effect. However, the number of secretions with a known function is limited, although such a role could be proposed if inhibitors were to cause infertility. Few such experiments have been conducted, but it has been shown that neither drastic reduction of the epididymal content of carnitine nor abolition of glucosidase activity in

the rat epididymis cause infertility. Surprisingly, despite the paracellular blood-epididymis barrier, immunisation against epididymal secretions (CRISP, P26h) have led to reduced fertility or complete infertility in rats, mice and hamsters, possibly by entry of immunoglobulins into the efferent ducts or by a transcellular vesicular route.

Initially, the most promising approach to functional sterility was employment of chlorinated antifertility compounds to inhibit sperm glycolysis, which depended on two facts; 1) that spermatozoa preparing for fertilisation are more dependent upon glycolysis than respiration for the production of energy, and ii) that glycolytic enzymes in spermatozoa differ in structure from those in somatic cells. For example, sperm glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has an additional domain involved in anchoring the enzyme to the axoneme, by which ATP would be produced directly at its site of utilisation. Chlorinated antifertility compounds such as α-chlorohydrin and ornidazole produce chlorinated structural analogues of the substrate of GAPDH which inhibit the enzyme. Although, when fed at antifertility doses, α-chlorohydrin and ornidazole had no toxic effects on rats over a period of 1 – 2 years (Jones and Cooper 1999), at the higher doses required for toxicological evaluation, central nervous effects were induced. To circumvent this problem, novel chlorinated compounds were synthesised that should generate, by the process of glycolysis itself, the same chlorinated inhibitor of GAPDH produced from α-chlorohydrin and ornidazole. Such “suicide” compounds were shown to be effective in vitro at inhibiting glycolysis and modulating motility of spermatozoa from rats, mice and men but they failed to induce infertility in rats as a result of dechlorination and urinary excretion. Compounds are being synthesised that should be rapidly accumulated within the epididymal lumen, thus avoiding excretory loss and dechlorination and limiting toxic side effects.

The difference in osmotic pressure between the male (high) and female (low) gamete tracts has long been known, but its significance has only recently been appreciated. Observations on certain infertile transgenic male mice have highlighted the importance of volume regulation in fertility, because spermatozoa that fail to regulate their volume cannot pass the uterotubal junction. The abnormal volume regulation exhibited by spermatozoa from infertile male mice reflects a failure in volume regulation, considered to result from insufficient intracellular osmolytes that are normally lost, together with intracellular water, to counter swelling that occurs as the spermatozoa enter the hypotonic fluids of the female. The epididymis has been hypothesised to provide osmolytes to spermatozoa during epididymal transit (Cooper and Yeung 2003), so that the reduced intrasperm osmolytes in the infertile males would reflect reduced provision of osmolytes by the epididymis which, in the infertile males, is abnormal due to the lack of the initial segment. Specifically, a glutamate transporter expressed in the initial segment is down-regulated in the knockout male and, as a consequence, less glutamate is present in epididymal spermatozoa. Other epithelial transporters are up- or down-regulated in this animal model and the

osmolyte and their channels are currently being sought in spermatozoa.

The infertility of some domestic animals that exhibit spermatozoa with a similar phenotype to that of the c-ros knockout male (e.g. Dag defect; Cooper 1992), suggests that poor sperm volume regulation could underlie unexplained infertility. Furthermore, as swollen human sperm display altered motility patterns and reduced entry into artificial mucus, it is likely that targeting either epididymal transporters so as to modify luminal fluid, or sperm channels to block osmolyte influx or efflux, could be a new, post-testicular, contraceptive approach for man as well as the elephant.

To date the studies on post-testicular contraceptives have involved daily oral administration so that rapid onset of action and reversal after withdrawal can be appreciated. While this regimen may be convenient for man, there is no reason why, like hormonal contraceptives, the post-testicular variety could not be formulated so as to be delivered continuously from a slow-release depot.

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Fertility control of brushtail possums in New Zealand

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Introduced brushtail possums (Trichosurus vulpecula) are major pests in New Zealand because of their impacts on conservation values, biodiversity and agriculture. Annually, about NZ$80 million is spent on spreading 2.5 tonnes of 1080 poison and increasing quantities of anticoagulants and other poisons. Currently, > 9 million hectares are under sustained possum control. Issues like ongoing costs, environmental contamination, non-target risks and changing public attitudes have driven research into alternative approaches, such as fertility control.

Fertility control research for possums is guided by a national strategy that is aimed at effecting long-lasting infertility and/or sterility in treated animals. Research on immunological blocks to fertility is focused principally on targeting proteins of the zona pellucida (ZP; Landcare Research), ovarian vesicle-associated proteins (AgResearch) and embryonic shell coat proteins (Melbourne University). An alternative approach is aimed at developing gonadotrophin-releasing hormone (GnRH)–toxin complexes that will target appropriate pituitary cells (AgResearch). Parallel research is underway on delivery, including mucosal immunology and uptake of materials from the possum gut. Genetically modified plants and bacterial ghosts are being assessed as potential oral vaccines. The potential for a combined vaccine based on BCG, that would immunise possums against infection with bovine tuberculosis and induce infertility simultaneously, is also being researched. Disseminating delivery systems are being investigated, with current focus on identifying and characterising possum viruses, and genetically modifying a possum-specific nematode parasite to express infertility-inducing proteins and/or epitopes.

Our research has concentrated on immunocontraception by targeting egg proteins of the zona pellucida (ZP). Trials of an injectable vaccine containing whole recombinant ZP3 and ZP2 proteins demonstrated 70-75% reduction in fertility of captive animals bred naturally or by artificial insemination. Immunisation with a ZP3 epitope that spanned the putative sperm-binding region resulted in a reduction of about 66% in the proportion of female possums that produced at least one embryo. Recent epitope mapping identified a ZP2 epitope which, on testing by injection, also reduced fertility significantly.

Two approaches are being taken to examine oral delivery of immunocontraceptives, by seeking expression of contraceptive antigens in transgenic plants, or bacterial ghosts. To date, immune responses, including antibodies detected in blood, reproductive tract secretions and in follicular fluid, have been demonstrated against a model antigen expressed in transgenic potato and Lucerne plants. Recombinant bacterial ghost vaccines expressing possum ZP3 stimulated humoral immune responses in serum, ovarian follicle fluid, and reproductive tract secretions in female possums when applied to mucosal surfaces of the eye and nose, or were administered orally. Cellular immune responses and effects on ovarian function and fertility following mucosal immunisation are now being assessed.

Finally, computer modelling based on field-derived parameter estimates of possum population dynamics has been used both to define minimum vaccine efficacy and to identify strategies for integration of fertility control baits and poison baits that are likely to produce benefits of reduced costs and reduced toxin use.

Immunomodulating GnRH to alter sexual and aggressive behaviour in the male: a bovine model

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The rationale for several decades of research into immunocontraception and immunocastration of farm, domestic and feral animals has been discussed extensively (see text box: Bonneau and Enright, 1995; Enright, 1995; Enright et al. 1995; Meloen, 1995; Becker et al. 1999; Delsink et al. 2002). If developed properly, active immunisation regimens can be cheap, practical and effective ways of generating effective antibody titres against a target molecule; however, the immune response can vary considerably between animals and a lag period is inevitable before the biological response is observed.

Possible uses of immunomodulation in animals

- Suppression of oestrous behaviour in heifers, racing fillies, cats, dogs
- Reduction of aggressive behaviour in bulls, stags, racing or show-jumping stallions, wild elephants, tomcats, dogs
- Prevention of boar taint
- Reduction of fertility in feral horses, wild elephants, pest species (rodents, rabbits in Australia), disease carrying species (possums in New Zealand, badgers), cats, dogs
- Increased litter size in farm species
- Improved efficiency and leanness in farm animals

There are many factors other than inter-animal variation that determine the success of an active immunisation approach, including species, age, structure of the hormone/peptide, type of carrier molecule and adjuvant used, conjugation procedure, dose of antigen and adjuvant, molar ratio of hormone to carrier, and frequency and relative timing of immunisation (see Crowe et al. 1995).

(Dis)Advantages of bull beef production

Aggressive behaviour and libido can be a serious problem during the husbandry of male cattle. Post-pubertal bulls are difficult to handle, cannot be grazed near heifers, cause pasture damage and are a threat to human safety. These problems create management difficulties in all commercial beef systems, including grass-based extensive and intensive housing/feedlot systems. In addition, because of their high activity level, bulls have a greater tendency to yield bruised and dark-cutting meat at slaughter. To overcome these problems, in many countries male cattle are castrated using either the burdizzo or conventional surgical methods. However, while castration renders male cattle docile, it also decreases growth and feed conversion efficiency by 15%, and increases the fat content of the meat. The challenge is to therefore to

reduce aggressive behaviour while maintaining the performance advantages of bulls. Immunisation against GnRH has proven a promising approach.

Immunisation of pre-pubertal bulls

In an initial study, Finnerty et al. (1994, 1996) immunised 47 three-month (mo) old Friesian bull calves against GnRH using various GnRH conjugate doses, adjuvants and booster intervals (1 or 2 mo post primary injection). Immunisation decreased LH and testosterone (T) secretion for 2 to 4 mo after the booster; thereafter, LH and T returned to levels approaching those of control bulls. Immunisation also decreased the incidence of aggressive and sexual behaviour at pasture, but had no effect on growth. In a follow-up study, Finnerty et al. (1997a, b) confirmed that GnRH immunisation prior to puberty (injections at 3 and 5, 3 and 7, 3 and 9, 3 and 11 or 3, 5, 7, 9 and 11 mo of age) transiently reduced T in all 50 animals (e.g. Figure 1) and reduced aggressive behaviour until slaughter at 21 mo of age (although not quite to the level of control steers). When a primary and a single booster were used (e.g. 5 and 7 mo group), growth was similar to that of control bulls and much superior to that of steers. Repeated immunisation (3, 5, 7, 9, 11 mo group) resulted in aggressive behaviour similar to that of steers for the duration of the study, including the last week (see Figure 2), while growth rate was similar to control bulls and considerably better than steers. Immunised bulls showed reduced homosexual behaviour at pasture at 9-12 mo of age, although there was no consistent effect at later ages.

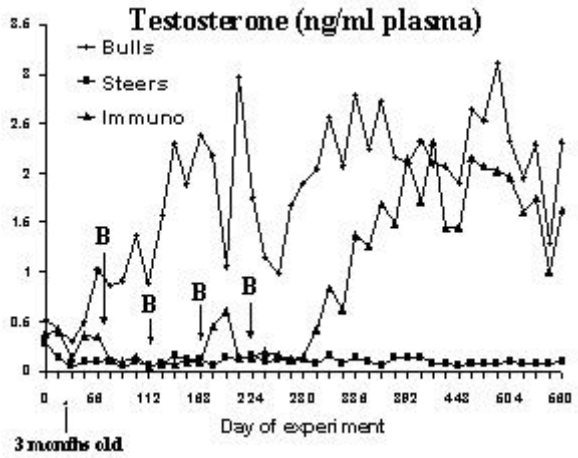


Fig. 1

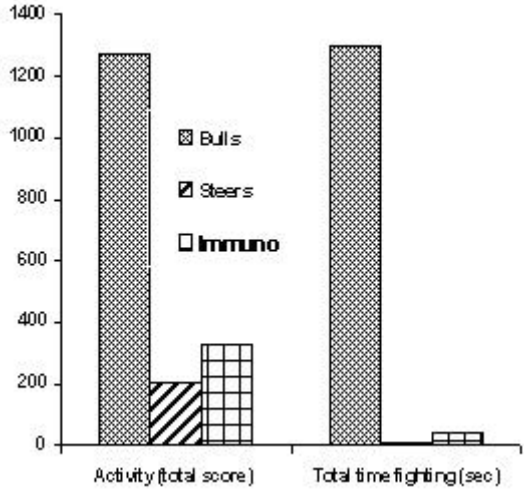


Fig. 2

In a similar study in New Zealand, Jago et al. (1997) looked at the effect of the timing of primary immunisation (as early as 2 mo of age, as late as 7.5 mo of age) and the number of booster immunisations. In all cases, immunisation delayed the prepubertal increase in T, and there was no difference in behaviour between the immunocastrate groups. From 10-17 mo of age, sexual behaviour of immunocastrates was intermediate between that of steers and bulls. Throughout the study aggressive behaviour was highest in bulls, while steers and immunocastrates were equal until 16 mo of age. Immediately prior to slaughter, at 18 mo of age, close monitoring during a 16 hr period of lairage revealed that steers now displayed much less sexual and aggressive behaviour, and that immunocastrates were only marginally less aggressive than bulls.

Immunisation of peri- and post-pubertal bulls

When 12-mo-old bulls were immunised against GnRH (Finnerty et al. 1995), T concentrations fell to at or near surgical castrate levels for the last 14 wk of a 20-wk study. During this time, sexual behaviour of the immunocastrates was also similar to that of surgical castrates while, compared to intact bulls, aggressive interactions were slightly reduced in duration but not frequency. Immunisation retarded growth to the same extent (≈25%) as surgical castration. In studies performed in California (Huxsoll et al. 1998; Price et al. 2003), bulls received a primary immunisation at 1, 4 or 6 mo of age and a booster at 12 mo. When observed at 16 mo of age, immunocastrates had aggressive behaviour similar to that of steers.

Conclusion

On the basis of the above studies, it is apparent that an optimised GnRH immunisation protocol for pre-pubertal bulls would be a viable option for obtaining the docile behaviour of steers but (close to) the growth rate of bulls. However, if GnRH immunisation is delayed until after puberty, growth rate is more likely to be adversely affected while the effects on behaviour may be inadequate. Furthermore, immunomodulation of endogenous hormone activity is an attractive alternative to

other methods of increasing the efficiency of animal production because vaccine residues are not perceived by consumers to be a problem, and because the approach is conducive to good animal welfare.

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Immunocontraception of African elephants using porcine zona pellucida vaccine

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Zona pellucida proteins isolated from the ovaries of pigs (pZP) have proven an effective basis for an immunocontraceptive vaccine in horses (Kirkpartrick et al. 1985, Kirkpatrick and Turner 2002; Turner et al. 2002) and a range of wild and captive non-domestic herbivores (Kirkpatrick et al. 1996a; Kirkpatrick et al. 1996b; Naugle et al. 2002; Shideler et al. 2002). In these species, the pZP vaccine was shown to be both safe and, where tested, reversible. The potential to use a pZP vaccine as a contraceptive in free-ranging African elephant populations has been recognised for some time, and the current paper documents a project carried out in three phases to investigate whether pZP vaccine represents a viable contraceptive for elephants in the Kruger National Park (KNP).

Phase 1

The aim of phase 1 was to establish whether there was a reasonable degree of homology between the zona pellucida proteins in pigs and African elephants. In 1995 during the last elephant cull performed in KNP, ovaries were collected from several elephant cows. After fixation, ovarian blocks were embedded in paraffin, and thin sections were cut and processed for immunocytochemistry. Immunostaining was then performed using a rabbit-anti-pZP primary antibody followed by a secondary antibody labelled with colloidal gold. Finally, the staining was enhanced with silver. Histological examination of the sections revealed distinct immunogold staining of the zona pellucida of oocytes in primary, secondary and tertiary follicles. This staining pattern was confirmed in samples examined by transmission electron microscopy. It was, therefore, established that the ZP proteins of pig and African elephant share significant epitopes, and this in turn suggested that antibodies to pZP should cross-react with elephant zona pellucida proteins (Fayrer-Hosken et al., 2000).

Phase 2

Phase 2 was designed to establish an effective pZP vaccination regime for African elephants. To this end, two elephant cows (both at zoos in the USA) were injected with 400 µg of pZP in 5mg of a synthetic trehalose dicorynnomycolate adjuvant.

Booster vaccinations (400 or 600 µg) were administered 4 weeks and 10 months later. The anti-pZP antibody concentrations achieved in both cows after the second booster were comparable to those recorded in horses that had been successfully contracepted with pZP vaccine (Fayrer-Hosken *et al*., 2000).

Phase 3

The objective of Phase 3 was to test the contraceptive efficacy of the pZP vaccine in a sample of free-ranging elephant cows in the KNP. In 1996, 41 cows (21 treatment and 20 controls) were selected for the first trial. Criteria for selection included a small calf (< 1 m high) at foot and a negative transrectal ultrasound pregnancy result. Cows in the Treatment Group were vaccinated with 600 µg of pZP combined with the adjuvant described above; controls were injected with placebo. All injected cows were radio-collared and, 6 weeks later, they were located and injected with a booster vaccination using drop-out darts fired from a helicopter. A second booster was administered similarly 6 months after the primary vaccination.

Twelve months after the primary vaccination, 19 treated and 18 control elephants were located, immobilised and examined ultrasonographically for pregnancy. One cow in the Treatment Group had to be excluded because the results demonstrated that she was already pregnant at the time of the primary vaccination. Of the remaining vaccinated cows, 8 (44 %) were pregnant compared to 16 (89 %) of the controls. Although this difference in pregnancy rate was statistically significant, the results were disappointing compared to those achieved in free-ranging horses. Therefore, a second trial was planned that included 10 vaccinated cows. This time the intervals between the primary vaccination and first and second boosters were seat at 2 and 6 weeks, respectively. In this trial, significantly fewer vaccinated cows became pregnant (2; 20 %). The trials also proved that the vaccine is safe to use in pregnant elephants, that contraception can be maintained by administration of an annual booster and, that if a booster is not administered, that cows recover fertility. These are important qualities when selecting a contraceptive for wildlife species

In conclusion, the results of these studies demonstrate that pZP vaccine is a safe and effective contraceptive for free-ranging African elephant cows. However, because the current vaccine requires three administrations during the first year, it is probably only suitable for use in small populations (< 100 cows).

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The elephant population problem in Zimbabwe: Can there be any alternative to culling?

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Aerial counting of elephant populations has been carried out in Zimbabwe since 1967. The latest census in 2001 revealed a total of approximately 88100 elephant in 4 separate populations (Table 1)

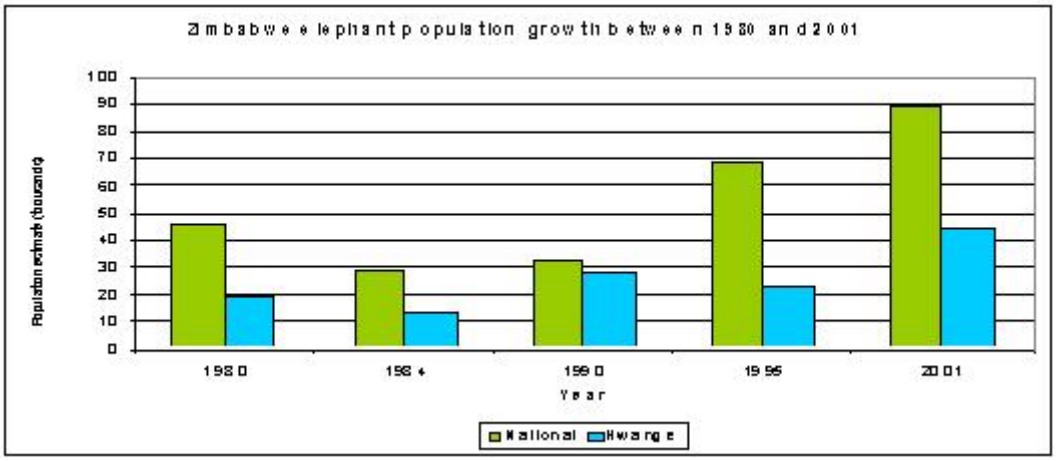
Table 1: Aerial Census of Zimbabwe's Elephant in 2001

Population sampled	Area (km^2)	Percent sampled	95% confidence limits	Population
North-west Matabeleland (includes Hwange NP)	25100	7.8	12.3	49300
Sebungwe	15600	13.8	15.2	14000
Zambezi Valley	17100	15.5	13.1	19300
South-east lowveld	8800	14.1-22.5	32.8 – 60.2	5500
TOTAL	66600		8.0	**88100**

The total population has increased from an estimated 47,000 animals in 1980, with the greatest percentage increase occurring in Hwange National Park (HNP) which, on its formation in 1928, was believed to contain between 1000 and 2500 elephant. Overall, Zimbabwe's elephant population has been increasing at approximately 5% per annum, and is therefore likely to be as high as 97,000 animals at the present time (Figure 1).

Figure 1: Elephant population increases in Zimbabwe, nationally and in Hwange

National Park



In Hwange National Park (HNP), the provision of some 70 artificial, perennial water points has increased the dry season foraging range of elephants from 35% to 75% of the Park's total area. Elsewhere, compression of the elephants' range by the increase in the human population from 0.5 million in 1900 to 12.5 million in 2001 and the recent "land crisis" has raised elephant densities to as high as 6 per km2. The major effects of this increase in elephant density have been erosion, vegetation damage and the loss of biodiversity. Erosion has occurred as a result of the loss of tree cover and has led to the siltation of water points. Vegetation damage has included a severe loss of trees which, combined with uncontrolled fires, has caused a loss of palatable species of shrubs and grasses. The effect of the elephant over-population on biodiversity has been less easy to monitor. It has been estimated that at densities of < 0.5 elephant per km2, forage and biodiversity may actually be enhanced, while higher densities lead to a loss of biodiversity. Of particular concern has been the effect on rhino in HNP. They are now unable to utilize areas under heavy elephant pressure and may even be prevented from access to some water points.

In addition, there has been more human-elephant conflict because of increased damage to crops and water facilities. These elephant attacks are likely to increase since 15% of Zimbabwe's elephant live outside the protected areas. The recommended elephant population for Zimbabwe is around 35000 - 40000 animals (0.6 per km2).

Efforts to control the elephant population

Cropping elephants by shooting was instituted in 1960, and by 1988 some 44000 elephants had been shot, mostly by eliminating whole breeding herds. Between 1989 and 1995, when elephant culling was stopped, approximately 2500 more animals were shot in problem animal control operations. In the 1980s, it was proposed that the number of elephant in HNP be reduced and held at 13000 (< 1 per km2). Although this target was reached in 1985, the policy was not continued and the

population has been allowed to increase unchecked, to the point where a “die-off” is likely to occur in the Park in the near future.

No plans exist to re-institute cropping of elephant in the protected areas. This has resulted from international pressure against elephant culling and a lack to resources within the Parks and Wildlife Management Authority of Zimbabwe to undertake such large-scale operations.

Options for the future

Options for future control of elephant in Zimbabwe include a laissez-faire approach, culling, translocation and contraception. Inactivity will lead to massive die-offs in some areas of the country such as HNP, Chizarira and Mana Pools National Parks. It would also result in an unacceptable loss of vegetation and bio-diversity. For culling to be reinstated, the necessary resources would have to be found in a country that already faces a severe economic crisis. Even if it could happen the task will be difficult. At the peak of culling in the 1980s, a maximum of 5340 elephant were shot in one year. If that total could now to be increased to, say, 10000 per annum, the recommended total population of 40000 would only be attained after 10 years of sustained effort.

However, an attraction of culling would be the sizeable economic return if the skin, ivory and meat could be recovered and successfully marketed. It has been estimated that a 5% off-take of excess elephant by a combination of safari hunting, culling and problem animal control could return as much as US$ 320 per km2. This compares favourably with the calculation of US$ 200 per km2 to manage an intensively protected wildlife area, particularly when only US$ 10 per km2 is presently available in Zimbabwe. However, culling is not generally acceptable to the international conservation community, and Zimbabwe would probably receive widespread condemnation if large numbers of elephant were shot.

Translocation of elephant breeding herds was undertaken in Zimbabwe in 1992/3, when around 1000 elephant were successfully moved from Gona-re-zhou National Park following a severe drought. Most were moved to the nearby Save Valley Conservancy, and 200 were taken to South Africa. Therefore, mass translocations are certainly feasible, but they are very expensive at > US$ 1500 per animal and there are few areas to which the elephant can now be moved, all of which are outside Zimbabwe. Translocation cannot therefore be considered seriously as an option for significant population reduction.

Contraception or sterilization of males and females is an attractive alternative for elephant population control. However, it has been shown by modelling that at least 75% of breeding females would have to rendered infertile for a period of >10 years to produce a significant drop in the population and that it might be more efficient to sterilize pre-breeding females. However, this is unlikely to be achievable with the drugs and technology presently available. An ideal contraceptive would be

deliverable as a single dose in a dart fired from a helicopter. Treated animals or their breeding groups would have to be identifiable, the cost per animal would be moderately high (at around US$ 150) and there would be no economic return for the effort. Problems with social and behavioural changes in treated individuals may occur, as well as the major disturbance during the treatment programme.

Ultimately, it appears likely that a combination of culling and contraception, with the latter having more application after elephant numbers have been reduced significantly, would find greatest acceptability. Those opposed to culling might be less vocal if contraception was being used simultaneously, while critics of population reduction without an economic return would be satisfied that the cropping option was not being ignored.

Conclusions

The following conclusions are pertinent to Zimbabwe's elephant overpopulation problem:

· The elephant population in Zimbabwe has now reached crisis proportions and large-scale die-offs are probably imminent and unavoidable.

· Severe impacts on the environment and bio-diversity will continue for some time, even if population-reduction measures could be initiated rapidly. However, the resources needed to implement effective control and subsequent monitoring are lacking.

· A combination of culling and some contraception or sterilization would probably be the best solution. Local opinion would favour culling alone but the arguments for this course are not generally supported internationally.

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Contraception by ZP immunisation: Organon's involvement

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Immunological disruption of gamete interaction by vaccinating against sperm and/or oocyte antigens is considered a promising alternative approach to human birth control. In this respect, considerable recent progress has been made in characterising the proteins that make up the mammalian zona pellucida (ZP), the extracellular matrix that surrounds the oocyte. And although the exact molecular composition of the ZP does vary between species, in most cases the ZP consists primarily of three to four major sulphated glycoproteins (Epifano and Dean 1994). Among these glycoproteins, ZP3 is generally believed to be responsible for the species-specificity of sperm-binding to the oocyte and for the induction of the acrosome reaction in bound sperm (Rosiere and Wassarman 1992). Not surprisingly, the oocyte-specific expression of their genes during oogenesis led to the suggestion that ZP proteins could be used as targets for vaccines to regulate both human and animal fertility. Indeed, numerous studies to assess this concept in animal models have resulted in the desired infertility. However, optimism about the feasibility of this approach for birth control in man has been tempered by numerous studies in a variety of mammalian species in which active immunisation against purified porcine ZP resulted in significant ovarian pathology. For example, immunization of the common marmoset (Callitrix jacchus) with purified pig ZP3 (pZP3) and deglycosylated pZP3b (32 kDa) induced ovarian degeneration characterized by a depletion of primordial follicles, appearance of follicular clusters lacking oocytes and depletion of the pool of recruited follicles (Paterson et al. 1992). By contrast, active immunization of rabbits with deglycosylated pZP3b did not result in any visible signs of ovarian pathology (Keenan et al. 1991). The reason for the immunopathology observed in some species is unknown, but it has been postulated to reside in contamination of supposedly purified ovarian-derived ZP3 preparations. To this end, recombinant human ZP3 (rec hZP3) was produced in Chinese hamster ovary (CHO) cells (Van Duin et al. 1994) but, in marmosets, even active immunization with this “pure” hZP3 disrupted ovarian function to the extent of complete cessation of ovarian cyclicity and the loss of primordial follicles (Paterson et al. 1996). This latter loss was completely unexpected, given that it was previously believed that ZP3 was absent from primordial follicles. Although the immunological mechanism underlying ZP3-triggered depletion of the primordial follicle pool was still unexplained, it was proposed that vaccinating with very specific immunocontraceptive ZP3b cell epitopes might circumvent the immunopathology. To verify this hypothesis, highly specific

mouse monoclonal antibodies were generated against different epitopes (linear and conformational peptides) of hZP3. These novel tools were then used to perform more in-depth immunohistological studies to map the temporal and spatial expression of ZP3 in rabbit, marmoset monkey and human ovaries. Immunoreactive staining of primordial follicles indicated the presence of ZP3 in early primordial follicles, thereby explaining why vaccination against ZP3 often disrupted all stages of folliculogenesis. Unfortunately, this finding simultaneously suggested that ZP3 is not a sufficiently stage-specific target for the development of a reversible form of immunocontraception (Grootenhuis et al. 1996).

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Modelling the effect of fertility control on elephant population dynamics

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Mathematical models have a long and rich tradition in epidemiology, population biology and ecology and are valuable tools for predicting the long-term, and to a lesser degree short-term, effects of various interventions on population dynamics. Models are particularly useful for studying the consequences at a population level of changes and mechanisms operating at an individual level. We propose that a model for elephant population dynamics could be designed to gauge the long-term effects and consequences of population management by immunocontraception. This model could then be used to determine whether immunocontraception is a viable alternative to culling or translocation for controlling population size and structure in a sustainable way.

We propose to use a matrix population model, a flexible and well-developed class of model that collates information on life-history characteristics across age classes and generates short, medium and long-term predictions of population growth and demography (see Caswell 2001). The long-term population dynamics are predicted by calculating the asymptotic growth rate of a population and the stable age distribution that is associated with that growth rate. Matrix models are simple in so far as the number of variables and parameters are small, however their major advantage is their mechanistic nature and the clarity of the assumptions that underlie them. Both density dependent (e.g. population self-regulation by changes in the age at first reproduction) and stochastic effects (both demographic and environmental) can be taken into account. In addition, it is possible to perform factor sensitivity analysis to determine which parameters most influence population growth rate. This helps to identify not only the key parameters of growth within a population, but highlights how different parameters may influence the steady-state age distribution.

To date relatively little elephant population modelling has been documented. Armbruster and Lande (1993) modelled elephant demographics using a Leslie model (a special class of matrix population model) to investigate the minimum reserve area required to sustain an elephant population. Their model included aspects of environmental stochasticity, such as occasional long periods of drought.

Dobson and Poole (1993) also used a Leslie matrix approach to model elephant

population dynamics, although they included density dependent rather than stochastic effects, and also took the social structure into account. The resulting model was used to analyse the impact of ivory poaching on East African elephant populations, and to estimate the time needed for a population to recover from heavy poaching pressure after it became fully protected. All of the parameters of their model were based on empirical data obtained from field studies, and one of their primary conclusions was that “social structure and reproductive physiology are crucial to understanding the demography of elephants”.

Dobson (1993) used the model designed by Dobson and Poole (1993) to predict the effects of immunocontraception or inducing abortion on elephant population dynamics. Importantly, the model included 2 parameters known to play a critical role in elephant population regulation, namely density dependent variation in the age at first reproduction and in the inter-calf interval. Based on data from the Amboseli population, it was predicted that an elephant population could be maintained at a stable level by any form of birth control that extended by two years both the age at first calving and the inter-calving interval.

More recently, a model similar to that of Dobson and Poole’s was used to investigate the effect of culling on the elephant population in the Kruger National Park (KNP: Van Aarde et al. 1999; Whyte et al. 1998). The authors discussed various contraceptive options but concluded that none were practical in a population as large as that in KNP (see Pimm and Van Aarde, 2001). Their model suggested that a large proportion (75%) of the female population would need to be contracepted successfully if zero population growth was to be achieved, and that population stagnation would be reached only after a considerable length of time. They, therefore, concluded that culling remains the best option available for controlling elephant numbers in the KNP.

Since the publication of Dobson’s population model ten years ago, there have been major advances in our knowledge of fertility control in elephants. Clearly, however, there remains considerable uncertainty about the likely knock-on effects of contraception or abortion on individual elephants (see Whyte et al. 1998; Pimm and Van Aarde 2001; Fayrer-Hosken et al. 2001); these effects need to be clarified. Nevertheless, our understanding of elephant population biology, demography, behaviour and social organisation has increased significantly during the last decade. Importantly, several detailed long-term studies have charted elephant population structure and behaviour, and these studies should play a major role in both building and validating a new model. Significantly, the data sets are drawn from different populations with very different age distributions (e.g. an aged population in Amboseli National Park and a younger population in Tarangire), and this should help clarify the impact of population structure on growth rate. We believe that it is now possible to extend the matrix model to study more thoroughly the impact of social, reproductive and behavioural changes resulting from procedures like immunocontraception. Our

aim is thus to collate information from the relevant experts to establish the effects of fertility control on individual elephants, and thereafter to design a model for predicting population dynamics in the face of various methods of population control.

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Aspects of male reproductive physiology and the phenomenon of temporary infertility in adult bulls

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Abstract
Worldwide, the success rate of breeding programmes for captive elephants is poor. In this respect, alongside undiagnosed reproductive disorders in females and fatal diseases like the newly discovered elephant herpes virus infection (EEHV: Richman et al., 1999), male infertility has been identified as a major factor contributing to the failure of captive populations to become self-sustaining (Hildebrandt et al., 2000). To investigate the possible causes of male reproductive inadequacy, approximately 400 ultrasonographic examinations of the internal genitalia combined with semen collection were performed in captive (n=15) and wild (n=13) African (Loxodonta africana) and captive (n=91) Asian (Elephas maximus) bull elephants, ranging in age from 4 to 50 years. Approximately half of the reproductive assessments were performed in protected contact situations with elephants handled in a restraint device, while the other half involved assessments of unrestrained trained Asian bulls, or examinations under general anesthesia. In the latter category, 14 examinations were performed in the field on wild adult African bull elephants immobilized during either translocation projects or GPS home-range studies.

Transrectal ultrasonography was used to characterise the morphology and functionality of the entire urogenital tract, including the size and developmental status of testes and accessory sex glands (Hildebrandt et al., 1997a; Hildebrandt et al., 1998, Hildebrandt et al., 2000a). In fact, transrectal ultrasonography enabled the identification of pre-pubertal and pubertal individuals, who were then excluded from further analysis (Hildebrandt et al., 1999). In addition, bulls were categorised on the basis of breeding status (breeders versus non-breeders) and, where possible, social rank (i.e. type of interaction with male and female conspecifics and keepers). Most of the bulls were adult non-breeders, and the rate of apparent infertility of non-organic origin (i.e. not due to specific anatomical abnormalities) in otherwise healthy animals was surprisingly high.

In general, breeding bulls had a fully developed genital tract, moderate blood testosterone concentrations (10 - 40 ng/ml) and delivered a fertile ejaculate of adequate volume (>200 ml) after manual stimulation (Hildebrandt et al., 1997b; Schmitt and Hildebrandt, 1998) or electro-ejaculation (Hildebrandt et al., 2000b). In

these breeding bulls, the seminal vesicles contained large volumes of fluid prior to ejaculation. With regard to social environment, captive breeding bulls were usually housed singularly with a group of female elephants or with other significantly younger bulls. In the wild, bulls identified as breeding animals by dint of their semen quality and the sonomorphologic appearance of their internal genital organs were never found associated with other adult bulls. By contrast, wild non-breeding bulls generally lived in bachelor groups of two or more individuals and were characterized reproductively by low ejaculate volumes, underdeveloped accessory sex glands and low circulating testosterone concentrations (<10 ng/ml). Captive non-breeding bulls were, in most cases, housed together with other adult males of a similar age or within a group where they were dominated by older cows. On occasions, captive bulls identified as non-breeders were apparently dominated only by zoo staff. This correlation between social rank and genital and spermatological findings was only overturned when a bull entered into musth. In musth bulls, blood testosterone concentrations were always high (up to 160 ng/ml) and the seminal vesicles were very large. However, semen collection from musth bulls was difficult and never resulted in a high quality ejaculate because of urine contamination.

On the basis of the correlations between semen quality, genital development and social status/situation, it is proposed that, in the wild, breeding bulls capable of impregnating cows are almost exclusively represented by dominant solitary animals. By contrast, adult males in bachelor groups are usually sexually inactive, presumably so that they can avoid social conflicts within their group until they have built up sufficient bodily resources (and experience) to enter an annual musth cycle and fight for a breeding-bull position. Preliminary results indicate a ratio, in the wild, of approximately 1 breeding bull to every 50-100 non-breeding bulls. The phenomenon of temporary or permanent social rank-induced sexual inactivity in bull elephants should be studied more intensively for possible future application to population management strategies for wild African elephants.

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Viral-vectored immunocontraception for the wild house mouse, mus domesticus, in Australia

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Introduction
Populations of the introduced house mouse erupt irregularly in the grain growing regions of South-eastern Australia, reaching densities greater than 1000 mice per hectare. These mouse plagues have major economic impacts as well as causing social stress within rural communities. Current control strategies usually involve the use of rodenticides that are not highly target specific, are costly to apply, can lead to environmental damage and are not humane.

As an alternative to current lethal agents, we are developing fertility control approaches whereby viruses are being assessed as delivery vectors. Non-toxic baits are also being considered. The research combines molecular biology, population ecology and epidemiology of the virus vector, as well as risk assessment of the use of genetically engineered organisms in the Australian landscape.

An ideal immunocontraceptive vaccine must evoke a sustained immune response which blocks a key reproductive process (e.g. fertilization), be species-specific, be delivered effectively throughout the pest population on a broad scale and be cost-effective, environmentally benign and publicly acceptable – a big challenge.

The components of the fertility control approach for the wild house mouse are mouse zona pellucida protein 3 (mZP3) as the reproductive antigen and mouse cytomegalovirus (MCMV) as the candidate delivery vector. This virus is highly prevalent in wild mice in Australia and is species-specific. It spreads by close contact and animals can become infected with multiple strains, which means that super-infection with a recombinant virus is feasible. The virus persists in the salivary gland and lung and can be reactivated during periods of stress. Since it is a DNA virus it can be engineered to carry additional genetic information.

Efficacy of an infertility vaccine.
We tested the effects of inoculating mice with an engineered Australian strain of MCMV expressing the mZP3 gene. Intraperitoneal (i.p.) inoculation with the recombinant virus resulted in stimulation of the host immune system with the

production of antibodies to both the virus and mouse ZP3. Treated mice remained infertile for up to 250 days. Three host strains of MCMV, G4 and N1 from Western Australia and K181, originally from USA, were engineered. All three recombinant MCMV-mZP3 vaccines induced reproducible infertility in laboratory mice following i.p. inoculation. Infertility, resulting from complete loss of primordial ovarian follicles is permanent. Two other recombinant MCMV vaccines expressing mouse zona pellucida protein 1 (mZP1) and a modified form of mouse ZP3 (ubi-mZP3), also caused infertility in female mice after infection. However, the recombinant MCMV-ZP3 derived from the Australian G4 isolate was chosen to be the first mouse vaccine product because it causes consistent and reproducible infertility. The final vaccine is being formulated using strict quality control protocols to remove all non-essential transgenic gene sequences.

Transmissibility of the vaccine.
The ability of a recombinant MCMV-mZP3 vaccine to spread and infect large numbers of mice in the field is critical for its success. We had shown only limited transmission from mice experimentally infected with the wild type MCMV strain G4 to uninfected wild mice within social groups. We are also investigating the transmission dynamics of an MCMV strain from wild-caught mice in eastern Australia. When naturally infected mice were placed in social groups with uninfected mice, this new strain of MCMV spread rapidly. We are currently isolating the strain for further characterisation and to use it as starting material for second-generation vaccines with enhanced transmission ability.

Species specificity and safety.
Following discussions with stakeholders, two major hazards were identified. First, the risk of inadvertently exporting a genetically modified virus overseas in infected live mice and, second, the species-specificity of the recMCMV-mZP3. To address the first concern, a mathematical model is being developed to assess the export risk during mouse plague conditions. For the second, Lewis rats were infected experimentally with a laboratory standard prototype vaccine, recMCMV-mZP3, using 20 times the dose used to sterilize mice. All the rats remained fertile and no recombinant virus was recovered from them by plaque assay or PCR of tissues. Similarly, 3 Australian native rodents have been infected with wild type MCMV and have not become infected.

Practicality in the field.
The ability to introduce a sterilizing virus that can spread effectively among wild mice is critical for the success of the vaccine. The use of non-toxic baits is being explored, and two oral inoculations with strain G4 recMCMV-mZP3 will effectively sterilize laboratory mice. The recombinant vaccine can be freeze-dried and/or applied to bait, with subsequent recovery of infectious recMCMV-mZP3. These results encourage the belief that bait delivery of infectious freeze-dried virus could be an effective means of inoculating field populations of mice. Further trials using freeze-dried

recombinant vaccine delivered on baits are in progress.

To determine the level of infertility required to reduce recruitment in mouse populations, enclosure studies using surgical sterilisation of varying proportions of the adult females are being undertaken to determine the rate of growth and the structure of confined populations. Surgical sterilisation of 67% of adult females significantly reduced the growth rate of the population. Mathematical models have also been used to simulate fertility control. These indicate that a level of 33% infertility for the duration of a breeding season, with each cohort of young being affected at this level, will reduce mouse numbers and maintain them below plague densities (< 200 mice per hectare in grain growing areas).

Conclusions.
Field and laboratory results, as well as computer modelling, all show excellent prospects for the use of viral vectored vaccines based on MCMV for managing eruptions of mouse populations. However, public acceptability of the technology has yet to be confirmed. The issues of species specificity, delivery system stability and other potential or perceived risks require open and wide-ranging debate, nationally and internationally, before field trials of a genetically modified virus to control populations of mammals could occur.

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Translocation as a management tool for control of elephant populations

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Background
Elephant translocation within South Africa has increased significantly in the last decade. Until 1993 only orphaned elephants from the Kruger National Park (KNP) culling operations were translocated in significant numbers. A number of newly established smaller reserves in South Africa such as Pilanesberg, Umfolozi/Hluhluwe and Madikwe were stocked with these juvenile elephants but, after a few years, aberrant social behaviour was noted in the reserves containing introduced elephants.

In 1993 Clem Coetzee developed a method to move adult elephant in Zimbabwe. He came to KNP in 1994 to help the local capture team develop the technique, after which South Africa National Parks (SANParks) adopted his method and refined the equipment to be able to move large numbers of elephants. Since then only complete family units and adult bulls have been translocated from KNP and this change of policy seems to have solved the behavioural problems seen previously in the populations introduced as youngsters. Over 750 elephants in family units and almost 100 mature bulls have been moved by the KNP capture team in the last 9 years.

Capture technique
Elephant capture should only be attempted by experienced personnel using the right equipment. Immobilisation of such a large mammal is a very specialist process, and this is followed by the considerable practical difficulty of raising the adult elephant off the ground and into the recovery vehicle so it can be transported to the new destination safely and humanely and without danger to people or traffic. The basic equipment required includes; i) a truck with a heavy-duty crane; ii) a recovery trailer and winch; iii) a recovery chamber for the elephant to wake up in; iv) a well-constructed transport container.

Experienced personnel should accompany elephants being transported over long distances as fighting may occur so that tranquilisation could be necessary.

Most elephants translocated currently from the KNP go to relatively small, but well fenced, reserves in Zimbabwe and South Africa. This means they must be exposed to electrification before they can be released onto the property. Initially they are

released into a small holding camp or “boma” of about 1-2 hectares in area so that they can become familiar with, and learn to respect, the electric fencing. The boma must be well constructed, securely fenced with cables and have a good and functional electrification system in place. The elephants should stay in the boma for at least 24 h but not so long that they become hungry or bored. The gates must be sturdy and should slide rather than swing open. They should also be electrified at all times. Supplementary food may be needed if more that one group are to be released from the same boma over a short period of time.

In summary, the capture and translocation of elephants can only be done by experienced personnel using specialised equipment. It is expensive (+/-R10,000 per elephant, excluding transport) and areas available for translocation are limited, both by ecological size and accessibility to the heavy equipment needed. A good management plan, a release boma and functional fences are prerequisites before elephants can be released into a reserve. Furthermore, planning for future population control should be in place. KNP cannot control its elephant population by translocation alone since there are not enough accessible areas available to absorb the numbers necessary to reduce the population sufficiently to avoid ecological damage.

For the future, few options are available to the KNP to reduce its elephant population before serious ecological damage is done. Expansion of the conservation area through well-managed Transfrontier Conservation Areas is one option being developed currently, but the fences have not yet been removed and the local population do not support the concept of large numbers of elephant moving back into the areas they now occupy. Application of practical contraceptive methods is a second option but this still needs research and refinement. Improved translocation equipment able to move large numbers of elephants to areas outside South Africa that have had their indigenous elephant population reduced by poaching would be desirable although the security of such areas still remains doubtful. Finally, organised and controlled culling must almost certainly be re-introduced to quickly stabilise the rapidly expanding population of elephant.

Elephant population trends and the challenges facing protected area managers

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The decline of Kenya's elephant population due to runaway poaching for ivory was halted in 1989 after the country's herds had been reduced to only 12% of their former numbers. Since then, protection, legislation and conservation of habitat have all been effective, such that Kenya's elephants today number approximately 30,000, up from 19,000 in 1989.

So successful have been these measures that the challenge of managing elephants nowadays involves conflict resolution where elephants are returning to traditional migrations in areas now occupied by people. Fencing elephants into protected areas and out of inhabited lands has resulted in damage to fragile and unique habitats in some areas, and it is now necessary to manage elephant numbers to protect biodiversity.

Unlike the situation in other parts of Africa, culling of elephant is not an option in Kenya. Meat and other products cannot be sold in local or international markets and elephant meat is not eaten by most local communities. Moreover, Kenya is home to some of the most intense elephant research in Africa, which has revealed the complex social and behavioural world of this species. The country is heavily dependent on tourism for foreign revenue and the negative impact of culling could damage this fragile industry.

To date, Kenya's policy on elephant management has involved the creation of corridors, elephant sanctuaries and barriers to elephant movement, where human lives and property are at risk. Translocation has been adopted as a humane alternative to culling, to reduce numbers or to remove problem animals, and contraception has been proposed for specific elephant populations.

Recent research in the Shimba Hills area revealed that the damaging effects of elephants are exaggerated by human activity in the form of logging. This population of forest-living elephants is divided into a distinct bull area in the dry forest, where habitat degradation from tree pushing is most severe, and a family area in the rain forest. The density of elephants in this one small protected area ranged from ± 6 per km2 to < 1 per km2.

In rain forest patches, the elephants induced changes in the composition and

structure of species beneath the canopy. Thus, while rain forests were not being converted to grasslands, there were concerns that elephant activity beneath the canopy would eventually prevent its re-establishment when old trees died. This effect was demonstrated in an experimental design in which exclusion and inclusion of elephants was compared. In forests in which gaps had been created by logging, elephant activity was intense due to the flush of re-growth.

In dry forest, elephant activity was much more dramatic and their debarking and pushing over of trees had resulted in complete change from forest to open bushland. Analysis of aerial photographs revealed that the decline in forest was due to elephant activity at the edges, especially by bulls.

The Shimba Hills is the first protected area in Kenya to be completely surrounded by a fence, and this has caused artificial concentration of elephants into a small area. Research findings recommended a multi pronged approach to reduce the impact of elephants. Namely; 1) Reduction of the population by translocation; 2) Contraception or complete sterilisation of females, because of the lack of poaching and the unique biodiversity in the area; 3) Expansion of the protected areas by acquisition of more land and the creation of corridors; 4) The cessation of logging.

Towards this end, an experimental translocation of 30 bulls in 1999 proved successful and a small expansion of the protected area was achieved concomitantly. Logging was prohibited, but the contraception work was not initiated, partly due to a lack of follow up and partly because the technology was unproven at that time.

Following the work in South Africa, it is now realistic for Kenya to initiate immuno-contraception in elephant populations. However, such a project would require a level of scientific sophistication that the government may not be able to provide. For example, the work would require individual recognition of the elephants, intense monitoring of their movements, behaviour and reproductive status, and it would involve radio-tracking, sample collection and analysis, aerial transport and staff accommodation.

Perhaps the most effective way to achieve successful contraception of elephants in Kenya would be a partnership between Kenya Wildlife Services and a respected non-governmental organisation like Save the Elephants. At least three populations of varying size living in different habitats could be used initially, including Sweetwaters, Shimba Hills and Mwea. This would give the opportunity to explore the application of different contraceptive methods under different conditions. Hopefully, a collaboration between Kenya and southern African countries could quickly provide guidance for other African countries with similar problems of high elephant density and different government policies for elephant management.

The public perception of contraception may be more difficult to accommodate in

South Africa than Kenya. Revenue from tourism and related activities is the key to community support for conservation in Kenya. For example, communities in the Shimba Hills made a commitment to conservation and the development of tourism in 1997 by setting aside land for conservation in the Mwaluganje Elephant Sanctuary, an area adjacent to the Shimba Hills Reserve. They now have a game lodge and two restaurants and they are responsible for running the sanctuary in conjunction with the KWS, a local NGO and the Forest Department. This could become Kenya's showcase for ethical management of elephant populations, with the consequent boost to tourism.

Hormonal approaches to male contraception

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A potential male contraceptive has to fulfil certain prerequisites before it can be considered as a reliable alternative for use in human patients (see Table 1: Nieschlag et al. 2000). Current male contraceptive approaches are either highly effective but potentially irreversible (vasectomy) or relatively ineffective (condom). For this reason, there is considerable interest in developing alternative, testicular and post-testicular, methods for male contraception. Of the various theoretical approaches considered, the closest to practical implementation is the administration of exogenous hormones.

Table 1: Prerequisites of an ideal male contraceptive

An ideal male contraceptive should:

- be administered independently of the sexual act
- be acceptable to both partners
- not interfere with libido, potency or sexual activity
- have no short or long-term toxic side-effects
- have no impact on any eventual offspring
- be rapidly effective and fully reversible
- be more effective than condoms

The aim of the hormonal approach to male contraception is to suppress spermatogenesis and sperm release from the testis, and thus induce azoospermia (no sperm in the ejaculate) or a severe oligoasthenozoospermia (few, predominantly immotile or poorly motile sperm in the ejaculate). The general principle of this approach is to interfere with the endocrine regulation of spermato-genesis. In contrast to rodents, normal spermatogenesis in primates is both quantitatively and qualitatively strongly dependent on the pituitary gonadotropins LH and FSH which, in turn, depend on hypothalamic GnRH for their secretion. FSH acts directly on spermatogenesis via specific FSH receptors in the Sertoli cells, whereas LH acts indirectly by stimulating testosterone production in the Leydig cells. For successful

male hormonal contraception, secretion of both gonadotropins needs to be suppressed profoundly, so as to arrest spermatogenesis at the spermatogonial stem cell level. On the other hand, the resulting extra-testicular androgen deficiency also needs to be addressed in order to maintain other androgen dependent functions. For this reason, androgens are an essential component of all hormone based male contraceptives (Nieschlag et al. 2000).

With these principles in mind, testosterone was an obvious first candidate as a hormonal contraceptive for men; it not only suppresses pituitary LH and FSH secretion but simultaneously corrects the circulating testosterone deficiency. Initial studies in the 1970s demonstrated that although the oligozoospermia (< 3 x 106/ml) induced by testosterone administration was not as effective as azoospermia in preventing pregnancy, it still offered better protection than condoms (World Health Organization, 1996). However, as in later studies of androgen-only hormonal contraceptives, azoospermia was induced in almost all East Asian men but only two thirds of Caucasians.

In an attempt to enhance the efficacy in Caucasians, testosterone was combined with other gonadotropin-suppressing substances, such as progestins or GnRH antagonists. The combination of testosterone and a GnRH antagonist leads to a more rapid onset and complete suppression of sperm production. Unfortunately, the preparations currently available are considered unacceptable for practical use. On the other hand, new depot preparations and studies suggesting that the GnRH antagonist may be needed only during the induction phase, mean that this approach may eventually become feasible in practice.

A combination of androgen and depo-medroxyprogesterone acetate (DMPA) has also shown good suppression of spermatogenesis in Caucasian males in some studies, but up to 12 % inadequate responders in others. In more recent studies using 300 mg DMPA (injected every 3 months) combined with testosterone implants (800 mg every 4 months), a rapid initial suppression of spermatogenesis and azoospermia was achieved in 49 out of 53 volunteers and no pregnancies (upper 95% confidence interval for contraceptive failure is 8%) have occurred in 35.5 person years (Turner et al. 2003). On the other hand, 15% of subjects discontinued the study because of failed suppression or problems with the implants, and the recovery to normal semen production appears to be much slower than with other progestins.

Nineteen-noresthisterone was one of the earliest progestins derived from testosterone, has the strongest androgenic activity of all 19-nortestosterone derivatives (approximately 10% that of testosterone) and induces profound, rapid and sustained suppression of serum FSH and testosterone concentrations in normal men. When norethisterone enanthate (NETE) or orally active norethisterone acetate (NETA) were combined with injectable testosterone undecanoate (TU), azoospermia was achieved in 51 out of 56 volunteers and sperm production dropped below 1 x

106/ml in a further 4 subjects (Kamischke et al. 2001, 2002). Since TU and NETE can be combined in a single injection (both are dissolved in castor oil), a combination of these 2 hormones injected at 6 - 10 weekly intervals offers great potential as a male contraceptive.

Orally administered desogestrel (DSG) and its implantable active substance etonorgestrel (ENG) have also been shown to powerfully suppress spermatogenesis when combined with short-acting testosterone enanthate (100 mg weekly) or testosterone pellets (400 mg every 12 weeks). And although azoospermia was achieved in only 78% of 102 Caucasian volunteers, the high proportion of men (92%) that achieved sperm concentrations < 1 x 106/ml compensated for the relatively low rate of azoospermia and confirmed ENG combined with implantable testosterone preparations as an interesting approach to male contraception (for review see Kamischke and Nieschlag, in press).

In conclusion, because the contraceptive efficacy of all self-administered androgen preparations so far tested has been disappointing, recent studies have focused on a combination of either androgen implants or long-acting injectable testosterone esters such as TU with potent progestins. In terms of general long-term acceptance, the ideal would be to combine both medications in a single application. To date, however, the combination of testosterone implants or long-term injectable esters with the progestins NET, DSG and DMPA have proven effective in small clinical trials and offer most potential for licensing.

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Elephant contraception: looking beyond the pharmacology

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It is easy to pharmacologically inhibit fertility in virtually any wildlife species. It is not easy to establish how to administer those pharmacological agents into a sufficient number of intelligent, mobile and often dangerous animals in any given population, such that biological goals are achieved in a publicly acceptable manner. Thus, the starting point for any wildlife contraceptive programme must be the development of a set of “ideal” characteristics for the theoretical antifertility drug for each particular species. Such “ideal” characteristics were developed for wild horses in North America in 1991 and, over the past 16 years, porcine zona pellucida (PZP) immunocontraception has been tested against those characteristics. Its efficacy in wild horses and elephants is based on 16 years of treatment of wild horses in > 12 distinct herds and from 4 years treatment of 31 female elephants in The Kruger National Park and 23 cows in Makalali Wildlife Park, both in South Africa.

For the wild horse herds in North America, and elephant groups in Africa, the “ideal” characteristics include i) an efficacy of > 90%; ii) the ability to deliver the drug remotely in 1.0 cc darts so as to reduce or eliminate handling or capture-related stress; iii) reversibility of contraceptive action to preserve the reproductive and genetic integrity of the target animals; iv) safety of administration to pregnant animals; v) inability of the agent to pass through the food chain to predators, scavengers and humans; vi) lack of significant effect upon social organization or behaviour; vii) a lack of harmful or debilitating long-term health side effects; viii) low cost. All these “ideals” have been met in both horses and elephants.

In addition, three levels of progress in the development of a practical wildlife contraceptive have been noted; i) demonstration that the contraceptive agent (PZP) is pharmacologically effective in the target species (proven for horses and elephants, as well as more than 100 other mammalian species), ii) demonstration that the contraceptive agent can be delivered to free-ranging wildlife under field conditions and, iii) demonstration that a sufficiently large portion of a population can be treated to achieve a true population goal.

It appears that by 2003, the only significant scientific hurdle left to overcome with the use of PZP vaccine is the need to deliver a year or more of contraceptive in a single inoculation. Currently, animals are treated twice during the first year, once annually

for the next 2 years and once in alternate years or every third year thereafter. This shortcoming can be overcome in two ways. First, a single “primer” dose of vaccine can be given in Year 1, without subsequent contraceptive action in that year but with the benefit that each animal so treated needs only a single annual booster inoculation thereafter. This is the simplest approach and its utility has been proven with wild horses. A second approach is to place the immunogen in a slow release matrix, and several materials exist which enable this to be accomplished. We have used lactide-glycolide pellets that fit into the 14-gauge needle of the dart with some success. In wild horses in Nevada, a single treatment resulted in 90% contraception in the first year, 83% in the second year and 60% in the third.

Wildlife contraception, particularly in wild horses and elephants, is attended by significant public scrutiny, concern and scepticism, which mean the research team must pay close attention to a proactive public education programme. Despite its high score in terms of “ideal” characteristics, PZP may not be the optimum contraceptive agent for elephants. The natural history, behaviour and even the prevailing public attitudes towards the target species, not to mention the logistic problems of administering the contraceptive agents to identifiable individuals in the field, must all be weighed carefully when attempting to construct a set of working characteristics with which to measure the utility of the product in the chosen target species.

Progestagen depot with or without testosterone: a strategy for regulating fertility in male elephants

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Progestins, notably medroxy-progesterone acetate (MPA), are potent inhibitors of gonadotrophin secretion and have been used widely in steroid-based contraceptive regimens in both man and animals. Moreover, we believe that MPA depot treatment represents a potentially effective method for controlling fertility and/or aggressive musth-related behaviour in male elephants (Loxodonta africana and Elephas maximus). MPA is cheap and readily available in large quantities, its actions are very well characterised and long lasting, and the contraceptive effects are reversible.

In man, MPA given as a subcutaneous depot (150-300mg in oil every 3 months) induces testicular regression, infertility and low androgen status. MPA can also be given in combination with testosterone to enhance effectiveness and maintain male sexual behaviour and other androgen-dependent characteristics, as is desired in human male contraception (Wu and Aitken 1989; Brady et al. 2003). MPA acts primarily through the progesterone receptor to suppress gonadotrophin secretion. In red deer (Cervus elaphus), a 150mg depot of MPA given to males in the sexually active phase of the annual reproductive cycle blocks gonadotrophin release and results, within a month, in the antler casting indicative of testicular inactivity (Muir et al. 1982; Suttie et al. 1995). The effect lasts for several months, as evidenced by reduced LH/testosterone responses to challenge with gonadotrophin releasing hormone (GnRH). The anti-androgenic progestin, cyproterone acetate (CPA), also blocks expression of overt rutting characteristics in red deer stags, but is less effective than MPA in suppressing testicular activity (Suttie et al.). MPA is used in veterinary practice to chronically block testosterone secretion, but may be less effective in some species (e.g. the dog; Paramo et al. 1993).

To date, there have been no robust studies of the long-term efficacy of MPA for inducing oligo/azoospermia in male elephants (MedLine Search). However, the anti-androgen flutamide (2500-7500mg/adult male) has been shown to suppress musth behaviour in captive/working Asian elephant bulls (Chandrasekharan and Cheeran 1996). Not only did the highest dose totally block musth behaviour, but the

subsequent musth did not occur until the normal time in the following season, thereby illustrating that it is possible to control the complete annual cycle with a single treatment. This has implications for the humane management of elephant bulls in captivity (Taylor and Poole 1998). Furthermore, we predict that a single dose of MPA (5000mg) would block the musth cycle and render a male elephant infertile for many months. A combined MPA and androgen/oestrogen treatment might be a more refined method to induce infertility but maintain male behaviour, allowing bulls to remain socially dominant.

The possible contraceptive/management protocol in the wild would take advantage of several aspects of elephant physiology and behaviour: 1) Bull elephants are very large and it is possible to follow musth cycles in individuals in the wild (Poole 1994). 2) Most matings are performed by the larger, older (30-50 year old) male elephants during the musth phase of the annual reproductive cycle (Poole 1994). Thus, only the big, dominant males need to be targeted for steroid-based contraception. 3) Dominant bulls suppress musth behaviour in younger subordinates (Slotow et al. 2000); this favours a treatment that does not affect dominance behaviour of the older bulls. 4) Musth appears to be driven by an endogenously generated circannual rhythm (Lincoln and Ratnasooriya 1996), which means that blocking the sexually active phase of the cycle should not affect the overall timing of an individual's cycle, thus simplifying long-term management. 5) Male elephants may be especially sensitive to the negative feedback effects of MPA, since females produce 5 alpha-reduced progestins, rather than progesterone, to regulate gonadotrophin secretion during the oestrous cycle and pregnancy (Hodges et al. 1997).

We propose that two types of experiments should be conducted:
A. Phase 1; in captive working/zoo adult Asiatic elephants. An experimental group of adult bulls that express musth cycles (e.g. n=10; 20-50 years old animals) would be treated with a MPA depot (e.g. 5000mg). Thereafter, the effects on the musth/testis cycles could be monitored by regular blood sampling to measure gonadotrophin and testosterone secretion (Lincoln and Ratnasooriya 1996; Niemuller and Liptrap 1991), ultrasonography to measure testis and accessory organ development (Hildebrandt et al. 1998), and behavioural observations to examine the expression of musth. To prove an effect of treatment, the same animals could be monitored in the preceding or following year as their own controls. In subsequent years, the efficacy of combined MPA and testosterone therapy could be examined.

B. Phase 2; would be based on the data from the captive Asian elephants, and performed in a well-monitored population of wild African elephants. A group of adult bull elephants that express musth cycles (e.g. n=10; 20-50 year old animals in a single area) would be identified and treated with a MPA depot (e.g. 5000 mg). The effects on musth and other social behaviours would be monitored. The treatment

would be continued for a minimum of 3 annual cycles, with additional data on birth rates in the local elephant population analysed to determine whether treatment was having the desired contraceptive effect.

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Evaluation of GnRH conjugated to a cytotoxic agent as a reproductive sterilant in mammals

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The goal of our research is to develop a treatment for chemically sterilizing animals that is both safe and effective. Ideally, we would like to identify a compound that: 1) is effective in males and females, 2) is effective in many species, 3) has permanent effects, 4) is easy to administer, e.g. injectable, and 5) will not sterilise predators of the target species.

With these characteristics in mind, the hypothalamus and anterior pituitary gland were considered more appropriate targets than the gonads. Not only are there obvious sex differences between the gonads, but there are also species differences in the structure of the hormones that stimulate them, i.e. follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Of course, the hypothalamus is protected by the blood-brain barrier and might not be accessible to compounds designed to inhibit reproduction. We therefore decided to target the gonadotropin-secreting cells of the anterior pituitary gland, which have receptors for gonadotropin-releasing hormone (GnRH), a decapeptide identical in structure across all mammalian species and that has been used to regulate reproductive processes in a wide range of species. A compound that interrupted reproduction at the level of the gonadotropes would satisfy criteria 1 and 2 above, i.e. it would work in both males and females across a range of species. The challenge was rather to find a method of permanently disabling gonadotrope function.

Several long-acting formulations of GnRH (or GnRH agonists) have been developed that suppress reproduction for as long as they are administered. Shortly after treatment ends, however, reproductive function recovers. Our approach was instead to conjugate a cytotoxic agent to an analogue of GnRH, and thereby target the cells for destruction. If all the gonadotropes could be destroyed, there would be no more stimulation to the gonads and the animal would become sterile. After considering several cytotoxic agents, we chose a ribosome-inhibiting protein derived from pokeweed (Phytolacca americana), pokeweed antiviral protein (PAP). PAP inhibits protein synthesis by inactivating 28S rRNA and, in turn, this inhibition of protein synthesis leads to cell death. If PAP could be delivered specifically to the gonadotropes by conjugation to a GnRH analogue it should allow specific elimination

of the gonadotropes (other pituitary cell types do not have GnRH binding capacity), and thereby act as a chemical sterilizing agent. Because the GnRH-PAP conjugate would be digested if administered orally, it would have to be injected. Overall, a GnRH-PAP conjugate would thus meet all 5 of our original criteria.

To test our hypothesis, we first conjugated a superagonist of GnRH (D-Lys6-GnRH) to PAP and examined the ability of the conjugate (GnRH-PAP) to bind to GnRH receptors in membranes isolated from bovine anterior pituitary glands. Although conjugation to PAP reduced the affinity of the superagonist for GnRH receptors by ~80%, the conjugate still bound to the receptor at a higher affinity than native GnRH. Next, we examined the ability of the conjugate to inhibit protein synthesis in an in vitro translation system. Conjugation only slightly reduced the ability of PAP to inhibit protein synthesis. At this point, we evaluated the ability of the conjugate to specifically inhibit synthesis of LH in cultured pituitary cells; the synthesis of LH was inhibited by > 95% by GnRH-PAP while the synthesis of prolactin was not affected. In short, the conjugate was able to prevent protein synthesis by gonadotropes, but did not appear to affect other pituitary cells. Since it was difficult to accurately evaluate cytotoxicity in a mixed population of pituitary cells, we next examined the effect of GnRH-PAP on Chinese Hamster Ovary (CHO) cells transfected with cDNA for the GnRH receptor. The conjugate was cytotoxic to GnRH receptor transfected cells in a dose and time dependent manner, whereas it had no effect on CHO cells lacking the GnRH receptor.

To examine the effects of GnRH-PAP in vivo, we treated rats with an amount of conjugate equivalent to 20ng of GnRH binding activity. In males, this led to a loss of spermatocytes and spermatids and a 50% reduction in testis weight, compared to vehicle treated controls ($p < 0.05$). Peripubertal females treated similarly failed to have estrous cycles and, 60 d after treatment, their ovaries contained only atretic follicles. Next, we used ovariectomized ewes to determine whether GnRH-PAP could reduce secretion of LH in vivo. After a single administration of GnRH-PAP, GnRH-induced LH secretion was reduced to approximately 50% of that in untreated sheep ($p < 0.05$) for at least 6 months. Finally, we examined the effect of GnRH-PAP on testis size, LH and testosterone secretion in male dogs. Four dogs received 0.0042 mg/kg GnRH-PAP hourly for 36 h (Hourly), while another four dogs were treated with a single daily bolus injection of 0.1 mg/kg GnRH-PAP for 3 consecutive days (Bolus). One further dog received a single bolus injection of GnRH-PAP (0.1 mg/kg; Single), and three control dogs were treated with GnRH alone (Controls). Twenty-five weeks after the initial treatment, all treated dogs were given a further single injection of 0.1 mg/kg GnRH-PAP while controls were injected with 0.0045 mg/kg of GnRH analogue. For nine months after treatment, testis size was measured and serum samples collected to monitor testosterone and LH concentrations by radioimmunoassay. To evaluate LH release capacity, the area under the serum LH concentration curve was examined after stimulation with 5 mg/kg GnRH. In summary, post-treatment serum testosterone concentrations and testicular volume were

significantly lower in Bolus and Hourly groups than in Controls ($p < 0.05$). In addition, basal LH concentrations during weeks 1 - 33 after treatment ($p < 0.05$) and LH release after GnRH stimulation were lower in the Bolus and Hourly than the Control group ($p < 0.05$). It is concluded that GnRH-PAP offers considerable promise as an agent for inducing chemical sterilization in a wide variety of mammalian species.

The efficacy of GnRH vaccines in controlling reproductive function in horses

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There are a number of situations in which suppressing the reproductive endocrine system in horses may be desirable. These include the competing animal whose tractability during training, or performance during athletic competition, is compromised by the expression of sexual or aggressive behaviour. Suppression of circulating testosterone concentrations has also been proposed as a treatment for stallions that shed equine arteritis virus (EAV) in their semen, because persistence of the virus in the accessory sex glands is testosterone-dependent, and continued shedding disqualifies that stallion and its semen from export to any of the important horse-breeding countries. Ablation of endogenous gonadotrophin stimulation may also provide a user-friendly means of preparing mares as sex steroid-inducible 'teasers' for semen collection or as recipients for embryo transfer (ET), and could, in the future, also represent a means of increasing the efficacy and reducing the variability in the response to superovulatory treatment of ET donor mares. In practice, the most important current indication for endocrine suppression is behavioural control, for which the alternative treatments include long-term progestagen therapy and surgical gonadectomy. Of course, the former is banned in athletic competition as potentially anabolic, whereas gonadectomy involves the risks of surgery and, if the animal in question turns out to be a gifted athlete, the loss of valuable genetic material from the breeding pool. In this light, the possibility of reversibly shutting down the reproductive endocrine system with a vaccine that induces anti-GnRH antibody formation is an attractive prospect. Unfortunately, while initial oil-based GnRH vaccines were effective in suppressing hormone production, they were unregistrable because of the severe local reactions that they induced. More recent vaccine formulations are water-based and induce less reaction, albeit at the cost of reduced reliability and a shorter duration of action.

Our own studies have involved the use of an ovalbumen conjugated GnRH-dimer vaccine (Oonk et al. 1998) to examine the efficacy and reversibility of GnRH vaccination in young stallions and in adult stallions and mares. In young stallions (3 years old), a course of two injections induced, from 2 weeks after the 'booster' injection, a dramatic increase in anti-GnRH antibody titres and an accompanying fall in systemic testosterone concentrations to baseline values. Treated stallions also showed mark reductions in testicle size, sperm production and sperm quality, and

depressed libido. However, while approximately 75% of treated animals responded to vaccination, the precise magnitude and duration of the effect differed markedly between individuals. Reversibility of treatment was demonstrated 11 months later when sperm quality and production parameters were similar to those in control animals. When the vaccine was tested in retired breeding stallions, only 4 of 8 responded with the expected fall in circulating testosterone concentrations after the initial course of two injections. And while two further stallions responded partially after a third injection 12 weeks later, the remaining two failed completely to respond even after a 4th vaccination. On the other hand, in the animals that did respond properly, testis size, sperm production capacity and sperm quality were reduced below those of both control stallions and poor responders. Disappointingly but not unexpectedly, response to vaccination had little effect on the intensity of sexual or aggressive behaviour in these older stallions. Eight months after the last vaccination, sperm production capacity had returned to pre-treatment levels, but sperm quality had yet to recover completely.

In adult mares, 3 successive vaccinations at 3-4 week intervals were needed to induce ovarian inactivity. Nevertheless, within 4 weeks after the third injection, 6 out of 7 mares entered a deep anoestrus characterized by a total absence of ultrasonographically visible ovarian follicular activity and circulating progesterone concentrations < 1ng/ml. Three of these mares restarted follicular development within 8 weeks (6-8 weeks) and one ovulated at this time. The remaining 3 mares were still completely acyclic 12 weeks after the final vaccination, although the onset of winter meant that it was not possible to distinguish clinically between a vaccine and a seasonal effect. The following spring, all 3 mares showed renewed ovarian activity. In a follow up trial aimed at ablating reproductive cyclicity for the entire breeding season, vaccination was performed in early March and repeated 3, 6 and 17 weeks later. As previously, 3 of 4 naive mares entered anoestrous within 2 weeks of the third vaccination. More surprisingly, while 2 of 4 previously vaccinated mares entered anoestrus soon after the first seasonal vaccination and remained acyclic for the entire breeding season, one previous responder failed totally to respond and another was rendered acyclic for only 4 weeks, shortly after the 3rd vaccination. In addition, treated mares showed irregular but occasionally strong oestrous behaviour, despite complete ovarian inactivity.

In conclusion, these studies confirm earlier reports that GnRH vaccination is a successful and reversible way of suppressing reproductive endocrine activity and sex-steroid induced behaviour in young horses. In older animals, the response rate tends to be lower and to require more injections to elicit a clinical effect of shorter duration. In addition, significant injection site reactions have been noted in older animals that would reduce its suitability in competing animals. Finally, older animals continued to exhibit sexual behaviour despite baseline gonadal steroid hormone

production. In terms of the utility of this treatment as a contraceptive, responding mares would be rendered temporarily infertile whereas, despite lowered sperm production and quality, most stallions would have remained capable of getting mares pregnant. In practical terms, the need for repeated injections to induce a clinical response would limit the use of this vaccine to only those feral or wild populations where individuals are readily tracked and identified.

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Effects of vaccination against gonadotropin releasing hormone (GnRH) on sexual development and fertility in mammals

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Successful immunization against gonadotropin releasing hormone (GnRH) results in the production of antibodies that bind to and inactivate the native hormone, and thereby suppresses fertility in mammals. Inactivation of GnRH results, in turn, in reduced production and secretion of luteinising hormone (LH) and, to a lesser degree, follicle-stimulating hormone (FSH). In female mammals, the reduced levels of FSH and LH lead to a failure of follicle growth and ovulation, respectively, while in males low LH and FSH levels lead to reduced testis function, e.g. suppressed testicular steroid production, and a resulting fall in both libido and sperm production.

However, because GnRH is a small ‘self’ molecule, it is difficult to induce an immune response sufficient to neutralize the release of GnRH from the hypothalamus. Consequently, vaccines based on native GnRH are only moderately effective, and induction of an acceptable response requires aggressive adjuvants and multiple injections. To solve this problem of low intrinsic antigenicity, we extended the GnRH antigen into a tandem dimer molecule, introduced foreign amino acids and conjugated the resulting peptide to a carrier protein.

Most of the clinical trials of the GnRH immunosterilization vaccine were performed on male piglets, for which there is a practical desire to prevent the development of “boar taint”, a phenomenon induced by testicular steroid hormones. These trials demonstrated that, after two immunisations, testosterone concentrations are reduced to undetectable levels, and the development and function of the genital glands is halted completely. Efficacy of the vaccine in young male piglets is almost 100%, and poorly responding pigs can easily be distinguished by the larger size of their testes and scrotum. In female piglets, vaccination resulted in lowered circulating LH concentrations and a cessation of ovarian function, as evidenced by low inhibin levels and the absence of follicles and corpora lutea on the ovaries.

In sexually mature pony stallions, a primary course of 2 injections was sufficient to reduce testosterone concentrations to baseline values in all 4 treated stallions for the duration of the experiment (13 weeks). In addition, testis function was suppressed in 3 out of the 4 stallions, as witnessed by reduced sperm motility, a lower Johnson score (a measure of spermatogenic activity), a reduction in seminiferous tubule

diameter and a substantial decrease in testis size.

Recently, we modified the GnRH immunosterilization vaccine for the treatment of prostate cancer in men. In fact, it transpired that the prostate cancer vaccine (PCV) was superior to the original immunosterilization vaccine (ISV) for suppressing reproductive function in sexually mature boars and male rats. In both of the latter species, the PCV induced a rapid suppression of serum testosterone levels and atrophy of the genital glands in all treated animals. In the pig study, 3-5 years old boars were immunized twice at a 4-week interval. None of the boars treated with ISV had responded after an initial course of 2 immunizations, whereas two out of four boars treated with PCV exhibited baseline serum testosterone concentrations within three weeks after the second injection. A third immunization caused the serum testosterone concentrations to drop to zero in all four boars in the PCV group. By contrast, a measurable biological response was effected in 3 out of 4 boars in the ISV group only after a fourth consecutive immunization. In the early responding (PCV) boars, GnRH antibody titres rose rapidly and remained high until the end of the study. Twenty-five weeks after the initial immunization, the boars were slaughtered and their testes and accessory genital glands were weighed. Testes of control boars consistently weighed in excess of 550 grams, whereas testes of all boars treated with PCV weighed less than 400 g. By contrast, only three of the boars treated with ISV had testes that weighed less than the control animals and the testes of the non-responder reached 580 g. Examination of seminal vesicle development revealed an even more pronounced effect of successful vaccination. Six out of 8 vaccinated boars (4 PCV and 2 ISV), had seminal vesicles weighing less than 200 g, whereas the seminal vesicles of control boars exceeded 800 g. In addition, libido was suppressed in 3 of the vaccinated boars (2 PCV and 1 ISV), while sperm quality was reduced in 3 other vaccinated animals (2 PCV and 1 ISV). In rats, similar results were observed with circulating testosterone concentrations beginning to drop 2 weeks after the second immunization with PCV (4-week interval between vaccinations) and reaching baseline levels a further 4 weeks later.

In conclusion, immunisation with the modified GnRH tandem-dimer vaccine leads to a rapid suppression of circulating testosterone concentrations. This vaccine, therefore, represents a promising potential treatment for hormone-dependent prostate cancer, and for suppressing fertility.

The feasibility of current options for the management of wild elephant populations

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There is now ample evidence that in game reserves in which elephants are adequately protected from poaching their numbers will increase to the point where they begin to have negative impacts on biodiversity. Initially this may only affect the structural diversity of habitats but as elephant numbers continue to increase, some other species will be lost from the system. Generally, woodlands are changed to grasslands so that all species which are dependant upon woodland habitats may be extirpated.

The decision whether elephant numbers in any reserve should be limited must be based on the management priorities set for the reserve. If biodiversity is a priority, something must be done to limit the elephant population to a level at which their impacts will not have negative consequences for other species. And if the priorities for such reserves have not been clearly defined, management will be directionless and muddled.

In reserves in which biodiversity has been defined as the management priority, what options are available for managing elephants? Three only – translocation, contraception and culling. While the first two non-lethal options seem more ethically acceptable, they have their problems.

Translocation
Opportunities for translocations are now very limited. Most areas in Southern Africa that could potentially accommodate elephants now have them, while those that still can (e.g. Limpopo National Park in Mozambique), will not be able to take the numbers that need to be moved. While this method of population management may seem to be ethically acceptable it can seldom be established with certainty whether family units are moved intact or some members are left behind. The effects of such separation are not yet well understood.

Contraception
Current contraception technology is of limited value for controlling large populations. While porcine zona pellucida (pZP) vaccines have been shown to be effective and acceptable in elephants, oestrogen (oestradiol-17ß) implants caused behavioural aberrations and other unacceptable post-treatment effects. But regardless, the logistics of both techniques preclude their use. To stabilize an elephant population,

75% of all breeding females (i.e. 35% of the total population) must be constantly under treatment. These animals all require “boosters” at specific times, which means they must be individually locatable and therefore fitted with a radio collar. These requirements all push the financial and logistic costs to an unaffordable level for most conservation agencies.

Sterilization has the major drawback of irreversibility. Any population being regulated in this manner would be severely at risk from epidemics of either poaching or disease and its ability to recover would be greatly impaired if most of the adults (females or males) had been permanently sterilized.

A final problem with all contraception techniques is that they cannot reduce a population in the short term. Once a population has been stabilized by contraception, there will be a time lag before natural mortalities will eventually begin to reduce it.

Culling

Culling is the third option and, currently, is the only one that can solve the problems of over-abundance in a large population. However, it is an extremely emotive subject, particularly when applied to elephants. While culling elephants may be an anathema to some, it is usually seen by those concerned with the maintenance of biodiversity as a necessary evil – one which must be used in situations where translocation and contraception are not implementable.

The advantages of culling are that, in large populations, the over-population problem can be addressed successfully. Also, the processed by-products of culling (meat, hides, ivory, etc.) can be sold profitably to provide conservation activities, such as anti-poaching, with much needed funding. Disadvantages? a certain amount of disturbance to nearby related groups, the degree of which will depend on proximity and will be worst when a large group must be split. However, such disturbance is identical to that which accompanies translocation. The impacts come from the disturbances of the operation itself and from the longer-term effects of the loss of bond group or family members.

The major ethical question associated with culling is: Is it morally justifiable to kill elephants? Some Western cultures may find culling ethically unacceptable. But the question must be asked, whose ethics should apply? To a rural African with little access to protein, an average Westerner, or an animal rights campaigner, ethical elephant management will mean very different things.

While many believe that culling is not ethical, it must be understood that the culling option has to be weighed up against the losses of whole populations of other species. Loss of a species from a system will have its effects on biodiversity and the system’s food webs, and such a loss may even mean the complete extinction of that species. These species losses will occur if elephant numbers are not limited by some

means. Therefore one must ask, is it ethical to allow species to be lost from a system when prevention is possible

Report

The First International Workshop on the “Control of Wild Elephant Populations” was convened at the Golden Tulip Hotel in Beekbergen, The Netherlands, during 7th and 8th November 2003. This workshop was initiated by Mr. Paul Fentener van Vlissingen, sponsored by the Prince Bernhard Nature Fund and organised by the Faculty of Veterinary Medicine of Utrecht University. The twenty-one experts that participated in the workshop were drawn from 9 countries and included veterinary scientists, biologists and wildlife managers. A total of 20 papers were presented on a range of topics that included: 1) management strategies and population control mechanisms already in place in major game parks in South Africa, Zimbabwe and Kenya; 2) ongoing experiments in Kruger National Park and Makalale Reserve in Northern Transvaal to suppress conception rates in female elephants by dart-delivered active immunisation against ZP3, the principal sperm binding protein in the zona pellucida; 3) current research programmes in academic institutions worldwide on immunocontraception in female and male large domestic and wild animal species, using various preparations of ZP3 or gonadotrophin-releasing hormone (GnRH) as immunogen and a range of synthetic oil or water based preparations as adjuvant; 4) novel methods of achieving contraception or infertility in pest species, such as the house mouse in Australia and possum in New Zealand, based on the incorporation of a reproductive immunogen into either the genetic material of a species-specific non-lethal virus or into bacterial ghosts; and 5) the actions of specific chemicals on the morphology and functionality of spermatozoa while in the epididymis, and the effects of exogenous androgens and progestagens or GnRH antagonists or vaccines on testicular function and libido in prepubertal and mature men and male animals.

In frank and wide-ranging discussion sessions that followed each group of papers, differing views and strategies were debated rigorously and, wherever possible, a consensus opinion was reached. As a result of the great deal of new information presented and the ensuing discussions, a number of broad conclusions and recommendations were reached:

1. Preliminary experiments to induce contraception in adult female elephants have yielded promising results. Selected females in the large herd of 11,000 elephants within the boundaries of the Kruger National Park and all 18 adult females in the much smaller herd of 60 elephants maintained in the Makalale Reserve, have been actively immunised against ZP3. At the same time, challenges with regard to immunogen delivery, preparation of the optimum adjuvant and recognition of immunised animals for monitoring and revaccination, are being actively investigated. The Workshop is unanimous in recommending strongly that adequate funding and practical help be made available to ensure that further vaccination field trials planned for Kruger Park and other smaller public and private game parks in South Africa and neighbouring Botswana can be undertaken.

2. Despite the considerable success of the relocation strategy used by Kruger

National Park in recent years, and justified optimism that an efficacious method for inducing harmless and reversible contraception in larger elephant herds will be developed in coming years, organised culling remains a necessary method for controlling unwanted increases in, or achieving rapid reductions in, certain elephant populations. Most notably, those populations in Kruger Park, Hwange Park and surrounding areas in Zimbabwe and in clearly defined areas in Botswana need urgent attention. The Workshop recommends that the expertise of the relocation teams in Kruger Park be made available to other game parks and countries in southern and eastern Africa to assist necessary culling operations. The Workshop is also very aware and approving of the considerable potential benefits that may and should accrue to local populations and park management authorities as a result of well-organised culling operations, including the provision of food and cash from the sale of ivory and other by-products.

3. The Workshop was much alarmed to learn of the dangerous elephant overpopulation of Hwange National Park and surrounding areas in Zimbabwe, and of the huge scale “die-off” that will almost certainly occur if drastic action is not taken to reduce the population by some 20,000 animals over the next 2-3 years. The negative effects of such an “elephant holocaust”, in terms of animal welfare and biodiversity of plant and other animal species in the area, are too horrendous to contemplate and the impending disaster simply cannot be allowed to occur. The Workshop therefore urges that every possible and necessary political, financial and logistic step be undertaken urgently to enable a major culling operation in Zimbabwe to relieve this intense overpopulation pressure.

4. The Workshop appreciates greatly the many university, research institute and industry-led research groups worldwide that are actively investigating novel methods for inducing effective contraception in male and female humans and domestic and wild animal species. Much of this work was presented and/or discussed at the Workshop and many useful scientific contacts were made. It is important that these “other species” research groups remain aware of, and interested in, the problems faced in elephant population control and continue in their willingness to share new ideas and techniques with colleagues at the “elephant coal-face”. It is, therefore, essential that non-elephant contraceptive experts remain an active part of the Workshop in future years.

5. In view of the practical and logistical difficulties associated with the identification and location of individual animals, which is a vital step in implementing existing contraceptive methods which require multiple carefully-timed injections of immunogen, the Workshop urges that time and money be devoted to developing and refining a method of multiple time-delayed repeated immunogen delivery within a single dart. The Workshop was also greatly impressed by recent advances in the technique for permanently and specifically ablating gonadtroph cells in the pituitary glands of target species using a single injection of a potent GnRH agonist conjugated to a cytotoxin such as the ribosome inhibiting protein, pokeweed anti-viral protein

(PAP). Such a single-administration, minimally invasive treatment that has the potential to induce permanent sterility in targeted animals could be applicable to wild elephants. For example, it could potentially be employed to ablate reproductive function and suppress oestrous cyclicity in every 3rd or 4th adult female in elephant family groups, thereby lowering overall fertility of the population while maintaining the necessary broad base to the pyramidal family structure. The Workshop thus suggests that, as soon as practically and scientifically possible, this strategy of gonadotroph ablation be tested in a suitable, small game park elephant population.

6. Although a variety of useful treatment regimes for inducing temporary infertility and down-regulating libido in adult males of large animal species like the horse and cow are available, the Workshop does not currently foresee an application of such techniques for contraceptive purposes in large herd situations. Ablation of musth and reproductive function in large mating bulls, even if it were possible, may simply mean that younger, less genetically desirable bulls quickly rise up and assume the mating role within that population. On the other hand, these treatments do offer considerable potential for controlling fertility in small herds, or sexual and aggressive behaviour in problem bulls.

7. Clearly, computer models based on available population growth and demographic data need to be created to accurately predict the number or proportion of animals that need to be removed, permanently or reversibly sterilised in order to maintain numbers in a given elephant population within desired limits for a prolonged period of time. These models should also take into account the fact that the chosen strategy must allow the population in question to retain the flexibility to respond to unpredictable challenges, such as severe drought or disease. Ideally, any new model should also be able to predict the effect of a given contraceptive strategy on family structure and social or sexual interactions.

In conclusion, the First Workshop on the Control of Wild Elephant Populations was a resounding success, both scientifically and socially. A mood of enthusiasm and scientific cooperation pervaded the meeting and there was unanimous agreement amongst the participants that considerable benefits could be gained from a further meeting in 2-3 years time. In the interim, the members are resolved to maintain close collaborative links and to do everything possible to further the aims expressed in the recommendations made above. Warm and very generous appreciation is extended to Mr. Paul van Vlissingen and the Prince Bernhard Nature Fund for their foresight and generosity in creating this useful gathering.



Conclusions

MANAGING AFRICAN ELEPHANT POPULATIONS: ACT OR LET DIE?

In several African countries, elephant populations are growing at an alarming rate, as high as 5-10% per annum. Such population expansion is not sustainable and, despite the huge tracts of land open to elephants, it is already having a devastating effect on a number of unique ecosystems and the biodiversity (plant and animal) within them. To avoid an ecological catastrophe culminating in a mass die-off of elephants, urgent action is needed to reduce elephant numbers. Even then, further long-term population control strategies must be implemented if the apocalypse is to be prevented rather than just postponed. With this in mind, an expert consultation on the "Control of Wild Elephant Populations" was organised by Utrecht University's Faculty of Veterinary Medicine in Beekbergen, The Netherlands on 7th – 8th November, 2003. Twenty-one international experts in fields ranging from reproductive physiology and contraception to population dynamics and game-park management attended the meeting, and came to the following conclusions:

1. In many of the more then 35 African countries that are home to wild elephants, populations are or have historically been endangered by poaching and habitat destruction. However, in several parks where the elephants are well protected, populations are now growing at such a rate that other animal species and plants are being driven to extinction and, due to destruction of the vegetation, a mass die-off of elephant is fast becoming inevitable.

2. The approach to controlling elephant numbers depends primarily on the aim of a given park; i.e. is it primarily an elephant sanctuary or an area dedicated to conserving as great as possible a diversity of species and habitats. If the aim is biodiversity and the elephant population density is already well above the carrying capacity, immediate population reduction is the only answer. Although this could, in theory, be achieved by translocating groups of elephants to new areas, translocation is expensive, time-consuming and logistically complex and, moreover, there are very few areas left to accommodate the large numbers of animals involved. Indeed, in some areas, the numbers of elephants are so large, that the only realistic way of bringing the population under control is culling.

3. Contraceptive techniques have been developed that successfully control population growth in feral horses and are being developed for pest species such as mice and possums. While such anti-fertility treatments are very useful for reducing the rate of population growth in the longer term, they are certainly not suitable for reducing numbers of a long-lived and late reproducing species, like the elephant, in the short term.

4. Techniques exist to exert reversible or irreversible contraception on male and/or female animals. Indeed, there are already encouraging results from field trials of anti-

zona pellucida vaccination of female elephants (a treatment that should block fertilisation) and pilot trials have begun with a GnRH vaccine in male elephants (vaccination should block sperm production and suppress sexual behaviour in males and prevent follicle development and ovulation in females). Further studies are needed to examine the effects, efficacy, reversibility and in-field practicality of these and other putative contraceptives. Ideally, the bulk of this development will be achieved using in vitro or other-species models, since the 4-year calving interval in elephants means that field trials yield meaningful results only slowly.

5. The way in which the presence and behaviour of a dominant bull suppresses reproductive activity in other male elephants and, at least in captivity, can lead to temporary infertility should be investigated. This may lead to novel contraceptive approaches tailored to male elephants.

6. Demographic models need to be developed, using existing and new, targeted field data, to predict the effects of elephant removal and/or contraception on population growth and conservation targets. Field data on the natural factors, such as food resources, that affect population growth will improve the accuracy of these models, which could then be used to identify the best strategy, and the proportion of animals that need to be included, to control a given population.

7. The expert group makes a call for international political will and financial support to:

a. Preserve eco-systems in Africa

· By protective measures for animals and plants,

· By urgent reduction of elephant numbers where needed, through translocation and culling.

· By reducing elephant population growth via fertility control

b. Study population dynamics in elephants and the factors that affect it, so as to enable reliable modelling of the effects of protective and control measures on population growth, social structure and behaviour.

c. Improve existing and develop novel methods of contraception for the elephant.

Disclaimer

The Conclusions are based on the general consensus of opinion reached during the

workshop and may, therefore, not express the personal opinion of all the individual participants.



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Major locus on ECA18 influences effectiveness of GonaCon vaccine in feral horses

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ABSTRACT

Contraceptive vaccines are used to reduce birth rates in wild and feral animal populations. While the immunocontraceptive GonaCon-Equine has proven effective in reducing fertility among female feral horses, there is individual variation in the duration of infertility following treatment. To identify genetic factors influencing the effectiveness of GonaCon-Equine, we conducted a genome-wide association study of 88 mares from a feral population genotyped using the Illumina GGP Equine 70k SNP array. Contraceptive treatment schedules and long-term foaling rates have been recorded for each individual. We used mixed linear models to control for relatedness among mares. We found a significant association ($p < 5 \times 10^{-8}$) with a locus on equine chromosome 18. The most likely candidate genes in this region are *STAT1* and *STAT4*, which are both involved in immune system function. Variation in STAT function could affect the immune response to the vaccine, leading to variation in contraceptive efficacy. Additional SNPs reaching a less stringent threshold of significance ($p < 5 \times 10^{-6}$) were located on other chromosomes near known immune system genes, supporting the hypothesis that variation in immunocontraceptive efficacy can be attributed to genetic variation in immune response rather than fertility genes.

1. Introduction

Immunocontraception has been used effectively to decrease birth rates in many different animal species, wild and domestic, with contraceptive vaccines targeting one of two proteins that are required for reproduction. Some vaccines use porcine zona pellucida protein (PZP) as an antigen (Kirkpatrick et al., 2009). Antibodies to PZP bind to the zona pellucida and block fertilization in immunized females. The second target protein is gonadotropin-releasing hormone (GnRH), a key hypothalamic hormone that regulates synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland.

Immunization against GnRH has been increasingly used to manage wild populations. The National Wildlife Research Center (USDA-APHIS Wildlife Services) developed an immunocontraceptive named GonaCon, which consists of a GnRH peptide combined with an adjuvant to trigger an immune response (Miller et al., 2004). This generates neutralizing antibodies against GnRH, thereby inhibiting FSH and LH synthesis and secretion. In females this vaccine blocks ovulation and estrus, resulting in infertility (Miller et al., 2004). In male white-tailed deer, GonaCon vaccination leads to reduced testosterone levels, reduction of testes size, and inhibition of reproductive behavior (Killian et al., 2005). However, infertility is temporary in both males and females, lasting only as long as GnRH-antibody titers remain high enough to block GnRH activity (Miller et al., 2000; Pinkham et al., 2022). The efficacy of GonaCon in reducing reproduction has been evaluated in a wide variety of mammalian species, such as prairie dogs and fox squirrels, wild boar, feral cats, and elk (Krause et al., 2014; Massei et al., 2012; Powers et al., 2014; Vansandt et al., 2017; Yoder and Miller, 2010).

GonaCon has also been considered for use in feral horse herds. Feral horses on public lands exhibit a high population growth rate with about 20% increase per year (National Research Council, 2013) and cause ecological damage when they reach high densities (Eldridge et al., 2020), necessitating some form of population control. Public sentiment favors contraception as a horse management tool over lethal methods or capture and removal, yet the public often responds poorly to permanent sterilization of horses. Reversibility of the GonaCon vaccine provides an appealing option for management of feral horse populations.

Baker et al. (2018) began research on the long-term effectiveness of GonaCon-Equine in free-roaming feral horses in Theodore Roosevelt

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National Park (TRNP) in 2009, with 25 mares receiving a primary vaccination that fall and a secondary vaccination four years later, by hand-injection at a roundup. Due to the success of the treatment, the study was expanded. To determine optimal reimmunization interval, 40 additional mares in three treatment groups were treated by remote delivery via dart, with the last group receiving their secondary vaccination in 2016 (Baker et al., manuscript in prep). Collectively, the reimmunization treatments were divided into groups based on the interval between primary and secondary injections: 4-year intervals, 2-year intervals, 1-year intervals, and 6-month intervals. A control group was treated with a saline injection on the same schedule as the 4-year group. Foaling rates of these mares were monitored from 2009 through 2021. While the vaccine was shown to be effective in reducing foaling rates, the duration of infertility was variable; some mares had one or two years of infertility while others remained infertile for the duration of the study. Factors influencing individual mares' responses to treatment are as yet unknown.

Individual variation in reproductive rates following immunocontraception could be due to many factors, both environmental and/or genetic. Some genetic components of fertility are known, but many aspects of reproduction are complex and not fully understood. In humans, genes have been identified for some female reproductive disorders/diseases as well as markers contributing to fertility traits such as reproductive lifespan and ovarian function (Gajbhiye et al., 2018). The genetics of fertility traits in dairy cattle has also been a subject of interest, with several candidate markers identified (Ma et al., 2019). However, genes that influence fertility in horses have yet to be identified (Laseca et al., 2021; Raudsepp, 2020).

Variation in efficacy of immunocontraceptives could also be due to variation in genes underlying immune responses. As with fertility, immune responses are complex and difficult to attribute to any single gene. Research into human disease-resistance has demonstrated that epigenomic changes in gene expression play a role beyond purely genetic factors (Yamamoto et al., 2021). In cattle, some quantitative trait loci have been associated with immune response to vaccination (Leach et al., 2012). However, no previous studies have identified genetic factors in response to any immunocontraceptive agent in horses or response to GonaCon in any other species.

Here, we use SNP genotypes to conduct a genome-wide association study (GWAS) in search of loci associated with variation in the long-term effectiveness of GonaCon-Equine vaccine. We hypothesize that genes involved with fertility and/or immune response will impact the duration of infertility following vaccination. This research will improve our understanding of the mechanism by which the vaccine works and inform future use of GonaCon-Equine in other feral herds. More broadly, findings from this study may also provide insight into why vaccine induced immunity persists or wanes over time, which is an outstanding question in immunology (Kennedy et al., 2020).

2. Materials and methods

2.1. Sample and data collection

Hair samples were collected from all study mares during a regularly scheduled gather in 2013. Individual horses were identified by coat color, markings, and conformation. In 2017, tissue samples were collected via biopsy dart from four mares that had evaded the 2013 capture and were included in the later GonaCon treatment groups. We collected an additional 18 tissue samples from young study mares born in 2014 by biopsy dart under research permit number THRO-2020-SCI-0013 and NPS IACUC approval (ND_THRO_McCann_HorseBiopsyDarting_2020.A1). This resulted in a total of 88 samples, which included all treated and control mares that had received both injections (Table 1). We used foaling data as collected by Baker et al. (2018), consisting of annual pregnancy status of all mares for the duration of the study. We also generated pedigrees from records for the herd going back to the 1980 s. Many pedigree relationships of the past two decades have been confirmed through genetic testing, and older relationships were recorded from behavioral observations of familial relationships of mares and their offspring.

Table 1
GonaCon treatment groups, named for time interval between primary and booster GonaCon injection. Control group received saline injections at the same schedule as 4-year group.

Treatment Groups	Number of Individuals
Control	25
4-year	25
2-year	11
1-year	14
0.5-year	13

2.2. DNA extraction and genotyping

Genomic DNA was extracted from hair follicle and tissue samples by the Animal Genetics Laboratory at Texas A&M University using Gentra Puregene Tissue kits (Qiagen) following manufacturer's protocols. Individuals were then genotyped at Neogen Genomics Laboratory (Lincoln, NE) for over 70k SNPs located evenly across the horse genome using the Illumina Equine GGP 70k BeadChip (Illumina Inc., San Diego, CA).

2.3. Data pruning

We performed quality control filtering of the genotypes using SNP & Variation Suite v8.9.0 (SVS) (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com). We removed markers with call rate ≤ 0.95 and with a minor allele frequency (MAF) of 0.05 or less. We removed markers with Hardy-Weinberg equilibrium (HWE) P-values $< 1 \times 10^{-6}$ as recommended by Marees et al. (2018). All samples had a call rate ≥ 0.90 and all but three had a call rate ≥ 0.98, so we retained all individuals due to the already limited sample size. We mapped the remaining SNPs to the horse reference genome EquCab3.0 (https://www.ncbi.nlm.nih.gov/assembly/GCF_002863925.1/). Since our dataset only included females, we retained the SNPs on the X-chromosome. The dataset contained a total of 47,495 SNPs after pruning.

2.4. Data analysis

We conducted a single-locus mixed linear model (MLM) GWAS using SVS's implementation of EMMAX (Kang et al., 2010; Vilhjalmsson, 2012). Infertility following GonaCon booster was treated as a quantitative trait. Because treatment groups had variable durations since the booster, we calculated a normalized measure of infertility as the proportion of years infertile: (# years infertile)/(total # of years since booster). Infertility values ranged from 1 for those mares that exhibited complete infertility to 0 for mares that exhibited complete fertility since the booster (Fig. 1). We included Treatment Group as a fixed effect interaction with genotype in the model. Because many of the mares were related to each other, we computed a relationship matrix in SVS using pedigree data. This kinship matrix was included as a random effect to account for relatedness among mares. The final model can be represented as Infertility = Genotype x Treatment Group + Kinship. The MLM analysis used an additive genetic model and included Bonferroni multiple testing corrections and false discovery rate (FDR) calculations. SVS also estimated narrow-sense heritability (h^2) of Infertility for each model following the method of Kang et al. (2010). We used $p = 5 \times 10^{-8}$ for a genome wide significance threshold but also considered SNPs with a less stringent threshold of $p = 5 \times 10^{-7}$ as suggested by Chen et al. (2021). We referenced EquCab3.0 to identify genes that contained significant SNPs and genes within broader regions of multiple significant SNPs. We

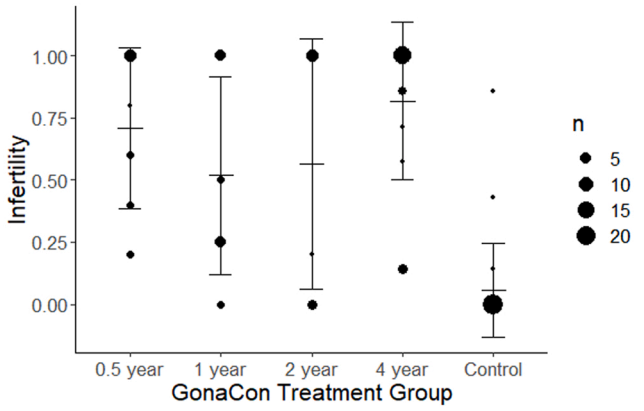


Fig. 1. Infertility after treatment with GonaCon-Equine for mares in each reimmunization group, with error bars indicating mean and standard deviation. Infertility was calculated as the proportion of years infertile following booster injection.

included genes found within 200 kb of significant SNPs as candidate loci (Brodie et al., 2016). To investigate biological function of these candidate genes we referenced The Human Gene Database (www.genecards.org).

We also evaluated several other variations of the MLM to test for potentially confounding variables. To verify that significant genetic associations were due to the treatment group by genotype interaction, we dropped the interaction from the model and only included main effects, summarized as Infertility = Genotype + Treatment Group + Kinship. To determine whether there were any fertility differences among mares prior to assignment to treatment groups, we tested an MLM with infertility prior to GonaCon immunization as the dependent variable. Infertility prior to the first injection was calculated in the same manner as infertility following the booster: (# years infertile)/(total # of years between sexual maturity and initial injection). Sexual maturity was defined as 3 years of age for the purposes of this study, based on age at first foaling typical for the herd. However, a few mares produced a foal as 2-year-olds, so their age at sexual maturity was considered 2 years. The model included the same interaction term and kinship matrix as above, summarized as Infertility Before Injection = Genotype x Treatment Group + Kinship. We also analyzed several models with additional covariates such as age at first injection and injection delivery method (hand injection or dart delivery). These covariates had minimal impact on the results of the simpler model reported below (Fig. S1).

3. Results

The mixed linear model GWAS for infertility after GonaCon treatment using the treatment group by genotype interaction yielded 10 significant SNPs at the $p = 5 \times 10^{-7}$ threshold, with one SNP significant at $p = 5 \times 10^{-8}$ (Table 2). These 10 SNPs were located within a 2.10 Mb region on chromosome 18 of the horse reference genome (Fig. 2). All 10 SNPs were also significant using a very stringent Bonferroni multiple testing correction, with corrected p-values < 0.05. The FDR for each was < 0.005. A Q-Q plot of observed versus expected p-values shows deviation of significant SNPs from values expected when assuming no association, with multiple SNPs deviating from the otherwise linear relationship (Fig. 3). This relationship strongly suggests that genetic variants truly influenced GonaCon effectiveness. Heritability of the Infertility trait was 0.277604 (SE = 0.238108) for this model, while heritability in the model evaluating infertility prior to treatment was 4.53979×10^{-5} (SE = 0.171988).

None of the significant SNPs were located within coding regions of genes, however one was located within an intron of NGFI-A binding protein 1 (*NAB1*) (Table 2). The region of ECA18 with significant SNPs contained ten annotated genes and two uncharacterized protein coding regions (Table 3). Two of those genes, signal transducer and activator of transcription 1 (*STAT1*) and signal transducer and activator of transcription 4 (*STAT4*), have known roles in the immune system.

Inspection of the Manhattan plot in Fig. 2 suggests additional regions of interest on chromosomes 4, 5, and 15, despite SNPs falling below the most stringent thresholds for genome-wide significance. Five SNPs within those regions had p-values $< 5 \times 10^{-6}$ (Table 4) and fourteen genes were located within a 200 kb window around those loci (Table 5). One of the SNPs on ECA4 is located within an intron of the IKAROS family zinc finger 1 (*IKZF1*) gene, which regulates lymphocyte differentiation. The nearest annotated gene to the SNP on ECA5 is protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), which regulates T-cell receptor signaling. There were no annotated genes located within the 200 kb window surrounding SNPs on ECA15.

Results from the alternate models yielded no significant SNPs for infertility. When the treatment group by genotype interaction was dropped from the model, SNPs on ECA4, ECA5, ECA15, and ECA18 were no longer significant (Fig. 4a). In addition, the SNPs identified in Fig. 2 were not associated with infertility in mares prior to their treatment with GonaCon (Fig. 4b). Addition of other covariates (e.g., age at immunization) to the model did not appreciably change the GWAS results, and so were not included in the final model described above (Fig. S1).

4. Discussion

This study demonstrates that genetic variants influenced the response of TRNP mares to GonaCon vaccination. The difference between models including vs. excluding the genotype by GonaCon interaction and models before vs. after GonaCon immunization strongly indicate that genetic variants specifically influence GonaCon efficacy. The lack of significant SNPs resulting after elimination of the interaction between GonaCon treatment and genotype from the model indicates that these associations cannot simply be explained by genotype or by GonaCon vaccination alone. In addition, there were no associations between genotype and infertility prior to GonaCon vaccination. The near-zero heritability of infertility prior to vaccination in contrast with moderate heritability after vaccination also points to genetic variation in

Table 2
SNPs that exhibit significant genome-wide associations with GonaCon response in TRNP mares using a mixed linear model with threshold $P < 5 \times 10^{-7}$.

EVA SNP ID	Chr.	Position	P-Value	-log$_{10}$ (P)	Bonferroni P	FDR	Gene
rs69127755	18	67111422	1.792E-08	7.747	0.0008	0.0008	NAB1
rs69130547	18	68859296	1.166E-07	6.933	0.0055	0.0027	–
rs69130542	18	68836692	1.957E-07	6.708	0.0092	0.0015	–
rs69130541	18	68836582	1.957E-07	6.708	0.0092	0.0018	–
–	18	68760139	1.957E-07	6.708	0.0092	0.0023	–
rs69130536	18	68737439	1.957E-07	6.708	0.0092	0.0031	–
rs69130578	18	69211986	3.058E-07	6.515	0.0144	0.0018	–
–	18	68647709	3.058E-07	6.515	0.0144	0.0021	–
rs69129190	18	68141026	4.022E-07	6.396	0.0189	0.0019	–
rs69129189	18	68125695	4.022E-07	6.396	0.0189	0.0021	–

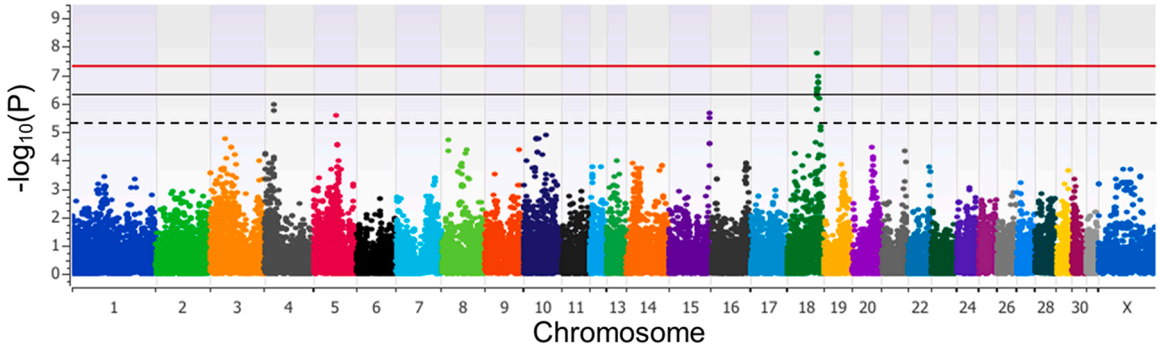


Fig. 2. Manhattan plot of SNP association with infertility in TRNP mares following treatment with GonaCon-Equine. Red line denotes the genome wide significance threshold of $p = 5 \times 10^{-8}$ where $-\log_{10}(P) = 7.3$. Black line at 6.3 denotes the genome wide significance threshold of $p = 5 \times 10^{-7}$. Dashed line at 5.3 denotes the relaxed threshold of $p = 5 \times 10^{-6}$ used in evaluating suggestive loci on other chromosomes.

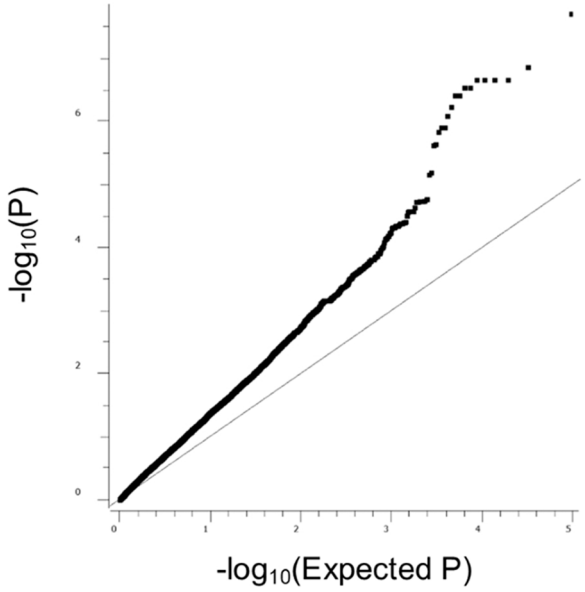


Fig. 3. Q-Q plot of observed versus expected P-values from GWAS for infertility in TRNP mares following treatment with GonaCon-Equine.

Table 3
Genes found within the 2.10 Mb ± 200 kb region on chromosome 18 of EquCab3.0 that contains SNPs with $P < 5 \times 10^{-7}$. Asterisks mark genes with known immune system function.

Chr.	Start	Stop	Gene Name
18	66908950	66981047	MFSD6
18	66984476	67008007	NEMP2
18	67096736	67106335	LOC102148954
18	67099791	67138617	NAB1
18	67177759	67210309	LOC102149070
18	67330485	67410571	GLS
18	67414096	67452831	STAT1 *
18	67464261	67559483	STAT4 *
18	67654558	67834776	MYO1B
18	68053863	68062353	NABP1
18	68189795	68203091	CAVIN2
18	68298154	68523485	TMEFF2

response to the vaccine. Taken together, these findings indicate that individual differences in infertility are the results of genetic variation in the immune response to the GonaCon vaccine. Indeed, genetic variants are a major factor influencing immune responses to pathogens and vaccines in humans (Scepanovic et al., 2018).

The most likely candidate genes to influence GonaCon effectiveness in TRNP mares are *STAT1* and *STAT4* on ECA18. The STAT family of genes are transcription activators with a wide range of functions, some of which include innate and adaptive immune response to a variety of pathogens (Horvath, 2000). Together with Janus kinases (JAK) they form the JAK-STAT signaling pathway by which cell surface receptors trigger transcription in the nucleus (Stark and Darnell, 2012). *STAT1* and *STAT4* variants have been shown to influence disease risk and immune responses in humans (Hedl et al., 2020). *STAT1* is involved in the signaling pathway that activates antiviral responses and reduced activity of *STAT1* is connected to increased infection susceptibility (Katze et al., 2002; Najjar and Fagard, 2010). Mutations in *STAT1* cause different clinical disorders depending on whether the mutations are recessive or dominant and whether they result in *STAT1* deficiency or *STAT1* gain-of-function (Mizoguchi and Okada, 2021). Loss of *STAT1* function leads to increased susceptibility to mycobacterial and viral infection, and gain-of-function is associated with autoimmune disease in addition to increased infection susceptibility (Boisson-Dupuis et al., 2012). *STAT1* plays a key role in inhibition of the IL-17 pathway, which can result in impaired immunity (Liu et al., 2011). The cytokine IL-17 induces tissue inflammation and is produced by Th17 memory cells (Korn et al., 2009), which are themselves a major cause of autoimmune disease in mice and humans when dysregulated (McGeachy, 2013). In horses, *STAT1* was upregulated following infection with African horse sickness virus (Wall et al., 2021). *STAT4*, which is adjacent to *STAT1* on ECA18, is also an important component in immune response to many diseases and is linked to inflammation and T-cell differentiation (Yang et al., 2020).

Genetic variation in *STAT1* and/or *STAT4* function within the TRNP herd could affect the mares' ability to mount a strong and long-lasting immune response to the GnRH peptide. However, the significant SNPs on ECA18 are not found within any of the candidate genes. The Equine GGP 70k chip only included one SNP within an intron of *STAT1* and one SNP within an exon of *STAT4*, neither of which reached the threshold for genome-wide significance ($p = 9.60\text{x}10^{-4}$ and $p = 5.21\text{x}10^{-2}$ respectively). In human response to an influenza vaccine, *STAT1* expression levels were linked to antibody response, with increased antibody titers strongly correlated with higher *STAT1* expression on the first day after vaccination (Bucasas et al., 2011). Thus, we hypothesize that the causal SNP or SNPs in the TRNP mares are likely to influence expression of *STAT1* and/or *STAT4* in basal conditions and/or in response to GonaCon vaccination. Future studies could test this hypothesis by drawing blood from mares and measuring *STAT1* and *STAT4* expression and activation in white blood cells under basal condition and in response to signaling molecules that activate STATs during immunization.

The observation that two of three suggestive loci on other chromosomes also contain genes related to immune function (Table 4) supports our initial hypothesis. *IKZF1* and *PTPN22* have both been implicated in a

Table 4
SNPs on other chromosomes which are above the threshold of $P = 5 \times 10^{-6}$. Asterisks mark genes with known immune system function and parentheses indicate a gene located ~34 kb away from that SNP.

EVA SNP ID	Chr.	Position	P-Value	-log$_{10}$ (P)	Bonferroni P	FDR	Gene
rs69610539	4	20272692	1.141E-06	5.943	0.054	0.0041	DDC
rs69609380	4	20206184	1.832E-06	5.737	0.087	0.0054	IKZF1 *
rs69568794	5	51399392	2.645E-06	5.577	0.125	0.0069	(PTPN22)*
rs69023808	15	92073632	2.305E-06	5.637	0.109	0.0064	–
–	15	92050892	3.260E-06	5.487	0.154	0.0081	–

Table 5
Genes found within 200 kb of SNPs which are above the threshold $P = 5 \times 10^{-6}$. Asterisks mark genes with known immune system function.

Chr.	Start	Stop	Gene Name
4	20122309	20219653	IKZF1 *
4	20258323	20265158	FIGNL1
4	20269062	20359213	DDC
4	20372622	20577702	GRB10
5	51253066	51256536	OLFML3
5	51257521	51306709	HIPK1
5	51319364	51360409	DCLRE1B
5	51327263	51336444	AP4B1
5	51363965	51411556	LOC100064949
5	51431066	51437642	BCL2L15
5	51423450	51492288	PTPN22 *
5	51493896	51531777	RSBN1
5	51534055	51581007	PHTF1
5	51587886	51811736	MAGI3
15	–	–	–

range of immunodeficiencies (Kuehn et al., 2021; Tizaoui et al., 2021). In humans, variants in *IKZF1*, *PTPN22*, and *STAT4* are associated with risk of developing the autoimmune disorder systemic lupus erythematosus (Ebrahimiyan et al., 2018; Hu et al., 2013; Kyogoku et al., 2004; Mohan and Putterman, 2015). There is some evidence of epistasis between *IKZF1*, *STAT4*, and other loci (Dang et al., 2014), and *IKZF1* may be a regulator of *STAT4* in human T cells (Hu et al., 2013). *PTPN22* has roles in both the adaptive and innate immune system as a signal inhibitor of T-cell activation and a signal enhancer promoting production of type I interferon in myeloid cells, respectively (Stanford and Bottini, 2014).

While we think that genetic variation in immune response to GonaCon vaccination is the most plausible hypothesis for variation in contraception, STAT family genes have also been associated with reproductive traits in cattle. *STAT1* and *STAT4* are located together on chromosome 2 in the cattle genome and were within a genome window associated with age at first calving in cattle (Dubon et al., 2021). *STAT1* and the paralog *STAT3* were linked to embryonic survival in cattle (Khatib et al., 2009). Several members of the STAT family, as part of the JAK-STAT pathway, have also been shown to play a role in milk production in dairy cattle (Khan et al., 2020). In horses, STAT genes were found to be differentially expressed in adult versus fetal ovaries (Hall et al., 2017), but not much is known about the association of STAT gene variation with horse reproductive traits. In mice, the JAK-STAT pathway has been shown to play a critical role in normal reproductive development and function. Cytokine receptors activate JAK-STAT signaling in GnRH neurons, which regulate the secretion of GnRH peptide. Knockout of the *JAK2* gene resulted in impaired fertility (Wu and Wolfe, 2012). Thus, altered STAT expression/activity in GnRH neurons could also potentially affect reproductive function in the mares. This hypothesis could be tested through experiments measuring STAT expression and activity in GnRH neurons in a rodent model of GonaCon immunocontraception.

Further work needs to be done to identify the specific locus responsible for the genetic association as well as the mechanism causing phenotypic variation in the long-term efficacy of GonaCon. Future studies should use a higher density of markers on a SNP chip or whole genome sequencing to fine map causal variant(s). If possible, future studies should also aim to use unrelated individuals to minimize the

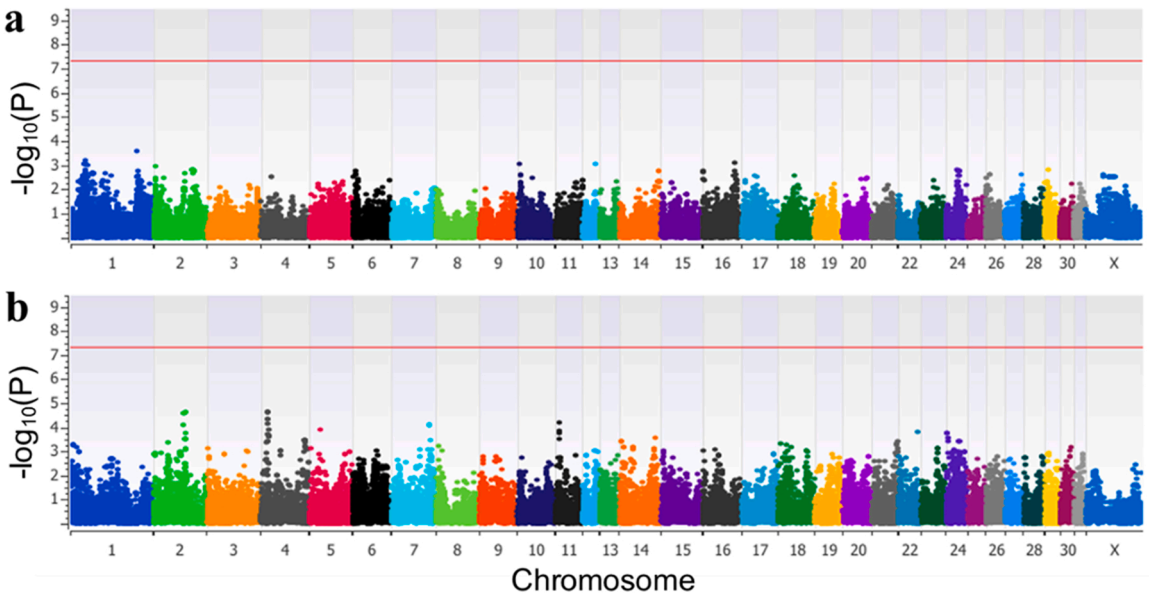


Fig. 4. Manhattan plots from other MLMs to test for SNP association with infertility in TRNP mares following treatment with GonaCon-Equine. Red line denotes the genome wide significance threshold of $p = 5 \times 10^{-8}$. a) SNP significance in infertility after GonaCon treatment when treatment group is included as a covariate rather than an interaction with genotype. b) SNP significance when infertility before first injection is used as dependent variable.

potential impact of family and population structure. Such studies would shed light on whether cause variants are within coding sequences (affecting protein sequence, structure, and function) or in cis-acting regulatory regions (affecting gene expression).

Given that this vaccine induces an immune response to the body's own reproductive hormone, it is reasonable to assume that genes in immune pathways are involved in either the initial response to GonaCon injection or the maintenance of immunity over time (i.e., Th17 memory cells). Although other loci did not cross the more stringent genome-wide thresholds of significance, the presence of other immune related genes within noticeable peaks suggests that genetic variation across multiple loci affects the immune response to GonaCon vaccination.

Finally, there are additional factors not explored here which may also be influencing efficacy of GonaCon injections. Baker et al. (manuscript in prep) evaluated differences between injection method and found that the two-year dart injection treatment group had a more variable response than the 4-year hand injection treatment group, though the difference was not statistically significant. Neither of the other dart injected groups (0.5-year and 1-year) were significantly different from the hand injected group. The use of darts does have the potential for greater tissue trauma at the injection site due to the velocity of both the dart impact and the expulsion of the dart's contents (Hampton et al., 2021). Differences in dart placement, ballistics, injection force, the animal's behavioral response, and individual physiological variation could influence the response to vaccine delivered via darting. In contrast, the placement and force of the injection can be more carefully regulated when vaccine is administered by hand. Baker et al. hypothesize that variation in contraceptive success between treatment groups is due to booster interval rather than injection delivery method; however, it is possible that tissue trauma caused by darting may influence the strength of the immune response to vaccination.

4.1. Implications

Our findings have important implications for population management plans, which should be aware of a significant genetic influence on the effectiveness of GonaCon in reducing foaling rates in feral mares. Over multiple generations the continued use of an immunocontraceptive within a population could result in unintended artificial selection for alleles that decrease responsiveness to the vaccine, reducing its effectiveness over time and potentially weakening the immune response to pathogens (Cooper and Larsen, 2006; Magiafoglou et al., 2003). Those managing feral horse herds should take into consideration their long-term goals for population management. Immunocontraception will likely need to be used in combination with other management techniques, balancing the benefits of slowing population growth with the potential for reduced efficacy of the vaccine over time.

While GonaCon immunocontraception is currently effective, selection for a reduced immune response to GonaCon may necessitate new approaches in the future. The genetic variants associated with GonaCon response may or may not affect response to the immunocontraceptive PZP, which is prepared using a different adjuvant to trigger immune response (Naz and Saver, 2016). A similar GWAS could be performed on mares treated with PZP to test whether the same loci are associated with PZP response. If PZP response is associated with different genetic variants than those identified in this study, selection pressure could be reduced by alternating GonaCon and PZP use in the population.

4.2. Conclusions

Genetic variants are influencing individual variation in infertility duration following treatment of feral horses with GonaCon. Rather than fertility-specific factors, this variation in immunocontraceptive efficacy can be attributed to genetic variation in immune response.

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Conflict of Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jri.2022.103779.

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Remotely delivered immunocontraception in free-roaming feral burros (*Equus asinus*)

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Regulation of local overpopulations of free-roaming feral equids is in demand worldwide for ecological balance and habitat preservation. Contraceptive vaccines have proven effective in feral horses, which breed seasonally, but no data are available for equids such as the burro, which is reproductively active all year round. In the present study, 27 individually identified female feral burros (*Equus asinus*) roaming free in Virgin Islands National Park (St John, US Virgin Islands; Lesser Antilles) were remotely treated with pig zonae pellucidae (PZP) vaccine. Between January and May, 16 burros were darted with a 1 ml emulsion of PZP plus Freund's adjuvant. Ten to twelve months later each treated burro was given a single booster injection of PZP plus adjuvant to maintain contraception through a second year. Eleven adult untreated jennies served as controls. Beginning one year after initial vaccination, these burros were monitored for pregnancy and foal production. Collection of data to determine treatment effect was not begun until 12 months after initial treatment to ensure that pregnancies existing before vaccination were not included. Pregnancy was assessed using previously validated methods for steroid metabolite measurement in fresh faecal samples. None of the PZP-treated burros produced foals between 0 and 12 months after the last inoculation. One PZP-treated burro tested positive for pregnancy at 10 months after the final inoculation. During this same period, six of 11 untreated burros tested pregnancy-positive, and four were observed with foals. There was no difference in pregnancy rates among treated, control and randomly sampled jennies between 12 and 24 months after the last inoculation. The results demonstrate that, in free-roaming feral burros that are reproductively active all year round: (1) burros can be accessed for remotely delivered PZP vaccination; (2) PZP contraception is effective; (3) PZP contraception is reversible; and (4) pregnancy can be reliably detected by faecal steroid analysis.

Introduction

Reducing fertility among free-roaming horses (*Equus caballus*) and feral burros (*Equus asinus*) has been the goal of numerous studies in the United States over the past two decades. Initial experiments focused on contraceptive steroids which were delivered as injectable, sustained-release (microencapsulated) compounds (Kirkpatrick *et al.*, 1982; Turner and Kirkpatrick, 1982) or Silastic® implants (Plotka *et al.*, 1989). All of these methods resulted in various degrees of pharmacological success but the microencapsulated steroids required extremely large doses and the Silastic® implants required restraint and surgery. In addition, there is concern about steroid-induced pathologies, alteration of behaviour, and the passage of steroids through the food chain when they are used to prevent conception in free-roaming animals (Turner and Kirkpatrick, 1991).

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An alternative to steroid-induced fertility control in equids is immunocontraception. Solubilized pig zonae pellucidae (PZP) injections delivered remotely inhibited fertility in captive feral mares (Liu *et al.*, 1989), and free-roaming wild mares (Kirkpatrick *et al.*, 1990a). In a follow-up experiment, 14 previously treated mares were given a single PZP booster inoculation, and 13 of the 14 failed to produce foals (Kirkpatrick *et al.*, 1991a). These studies also showed that immunosuppression, which usually accompanies pregnancy in mammals, does not interfere with the contraceptive effects of the vaccine and that the vaccine is safe for use in animals that are already pregnant.

Another advantage of the PZP vaccine is the protein nature of the contraceptive antigen. This characteristic precludes the possibility of passage of the antifertility agent through the food chain. In any realistic management by contraception, some treated animals will die from natural causes and a variety of scavengers will feed upon the carcasses. Protein, unlike steroids (particularly synthetic steroids), cannot be passed

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along intact to the predators' tissues and cannot accumulate therein. This is an important environmental consideration where large-scale management of wild or feral equids is contemplated (Turner and Kirkpatrick, 1991).

The success of PZP vaccine in suppressing fertility appears to be via its ability to inhibit fertilization (Sacco *et al.*, 1984). The PZP consists of four glycoproteins. At least one of these, ZP3, is the receptor molecule for sperm surface molecules (Florman and Wassarman, 1985). Antibodies raised against PZP are thought to block the sperm receptor sites on the equine zona pellucida, possibly by steric hindrance (Skinner *et al.*, 1984), therefore preventing fertilization.

An equid that has not been tested for effectiveness of PZP contraception is the burro. This hardy equid, with a gestation of 11 months, has been a successful reproducer in many harsh environments (McKnight, 1958), and is a problem feral animal in several parts of the world including North America, Australia and Africa (Woodward, 1979). Presently there is a feral burro population inhabiting the island of St John, United States Virgin Islands, which is the site of Virgin Islands National Park. The burros in this population, locally referred to as 'donkeys', are *Equus asinus*, and no mules are known to be present. The population is reportedly in excess of 250 and is expanding (Nellis *et al.*, 1986). The circumstances of fixed land mass and expanding populations, both human and burro, have raised interest on the part of the National Park Service to investigate means of regulating feral burro numbers on St John. This situation offered an opportunity to evaluate PZP immunocontraception in an equid that is reproductively active throughout the year (Nellis *et al.*, 1986). An additional opportunity available with this burro population was to determine whether pregnancy could be detected via measurement of concentrations of specific steroid metabolites in faecal samples. Previous studies have shown that pregnancy is consistently associated with high concentrations of oestrone sulfate, a group of progesterone metabolites, and total oestrogens in extracts of fresh faeces in free-roaming feral horses (Kirkpatrick *et al.*, 1990b, 1991b) and in several other species (Loskutoff *et al.*, 1982; Lasley and Kirkpatrick, 1991).

The goals of the present study of immunocontraception of feral burros inhabiting Virgin Islands National Park (VINP) were to determine: (1) the accessibility to feral burros for remote vaccination; (2) the deliverability of PZP vaccine to feral burros by dart; (3) the contraceptive effectiveness of the PZP vaccine in feral burros; (4) the reversibility of the contraceptive effect in feral burros; and (5) the capability for pregnancy testing of feral burros through faecal steroid measurement.

Materials and Methods

Study area

The study was carried out on the Caribbean island of St John in the US Virgin Islands, a part of the Lesser Antilles island group, located at 18°20'N latitude and 65°45'W longitude. St John is approximately 11 km × 5 km, covering an area of 5180 ha. The majority of the burros included in the study inhabit Virgin Islands National Park, which, in somewhat checkerboard fashion, comprises 57% of the land area of St John. The climate is relatively constant throughout the year with steady trade winds, temperatures normally ranging from 18° to 32°C, and an average 75% humidity. The topography is rugged, characterized by deep valleys, high ridges and several major drainage basins. Sixty-three per cent of the island is covered by four types of dry evergreen forest, and the remainder is covered by moist forest, vegetation secondary to recent human activity, pasture and urban development. Burros tend to be concentrated in basin areas, but can be found in every habitat type, including urban areas. The drainage basins contain fresh and brackish water ponds, which the burros use for drinking water.

Burro identification and access

The present study used 27 adult female burros ranging in weight from approximately 90 kg to 115 kg. Sixteen of these were treated with contraceptive, and 11 were untreated controls. Half of the control jennies were in the same family groups as treated jennies. Individual burros were identified on the basis of coat colour and shading, body and head size and shape, and unique markings or permanent scars (Turner *et al.*, 1981). This method of identification was initially time intensive but proved highly reliable and consistent across the study period. Access to burros to provide treatment via remotely delivered dart was accomplished by the establishment and regular use of bait stations. These 'stations' were merely ground sites along paths frequented by burros, usually in flat areas and within 10 m of brushy sanctuary. A mixture of grain, hay and molasses was placed at the sites daily. Regular use of these bait stations occurred within 2 weeks.

Pig zonae pellucidae vaccine preparation

The pig zonae pellucidae vaccine was prepared as described by Liu *et al.* (1989) and stored frozen in 0.5 ml aliquots until used. Burros were given an initial one- or two-injection PZP treatment and, after 10–12 months, were given a one-injection PZP booster treatment. Initial treatment consisted of: (1) two separate injections (3 weeks apart) of a 1.0 ml emulsion, containing 65 μg PZP plus Freund's complete (first injection) or incomplete (second injection) adjuvant ($n = 13$); or (2) a single injection containing 130 μg PZP emulsified in Freund's complete adjuvant. The PZP for the single injection was present in two forms: 65 μg unsequestered and 65 μg sequestered in 10–50 μm diameter microspheres of biodegradable lactide and glycolide in a 1:1 ratio ($n = 3$). The polymer microspheres were prepared by R. Linhardt and D. Flanagan (University of Iowa) using a coacervation method described by Wang *et al.* (1991). Release rates were projected to be continuous across 4 weeks, with greatest release in weeks 1 and 4 (Wang *et al.*, 1990). Booster treatment format consisted of a single injection of 65 μg PZP (0.5 ml) emulsified with 0.5 ml Freund's incomplete adjuvant.

Each treatment was administered remotely by a dart fired from a rifle. The most reliable delivery was obtained using the Pneu-Dart system (Williamsport, PA), which uses a .22 caliber rifle with scope and powder-charged, 1.0 cc, self-injecting darts. All darts were recovered after injection.

Pregnancy testing and foal counting

All burros in the study were monitored for effectiveness of treatment and reversibility of treatment as determined by foal counts and by pregnancy testing. Collection of data to determine treatment effect was not begun until 12 months after initial treatment to ensure that pregnancies existing before vaccination were not included. Collection of data to determine reversibility of treatment effect was between 12 and 24 months after the last treatment. Pregnancy was assessed via measurement of concentrations of total faecal oestrogens (TFE), oestrone conjugates (E_1C) and immunoreactive pregnanediol-glucuronide-like metabolites (iPdG) in extracts from fresh faeces (Kirkpatrick *et al.*, 1991b). Individually identified treated and control jennies were monitored until they defecated, and samples were immediately collected and kept frozen until assayed. Pregnancy data were generated for up to five faecal collection periods, which were approximately 12, 21, 24, 36 and 48 months after initial PZP vaccination. Pregnancy data are presented as means ± SEM. Statistical hypothesis testing for foaling data used binomial probability distribution (BPD) and, for pregnancy-test data, used Student's *t* test or Tukey's test for multiple proportions (Zar, 1984).

Results

Concentrations of steroid metabolites indicative of pregnancy were compared between burros that produced foals and burros that did not produce foals during the period from February 1993 to February 1994 (Table 1). Two non-foaling burros, one PZP-treated and one control, showed conflicting steroid results (pregnant versus nonpregnant) among sample periods. It is possible that a pregnancy occurred between samplings, but no foals were produced by these burros during the sampling period. The average concentrations (ng g^{-1} faeces ± SEM) of faecal steroids in samples from nonfoaling burros ($n = 10$) were 15.4 ± 1.6 for TFE, 24.2 ± 1.8 for E_1C, and 849.9 ± 289.1 for iPdG. In contrast, burros that produced foals ($n = 6$) exhibited average faecal steroid concentrations during pregnancy of 286.5 ± 100.2 for TFE, 145.4 ± 57.6 for E_1C, and 2950.7 ± 160.7 for iPdG. The differences between foaling and nonfoaling burros were significant ($P < 0.05$, Student's *t* test) for all three steroid measures. In PZP-treated and control burros for which reproductive status was known for at least one full gestation period ($n = 18$), pregnancy test data correlated 100% with eventual presence or absence of foals.

At the beginning of the study (before any treatments), six of the 16 burros scheduled for PZP treatment had offspring ranging from approximately 2 to 12 months of age. By 1 year after treatment, when monitoring for treatment effect was begun, an additional four jennies had produced foals. In the period from 12 to 24 months after PZP treatment, none of the 16 treated burros was observed with a foal, and one burro tested positive for pregnancy at 22 months after immunization (Table 2). In contrast, four of the 11 control burros were observed with offspring, and six (including three of the above four) of the 11 tested positive for pregnancy during this period. In the four jennies that had been given a booster inoculation after 1 year, there were no positive pregnancy tests and no foals were produced. The proportions of burros producing foals in the PZP-treated versus control groups were different ($P < 0.01$; BPD) in the 11–24 months after treatment. In addition, the proportion of burros producing foals before treatment and during the test period for treatment effectiveness (12–24 months) was different ($P < 0.01$; BPD). All burros in the study appeared subjectively to remain healthy for the entire study period, based on physical appearance, demeanour and general behaviour as indices of condition.

Reversibility of treatment effect was assessed by faecal pregnancy testing only. For reversibility testing we were able to locate only 13 of 16 treated burros, and six of 11 control burros. Six of 13 (46.1%) PZP-treated and three of six (50.0%) control burros tested pregnancy positive (Table 3). Faecal samples were also collected from 33 randomly chosen jennies that were not in the study. Fifteen of these 33 jennies (45.5%) tested pregnancy positive.

Mating behaviour, which included courtship, female oestrous behaviour, and male mounting and copulatory behaviour (intromission with or without ejaculation) were observed on 21 occasions during the study period. No subjectively discernible differences in reproductive behaviour were observed between PZP-treated and control burros. Breeding was recorded in all months studied. Across 3 years, the approximate frequency of reproductive behaviour occurrence per 100 h of observation was 7.5 in January, 7.5 in February, 12 in April and 4.5 in November. Observations were not made in other months.

Table 1. Correlation of foal production and total faecal oestrogens (TFE), oestrone conjugates (E_1C) and progesterone metabolites (iPdG) concentrations in feral burros

		Faecal steroid concentration (ng g^{-1} dry mass ± SE)		
Condition[a]	*n*	TFE	E_1C	iPdG
Burros that produced foals[b]	6	286.5 ± 100.2	145.4 ± 57.6	2950.7 ± 160.7
Burros that did not produce foals	10	15.4 ± 1.6	24.2 ± 1.8	849.9 ± 289.1

[a]Samples were collected over 12 months.
[b]Samples were collected from 3–9 months before foal was first observed.

Table 2. Effect of pig zonae pellucidae (PZP) immunocontraception on the incidence of pregnancy in free-roaming feral burros

Condition	*n*	Number (%) of burros that were pregnant
PZP-treated (2 injections)	13	0 (0)[a]
PZP-treated (1 injection)	3	1 (33.0)[b]
Untreated control	11	6 (54.4)[b]

Pregnancy was determined via measurement of concentrations of total faecal oestrogens (TFE), oestrone conjugates (E_1C) and progesterone metabolites (iPdG) in extracts of faecal samples obtained from individually identified burros. Samples were taken 0–12 months after the last PZP treatment.
Values with different superscripts are significantly different from each other; $P < 0.05$ Tukey's test for multiple proportions.

Table 3. Reversibility of pig zonae pellucidae (PZP) immunocontraception in free-roaming feral burros

Condition	*n*	Number (%) of burros that were pregnant
PZP-treated[a]	13	6 (46.1)
Untreated control	6	3 (50.0)
Random sampling	33	15 (45.5)

Pregnancy was determined via measurement of concentrations of total faecal oestrogens (TFE), oestrone conjugates (E_1C) and progesterone metabolites (iPdG). Samples were taken 12–24 months after the last PZP treatment.
No significant differences across groups; $P > 0.05$, Tukey test for multiple proportions.
[a]Values for jennies given one injection and two injections were pooled, since pregnancy incidence did not differ in these groups.

Discussion

The results of this study demonstrate that PZP immunocontraception can be delivered to free-roaming feral burros, is highly effective in preventing pregnancy in this species, and is reversible in its effect. As stated previously, the first 12 months after immunization were not included in the assessment of treatment efficacy on the premise that some burros were already pregnant at immunization time. This turned out to be the case for four burros which foaled in the 12 months following immunization. The foals were nursed and grew normally. Since the gestation period is approximately 11 months in burros (McKnight, 1958), these pregnancies had begun prior to the PZP treatment. Thus, in burros, as in other species studied to date (Turner and Kirkpatrick, 1991), PZP contraception does not disrupt existing pregnancies.

The two-injection protocol was more effective than the single-injection, controlled-release PZP protocol in preventing pregnancy. These results are similar to contraceptive effects of the two-injection PZP protocol reported for horses (Kirkpatrick *et al.*, 1990a) and for white-tailed deer (Turner et al., 1992). The refinement of a high-efficacy, single-injection treatment, which is underway, will be necessary before immunocontraception can be used for routine management of burros.

It appears that burros on St John exhibit reproductive behaviour throughout the year. However, the data from the present study showed that breeding frequency is greater in spring than in other seasons. November was a breeding nadir among the months studied. Assuming that the contraceptive effect is of similar duration across treated individuals, the year-round nature of burro reproduction on St John makes it necessary to consider spreading contraceptive treatment across the year. This would prevent the bunching of foal production at one time of year, and ensure a more natural temporal pattern in foaling.

Obtaining access to the burros for treatment was accomplished with high success by the use of bait stations. It was necessary to maintain a regular daily baiting schedule to ensure regular return of burros to the site, and even with a regular baiting schedule, almost 40% of the burros were not daily visitors. However, over 4–5 days, most study burros would visit their respective bait stations at least once. While almost all burros consumed bait when it was available, a definite pecking order developed regarding consumption of limited bait. At a given station, a few burros would eat the bait while the remainder of the group watched. Placing two or three piles of bait several metres from each other permitted access for more individuals.

Burro family groups appeared to prefer certain limited areas (home ranges) of the island and showed fairly regular patterns of movement within these areas. This is consistent with the findings of Nellis *et al.* (1986). Among burros in the present study, each home range included at least one drainage basin with a clearing, fresh water supply, and at least 1 km of dirt or paved road. Some burro groups moved back and forth regularly between two different drainage basins and were observable in the clearings. Many road areas were lined with vegetation that appealed to burros, and they were often observable along the roads. All except two of the jennies in the study remained throughout the 3 year study within the home range where they were first observed. The home-range stability and frequent use of roads and bait stations by burros facilitated the determination of burro identification, movement patterns and habits.

The use of counts of young offspring has been a classic approach to determining fecundity (Seber, 1982; Wolfe *et al.*, 1989). Unfortunately, counts of young can often yield underestimates of reproductive rates and productivity, since fetal losses and neonatal losses, respectively, may be missed (Berger, 1986). On St John, the jennies rarely bring young foals out of the dense bush. Most foals are not seen until they are 2–4 months of age. Thus, counting of foals is not an optimal method for assessing contraceptive effectiveness in St John burros.

In contrast, burro pregnancy testing via measurement of steroid metabolites in faeces appears to be reliable. The differences in faecal steroid concentrations between pregnant and nonpregnant burros were large, ranging from an average of 3.5-fold for iPdG to 18.5-fold for TFE. These differences were similar to those reported in horses (Kirkpatrick *et al.*, 1990b, 1991b). All burros that produced foals had previously tested positive for pregnancy, and none of the burros that tested negative for pregnancy have produced foals. This

reliability in testing for pregnancy in burros suggests potential usefulness for assessing various aspects of burro reproduction, including population fertility rates, fetal loss rates, seasonal reproductive patterns and individual oestrous cycles. In two burros showing both pregnancy-positive and pregnancy-negative steroid data across their samples, the pregnancy-positive data were late in the sample period. These burros may have become pregnant between early and late samples.

In summary, the present study has demonstrated in free-roaming feral burros on St John, which breed all year round that: (1) burros are accessible for PZP treatment by use of bait stations; (2) PZP vaccine can be remotely delivered by dart; (3) PZP vaccine is effective in preventing pregnancy; (4) PZP treatment effect is reversible; and (5) pregnancy can be detected reliably by faecal steroid analysis.

The execution of this project has involved a cooperative effort on the part of the Virgin Islands National Park, The Humane Society of the United States, the Medical College of Ohio, and the Virgin Islands Environmental Resource Station of the University of the Virgin Islands. Special thanks are accorded T. Kelley and J. Bjork of Virgin Islands National Park for their project coordination efforts and field assistance on site. Funding of the project was undertaken by Virgin Islands National Park and the Humane Society of the United States. Special thanks to M. Bernoco for preparation of the PZP vaccine and to E. V. Berkeley for faecal steroid analysis.

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IMMUNOCONTRACEPTION LIMITS FOAL PRODUCTION IN FREE-ROAMING FERAL HORSES IN NEVADA

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Abstract: We determined contraceptive effectiveness and duration of effect after porcine zona pellucida (PZP) vaccination in free-roaming feral horses in Nevada. We captured, freeze-branded, treated and subsequently released 132 adult feral mares. Treatment consisted of 1 or 2 injections (1 month apart) of vaccine emulsion consisting of aqueous PZP and Freund's complete adjuvant (Sigma, St. Louis, Mo.; FCA; inj. 1) or Freund's incomplete adjuvant (FIA; inj. 2). We gave controls saline and Freund's adjuvant as above. Among 1-injection mares, we gave one group a single dose of raw antigen, and we gave the other group raw antigen plus a second dose of antigen sequestered in controlled-release microspheres. We also studied 63 untreated mares. We administered inoculations in December 1992, and monitored the mares via fecal analysis for pregnancy and via helicopter and ground survey for foal production through September 1994. We determined pregnancy via measurement of estrone sulfate, progesterone metabolites and total estrogens, in fresh feces collected from the ground. Among antigen-treated mares, reproductive success across 1 year was 4.5% (2-inj.), 20.0% (1-inj. + microspheres) and 28.6% (1-inj., no microspheres). Reproductive success in placebo (55.0%) and untreated (53.9%) mares was significantly greater than in treated groups. The following year, without further treatment, reproductive success was 44.0% (2-inj.), and 54.5% (untreated) and these values were not different. Data from other groups were insufficient for comparison. The results of this study confirm the feasibility of capturing large numbers of mares in the field for treatment and demonstrate: (1) 1-year contraceptive efficacy of several PZP vaccine protocols, (2) greater effectiveness in 2-injection protocols than in 1-injection protocols, and (3) a return to fertility in the second year after vaccination.

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Key words: antibody titers, contraceptive vaccine, controlled-release, equids, feral horses, fertility, horse immunocontraception, mares, microspheres, population control, reversibility.

Since the late 1970s, fertility control has been investigated as a potential tool for regulating feral horse (*Equus caballus*) numbers. In early studies, various steroids were the focal contraceptive agents, with controlled-release androgens used in stallions (Turner and Kirkpatrick 1982, Kirkpatrick et al. 1982) and estrogenic and progestagenic steroids used in mares (Plotka et al. 1988, 1992). Despite contraceptive efficacy obtained with some steroids, interest in these agents waned in the face of undesirable behavioral effects and potential environmental hazards of such steroids (Turner and Kirkpatrick 1991, Kirkpatrick and Turner 1991). In 1987, the antifertility effect of a PZP vaccine was tested successfully in free-roaming feral mares on Assateague Island National Seashore (Kirkpatrick et al. 1990*a*). Previously this PZP vaccine had prevented pregnancy in 13 of 14 domestic or captive feral mares (Liu et al. 1989). In the Assateague study the dart-delivered vaccine was >95% effective and had no apparent effects on pregnancies in progress, the health of offspring, or the behavior of treated mares.

The effectiveness of the vaccine appears to reside in the antigen-stimulated production of anti-PZP antibodies in the vaccinated animal. These antibodies bind to the zona pellucida surface of the recipients' ovulated egg, presumably preventing sperm attachment (Liu et al. 1989). In ungulates studied to date, 2 separate exposures to PZP in one month have been used to obtain maximal, sustained anti-PZP titers for the first year of contraception (Kirkpatrick et al. 1990*a*, Turner et al. 1992*a*). Thereafter, a single annual booster injection has been sufficient to maintain fertility from year to year (Kirkpatrick et al. 1991*a*, 1992*a*).

Because it is impractical on a management scale to give a 2-injection sequence to free-roaming wildlife, the potential use of polymers that permit a controlled, gradual, or pulsed release of the vaccine has been investigated. One

approach involves forming a homogenous mixture of the drug with a biodegradable nontoxic material, in the form of microspheres (Eldridge et al. 1989, Wang et al. 1990). Upon intramuscular injection and contact with tissue fluids, the biodegradable material erodes and releases the drug over a predetermined period of time (Wang et al. 1991). One goal of the present study was to compare the efficacy of PZP vaccine administered as 2 separate injections with the efficacy of PZP vaccine administered as a single injection with and without a controlled-release PZP component.

Federal agencies have considered contraception for a potential management role in regulating some Western feral horse populations. While barrier-island feral horses and Western feral horses are the same species, there are many regional differences in horse behavior and habitat (Turner and Kirkpatrick 1982, Keiper and Houpt 1984, Berger 1986, Rutberg 1990), as well as agency differences in horse management techniques (T. Pogacnik, Bur. Land Manage., pers. commun.; B. Underwood, Natl. Park Serv., pers. commun.). Techniques for accessing and contracepting barrier-island horses may not be effective with Western horses. Therefore, an additional goal of the present study was to determine field accessibility to Western feral horses for PZP contraception.

We thank: M. Bernoco for PZP preparation and anti-PZP antibody titer analysis, and B.L. Lasley for E_1C antibodies; U.S. Bureau of Land Management (BLM) personnel and associates for the gathering, holding and releasing the horses, with appreciation to W. Baker, R. Brown, D. Cattoor, B. Dawson, C. Heaverne, J. Hicks, K. McKinstry, S. Luttrell, T. Pogacnik, M. Ravnikar, R. Sanford, J. Smith, K. Walker, and F. Wyatt. This study was performed under the guidelines of (1) BLM-approved procedures for the gathering and handling of feral horses as detailed in the Capture/Removal Plans for the Antelope and Antelope Valley Herd Management Areas, and (2) animal welfare protocol #91–58, approved by the Medical College of Ohio. Primary funding for this study was provided by BLM Cooperative Agreement #1422F950A20002, U.S. Department of Interior, with additional support by the Humane Society of the United States.

METHODS

Experimental Design

The study examined the antifertility effectiveness and duration of effect of 3 variations of injected PZP contraceptive vaccine in comparison to placebo and untreated control conditions. We (the research team) randomly assigned to groups, adult mares previously identified by freeze-branding. Investigators inoculating the animals were unaware of the content of a given injection. From previously identified individuals, we determined treatment effectiveness and duration of effect by foal counts and pregnancy testing of fecal samples collected upon defecation. Mare groups consisted of: (1) 2 separate injections 4 weeks apart of vaccine containing adjuvant and native PZP, (n = 60); (2) 1 injection of vaccine containing adjuvant and native PZP (n = 21); (3) 1 injection of vaccine containing adjuvant and native PZP plus an additional, controlled-release PZP (no controlled-release adjuvant) dose (n = 22); (4) 2-injection placebo (adjuvant and saline, without PZP) (n = 19), and (5) 1-injection placebo (n = 10). In addition to these groups, we tested for pregnancy or observed for presence of a foal 63 unmarked and untreated mares (that had not been captured) during the study. To prevent duplication of data collection among these 63 mares, we identified the mares by coat color, face markings, and by association with other horses in their family band (Turner et al. 1981). Contraceptive treatment was administered in December 1992. We collected fresh feces for pregnancy testing in September–October 1993 and 1994. We counted foals in May, August, September, and October of 1993 and 1994.

STUDY AREA

The study area encompassed about 1,825 km^2 in northeastern Nevada centered at 40°15′N and 113°30′W near the Dolly Varden Mountains. Elevations ranged from 1,600 to 2,400 m. Vegetation on the study area was composed largely of shrub-steppe communities with sagebrush (*Artemisia* sp.), rabbitbrush (*Chrysothamnus* sp.), and bitterbrush (*Purshia tridentata*) dominating. Areas of pinyon pine (*Pinus edulis*) were present at higher elevations. Average annual precipitation was 25 cm at 1,500-m elevation, with increasing amounts (mostly as snowfall) at higher elevations. Water was available from permanent springs scattered throughout the study area. The northern and southern portions of the study area were administered by the Elko and Ely districts of the Nevada BLM.

Horse Population

The study area was inhabited by about 700 horses with a harem band social structure (Turner et al. 1981, Berger 1986). The peak breeding period was May and June, and the peak foaling period was April and May. We subjectively estimated the physical condition of the mares according to the condition scale (1–10) developed by Henneke et al. (1983). Mare condition ranged from fair (3) to very good (7) during the study. We rated well-fleshed animals with a sleek coat in very good condition. We rated animals with noticeable ribs, mild hip and shoulder bone protrusion and low quality coat in fair condition.

PZP Vaccine Preparation

We prepared PZP from porcine ovaries (Liu et al. 1989). The vaccine consisted of 0.5-cc standard phosphate buffer solution containing 65-μg PZP, which was emulsified with 0.5-cc FCA. We emulsified PZP with FIA for the second injection. We prepared the emulsion within 12 hours of injection, using 2 10-cc glass syringes joined with a plastic connector. After 100 plunger strokes, we loaded the emulsion into a 3-cc plastic syringe for injection via a 19-gauge, 3.7-cm needle. The needle and injection site were prewashed with 70% ethanol.

In an attempt to mimic the 2-injection condition with a single injection, we gave one group of mares an injection, with an additional component of controlled-release microspheres containing 65-μg PZP with no adjuvant. While we recognized that the presence of adjuvant was desirable in the microspheres for maximizing the immune response, the technology for preparing adjuvant-containing microspheres was unavailable. The microspheres were 10–50 μm in diameter and composed of bioerodable lactide and glycolide polymers. They were prepared by D. Flanagan, R. Linhardt and E. Schmitt at University of Iowa using a coacervation method (Wang et al. 1991). We projected release rates to be continuous over about 4 weeks, with greater release in weeks 1 and 4 (Wang et al. 1990, 1991).

Accessing, Handling, and Inoculating of Horses

The BLM gathered horses by helicopter, permitting injection by hand. We chose this method because access to the horses was possible as a part of scheduled roundup for the BLM horse adoption program.

Between 2 and 12 December 1992, the BLM gathered about 500 horses by helicopter into portable corrals. In a cooperative effort, we and the BLM: (1) separated the gathered stallions and mares, (2) moved them singly through a chute, (3) determined aged and physical condition, (4) gave a 1-cc prophylactic Strepguard® (Miles Lab., Shawnee Mission, Kans.) injection intramuscularly, and (5) permanently marked healthy mares (n = 132) between the ages of 5 and 15 (prime reproductive age) with consecutive numbers by freeze-branding. The brands were located on the upper left hip, were 10-cm in height and were readable from a helicopter and through a spotting scope at >500-m distance on the ground.

We hand-injected intramuscularly 1 cc of freshly prepared emulsion of buffered PZP (or buffer alone) and adjuvant into the left gluteus muscle and photographed each marked mare. The BLM maintained mares given a single injection in the portable corrals on grass-hay and water ad libitum for up to 72 hours and then released them into the range area from which they had been gathered. Before release, the BLM separated the mares and their foals and retained the foals for eventual adoption. They separated mares in the 2-injection protocol from the foals and transported them by truck to the Palomino Valley BLM corral facility. The BLM maintained the mares, under observation, for 4 weeks on a grass-hay and water (ad libitum) diet until we gave the second PZP injection. The BLM then returned the mares to their home range area and released them. Members of the same harem band, previously marked with a same-color grease pencil, were released together. Within 24 hours of the release, representatives of the BLM and the research team surveyed the study area by helicopter to determine the well-being and dispersal of the mares.

Antibody Titers

Before release from captivity and after the second inoculation, we examined each mare for possible injection-site abscess, and obtained a 10-cc blood sample by jugular venipuncture for antibody titer analysis from randomly chosen PZP-treated (n = 19) and placebo (n = 8) mares. We harvested the serum and froze it at −20 C until assay. We determined anti-PZP antibody titers in the above serum samples by an

enzyme-linked immunosorbent assay (ELISA; Voller et al. 1986), with modification reported previously for horses (Liu et al. 1989). We assayed the sera in duplicate and expressed the results as a percentage of the horse positive reference serum, which consisted of a pool of sera that had demonstrated near-maximal titers, about 4–6 weeks after an initial PZP injection (Liu et al. 1989). Pooled preimmunization sera served as a control (no anti-PZP antibody).

Pregnancy Testing by Fecal Steroid Analysis

Pregnancy testing avoids the potential problem of incorrectly assessing contraceptive effectiveness due to undetected stillbirths or mortality of neonates. In the study area, most foaling occurs in April and May, and breeding is uncommon after mid-June. Pregnancy can be detected reliably with fecal analysis within 80 days of conception (Kirkpatrick et al. 1991*b*). We sampled mares for pregnancy detection in fall 1993 (to determine treatment efficacy) and fall 1994 (to determine duration of effect). Between mid-September and mid-October of 1993 and 1994, we observed mares for defecation using binoculars and 20–40× spotting scopes. We insured that each sample collected was from the desired mare by pairing observers such that one person maintained view of the sample through the scope while hand-signalling the other observer to the sample. In any case where the location or specificity of the sample was in questions, none was collected. We collected freshly dropped fecal balls (2–3) in a sealable plastic bag, labelled them, and stored them on ice until they could be placed in a freezer (within 72 hr). We determined pregnancy in each sample via measurement of estrone sulfate (E_1C) and immunoreactive progesterone metabolites (iPdG) by ELISA (Kirkpatrick et al. 1991*b*) and total estrogen (TE) by radioimmunoassay (Kirkpatrick et al. 1990*b*). The combined measures have proven nearly 100% accurate in pregnancy diagnosis in several species (Lasley and Kirkpatrick 1991), including the feral horse. We considered a mare pregnant when values for a given sample were $E_1C > 27.9$, iPdG > 4000.0 and TE > 250.0 ng/g of dry-weight feces.

Foal Counts

We determined the presence of a foal with a given marked mare by helicopter and ground observations in May and September of 1993 and 1994. We verified which foal was with which mare in ground surveys by observing the horses until the foal clearly associated with a given mare by its repeated proximity to her during grazing and travelling and/or by nursing from her. In the helicopter surveys we verified parentage by maintaining sufficient helicopter distance from the family groups of horses to permit foals to associate closely with the mother as they moved away from the helicopter. The latter verification method has correlated well with subsequent ground survey data in previous studies (Turner et al. 1992*b*). We considered a mare to be successful reproductively if she tested positive for pregnancy and/or was verified to have a foal.

Statistical Analysis

Where appropriate, we have presented data as mean ±SE. We employed Student's *t*-test for statistical analysis of fecal and titer data. We determined possible group differences among reproductive success rates using a Tukey-type multiple comparison test for proportions or a binomial probability distribution (BPD; Zar 1984).

RESULTS

During one month of captivity and at the time of their release in January 1993, none of the 60 2-injection mares showed abscesses. None of the mares observed in the field thereafter showed injection-site marks, suggesting that abscesses had not occurred. Following the treatment of mares in December 1992, severe winter conditions prevailed in northern Nevada through March 1993. However, we observed 93 of the 130 marked mares (71.5%) between May and October 1993. We found an additional 8 marked mares not seen in 1993 between May and October 1994, yielding a total reidentification rate of 77.7% during the study.

In 1993 we collected fecal samples from 51 marked mares. In the 1993 samples obtained from mares that were reidentified and had produced foals in 1994 ($n = 9$) the average values for E_1C, iPdG, and TE were 18.9, 38.7, and 22.2 × greater than the respective average values associated with mares that did not produce foals ($n = 19$) in 1994 (Table 1). For each of the 3 steroids measured, the average value for mares that did produce foals was significantly greater ($P < 0.01$) than for mares that did not

Table 1. Association of fecal steroid metabolite levels in feral mares with subsequent presence or absence of foals[a]

Mare condition	No. of mares	Steroid metabolites concentration[b] (ng/g feces dry wt ± SE) E_1C	iPdG	TE
Without foal	19	14.6 ± 8.3	378.0 ± 217.4	53.9 ± 21.4
With foal[c]	9	276.2 ± 69.8	14,637.1 ± 2,250.0	1,197.1 ± 214.0

[a] Fecal samples collected in Sep 1993, foal counts performed May–Sep 1994.
[b] E_1C = estrone sulfate, iPdG = immunoreactive pregnanediol glucuronide, TE = total estrogens.
[c] Values between conditions for all steroids are significantly different; $P < 0.01$, Student's t-test.

produce foals. Among the mares tested, the values for all 3 steroids were consistent, i.e., all were elevated or all were depressed. All mares that were diagnosed as pregnant (by fecal steroids) and were reidentified the following spring were observed to have a foal. We observed that 2 of 19 mares that were diagnosed as not pregnant had a foal the following spring. Overall accuracy was 26/28 (93%).

Of 44 reidentified mares in the 2-injection PZP group, only 2 mares were reproductively successful in the 1993 breeding season. Eleven of 20 mares were reproductively successful in the 2-injection placebo group. In the 1-injection groups reproductive success occurred in 4 of 14 mares (PZP, no microspheres), 3 of 15 mares (PZP plus PZP microspheres), and 2 of 4 mares (placebo). Reproductive success was achieved in 34 of 63 unbranded mares that were studied (Table 2). The proportion of reproductively successful mares in all treatment groups was significantly lower ($P < 0.05$) than in both placebo and untreated control groups, the proportions of which were not different from each other. In addition, the proportion of reproductively successful mares in the 2-injection PZP group was significantly lower ($P < 0.05$) than the proportions in both 1-injection PZP groups. The proportions in the 1-injection PZP groups were not different.

Table 2. Effect of PZP immunocontraception on fertility of free-roaming feral mares in Nevada.

Treatment[a]	No. of mares located[b]	No. of mares reproductively successful[c]	% of mares reproductively successful[c,d]
Untreated	63	34	53.9[q]
Placebo (1- or 2-inj.)	20	11	55.0[q]
PZP (2-inj.)	44	2	4.5[r]
PZP (1-inj. + microspheres)[e]	15	3	20.0[s]
PZP (1-inj.)	14	4	28.6[s]

[a] A given treatment (Dec 1992) consisted of i.m. injection of an emulsion of 0.5-cc Freund's adjuvant and 0.5-cc phosphate buffer solution with or without (placebo) 65-μg PZP.
[b] Mares that were located for sample collection and/or observation between May 1993 and Oct 1994.
[c] A positive fecal pregnancy test or presence of a foal were criteria for reproductive success.
[d] Values with different superscript ([q,r,s]) are significantly different from each other; $P < 0.05$, Tukey-type multiple comparison test for proportions.
[e] One dose of microspheres contained 65-μg PZP for controlled release and was incorporated into the injection.

Anti-PZP antibody titers in 2-injection mares at 4 weeks after initial inoculation averaged 86.2% of positive reference control titers in 19 PZP-treated and 0.9% in 8 placebo-treated animals ($P < 0.01$). Control titers were 0–2%. Thirteen of the PZP-treated mares showed titers >90%, and 6 were between 46 and 75%. Sixteen of the titer-tested PZP mares were reidentified for subsequent fertility evaluation. All of these had shown titers >52% at 4 weeks posttreatment, and all were infertile in the first year after treatment.

We determined duration of treatment effect on the basis of 1994 pregnancy testing only. This basis was considered acceptable, because the 1993 correlation between pregnancy-test and foal-count data reported was 0.93 ($n = 28$). In the 2-injection PZP group (treated Dec 1992) fecal samples from 11 of 25 mares showed pregnancy-positive levels of steroid metabolites in the second year (Sep 1994) after treatment. We obtained a positive pregnancy test in fecal samples from 12 of 22 unbranded mares that we had sampled during the same period as for branded mares. The pregnancy rates were not different ($P > 0.05$, BPD) between these groups (Table 3). Among 2-injection placebo mares, samples (Sep 1994) from 2 of 4 indicated pregnancy. In the 1-injection PZP and 1-injection PZP-plus-PZP microspheres groups, samples (Sep 1994) from 1 of 2 and 1 of 2 mares, indicated pregnancy. We did not obtain samples from mares in the 1-injection placebo group. Sample sizes for 1-injection groups were insufficient for statistical analysis.

Table 3. Reversibility of PZP contraception in free-roaming feral mares.

Condition	No. of mares sampled[a]	No. of pregnant mares[b]	% of mares pregnant[c]
PZP (2-inj.)	25	11	44.0
Untreated	22	12	54.5

[a] Treatment in Dec 1992; fecal samples collected in Sep 1994.
[b] Based on pregnancy test criteria as presented in Table 1.
[c] No differences among groups; ($P > 0.05$) binomial probability distribution.

DISCUSSION

Inoculation of captured and released feral mares with PZP vaccine resulted in a marked suppression of fertility across one breeding season. While a fertility rate of 4.5% occurred with use of the 2-injection protocol, fertility rates in both single-injection groups were higher (20–28%). Nonetheless, single-injection fertility rates were only 1/3–1/2 of fertility rates observed in control (placebo and untreated) mares, and this difference was statistically significant. Fertility rates among control mares were similar to those reported for other feral horse populations (Wolfe et al. 1989).

The results indicate that the controlled-release microspheres neither mimicked the condition of 2 separate injections, nor produced significantly greater infertility than a single injection alone. Exposure of the mares to PZP via microspheres differed from a second vaccine injection in that the PZP in the former was not released as a bolus and was not accompanied by adjuvant. In a previous equid study employing FCA (Kirkpatrick et al. 1990*a*), we observed abscesses in 3 of 26 treated mares. However, abscesses occurred only in mares given 3 injections of vaccine (1 = FCA, 2 = FIA, 3 = FIA), not in mares given 2 injections (1 = FCA, 2 = FIA). Had there been abscesses in 2-injection mares in the present study, the abscesses should have appeared during the month of captivity. Thus, it appears that, in the 60 mares given 2 injections, abscesses did not occur. Abscesses seemed unlikely in mares given a single injection, since they received less exposure to adjuvant than 2-injection mares.

The presence of well-elevated anti-PZP antibody titers in all of the PZP-treated mares and in none of the placebo-treated mares that were sampled is consistent with previous reports in horses (Liu et al. 1989). Also, these titers were consistent with fertility data, i.e., no mares with elevated titers produced foals in all of the mares ($n = 16$) for which the assessment could be made.

The presence of anti-PZP antibody titers and infertility are not necessarily related. However, elevated titers consistently have been associated with infertility in the horse. In a previous study, none of 13 mares exhibiting peak (4–6 weeks post-inoculation) titer levels >65% of positive reference serum titers before the breeding season became pregnant (Liu et al. 1989). In the present study, none of 16 mares showing titers >52% at 4 weeks post-inoculation was reproductively successful during the subsequent breeding season.

The effect of PZP treatment in 2-injection mares was sustained through 1, but not 2, breeding seasons, indicating a return to fertility after 1 year. Forty-four percent of 2-injection, PZP-treated mares that were relocated in the fall of 1994 were pregnant. This finding is similar to the pregnancy rates in placebo and untreated control mares, which averaged 56 and 54% in 1993, and 50 and 54% in 1994. We focused the reidentification effort to test duration of infertility on the 2-injection mares, since they were exposed to the greatest stimulation by PZP. We expected that the possibility of extended infertility would be greatest in this group. Also, the 2-injection mares composed the largest group in the study, offering the best chances for reidentifying a large number of these mares. Although the 1-injection data are consistent with the 2-injection data regarding reversibility, the small number of reidentified mares in the 1-injection groups precludes conclusions in these groups.

This study confirms the high accuracy of pregnancy diagnosis via fecal steroid metabolites in feral horses (Kirkpatrick et al. 1991*b*). The minimum 18-fold difference observed between non-pregnant and pregnant state in the measured metabolite levels favors reliable differentiation of pregnant and non-pregnant state. Although no discrepant values were observed in the present study, an occasional (<2%) discrepant high or low value is possible among the 3 metabolites we have used (Lasley and Kirkpatrick 1991). By measuring all 3 metabolites (E_1C, iPdG and TE), we minimized the chances of misdiagnosis. Among 28 mares for which we obtained both pregnancy and foaling data, agreement was 93%. Both cases of disagreement were false negatives, i.e., pregnancy

diagnosis was negative but a foal was present. We were unable to determine whether the pregnancy test was incorrect or whether we mismatched the foal with the mare or whether conception occurred too late in the year to be detectable at the time of fecal collection.

MANAGEMENT IMPLICATIONS

The study was carried out in Nevada, where more than 60% of Western feral horses reside. Helicopter roundup of horses is a well-established method for accessing feral horses in Nevada and other Western states for roundup/adoption programs, and has been used for other contraception studies (Eagle et al. 1992, Plotka et al. 1992). Incorporation of PZP vaccination into the existing roundup procedures greatly reduces the cost of accessing horses for immunocontraception in years when gathers are performed. In the BLM roundup/adoption program, mares more than 5 years of age are returned to the range. An added advantage of using scheduled periodic roundups for administering immunocontraception is that only mares destined to remain on the range will be treated; i.e., treatment will not be wasted on mares aged 3–5 years, which will be removed and adopted. Options for horses for single-injection immunocontraception in years when adoption roundups are not performed include gathering by helicopter, and remote delivery by dart from helicopter (Turner and Kirkpatrick 1991).

The use of contraception for management purposes has the potential to reduce management costs by reducing the frequency of roundups and eliminating the need to maintain unadoptable horses in captivity. Contraception can also enhance the humaneness of management, since roundups and extended captivity are stressful to the horses. However, Garrott (1991) has concluded from feral horse modelling studies that each population (herd area) is unique and must be considered individually for potential use of contraception. We agree with this point. Also, as suggested by Garrott (1991), the issues of both cost-effectiveness and limitation of population growth must be included in assessing long-term contraceptive management potential.

A 2-injection protocol presents logistical and economic problems for routine use in feral horses, because of the need to keep gathered horses for 3–4 weeks. However, single-injection methodology presently could be used in the field, with the caveat that the first year after treatment the expected fertility rate will be 20–28%. An annual booster in subsequent years should yield >90% efficacy, because Kirkpatrick et al. (1992*a*) have observed <5% fertility in year 3 among mares given a single injection of PZP vaccine in years 1 and 2. In other words, despite a submaximal response to antigen in year 1, the response to booster antigen in year 2 can be maximal.

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Porcine zona pellucida (PZP) immunocontraception of wild horses (*Equus caballus*) in Nevada: a 10 year study

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Porcine zona pellucida (PZP) immunocontraception was investigated for possible use in free-roaming wild horses in the western USA. A protocol of two injections (3–4 weeks apart) of vaccine lasting 1 year was first used and a single-injection controlled-release vaccine of 1 year duration was developed and tested in the field. Studies of a presumptive vaccine of 2 year duration were initiated. The parameters of anti-PZP antibody titre response, pregnancy testing and offspring production were used, and PZP vaccine was found to provide up to 94% infertility in free-roaming wild mares. In addition, a single-injection PZP vaccine of 1 year duration and containing a controlled-release component of PZP in a polymer matrix can provide infertility equivalent to the two-injection PZP vaccine. All the PZP vaccine preparations tested were associated with a return to normal fertility within 1 year. During the course of these studies, attention was given to practical aspects of management application of PZP contraception. Preparation of the controlled-release portion of the vaccine in pellets, which fit into the needle of a dart or syringe, has simplified vaccine handling and permitted long-term storage of the controlled-release component. Vaccine delivery is now performed using a jabstick on captured mares restrained in a field stock chute during routine horse gathers. Provision of a vaccine-training programme has maximized personnel safety during vaccine preparation and use.

Introduction

The first study of immunocontraception of free-roaming wildlife took place in 1988 on wild horses (*Equus caballus)* on Assateague Island National Seashore (ASIS) off the Maryland coast. Successful contraception was achieved with a porcine zona pellucida (PZP) vaccine on ASIS (Kirkpatrick *et al.*, 1990) and in captive mares (Liu *et al.*, 1989). These studies encouraged pursuit of immunocontraception as a humane and cost-effective tool for managing wild horses in the western USA. Since 1991, with funding provided by the US Department of the Interior,

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Bureau of Land Management (BLM), we have investigated long-term fertility control of free-roaming wild horses using PZP immunocontraception.

This research has focused on three primary criteria that must be satisfied to enable practical wildlife contraception: (i) high efficacy with reversibility; (ii) access to animals in the field; and (iii) ability to limit population growth. The specific aims of these studies were: (i) to convert the existing two-injection (1 month apart) vaccine with a 1 year duration into a single-injection vaccine with high efficacy and 2–3 years duration; (ii) to ensure return to fertility in treated animals; and (iii) to design the vaccine for practical application in wild horse management. In this article, research toward these aims in the period from 1991 to 2001 is presented.

Materials and Methods

General

This work has involved several sets of laboratory experiments followed by field trials in free-roaming wild horses. Laboratory work involved: (i) vaccine formulation; (ii) *in vitro* testing of controlled-release vaccine characteristics; and (iii) monitoring of *in vivo* antibody production in response to immunization. Subjects for laboratory studies were horses (captive-wild or domestic) and rabbits, with group size ranging from 5 to 10 unless stated otherwise. Field trials used specific herds of free-roaming wild horses in Nevada, the location of approximately 65% of wild horses in the western USA. The reference immunization for all studies consisted of two i.m. (gluteus) PZP (65 μg)–adjuvant injections, 1 cc each, administered 3–4 weeks apart as described by Liu *et al.* (1989). All treated mares in field studies were freeze-marked on the left hip for future identification from the ground or helicopter, and subsequent foals were counted by ground and aerial survey. Efficacy and reversibility of contraceptive treatment was determined by antibody titres in captive mares using a serum ELISA specific for anti-PZP antibodies (Liu *et al.*, 1989) and by pregnancy determination in free-roaming mares based on faecal steroid assay (Kirkpatrick *et al.*, 1991) or foal counts or both.

Controlled-release vaccine

The high costs and formidable logistics of maintaining wild horses in captivity for the required 3–4 weeks between injections make a two-injection vaccine protocol impractical. Thus, a primary aim was to develop a single-injection PZP vaccine. Initially, it appeared that controlled-release technology, already used for the delivery of a number of agents, including cancer chemotherapeutics and various vaccines (Chang, 1976; Carter *et al.*, 1988; Linhardt, 1989; Wang *et al.*, 1990), could be applied to the PZP vaccine to meet the requirement for a single-injection vaccine. This technology was used to sequester PZP or adjuvant or both within inert, non-toxic poly L-lactide co-glycolide (PLG) polymer microspheres or pellets (mini-rods), which would release their contents upon injection and exposure to an aqueous environment in a fashion dependent on the formulation.

PLG controlled-release preparations were performed using a coacervation technique (Wang *et al.*, 1991). PZP comprised 1% of final microsphere weight, and duration of delay in PZP release (1, 3 or 12 months) was adjusted by altering the lactide:glycolide ratio (from 50:50 to 100:0). Initial studies focused on microspheres of several types, including microspheres with slow, continual release and microspheres showing a delayed bolus release at 1–6 months, as desired.

Preliminary *in vitro* studies were performed with controlled-release preparations. Although release characteristics of these preparations were not likely to be identical *in vivo* and *in vitro*,

the latter provided reasonable, inexpensive estimates of release patterns. The release of PZP from the controlled-release PZP preparations was monitored by solid-phase protein assay (SPA) ELISA of biweekly aliquots of an incubation medium of phosphate-buffered saline kept at 37°C under gentle, continuous shaking.

For immunization of animals with appropriate releasing preparations, microspheres were suspended in an inert liquid carrier (carboxymethylcellulose) and injected by hand or dart. Controlled-release vaccines consisted of a standard, 1 cc PZP–Freund's complete adjuvant (FCA) emulsion plus the controlled-release component. Initial microspheres did not contain adjuvant, as the requirement for water solubility for incorporation into the polymer preparations precluded the use of Freund's adjuvant. Later microspheres were prepared containing the water-soluble adjuvant Carbopol 934 (CAR) (B. F. Goodrich, Cleveland, OH).

In addition to microspheres, controlled-release PLG pellets containing PZP or adjuvant or both were also prepared and tested. Pellets were prepared using a physical compression or thermal extrusion method (Sanders *et al.*, 1986) and were approximately 6.0 mm × 1.5 mm in diameter, and designed to fit into the barrel of a 14-gauge hypodermic syringe. Pellets contained 70–90 µg PZP or 150–175 µg QS-21 (water-soluble saponin adjuvant; Aquila Biopharmaceuticals, Framingham, MA) or both and exhibited a bolus release similar to that observed in bolus-release microspheres.

Conjugate vaccine

Conjugate vaccines may provide an ideal way to increase T-cell activation and cytokine production essential for B-cell activation and antibody production after immunization. As most carrier proteins are stronger T-cell stimulators than are zona pellucida antigens, conjugation of antigen to carrier protein can potentially increase antibody affinity maturation and duration of immunological memory. It was hypothesized that the effects of conjugation could play a key role in developing a single-injection vaccine of 1 year duration.

For this study, captive wild mares were immunized (left hip, 1 cc, by syringe) with PZP conjugated to keyhole limpet haemocyanin (KLH) at a 1:1 mass ratio (Pierce Chemical Company, Rockford, IL) prepared by R. Fayrer-Hosken (University of Georgia). The study compared several combinations of conjugated PZP–KLH and non-conjugated PZP (each emulsified with FCA or Freund's incomplete adjuvant (FIA)) against the standard two-injection PZP–FCA/FIA vaccine.

Results and Discussion

Vaccine efficacy

Controlled-release studies. An example of release data obtained from *in vitro* incubation of a PZP microsphere formulation (Turner *et al.*, 2001) and an example of a PZP pellet release pattern are shown (Fig. 1a and 1b, respectively). Release characteristics have been examined for microspheres and pellets with 1–3 and 3–6 month release delays. Assessment of PZP release from pellets designed for release after 12 months is still in progress.

Rabbits were used for initial *in vivo* testing to determine antibody titre response, with subsequent field testing of early microsphere preparations in 132 mares of the Antelope Valley Herd Management Area (HMA) in Nevada (Turner *et al.*, 1997). A summary of various controlled-release vaccines and their contraceptive efficacy in the field, including Antelope Valley HMA, is shown (Table 1).

The results of the Antelope Valley HMA study were encouraging in several aspects. Firstly, they established the feasibility of treating large numbers of mares in the field during routine

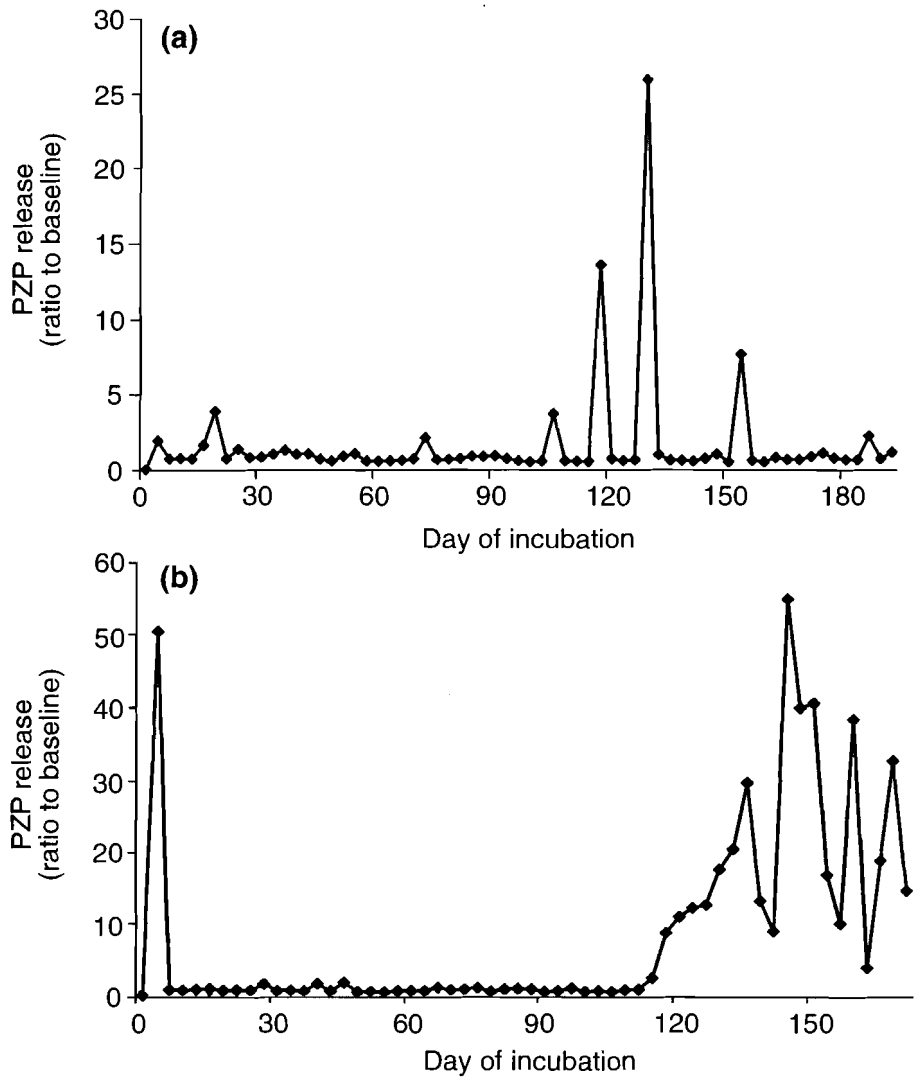


Fig. 1. (a) *In vitro* release pattern for three identical, separately incubated lactide:glycolide (ratio 65:35) microsphere populations containing 80 μg porcine zona pellucida (PZP) per population. Each microsphere population exhibited one major bolus release during the incubation. (b) *In vitro* release pattern for a single lactide:glycolide (85:15 ratio) pellet containing 80 μg PZP incubated at 37°C in phosphate buffered saline and designed to release after approximately 3 months. Note initial release associated with PZP on or near pellet surface.

BLM horse gathers. Secondly, they demonstrated that the fertility rate in mares given the single-injection microsphere vaccine was lower than in untreated controls. Finally, they demonstrated that treated mares showed infertility of one breeding season in duration, with a return to fertility in the second year after vaccination (Turner *et al.*, 1997).

However, the single-injection (with microspheres) treatment was less effective than the standard two-injection vaccine. There were two likely shortcomings of the original microsphere formulation. Firstly, continuous release rather than bolus release of antigen was a poor mimic of a second injection. Secondly, the absence of adjuvant in the second exposure resulted in a submaximal response. Therefore, the microspheres were reformulated to allow for bolus release of antigen and inclusion of adjuvant, and these were tested. As water-

Table 1. Effects of various PZP vaccine formulations on fertility in free-roaming feral horses in Nevada

Vaccine type[a]	Injection type	Adjuvant[b]	Study site (HMA)	Number of mares	Fertility rate[c] (%)	
					Treatment	After treatment[d]
Untreated control[e]	Hand		Composite	122	55	63
Standard two-inoculation[f]	Hand	FIA	Composite	376	8	60
Microspheres (1 month)	Hand	None	Antelope Valley	15	20	44
Microspheres (1 month)	Hand	CAR	Nevada Range	97	11	–
Microspheres (1 and 3 month)	Hand	QS-21	Fishcreek	40	10	65
Pellets (1 and 3 month)	Hand	QS-21	Kamma Mountains	18	11	62
Pellets (bolus 1 and 3 month)	Dart	QS-21	Monte Cristo	57	24	54
Pellets (bolus 1 and 3 month)	Dart	QS-21	Dolly Varden	57	26	46
Pellets (bolus 1, 3 and 12 month)	Jabstick	QS-21	Clan Alpine	32	6	–

[a]All vaccines contained an initial injection with porcine zona pellucida–Freund's complete adjuvant (PZP–FCA) emulsion. Controlled-release pattern was gradual for Antelope Valley and bolus for all sites thereafter.

[b]Adjuvant for second exposure to PZP (FIA: Freund's incomplete adjuvant).

[c]Based on foal counts or faecal steroid-based pregnancy test or both.

[d]Pregnancy rates in treated mares were determined 2 years after treatment, and sample size ranged from 16 to 38.

[e]Composite value comprised untreated horses from each Herd Management Area (HMA) listed in this Table.

[f]Some treated marked populations included mares given the standard two-injection vaccine.

soluble adjuvants show similar solubility to PZP, no testing of these adjuvants *in vitro* was performed.

Revised microsphere formulations were evaluated in horse and rabbit studies that lasted up to 68 weeks using anti-PZP antibody titres as an indicator of vaccine efficacy (J. W. Turner, Jr and I. K. M. Liu, unpublished). These studies showed that: (i) some animals respond vigorously and others only moderately to the same dose of PZP vaccine; (ii) increasing PZP doses by up to three times in rabbits or up to six times in horses did not increase either antibody titre response or the percentage of animals responding; and (iii) microspheres given alone, without a PZP–FCA priming dose, did not elicit sufficient antibody titres.

On the basis of the above studies, a field trial was initiated in January 1996 in Nevada to test a presumptive single-injection, 1 year PZP vaccine using bolus-release PZP–CAR microspheres against the standard two-injection vaccine (Turner *et al.*, 2001). Adult wild mares ($n = 267$) were captured, freeze-marked, treated and released. Reproductive success across 1 year was 12.8% in mares receiving the two-injection vaccine, 11.3% in mares given the single-injection vaccine containing microspheres and 62.5% in untreated control mares. This study revealed equivalent 1 year contraceptive efficacy between single- and two-injection PZP vaccines, indicating that controlled-release technology can replace a second injection.

Despite the contraceptive success of the controlled-release vaccine, some disadvantages of CAR adjuvant and of microspheres became evident during field trials. As previously stated, microspheres were delivered by suspending them in an inert liquid in a syringe. Unless the injection was made within 30 s of suspension, microspheres clumped at the tip of the syringe

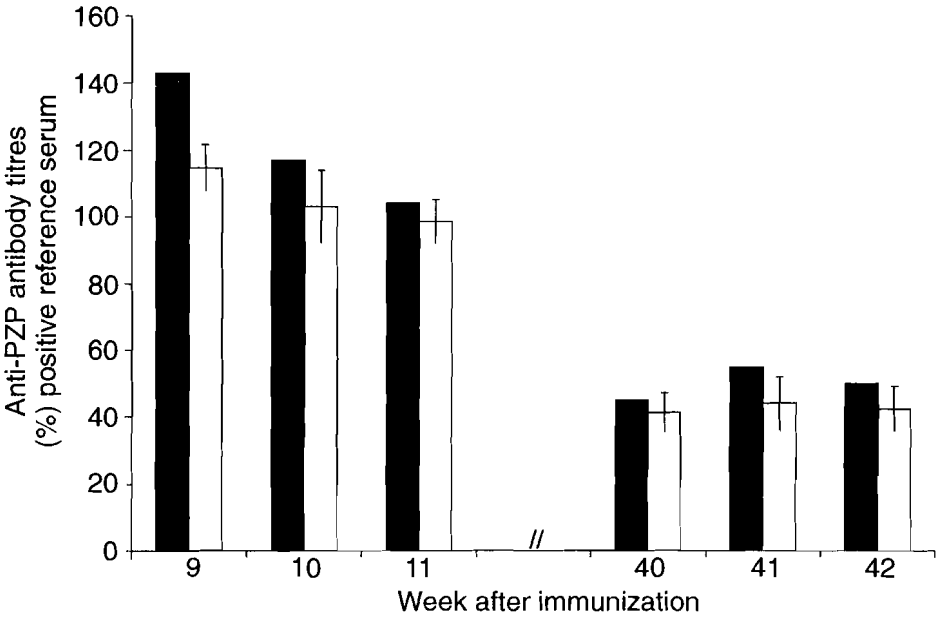


Fig. 2. Comparison of antibody response in domestic mares to standard two-injection porcine zona pellucida (PZP) vaccine (■) versus controlled-release PZP vaccine (□). Two-injection vaccine ($n = 2$) consisted of 65 μg PZP–Freund's complete adjuvant (FCA) followed in 3 weeks by 65 μg PZP–Freund's incomplete adjuvant (FIA). Controlled-release vaccine ($n = 5$) consisted of a single injection containing 65 μg PZP–FCA emulsion plus 1 month pellet (70 μg PZP and 150 μg QS-21 adjuvant) and 3 month pellet (90 μg PZP and 200 μg QS-21 (water-soluble saponin) adjuvant). Reference serum was obtained at 8 weeks after immunization from a mare previously given a standard two-injection vaccine.

and prevented injection. In addition, the CAR adjuvant had to be delivered in mg amounts, which required either overloading the microspheres or using a 2–3 cc injection volume. Thus, the use of pellets as a controlled-release medium and QS-21 as an alternative water-soluble adjuvant was investigated.

In 1998, as part of a 45 week study of 25 domestic mares, the standard two-injection PZP vaccine was compared with a presumptive single-injection vaccine, using PZP and QS-21 pellets in place of the second injection (Fig. 2). The PZP–QS-21 pellet vaccine consistently produced titres similar to the two-injection PZP treatment group during the 45 weeks that were monitored. Importantly, the PZP–QS-21 pellet-treated mares consistently showed titres in the range usually associated with contraceptive effects in mares, as determined in previous experiments by Liu *et al.* (1989). This finding indicated that an effective single-injection contraceptive vaccine with approximately 1 year duration had been developed.

Early in 1999, a series of tests was initiated in five separate wild horse populations to assess the impact of the single-injection, 1 year vaccine containing PZP–QS-21 pellets on population growth. Although the population-impact portion of these tests is still in progress, data from these tests on vaccine efficacy and reversibility are shown (Table 1). It is noteworthy that the fertility rates in treated mares in the Monte Cristo and Dolly Varden populations were higher than in other treated populations. This lower efficacy was purportedly due to a significant incidence of failed injection of vaccine by darts used for remote delivery in these two studies (other studies used hand injection or jabstick). A similar efficacy problem has been encountered in studies using dart delivery of PZP vaccine in white-tailed deer (A. Rutberg and R. Naugle, personal communication).

Results of the studies using a single-injection, 1 year vaccine encouraged exploration of a vaccine containing a controlled-release annual booster. A pellet projected to release PZP–QS-21 at about 12 months after injection was designed, with the amounts of PZP and QS-21 increased to 250 μg and 500 μg, respectively, to account for expected biological degradation and cumulative gradual loss from the pellets across time. In January 2000, wild mares (n = 96) in the Clan Alpine HMA were treated and released using a vaccine consisting of a priming dose of PZP in FCA and 1-month, 3-month and presumptive 12-month releasing pellets of PZP–QS-21. This vaccine was expected to provide 2 continuous years of infertility. Results after 1 year revealed 6% fertility among 32 treated mares that were re-identified and 59% fertility in 33 untreated mares (Table 1). Year 2 data will be available from foal counts in 2002.

Conjugate studies. Captive wild mares (n = 42) were divided into several treatment groups (n = 7–9 per group) to allow comparison of the PZP–KLH conjugate vaccine with standard PZP and control vaccines. All injections were delivered by hand in December 1998, with a booster at week 4 and antigen challenge with PZP alone at week 44. All mares in the study underwent normal ovarian cycles as demonstrated by faecal ovarian hormone concentrations measured from April to June. Delayed-type hypersensitivity skin test results revealed no increase in Th1 T-cell activation as determined by wheal diameter (mm) in response to intradermal injection of PZP, indicating that the risk of autoimmune ovarian disease typically associated with ovary-specific $CD4^+$ Th1 cells (Bagavant *et al.*, 1999) was not increased.

Primary immunization of mares with PZP–KLH in FIA followed by boosts with PZP in FIA resulted in increased, sustained anti-PZP antibody titres that were not significantly different from titres in mares immunized similarly using PZP alone. Mares immunized with the PZP–KLH sequence and mares immunized with PZP alone sequence had significantly ($P < 0.05$, binomial probability distribution) lower pregnancy rates, 33% and 29%, respectively, compared with a KLH–adjuvant control pregnancy rate of 75%.

This study is the first to demonstrate an immunization protocol in horses that is as effective as the standard two-injection PZP vaccine without requiring the use of FCA. As FCA is not indicated for widespread use in the field, the PZP–KLH conjugate used with permissible oil adjuvants or other delivery systems may have high potential for routine management use. The PZP–KLH conjugate may also prove to be an ideal priming dose in deer and other species in which routine testing for tuberculosis (TB) infection is performed, as vaccines that include FCA may result in a false-positive TB test.

The PZP–KLH conjugate may also have potential in controlled-release preparations. As the immune response to PZP is not vigorous, a strong adjuvant such as FCA has been necessary in the priming dose of PZP. However, the oil-based FCA cannot be used in controlled-release pellets, and no water-soluble adjuvants to date have equalled the potency of lipid-based FCA. If PZP–KLH conjugate is proven compatible with existing rapid-release (from hours to days) polymers, an attractive immunization protocol would be a priming dose of lyophilized PZP–KLH and water-soluble dry adjuvant in a rapid-release pellet with secondary PZP doses in delayed-release pellets. This would allow a completely pelletized, dry vaccine of controlled-release components and elimination of soluble vaccine containing FCA. Without a liquid component, the vaccine would have a long shelf life (years) and neither on-site emulsification nor cold storage of components would be required.

Reversibility

Short-term recovery of fertility after administration of anti-fertility treatment has been

determined in our field trials by helicopter and ground surveys (Table 1). In most cases, fertility rates were determined among treated and untreated mares in the year that infertility was elicited, as well as in treated mares in the year after contraceptive effect, to determine reversibility. Fertility rates among different populations were similar, ranging from 44% to 65% in the year of return to fertility. This range was similar among the same populations monitored in the year before contraceptive effect. In addition, in populations containing a percentage of treated mares, the range of fertility rates in untreated mares was from 54% to 67%. These data support the conclusion that a return to normal fertility occurred within 1 year after the period of active contraception.

As routine horse gathers take place a minimum of 3 years apart, the BLM has encouraged the development of a single-injection vaccine with 3 year duration. The theory is that this would ensure continuous contraceptive coverage. However, perspectives on this matter continue to change as a result of the association of consecutive treatment for 3 or more years (to provide continuous infertility) with delayed return to fertility in 69% of treated mares across 4 years in the studies on ASIS (Kirkpatrick *et al.*, 1992).

The development of a PZP vaccine with effects up to 3 years in duration is warranted, but use for management purposes should be contingent on establishment of a strong database on the population impact of treating specific percentages of a population with vaccines of specific duration. To date, our studies have addressed only a 1 year PZP vaccine in this regard and results are incomplete. An additional complicating factor is the size of the population being treated. Hobbs *et al.* (2000) have used computer modelling for ungulate populations to demonstrate that long-lived contraceptives have the potential to reduce small populations to the point that they cannot recover. One way to minimize this risk is to use a contraceptive of shorter duration in smaller populations, which would enable more responsive and adaptable management.

Another important aspect of the reversibility issue related to management is the gene pool and the aim of maintaining genetically viable populations. Unimpeded natural selection will yield the best fit of horse to habitat, and the value of the wild horse gene pool lies in that fit. The current gather programme regularly and permanently removes genes and manipulates the gene pool artificially. In contrast, reversible contraception does not eliminate genes and, thus, leaves more material for natural selection.

Management issues

There are issues other than efficacy and reversibility that are relevant to a viable vaccine for use in the field. These include field access to animals and safety of use for administrator and recipient.

Field access. A highly effective vaccine is worthless until it is in the target animal; thus, access is a critical component of any wildlife fertility control programme. The most common access methods are immobilization or trapping (to enable immunization by direct injection) and remote delivery (via dart or biobullet) in the open field or at bait stations. Oral delivery in feed or bait is also under consideration, although this method is faced with the serious challenge of assuring species specificity in the consumer. Early studies on ASIS involved accessing mares by tracking them on foot followed by darting. This method was also used effectively in a separate project in free-roaming wild burros (Turner *et al.*, 1996). Tracking and darting were so successful that we initially considered remote-delivery darting of western horses from a blind (camouflaged hiding) spot at waterholes or from helicopters.

Although the feasibility of these approaches has been documented (Kirkpatrick *et al.*, 1991), the ongoing gather and adoption programme of the BLM guaranteed access to mares in the field. Horses were gathered by helicopter and placed individually into a stock chute to be aged by dental examination. Mares older than a BLM-specific age were immunized with the PZP vaccine and returned to the range. Several methods were examined for delivery of PZP pellets, including syringe/trocar, self-injecting dart and jabstick, with the PZP–FCA emulsion portion of the vaccine in the syringe or dart propelling the pellets into the muscle in all delivery methods. The jabstick emerged as the most suitable delivery method for use in routine horse gathers. The adoption programme is expected to continue indefinitely, with associated horse gathers guaranteeing access to mares for PZP vaccine delivery. An important advantage of this method of access is that the only cost associated with ongoing fertility control in this situation is the vaccine and its delivery equipment.

Safety and training. The biological safety of the PZP vaccine in target animals has been documented through the observed normality of physiological and behavioural function in treated animals of various species including deer (McShea *et al.*, 1997), elephants (Delsink *et al.*, this supplement) and wild horses (Kirkpatrick *et al.*, 1995; Powell, 1999; Turner and Kirkpatrick, this supplement). Qualitative observations of PZP-treated and control horses in Nevada during the past 10 years have been consistent with these reports. Furthermore, the obvious improved nutritional status of females freed periodically from the metabolic demands of pregnancy and lactation (McShea *et al.*, 1997) has affected individual lifespan and overall health positively (Turner and Kirkpatrick, this supplement).

Ensuring the safety of personnel who prepare and administer the vaccine is important and can best be achieved by training. In early studies, all fieldwork was performed by the investigators. However, the increase in both the number of study sites and number of BLM personnel involved in these studies led to provision of a formal 4 day training programme on the topic of wild horse immunocontraception at ZooMontana (Billings, MT; instructors: J. Kirkpatrick, K. Frank, R. Lyda and F. Deigert) in 1999 and 2000. This training has maximized the assurance of safety and comfort with vaccine preparation and use.

Conclusion

Considerable progress has been made in wild horse contraception since 1991. A single-injection controlled-release PZP vaccine has been developed that provides 1 year of infertility followed by a high return to fertility. Efficient access to wild mares has been demonstrated during routine BLM gathers of wild horses, and reasonable vaccine delivery and safety have been provided for both administrator and recipient. PZP vaccine appears viable for management application, pending results of ongoing studies, which address contraceptive limitation of population growth. While PZP vaccine can be used effectively in its present (1 year) form, it has additional potential pending the outcome of current studies to extend its duration to 2 years and to simplify its preparation and storage.

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Immunocontraception in Wild Horses: One Inoculation Provides Two Years of Infertility

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ABSTRACT Previous studies reported one year of contraception associated with a 1-injection porcine zona pellucida (PZP) vaccine. We have subsequently determined contraceptive effectiveness of a presumptive 1-injection, 2-year-duration PZP vaccine in free-roaming wild horses (*Equus caballus*) in Nevada, USA. In January 2000, we captured, freeze-branded, treated, and subsequently released 96 adult females that received 1) a primary dose of vaccine emulsion consisting of aqueous PZP and Freund's Complete Adjuvant, and 2) booster doses of PZP and adjuvant in controlled-release polymer pellets. We determined PZP release characteristics of pellets in vitro, prior to field use. We determined reproductive success in treated and untreated females through October 2004 via measurement of estrone sulfate and progesterone metabolites in fresh feces collected from the ground and by twice-annual foal counts. Among treated females, annual reproductive success from 2001 though 2004 sequentially was 5.9%, 14.0%, 32.0%, and 47.5%. Untreated females showed average reproductive success of 53.8 ± 1.3% across this period. This study revealed that: 1) PZP acted as an effective contraceptive for 2 years posttreatment; 2) some residual contraceptive effect remained in year 3; and 3) fertility returned to control levels by year 4 posttreatment. It appears that controlled-release technology can replace both the second (1-month) and annual booster injection of PZP vaccine, thereby decreasing cost and increasing efficiency of use of this vaccine in wild horse management. (JOURNAL OF WILDLIFE MANAGEMENT 71(2):662–667; 2007)

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KEY WORDS adjuvant, controlled-release vaccine contraception, *Equus caballus,* field study, free-roaming wild horse, pregnancy testing, zona pellucida antigen.

Since the passage of the Free-Roaming Wild Horse and Burro Protection Act in 1971, management of wild horses (*Equus caballus*) on public lands has proven biologically and politically challenging. Under its multiple-use mandate, the Bureau of Land Management (BLM) must maintain healthy, viable wild horse herds in a thriving ecological balance with many competing uses, including wildlife, livestock, and other commercial and recreational activities. With lethal horse population control being illegal and broadly unacceptable to the public, the BLM has relied principally on gathering, removing, and adopting wild horses to control their numbers on the range. However, the supply of wild horses has markedly exceeded adoption demand, forcing the BLM to hold horses in sanctuaries and other long-term holding facilities, primarily at taxpayer expense.

To help reduce population pressure on public lands and the adoption program, the BLM has been supporting horse contraception research since the 1970s (Kirkpatrick 2005). The use of porcine zona pellucida (PZP) immunocontraception for the purpose of safe and cost-effective regulation of free-roaming wild horse populations has been under investigation since 1988 (Turner and Kirkpatrick 1991). Porcine zona pellucida vaccine use in the horse was first reported by Liu et al. (1989). The vaccine appears to act by stimulating anti-PZP antibodies that bind to the surface of the ovulated egg, preventing sperm attachment (Liu et al. 1989).

In initial studies, a vaccination protocol of 2–3 separate inoculations about a month apart yielded reversible infertility for one breeding season in a number of species including horses (Liu et al. 1989, Kirkpatrick et al. 1990), burros (*Equus asinus*; Turner et al. 1996), white-tailed deer (*Odocoileus virginianus*; Turner et al. 1992), and African elephants (*Loxodonta africana*; Fayrer-Hosken et al. 1997). However, for managing free-ranging wildlife a 1-injection vaccine effective for 1–3 years is desirable.

One approach to a one-injection, multiple-year vaccine is to mimic the effects of booster injections by incorporating vaccine into controlled-release polymers. This methodology has involved forming a homogenous mixture of bioactive ingredients with a biodegradable, nontoxic lactide-glycolide polymer in the form of microspheres (Eldridge et al. 1989, Wang et al. 1990). Upon intramuscular injection and contact with tissue fluids, the polymer material gradually erodes and releases the bioactive contents (Wang et al. 1991).

In previous studies with free-roaming wild horses, a single injection containing primer PZP adjuvant and booster PZP (no adjuvant) in controlled-release preparations of lactide-glycolide microspheres reduced fertility but was less effective than 2 separate inoculations (Turner et al. 1997). Since an important function of adjuvant is to enhance immuno-

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recognition of antigen and consequent antibody production, we expected that adjuvant presence in the controlled-release component of the vaccine would boost the effectiveness of this component. In a subsequent study, a vaccine preparation in which a carbomer adjuvant was added to the PZP-containing polymer microspheres proved as effective as 2 separate injections of PZP adjuvant (Turner et al. 2000). Further refinement of controlled-release components led to replacement of gradual-release polymer microspheres with bolus-release polymer mini-rods (pellets) in the 1-injection, 1-year vaccine (Turner et al. 2002). We hypothesized that these conditions would resemble multiple, separate injections of vaccine and would yield infertility similar to that obtained with multiple booster inoculations.

Our present objective was to test the effectiveness of a presumptive 2-year PZP vaccine that utilized a controlled-release PZP adjuvant component to provide multiple bolus-release booster events.

STUDY AREA

The study area was part of the Clan Alpine Herd Management Area in central Nevada, USA, and comprised about 7,800 km^2 centered at 39°32′N latitude and 117°55′W longitude. Elevations ranged from 1,300 m to 3,066 m. Vegetation on the portion of the range most used by study horses was composed largely of sagebrush (*Artemisia* sp.) communities, with pinyon pine (*Pinus edulis*) and juniper (*Juniperus* sp.) occurring at higher elevations. Grasses commonly utilized by horses in these communities were Indian rice grass (*Achnatherum hymenoides*), galleta (*Pleuraphis jamesii*), and desert needlegrass (*Achnatherum speciosum*). Average annual precipitation (2000–2004) was <22 cm at 2,000 m elevation. Water was available from several permanent springs in the study area. During the study period, we made >80% of the observations by aerial and ground surveys in 4 distinct areas comprising <3,000 km^2 and favored by the horses due to feed and water availability. The Carson City (Nevada) District of the Bureau of Land Management (BLM), United States Department of Interior administered the study area.

METHODS

Experimental Design

The study examined the antifertility effectiveness and longevity of a single-injection PZP vaccine containing a controlled-release component exhibiting several release windows. Every vaccine treatment contained an emulsion of aqueous PZP, Freund's Complete Adjuvant (FCA), and a controlled-release component containing PZP and the saponin adjuvant QS-21. We applied contraceptive treatment in the field (Jan 2000) after we randomly assigned >200 captured adult wild females to a vaccine treatment group ($n = 96$) or to an untreated control group ($n > 100$). We did not include a sham control group because we have previously shown (Turner et al. 1997) that wild females captured, handled, and given a placebo injection exhibited fertility rates that did not differ from those of untreated females not captured. We estimated fertility rates in the absence of contraceptive effects from foal counts made among treated and untreated females in May and October of 2000 (i.e., foals born to both were conceived before treatment in Jan 2000). We determined the effectiveness and duration of treatment by assessing reproduction via ground and aerial foal counts and fecal-hormone pregnancy tests at various times across the period from autumn 2000 through autumn 2004. We considered a female to have successful reproduction if she tested positive for pregnancy or was observed with her foal or both.

Horse population.—We performed this study under auspices of Animal Care and Use protocol (no. 100742) of the Medical University of Ohio and the standard animal management practices of the Bureau of Land Management. The study area was inhabited by about 600 horses with a harem-band social structure (Turner et al. 1981, Berger 1986). The peak breeding period was May and June, and the peak foaling period was April and May. We estimated the physical condition of females in the corral prior to inoculation and in the field during subsequent observations using the condition scale (1–10) developed by Henneke et al. (1983). Initial female condition ranged from fair (3) to good (6). Condition-3 females comprised <30% of those treated. None of the females appeared to deteriorate in condition across the study and many apparently improved, possibly due to increased feed availability after a January 2000 permanent removal of 233 (329 initially removed, 96 returned to range) horses from the range.

Because BLM selective-removal policies in place at the time dictated the removal of all females under 10 years of age, all females receiving treatment were >10 years of age. Six treated females were aged >20 years. In some wild horse herds, reproduction may be reduced in females >20 years old (T. Pogacnik, BLM Wild Horse and Burro Program files, unpublished data). However, data for the Nevada Wild Horse Range in central Nevada, where female physical condition was generally inferior to the Clan Alpine herd, showed fertility rates in females aged 10–20 were similar to those rates in females >20 (Turner et al. 2000).

PZP vaccine emulsion preparation.—We prepared PZP from porcine ovaries as previously described (Liu et al. 1989). The primer portion of the vaccine consisted of an emulsion of 0.5 cc FCA with 65 μg PZP in 0.5 cc phosphate buffer solution (PBS). We prepared the emulsion, 8 doses at a time within 24 hours of injection using 10-cc glass syringes joined with a plastic connector. After 100 plunger strokes, we loaded the emulsion into 10-cc plastic syringes for injection via a 14-gauge, 3.7-cm needle. We prewashed the needle (new needle each injection) and the injection site with 70% ethanol. The emulsion was kept chilled, but we hand-warmed it prior to injection.

Controlled-release pellet preparation.—We prepared the pellets by a heat–extrusion process employing a Dynisco extruder (Dynisco, Morgantown, PA). The pellets consisted of lyophilized PZP and QS-21 (a water-soluble saponin adjuvant) matrixed in homogenous lactide and glycolide

Table 1. Polymer pellet composition and in vitro bolus controlled-release characteristics for porcine zona pellucida (PZP) vaccine used in female wild horses in Nevada, USA, in 2000.

Pellet type based on exp release delay (months)	No. of pellets tested	Pellet mass (mg)	Polymer ratio[a] (lactide:glycolide)	PZP mass (μg)	QS-21[b] mass (μg)	Loading rate (PZP + QS-21) polymer (%)	Obs in vitro release delay[c] (months)	
							$\bar{x}$	Range
1	6	7	65:35	70	175	3.5	1.5	0.9–2.1
3	6	7	85:15	90	200	4.1	3.9	3.0–5.2
12	6	8.5	100:0	250	500	9.4	11.5	10.3–14.2

[a] Lactide used is a mixture of dextro- and levo-rotatory forms.
[b] QS-21 is a water-soluble saponin adjuvant.
[c] $\bar{x}$ and range are based on when >80% of total PZP release occurred during entire in vitro incubation period.

polymers. Pellet preparation and release characteristics (Table 1) are summarized below. We regulated the delay period for release of active ingredients from pellets by altering the ratio of lactide to glycolide. We chose to release windows at approximately 1 month and 3 months to mimic a second and third inoculation, which had yielded effective contraception for one breeding season in an earlier study (Kirkpatrick et al. 1990). Likewise, we chose an approximate 12-month window to mimic an annual booster inoculation shown previously to provide a second year of contraception (Kirkpatrick et al. 1992). We extruded pellets from a melted (<95° C) mixture of lactide and glycolide containing micronized PZP and QS-21. The cylindrical pellets, designed to fit inside the barrel of the 14-gauge hypodermic needle, were approximately 0.6 × 1.5 mm with an average weight of 7.5 mg. Pellets were of 3 types (Table 1), and we determined characteristics of PZP release at several polymer ratios in vitro by measuring PZP concentrations in the incubation medium across time using a sandwich-type enzyme-linked immunosorbent assay (ELISA) for PZP (Turner et al. 2002). The in vitro environment for a given pellet consisted of 3 cc of 37° C bacteriostatic PBS – 1% horse serum gently and continuously shaken (2 back-and-forth motions/sec). Every 5–7 days for up to 14 months, we removed the medium for assay and added 3 cc of new medium to the pellet vial. We performed the ELISA on 100 μl of each sample of medium. We did not measure the release of QS-21 from pellets. However, we expected release characteristics to be similar to PZP, based on similar water solubility (C. Kensil, Antigenics, Inc., personal communication).

Accessing, handling, and inoculating of horses.—The BLM gathered horses by helicopter, which permitted injection by hand. We chose this method because access to the horses was possible as a part of scheduled roundup for the BLM horse adoption program. Between 2 January and 9 January 2000, the BLM gathered 329 horses by helicopter into portable corrals. In the corral system the BLM 1) separated the gathered males and females, 2) moved them singly through a stock chute, 3) estimated age through dentition and physical condition, 4) gave a 1-cc prophylactic Strepguard® (Miles Laboratories, Shawnee Mission, KS) injection intramuscularly, and 5) permanently marked 96 healthy females >10 years of age with consecutive numbers by freeze-branding. The brands were located on the upper left hip, were 8 cm in height, and were readable from a helicopter and through a spotting scope at >500 m distance on the ground.

We used a jabstick to inject 1 cc of freshly prepared emulsion of buffered PZP adjuvant (described above) into the left gluteus muscle and we simultaneously injected 1-month, 3-month, and 12-month pellets, which were sequestered in the needle barrel. We examined each needle postinjection to ensure pellet delivery. Three of the 96 deliveries by this method failed, and in those cases, we delivered the pellets by a second injection using PBS to propel the pellets. We photographed each marked female prior to release. The BLM maintained all females (treated or untreated) in portable corrals on grass–hay and water ad libitum for up to 4 days, and then released them into the range area from which they had gathered them. Before release, the BLM separated the females and their foals and retained the foals for eventual adoption. Within 24 hours of the release, representatives of the BLM surveyed the study area by helicopter to determine the horses' well-being and dispersal.

Pregnancy testing by fecal steroid analysis.—In the study area, most foaling occurs in April and May, and breeding is uncommon after mid-June. Pregnancy can be detected reliably with fecal analysis within 80 days postconception (Kirkpatrick et al. 1991). We sampled females for pregnancy detection in autumn 2001 to determine treatment efficacy for year 2 and to verify for this herd the validity of foal counts for assessing reproductive rates, as previously reported for other herds (Turner et al. 1996, Turner and Morrison 2001). Between mid-September and mid-October of 2001, we observed females for defecation using binoculars and 20–40× spotting scopes. We took photographs of untreated females at observation when possible to enable later identification for foal presence or absence. We ensured that each sample collected was from the desired female by pairing observers such that one person maintained view of the sample through the scope while hand- or radio-signaling the other observer to the sample. If the location or specificity of the sample was in question, we did not collect the sample. We collected 2 freshly dropped fecal balls in a sealable plastic bag, labeled it, and stored it on ice until it could be placed in a freezer (within 72 hr). We determined pregnancy in each sample via measurement of estrone sulfate (E1C) and immunoreactive progesterone metabolites (iPdG) by ELISA (Kirkpatrick et al. 1991). The combined measures

Table 2. Association of steroid metabolite levels in fecal samples collected in September and October 2001 with the presence of foals the following summer in female wild horses in Nevada, USA.

F condition	n[a]	Fecal metabolite concentration[b] E_1C[c] (pg/g feces wet wt) $\bar{x} \pm$ SE	iPdG[c] (ng/g feces wet wt) $\bar{x} \pm$ SE
Without foal	24	3.0 ± 0.1	649.3 ± 107.6
With foal	14	6.8 ± 0.9	10,083.0 ± 2,153.1

[a] F included freeze-marked ($n = 17$) and unmarked, but uniquely identifiable ($n = 21$), individuals.

[b] E_1C = estrone sulfate, iPdG = immunoreactive pregnanediol glucuronide.

[c] Values with vs. without foal were different ($t = 2.74$, $P < 0.01$).

Table 3. Hormonally determined pregnancy rates in the second year of treatment for free-roaming female wild horses in Nevada, USA, given in 2000 a single-injection porcine zona pellucida (PZP) contraceptive vaccine of presumptive 2-year duration.[a]

Condition	Total no. F	No. pregnant	No. not pregnant	Pregnancy rate (%)
PZP-treated[b]	17	3	14	16.6
Untreated control	21	11	10	55.0

[a] We immunized F in January 2000 and we collected fecals for pregnancy testing in September and October 2001. We considered a F to be pregnant if fecal estrone conjugates were >3.0 ng/g and immunoreactive pregnanediol glucuronide was >2,400 ng/g.

[b] Vaccine treatment consisted of intramuscular injections of liquid (PZP/Freund's Complete Adjuvant) plus 3 controlled-release pellets (PZP/QS-21 [a water-soluble saponin adjuvant]; 1-month, 3-month, or 12-month delay in release).

have proven >93% accurate in pregnancy diagnosis in several species (Lasley and Kirkpatrick 1991), including the wild horse. We considered a female pregnant when values for a given sample were E1C > 3.1 ng wet feces and iPdG > 2,400 ng/g wet feces.

Foal counts.—The presence of a foal with a given marked female was determined by ground observations in May and October of 2000–2004. We concurrently counted foals among untreated, unmarked females. Double-counting of a given unmarked female was unlikely in the context of unique color and marking patterns across a given band of horses. In questionable cases, we compared our immunization-day photo-identification file with video of target females taken during field collections. We verified which foal was with which female in ground surveys by observing the horses until the foal clearly evidenced association with a given female by its repeated proximity to her during grazing and traveling or by nursing from her or both. We performed aerial counts of horses using a Bell 47 (Ft. Worth, TX) or Hughes Jet Ranger (Seattle, WA) helicopter, with both pilot and observer (1 or 2) having prior experience in aerial survey of horses. We established maternity by applying minimal pressure on a given band of horses initially, which permitted foals to pair with mothers as the band formed up and moved away from the helicopter. We approached more closely to permit reading of freeze-mark numbers, and then withdrew to permit reobservations of mother–foal pairings. We determined reliability of mother–foal pairing behavior as a basis for contraceptive assessment to be >95% when we reidentified and verified marked females with and without foals in ground surveys.

Statistical analysis.—Where appropriate, we have presented data as mean ± standard error. We employed Student's *t*-test for statistical analysis of fecal data. We determined possible group differences among reproductive success rates using a Tukey-type multiple-comparison test for proportions or a binomial probability distribution (BPD; Zar 1984).

RESULTS

In vitro incubation of PZP-containing pellets yielded some PZP release (<10% of total) in the first 10 days, with no detectable release thereafter until a primary release event (72–85% of total release) for a given pellet. Considerable variation occurred in delay of release onset among pellets of a given type (Table 1). Duration of release averaged 8 days, 14 days, and 20 days for 1-month, 3-month, and 12-month pellets, respectively.

Among marked females, 18% ($n = 17$) were both pregnancy tested (Oct 2001) and assessed by foal counts (May and Oct 2002). In addition, among unmarked (untreated) females that were uniquely identifiable (from photographs taken during fecal collections), we assessed 21 individuals by both pregnancy test and foal presence or absence. Thus, we assessed 38 females both hormonally and by foal observation (Tables 2, 3). Average values for E_1C and iPdG were 2.3-fold and 15.5-fold, respectively, greater among females that produced foals than corresponding values for these metabolites in females that did not produce foals (Table 2). For both steroids measured, the average value for females that did produce foals was significantly greater ($t = 2.74$, $P < 0.01$) than for females that did not produce foals. In the spring of 2002, we were able to locate and reidentify 12 of the females that had tested pregnancy-positive and 8 of the females that had tested pregnancy-negative in October 2001. We observed all 12 of the pregnancy-positives and 1 of the 8 pregnancy-negatives to have a foal the following spring. Thus, diagnostic accuracy of hormone versus observation-based fertility assessment among these females was 19/20 (95%).

The percentages of reproductive success in 2001 were 5.2% for treated and 53.6% for untreated females (Table 4). These rates in 2002 were 14.9% (treated) and 58.5% (untreated) by observation, and 16.6% and 55.0%, respectively, by pregnancy test (Table 3). Statistics applied to observational rates revealed a significant difference between treated and untreated averages in both years (2001: $z = 5.66$, $P < 0.001$; 2002: $z = 4.81$, $P < 0.001$; Table 4). Reproductive success rates in treated females remained 19.5% below rates in untreated females in 2003, but this was not significant ($z = 1.15$, $P = 0.252$). In 2004, reproductive success rates in treated and untreated females were nearly identical (Table 4). Among untreated females,

Table 4. Fertility rates from 2000–2004 for free-roaming female wild horses in Nevada, USA, given a single-injection porcine zona pellucida (PZP) contraceptive vaccine of presumptive 2-year duration.

Yr[a]	Total ad observed[b]	Total foals observed	Untreated F		Treated F		Fertility rate (%)	
			With foal	No foal	With foal	No foal	Untreated	Treated
2000	107	27	11	9	16	17	52.4	48.5[c]
2001	127	40	37	32	3	55	53.6	5.2[d]
2002	236	41	31	22	10	57	58.5	14.9[d]
2003	128	28	22	21	6	13	51.1	31.6
2004	187	46	28	26	18	21	51.8	46.2

[a] Treatment was in Jan 2000; assessment period was approx. 2 weeks between May and Oct of each yr.
[b] No. includes M and F, representing the base population from which foal data were obtained each yr.
[c] No treatment effect expected on 2000 foal counts, because F were bred 6–8 months prior to treatment.
[d] Values for treated vs. untreated were different (2001: $z = 5.66$, $P < 0.001$; 2002: $z = 4.81$, $P < 0.001$).

the reproductive success rate averaged 53.5% across the entire study period. Females observed for foal presence across seasons and years were not necessarily the same individuals.

DISCUSSION

Inoculation of captured and released wild females with PZP vaccine resulted in a marked suppression of fertility that was significant across 2 breeding seasons, although year-2 infertility was not as great as in year 1. Although the reproductive rate in year 3 for treated females was not significantly below the rate in untreated females, the 19.5% absolute rate difference suggests a partial contraceptive carryover through year 3. The vaccine format employing controlled-release pellets was as effective in year 1 as that reported previously (Turner et al. 2000) for 2 separate inoculations. Fertility rates among both treated and untreated females foaling in 2000 (before vaccine effects) and among untreated females in other years were similar to those reported for other wild horse populations (Wolfe et al. 1989, Turner et al. 2000).

This study reconfirms the high accuracy of pregnancy diagnosis via fecal steroid metabolites in wild horses (Kirkpatrick et al. 1991, Turner et al. 2000), with pregnancy data versus foaling data in 95% agreement. These data are also evidence that fetal loss was unlikely to have inflated infertility rates since pregnancy diagnosis was well-correlated with foal counts. By measuring both E_1C and iPdG (Table 2), we minimized the chances of misdiagnosis.

Polymer pellet composition and characteristics (Table 1), and data obtained from in vitro incubation of PZP-containing pellets (previously reported by Turner et al. 2002) showed that all 3 pellet types released PZP in an approximately bolus fashion, with up to 80% of the PZP being released across a 7–10-day period for a given 1-month pellet and across a 12–30-day period for 3-month and 12-month pellets. While the in vitro pellet-release data may provide a reasonable approximation of in vivo activity, we cannot precisely extrapolate it to in vivo conditions (Sanders et al. 1986, Tracy et al. 1999); that is, actual release characteristics of the previous pellets in the females of this study are undetermined. However, the marked infertility in both years 1 and 2 indicate that pellet content release was sufficient to engage immune system memory of the PZP antigen (Kirkpatrick et al. 1992, Newman and Powell 1995).

Despite an increase in reproductive success across years 1–3 posttreatment in the population of treated females, the average reproductive success rate in treated females remained below untreated females. In year 4 posttreatment, the rates in treated and control females were nearly identical, indicating complete return to fertility. It is likely that slower return to fertility in some females reflects individual differences in immune system responsiveness or sensitivity across the population.

MANAGEMENT IMPLICATIONS

As suggested by Garrott (1991), the issues of cost-effectiveness and limitation of population growth must be included in assessing long-term contraceptive management potential. The availability of a 1-inoculation, multi-year PZP vaccine allows contraception to be incorporated at low cost into standard BLM management practices, which include the regular round up (gather) of wild horses for the purpose of removing some from the range. Administering a single-injection, multi-year contraceptive to females being returned to the range has the potential to limit management program costs by reducing frequency of horse roundups, number of horses entering the adoption program (or maintained in long-term holding facilities), and the number of horses using the range (Gross 2000). Associated benefits will include improved range quality and reduced stress to wild horse populations (due to better habitat and less human intervention).

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Controlled-release components of PZP contraceptive vaccine extend duration of infertility

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Abstract. Successful immunocontraception of wildlife relying on repeated access to individuals for boosters has highlighted the need to incorporate primer and booster immunisations into one injection. We have investigated use of controlled-release polymers (lactide–glycolide) in small pellets to provide delayed *in vivo* delivery of booster porcine zona pellucida (PZP) antigen and adjuvant. This report reviews pellet-making methodology, *in vitro* testing of controlled-release pellets and *in vivo* effects of controlled-release PZP vaccine. We assessed 3 different manufacturing approaches for producing reliable, cost-effective pellets: (1) polymer melting and extrusion; (2) solvent evaporation from polymer solution; and (3) punch and die polymer moulding. *In vitro* testing of release patterns of controlled-release formulations, towards development of a 3-year duration vaccine, provided estimates for *in vivo* use of pellet preparations. These *in vitro* studies demonstrated protein release delay up to 22 months using 100% *l*-lactide or polycaprolactone polymers. For *in vivo* tests, pellets (1-, 3-, and 12-month release delay) serving as boosters were administered intramuscularly with PZP/adjuvant liquid primer to wild horses (*Equus caballus*), white-tailed deer (*Odocoileus virginanus*) and African elephants (*Loxodonta africana*). Horse field studies assessed fertility via offspring counts and/or faecal-hormone pregnancy testing. Treatment decreased fertility 5.3–9.3-fold in Year 1 and 3.6-fold in Year 2. In preliminary testing in deer, offspring counts revealed treatment-associated fertility reduction of 7.1-fold Year 1 and 3.3-fold Year 2. In elephants, treatment elevated anti-PZP titres 4.5–6.9-fold from pretreatment (no fertility data).

Introduction

Contraception has become a substantive means of managing several species of free-roaming wildlife and captive exotics. In the light of growing interest in this area, the need for agents that are maximally both user and recipient friendly has become apparent. Two agent characteristics that partially address this need are efficient agent delivery and extended duration of effectiveness in a single treatment. The impetus for developing a controlled-release, extended-duration immunocontraceptive originated from studies with free-roaming wild horses. Early studies employed a protocol of two or three separate immunisations (primer plus one or two boosters) about 1-month apart (Kirkpatrick *et al.* 1990). This protocol was necessary to satisfy requirements for optimal and sustained immune response with maximal immunomemory for subsequent annual boosters. Although the treatments were effective, the high costs and daunting logistics of (1) multiple accessing of wild horses for darting or (2) maintaining wild horses in captivity between injections made the multiple-injection protocol impractical for most circumstances. Since controlled-release polymer technology was already in use for delivery of cancer chemotherapeutics and some vaccines (Chang 1976; Carter *et al.* 1988; Linhardt 1989; Wang *et al.* 1990, 1991), we began investigations of this technology for possible application in a single-injection porcine zona pellucida (PZP) immunocontraceptive.

This report reviews and updates salient aspects of the testing of a one-injection, multi-year immunocontraceptive that employs controlled-release polymer components to achieve efficient vaccine delivery and extended vaccine effect. The vaccine utilises PZP antigen plus adjuvant to provide contraception (Kirkpatrick *et al.* 1990, 1991*b*). Polymer-based controlled-release vaccine delivery has been studied predominantly in horses to date (Turner *et al.* 2001, 2002, 2007), and this is reflected in the present report. However, preliminary data applying this technology to white-tailed deer and African elephants are also presented for broader perspective. The

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content of this review is divided into three sections: (1) *in vitro* studies; (2) *in vivo* studies; and (3) research assessment and management implications.

In vitro studies

Methods

The endpoint for *in vitro* testing has been the temporal pattern of protein release from controlled-release formulations into incubation medium. Early *in vitro* studies (1992–1996) focussed on controlled-release polymers mixed with vaccine and formulated as microspheres suspended in liquid for delivery (Turner *et al.* 2002). However, repeated difficulties were encountered with delivery of microspheres, due to the tendency of these particles to settle out of the liquid vehicle, clump in the syringe needle and clog it. This persistent and unresolvable problem led to development of solid pellets as the delivery medium, and all subsequent studies have utilised pellet formulations.

Polymers and pellets

The polymers used to make pellets (and information regarding their projected release attributes) were obtained from Birmingham Polymers (Birmingham, AL; now Lactel/Durect, Inc., Pelham, AL). Polymer information is presented in Table 1. Lyophilised PZP and water-soluble saponin adjuvant (QS-21, human-grade; QA-21, veterinary-grade Vrbac Corp., Brussels, Belgium) were mixed by trituration to form a homogeneous matrix with the inert, non-toxic polymers. The mixture was then pelletised by one of three methods briefly described below: heat/extrusion (H/X), cold/evaporation (C/V) or pressure/moulded (P/M).

The H/X method involved melting the polymer–vaccine mixture in a Dynisco extruder (Morgantown, PA) and pushing it through a small aperture to form a long strand of diameter 0.6 mm (to fit inside a 14-gauge hypodermic needle). The strand was then cut to ~1.6-mm length pellets. This method had the advantage of bulk pelleting capability, but ~50% of the ingredients were lost during manufacture, and the heat (100–113°C) could partially degrade the PZP. For this reason we examined two alternate methods that did not employ heat. In assessing the viability of these two methods, we used H/X pellets (of proven efficacy) as a reference base.

The C/V pellets were formed at <20°C by homogeneous suspension of vaccine ingredients in polymer that was rendered as a viscous liquid in the organic solvent dichloromethane. This liquid was then injected into 14-gauge Silastic tubing moulds 5 cm each in length. Gradual evaporation of solvent from the moulded polymer–vaccine mixture across 48 h at 4°C yielded a firm strand inside the tubing. The mould was cut away, and the cylindrical strand was then cut into 1.6-mm lengths, yielding 26–30 pellets. Loss of ingredients during pellet manufacture was 15–20%. This method required ~3-fold more personnel time per pellet than the H/X method.

The P/M pellet-making method employed a low-pressure punch-and-die system. We designed an aluminum mould with 40-pellet capacity that was based on a standard mould used for small-scale pharmaceutical tablet/suppository production. Each half of the mould contained a row of half cylinders, which became whole cylinders for pellets when the mould halves were clamped together. We had the mould manufactured locally (Bollinger Tool and Die Co., Toledo, OH). The pellet-making process consisted of (1) thorough mixing (trituration) of finely powdered vaccine ingredients, polymer and colour dye, (2) loading the powder into the mould, (3) warming the mould in an incubator for 20–30 min while periodically applying pressure to the die chambers by a spring-loaded punch array. This method employed a temperature of 80–85°C, which was well below the denaturation temperature for this vaccine (J. Turner, unpubl. data). The polymer–vaccine mixture became pliable at this temperature, allowing pressure from the punch array to form the pellets in the die cylinders. The halves of the multi-pellet mould were then separated, and the exposed pellets were removed. Relative to H/X pellets, these product pellets were of similar hardness (based on force required to cut with a single-edged razor blade and to crush in a mortar and pestle) and similar physical appearance at 10× magnification (similar translucence and lack of air bubbles or fracture lines). The loss of materials was 8–14% (compared with >50% for H/X method), and the per-pellet personnel time was ~1.5-fold greater than for H/X pellets. An advantage of the P/M method is that no minimum number of pellets must be produced to obtain reasonable production efficiency (the H/X method requires a 100-pellet minimum of each type). Thus, the P/M method can be advantageous for short-notice production and for application to small numbers of animals.

Table 1. Characteristics of polymer pellets employed in one-injection controlled-release porcine zona pellucida (PZP) vaccine of known effectiveness

Pellet type (expected release delay)	Actual release delay (days)	Polymers	Ratio (%)	PZP (μg)	Adjuvant (μg) (QA-21)
1 month	30–60	Lactide : glycolide	65 : 35	100	200
3 months	75–100	Lactide : glycolide	85 : 15	100	200
12 months	340–370	*d*, *l* lactide	100	250	500

Controlled-release pellet contents

Three polymer–vaccine formulations were prepared by the H/X method and tested *in vitro*. Contents of the various pellets are presented in Table 1. Lyophilised PZP was prepared as previously described by Liu *et al.* (1989). The adjuvant was QS-21 or QA-21. Dyes for colour coding were commercial non-toxic food dyes. Polymers were finely ground and passed through a #60 sieve (250 μm). These were projected to release respectively in windows of 1–3 months, 3–5 months and 10–12 months. For all pellets, homogeneity was assured by trituration mixing of powdered ingredients (polymer, PZP, adjuvant, dye) using a mortar and pestle and examination of produced powder by microscope (10–20×). The cylindrical pellets (~0.6 mm × 1.6 mm) weighed 7.5–10.0 mg and the ratio of vaccine ingredients to polymer (termed '% loading') was <9%. For optimal pellet performance maximal loading should be <10% (D. Flanagan, pers. comm.). Pellets were stable at room

temperature but were nonetheless refrigerated for extended storage.

Since the pellet-making process involved potential protein-denaturing conditions (heat for H/X process; organic solvent for C/E process), we assessed viability of PZP exposed to these pellet-making conditions (P/M-type pellets have not been tested to date). We determined conservation of immunoreactivity by Ochterlony immuno-diffusion and titre response of mares immunised with these PZP preparations. No significant effect on PZP of heat or solvent exposure was observed (Liu *et al.* 2005; Turner *et al.* 2007).

In vitro *testing methods*

For all pellets, we determined characteristics of PZP release by measuring PZP concentrations in the incubation medium across time using a sandwich-type enzyme-linked immunosorbent assay (ELISA) for PZP (Turner *et al.* 2002). The *in vitro* environment for a given pellet consisted of 1–3 cc of 37°C bacteriostatic PBS gently and continuously rocked back and forth (one motion per second). Every 3–5 days for up to 23 months, we removed the medium for assay and added the same volume of new medium to the pellet vial. We performed the ELISA on 100 µL of each medium sample. We did not measure the release of adjuvant from pellets, but expected release characteristics to be similar to PZP based on similar water solubility (C. Kensil, Antigenics, Inc., pers. comm.). Some testing of pellets employed bovine serum albumin (molecular weight similar to PZP) in place of PZP. Protein release in those tests was measured by commercial protein assay (Pierce BCA Kit, Rockford, IL). Protein assay sensitivity was 0.5 µg mL^{-1} incubation fluid.

In vitro group data are presented as mean values ± standard error (s.e.). However, statistical analysis was not applied, since the purpose of these studies was to examine release patterns, which were visually obvious.

In vitro *findings*

Release patterns for PZP vaccine-containing pellets have been previously reported (Turner *et al.* 2002). However, in light of the focus of this report on pellets, relevant details are provided in Table 1, and a sample *in vitro* PZP release pattern is presented in Fig. 1.

C/V pellets

We examined the C/V method towards avoiding the disadvantages of H/X method: heat denaturation of PZP and high materials loss. Although the C/V method did achieve these goals, C/V pellets performed suboptimally *in vitro*, releasing ~80% of the total PZP before the desired release window (Fig. 2). Nonetheless, these studies included release characteristics of extremely water-resistant polymers (100 *l*-lactide and polycaprolactone) in pellets that could theoretically delay vaccine release by up to 2 years. One useful outcome of the *in vitro* testing of these polymers in C/V-type pellets was the finding during a 23-month incubation of presumptive 20-month pellets that both polymers can delay some protein release for up to 20 months (Fig. 2). Although only ~20% of the protein remained in these pellets after the first 18 months of incubation, across the next 5 months more than half of the remaining protein was released during only 1 month (Month 20 for polycaprolactone; Month 21 for 100 *l*-lactide).

P/M pellets

In vitro protein-release testing of 1-month P/M-type pellets containing bovine serum albumin (an inexpensive stand-in for PZP) was carried out as previously described for H/X pellets. The protein release pattern showed <12% initial release followed by <5% thereafter (except 7% on Day 20), with the major release (45.2%) occurring across Days 40–48 (Fig. 3). Release returned to pre-surge values thereafter.

***In vivo* studies**

Animals

These studies report effectiveness of controlled-release PZP vaccine in domestic and free-roaming wild horses (*Equus caballus*) and in free-roaming white-tailed deer (*Odocoelius virginanus*) and African elephants (*Loxodonta africana*). The principal investigators were J. Turner (horse), A. Rutberg (deer) and H. Bertschinger (elephant). Horse study sites were USA Department of Interior Bureau of Land Management (BLM) Herd Management Areas in Nevada (USA) for free-roaming horses and University of California, Davis (I.K.M. Liu) for domestic horses. The deer study site was Fripp. Island, SC (USA). The elephant study site was in Limpopo Province, South

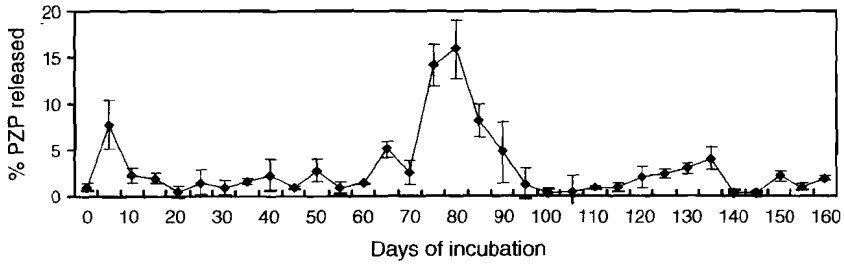


Fig. 1. *In vitro* release pattern of porcine zona pellucida (PZP) from heat/extrusion (H/X) pellets of presumptive 3-month release delay. Each point is the average of 6 pellets (8 mg per pellet). Each pellet was a matrix of lactide : glycolide polymer and PZP (100 µg) incubated at 37°C in phosphate-buffered saline (PBS; pH 7.2) with gentle rocking motion (one per second). Assay was by enzyme-linked immunosorbent assay (ELISA).

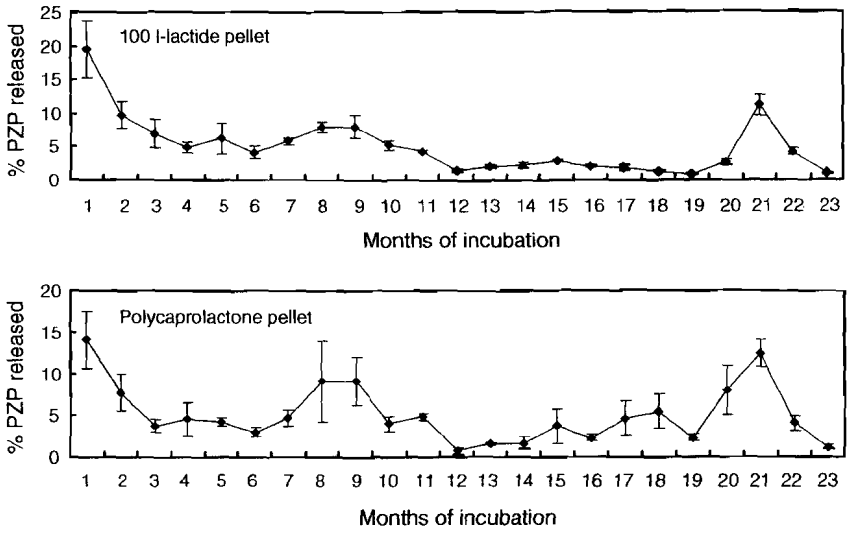


Fig. 2. *In vitro* porcine zona pellucida (PZP) release (as percentage of total pelleted PZP) from polymer pellets of cold/evaporation (C/V) type with presumptive release delay of up to 20 months. Each pellet contained PZP (400 μg) plus QA-21 adjuvant (500 μg). Each monthly value is the average from 10 pellets across four separate 7-day samplings of incubation medium and is presented as % of total PZP placed in each pellet. Lower limit of detection was 0.5 μg protein per mL phosphate-buffered saline (PBS) measured by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

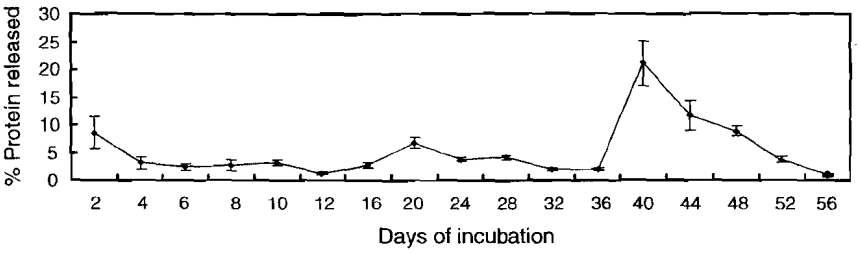


Fig. 3. Percentage of total pelleted bovine serum albumin (BS) released from P/M-type pellets. Each point is the average of 6 pellets (mass = 10 mg/pellet), which were derived from a mixture of lactide : glycolide (50 : 50) and bovine serum albumin (BSA; 750 μg), incubated at 37°C in phosphate-buffered saline (PBS; pH 7.2) with gentle rocking motion (one per second).

Africa. All animals were studied under auspices of respective specific Animal Care and Use protocols for each organisation. Data for horses include both new and previously published files, with the latter clearly referenced. Deer and elephant data represent preliminary studies.

Vaccine

PZP controlled-release vaccine was delivered via hip i.m. injection composed of (1) a primer dose of 100-μg PZP in 0.5 cc phosphate-buffer solution (PBS) emulsified with 0.5 cc Freud's adjuvant (complete = FCA or modified = FMA) and (2) 1-month and 3-month pellets (for 1-year vaccine) or 1-month, 3-month and 12-month pellets (for 2-year vaccine). All pellets used *in vivo* were prepared by the H/X method (except C/V type in one deer group) and contents of each pellet type were as previously described.

Statistics

Titre data are presented as mean value ± s.e. Domestic horse titre data for treatment *v.* control were sufficiently different to obviate statistical assessment. Wild horse data were previously published and statistically assessed in those reports (Turner *et al.* 2002, 2007). Elephant titre data were assessed by *t*-test and deer fertility data were assessed for treated *v.* control differences by binomial probability distribution, (Zar 1984).

Horse studies

Subsequent to *in vitro* testing and before testing in free-roaming horses, *in vivo* assessment of presumptive 1-year and 2-year controlled-release vaccine was performed in captive mares at U.C., Davis. Mares were titre tested via periodic jugular blood sampling across 42 weeks (for 1-year vaccine) and 24 months (for 2-year vaccine). Anti-PZP antibody titres were measured using an

ELISA developed for this purpose and previously described (Liu *et al.* 1989). The positive reference titre was produced by standard two-injection vaccine averaged across six mares from a prior study (Liu *et al.* 1989).

We previously reported *in vivo* anti-PZP titre response to 1-year vaccine containing 1- and 3-month pellets (Turner *et al.* 2002). To summarise those data: relative to a standard two-injection protocol (known to provide 1 year of infertility), the 1-year vaccine was not different in titres across a 42-week monitoring period. By Week 3, the titre response to both vaccine types was >100% of positive reference control and remained so through Week 10. By Week 42, levels were 49–55%. Previous studies comparing titres and fertility in the same mare have shown excellent contraception associated with titres near or above 60% of positive reference control (Turner *et al.* 2002). In a subsequent study, not previously reported, we assessed titre response pattern in domestic mares given 2-year vaccine (containing 1-, 3-, 12-month pellets). Treated mares exhibited a rapid anti-PZP titre rise across 3 weeks to reach 1.4 × positive reference control titre (Fig. 4). These titres gradually decreased but remained above or near positive reference control (100%) for 45 weeks. Thereafter titres declined more rapidly, but remained elevated (54%) at 53 weeks. Control mares, given saline plus blank pellets, showed titres near or below reliable detection throughout the study.

In addition to studies in domestic horses, free-roaming wild mares were field-tested with PZP vaccine. Several herds in Nevada were given 1-year controlled-release vaccine between 1995 and 2001, and these studies have been previously reported (Turner *et al.* 2001, 2002). Likewise, a field test of 2-year controlled-release vaccine was performed in Nevada from 2000 to 2005 (Turner *et al.* 2007). Methods and Results of these studies are summarised below in the context of controlled-release vaccine efficacy. All studies utilised adult horses captured in routine BLM gathers, and mares scheduled for return to the range were treated, by hand-injection or by darting in field corrals. The 1-year and 2-year PZP vaccine used in these studies was the same as described elsewhere in this review. Fertility was determined by foal counts and/or faecal-sample pregnancy test (Turner *et al.* 2001). Pregnancy was assessed by the concentrations of oestrone conjugates and immunoreactive pregnanediol glucuronide in aqueous extracts of faecal material from individually identified mares. These conjugates were measured via ELISA as described by Kirkpatrick *et al.* (1991*a*).

Summary data compiled from our previously reported field studies with 1-year vaccine (Turner *et al.* 2001, 2002) are presented in Table 2. Mares given 1-injection, 1-year vaccine by hand or by dart had fertility rates 0.19 and 0.39, respectively, compared with those of untreated mares (0.61–0.63). Mares given standard two-injection, 1-year vaccine averaged 0.12 of the fertility of untreated mares. All treated groups returned to normal fertility the following year.

More recently, in our field study of hand-injected 2-year PZP vaccine effectiveness in 96 mares (Turner *et al.* 2007), we observed fertility rates of 53.6% for pretreatment and 5.2, 14.9, 31.6 and 46.2% across the ensuing 4 years, evidencing marked infertility effect of 2-year vaccine in Years 1 and 2, partial infertility remaining in Year 3 and return to normal fertility in Year 4.

Deer study

As part of a larger study of one-injection PZP preparations performed on Fripp. Island, white-tailed deer were treated with pellet-based controlled-release PZP vaccine. In February and March (2005, 2006), adult female deer were captured via chemical immobilisation, ear-tagged, measured, weighed, blood-sampled and hand-injected with one of several PZP

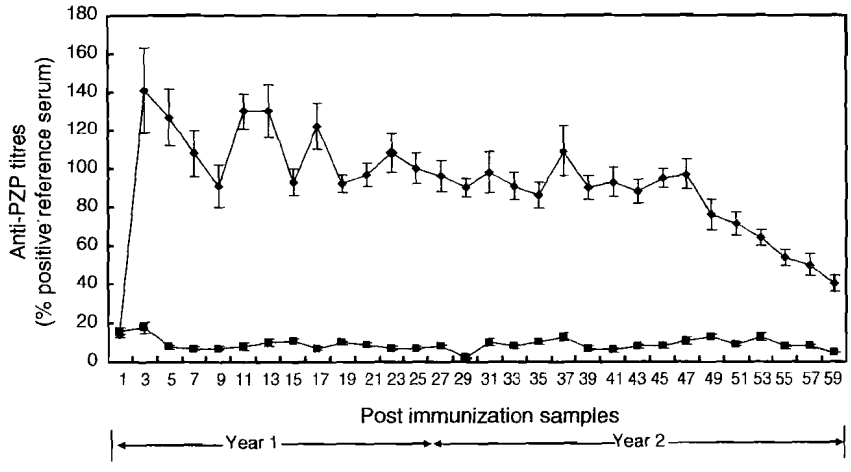


Fig. 4. Antibody response in domestic mares to controlled-release porcine zona pellucida (PZP) vaccine of presumptive 2-year duration. Treatment was standard PZP/adjuvant primer injection plus 1-, 3-, 12-month pellets ($n = 10$; ◆). Control was saline injection plus blank pellets ($n = 5$; ■). Positive reference serum (100%) was highest titre average of six previously studied mares given standard two-injection PZP vaccine. First sample was on the day of immunisation.

Table 2. Survey of field tests of 1-year controlled-release vaccine in wild horses[A]

No. of mares[B]	Vaccine type[C]	Delivery format[D]	Fertility (% foaling) Year-1	Year-2
295	Untreated	None	64	61
376	Standard 2-injection (no pellets)	Hand injection	8	60
48	Primer plus 1-, 3 months pellets	Hand injection	12	58
114	Primer plus 1-, 3 months pellets	Dart	25	50

[A]Modified from Turner *et al.* (2002).
[B]Data are composite of 1–4 separate study sites in Nevada.
[C]Adjuvant in all pellets was a saponin (QS-21).
[D]All mares treated in field corrals or stock chute during routine horse gathers associated with US government wild horse and burro program.

preparations. At capture, females received a priming injection of 100-μg PZP/FMA and a set of one each 1-month, 3-month and 12-month H/X or C/V pellets, hand-injected via trocar. Treated deer that could be located again were recaptured 1 and 2 years later (2006, 2007), and blood was drawn for pregnancy testing via an ELISA for pregnancy-specific protein B (Biotracking, Moscow, ID).

In 160 adult white-tailed does that were captured and pregnancy tested before the treatment period, 133 (83%) were pregnant (Table 3). Of 32 does given 2-year vaccine containing H/X-type pellets, nine were recaptured 1 year later and none of these was pregnant (0% fertility). Of 12 does given 2-year vaccine with C/V-type pellets, two of eight recaptured were pregnant (25.0% fertility). Four does (three H/X, one C/V) were recaptured in Year 2, and one doe (H/X) was pregnant (overall 25.0% fertility). Thus, among all does given pellet-containing 2-year vaccine, fertility rates were 11.7% in Year 1 and 25.0% in Year 2. Relative to pretreatment, Year-1 fertility rates were reduced in treated deer ($P<0.01$ for H/X, $P<0.05$ for C/V; binomial probability distribution). Year-2 data were insufficient for analysis. Return to fertility remains to be determined.

Elephant study

Two groups of elephant cows were treated in South Africa. One ($n=6$) was given standard two-injection PZP vaccine, albeit using 400-μg PZP (instead of 100 μg) in the primer dose and 200 μg (instead of 100 μg) in the booster. The larger doses were used in consideration of large elephant bodyweight. These free-roaming animals were accessed by remote darting from a helicopter. The second group ($n=3$) was captive and hand injected with 1-year controlled-release vaccine consisting of primer (400-μg PZP/FMA) plus two pellets each of the 1-month, 3-month and 12-month type per cow.

Titres were calculated as percentage of positive reference control value. A peak titre value of 100% was assigned to the six two-injection elephant cows in the present study, since the same vaccine dose and protocol provided up to 80% contraceptive efficacy in previous elephant studies (Fayrer-Hosken *et al.* 2000). Thus, these two-injection cows averaged anti-PZP titres of 100%, at 3 months post immunisation. In the three cows given 1-year (pellet-containing) PZP vaccine, anti-PZP titres were 162.1 after 3 months (Table 4). The titre difference was significant ($P<0.01$, *t*-test), giving preliminary indication of more vigorous effect of pelleted vaccine than standard two-injection vaccine in this species. Titre monitoring remains in progress and fertility has not been assessed.

Research assessment and management implications

Considerable progress has been made in the use of controlled-release technology to improve PZP immunocontraception for wildlife. A major technical development in this area has been the

Table 3. Effect of one-injection, 2-year controlled-release porcine zona pellucida (PZP) vaccine on fertility in white-tailed deer

Condition	Doe fertility[A] Year 1		Year 2	
Untreated[B]	133/160	(83.1%)	–	
H/X treated[C]	1/9	(0%)[D]	**1/3**	
C/V treated[C]	2/8	(25.0%)[E]	**0/1**	(25.0%)

[A]Determined by pregnancy-specific β-protein enzyme-linked immunosorbent assay (ELISA; Biotracking, Moscow, ID): denominator = total doe *n*; numerator = fertile doe *n*; parentheses = % of does producing fawns.
[B]Fertility tested at original capture.
[C]Recaptured 1 or 2 years later (H/X = heat-extruded pellets; C/V = cold-evaporation pellets).
[D]Different from untreated ($P<0.01$, binomial probability distribution).
[E]Different from untreated ($P<0.05$, binomial probability distribution).

Table 4. Anti-porcine zona pellucida (PZP) titre response in African elephants given PZP vaccine

Vaccine type	*n*	Anti-PZP titres[C] (% positive reference control ± s.e.) PRE(zero time)	POST(3 months)
Two-injection vaccine[A]	6	–	100.0 ± 14.2
Controlled release[B]	3	16.2 ± 3.3	162.1 ± 16.3[D]

[A]Delivery by dart; primer (400 μg PZP/ Freund's modified adjuvant (FMA)), booster (200 μg PZP/Freund's incomplete adjuvant (FIA)).
[B]Delivery by hand injection; primer (400 μg PZP/Freund's modified adjuvant (FMA)), pellets (two each of 1 month and 3 months type). One- and 3 months pellet = 100 μg PZP/200 μg QA-21; 12 months pellet = 250 μg PZP/500 μg/ QA-21).
[C]Assay via enzyme-linked immunosorbent assay (ELISA) at 1 : 10 serum dilution; measurement via microtitre plate reader.
[D]Different from two-injection value and pretreatment value ($P<0.01$, *t*-test).

transition from microspheres to pellets. A major functional development has been the use of pellet-based vaccine boosters to enable 2 years of contraception in a single injection, thus limiting the need to re-access animals for treatment. *In vitro* assessment of various controlled-release polymer formulations has been a beneficial and cost-efficient weeding-out step in pellet development. Despite the inability to project *in vivo* performance from *in vitro* data, reasonable estimates can be made. The need for *in vivo* testing remains essential and should include both antibody titre measurement in blood and actual fertility assessment via pregnancy test and/or offspring counts. *In vivo* assessment should include pretreatment, treatment (contraceptive effect) and follow-up (return to fertility) data. To date, positive outcomes of *in vitro* release-delay studies and *in vivo* titre studies associated with controlled-release pellets have correlated well with field-treatment contraceptive success in horses (Turner *et al.* 2002; Liu *et al.* 2005) and deer (Turner *et al.* 1992; Rutberg *et al.* 2004). Although controlled-release pellet technology has shown promise for eventual routine management use, several obstacles remain to be surmounted. An issue that faces use of controlled-release, multi-year PZP vaccine is possible delay in return to fertility (Kirkpatrick *et al.* 1995; Turner *et al.* 2007). Thus, controlled-release PZP vaccine must be assessed regarding population-level effects of both initial treatment and retreatment. If controlled-release capability can eventually extend infertility to 3 or 4 years, this issue will likely become even more pressing. Another, perhaps simpler, issue is the reliable delivery of vaccine by remote darting. Currently available darts cannot reliably provide sufficient or rapid enough pressure to propel both vaccine primer emulsion and two pellets into muscle tissue. Partial loss of vaccine ingredients sometimes occurs owing to backflow from the injection site or incomplete expulsion from the dart needle or both. This is evidenced by data in Table 2 showing half the contraceptive efficacy in dart-injected *v.* hand-injected mares given 1-year PZP vaccine. This circumstance for darting is both costly and time consuming. In treating wild horses gathered in routine BLM horse management, the delivery problem has been surmounted by separate hand-injections of primer emulsion by syringe and pellets by trocar. The latter has been 100% effective in pellet delivery in >200 horses (J. Turner, unpubl. data). Development of a trocar-containing dart (now in progress) may resolve this issue. In addition to delivering primer vaccine emulsion, this dart also propels the pellets in the needle barrel with a metal rod upon dart firing.

The database for pellet use in horses indicates that controlled-release PZP vaccine is a viable option in this species. Because controlled-release pellets of the 2-year vaccine provide <24 months (21–22 months) of contraceptive-level titres (Fig. 4), application of vaccine is best done in the autumn or winter of a given year to maximise infertility for two breeding seasons. The same preference may eventuate for 3-year vaccine, since the longest-term pellets for this version of vaccine release at 19–21 months rather than 24 months. Although effectiveness of PZP contraception has been previously established for deer (Turner *et al.* 1992; Rutberg *et al.* 2004) and elephants (Fayrer-Hosken *et al.* 2000; Delsink *et al.* 2002), use of controlled-release pellets as boosters has not been reported. Controlled-release vaccine data for deer and elephant in this report are preliminary but encouraging. In deer the limited evidence shows markedly reduced fertility in Year 1 with controlled-release, 2-year vaccine. Although the finding of only one in four recaptured does with fawns in Year 2 is promising, no conclusions can be drawn from this small '*n*'. Interestingly, C/V pellets, which did not maintain anti-PZP titres in mares, yielded a 58% reduction in doe fertility. The reason for this difference is unknown but may lie in species differences in responsiveness to a given PZP exposure pattern. Elephant data to date are insufficient for conclusions regarding pelleted vaccine, but the vigorous titre response (62% greater than for 2-injection vaccine) is encouraging. It is unlikely that this difference was due to incomplete two-injection vaccine delivery by dart, since previous studies of PZP vaccine delivered to elephants by dart have shown effective (2–4-fold) fertility reduction (Fayrer-Hosken *et al.* 2000; Delsink *et al.* 2002).

Although controlled-release technology is not new, the extended release-delay intervals to which PZP vaccine use has aspired are unexplored territory. Much of the field success has stemmed from trial and error forays and *in vitro* inferences. However, emergent from this has been the efficacious transition from a two-injection contraceptive lasting 1 year to a one-injection contraceptive lasting 2 years. Continued efforts should target refinements in controlled-release capabilities, ease of use and more extended contraceptive duration through controlled-release technology.

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Effects of immunocontraception on population, longevity and body condition in wild mares (*Equus caballus*)

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Contraception is becoming a common approach for the management of captive and wild ungulates yet there are few data for contraceptive effects on entire populations. Management-level treatment of mares with porcine zona pellucida (PZP) vaccine resulted in zero population growth of the Assateague Island wild horse population within 1 year of initiation of treatment. Contraceptive efficacy was 90% for mares treated twice in the first year and annually thereafter. For mares given a single initial inoculation, contraceptive efficacy was 78%. The effort required to achieve zero population growth decreased, as 95, 83 and 84% of all adult mares were treated in each of the first 3 years, compared with 59 and 52% during the last 2 years. Mortality rates for mares and foals after the initiation of management-level treatments decreased below historic and pretreatment mortality rates of approximately 5%. Two new age classes have appeared among treated animals (21–25 years and > 25 years), indicating an increase in longevity among treated animals. Body condition scores for all horses, all adult mares and non-lactating mares increased significantly between summer 1989 and autumn 1999 but did not change significantly in lactating mares. These results provide reliable data for the construction of realistic models for contraceptive management of free-roaming or captive ungulate populations.

Introduction

The management of free-roaming and captive ungulate populations often relies on some form of contraception. The use of porcine zona pellucida (PZP) vaccine to inhibit fertility among various free-roaming wildlife (Kirkpatrick *et al.*, 1990a, 1991a, 1992, 1995a; Kirkpatrick, 1995) and captive exotic (Kirkpatrick *et al.*, 1995b, 1996) species has been well documented. Until 1994, studies had focused on contraceptive efficacy and short-term (< 3 years of treatment) safety of the vaccine, and they did not address the larger issue of changes among entire populations and long-term safety (> 3 years of treatment). One of the ultimate goals of any wildlife contraceptive effort, whether captive or otherwise, is to alter entire populations in some manner, which will positively affect habitat, other species or the target population itself.

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In the case of Assateague Island National Seashore, MD (ASIS), the National Park Service (NPS) sought to protect fragile barrier island habitat from a rapidly growing wild horse population (Rodgers, 1985; Stribling, 1989, 1990; Furbish, 1990, 1994) and to protect the health of the wild horses themselves, by limiting population growth, while at the same time protecting the genetic integrity of the population.

From 1988 to 1993, between 10 and 34 mares in a total population that ranged from 129 to 156 animals were treated annually to test the efficacy and safety of the PZP vaccine. In 1994, ASIS began management-level application of PZP immunocontraception with the short-term goal of stabilizing the population at or near 166 animals. This paper reports the efficacy of PZP contraception over an 11 year period, population changes over 5 management years, and associated changes in mortality, the percentage of mares treated annually, and the occurrence of increased longevity and body condition among treated animals. The working hypotheses were that: (i) the population could be stabilized in < 5 years; (ii) the body condition scores of treated mares would increase; and (iii) that annual effort to achieve these goals would decrease over the 5 years.

Materials and Methods

Management plan

In 1994, a management plan was developed by the ASIS Resource Management staff to maximize the potential population-level effects of immunocontraception while protecting the genetic integrity of the wild horse herd. A treatment priority was established: (i) to inhibit older mares (those with three living offspring or two or more surviving generations) from breeding for the remainder of their lives; (ii) to limit treatment of mares with fewer than two living offspring to no more than 3 consecutive years of treatment, after which they would be permitted to have a foal; and (iii) to prohibit any treatment of older mares with no living offspring. The 3 year limit was imposed because it was clear in 1994 that PZP contraception was reversible after a few consecutive annual treatments (Kirkpatrick *et al.*, 1995a), but fewer data were available for reversibility after treatment for longer periods. The long-term treatment of mares was liberalized in 1998 to two surviving offspring or two generations, where offspring was defined as a foal that survived through its first winter. At the onset of management-level contraceptive treatment in March 1994, the population was 166.

Immunocontraception

Contraception was carried out with PZP vaccine, as previously described (Liu *et al.*, 1989; Kirkpatrick *et al.*, 1990a, 1991a, 1995a; Kirkpatrick, 1995). The vaccine consisted of 0.5 ml PZP, at a concentration of 65 μg protein per 0.5 ml phosphate-buffered saline (PBS), emulsified in 0.5 ml of either Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA). FCA was used for the initial inoculation only and FIA was used exclusively thereafter. The total 1.0 ml of PZP–adjuvant was administered via a barbless 1.0 cc dart with a 14 g, 3.8 cm needle, and was delivered by a Model 193 Pneu-Dart® capture gun (Pneu-Dart, Inc., Williamsport, PA). Each mare was located on foot or by vehicle and darted at a range of 20–50 m to inject the antigen i.m. in the hip or gluteal muscles. Darts were recovered and inspected to make sure they had injected all their contents. All mares on ASIS are identified by physical markings and colour patterns, and the locations and band affiliations of each animal are monitored monthly by ASIS personnel. Initial treatments were carried out in March of each year, or in August or September in the case of mares that had already been treated for at least 2 years.

In March 1994, there were 82 mares aged ≥ 2 years. Of these mares, 41 had never been treated during the previous 6 years of contraceptive research; 33 had been treated previously in the course of the research; and eight mature mares had been treated previously but were not to be treated again until after they had foaled. The 41 untreated mares were given a single PZP–FCA inoculation, the aim of which was not to achieve contraception, but rather to establish antigen recognition and thereby make them 'single-inoculation' animals in preparation for subsequent management-level application of the vaccine. One year later, in March 1995, all mares designated for treatment under the management plan were treated with the PZP vaccine as described above. Two-year-old mares received only a single dose treatment in 1995, but in 1996, the 2-year-olds received an initial PZP–FCA inoculation followed by a second PZP—FIA inoculation approximately 2 weeks later. All other mares included in the management plan received a single booster inoculation of PZP–FIA.

Reproductive success was determined as the birth of a foal in the year after treatment, or a positive pregnancy test during the autumn months after treatment, by analysing concentrations of hormone metabolites in urine and faecal samples (Kirkpatrick *et al.*, 1990b, 1991b; Lasley and Kirkpatrick, 1991). Contraceptive effectiveness was measured by the lack of reproductive success during the 12 months after each mare was treated.

Body condition scores

The body condition score of each mare was assessed on a scale of 0 to 5 according to the shape of the rump and, therefore, the amount of fat deposited in the rump area: 0 indicated an animal in very poor condition and 5 represented extreme fat deposits (Rudman and Keiper, 1991). These scores were assessed between July and November in 1989 and again in 1999 by the same method, and the findings were compared.

Results

Overall contraceptive effectiveness during 1988–1999 for the animals given either the standard double-inoculation protocol, then given a single booster inoculation, and the 41 mares given a single-inoculation initial treatment only in 1994, was 88.7% (43 foals produced after 381 treatments). The effectiveness of contraception was 90% (34 foals produced after 340 treatments) when the 41 single-inoculation mares were excluded. The contraceptive effectiveness among the 41 mares treated with single-inoculation treatment in 1994 was 78% (nine foals produced after 41 treatments).

From 1994 to March 1999, the original population of 166 horses remained stable, ranging between 172 and 164 (Fig. 1). On the basis of historic population increases of 5% annually (Keiper and Houpt, 1984), the number of horses in this herd would have increased to 240–260 by March 2000 had immunocontraception not been used.

The percentage of the total number of mares of all ages that required treatment under the ASIS management plan decreased from 95% to 52% in 1999. This decrease was brought about by cessation of treatment of mares after they had finished their third consecutive treatment, as dictated by the management plan, and by not inoculating other animals that had already been treated for several consecutive years.

The mortality rate in the total population of mares and foals decreased between 1990 and 1999 despite an overall increase in population during that period (Fig. 2). During the 4 years preceding the introduction of management-level contraception, the mortality rate for all adult horses was > 10% and was approximately 3% for foals. During the 4 years after the start of the management-level contraception, overall adult mortality decreased to < 4%; mare mortality dropped to < 4% and foal deaths were only about 1%. The unusually high mortality rates in

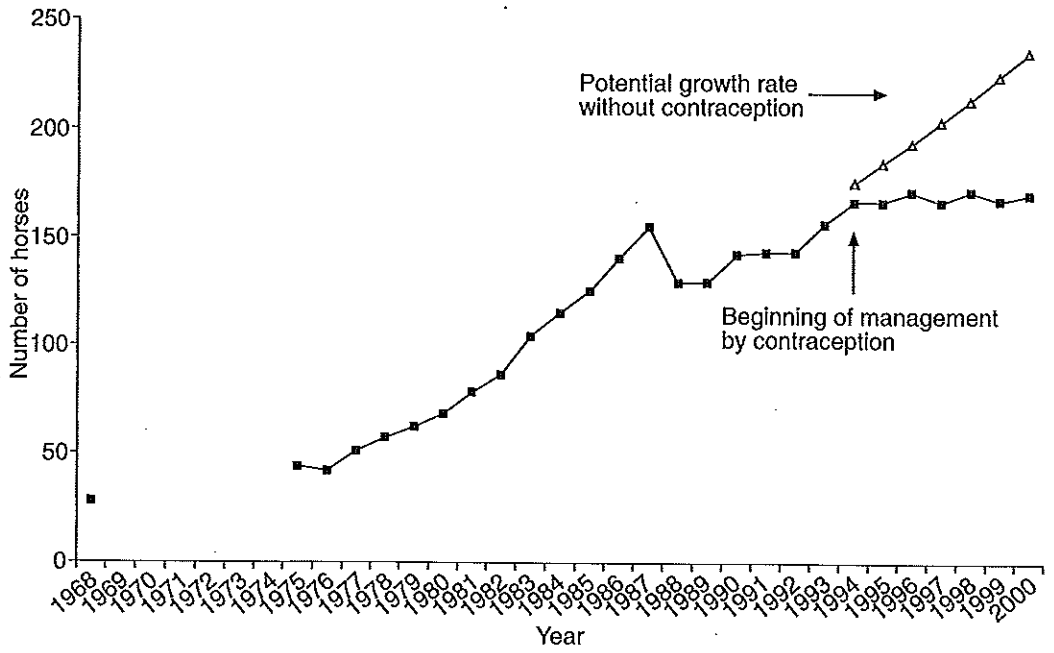


Fig. 1. Population growth curve for the Assateague wild horses. The sharp decrease between 1988 and 1990 was caused by an outbreak of Eastern equine encephalitis. The absence of growth between 1990 and 1993 was the result of additional mares being treated during the research phase of the study and storm-related deaths. The projected increase in population without contraception, beginning in 1994, was based on historic foaling and mortality data.

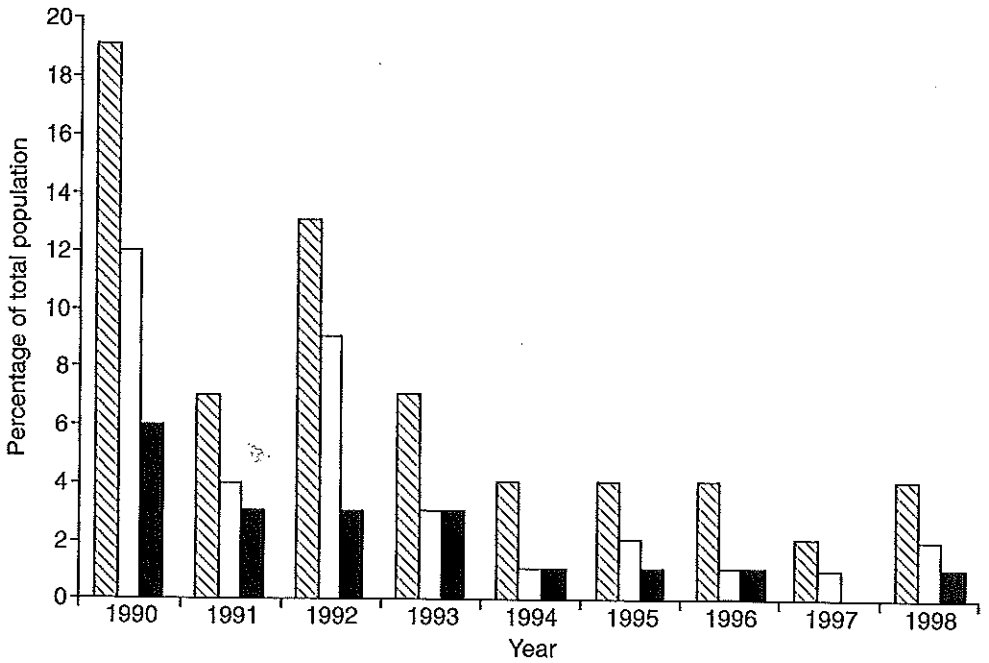


Fig. 2. Mortality rates among Assateague wild horses. The unusually high rates in 1990 and 1992 were the result of an Eastern equine encephalitis outbreak and a large number of storm-related deaths, respectively. ⧅: Total population; □: mares only; ■: foals only.

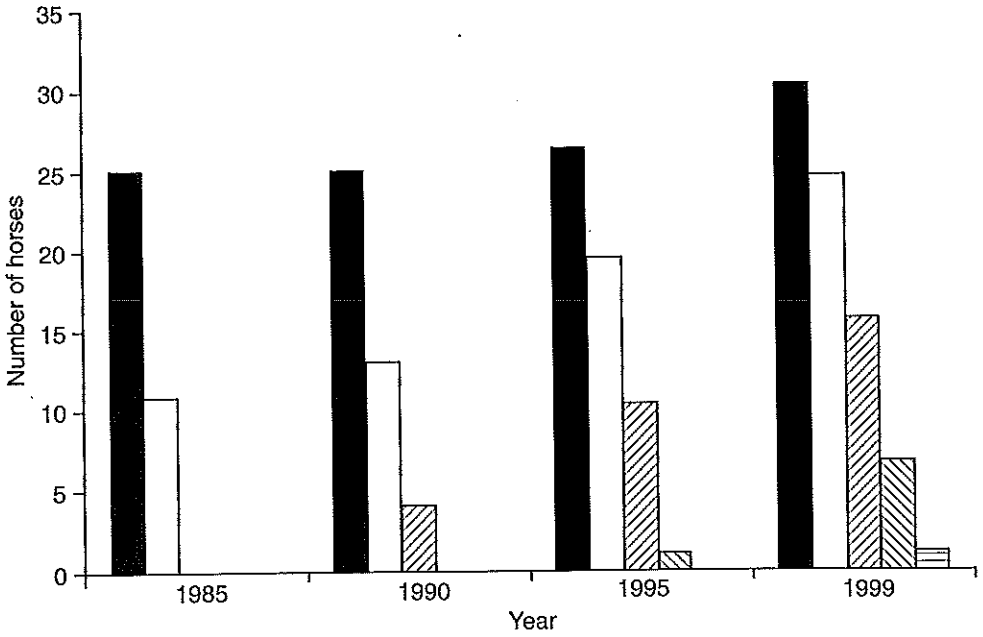


Fig. 3. Changes in the age class structure of the Assateague wild horse herd. The marked change in population trends between 1986 and 1990 was the collective result of horse removals, an outbreak of equine encephalitis, contraception of 26–33 mares for research purposes and storm-related deaths. ■: Age 6–10 years; □: age 11–15 years; ▨: age 16–20 years; ▧: age 21–25 years; ⊟: age > 25 years.

1990 and 1992 were the result of an outbreak of Eastern equine encephalitis on the island in 1990 and severe storms in 1992. The 1991 and 1993 mortality rates were normal and representative of the 8-year period before the start of immunocontraception (Keiper and Houpt, 1984).

Changes in the age classes of the entire herd shifted upwards, indicating increased survival (Fig. 3). These changes were not just an expected increase in the age profiles of the treated herd, but rather animals were reaching new and older age classes, indicating increased longevity. The change was greater among mares than stallions, with the former accounting for 83% of the animals reaching the 16–25 year group in 1995, and 67.7% of the animals reaching the same age group in March 1999.

Body condition scores were significantly higher in 1999 than 1988. For example, the mean ± SEM scores for all horses in 1999 and 1988 were 3.04 ± 0.06 and 2.47 ± 0.43, respectively ($t = 2.85$, 46 df, $P = 0.0064$).

For all adult mares, the mean body condition scores were 2.96 ± 0.08 and 2.33 ± 0.35, respectively ($t = 4.01$, 31 df, $P = 0.0004$) for these 2 years, and for non-lactating mares the scores were 3.07 ± 0.05 and 2.42 ± 0.38, respectively ($t = 5.71$, 18 df, $P = 0.0001$). However, the body condition scores for lactating females did not differ significantly between 1999 and 1988 (2.0 ± 0.55 and 1.96 ± 0.60, respectively; $t = 1.0$, 4 df, $P = 0.373$).

Discussion

Contraceptive efficacy after management-level treatment with PZP was about the same, approximately 90%, as in earlier research trials with the ASIS horses (Kirkpatrick *et al.*, 1990a, 1991a, 1995a) and captive and wild cervids (Turner *et al.*, 1992, 1996; McShea *et al.*, 1997).

The high degree of efficacy of the single-inoculation treatment given to the 41 previously untreated mares in 1994 (78%) was unexpected, and was probably a result of three factors. Firstly, the initial inoculations were given in March, just before the breeding season. Keiper and Houpt (1984) showed that 75% of ASIS foals are born between April and June, so that the single inoculation of the PZP vaccine in March would only need to stimulate contraceptive antibody titres for a period of 3 months to achieve significant inhibition of fertility. A second factor is that wild horses, unlike white-tailed deer, do not appear to have an extended breeding season after contraception. McShea *et al.* (1997) demonstrated that the breeding season of PZP-treated white-tailed deer can be extended by as much as 2 months. In contrast, it was demonstrated that wild horses have a very discrete breeding season that does not appear to lengthen even when nutritional planes are increased or pregnancy does not result (Kirkpatrick and Turner, 1983). Thus, the onset and cessation of the breeding season for ASIS mares are rigidly set and were not extended by contraception.

A third possible reason for the success of the single-inoculation treatment was the use of FCA as the adjuvant. This particular adjuvant is considered widely to be the best available with regard to the ability to stimulate significant antibody titres (Leenaars *et al.*, 1994). The use of FCA is contra-indicated in some other animal species because it may cause false positive tuberculosis tests, but as no definitive test exists for equids, FCA has no health testing implications in horses. Some concern has been expressed over possible abscess formation resulting from the use of FCA. However, after 381 treatments, only two abscesses were noted among the treated ASIS animals, one of which occurred after treatment with FIA rather than FCA. In both cases, the abscesses were < 2.5 cm in diameter and they drained and reduced within a week of formation. We attribute the low rate of abscess formation to the exclusive inoculation into hip and gluteal muscles rather than the neck muscles. In other species, the adjuvant of choice is modified Freund's Adjuvant (Calbiochem, La Jolla, CA), which no longer results in false tuberculin tests and which appears to impart contraceptive effectiveness when given with PZP (J. F. Kirkpatrick, unpublished).

Both the 90% effectiveness of the PZP contraception over 5 years and the unexpected high efficacy of the single-inoculation PZP–FCA treatment regimen in 1994 are important observations when considering the development of population models for captive or wild ungulate fertility control. Estimates of contraceptive efficacy in the past have been theoretical (Garrott *et al.*, 1992; Hone, 1992; Garrott, 1995; McCullough, 1996; Muller *et al.*, 1997), but these data provide a sound factual basis for using a 90% effectiveness figure. In addition, herds of wild horses will exist where the administration of two inoculations in the first year is not easy or even possible, whereas a single inoculation may be feasible. The contraceptive efficacy achieved by the single inoculation, if given in March, provides additional data for population models.

The effect of contraception on the total ASIS population has been marked, with achievement of zero-population growth within the first year of management, and continued over 5 years. Even within an extremely conservative management plan that permits a certain level of successful reproduction for genetic reasons, it was possible to stabilize growth of the herd and thereby meet the first management objective.

The failure to reduce the size of the population after 5 years of management-level treatment has three obvious causes. The first is the fairly conservative treatment schedule, which permits a low level of successful reproduction to ensure genetic integrity of the herd. It ensures that all mares have an equal opportunity to make a genetic contribution to the herd by permitting some reproduction. A second cause is the increased longevity occurring among the treated mares. It is to be expected that the mean age of a population undergoing management by contraception will increase and that the percentage of animals in older age classes will

increase. However, the creation of entirely new age classes within the population was unexpected. The cessation of foaling and lactation as a result of contraception, particularly among older mares, which were already losing body mass as a result of the ageing process, was associated with the attainment of new and older age classes, which, in turn, will delay mortality. This increase in longevity has important implications for the use of immunocontraception in wild and captive ungulates. Basically, contraception can still be used to decrease the size of a herd, but the time needed to achieve a significant decrease will be longer than originally thought, particularly when there is concern about the genetic integrity of the herd. In the case of the ASIS herd, significant mortality due to ageing has been delayed and significant decreases in population size will not occur until a new 'age of mortality' is reached. On the basis of available data, this new mortality peak will probably occur between 20 and 25 years of age, rather than at 15–20 years.

The third reason for failing to reduce the size of the population rapidly relates to mortality rates. Normally, as population size increases in most mammalian species, mortality, and particularly mortality of the young, also increases (Christian, 1971; Krebs, 1978). The opposite scenario has occurred on ASIS. During the years of management-level contraception (1994–1999), the population remained static, but between 1988 and 1994 it increased from 126 to 166. Thus, mortality rates should have increased correspondingly or at least remained the same. The tendency for reduced mortality was primarily among mares, indicating that the reduction in physiological stress from not undergoing pregnancy or lactation underlies the higher survival rates in this group. The reduction in foal mortality is harder to explain, but may be related to better body condition of the mothers. Assateague Island is a harsh environment and an earlier study of body condition of all ASIS horses (Rudman and Keiper, 1991) showed that adult females and non-lactating adult females were in fair to good condition, whereas lactating females were classed as thin. In 1999, by comparison, scores had improved to fat for all horses, including adult females and non-lactating females. Clearly, something had improved body condition in the ASIS horses, despite an increase in the number of animals. This finding indicates that continued contraception had a positive effect on body condition scores and, by implication, the health of the horses.

The effort necessary to achieve the desired degree of change in the population within the context of this management plan is clearly intense during the first 3 years, but after this time the effort necessary to maintain the effect decreases as a result of the long interval between the last of the three consecutive treatments in younger mares and the time at which they produce their first post-treatment foal. Apart from a reduction in the number of animals to be treated, the timing of the treatments (March versus August) increases efficiency. In March, there are few humans on ASIS and even fewer on the marshes of the island, where the horses spend most of their time. At this time of year the horses are extremely wary of humans, so successful treatment requires hours of effort for each horse. It appears that after 2 consecutive years of treatment, the third treatment can be carried out in August or September, or probably at any time of the year, and the contraceptive effect will extend throughout the following breeding season. In August and September the horses spend much of their time on the beaches to avoid insects and even in the camping areas in close association with humans. Thus, treatment requires much less effort than in March.

The reduction in effort may be temporary. The management plan was liberalized to permit the long-term treatment of mares with only two surviving offspring to avoid resurgent breeding. However, even with that added burden, the new and older mortality age of an increasing number of mares is approaching and the death of these animals should reduce the annual effort so as to balance the increase from the need to treat a greater number of younger mares.

Conclusion

This study of 5 years of treatment under a management plan provides the first population-level data for contraception in wild horses, and may be useful in developing new models. Previous models have not accounted for changes in age class structure, mortality or the degree of effort required to achieve particular goals. Models can now be refined and improved on the basis of these data.

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Federal Docket No. FWS–R8–ES–2015–0049

90-Day Finding on a Petition to List a Distinct Population Segment of North American Wild Horses on all U.S. Federal Public Lands as an Endangered or Threatened Species

Background

Section 4(b)(3)(A) of the Act requires that we make a finding on whether a petition to list, delist, or reclassify a species presents substantial scientific or commercial information indicating that the petitioned action may be warranted. We are to base this finding on information provided in the petition and supporting information submitted with the petition.

Our standard for substantial scientific or commercial information within the Code of Federal Regulations (CFR) with regard to a 90-day petition finding is "that amount of information that would lead a reasonable person to believe that the measure proposed in the petition may be warranted" (50 CFR 424.14(b)).

Petition History

On June 17, 2014, we received a petition, dated June 10, 2014, from Friends of Animals and The Cloud Foundation, requesting that the distinct population segment (DPS) of North American wild horses on all U.S. Federal public lands be listed as an endangered or threatened species under the Act. The petition clearly identified itself as such and included the requisite identification information for the petitioner(s), as required by 50 CFR 424.14(a). In an October 3, 2014, letter to the petitioner, we responded that we reviewed the information presented in the petition and did not find that the petition warranted an emergency listing. This finding addresses the petition.

Evaluation of Petition to List the Distinct Population Segment of North American Wild Horses on all U.S. Federal Public Lands

Species and Range

Does the petition identify an entity that is eligible for listing (i.e., is the entity a species, subspecies, or DPS)?

☐Yes

X No

The petitioners requested that we list the North American wild horse on all U.S. Federal public lands as a DPS. The petition classifies the North American wild horse as a population of the species *Equus caballus*.

The petition requests that the Service list the DPS of North American wild horses on all U.S. Federal public lands (intended to encompass those lands owned or administered by the Bureau of

Land Management (BLM), the Service, the National Park Service (NPS), and the U.S. Forest Service (USFS). However, the information provided in the petition appears to focus on the wild horses covered under the Wild Free-Roaming Horse and Burro Act of 1971 (WHBA).

The WHBA defines ""wild free-roaming horses and burros" to mean "all unbranded and unclaimed horses and burros on public lands of the United States" and defines "public lands" to mean "any lands administered by the Secretary of the Interior through the Bureau of Land Management or by the Secretary of Agriculture through the Forest Service." (16 USC 1332 (b) and (e)). The WHBA does not apply to management of feral horses or burros within National Wildlife Refuges, which are managed by the Service, or management of feral horses or burros in National Parks, which are managed by the NPS.

However, because the petition requested that we consider North American wild horses on all U.S. Federal public lands, we are evaluating the petition based on this request. Our evaluation considers whether the petition presents substantial information that the North American wild horse found on U.S. Federal public lands may meet the criteria to be considered a distinct population segment.

Evaluation of the Population of North American Wild Horse Found on Public Lands as a Distinct Population Segment

To interpret and implement the DPS provisions of the Act, the Service and the National Oceanic and Atmospheric Administration published the Policy Regarding the Recognition of Distinct Vertebrate Population Segments Under the Endangered Species Act in the Federal Register on February 7, 1996 (61 FR 4722). Under the DPS Policy, three elements are considered in the decision regarding the establishment and classification of a population of a vertebrate species as a possible DPS: (1) The discreteness of a population segment in relation to the remainder of the species to which it belongs; (2) the significance of the population segment to the species to which it belongs; and (3) the population segment's conservation status in relation to the Act's standards for listing, delisting, or reclassification. Both discreteness and significance are used to determine whether the population segment constitutes a valid DPS. If it does, then the population segment's conservation status is used to consider whether that DPS warrants listing. We address these elements with respect to the North American wild horse found on public lands.

Discreteness

Under the DPS policy, a population segment of a vertebrate species may be considered discrete if it satisfies either one of the following conditions: (1) It is markedly separated from other populations of the same taxon as a consequence of physical, physiological, ecological, or behavioral factors (quantitative measures of genetic or morphological discontinuity may provide evidence of this separation); or (2) it is delimited by international governmental boundaries within which differences in control of exploitation, management of habitat, conservation status, or regulatory mechanisms exist that are significant in light of section 4(a)(1)(D) of the Act.

Markedly Separated

Information Provided in the Petition

The petitioners assert that the population of North American wild horses on U.S. Federal public lands is markedly separated from other populations of horses throughout its range due to physical separation as well as ecological, physiological, and behavioral factors.
The petitioners assert that the BLM-designated Herd Management Areas (HMAs) separate the population of wild horses on public lands from domestic horses on private property as well as wild horses occurring on tribal lands. In 2012, there were 179 HMAs that occurred in ten states – Arizona, California, Colorado, Idaho, Montana, Nevada, New Mexico, Oregon, Utah, and Wyoming; 171 of the HMAs were occupied by either horses or burros (National Research Council, 2013, p.21-24). Some HMAs are connected to other HMAs and the animals can move between these areas; these areas often managed as a complex (National Research Council, 2013, p.22). The BLM often removes horses from HMAs and places them in short-term or long-term holding areas (National Research Council, 2013, p.2). These animals may be adopted out to private owners. The horses that are removed may also be gelded or treated with a contraceptive and returned back to an HMA (National Research Council, 2013, p. 25).

The horses occurring on HMAs may disperse or migrate out of the HMA. According to the 2013 National Research Council report: "A designated HMA may constitute the core range of a herd or population, but dispersal movements outside the HMA are possible" (p. 84).

Although the citations provided by the petitioner speak to horses occurring on BLM-lands, it is not clear that they apply to all North American horses that occur on U.S. Federal public lands. Therefore, we find the petition does not provide substantial information that the population of North American wild horse on Federal public lands may be discrete based on physical separation.

The petitioners assert that the wild horses occurring on Federal public lands in the Western States occupy a unique ecological setting. The petition describes the horses on Federal lands in the west as living in areas that "can be extremely harsh with very hot summers, cold winters, sparse vegetation." The petition asserts that domesticated horses that live on private properties are provided food and water by humans and do not face the same conditions that horses on private lands do. We note that the consideration of a population occurring in a unique ecological setting is generally addressed under the evaluation of Significance (see below). However, in evaluating discreteness, we consider how physiological and behavioral differences may result in the marked separation of populations of the taxon. Thus, although there may be physiological or behavioral differences between populations, these differences are only relevant for discreteness if they are a cause of the population being markedly separate from other populations of the taxon.

The petition asserts that wild horses have physiological differences that make them more suitable for living in the wild when compared to domestic horses. For example, the petition asserts that wild horses may be able to survive longer without water than domestic horses; however, the citation to support this claim indicates that this contention "has not been demonstrated with empirical data" (Beever 2003, p. 892). The petition also points to a 2008 article by Jaime Jackson that discusses the differences between wild and domestic horse hooves. Although the article provides a description of common characteristics of the wild horse foot, we find that it does not provide substantial information that the North American wild horse on Federal public lands may be markedly separate from other populations of the taxon based on physiological

factors related to hoof structure. The petition also identifies the "long limbs and single-unit soliped hooves" of wild horses that allow them to move long distances in order to obtain food and water. The provided citation (Downer 2014) does not indicate that this is a physiological difference between the wild horse on Federal public lands and other populations of the taxon. In fact, all equids have soliped (single) hooves on each foot. Therefore, based on our review of the petition and information provided by the petitioner, we find that the petition does not provide substantial information indicating that the wild horse occurring on Federal public lands may be markedly separate from other populations of the taxon based on physiological differences.

The petition asserts that wild horses have different behaviors than domestic horses. Specifically, the petition asserts that wild horses live in family groups (or bands) consisting of a lead stallion and several mares. The petition provides a discussion of the behavior of the bands, as well as a discussion of the communication patterns of wild horses and aggressive encounters between stallions of wild horses. However, the petitioner does not provide citations to indicate that these communication patterns or aggressive encounters do not occur with wild horses occurring outside of Federal lands or among domesticated horses. The petitioner asserts that a wild horse has a "highly refined fight or flight reaction," but the citation provided (Nock, 2010) does not discuss how or why the fight or flight response differs between North American wild horses and other populations of horse. While the petitioner provides a quote from a 2014 perspective piece (Marshall, Dobney, Denham & Capriles) that states domestication "has resulted in diverse phenotypic and behavioral changes to wild animals, including decrease flight responses, increased sociality, earlier reproduction, and modification of endocrine and metabolic systems," the article's authors cite four additional articles associated with the quote. These articles were not provided to the Service by the petitioners. Furthermore, the focus of the Marshall, et al. 2014 article is on assumptions of gene flow between wild and domestic stocks of animals (including horses, donkeys, cats, dogs, etc.) and the new data that are showing such gene flow existed for longer periods of time than previously thought. Although behaviors between domestic and wild (or feral) animals of the same species may differ, based on our review of the information in the petition, we find that the petition does not present substantial information that the North American wild horse may be markedly separate from other populations of horse as a consequence of behavioral differences.

The petition asserts that the population of wild horses is discrete because the WHBA applies regulations to horses on public lands that are not applied to horses on private or tribal lands. The petition asserts that this difference in management is important with regard to section 4(a)(1)(D) of the Act. Under our discreteness analysis, we consider whether a population is delimited by international governmental boundaries within which differences in control of exploitation, management of habitat, conservation status, or regulatory mechanisms exist that are significant in light of section 4(a)(1)(D) of the Act. The discreteness analysis does not consider whether a regulatory mechanism might apply to only specific populations of a species or subspecies that occur within the United States. Because the horses addressed in the petition occur within the United States and are not delimited by international governmental boundaries, the second condition of discreteness is not met.

Following a review of the petition and the sources provided, we find that the petition does not present substantial information that the population of North American wild horse may be discrete under our DPS policy.

Significance

Under the DPS policy, a discrete population segment of a vertebrate species may be considered significant if there is: (1) Persistence of the discrete population segment in an ecological setting unusual or unique for the taxon; (2) evidence that loss of the discrete population segment would result in a significant gap in the range of the taxon; (3) evidence that the discrete population segment represents the only surviving natural occurrence of a taxon that may be more abundant elsewhere as an introduced population outside its historical range; or (4) evidence that the discrete population segment differs markedly from other populations of the species in its genetic characteristics.

We concluded in the previous section that the population of North American wild horse did not meet the discreteness criteria. Therefore, we do not need to evaluate the significance criteria. Based on our review of the petition and the information provided, we find there is not substantial information that the North American wild horse occurring on Federal public lands may meet the discreteness criteria outlined in our 1996 policy, and therefore the petitioned entity is not a DPS.

Petition Finding

Based on our review of the petition and sources cited in the petition, we find that the petitioned entity is not a listable entity under the Act.

In summary, the petition does not present substantial information supporting the characterization of North American wild horses on all U.S. Federal public lands as a DPS, because the discreteness criteria were not met. Therefore, this population is not a valid listable entity under section 3(16) of the Act.

On the basis of our determination under section 4(b)(3)(A) of the Act, we conclude that the petition does not present substantial scientific or commercial information to indicate that listing North American wild horses on all U.S. Federal public lands as a DPS as a threatened or endangered species under the Act may be warranted at this time.

Author

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Date: 6/22/15

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Human Health and Ecological Risk Assessment
for the Use of Wildlife Damage Management Methods
by APHIS-Wildlife Services

Chapter XXIV

Use of GonaCon

in Wildlife Damage Management

June 2017

USE OF GONACON IN WILDLIFE DAMAGE MANAGEMENT

EXECUTIVE SUMMARY

Gonadotropin releasing hormone (GnRH) is a naturally occurring hormone that stimulates production of sex hormones such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH regulate gamete and steroid hormone production by the ovaries and testes and are critical in the reproduction of vertebrates. USDA APHIS Wildlife Services has developed an immunocontraceptive vaccine against GnRH, called GonaCon, when injected into a target animal, GonaCon induces the body to make antibodies against its own GnRH, resulting in infertility. GonaCon is an effective tool in managing wild and feral animal fertility.

GonaCon is preloaded into syringes which is injected into target animals by hand, or transferred into jab stick hypodermic needles or darts for remote delivery. GonaCon is registered for use in female wild or feral equids and white-tailed deer, and shows potential use in bison and feral swine as well as other mammals where it may eventually be registered for use.

USDA APHIS evaluated the potential human health and ecological risks from the proposed use of GonaCon in this assessment and determined that the risks to human health and the environment are negligible. Risks to workers as well as the general population, including hunters who may harvest injected animals, are low based on the method of application, the mode of action of GnRH, and label requirements. Similarly, risks are negligible for nontarget fish and wildlife based on how GnRH is applied, its fate, and label requirements.

Since GnRH is mostly used from preloaded syringes, with the contents discharged into the target animal, adverse exposure potential is reduced. Remote delivery and jab sticks have an increased risk because the jab stick and darts have to be loaded from preloaded syringes with a potential, albeit low, for exposure. When a remote delivery application misses the intended target or when a syringe or dart is dislodged from an animal, potential exposure increases. The label requires personnel to attempt to be retrieve all darts that miss or fall out of animals. Thus, retrieving darts, the low volume of GnRH in each syringe or dart, the expected short half-life of GnRH, and the infrequent occurrence of a remote delivery miss would be expected to result in negligible risk to human health and nontarget fish and wildlife. Risk to humans and nontarget organisms that may consume animals injected with GonaCon is also negligible due to the low toxicity of GnRH, its short half-life, and its degradation in the gut.

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1 INTRODUCTION

GonaCon is an immunocontraceptive vaccine that was developed and could be used operationally by the U.S. Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Wildlife Services (WS) in the management of certain wildlife and feral vertebrate animal populations. This risk assessment provides a qualitative evaluation of potential risks and hazards to human health, nontarget fish and wildlife as a result of exposure to GonaCon under the proposed uses in the APHIS and WS Program. The results of this human health and ecological risk assessment will help WS determine an appropriate risk management strategy to achieve its program management goal.

The methods used to assess potential human health effects follow standard regulatory guidance and methodologies (National Research Council 1983, USEPA 2017). The methods used to assess potential ecological risk to nontarget fish and wildlife follow U.S. Environmental Protection Agency (USEPA) methods, as well as other published methodologies where appropriate. Data used in this risk assessment were obtained from USEPA registration-related and other peer-reviewed documents, other published literature, and online searches for relevant data.

The following risk assessment is divided into four sections: problem formulation (identifying hazard), toxicity assessment (the dose-response assessment), exposure assessment (identifying potentially exposed populations and determining potential exposure pathways for these populations), and the integration of the exposure and toxicity assessments, or risk characterization.

2 PROBLEM FORMULATION

Wildlife and feral animals can reach population levels that are harmful to ecosystems or the human environment. WS employs several lethal and nonlethal methods for managing these populations depending on the particular problem. GonaCon is one nonlethal population control option -- an immunocontraceptive vaccine that renders female animals temporarily infertile. To date, APHIS has obtained USEPA pesticide registrations for use of GonaCon to control populations of white-tailed deer[1], wild and feral horses (*Equus caballus*); and burros (*Equus asinus*). APHIS is also investigating the potential for obtaining USEPA registrations to use GonaCon to manage populations of bison and feral swine. In experimental studies, GonaCon has also been shown to prevent pregnancy in other species including elk, domestic and feral swine, bison, domestic cats, California ground squirrels, prairie dogs and brown (Norway) rats (Miller et al. 2008).

2.1 Chemical Description and Product Use

GonaCon is an injectable immunocontraceptive vaccine, containing the active ingredient mammalian gonadotropin releasing hormone (GnRH), developed by the WS National Wildlife Research Center (NWRC) (Eisemann et al. 2006). GnRH is a 10 amino acid peptide hormone with the same amino acid sequence conserved across most mammals (USEPA 2009a). The U.S. Food and Drug Administration (USFDA) formerly regulated GonaCon as an investigational new animal drug, but the regulatory jurisdiction for the product changed to USEPA once research and development of the product by APHIS WS moved forward as a registered wildlife management method. After interagency discussions between USFDA and USEPA, responsibility for regulatory approval was moved to the USEPA, Office of Pesticide Programs (OPP). USEPA regulates GonaCon as a pesticide for contraceptives in wild and feral animals (USEPA 2009a). APHIS is the registrant of two GonaCon end-use products:

- USEPA Reg. No. 56228-40: GonaCon Immunocontraceptive Vaccine for white-tailed deer (registered in September 2009)[2]

[1] Scientific names are given in the Risk Assessment Introduction Chapter I, or given if not in that Chapter.

[2] Latest label @ https://iaspub.epa.gov/apex/pesticides/f?p=PPLS:8:::NO::P8_PUID,P8_RINUM:496016,56228-40.

- USEPA Reg. No. 56228-41: GonaCon Equine (registered in January 2013)[3]

Both products are GnRH-based water-in-oil emulsions with the same active ingredient (0.03% GnRH). GnRH (also known as luteinizing hormone-releasing hormone (LHRH)) is a reproductive hormone produced in the arcuate nuclei of the hypothalamus. GnRH stimulates production and secretion of two gonadotropins (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) by the anterior pituitary gland. LH and FSH regulate gamete and steroid hormone production by the ovaries and testes (i.e., estrogen, progesterone, and testosterone).

The formulation is comprised of two primary components that are mixed together to form an emulsion for injection. The first component is AdjuVac™ (a mineral oil-based adjuvant that contains killed *Mycobacterium avium*) to induce a localized inflammatory response that triggers a rapid, strong, and sustained immune response. The second is a mollusk stabilizing buffer (Gionfriddo et al. 2011) containing GnRH conjugated to a mollusk-derived carrier protein (i.e., keyhole limpet hemocyanin or blue protein) (Bender et al. 2009). Three additional amino acids are added to the GnRH to act as a spacer and to facilitate conjugation to the carrier protein. Despite GnRH being a "self" protein, GnRH conjugated to the mollusk carrier protein causes the body to recognize GnRH as "foreign," stimulating an immune response and production of specific antibodies against it. This immune response prevents GnRH from stimulating production of additional sex hormones (LH and FSH) and blocks ovulation. The targeted animals become temporarily infertile, and because the production of sex steroids is also reduced, reproductive behaviors are also often suppressed. (USEPA 2009b, Killian et al. 2006).

For deer, 1-mL doses of GonaCon are packaged and pre-loaded in 3-mL plastic syringes. Applicators (limited to APHIS WS personnel, state wildlife management personnel, or persons working under their authority) administer GonaCon by hand injection.

According to the label for USEPA registration No. 56228-40, GonaCon Immunocontraceptive Vaccine induces temporary infertility in female white-tailed deer to control its populations. On the current deer label, application is by hand-injection only. Studies are ongoing to investigate remote darting as another application method. If approved, issues relating to risks of darting application would be similar to those discussed below for the equine label, and discussed further in the Use of Immobilization and Euthanasia and Firearms Risk Assessments.

Applicators inject a single dose (1 mL) of GonaCon at least 2 to 3 months prior to the onset of rut (USEPA 2009b). Applicators administer GonaCon by hand injection in a large muscle mass using an 18G (gauge) or 19G stainless steel hypodermic needle (USEPA 2009b). In studies, researchers injected GonaCon into the upper hind limb of deer (Gionfriddo et al. 2011). In deer, a single vaccination can induce infertility for multiple years.

GonaCon Equine is approved for fertility control in female wild or feral horses and burros according to the label for USEPA Reg. No. 56228-41 dated January 2013. Horses and burros receive a single dose (2 mL) of GonaCon Equine at least 2 to 3 months prior to the onset of breeding. A single vaccination can induce infertility for multiple years in equine and other ungulates (Gray et al. 2010, Fagerstone et al. 2010). GonaCon Equine is administered by hand injection, jab stick, or remote delivery (darting) under the following directions:

- Hand injection: Inject 2.0 mL of GonaCon by intramuscular injection into a large muscle mass (e.g., rump, neck) using the preloaded syringe and a 1.5-inch 18G or 19G stainless steel hypodermic needle

- Jab Stick: Transfer 2 mL GonaCon from the preloaded syringe. Inject intramuscularly using a 1.5-inch 14G stainless steel hypodermic needle

[3] Latest label @ https://iaspub.epa.gov/apex/pesticides/f?p=105:8:::NO::P8_PUID,P8_RINUM:508549,56228-41.

- Remote Darting: Recommended dart specifications are 2 cc dart with a 1.25- or 1.5-inch 14G gelatin barb needle. The 2 mL GonaCon in a preloaded syringe must be transferred into the dart. Deliver intramuscularly using the appropriate projection device.

- If remote delivery is used, the applicator must make every attempt to recover the dart, whether it missed or fell out of the equid. If "weathered-out" such as inclement weather, darkness, or safety concerns, the applicator can make note of the location and retrieve the dart at a later date.

GnRH potentially causes injection-site reactions and in lymph nodes, which includes abscesses, nodules, swelling and stiffness from the water-in-oil emulsions containing mycobacteria such as AdjuVac (Gionfriddo et al. 2011), but not in all studies (Gray et al. 2010). Injection site and lymph node reactions may be a necessary factor of the immune response that causes infertility (Gionfriddo et al. 2011). Vacuoles in the lymph nodes were found in cytoplasm or among macrophages and multinucleated giant cells, which were likely mineral oil droplets from the AdjuVac. However, injection site reactions to the vaccine did not result in observed differences in an animal's range of movement or locomotor patterns. Thus, a side effect can be injection site reactions, but did not have any noted physical effects.

The method of drug delivery may be responsible for some injection-site reactions. More injection-site reactions were observed in remote delivery compared to syringe injection with porcine zona pellucida (PZP) (Roelle and Ransom 2009), another immunocontraceptive agent. Rifle darts caused less injuries than blowpipes. Roelle and Ransom (2009) determined that the method of delivering the drug was likely the cause of injuries since hand injection resulted in few injuries whereas dart trauma was much higher.

Both GonaCon products are classified by USEPA as restricted-use pesticides. The labels prohibit GonaCon from direct use in water, in areas where surface water is present, or in intertidal areas below the mean high water mark. Since these products are restricted-use, all users must be certified pesticide applicators or be under the supervision of a certified pesticide applicator. For both USEPA approved GonaCon labels, use of the deer label is further restricted to USDA APHIS Wildlife Services or state wildlife management agency personnel or persons working under their authority, and for the equine label employees of USDA APHIS Wildlife Services and Veterinary Services, U.S. Bureau of Land Management, U.S. Fish and Wildlife Service, U.S. National Park Service, U.S. Department of Defense, federally recognized Indian Tribes, state agencies responsible for wild or feral horse and burro management, public and private wild horse sanctuaries, or persons working under their authority (equine label). In addition, the labels specify that applicators are not to use these products near humans, domestic animals, or pets. Use of these pesticide products also requires pesticide registration by States. As of April 2017, New Jersey, North Carolina, and North Dakota have approved use of the deer label (EPA Reg. No. 56228-40) and Arizona, Louisiana, Nevada, North Dakota, and Pennsylvania of the equine label (EPA Reg. No. 56228-41). The labels also specify that users must consult State regulations to determine whether they need additional permits or approval prior to use.

2.2 Physical and Chemical Properties

GnRH (CAS Number: 9034-40-6, and molecular formula: $C_{55}H_{75}N_{17}O_{13}$) is a white odorless powder, solid at room temperature (USEPA 2009b), with a molecular weight of 1182.31 (Merck 2013). Melting point, boiling point, stability to normal and elevated temperatures, metal and metal ions, oxidation/reduction action, flammability, explodability and vapor pressure are not available (USEPA 2009b, Merck 2013). Preliminary testing of the GnRH peptide used in GonaCon indicates that it is very water-soluble (≥100 mg/mL) (Warren and Stephens 2008). Storage stability and corrosion characteristics are not applicable for GnRH because the product's shelf life is less than 6 months (USEPA 2009b).

2.3 Environmental Fate

As part of the data package required by USEPA for registration of GonaCon, APHIS submitted waivers for the environmental fate studies because, when used according to label instructions, there is limited potential for

environmental release from direct injection of the product into the target animal. USEPA-OPP granted waivers for environmental fate sturolledies including hydrolysis, photolysis, soil dissipation, aerobic soil metabolism, and adsorption/desorption due to the low potential for release to soil and water (USEPA 2009b).

2.4 Hazard Identification

The role of GnRH within the reproductive system has been intensively studied. However, mammalian toxicology data on GnRH is limited to a few acute toxicity studies. The toxicity study in deer performed during the safety and toxicity evaluation of GonaCon showed no significant contraindications or toxic effects in female deer in weeks following vaccination with GonaCon, except for the formation of granulomata at the injection site (Killian et al. 2006). Behavioral studies in white-tailed deer showed that treated animals are not permanently sterilized, but they exhibit fewer breeding behaviors due to the decline in serum sex steroids (Killian and Miller 2001). A field study in free-roaming feral horses showed that GnRH vaccine can significantly reduce fertility for several years with a single injection. However, no significant effects were noted from contraceptive treatment on the sex ratio of foals, birthing season, or foal survival (Gray et al. 2010). With elk, male precopulatory behavior rates toward cows treated with a GnRH vaccine was greater than controls and could be problematic; treatment did not affect existing pregnancies or calf survival (Powers et al. 2011)

Acute oral and dermal studies in the rat showed that all tested animals survived a 1-mL exposure. A primary eye irritation study in rabbits did not show corneal opacity or iritis. Treated eyes exhibited conjunctival redness and discharge within one-hour after treatment, but there were no "positive" grade irritations at 24 hours. Rabbits were free of all eye irritation after 72 hours. A primary skin irritation study in rabbits did not show swelling at any treated site. Initially, treatment resulted in a very slight superficial reddening of skin that dissipated within 72 hours. For these reasons, USEPA-OPP classified GnRH as a Toxicity Category IV (no precautions required) for acute oral/dermal toxicity and eye and skin irritation exposures (USEPA 2009b). Chronic toxicity of GnRH is not expected since it is rapidly metabolized in animals.

When GonaCon is used according to the label, human exposure to GnRH is unlikely. First, certified applicators deliver GonaCon while wearing personal protective equipment, which includes long-sleeved shirts, long pants, gloves, and shoes with socks. Secondly, GonaCon is pre-packaged in syringes and the preloaded syringes significantly reduce exposure or transferring it to jabstick syringes or darts presents minimal potential for exposure. Lastly, certified applicators apply GonaCon through hand injection (most equids are actually injected with from a syringe since they are in chutes following roundups) or remote delivery. As a result of these attributes, certified applicators are unlikely to make dermal contact with GnRH or inhale it during its intended use. Even so, remote delivery poses minimal risks. Thus, USEPA-OPP waived several study requirements, including:

- The acute inhalation study in the rat, because inhalation is unlikely to occur;
- The dermal sensitization study, because dermal contact is not expected;
- The studies on gene mutation, structural chromosomal aberration, and other genotoxic effects, because the intended use precludes human exposure;
- The acute delayed neurotoxicity study in the hen, because GonaCon is not an organophosphate pesticide and its structure is not similar to products causing delayed neurotoxic effects; and
- The chronic dietary toxicity studies for mammalian and non-mammalian species, because GonaCon use will not result in repeated human exposure during its intended use.

USEPA-OPP applied a Category IV to the acute inhalation toxicity, the same category as the acute oral toxicity.

If a pesticide is categorized as a food-use product by USEPA and it accumulates in animal or plant materials that will be consumed by humans, data to determine a safe level for consumption (a tolerance) are required for registration. A tolerance is not required for this product because it is not a food-use product and it does not accumulate in the tissues of treated animals.

The previously described use pattern for GonaCon also resulted in the waiver of ecological effects studies required by USEPA-OPP for pesticide registration including (USEPA 2009c):

- Seedling Germination/Vegetative Vigor (Guideline 122-1)
- Aquatic Plant Growth (Guideline 122-2)
- Avian Oral Toxicity (LD50) (Guideline 850.1200)
- Avian Dietary Toxicity (LC50) (Guideline 850.2200)
- Acute Toxicity Freshwater Invertebrates (EC50) (Guideline 850.1010)
- Freshwater Fish Toxicity (LC50) (Guideline 850.1075)

USEPA granted waivers for these studies due to the low potential for environmental release of GnRH from the proposed use pattern in this assessment.

3 DOSE-RESPONSE ASSESSMENT

3.1 Human Health Dose-Response Assessment

A dose-response assessment evaluates the dose levels (toxicity criteria) for potential human health effects including acute and chronic toxicities.

USEPA did not establish a reference dose for GnRH. USEPA assigned GnRH to the low toxicity Category IV for acute oral, dermal, inhalation, and ocular exposure routes. USEPA-OPP waived the requirement for an acute inhalation study during the registration of GonaCon (USEPA 2009b); thus, these studies are not available. With the use pattern of GonaCon, inhalation exposure is not expected to occur.

3.2 Ecological Exposure Dose-Response Assessment

USEPA-OPP waived the aquatic and terrestrial ecological effects data requirements for GonaCon due to its restricted use, method of application, mode of action, and lack of potential exposure to nontarget fish and wildlife. Currently the only available toxicity data applicable to the effects analysis for nontarget wildlife is an acute oral limit toxicity study using the rat. No lethal or sublethal effects were noted during a 14-day period at a dose of 1-mL solution containing 0.03% gonadotropin releasing hormone (Eurofins 2008). GnRH belongs to a large group of peptide-based endocrine-related hormones that are present in all vertebrates and are critical for proper reproductive function (Somoza et al. 2002). Sublethal effects would be expected in nontarget vertebrates under the appropriate exposure pathway and in cases where GnRH is non-specific to binding receptors; however, this is unlikely in nontarget fish[4] and wildlife since exposure would have to occur through intramuscular injection. GnRH and similar peptides have been noted in some invertebrates; however, their role in reproduction and other physiological functions is not fully understood (Tsai 2006). Studies regarding these peptides are conducted *in-vitro* and no dose-response data for invertebrates appear to be available in the literature.

4 EXPOSURE ASSESSMENT

4.1 Human Health Exposure Assessment

Exposure assessment estimates the potential exposure of humans to GnRH. The exposure assessment begins with the use and application methods of the GnRH products. A complete exposure pathway for GnRH includes (1) release from a GnRH source, (2) an exposure point where human contact can occur, and (3) an exposure route for the contact such as ingestion, inhalation or dermal contact (USEPA 1989). In this way, the potentially exposed human populations and complete exposure pathways are identified and qualitatively evaluated.

4.1.1 Identification of Potentially Exposed Human Populations and Complete Exposure Pathways

Exposure of certified pesticide applicators to GnRH is unlikely during proper use; however, there are potential exposures for trained applicators during an accidental event, or if the product is not used according to label directions and precautions.

The public would not have access to the GnRH vaccine. Exposure through consumption of treated animals by the public or hunters who consume a recently injected animal is unlikely because GnRH is rapidly metabolized and has a short half-life (under one hour) so dietary ingestion of GnRH from treated animals is expected to be minimal (see discussion below in Exposure Evaluation for additional information on oral exposure). If remote delivery is used, the label requires that applicators make every attempt to recover all darts. In addition, the applicators examine all fired darts after recovery to determine if the charge fired and the plunger fully expelled its vaccine content. Therefore, the

[4] Fish utilize a different form of GnRH, which reduces the risk even further.

potential for the general public to encounter a loaded dart, either from failing to recover darts that fall out of treated animals or missing the animal with the dart, is low.

Release of GnRH vaccine into soil is unlikely because the vaccine is contained within syringes and is injected directly into target animals. The lack of product reaching the soil means that subsequent movement and impacts to groundwater via leaching, surface water via runoff, or plant uptake are not expected. This in general excludes the exposure pathway for soil, groundwater, and surface water media or plant uptake. Loaded darts may occasionally fall out of or miss target animals and remain on the ground if applicators fail to locate and remove them. If this happens, a dose may leak onto the soil, but the volume released would be small. These events would be infrequent because not all animals are treated using remote delivery methods such as darts, but when they are, the chance that WS employees would miss their target is small because employees are trained and skilled in remote delivery methods.

4.1.2 Exposure Evaluation

This section qualitatively evaluates the worker exposure in direct contact pathways associated with applying GnRH formulations. It also discusses exposure through dietary consumption for hunters.

As discussed in Sections 2.4 and 3.1, GnRH has low toxicity (Category IV) via the oral, dermal, ocular, and inhalation routes of exposure. The direct contact exposures via incidental ingestion, inhalation, and dermal contact to trained workers wearing personal protective equipment (PPE) are minimal. PPEs for applicators include long-sleeved shirts and pants, gloves, and shoes with socks. In addition, the label prohibits pregnant women from handling or injecting GonaCon.

There is potential for humans to eat a GonaCon-treated animal harvested through hunting. However, little likelihood of exposure through dietary ingestion of game animals is expected for humans based on the short half-life (less than 1 hour) of GnRH in the animal relative to the harvest date and degradation in the digestive tract prior to absorption. The half-life of synthetic luteinizing hormone-releasing hormone (LH-RH) was reported as 4 to 12 minutes (Warren 2006). A short half-life indicates that GnRH is rapidly metabolized in treated animals and would not be available for human exposure by the time of dietary consumption. The half-life of the conjugated GnRH is expected to be longer at the injection site when compared to GnRH, but conjugated GnRH would not be biologically active and still degrade prior to the animal being harvested. Even if a human consumed a treated game animal shortly after administration, it is unlikely that he or she would be adversely affected because the active ingredient GnRH is a protein that is digested into its component amino acids instead of absorbed intact in the digestive tract of mammals. Therefore, oral exposure to GonaCon products is not an effective exposure route for humans and animals compared to the injection route (USEPA 2009a).

It is possible that the public could encounter a dart that has fallen on the ground from a targeted animal (either the dart falls out of the animal or the applicator misses the animal with the dart); however, the active ingredient would likely be contained within the syringe or have been evacuated as a result of contact.

4.2 Ecological Exposure Assessment

Aquatic exposure to GnRH is unlikely based on the method of application and short half-life once animals are injected. A scenario where an animal is injected and subsequently dies, due to other causes, no residues are expected that could runoff or leach into sediment or water. Secondary exposure to terrestrial nontarget wildlife should be considered. However, the extremely short half-life and the lack of effects from ingestion of GnRH due to breakdown in the gut would not result in an exposure pathway that could impact endocrine function in vertebrates. The short half-life would also reduce the potential for exposure to any invertebrates that may feed on injected animals or carcasses. Loaded darts may fall out of or miss target animals and remain on the ground if applicators fail to locate and remove them. Loaded darts that fall out of an animal would not be expected to contain GnRH since it would most likely have been discharged when the dart made contact with the target animal. In cases where the contents from a remote delivery are not

discharged, for example if the target animal is missed, the probability of exposure would also be low since it would be unlikely that nontarget wildlife would ingest the syringe and it is likely the contents would have been evacuated when the dart strikes the ground or other object.

5 RISK CHARACTERIZATION

This section qualitatively characterizes risks associated with human health and nontarget fish and wildlife. Under the anticipated uses, GonaCon should pose minimal risks to human health as well as nontarget fish and wildlife.

GonaCon products contain only 0.03% GnRH as active ingredient, and the remaining 99.97% of the formulations are other ingredients that cause an immune response and enhance the stability of the vaccine in the treated animal. Applicators administer the vaccine by injection (hand, jab stick, or remote darting). Trained applicators must use required PPE and follow other label directions to minimize exposure and risk. GnRH has a Toxicity Category IV for the registered uses regarding acute oral, dermal, inhalation, and ocular and skin irritation toxicities. The low potential for exposure to GnRH during intended use combined with the use of PPE and low toxicity suggest that adverse health risk to applicators is not expected.

Risks to consumers of hunted game animals are minimal based on the exposure evaluation discussed in Section 4.1.2.

GonaCon products pose negligible risk to terrestrial and aquatic nontarget plants and animals. The lack of ecological risk is due primarily to the proposed use pattern of GonaCon, which includes hand injection, jab stick, or remote darting, which greatly reduces the potential for any exposure to nontarget fish and wildlife either directly or from residues on soil, water, or plant material. The requirement for intramuscular injection for activation as well as the short half-life further reduces the risk to nontarget organisms. Direct risk to nontarget fish and wildlife and any impacts to habitat or prey items (indirect risk) would not be expected to occur under the proposed use pattern for GonaCon.

6 UNCERTAINTIES AND CUMULATIVE IMPACTS

The uncertainties associated with this risk evaluation arise primarily from the unpredictability associated with human behavior, and can only be discussed qualitatively. Accidental release of a small amount of GonaCon from a hand application may occur when GonaCon in the preloaded syringe is not accurately delivered to the target animal or the loaded syringe becomes detached. Accidental self-injection when administering GonaCon to the target animal may also occur. However, the potential for these accidental exposures is very low with trained and experienced applicators. Accidental exposure from transferring the contents of a preloaded syringe into a jab stick hypodermic needle or a dart could occur with similar risks as accidental self-injection. These risks are expected to be very low. Finally, accidental injection could occur as a result of the dart missing its target. However, this would be negligible since WS applicators are well-adept at using firearms (certified) and know what their backstop is should the dart miss.

WS does not necessarily record the application method (hand injection, jab stick, or dart) used to treat target animals with GnRH or other drugs, just that it was treated with GnRH. As this is unknown, and data is unavailable on the frequency with which darts fall from or miss target animals, and how often applicators are able to recover these darts, APHIS WS cannot exactly determine how much GnRH would end up in the environment. However, the amount is expected to be small because APHIS applicators make every attempt to recover all darts. In addition, the low volume of GnRH in each syringe, the expected short half-life, and the infrequent occurrence of a remote delivery miss where the dart is not collected, would equate to minimal amounts of GnRH in the environment; in all likelihood discharges in the environment would be expected to quickly result in undetectable residues. Any GnRH that would occur in the environment from this type of release would be localized to the area immediately adjacent to a compromised syringe and would be expected to quickly dissipate.

The potential impacts to human health and the environment of the GonaCon formulation have not been determined in toxicity studies. Previously discussed inert ingredients in the formulation occur in minor quantities. Other inert materials that occur in larger quantities have been evaluated and determined to be practically nontoxic to humans and nontarget organisms. In addition, potential exposure of humans and nontarget organisms to the formulation, due to the low volume and method of application, as discussed is low, and therefore the risk from the formulation is expected to be negligible.

Potential cumulative effects could be associated with: (1) repeated worker exposure to GnRH, (2) co-exposures to other pesticides within the program with respect to their toxicity, and (3) exposures to other chemicals affecting the toxicity of GnRH. Repeated exposure to GnRH by applicators wearing appropriate PPE is not expected. In addition, vaccinations are injected using prefilled syringes. An accidental exposure may occur if a syringe breaks and the contents spill. However, this occasional accidental exposure will not lead to accumulation in the human body. WS does not use other pesticides with the same active ingredient as GnRH, so exposure by applicators to multiple pesticides with the same mode of action will not occur. Cumulative effects involving other chemicals such as endocrine disruptors are possible for human and ecological exposures. However, it is highly unlikely for WS personnel to be simultaneously exposed to GonaCon and these other types of agents. Similarly, the low potential for GnRH to occur in soil, water, or plants makes cumulative impacts from exposure to other endocrine modulating chemicals and GnRH highly unlikely for nontarget fish and wildlife.

7 SUMMARY

This risk assessment has determined that the potential human health and ecological risks from the proposed use of GonaCon are low and that the risks to human health and the environment are negligible. Risks to workers as well as the general population, including hunters who may harvest treated animals, are low based on the method of application, the mode of action of GnRH, and label requirements. Similarly, risks are negligible for nontarget fish and wildlife based on how GnRH is applied, its fate, and label requirements.

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EVOLUTION IN MENDELIAN POPULATIONS

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THEORIES OF EVOLUTION

One of the major incentives in the pioneer studies of heredity and variation which led to modern genetics was the hope of obtaining a deeper insight into the evolutionary process. Following the rediscovery of the Mendelian mechanism, there came a feeling that the solution of problems of evolution and of the control of the process, in animal and plant breeding

and in the human species, was at last well within reach. There has been no halt in the expansion of knowledge of heredity but the advances in the field of evolution have, perhaps, seemed disappointingly small. One finds the subject still frequently presented in essentially the same form as before 1900, with merely what seems a rather irrelevant addendum on Mendelian heredity.

The difficulty seems to be the tendency to overlook the fact that the evolutionary process is concerned, not with individuals, but with the species, an intricate network of living matter, physically continuous in space-time, and with modes of response to external conditions which it appears can be related to the genetics of individuals only as statistical consequences of the latter. From a still broader viewpoint (compare LOTKA 1925) the species itself is merely an element in a much more extensive evolving pattern but this is a phase of the matter which need not concern us here.

The earlier evolutionists, especially LAMARCK, assumed that the somatic effects of physiological responses of individuals to their environments were transmissible to later generations, and thus brought about a directed evolution of the species as a whole. The theory remains an attractive one to certain schools of biologists but the experimental evidence from genetics is so overwhelmingly against it as a general phenomenon as to render it unavailable in present thought on the subject.

DARWIN was the first to present effectively the view of evolution as primarily a statistical process in which random hereditary variation merely furnishes the raw material. He emphasized differential survival and fecundity as the major statistical factors of evolution. A few years later, the importance of another aspect of group biology, the effect of isolation, was brought to the fore by WAGNER. Systematic biologists have continued to insist that isolation is the major species forming factor. As with natural selection, a connection with the genetics of individuals can be based on statistical considerations.

There were many attempts in the latter part of the nineteenth century to develop theories of direct evolution in opposition to the statistical viewpoint. Most of the theories of orthogenesis (for example, those of EIMER and of COPE) implied the inheritance of "acquired characters." NÄGELI postulated a slow but self contained developmental process within protoplasm; practically a denial of the possibility of a scientific treatment of the problem. Differing from these in its appeal to experimental evidence and from the statistical theories in its directness, was DE VRIES' theory of the abrupt origin of species by "mutations." A statistical process, selec-

tion or isolation, was indeed necessary to bring the new species into predominance, but the center of interest, as with Lamarckism, was in the physiology of the mutation process.

The rediscovery of Mendelian heredity in 1900 came as a direct consequence of DE VRIES' investigations. Major Mendelian differences were naturally the first to attract attention. It is not therefore surprising that the phenomena of Mendelian heredity were looked upon as confirming DE VRIES' theory. They supplemented the latter by revealing the possibilities of hybridization as a factor bringing about an extensive recombination of mutant changes and thus a multiplication of incipient species, a phase emphasized especially by LOTSY. JOHANNSEN'S study of pure lines was interpreted as meaning that DARWIN'S selection of small random variations was not a true evolutionary factor.

A reaction from this viewpoint was led by CASTLE, who demonstrated the effectiveness of selection of small variations in carrying the average of a stock beyond the original limits of variation. This effectiveness turned out to depend not so much on variability of the principal genes concerned as on residual heredity. As genetic studies continued, ever smaller differences were found to mendelize, and any character, sufficiently investigated, turned out to be affected by many factors. The work of NILSSON-EHLE, EAST, SHULL, and others established on a firm basis the multiple factor hypothesis in cases of apparent blending inheritance of quantitative variation.

The work of MORGAN and his school securely identified Mendelian heredity with chromosomal behavior and made possible researches which further strengthened the view that the Mendelian mechanism is the general mechanism of heredity in sexually reproducing organisms. The only exceptions so far discovered have been a few plastid characters of plants. That differences between species, as well as within them, are Mendelian, in the broad sense of chromosomal, has been indicated by the close parallelism between the frequently irregular chromosome behavior and the genetic phenomena of species crosses (FEDERLEY, GOODSPEED and CLAUSEN, etc.). Most of DE VRIES' mutations have turned out to be chromosome aberrations, of occasional evolutionary significance, no doubt, in increasing the number of genes and in leading to sterility of hybrids and thus isolation, but of secondary importance to gene mutation as regards character changes. As to gene mutation, observation of those which have occurred naturally as well as of those which MULLER, STADLER, and others have recently been able to produce wholesale by X-rays, reveals characteristics which seem as far as possible from those required for a directly adaptive evolutionary process. The conclusion nevertheless seems warranted by

the present status of genetics that any theory of evolution must be based on the properties of Mendelian factors, and beyond this, must be concerned largely with the statistical situation in the species.

VARIATION OF GENE FREQUENCY

Simple Mendelian equilibrium

The starting point for any discussion of the statistical situation in Mendelian populations is the rather obvious consideration that in an indefinitely large population the relative frequencies of allelomorphic genes remain constant if unaffected by disturbing factors such as mutation, migration, or selection. If $[(1-q)a+qA]$ represents the frequencies of two allelomorphs, (a, A) the frequencies of the zygotes reach equilibrium according to the expansion of $[(1-q)a+qA]^2$ within at least two generations,[1] whatever the initial composition of the population (HARDY 1908). Combinations of different series are in equilibrium when these are combined at random, but as WEINBERG (1909) and later, in more detail, ROBBINS (1918) have shown, equilibrium is not reached at once but is approached asymptotically through an infinite number of generations. Linkage slows down the approach to equilibrium but has no effect on the ultimate frequencies.

Mutation pressure

The effects of different simple types of evolutionary pressure on gene frequencies are easily determined. Irreversible mutation of a gene at the rate u per generation changes gene frequency (q) at the rate $\Delta q = -uq$. With reverse mutation at rate v the change in gene frequency is $\Delta q = -uq+v(1-q)$. In the absence of other pressures, an equilibrium is reached between the two mutation rates when $\Delta q = 0$, giving $q = v/(u+v)$.

Migration pressure

The frequency of a gene in a given population may be modified by migration as well as by mutation. As an ideal case, suppose that a large population with average frequency q_m for a particular gene, is composed of subgroups each exchanging the proportion m of its population with a random sample of the whole population. For such a subgroup, $\Delta q = -m(q-q_m)$.

The conditions postulated above are rather artificial since, in an actual species, subgroups exchange individuals with neighboring subgroups rather

[1] This statement assumes that there is no overlapping of generations which may bring about some delay in the attainment of equilibrium.

than with a random sample of the whole species and the change in q will be only a fraction of that given above. The fraction is the average degree of departure of the neighboring subgroups toward the population average. The formula may be retained by letting q_m stand for the gene frequency of immigrants rather than of the whole species.

Selection pressure

Selection, whether in mortality, mating or fecundity, applies to the organism as a whole and thus to the effects of the entire gene system rather than to single genes. A gene which is more favorable than its allelomorph in one combination may be less favorable in another. Even in the case of cumulative effects, there is generally an optimum grade of development of the character and a given plus gene will be favorably selected in combinations below the optimum but selected against in combinations above the optimum. Again the greater the number of unfixed genes in a population, the smaller must be the average effectiveness of selection for each one of them. The more intense the selection in one respect, the less effective it can be in others. The selection coefficient for a gene is thus in general a function of the entire system of gene frequencies. As a first approximation, relating to a given population at a given moment, one may, however, assume a constant net selection coefficient for each gene. Assume that the genes a and A tend to be reproduced in the ratio $(1-s):1$ per generation. The gene array $[(1-q)a+qA]$ becomes $[(1-s)(1-q)a+qA]/[1-s(1-q)]$. The change in the frequency of A is $\Delta q = [sq(1-q)]/[1-s(1-q)]$ or with sufficiently close approximation $\Delta q = sq(1-q)$ if the selection coefficient is small.

A second approximation may be obtained by considering the zygotic frequencies. Assume that the types aa, Aa, and AA reproduce in the ratio $(1-s') : (1-hs') : 1$ per generation. The change in the frequency of A to a sufficiently close approximation is $\Delta q = s'q(1-q)[1-q+h(2q-1)]$ (WRIGHT 1929). In the case of selection for or against a complete recessive ($h=0$, s' negative or positive respectively), $\Delta q = s'q(1-q)^2$.

The case of no dominance ($h=\frac{1}{2}$) is the same as the case of genic selection except that the selection against the gene is $s'/2$ instead of s.

The two factor case in which the phenotypes aabb, $aaB-$, $A-bb$ and $A-B-$ reproduce at the rates $(1-s_{ab}):(1-s_a):(1-s_b):1$ respectively yields (for low values of the selection coefficients):

$$\Delta q_A = q_A(1 - q_A)^2[s_a + (s_{ab} - s_a - s_b)(1 - q_B)^2].$$

The frequency of A depends on the frequency and selection of B, becom-

ing independent only if $s_{ab} = s_a + s_b$, that is, if the two series of genes are cumulative with respect to selection. It does not seem profitable to pursue this subject further for the purpose of the present paper, since in the general case, each selection coefficient is a complicated function of the entire system of gene frequencies and can only be dealt with qualitatively. Attention may, however, be called to HALDANE'S (1924–1927) studies of selection rates and of the consequent number of generations required for unopposed selection to bring about any required change in gene frequency under various assumptions with respect to mode of inheritance and system of mating.

Equilibrium under selection

There may be equilibrium between allelomorphs as a result wholly of selection, namely, selection against both homozygotes in favor of the heterozygous type. Putting $\Delta q = s'q(1-q)[1-q+h(2q-1)] = 0$ gives $q = [1-h]/[1-2h]$ as the condition.[2] This includes the case of selection against both homozygotes and also that in favor of them, but examination of the signs of Δq above and below the equilibrium point shows that only the former is in stable equilibrium in agreement with FISHER (1922). The linkage of a favorable dominant with an unfavorable recessive of another series is a case in which selection would be against both homozygotes as JONES (1917) has pointed out, and stressed as a factor in the vigor of heterozygosis. In a population produced by the intermingling of types in which different deleterious recessives have become fixed, there will be a temporary selection in favor of the heterozygotes even without any linkage at all. Unless the linkage is very strong, however, this effect does not persist long enough to have much effect on gene equilibrium. The extreme case of equilibrium of the sort discussed here is, of course, that of balanced lethals, found in nature in Oenothera.

In the two factor case, discussed in the preceding section,

$$\Delta q_A = 0 \text{ if } q_B = 1 - \sqrt{\frac{s_a}{s_a + s_b - s_{ab}}}$$

$$\text{and } \Delta q_B = 0 \text{ if } q_A = 1 - \sqrt{\frac{s_b}{s_a + s_b - s_{ab}}}.$$

[2] The condition can be expressed in a more symmetrical form by using a different form of statement of the selection coefficients. Assume that the rates of reproduction of the three types aa, Aa and AA are as $(1-s_a):1:(1-s_A)$. The value of q at equilibrium comes out $q = \frac{s_a}{s_A + s_a}$ with stable equilibrium only for positive values of the two selection coefficients.

There may be equilibrium here, if s_a and s_b are alike in sign, and s_{ab} is either opposite in sign or of the same sign and smaller, but it is an unstable equilibrium. Of more general importance, perhaps, is the equilibrium reached by a deleterious mutant gene. For mutation opposed by genic selection $\Delta q = -uq+sq(1-q)=0$, $q=1-u/s$. For mutation opposed by zygotic selection (aa, Aa and AA reproducing at rates $(1-s'):(1-hs'):1$ it is easily shown that $q=1-u/hs'$ (WRIGHT 1929), unless h approaches 0. Thus with no dominance, $q=1-2u/s'$, and for selection against a dominant mutation, $q=1-u/s'$. The important case of selection against a recessive is that in which $h=0$. The formula becomes $q=1-\sqrt{u/s'}$. All of these cases are illustrated in figure 1 in which the ordinates show the selection pressure as related to factor frequency, under the different conditions of selection. The intersections with the straight line representing mutation pressure give the points of equilibrium. If deleterious dominant and recessive mutations occur with equal frequency and are subject to the same degree of selection, the frequency of the recessive mutant genes will be greater than that of the dominant ones in the ratio $\sqrt{u/s'}$ to u/s'. The corresponding figure for factors lacking dominance is $2u/s'$, where s' is the selection against the homozygote. These considerations alone should lead to a marked correlation in nature between recessiveness and deleterious effect. This correlation is further increased by the greater frequency of recessive mutation which seems to be a general phenomenon. It is this correlation which gives the theoretical basis for the immediate degeneration which usually accompanies inbreeding, a process which increases the proportion of recessive phenotypes.

The amount to which gene frequency in a subgroup may depart from the species average as a result of local selection held in check by population interchange with other regions may be calculated by solving the quadratic $\Delta q = sq(1-q)-m(q-q_m)=0$. If the local selection coefficient is much greater than the proportion of migration ($s > m$), $q=1-\frac{m}{s}(1-q_m)$ or $-mq_m/s$ depending on the direction of selection, formulae analogous to those for the equilibrium between mutation and selection. If, on the other hand, selection is weak compared with migration ($s < m$), the departure from q_m is small and $q=q_m[1+\frac{s}{m}(1-q_m)]$. This case is doubtless the more important in nature. Large subgroups living under different selection pressures should show gene frequencies clustering about the average according to this expression. The effect of small size of the subgroups in bringing about random deviation in this and other cases is not here con-

sidered. The case in which s and m are of the same order of magnitude may be illustrated by the case of exact equality. Here $q = \sqrt{q_m}$ or $1 - \sqrt{1 - q_m}$ depending on the direction of selection.

Multiple allelomorphs

The foregoing discussion has dealt formally only with pairs of allelomorphs, a wholly inadequate basis for consideration of the evolutionary pro-

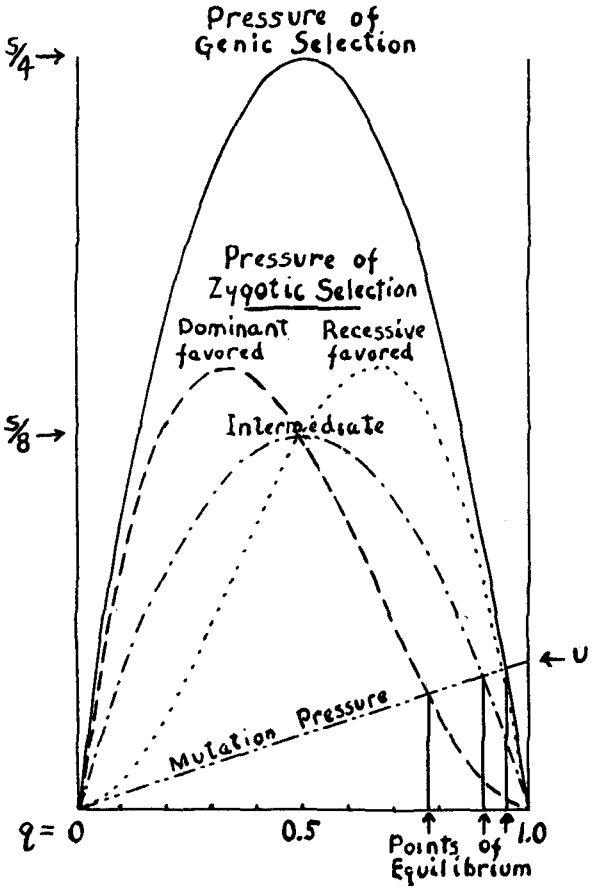


FIGURE 1.—Rate of change of gene frequency under selection or mutation. Genic selection (A, a reproducing at rates 1:1−s); Zygotic selection: dominant (B-, bb at rates 1:1−s), recessive (cc, C− at rates 1:1−s), intermediate (DD, Dd, dd at rates $1:1-\frac{1}{2}s:1-s$), Mutation such that $u = -0.05$ s. Intersections of line of mutation pressure with those for selection pressure determine the equilibrium frequencies.

cess unless extension can be made to multiple allelomorphs. Among the laboratory rodents some 40 percent of the known series of factors affect-

ing coat color are already known to be multiple. The number of multiple series is large in other organisms, for example, Drosophila (MORGAN, STURTEVANT and BRIDGES 1925). It is not unlikely that further study will indicate that all series are potentially multiple. In this case, each gene has a history which is not a mere oscillation between approximate fixation of two conditions but a real evolutionary process in its internal structure. Presumably any particular gene of such an indefinitely extended series can arise at a step from only a few of the others[3] and in turn mutate to only a few. Since genes as a rule have multiple effects and change in one effect need not involve others, it is probable that in time a gene may come to produce its major effects on wholly different characters than at first. Continuing this line of thought, it indeed seems possible that all genes of all organisms may ultimately be traced to a common source, mitotic irregularities furnishing the basis for multiplication of genes.

The relative frequencies of all allelomorphs in a series tend, of course, to remain constant in the absence of disturbing forces. The zygotes reach the equilibrium of random combination of the genes in pairs by the second generation from any initial constitution of the population. The effects of the various kinds of evolutionary pressure on the frequency of each gene may be treated as before by contrasting each gene with the totality of its allelomorphs. In the binomial expression $[(1-q)a+qA]$, A may be understood as representing any gene, and a as including all others of its series. Such treatment, however, requires further qualification with regard to the constancy of the various coefficients. It may still be assumed that the rate (u) of mutational breakdown of the gene in question (A) is reasonably constant, but its rate (v) of mutational origin from allelomorphs must be expected to change. This may be expected to rise to a maximum, as genes closely allied to A in structure become frequent, and to fall off to zero as changes accumulate in the locus. Even at its maximum, however, the rate of formation should in general be of the second order compared with the rate of change to something else, simply because it is one and its alternatives many. Moreover, there is an indication that the genes which become more or less established in a population are not a random sample of the types of mutations which occur. It has been the common experience that mutations are usually recessive. Recessiveness is most simply interpreted physiologically as due to inactivation which may well be the commonest type of mutational change. But the evolutionary process presumably involves in-

[3] Those most closely related genetically, however, need not always be closest in effect. The complete inactivation of a gene in a particular respect may for example occur more freely than a partial inactivation.

crease in activity of genes at least as frequently as inactivation with the consequence that the rate of formation (v) of genes of evolutionary significance becomes negligibly small in comparison with rate of breakdown (u) of such genes. It should be said that FISHER has advanced an alternative hypothesis according to which genes originally without dominance become dominant through a process of selection of modifiers (FISHER 1928, 1929, WRIGHT 1929, 1929a).

The selection coefficient, s, relating to a gene A cannot be expected to be constant if the alternative term a includes more than one gene. The coefficient should rise to a maximum positive value as A replaces less useful genes but should fall off and ultimately become negative as the group of allelomorphs comes to include still more useful genes. But as already discussed, even if A has only one allelomorph, the dependence of the selection coefficient on the frequencies and selection coefficients of non-allelomorphs keeps it from being constant. The existence of multiple allelomorphs merely adds another cause of variation.

Random variation of gene frequency

There remains one factor of the greatest importance in understanding the evolution of a Mendelian system. This is the size of the population. The constancy of gene frequencies in the absence of selection, mutation or migration cannot for example be expected to be absolute in populations of limited size. Merely by chance one or the other of the allelomorphs may be expected to increase its frequency in a given generation and in time the proportions may drift a long way from the original values. The decrease in heterozygosis following inbreeding is a well known statistical consequence of such chance variation. The extreme case is that of a line propagating by self fertilization which may be looked upon as a self contained population of one. In this case, 50 percent of the factors with equal representation of two allelomorphs (that is, in which the individual is heterozygous) shift to exclusive representation of one of the allelomorphs in the following generation merely as a result of random sampling among the gametes. From the series of fractions given by JENNINGS (1916) for the change in heterozygosis under brother-sister mating (population of two) it may be deduced that the rate of loss in this case is a little less than 20 percent per generation. A general method for determining the decrease in heterozygosis under inbreeding has been presented in a previous paper (WRIGHT 1921). It can be shown from this that there is a rate of loss of about 1/2N in the case of a breeding population of N individuals whether equally divided between males and females or composed of monoecious individ-

uals, assuming pairs of allelomorphs. HAGEDOORN (1921) has urged the importance of such random fixation as a factor in evolution.

Another phase of this question was opened by FISHER (1922) who attempted to discover the distribution of gene frequencies ultimately reached in a population as a result of the above process. He studied a number of conditions relative to mutation and selection. He does not state the rate of decrease in heterozygosis (where any) which would follow from the solutions which he reached but this can be deduced very directly from them. It comes out 1/4N for a population of N breeding individuals in the absence of selection or mutation. This is just half the rate indicated by the method referred to above.

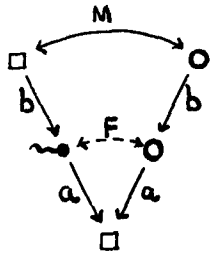


FIGURE 2.

Rate of decrease in heterozygosis

The following symbols and formulae were used in the previous paper in determining the consequences of systems of inbreeding. Primes were used to indicate the number of generations preceding the one in question. Only pairs of allelomorphs are considered here.

M correlation between genotypes of mates

b $(=\sqrt{\tfrac{1}{2}(1+F')})$ path coefficient, zygote to gamete

a $\left(=\sqrt{\dfrac{1}{2(1+F)}}\right)$ path coefficient, gamete to fertilized egg

F $(=b^2M)$ correlation between uniting egg and sperm, also, total proportional change in heterozygosis.

P $(=2q(1-q)(1-F))$ proportion of heterozygosis.

The general formula for the correlation between uniting gametes is easily deduced and has been used as a coefficient of inbreeding in dealing with complex livestock pedigrees (WRIGHT 1922, 1923, 1925, MCPHEE and WRIGHT 1925, 1926),

$$F = \Sigma\left[\left(\tfrac{1}{2}\right)^{n_s+n_d+1}(1 + F_A)\right].$$

Here F_A is the coefficient of inbreeding of any common ancestor that

makes the connecting link between a line of ancestry tracing back from the sire and one tracing back from the dam. The numbers of generations from sire and dam to such a common ancestor are designated n_s and n_d respectively. The contribution of a particular tie between the pedigrees of sire and dam is $(\frac{1}{2})^{n_s+n_d+1}(1+F_A)$ and *the total coefficient is simply the sum* of all such contributions. This formula makes it possible to compare quantitatively the statistical situation in actual populations with that in ideal populations.

In dealing with regular systems of mating the method of analysis consists in expressing the correlation between mated individuals in terms of path coefficients and correlations pertaining to the preceding generation ($M=\phi(a,'b,'M')$ and from this obtaining expression for F in terms of the F's of the preceding generations.

Consider a population in which there are N_m breeding males and N_f breeding females, and random mating. The proportion of matings between full brother and sister will be $1/(N_mN_f)$, that between half brother and sister $(N_m+N_f-2)/(N_mN_f)$, and that between less closely related individuals $(N_m-1)(N_f-1)/(N_mN_f)$. The correlation between mated individuals may be written as follows, giving due weight to these three possibilities:

$$M = a'^2b'^2\left[\frac{1}{N_mN_f}(2+2M') + \frac{N_m+N_f-2}{N_mN_f}(1+3M') + \frac{(N_m-1)(N_f-1)}{N_mN_f}4M'\right].$$

This leads to the following formula for proportional change in heterozygosis since the foundation period:

$$F = F' + \left(\frac{N_m+N_f}{8N_mN_f}\right)(1-2F'+F'').$$

The proportion of heterozygosis may be written, relative to that of preceding generations:

$$P = P' - \left(\frac{N_m+N_f}{8N_mN_f}\right)(2P'-P'').$$

It is to be expected that the proportional change per generation will reach approximate constancy. This rate may be found by equating P/P' to P/P''

$$-\frac{\Delta P}{P'} = \tfrac{1}{2}\left(1+\frac{N_m+N_f}{4N_mN_f}\right) - \tfrac{1}{2}\sqrt{1+\left(\frac{N_m+N_f}{4N_mN_f}\right)^2}.$$

This gives $(1/8N_m+1/8N_f)(1-1/8N_m-1/8N_f)$ as a close approximation even for the smallest populations while for reasonably large ones the form $1/8N_m+1/8N_f$ is sufficiently accurate.

The simplest case is that of continued mating of brother with sister ($N_m=N_f=1$). The rate of loss of heterozygosis comes out $\frac{1}{4}(3-\sqrt{5})$ or 19.1 percent per generation. The formula for proportion of heterozygosis takes the form $P=\frac{1}{2}P'+\frac{1}{4}P''$ as given in the previous paper, with results in exact agreement with those derived by JENNINGS (1916) by working out in detail the consequences of every possible mating from generation to generation.

Another simple case is that in which one male is mated with an indefinitely large number of half-sisters. This is approximately the system of breeding continuously within one herd, headed always by just one male. In this case $N_m=1$, $N_f\doteq\infty$, with rate of loss of heterozygosis of 11.0 percent per generation in agreement with previous results (WRIGHT 1921).

With a relatively limited number of males but unlimited number of females, the rate becomes approximately $1/8N_m$.

An especially important case is that in which the population is equally divided between males and females. Here $N_m=N_f=\frac{1}{2}N$ and the rate of loss is approximately $1/2N$ (or somewhat more closely $1/(2N+1)$) where N is the total size of the breeding population.

It is not, perhaps, clear at first sight that a population of N monoecious organisms, in which self fertilization is prevented, should show a decrease in heterozygosis exactly equal to that in a population of the same size equally divided between males and females. The chance that uniting gametes come from full sisters is $2/[N(N-1)]$, the chance that they come from half sisters is $4(N-2)/[N(N-1)]$ while the chance that they come from less closely related individuals is $(N-2)(N-3)/[N(N-1)]$ giving

$$M=\frac{a'^2b'^2}{N(N-1)}[2(2+2M')+4(N-2)(1+3M')+(N-2)(N-3)4M']$$

$$P=P'-\frac{1}{2N}(2P'-P'')$$

exactly as in the preceding case.

The somewhat arbitrary case in which the gametes produced by N monoecious individuals unite wholly at random is that which can be compared directly with FISHER'S results. The gametes have a chance $1/N$ of coming from the same individual and of $(N-1)/N$ of coming from different individuals. The correlation between uniting gametes may thus be written

$$F = \frac{1}{N}b^2 + \left(\frac{N-1}{N}\right)4b^2a'^2F'$$

$$P = \frac{(2N-1)}{2N}P'.$$

As might be expected, the result does not differ appreciably from that of the preceding case. The rate of loss of heterozygosis is exactly 1/2N instead of merely approximately this figure. The simplest special case is, of course, continued self fertilization in which N = 1 and the formula gives the obviously correct result of 50 percent loss of heterozygosis per generation.

From the mode of analysis it might be thought that the loss in heterozygosis is wholly the consequence of the occasional matings between very close relatives. This, however, is not the case. If instead of random sampling of the gametes produced by the population it is assumed that all individuals reproduce equally and that inbreeding is consistently avoided as much as possible, the percentage of heterozygosis still falls off. The rate of loss is, however, only about half as rapid (approximately 1/4N) in a reasonably large population equally divided between males and females. The cases of N = 2, 4, 8 and 16 have been given previously (WRIGHT 1921).

In dealing with heterozygosis in the foregoing, it has been assumed for simplicity that each locus was represented by only two allelomorphs in the population in question and that either complete fixation or complete loss of a particular gene means homozygosis of all individuals with respect to the locus. But in any case beyond that of self fertilization, more than two allelomorphs may be present and complete loss of the gene no longer implies homozygosis of the locus. The initial rate of loss of heterozygosis in a large population may thus be only 1/4N with gradual approach to 1/2N as the number of loci with only two remaining allelomorphs increases. The rate of decay of the distribution of gene frequencies is 1/2N regardless of number of allelomorphs.

The population number

It will be well to discuss more fully, before going on, what is to be understood by the symbol N used here for population number. The conception is that of two random samples of gametes, N sperms and N eggs, drawn from the total gametes produced by the generation in question (N/2 males and N/2 females each with a double representation from each series of allelomorphs). Obviously N applies only to the breeding population and not to the total number of individuals of all ages. If the population fluctu-

ates greatly, the effective N is much closer to the minimum number than to the maximum number. If there is a great difference between the number of mature males and females, it is closer to the smaller number than to the larger. In fact, as just shown, a population of N_m males and an indefinitely large number of females is approximately equivalent to a population of $4N_m$ individuals equally divided between males and females.

The conditions of random sampling of gametes will seldom be closely approached. The number of surviving offspring left by different parents may vary tremendously either through selection or merely accidental causes, a condition which tends to reduce the effective N far below the actual number of parents or even of grandparents. How small the effective N of a population may be is indicated by recent studies of SMITH and CALDER (1927) on the Clydesdale breed of horses in Scotland, in which they find a steady increase in the degree of inbreeding (Coefficient F) equivalent to that in population headed by only about a dozen stallions. Even more striking is the rapid increase in the coefficient of inbreeding in the early history of the Shorthorn breed of cattle (MCPHEE and WRIGHT 1925).

THE DISTRIBUTION OF GENE FREQUENCIES AND ITS IMMEDIATE CONSEQUENCES

No mutation, migration or selection

On making a cross between two homozygous strains a population is produced in which the members of each pair of allelomorphs in which the strains differ are necessarily equally numerous. The proportional frequency of each allelomorph in unfixed series is $q = 0.50$. In an indefinitely large population, there should be no change in this frequency in later generations (except by recurrent mutation or selection). In any finite population, however, some genes will come to be more frequent than their allelomorphs merely by chance. This means a decrease in heterozygosis, since the proportion of heterozygosis under random mating is $2q(1-q)$, and this quantity is maximum when $q = 0.50$. As time goes on, divergences in the frequencies of factors may be expected to increase more and more until at last some are either completely fixed or completely lost from the population. The distribution curve of gene frequencies should, however, approach a definite form if the genes which have been wholly fixed or lost are left out of consideration. This can easily be seen by considering a case opposite in a sense to that considered above. Suppose that a large number of different mutations occur in a previously pure line. The frequency ratio of mutant to type allelomorphs is initially $(1/2N):(2N-1)/2N$ where N is the number of individuals. The great majority of such muta-

tions will be lost, by the chances of sampling, as FISHER (1922) points out. Those which persist are largely those for which there has been a chance increase in frequency. The distribution curve of frequencies of persisting mutations will thus continually spread toward higher frequencies. There must be a position of equilibrium as far as form is concerned between this situation and that first considered, although a uniform decline in absolute numbers.

As noted above, decrease in heterozygosis takes place in the early generations following a cross without any appreciable fixation or loss of genes. But after equilibrium has been reached in the form of the distribution curve, further loss in heterozygosis must be identical in rate with fixation plus loss.

In simple cases, the equilibrium distribution of gene frequencies can easily be worked out directly. Under brother-sister mating, for example, the following relative frequencies of the 4 possible types of mating involving unfixed factors are in equilibrium although the absolute frequencies of all are falling off 19.1 percent ($=\frac{1}{4}(3-\sqrt{5})$) each generation as new genes enter the fixed states, AA × AA or aa × aa.

Mating	*Relative Frequency*
	Percent
AA×Aa	$7-3\sqrt{5}=$ 29.2
Aa ×Aa	$-22+10\sqrt{5}=$ 36.1
AA×aa	$9-4\sqrt{5}=$ 5.6
Aa ×aa	$7-3\sqrt{5}=$ 29.2
	100.1

Similarly in populations of 2 and 3 monoecious individuals with random union of gametes, the following relative frequencies are in equilibrium although the absolute frequencies are decreasing in each generation by exactly 25 percent and 16⅔ percent respectively verifying the 1/2N of theory.

Gene Frequency	*Class Frequency*	
	Percent	
3A:1a	32	Case of 2 monoecious
2A:2a	36	individuals per gen-
1A:3a	32	eration
Gene Frequency	*Class Frequency*	
	Percent	
5A:1a	18.3	
4A:2a	21.0	Case of 3 monoecious
3A:3a	21.4	individuals per gen-
2A:4a	21.0	eration
1A:5a	18.3	
	100.0	

In order to determine generally the distribution of gene frequencies, consider the way in which genes (A) with frequency q are distributed after one generation of random mating. In a population of N breeding individuals, each of the specified genes will have 2Nq representatives among the zygotes and their allelomorphs $2N(1-q)$. A random sample of the same size will be distributed according to the expression $[(1-q)a+qA]^{2N}$. The contribution of this sample to the frequency class with an allelomorphic ratio of $q_1:(1-q_1)$ will be in proportion to the $2Nq_1$'th term of the above expression and to the number of genes included in the contributing class (f). The sum of contributions from all such classes should give the $2Nq_1$'th term an absolute frequency smaller than its value in the preceding generation (f_1) by the amount $1/(2N+1)$ deduced above. Following is the equation to be solved for f as a function of q.

$$f_1\left(1-\frac{1}{2N+1}\right)=\frac{\lfloor 2N}{\lfloor 2Nq_1 \ \lfloor 2N(1-q_1)}\Sigma q^{2Nq_1}(1-q)^{2N(1-q_1)}f$$

Replacing summation by integration and letting $f=\phi(q)/2N=\phi(q)dq$ we have[4]

$$\frac{\phi(q_1)}{2N+1}=\frac{\lfloor 2N}{\lfloor 2Nq_1 \ \lfloor 2N(1-q_1)}\int_0^1 q^{2Nq_1}(1-q)^{2N(1-q_1)}\phi(q)dq.$$

[3]The cases of 2 and 3 monoecious individuals as worked out by simple algebra suggest an approach to a uniform distribution. As a trial let $\phi(q)=C$. It will be found that this makes the right and left members of the equation identical and is thus a solution.

$$\frac{C}{2N+1}=\frac{C\lfloor 2N}{\lfloor 2Nq_1 \ \lfloor 2N(1-q_1)}\frac{\Gamma(2Nq_1+1)\Gamma(2N-2Nq_1+1)}{\Gamma(2N+2)}=\frac{C}{2N+1}.$$

The case of loss at rate 1/2N should not differ appreciably from that at rate $1/(2N+1)$. It would appear that after a cross, the gene frequencies will spread out from 50 percent toward fixation and loss until a practically uniform distribution is reached. The frequencies of all classes will then slump at a rate of about 1/2N as 1/4N of the genes become fixed and the same number lost per generation. Figure 3 is intended to illustrate this situation.

[4] f must be equated to $\phi(q)/2N$ here, rather than $\phi(q)/(2N-1)$, if the convenient limits 0 and 1 are to be used for integration in place of the limits 1/2N and $(2N-1)/2N$ of the summation with its $2N-1$ terms.

Before finally accepting this solution, however, it will be well to examine the terminal conditions. The amount of fixation at the extremes if N is large can be found directly from the Poisson series according to which the chance of drawing 0 where m is the mean number in a sample is e^{-m}. The contribution to the 0 class will thus be $(e^{-1}+e^{-2}+e^{-3}\cdots)f = \frac{e^{-1}}{1-e^{-1}} f, = 0.582f.$

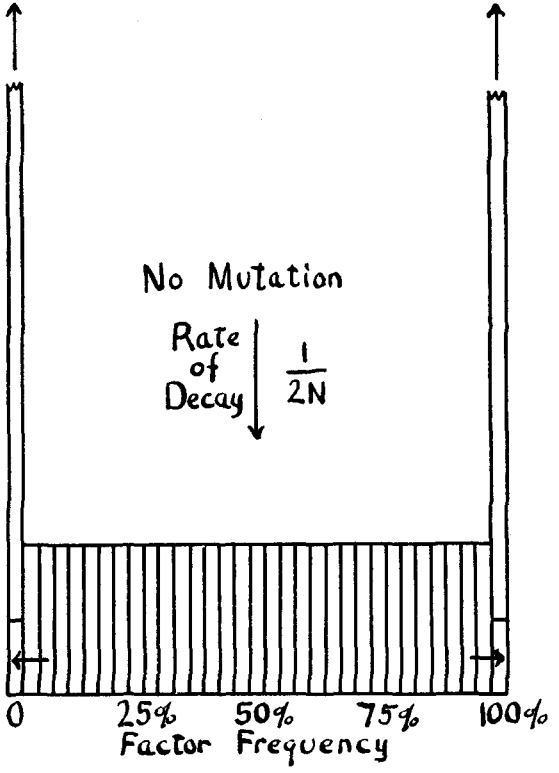


FIGURE 3.—Distribution of gene frequencies in an isolated population in which fixation and loss of genes each is proceeding at the rate 1/4N in the absence of appreciable selection or mutation. $y = L_0 e^{-T/2N}$.

This is a little larger than the $\frac{1}{2}f$ deduced above and indicates a small amount of distortion near the ends due to the element of approximation involved in substituting integration for summation. The nature and amount of this distortion are indicated by the exact distributions obtained in the extreme cases of only 2 and 3 monoecious individuals.

Letting L_0 be the initial number of unfixed loci (pairs of allelomorphs) and T the number of generations we have approximately

Unfixed loci in the T 'th generation $L_T = L_0 e^{-T/2N}$

An analogous formula holds for genes in multiple series, but in this case, as previously noted, the rate of fixation of loci is only half that given above.

The amount of genetic variation with respect to cumulative characters is easily calculated assuming for simplicity pairs of allelomorphs. The contribution of each factor pair to variance, in the case of no dominance, is $2a^2q(1-q)$ where a is the average difference in effect between plus and minus allelomorphs. The general formula for variance in this or any other distribution is thus $\sigma^2 = 2a^2\int_0^1 q(1-q)\phi(q)dq$. In the present case in which $\phi(q) = L$ this reduces to $\sigma^2 = \frac{1}{3}La^2$. PEARSON'S β_2 comes out with a value 2.8 a slightly platykurtic distribution. Since the percentage of heterozygosis for a given factor frequency, q, is $2q(1-q)$, the formula for heterozygosis is the same as that for variance except that a^2 is to be omitted.

Similarly in the case of dominance, the contribution of a single factor pair to variance is $4a^2(1-q)^2(2q-q^2)$ where a is half the average difference in effect between dominant and recessive zygotes. The total variance with perfect dominance is thus in general

$$\sigma^2 = 4a^2 \int_0^1 (1 - q)^2(2q - q^2)\phi(q)dq.$$

In the case of a uniform distribution this gives $\sigma^2 = 8/15\ La.^2$

FISHER (1918) has emphasized the importance of a characteristic of the population which he calls the dominance ratio. He analyzes the variance of characters into three portions, that due to genetic segregation (τ^2) that due to dominance, as something which causes deviations of the phenotype from the closest possible linear relation with the genotype (ϵ^2), and that due to environment. Assuming environment constant, $\sigma^2 = \tau^2 + \epsilon^2$. The simple formulae for the correlations between relatives, to be found if there is random mating and no dominance, must be modified, if dominance is present depending on the value of the dominance ratio defined as ϵ^2/σ^2. Following are examples which he gives:[5]

[5] The author wishes here to correct an error in his 1921 paper which was written without knowledge of FISHER'S results cited above. In this paper it was assumed that the correlation with no dominance needed merely to be multiplied by the squared correlation between genotype and phenotype, the same as FISHER'S $\tau^2/\sigma^2 = (1-\epsilon^2/\sigma^2)$, to obtain that with dominance. This gives correct results (if there is no assortative mating) in the case of offspring with parents, all other ancestors and also in the case of collaterals where one of the individuals is related to the other through only one parent but it is more or less in error in other cases, the fraternal correlation being that most affected. The reasoning followed was not exact because a correlation in the deviations due to dominance in the cases indicated was overlooked.

	Correlation	
	No dominance	*Dominance*
Parent and offspring	$\frac{1}{2}$	$\frac{1}{2}(1-(\epsilon^2/\sigma^2))$
Brothers	$\frac{1}{2}$	$\frac{1}{2}(1-(\epsilon^2/2\sigma^2))$
Uncle and nephew	$\frac{1}{4}$	$\frac{1}{4}(1-(\epsilon^2/\sigma^2))$
Double first cousins	$\frac{1}{4}$	$\frac{1}{4}(1-(3\epsilon^2/4\sigma^2))$

FISHER has shown that the contribution of a single factor to ϵ^2, if there is complete dominance, may be written $\delta^2 = 4q^2(1-q)^2a^2$ where q is the frequency for dominant allelomorphs. Whether a particular dominant gene has a plus or minus effect on the character under consideration is immaterial. The contribution due to genetic segregation he gives as $\beta^2 = 8q(1-q)^3a^2$ thus

$$\frac{\epsilon^2}{\sigma^2} = \frac{\Sigma\delta^2}{\Sigma(\delta^2+\beta^2)} = \frac{\int_0^1 q^2(1-q)^2\phi(q)dq}{2\int_0^1 q(1-q)^3\phi(q)dq + \int_0^1 q^2(1-q)^2\phi(q)dq}.$$

In the present case this reduces to $\frac{1}{4}$ as given by FISHER who also obtains a uniform distribution of factor frequencies for the case of no mutation or selection, although a different rate of decay.

Nonrecurrent mutation

If mutation is occurring, however low the rate, the decline in heterozygosis, following isolation of a relatively small group from a large population, cannot go on indefinitely. There will come a time when the chance elimination of genes will be exactly balanced by new genes arising by mutation. The equation to be solved is obviously as follows:

$$\frac{\phi(q_1)}{2N} = \frac{\lfloor 2N}{\lfloor 2Nq_1 \ \lfloor 2N(1-q_1)} \int_0^1 q^{2Nq_1}(1-q)^{2N(1-q_1)}\phi(q)dq.$$

It may be found by trial that the expression $\phi(q) = C_1q^{-1} + C_2(1-q)^{-1}$ is a solution. The terminal condition, reduction of the class of fixed genes ($q=1$) by an occasional mutation (contributing to the class $q=(2N-1)/2N$ necessarily involves the appearance of new genes (contributing to the class $q=1/2N$) and therefore means that only the symmetrical solution $\phi(q) = Cq^{-1}(1-q)^{-1}$ can be accepted as descriptive of the distribution of the entire array of genes at equilibrium (under the rather arbitrary postulated condition, no selection, no migration, no recurrence of the same mutations). Letting $f = (C/2N)\ q^{-1}(1-q)^{-1}$ and making $\sum f = 1$,

$$C = \frac{1}{2[(0.577 + \log(2N-1)]}$$

or approximately $C = 1/(2 \log 3.6N)$ (compare figure 5).

Before attainment of equilibrium with respect to heterozygosis the distribution will pass through phases of approximately the form $\phi(q) = C_1 q^{-1}(1-q)^{-1} + C_3$ in which the term C_1 gradually displaces C_3 as the number of temporarily fixed genes approaches equilibrium with mutation.

Each particular gene has a probability distribution for the future which spreads in time from the initial frequency in curves which are at first approximately normal in form but later (if the initial q was not too close to 1) become flat, the chances of complete fixation or complete loss each increasing by 1/4N each generation. As the chances of complete fixation increase, the chance of mutation must be taken into account. The distribution passes through phases of the type $C_2(1-q)^{-1} + C_3$, C_2 gradually displacing C_3, relatively, but itself beginning to decline as the chance of complete loss increases. With initial q equal (or close) to 1, equilibrium with mutation, and hence the hyperbolic distribution, is reached directly. The ultimate result in any case is complete loss of the gene in question (still assuming no recurrence of the same mutation and hence mutation *of* the gene but not *to* it). If there is reverse mutation, but at very low rate, a term $C_1 q^{-1}$ must be added to the formula, and an equilibrium will be reached in the form $Cq^{-1}(1-q)^{-1}$. This last formula means that in the long run (assuming no disturbances from selection, migration, etc.) the gene will usually be found either completely fixed or completely absent from the population (with frequencies proportional to the mutation rates to and from the gene respectively) but that occasionally fixation or absence will not be quite complete and that at extremely rare intervals the gene will drift from one state to the other.

The turnover among genes in equilibrium in the distribution $Cq^{-1}(1-q)^{-1}$ can be determined from consideration of the variance of q, and independently by application of the Poisson law.

Let $\sigma_q^2 = \Sigma(q - \frac{1}{2})^2 f / \Sigma f$ be the variance of q, excluding the terminal classes, the summation including $2N-1$ terms. This variance is increased in the following generation by the spreading out of each frequency class as a result of random sampling. The variance from the spreading of a single class is $q(1-q)/2N$ and the average is thus

$$\Delta\sigma_q^2 = \frac{\Sigma q(1-q)f}{2N\Sigma f} = \frac{1}{2N}\left(\frac{1}{4} - \sigma_q^2\right) = \frac{2N-1}{(2N)^2}C.$$

The sum $\sigma_q^2+\Delta\sigma_q^2$ includes the newly fixed factors whose contribution is $\frac{1}{4}K$ where K is the rate of fixation, plus loss, but excludes mutation.

Digressing for a moment to the case of no mutation but equilibrium of form, we have at once

$$\sigma_q^2 + \Delta\sigma_q^2 = K\tfrac{1}{4} + (1 - K)\sigma_q^2$$

$\left(K-\frac{1}{2N}\right)\left(\sigma_q^2-\frac{1}{4}\right) = 0$ giving an independent demonstration that the rate of decay is $1/2N$ in this case.

Returning to the case of equilibrium under mutation, the contribution of new mutations to variance is $K(N-1)^2/(2N)^2$.

$$\sigma_q^2 + \Delta\sigma_q^2 - \tfrac{1}{4}K + K\left(\frac{N-1}{2N}\right)^2 = \sigma_q^2$$

$$K = C = \frac{1}{2\log 3.6N}.$$

The proportion exchanged at each extreme is thus about half the corresponding subterminal class where N is large ($f_1 = f_{2N-1} = 2NC/(2N-1)$ by this method. This compares fairly well with the proportion as determined by the Poisson law, which is 0.46 times the subterminal class instead of 0.50.

The equilibrium frequencies can be worked out algebraically in simple cases. The figures below give the results in the case of a population of 3 monoecious individuals for comparison with the theoretical values deduced above. The rate of exchange at each extreme is actually 10.8 percent in comparison with 11.0 percent as $\frac{5}{12}\left(=\frac{2N-1}{4N}\right)$ the subterminal class, or 11.4 percent from the formula $\frac{1}{4(.577+\log 5)}$. The case of irreversible mutation is also given.

	Reversible Mutation		*Irreversible Mutation*	
Gene frequency	*Exact equilibrium*	$Cq^{-1}(1-q)^{-1}$	*Exact*	$C(1-q)^{-1}$
5A:1a	27.5	26.3	47.7	43.8
4A:2a	15.4	16.4	20.6	21.9
3A:3a	14.1	14.6	14.1	14.6
2A:4a	15.4	16.4	10.2	10.9
1A:5a	27.5	26.3	7.3	8.8
Totals	99.9	100.0	99.9	100.0
Terminal exchange	10.8	11.0	18.0	18.25
Loss			3.6	3.65

The number of unfixed loci (L) which a given mutation rate per individual (μ) will support in a population is easily found, assuming only pairs of allelomorphs. The number of mutations is KL as well as $N\mu$. Therefore $L = N\mu/K = 2N\mu \log 3.6N$. The variance of cumulatively determined characters worked out as in the preceding case comes out $2N\mu a^2$ in the case of no dominance and $10/3\ N\mu a^2$ in the case of dominance, in both cases, directly proportional to the size of population[6] and to the mutation rate. In view of the piling up of new mutations, one might perhaps, expect to find a leptokurtic distribution for characters. This, however, turns out not to be the case: PEARSON'S β_2 comes out exactly 3 in the case of no dominance on substitution in the general formula

$$\beta_2 = 3 + \frac{\int_0^1 q(1-q)[1-6q(1-q)]\phi(q)dq}{\left[\int_0^1 q(1-q)\phi(q)dq\right]^2}$$

FISHER'S dominance ratio comes out 1/5 in this case.

The preceding results differ somewhat from those presented by FISHER (1922). The latter's analysis was based on a transformation of the scale of factor frequencies designed to make the variance due to random sampling uniform at all points. The variance at a given value of q is $q(1-q)/2N$. FISHER assumes that if the ratio of small differences on the q scale to the corresponding differences on a new θ scale be made proportional to the varying standard deviation of q, the standard deviation on the θ scale will be uniform. Letting $dq/d\theta = \sqrt{q(1-q)}$ leads to the transformation $\theta = \cos^{-1}(1-2q)$ with uniform variance of factor frequencies of $1/2N$. Letting $y = F(\theta)$ be the distribution of factor frequencies in one generation, he wrote that in the next as

$$y + \Delta y = \int_0^\pi \frac{1}{\sigma\sqrt{2\pi}} e^{-\delta\theta^2/2\sigma^2}\left(y + y'\delta\theta + \frac{\delta\theta^2}{\underline{|2}}y'' \cdots\right)$$

and measuring time in generations (T) he reached the equation

$$\frac{\partial y}{\partial T} = \frac{1}{4N}\frac{\partial^2 y}{\partial \theta^2}.$$

After noting that the solution for the symmetrical stationary case is

[6] These estimates of number of unfixed loci and of variance depend, of course, on the validity of the conditions on which the formula of the distribution curve is based. How far the mutation rate per locus can be considered negligibly small as size of population increases is discussed later.

$y = L/\pi$, he proceeded to derive the formulae for increasing and decreasing y. Considering the latter, $dy/dT = -Ky$ where K is the rate of decay, giving $1/4N\ d^2y/d\theta^2 = -Ky$ as the equation to be solved. In the symmetrical case this yields $y = C \cos [\sqrt{4NK}(\theta - \pi/2)]$ where $C = \sqrt{4NK}/[2 \sin (\frac{1}{2}\pi\sqrt{4NK})]$ in order to give a total frequency of unity and is to be multiplied further by L_0e^{-KT} to show change from the initial frequency of L_0.

The maximum value which K can take without giving negative frequencies within the range is obviously 1/4N and FISHER found reason for accepting this as the value in the case of no mutation. The formula for the distribution in this case reduces to $y = \frac{1}{2} \sin \theta$. FISHER transformed these equations to the scale $Z = \log [q/(1-q)]$ in which the case of no mutation becomes $y = \frac{1}{4} \operatorname{sech}^2 \frac{1}{2}Z$ and the case of loss balanced by mutation becomes $y = 1/2\pi \operatorname{sech} \frac{1}{2}Z$. This transformation brings the curves into an approach to the form of the normal probability curve. For our present purpose it is preferable to transform to the scale of actual factor frequencies. The case of steady decay becomes $y = 1$ with which my results are in agreement, although in disagreement as to rate of decay. In the case of loss balanced by mutation, FISHER'S formula transforms into $y = 1/[\pi\sqrt{q(1-q)}]$ instead of $1/[2(\log 3.6N)q(1-q)]$ as developed in the present paper. FISHER obtained $\sqrt{\pi/2}N^{3/2}\mu$ for the number of unfixed factors, in contrast with $2N\mu \log 3.6N$; and $\sqrt{\frac{2}{\pi N}}$ for the factor turnover in contrast with $1/[2 \log 3.6N]$. It will be seen that FISHER'S solution leads to a smaller number of unfixed factors with more rapid turnover in very small populations (less than 81) but to a larger number of such factors with slower turnover in larger populations. In a breeding population of one million with one mutation per 1000 individuals, FISHER'S formula gives 1,250,000 unfixed factors with a turnover of 0.08 percent while I get 30,000 unfixed factors with a turnover of 3.3 percent.

The exact harmonizing of the results of the two methods of attack has been a somewhat puzzling matter, but Doctor FISHER, on examination of the manuscript of the present paper, has written to me the following which I quote at his suggestion. ". . . . I have now fully convinced myself that your solution is the right one. It may be of some interest that my original error lay in the differential equation

$$\frac{\partial y}{\partial T} = \frac{1}{4N} \frac{\partial^2 v}{\partial \theta^2}$$

which ought to have been

$$\frac{\partial v}{\partial T} = \frac{1}{4N} \frac{\partial}{\partial \theta}(y \cot \theta) + \frac{1}{4N} \frac{\partial^2 y}{\partial \theta^2}$$

the new term coming in from the fact that the mean value of δp in any generation from a group of factors with gene frequency p is exactly zero,[7] and consequently the mean value of $\delta\theta$ is not exactly zero but involves a minute term $-1/4N \cot \theta$. With this correction, I find myself in entire agreement, with your value 2N, for the time of relaxation and with your corrected distribution for factors in the absence of selection."

Reversible recurrent mutation

It only requires a very moderate mutation rate in a large population for the number of unfixed loci to become enormous. This raises the question as to the effect of a limitation in the number of mutable loci, and recurrence of mutations.

Consider now the case of genes with uniform rates of recurrence of mutation and reverse mutation. Let u be the rate per generation for break down of the gene A and v that for origin from allelomorphs. A class of genes with frequency q (that of all allelomorphs, $1-q$) will be distributed in the following generation under random sampling according to the expansion of the expression

$$\{[(1-q)-v(1-q)+uq]a+[q+v(1-q)-uq]A\}^{2N}f.$$

Equating the total contribution to a given class, to the frequency of this class in the parent generation, reduced by the proportion K, if there is a uniform rate of decay, gives as the equation to be solved:

$$\phi(q_1)\frac{1-K}{2N}=\frac{\underline{|2N}}{\underline{|2Nq_1}\ \underline{|2N(1-q_1)}}\int_0^1[q(1-u-v)+v]^{2Nq_1}$$
$$[1-q(1-u-v)-v]^{2N(1-q_1)}\phi(q)dq.$$

It will be found by trial that the right and left members became identical in certain cases in which $\phi(q)$ is of the form $q^s(1-q)^t$

Let $x=q(1-u-v)+v$

$$q=\frac{x-v}{1-u-v} \qquad dq=\frac{dx}{1-u-v}$$
$$q^s=\frac{x^s-svx^{s-1}\cdots}{(1-u-v)^s} \qquad (1-q)^t=\frac{(1-x)^t-tu(1-x)^{t-1}}{(1-u-v)^t}.$$

[7] p is the q of the present paper. Since the above was written, FISHER has published this revision of his results in *The genetical theory of natural selection*, 1930.

The small amount of spread from a given class will justify retention of the untransformed limits of integration.

Noting that $\Gamma(c+s+1)=\underline{|c}\ c^s\left(1+\frac{s(1+s)}{2c}\right)$ approximately when c is an integer and s is small compared with c, and making use of the following derived relation

$$\frac{2N\underline{|2N}}{\underline{|2Nq_1}\ \underline{|2N(1-q_1)}}\int_0^1 x^{2Nq_1+s}(1-x)^{2N(1-q_1)+t}dx$$
$$=\frac{4N+s(s+1)q_1^{-1}+t(t+1)(1-q_1)^{-1}}{4N+(s+t+1)(s+t+2)}q_1^s(1-q_1)^t$$

the equation may be written as followsf or small values of s and t (compared with N) and values of u and v of a still lower order of size.

$$(1-K)q_1^s(1-q_1)^t$$
$$=\frac{1}{1-(u+v)(s+t+1)}\left[\frac{4N}{4N+(s+t+1)(s+t+2)}q_1^s(1-q_1)^t\right.$$
$$+\left(\frac{s(s+1)}{4N+(s+t+1)(s+t+2)}-\frac{4Nsv}{4N+(s+t)(s+t+1)}\right)q_1^{s-1}(1-q_1)^t$$
$$\left.+\left(\frac{t(t+1)}{4N+(s+t+1)(s+t+2)}-\frac{4Ntu}{4N+(s+t)(s+t+1)}\right)q_1^s(1-q_1)^{t-1}\right]$$

The coefficients of $q_1^{s-1}(1-q_1)^t$ and of $q_1^s(1-q_1)^{t-1}$ must equal 0 either under complete equilibrium or equilibrium merely in form of distribution. Neglecting small quantities:

$$s=0 \quad \text{or} \quad s=4Nv-1$$
$$t=0 \quad \text{or} \quad t=4Nu-1.$$

In the case of complete equilibrium ($K=0$), it turns out that the coefficients of $q_1^s(1-q_1)^t$ *in the left and right members are also satisfied to a* first approximation by $s=4Nv-1$, $t=4Nu-1$. They are also satisfied by letting $s=0$, $t=0$ provided that $u+v=1/2N$. The relation between the fixed terminal and the unfixed subterminal classes, however, requires that $u=v=1/4N$ in this case, which thus becomes merely a special case of the first solution. Similarly, the solutions $s=0$, $t=4Nu-1$ and $s=4Nv-1$, $t=0$ require that $v=1/4N$ and $u=1/4N$ respectively and thus also reduce to special cases of the first solution. It appears then that the distribution of gene frequencies in equilibrium under mutation and reverse mutation may be represented approximately by curves of PEARSON'S Type I,

$$\phi(q) = \frac{\Gamma(4Nu + 4Nv)}{\Gamma(4Nu)\Gamma(4Nv)} q^{4Nv-1}(1 - q)^{4Nu-1}.$$

The terminal conditions are of interest in this and other cases to be considered. The factor turnover at each extreme may be written

$$K_0 = \tfrac{1}{2}f_1 = 2Nvf_0$$
$$K_{2N} = \tfrac{1}{2}f_{2N-1} = 2Nuf_{2N}$$

where the subterminal classes have the frequencies

$$f_1 = \frac{1}{2N}\phi\left(\frac{1}{2N}\right)$$
$$f_{2N-1} = \frac{1}{2N}\phi\left(1 - \frac{1}{2N}\right).$$

In the present case, the terminal classes have the frequencies $f_0 = C/[4Nv(2N)^{4Nv}]$ and $f_{2N} = C/[4Nn(2N)^{4Nu}]$ where C is the coefficient in the expression for $\phi(q)$.

It will be seen that the form of the curve depends not only on the rates of mutation of the genes but also on the size of the breeding population. With small populations or rare recurrence of mutations, the distribution approaches the symmetrical form $y = 1/(2 \log 3.6N)\, q^{-1}(1-q)^{-1}$ already discussed (figure 5). The ratio of the class of temporarily fixed genes (f_{2N}) to the class of complete absence (f_0) must be approximately v:u in this case in order that the number of mutations at each extreme of the symmetrical distribution of unfixed factors may be equal.

With increase in size of the population, the gene frequencies tend in general to be distributed in asymmetrical U— or even I— or J—shaped curves. For example, if the size of population reaches 1/4u and v is much smaller than u, the distribution will be the hyperbola $\phi(q) = Cq^{-1}$ with a piling up of factors with few or no plus representatives.

With sufficient increase in the size of population, the distribution at length takes a form approaching that of the normal probability curve; centered about the point $\bar{q} = v/(u+v)$ which, indeed, is always the mean

$$\left(\bar{q} = \int_0^1 q\phi(q)dq = \frac{v}{u + v}\right).$$

The variance of gene frequencies, $\sigma_q^2 = \int_0^1 (q-\bar{q})^2\phi(q)dq$ is

$$\frac{\bar{q}(1 - \bar{q})}{4N(u + v) + 1}.$$

The amount of genetic variation of cumulative characters may be calculated as before. In the case of no dominance and paired allelomorphs

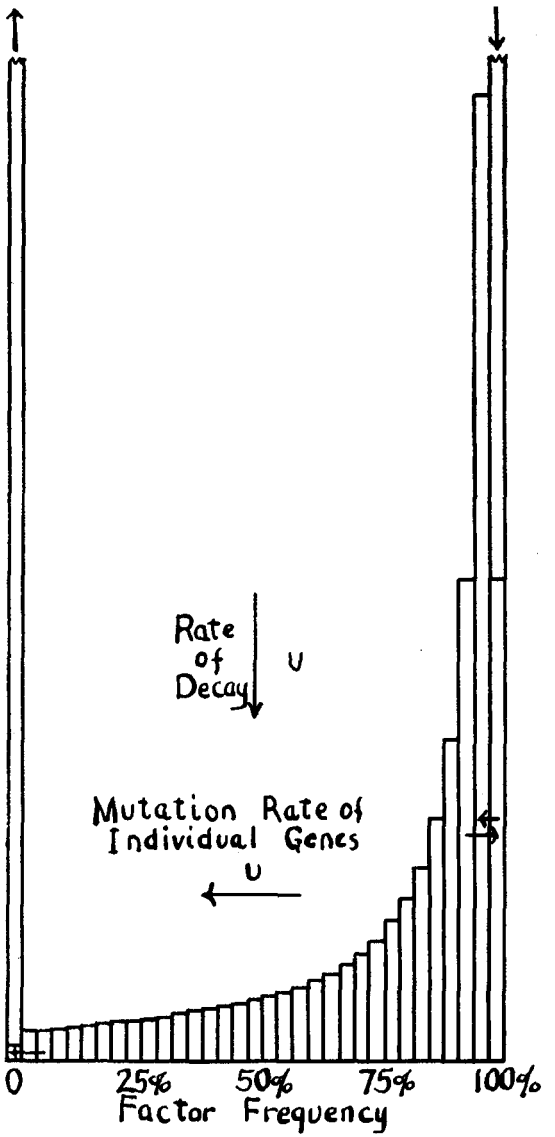


FIGURE 4.—Distribution of type genes in an isolated population in which equilibrium has been reached with destructive mutation but has not been approached with respect to formative mutation. $y=4NuL_0e^{-uT}(1-q)^{4Nu-1}$ with 4Nu much smaller than 1 and the formula approximately $\frac{L_0(1-q)^{-1}}{\log 3.6N}$.

it is $\sigma^2 = 2La^2\ 4Nuq/(4Nu+4Nv+1)$ or $2La^2[\bar{q}(1-q)-\sigma_q{}^2]$. Where u is much greater than v, this can be written approximately $\sigma^2 = 2La^2 4Nv/(4Nu+1)$ approaching $2La^2\bar{q}$ as a limit, as N increases and L comes to include all loci.

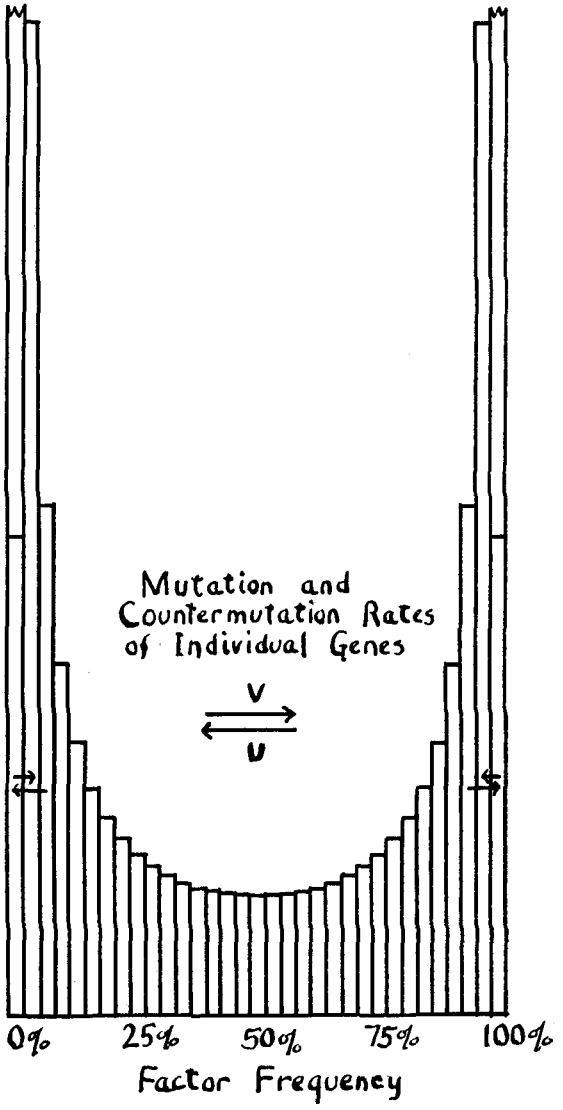


FIGURE 5.—Distribution of gene frequencies (or probability array of gene) where equilibrium with mutation has been attained. Population so small that the terms 4Nu and 4Nv are both much smaller than 1. $y = Cq^{4Nv-1}(1-q)^{4Nu-1}$, approximately $\frac{q^{-1}(1-q)^{-1}}{2 \log 3.6N}$.

As the formula for this case was derived on the assumption of small values of u and v, it is desirable to obtain an independent test of its applicability to large values. This can be done as follows: the increase in variance of q due to random sampling is

$$\frac{1}{2N}\int_0^1 q(1-q)\phi(q)\,dq = \frac{\bar{q}(1-\bar{q})-\sigma^2_q}{2N}.$$

Letting $\Delta q = -uq+v(1-q)$ be, as before, the change in q due to mutation, $q+\Delta q-\bar{q} = (q-\bar{q})(1-u-v)$. Thus the effect of mutation is as if all deviations from the mean were reduced in the proportion $(1-u-v)$. The decrease in the variance of q, due to mutation is therefore $\sigma_q^2[1-(1-u-v)^2]$. At equilibrium the increase in σ_q^2 due to random sampling is exactly balanced by the decrease due to mutation yielding:

$$\sigma^2_q = \frac{\bar{q}(1-q)}{4N(u+v)-2N(u+v)^2+1.}$$

The term $-2N(u+v)^2$ in the denominator is important only when $(u+v)$ has a large absolute value. Omitting this, the formula is identical with that deduced by the first method and thus gives an independent demonstration of its validity. As mutation approaches its maximum value $(u+v=1)$, the variance of q approaches $\bar{q}(1-\bar{q})/2N$, that due to random sampling alone.

Migration

The distribution of gene frequencies in an incompletely isolated subgroup of a large population can be obtained immediately from the preceding results. The change in gene frequency per generation under migration $\Delta q = -m(q-q_m)$ can be written $-m(1-q_m)q+mq_m(1-q)$ which is in the same form as the change of q under mutation, $\Delta q = -uq+v(1-q)$. We may write at once for the distribution under negligible mutation rates:

$$\phi(q) = \frac{\Gamma(4Nm)}{\Gamma[4Nmq_m]\Gamma[4Nm(1-q_m)]}q^{4Nmq_m-1}(1-q)^{4Nm(1-q_m)-1}.$$

The mutation terms 4Nu and 4Nv can be inserted, if mutation rates are not negligible.

Figure 6 shows how the form of the distribution changes with change in m or N. Where m is less than 1/2N there is a tendency toward chance fixation of one or the other allelomorph. Greater migration prevents such fixation. How little interchange would appear necessary to hold a large population together may be seen from the consideration that $m = 1/2N$

means an interchange of only one individual every other generation, regardless of the size of the subgroup. However, this estimate must be much qualified by the consideration that the effective N of the formula is in general much smaller than the actual size of the population or even than the breeding stock, and by the further consideration that q_m of the formula refers to the gene frequency of actual migrants and that a further factor must be included if q_m is to refer to the species as a whole. Taking both of these into account, it would appear that an interchange of the order of thousands of individuals per generation between neighboring subgroups of a widely distributed species might well be insufficient to prevent a considerable random drifting apart in their genetic compositions. Of course,

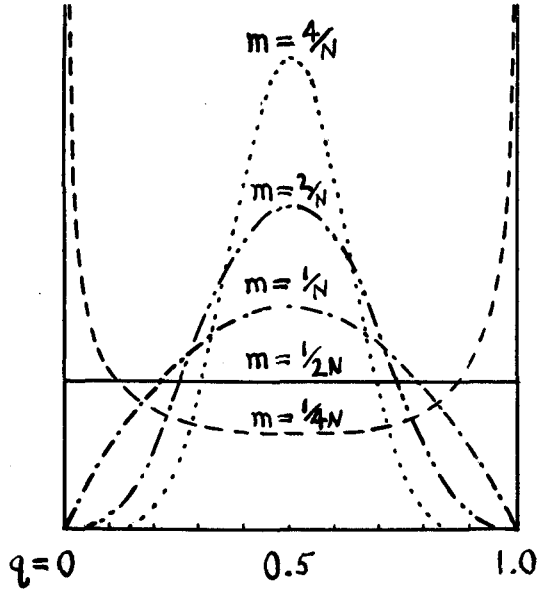


FIGURE 6.—Distribution of frequencies of a gene among subdivisions of a population in which $q_m = 1/2$ (or probability array of gene within a subdivision) under various amounts of intermigration. $y = Cq^{4Nmq_m-1}(1-q)^{4Nm(1-q_m)-1}$.

differences in the condition of selection among the subgroups may greatly increase this divergence. It appears, however, that the actual differences among natural geographical races and subspecies are to a large extent of the nonadaptive sort expected from random drifting apart. An interesting example, apparently nonadaptive, is the racial distribution of the 3 allelomorphs which determine human blood groups (BERNSTEIN 1925).

The variance of distribution of values of q among subgroups (in the ideal

case) is $\sigma_q^2 = q_m(1-q_m)/(4Nm+1)$ by substitution in the formula for the preceding case.

The zygotic distribution $[(1-q)a+qA]^2$ cannot be expected to hold in a population made up of isolated groups among which gene frequency varies. WAHLUND (1928) has shown that the proportions in each homozygous class are increased at the expense of the heterozygotes by the amount of the variance of the gene frequencies among the subgroups,[8] the proportions becoming $[(1-q)^2+\sigma_q^2]aa+[2q(1-q)-2\sigma_q^2]\ Aa+[q^2+\sigma_q^2]\ AA$. By substituting the expression for σ_q^2, given above, in WAHLUND'S formula one might determine empirically the effective value of 4Nm for the population, except that it would be difficult to rule out the possibility that some of the variance of gene frequencies might be due to differences in the selection coefficients among the subgroups instead of merely to random drifting apart.

Irreversible recurrent mutation

The solution $s=0$, $t=4Nu-1$ for the equation reached in the case of recurrent mutation satisfies the conditions for equilibrium of form under irreversible mutation ($v=0$), with decay at rate $K=u$.

$$\phi(q) = 4NuL_0e^{-uT}(1-q)^{4Nu-1}.$$

The proportional frequency of the unfixed subterminal class which is not replenished by mutation is $f_1/(L_0e^{-uT}) = 2u$, twice the rate of decay and thus approximately satisfying the necessary terminal condition.

For values of u as small as $1/(2N \log 3.6N)$ the coefficient in the expression for $\phi(q)$ must be calculated to a closer approximation $\dfrac{4NuL_0e^{-uT}}{1-\left(\dfrac{1}{2N}\right)^{4Nu}}$ which approaches $\dfrac{L}{\log 3.6N}$ as u approaches zero.

The distribution of gene frequencies under irreversible mutation is illustrated in figure 4.

This case is of most interest as representing for a long time the distribution of type genes in a small group isolated from a large one in which all type genes are close to fixation. The release of deleterious mutation pressure from equilibrium with selection will result in approximate equi-

[8] The percentage of heterozygotes is $2\int_0^1 q(1-q)\phi(q)dq$ where $\phi(q)$ is the distribution of values of q among the subgroups. As shown above this reduces to $2\bar{q}(1-\bar{q})-2\sigma_q^2$, thus demonstrating WAHLUND'S principle.

librium of the form described above. With decay at the rate u, it may be a very long time before effects of reverse mutation become appreciable and the final equilibrium $y = Cq^{-1}(1-q)^{-1}$ approached. Assuming that type genes are dominant, the dominance ratio in this case is 1/3.

Selection[9]

Using $\Delta q = sq(1-q)$ as the measure of the effect of genic selection, the class of genes with frequency $(1-q)a : qA$ is distributed after one generation according to the expression:

$$\{(1 - q)(1 - sq)a + q[1 + s(1 - q)]A\}^{2N}.$$

The distribution of gene frequencies which is in equilibrium may be obtained from the following equation which represents the total contribution to class q_1 after one generation, as equal to its previous frequency.

$$\frac{\phi(q_1)}{2N} = \frac{\lfloor 2N}{\lfloor 2Nq_1 \ \lfloor 2N(1 - q_1)} \int_0^1 q^{2Nq_1}(1 - q)^{2N(1-q_1)}(1 + s(1 - q))^{2Nq_1}(1 - sq)^{2N(1-q_1)}\phi(q)dq$$

To a first approximation, the selection terms approach the value $e^{2Ns(q_1 - q)}$. The introduction of a factor e^{2Nsq} into the previously reached formula for $\phi(q)$ gives a solution of the equation (for very small values of s) since it cancels the new term e^{-2Nsq} in the integral, and leaves e^{2Nsq_1} as a factor in $\phi(q_1)$. This was the basis for the formula published (WRIGHT 1929a) as $\phi(q) = Ce^{2Nsq}q^{4Nv-1}(1-q)^{4Nu-1}$ intended to exhibit in combination the effects of selection, mutation in both directions and size of population. Further consideration reveals that this solution is the correct one only for the case of irreversible mutation and then only when the selection coefficient is exceedingly small, less than 1/2N in fact. FISHER (1930) in his recently published revision of the results of his method of attack on this problem has given a formula for a special case of selection, equilibrium of flux from an inexhaustible supply of mutating genes. This is given as accurate as long as Ns^2 is small. Assuming one mutation per generation, he writes:

$$y = \frac{2dp(1 - e^{-4anq})}{pq(1 - e^{-4an})}.$$

[9] This and the following section have been rewritten since submission of the manuscript in order to take account of the correction of my formula, suggested by FISHER'S results in *The genetical theory of natural selection*, 1930 as noted herein.

In this formula, $a(=-s)$ is the selection coefficient, $p(=1-q)$ is frequency of mutant genes and dp may be taken as $1/2N$ numerically. This agrees with my previous formula for irreversible mutation, $y=Ce^{2Nsq}(1-q)^{-1}$ only when s is less than $1/2N$, above which value my formula rapidly leads to impossible results. On reexamination of my method, however, I find that the same degree of approximation can be reached by it. The expansion of $[1+s(1-q)]^{2Nq_1}[1-sq]^{2N(1-q_1)}$ yields series of terms which condense into the expression $e^{2Ns(q_1-q)}\{1-Ns^2[q_1(1-q_1)+(q_1-q)^2]\}$ taking into account terms in Ns^2, N^2s^3, N^3s^4, N^4s^5 as well as those in which N and s have the same exponent. Since the random deviations of q have a variance of $q_1(1-q_1)/2N$ the term (q_1-q) is of the order $\sqrt{1/2N}$. A second order approximation should be obtainable by retaining the term $Ns^2q_1(1-q_1)$ while that in $Ns^2(q_1-q)^2$ may be dropped. The equation to be solved can now be written.

$$\phi(q_1) = \frac{2N\,\underline{|2N}}{\underline{|2Nq_1}\;\;\underline{|2N(1-q_1)}}e^{2Nsq_1}[1 - Ns^2q_1(1 - q_1)] \int_0^1 q^{2Nq_1}(1 - q)^{2N(1-q_1)}e^{-2Nsq}\phi(q)dq.$$

Let $\phi(q)=e^{2Nsq}q^{-1}(1-q)^{-1}(a+bq+cq^2+dq^3\cdots)$.

The exponential term in the integral being cancelled, it becomes possible to carry out the integration by means of the approximate formula already used in the case of mutation (page 122).

$$\frac{2N\,\underline{|2N}}{\underline{|2Nq_1}\;\;\underline{|2N(1-q_1)}}\int_0^1 q^{2Nq1+z-1}(1 - q)^{2N(1-q_1)-1}dq = \frac{4N + z(z - 1)q_1^{-1}}{4N + z(z - 1)}q_1^{z-1}(1 - q_1)^{-1}.$$

The resulting coefficients of the powers of q_1 on the right side of the equation may now be equated separately to those of $\phi(q_1)$. To a sufficient approximation it turns out that $c = \frac{(2Ns)^2}{\underline{|2}}a$, $d = \frac{(2Ns)^2}{\underline{|3}}b$, $e = \frac{(2Ns)^4}{\underline{|4}}a$, $f = \frac{(2Ns)^4}{\underline{|5}}b$, $g = \frac{(2Ns)^6}{\underline{|6}}a$, etc.

Letting $C_1 = a/2$ and $C_2 = \frac{b}{4Ns}$

$$\phi(q) = 2e^{2Nsq}q^{-1}(1 - q)^{-1}[C_1 \cosh 2Nsq + C_2 \sinh 2Nsq].$$

From considerations of symmetry, it is obvious that another solution may be obtained by replacing q by $(1-q)$ and s by $-s$. The full solution may be written in the form

$$\phi(q) = q^{-1}(1-q)^{-1}[C_1(e^{4Nsq}+1) + C_2(e^{4Nsq}-1) + C_3(1+e^{-4Ns(1-q)}) + C_4(1-e^{-4Ns(1-q)})].$$

The relative values of the coefficients in the case of equilibrium can be obtained by setting up the equation for the absence of flux. Each group of genes, $f=\phi(q)dq$ tends to be shifted by the amount $\Delta q = sq(1-q)$ in a generation. There is thus a total flux measured by $\int_0^1 \phi(q)\Delta q dq$ unless there is counterbalancing mutation. The amount of mutation in each direction (assuming the rates of recurrence to be very small compared with 1/4N) is approximately half the respective subterminal classes, as demonstrated in the preceding cases.

$$f_1 = 2C_1 + 2sC_2 + (1+e^{-4Ns})C_3 + (1-e^{-4Ns})C_4$$
$$f_{2N-1} = (e^{4Ns}+1)C_1 + (e^{4Ns}-1)C_2 + 2C_3 + 2sC_4.$$

Since mutation moves genes from the fixed classes to the subterminal classes with gene frequencies of 1/2N and $\left(1-\frac{1}{2N}\right)$ respectively, it creates a net flux of $\frac{f_{2N-1}}{4N} - \frac{f_1}{4N}$ which at equilibrium should balance that due to selection

$$\int_0^1 \phi(q)\Delta q dq - \frac{f_{2N-1}}{4N} + \frac{f_1}{4N} = 0.$$

Substitution of the values given above leads to the condition $C_1-C_2+C_3+C_4=0$. Under this condition the formula simplifies greatly, becoming for all values of s $\left(\text{of lower order than } \frac{1}{\sqrt{N}}\right)$

$$\phi(q) = Ce^{4Nsq}q^{-1}(1-q)^{-1}.$$

The effect of selection in this case is perhaps best exhibited in the ratio of the classes of alternative fixed genes in the highly artificial case of equality in the rates of mutation in opposite directions. This ratio is e^{4Ns}. More generally, $f_0 = \frac{C}{4Nv}$ and $f_{2N} = \frac{Ce^{4Ns}}{4Nu}$ where u and v, both assumed to

be very small compared with 1/4N, are the opposing mutation rates. The number of unfixed loci (pairs of allelomorphs) takes the form

$$L = \frac{2N\mu}{f_1 + f_{2N-1}} = \frac{2N\mu}{C(e^{4Ns} + 1)}$$

where μ is the mutation rate per individual and C is chosen so that $\int_0^1 \phi(q)dq = 1$. The effect of selection on the variance of cumulative characters (pairs of allelomorphs) may be seen by comparing the formula

$$\sigma^2 = 2\mu a^2\left(\frac{e^{4Ns} - 1}{2s(e^{4Ns} + 1)}\right)$$

with the previously given form $2N\mu a^2$ which it approaches as s approaches 0.

In the case treated by FISHER, there is assumed to be irreversible mutation at the rate of one per generation from an inexhaustible supply. As each new gene becomes fixed, it may be considered as transferred to the type class, ready to mutate to new allelomorphs in its series. Thus in place of a return flux of $\frac{f_1}{4N}$, due to reversible mutation, we must write $\frac{f_1}{2}$ (if v = 0)

$$\int_0^1 \phi(q)\Delta q dq - \frac{f_{2N-1}}{4N} + \frac{f_1}{2} = 0.$$

This is solved if $C_1 = C_3 = C_4 = 0$ and

$$\phi(q) = C_2(e^{4Nsq} - 1)q^{-1}(1 - q)^{-1}.$$

In case the direction of mutation coincides with that of selection (u = 0), the mutational terms must be written $\frac{f_{2N-1}}{2} - \frac{f_1}{4N}$ giving the solution

$$\phi(q) = C_4(1 - e^{-4Ns(1-q)})q^{-1}(1 - q)^{-1}.$$

These are identical with FISHER's results on proper choice of the coefficient.

An interesting question which FISHER has discussed, is the chance of fixation of a single mutation. This is given by the ratio of the subterminal classes in the formulae just considered. Where selection opposes

mutation, $\frac{f_1}{f_{2N-1}} = \frac{2s}{e^{4Ns} - 1}$, always less than 1/2N. In the case of favorable mutations, $\frac{f_{2N-1}}{f_1} = \frac{2s}{1 - e^{-4Ns}}$, or approximately 2s. FISHER also gives an independent derivation of the last figure.

General formula

It is of especial importance to assemble the effects of all evolutionary factors into a single formula. Unfortunately, the equation of equilibrium of class frequencies becomes rather complicated and has not yet been worked through. Presumably the form is given at least approximately by a formula of the type $Ce^{aq}q^{4Nv-1}(1-q)^{4Nu-1}$ in the case of reversible mutation. In order that there may be no flux, $\int_0^1 \phi(q)\Delta q dq = 0$. It is not necessary to consider the terminal classes in this case. Thus

$$C\int_0^1 e^{aq}q^{4Nv-1}(1-q)^{4Nu-1}[-uq + v(1-q) + sq(1-q)]dq = 0.$$

Integration of the first term (that in $-uq$) by parts gives an expression which is immediately solved by letting $a = 4N$. Thus the selection term appears to be e^{4Nsq} regardless of the rates of mutation provided there is reversibility. It is approximately of this value in the case of irreversible mutation, discussed above, provided that s is considerably larger than 1/4N. The conclusions based on the previously presented value e^{2Nsq} still hold,[10] except that they should be applied to selection intensities just half as great.

The position of the mode of the I-shaped distribution curve given when u and s are greater than 1/4N can be found by equating the differential coefficient of the logarithm of the formula to zero.

$$4Ns + \frac{4Nv - 1}{q} - \frac{4Nu - 1}{1 - q} = 0.$$

When v is small but u and s are both large, q approaches the value $1 - \frac{u}{s}$ already given as the equilibrium point. The mean would be somewhat below this point, as expected from the curvilinear relation of selection

[10] These conclusions were presented at the meeting of the AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE for 1929 and were summarized in the abstracts (WRIGHT 1929b).

pressure to gene frequency and in contrast with the case of equilibrium between opposing mutation pressures (but no selection) in which the mean is always the equilibrium point $\frac{v}{u+v}$ and the mode is more extreme than this figure, $\frac{4Nv-1}{4N(u+v)-2}$.

Migration pressure introduces no other complications. Combining all factors:

$$\phi(q) = Ce^{4Nsq}q^{4N(mq_m+v)-1}(1-q)^{4N[m(1-q_m)+u]-1}.$$

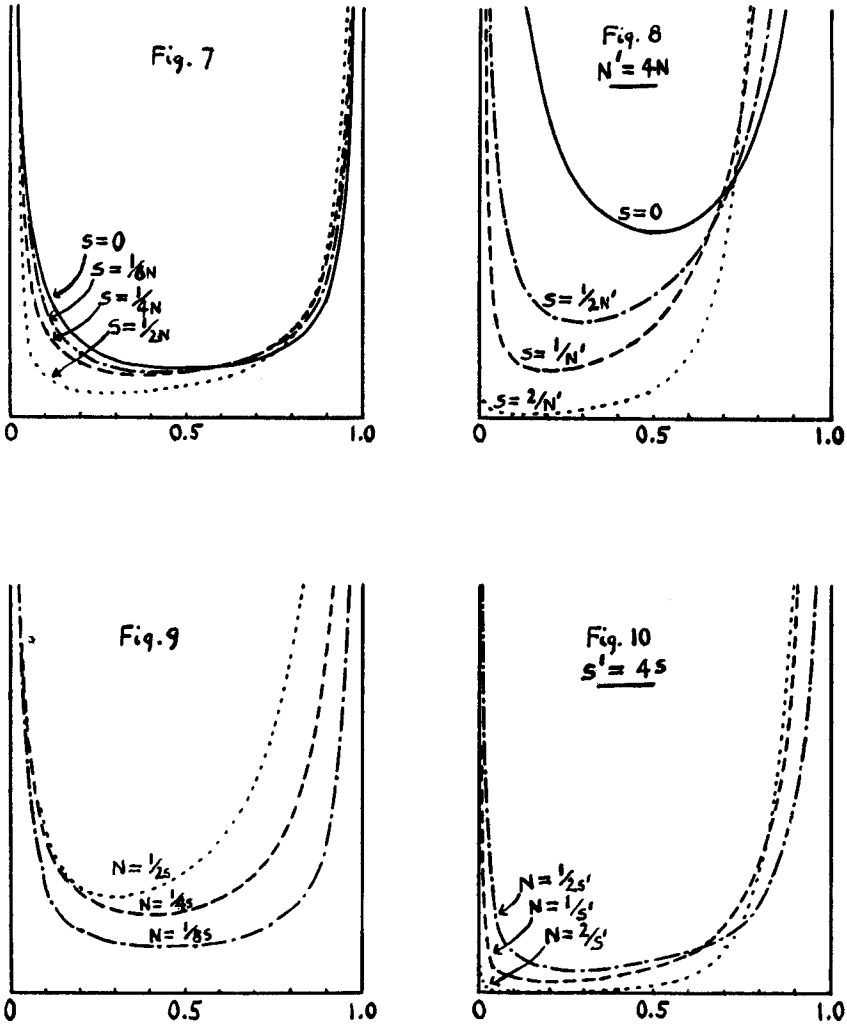
The selection coefficient refers here to the difference between the selection in the group under consideration and that in the species as a whole, the effect of the latter being taken account of in the mean gene frequency of the species q_m.

The distribution curves

Some of the forms taken by the probability array of gene frequencies, in cases involving selection, are illustrated in figures 7 to 14. Figures 7 to 10 deal with the case in which the rates of mutation are negligibly small compared with 1/4N. The curves are thus all variants of the form $y = Ce^{4Nsq}q^{-1}(1-q)^{-1}$. Figure 7 illustrates the relatively slight effect of selection below a certain relation to size of population. All conditions are the same in figure 8 except that the populations are four times as large as in figure 7. Thus while the absolute intensities of the selection coefficients are the same, the relations to size of population are altered.[11] The curves bring out the great effect of selection beyond the critical point, $s = \frac{1}{2N}$ (where mutation rates are low). Figures 9 and 10 are intended to show the effects of change in size of population where the intensity of selection remains constant (low in figure 9, four times as severe in figure 10). Up to a certain point $\left(N = \frac{1}{2s}, \text{ figure } 9\right)$ increase in population raises the middle portion of the curve. Above this point (figure 10) increase in population depresses the middle portion. In the former case, the increase in unfixed factors brings increased variability of cumulative characters, in the latter there is little change of variability in relation to population size, the depression among middle frequencies being balanced by the accumulation of nearly but not quite fixed factors. All of these fig-

[11] The probability that increase in the number of unfixed genes would react on the individual gene selection coefficients, reducing them, is here ignored.

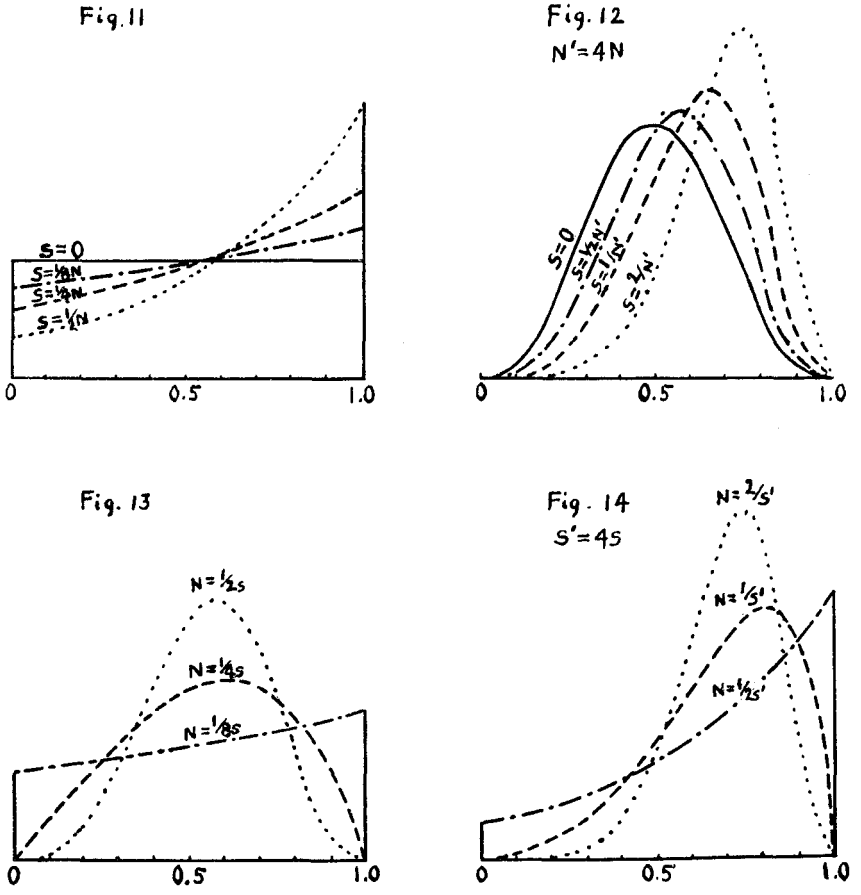
ures (7 to 10) may be taken as representative of conditions in small inbred populations which have been isolated sufficiently long to reach equilibrium in relation to mutation. It will be recalled that figures 3 and 4 represent successive stages preceding the attainment of such equilibrium.



FIGURES 7, 8, 9, and 10.—Distribution of gene frequencies in relation to size of population and intensity of selection where rates of mutation and migration are small compared with 1/4N. Formulae all of type $y = Cy^{4Nsq}q^{-1}(1-q)^{-1}$.

Figure 7. Small population, four degrees of selection. Figure 8. Population four times as large as in figure 7 under the same four (absolute) degrees of selection. Figure 9. Three sizes of population under given weak selection. Figure 10. Same three sizes of population as in figure 9, under selection four times as severe.

Figures 11 to 14 present exactly the same series of comparisons as figures 7 to 10, for small populations that are not completely isolated from the main body of a species.[12] In all cases the gene frequency (q_m) of the



FIGURES 11 to 14.—Distribution of gene frequencies in subgroups of large population (mean frequency $q_m = 1/2$) in relation to size of population and intensity of selection. Formulae all of the type $y = Ce^{4Nsq}q^{4Nmq_m-1}(1-q)^{4Nm(1-q_m)-1}$ Same comparisons as in figures 7 to 10.

Figure 11. Small subgroups ($2Nm = 1$), four degrees of selection. Figure 12. Subgroups four times as large as in figure 11, under same four (absolute) degrees of selection. Figure 13. Subgroups of three sizes under given weak selection. Figure 14. Same three sizes as in figure 13 under selection four times as severe.

The figures may also be used to illustrate cases of equal mutation to and from a gene ($u = v$). $y = Ce^{4Nsq}q^{4Nv-1}(1-q)^{4Nu-1}$

[12] These figures also represent the distribution of gene frequencies in population in which mutation and reverse mutation are equally frequent, but this seems to be so exceptional a case especially under multiple allelomorphism, as to be of little importance.

whole species is assumed to be 1/2. The relations of migration to size of population are such that there is very little complete fixation of genes. In figure 11, $m = 1/2N$ and the purely exponential curves show how increasing intensity of genic selection shifts a uniform distribution in the direction favored by the selection. The fourfold greater population of figure 12 brings about concentration, in curves approaching the normal in form. Figure 13 brings out the concentrating effect of increase in population in the case of weak selection while figure 14 does the same for the case of selection four times as severe.

The important case in which mutation is balanced by selection in a moderately large population (both s and u large compared with 1/4N) is illustrated in figure 19. The four curves represent four degrees of selection, rising by doubling of severity at each step from a case in which mutation pressure practically overwhelms the effect of selection to the reverse situation. The limiting condition in populations so large that 1/4N is very small compared with both s and u is that of concentration of factor frequency almost at a single value (figure 20, page 148).

Dominance ratio

The form of the distribution of the frequencies of the dominant genes affecting a character is of interest in connection with the dominance ratio. Since different genes have different mutation rates and selection coefficients, this distribution is a composite of curves of the types discussed. In small populations which have reached equilibrium, all of these arrays and hence their composite are of the type $Cq^{-1}(1-q)^{-1}$. The dominance ratio is 1/5 in so far as dominance is complete. FISHER gives the value as $3/13 = 0.23$ for the case "when in the absence of selection, sufficient mutation takes place to counteract the effect of random survival." The difference from the value 0.20 given above is due solely to the difference in the formula for the curve, discussed earlier.

In the case of the isolation of a small part of a large population, the dominance ratio takes the value 1/3 in so far as dependent on dominant type genes in equilibrium with recessive mutation but not with reverse mutation ($y = C/(1-q)$). Where following isolation both fixation and loss are substantially irreversible ($y = 1$) the dominance ratio is 1/4 in agreement with FISHER'S result. In both of these cases, of course, the dominance ratio falls to 1/5 when equilibrium is finally attained.

The foregoing discussion applies practically only to very small completely isolated populations. In large populations where the distribution of gene frequencies, even in partially isolated subgroups, tends to approach

the normal type, the dominance ratio comes to depend mainly on the mean gene frequency which depends on the relation of selection to mutation, or on selection against both homozygotes in favor of heterozygotes. In the extreme case in which the gene frequency is reduced to a single value, the dominance ratio is $q/(2-q)$. Values close to unity should not be uncommon, especially where gene frequency is controlled by the balance of selection and mutation. Such a dominance ratio has rather surprising effects on the correlation between relatives. The correlation between parent and offspring approaches 0 although that between brothers may remain as high as 0.25. However, the occurrence of an appreciable number of genes at lower frequencies, for example, held in equilibrium by selection favoring the heterozygote against both homozygotes would greatly lower the dominance ratio.

All of these figures are on the assumption that dominance is complete. Dominance, however, is frequently not complete. Among 22 heterozygotes in the guinea pig which have been studied with some care, at least 9 or about 40 percent are to some extent intermediate. Most of these have to do with color characters. It is not unlikely that incomplete dominance will be found to be even more frequent on careful study of size characters.

FISHER (1922) comes rather definitely to the conclusion that the dominance ratio is typically in the neighborhood of 1/3. This was based primarily on a distribution of factor frequencies which he reached for the case of selection against a recessive,[13] with which the results of the present study are not at all in agreement. He also finds, however, that the differences between fraternal and parent-offspring correlations in data which he analyzes indicate the same figure. The analysis of a large number of correlations of these sorts would undoubtedly furnish valuable information with regard to the statistical situation in populations. It is to be noted, however, that similarity in the environment of brothers as compared with parent and offspring may also contribute to a higher fraternal correlation and that in any case one cannot reason from the dominance ratio deduced from correlations to the distribution of factor frequencies without making some assumption as to the prevalence of dominance.

About all that seems justified by the present analysis, is the statement that for permanently small populations under low selection the value should

[13] This was given as $df \propto \frac{d\theta}{\sin \frac{1}{2}\theta \cos^3 \frac{1}{2}\theta}$ or $\phi(q) = Cq^{-1}(1-q)^{-2}$ on transformation of scale.

FISHER does not discuss dominance ratio in connection with his recent revision of his results in *The genetical theory of natural selection*, 1930.

be less and probably considerably less than 0.20 but that this figure may be raised by severe selection (favoring dominants) and especially by increase in size of population. It may even approach unity in very large populations under severe selection, if complete dominance is the rule.

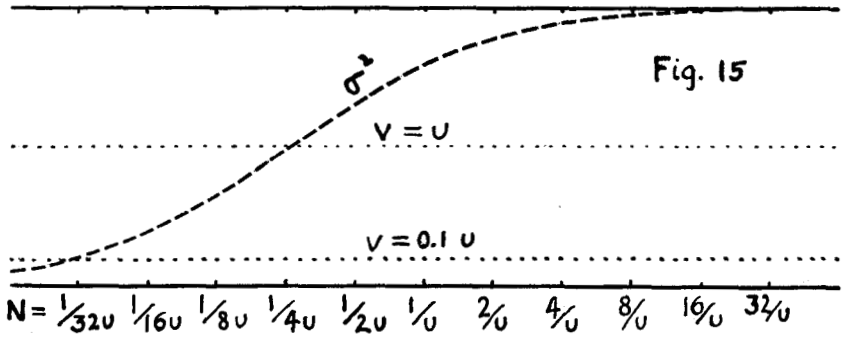
Mean and variability of characters

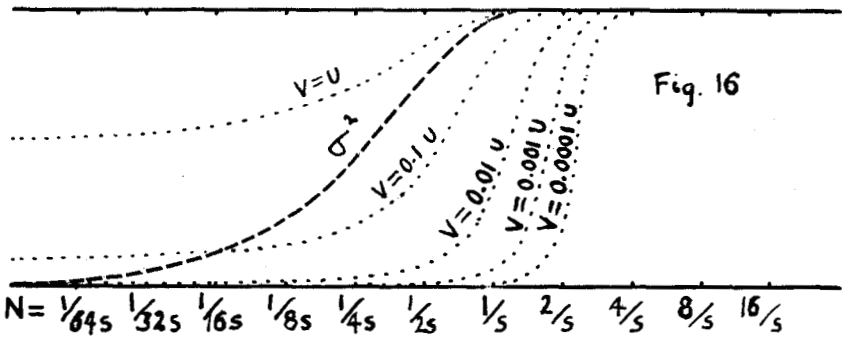
In the case of genes which are indifferent to selection (s less than u), the mean frequency $\bar{q} = v/(u+v)$ remains unchanged through all transformations from a U-shaped distribution in small populations to an I-shaped one in large populations. The variance, due to such genes, is small in small populations, rises in nearly direct proportion to size of population up to a certain critical point (about $N = 1/4u$) and then approaches a limiting value. For the case in which mutations in one direction (u) occur at a much greater rate than in the other (v), the general formula reduces to $\sigma^2 = \sigma_\infty^2\left(\frac{4Nu}{1+4Nu}\right)$, in which $\sigma_\infty^2 (= 2La^2v/u)$ is the limiting value. This case is illustrated in figure 15. The dotted lines represent mean gene frequencies and the line of dashes the variance.

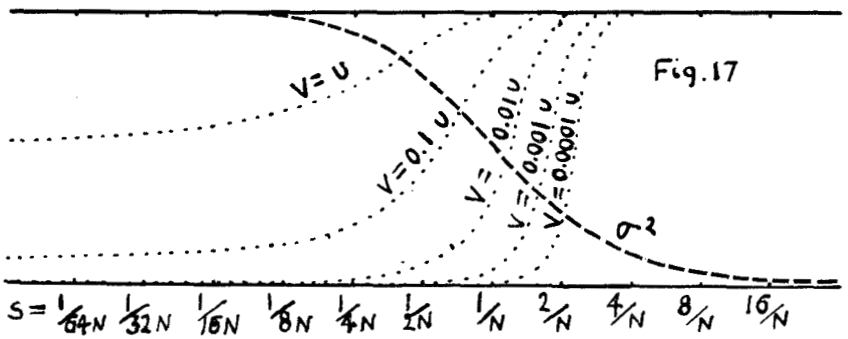
Actual changes in the size of a given population are not of course accompanied by instant adjustment of the distribution of gene frequencies. A decrease in size to a point well below the critical value is followed by decrease in heterozygosis and variance at a rate between 1/4N and 1/2N per generation depending on the number of allelomorphs. This may be a fairly rapid process in terms of geologic time but the recovery of heterozygosis through growth of the population to its original size occurs more slowly, since this depends on mutation pressure. On the other hand, the intercrossing of a number of isolated strains, in each of which the reduction of variance has occurred, is followed by immediate recovery of the original statistical situation (except with respect to factor combinations in which there is some delay).

In the opposite case of genes under vigorous selection (s much greater than u) mean frequency as well as variance is affected by size of population and by severity of selection. As in the preceding case, variance is small in small populations, rises in nearly direct proportion to growth of population[14] until a critical point is approached (here about $N = 1/4s$) and then rapidly approaches a limiting value $\sigma^2 = \sigma_\infty^2\left(\frac{e^{4Ns}-1}{e^{4Ns}+1}\right)$ where

[14] As before, the probability that the increase in variance, due to growth of population, would react on the selection coefficients of the individual genes, reducing them, requires some qualification of this statement in application to actual populations.







FIGURES 15 to 17.—The variance (σ^2) and mean gene frequencies (dotted lines) at equilibrium under various conditions of mutation, selection and size of population. Figure 15. Effects of increasing population where selection is negligible relative to mutation. Figure 16. Effects of increasing population where mutation rates are small compared with 1/4N. Figure 17. Effects of increasing selection where mutation rates are small compared with 1/4N.

$\sigma_\infty^2 = \mu a^2/s$. The mean factor frequency in large populations ($\bar{q} = 1 - u/s$) is close to 1, nearly complete fixation of the favorable gene. In small populations, on the other hand, the equilibrium point approaches that of the opposing mutation pressures ($\bar{q} = v/(u+v)$) and hence practically 0, with complete loss ($v = 0$) as the inevitable ultimate fate in an extended multiple allelomorphic series. Up to the point at which mutation pressure seriously disturbs the form of the distribution curve, the mean gene frequencies are simply the ratios of the chances of fixation at each extreme, namely, $ve^{4Ns}/(u+ve^{4Ns})$.

The relations of mean frequency and variance to size of population in this case are both shown in figure 16, the former for various relative values of u and v. Inspection of figures 9 and 10 may also be of assistance in understanding this situation.

As in the other case, actual change in size of population is not accompanied by immediate attainment of the new equilibrium. Decrease in population to a number well below the critical point is followed by decrease in heterozygosis at the rate described, bringing with it at the same rate the well known inbreeding effects, loss of variance and, in general, decline in vigor toward a new level. This immediate decline in vigor is not due to change in mean gene frequency, but merely to the greater proportion of recessive phenotypes as homozygosis increases, and thus comes to an end when the degree of homozygosis has reached equilibrium. The change in mean gene frequency proceeds more slowly since it depends on mutation pressure. Long continued isolation should thus involve two distinct degeneration processes, a rapid but soon completed process of fixation and a very slow process of accumulation of injurious genes. The recovery on increase in size of population is slow in both cases, depending on mutation pressure. The intercrossing of isolated lines, on the other hand, is followed by immediate return to the original status of the population if only the immediate inbreeding effect has occurred, but must wait on favorable mutations if there has been time for the slower process.

The effects of different intensities of selection on mean gene frequency and variance (population size constant) are illustrated in figure 17, still assuming that the selection coefficient is of higher order than mutation rate. Figures 7 and 8 showing the distribution of gene frequencies in this case may also be of assistance here. Selection has little effect on variability until it reaches about the value 1/8N, about half the variance is eliminated when selection reaches 1/N and most of it at $s = 4/N$. The formula is $\sigma^2 = \sigma_0^2\left(\frac{e^{4Ns}-1}{2Ns(e^{4Ns}+1)}\right)$. Selection, of course, affects the mean gene

frequency, the formula being the same as that given above under the effect of size of population. On actual increase in the intensity of selection, the rate of change toward the new equilibrium both in mean and variance is controlled by selection pressure and may thus be fairly rapid in terms of geologic time in a large population. This is the case in which HALDANE'S formulae for progress under selection are most applicable. The increase in variance and in the proportion of unfavorable genes following relaxation of selection, on the other hand, are controlled by mutation pressure and thus approach equilibrium relatively slowly. A shift in gene frequency at rate uq may well mean no more than 0.000,001 per generation.

The type of result where the selection coefficient is of the same order of magnitude as mutation rate can be inferred, qualitatively, at least from the preceding extreme cases. Inspection of figure 19 may also be of assistance here.

THE EVOLUTION OF MENDELIAN SYSTEMS

Classification of the factors of evolution

In attempting to draw conclusions with respect to evolution one is apt, perhaps, to assume that factors which make for great variation are necessarily favorable while those which reduce variation are unfavorable. Evolution, however, is not merely change, it is a process of *cumulative* change: fixation in some respects is as important as variation in others. Live stock breeders like to compare their work to that of a modeller in clay. They speak of moulding the type toward the ideal which they have in mind. The analogy is a good one in suggesting that in both cases it is a certain intermediate degree of plasticity that is required.

The basic cumulative factor in evolution is the extraordinary persistence of gene specificity. This doubtless rests on a tendency to precise duplication of gene structure in the proper environment. The basic change factor is gene mutation, the occasional failure of precise duplication. Since the time of LAMARCK, a school of biologists have held that the primary changes in hereditary constitution must be adaptive in direction in order to account for evolutionary advance. Unfortunately, the results of experimental study have given no support to this view. Instead, the characteristics of actually observed gene mutations seem about as unfavorable as could be imagined for adaptive evolution. In the first place, is their fortuitous occurrence. No correlation has been found between external conditions and direction of mutation, and those few agents which have been found to affect the rate (X-ray, radium, and to a relatively unimportant extent, temperature) merely speed up the rate of random mutation. The great

majority of mutations are either definitely injurious to the organism or produce such small effects as to be seemingly negligible. MULLER has graphically compared the range of mutations to a spectrum in which the nonlethal conspicuous mutations form a narrow field between broad regions of individually inconspicuous mutations on the one hand and of sublethal and lethal mutations on the other. In addition, the great majority of mutations are more or less completely recessive to the type genes from which they arise. These effects are easily understood if mutation is an accidental process. Random changes in a complex organization are more likely to injure than to improve it, and with respect to the immediate products of the gene, random changes are more likely to be of the nature inactivation (and hence probably recessive) than of increased activation. Finally is to be mentioned the extreme rarity of gene mutation. Even in Drosophila, mutation rates as high as $u = 10^{-5}$ per locus seem to be exceptional and 10^{-6} or less more characteristic. This infrequency seems unfavorable to rapid evolution, yet it is a necessary corollary of the usually injurious effect, if life is to persist at all. Moreover, the more advanced the evolution, the slower must be the time rate of mutation. In one-celled organisms, dividing several times a day, a rapid time rate of mutation will not prevent the production of sufficient normal offspring to maintain the species. The same time rate in Drosophila with an interval of some two weeks between generations would mean such an accumulation of lethals in every gamete that the species would come to an abrupt end. The time rate of lethal mutation in Drosophila (7 per 1000 chromosomes per month under ordinary conditions according to MULLER (1928)) would be quite impossible in the human species. The problem is to determine how an adaptive evolutionary process may be derived from such unfavorable raw material as the infrequent, fortuitous and usually injurious gene mutations.

It will be convenient here to classify factors of evolution according as they tend toward genetic homogeneity or heterogeneity of the species. They are grouped below in more or less definitely opposing pairs.

Factors of Genetic Homogeneity	*Factors of Genetic Heterogeneity*
Gene duplication	Gene mutation (u, v)
Gene aggregation	Random division of aggregate
Mitosis	Chromosome aberration
Conjugation	Reduction (meiosis)
Linkage	Crossing over
Restriction of population size (1/2N)	Hybridization (m)
Environmental pressure (s)	Individual adaptability
Crossbreeding among subgroups (m_1)	Subdivision of group ($1/2N_1$)
Individual adaptability	Local environments of subgroups (s_1)

The first pair have been discussed above. They enter into the formulae through the mutation rates u and v. MULLER has pointed out the necessary similarity, in order of size, of genes and of filterable viruses and has suggested the possibility that the latter may consist of single genes. If so, their evolution rests wholly on a not too high rate of mutation, and selection, which seems possible enough in organisms as simple as these presumably are, especially as in this case where the gene is the organism, the mutation of the gene need not be expected to be as fortuitously related to the activities of the organism as in more complex cases.

Presumably the first step toward higher organisms is the aggregation of such genes with multiplication of the aggregate by random division. Given occasional gene mutation, this leads to a new kind of variation, that in proportional abundance of the different kinds of genic material. The larger the aggregate, the less violent the variation. Large aggregates present a labile system capable of quantitative variation in response (perhaps physiologically as well as through selection) to changing conditions. As far as observation goes, the bacteria, and blue green algae have no mechanism of division beyond a random division of the protoplasmic constituents. Such apportionment of more or less autonomous materials may also be important in the differentiating cell lineages of multicellular organisms, but, except for a few plastid characters, seems to play no important role in heredity from generation to generation, as far as has been determined by experiment. There seems here an adequate basis for an evolutionary process in organisms so simple that the handing on of a few different protoplasmic constituents can determine all of the characteristics of the species but the conditions are not favorable for an extensive cumulative process.

Mitosis provides the mechanism by which an indefinitely large number of qualitatively different elements may be maintained in the same proportions. But it provides so perfectly for the persistence of complex organization that further change is difficult. Irregularities in mitosis provide a source of variation but of so violent a nature for the most part as to be of infrequent evolutionary importance, although the differences in chromosome numbers of related species demonstrate that they play a genuine rôle. Complete duplication (tetraploidy) is important in doubling the possible number of different kinds of genes. Other aberrations, especially translocations, are probably more important in isolating types, than for the character changes which they bring. Gene mutation remains the principal factor of variation, but seems inadequate as the basis of an evolutionary process under exclusively mitotic (asexual) reproduction.

The most important factor in transcending the evolutionary difficulties

inherent in the characteristics of gene mutation is undoubtedly the attainment of biparental reproduction (EAST 1918). This involves two phases, conjugation, a factor which makes the entire interbreeding group a physiological unit in evolution, and meiosis, with its consequence, Mendelian recombination which enormously increases the amount of variability within the limits of the species. Each additional viable mutation in an asexually reproducing form merely adds one to the number of types subject to natural selection. The chance that two or more indifferent or injurious mutations may combine in one line to produce a possibly favorable change is of the second or higher order. Under biparental reproduction, each new mutation doubles the number of potential variations which may be tried out. The contrast is between $n+1$ and 2^n types from n viable mutations.

Biparental reproduction solves the evolutionary requirement of a rich field of variation. But by itself it provides rather too much plasticity. It makes a highly adaptable species, capable of producing types fitted to each of a variety of conditions, but a successful combination of characteristics is attained in individuals only to be broken up in the next generation by the mechanism of meiosis itself.

An excellent illustration of the principle that a balance between factors of homogeneity and of heterogeneity may provide a more favorable condition for evolution than iether factor by itself may be found in the effects of an alternation of a series of asexual generations with an occasional sexual generation. Evolution is restrained under exclusive asexual reproduction by the absence of sufficient variation, and under exclusive sexual reproduction by the noncumulative character of the variation, but, on alternating with each other, any variety in the wide range of combinations provided by a cross may be multiplied indefinitely by asexual reproduction. The selection of individuals is replaced by the much more effective selection of clones and leads to rapid statistical advance which, however, comes to an end with reduction to a single successful clone. On the other hand a new cross (before reduction to a single clone) may provide a new field of variation making possible a repetition of the process at a higher level. This method has been a favorite of the plant breeder and is perhaps the most successful yet devised for human control of evolution in those cases to which it can be applied at all. Under natural conditions, alternation of asexual and sexual reproduction is characteristic of many organisms and doubtless has played an important rôle in their evolution.

The demonstration of the evolutionary advantages of an alternation of the two modes of reproduction seems to prove too much. Asexual re-

production is practically absent in the most complex group of animals, the vertebrates, and is rather sporadic in its occurrence elsewhere. The purpose of the present paper has been to investigate the statistical situation in a population under exclusive sexual reproduction in order to obtain a clear idea of the conditions for a degree of plasticity in a species which may make the evolutionary process an intelligible one.

First may be mentioned briefly a modification of the meiotic mechanism which has been introduced only qualitatively into the investigation where at all. This is the aggregation of genes into more or less persistent systems, the chromosomes. Complete linkage cuts down variability by preventing recombination. Wholly random assortment gives maximum recombination but does not allow any important degree of persistence of combinations once reached. An intermediate condition permits every combination to be formed sooner or later and gives sufficient persistence of such combinations to give a little more scope to selection than in the case of random assortment. Close linkage, moreover, brings about a condition in which selection tends to favor the heterozygote against both homozygotes and so helps in maintaining a store of unfixed factors in the population.

Resrtiction of size of population, measured by $1/2N$, is a factor of homogeneity and conversely with increase of size. The effects of restricted size may also be balanced by those of occasional external hybridization, measured by m.

Environmental pressure on the species as a whole is a factor of homogeneity. It has been urged by some that because natural selection is a factor which reduces variability, and most conspicuously by eliminating extreme types, it cannot be the guiding principle in adaptive evolution. From the viewpoint of evolution as a moving equilibrium, however, the guiding principle may be found on the conservative as well as on the radical side. The selection coefficient, s, depends on the balance between environmental pressure and individual adaptability. High development of the latter permits the survival of genetically diverse types in the face of severe pressure.

Subdivision of a population into almost completely isolated groups, whether by prevailing self fertilization, close inbreeding, assortative mating, by habitat or by geographic barriers is a factor of heterogeneity with effects measured by $1/2N_1$, N_1 being here the size of the subgroup. This factor may be balanced by crossbreeding between such groups, measured by m_1.

It is interesting to note that restriction of population size is a factor of homogeneity or of heterogeneity for the species, depending on whether it relates to the species as a whole or to subgroups and conversely with the crossbreeding coefficient. Similarly the selection pressures of varied environments within the range of the species (s_1) constitute factors of heterogeneity, restrained from excessive genetic effect by the same individual adaptability which appears in the opposite column in relation to the general environment of the species. Individual adaptability is, in fact, distinctly a factor of evolutionary poise. It is not only of the greatest significance as a factor of evolution in damping the effects of selection and keeping these down to an order not too great in comparison with 1/4N and u, but is itself perhaps the chief object of selection. The evolution of complex organisms rests on the attainment of gene combinations which determine a varied repertoire of adaptive cell responses in relation to external conditions. The older writers on evolution were often staggered by the seeming necessity of accounting for the evolution of fine details of an adaptive nature, for example, the fine structure of all of the bones. From the view that structure is never inherited as such, but merely types of adaptive cell behavior which lead to particular structures under particular conditions, the difficulty to a considerable extent disappears. The present difficulty is rather in tracing the inheritance of highly localized structural details to a more immediate inheritance of certain types of cell behavior.

Lability as the condition for evolution

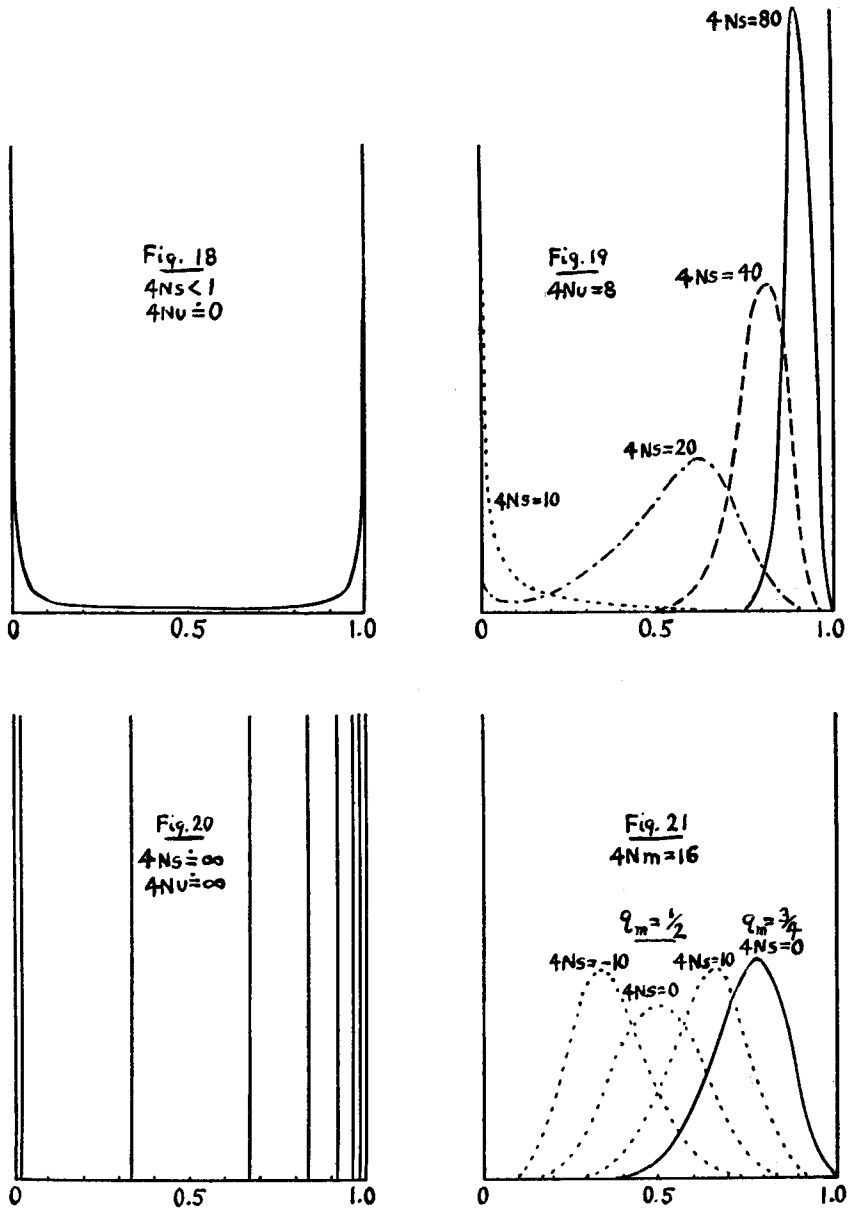
The statistical effects of the more important of these factors in a freely interbreeding population are brought together in the formula

$$y = Ce^{4Nsq}q^{-1}(1 - q)^{4Nu-1}.$$

The term 4Nv in the exponent of q is here assumed to be negligible and the terms applicable in case of external hybridization are also omitted.

Consider first the situation in a small population in which 1/4N is much greater than u and than s (figure 18). Nearly all genes are fixed in one phase or another. Even rather severe selection is without effect. There is no equilibrium for individual genes. They drift from one state of fixation to another in time regardless of selection, but the rate of transfer is extremely slow. Such evolution as there is, is random in direction and tends toward extinction of the group.

Consider next the opposite extreme, a very large undivided population under severe selection. Assume that s is in general much greater than u and that the latter is much greater than 1/4N. There is almost complete



FIGURES 18 to 21.—Distributions of gene frequencies in relation to size of population, selection, mutation and state of subdivision. Figure 18. Small population, random fixation or loss of genes ($y = Cq^{-1}(1-q)^{-1}$. Figure 19. Intermediate size of population, random variaton of gene frequencies about modal values due to opposing mutation and selection ($y = Ce^{4Nsqq-1}(1-q)^{4Nu-1}$. Figure 20. Large population, gene frequencies in equilibrium between mutation and selection ($q = 1-u/s$, etc.). Figure 21. Subdivisions of large population, random variation of gene frequencies about modal values due to immigration and selection. ($y = Ce^{4Nsqq_4Nmq_m-1}(1-q)^{4Nm(1-q_m)-1}$.

fixation of the favored gene for each locus. Here also there is little possibility of evolution. There would be complete equilibrium under uniform conditions if the number of allelomorphs at each locus were limited. With an unlimited chain of possible gene transformations, new favorable mutations should arise from time to time and gradually displace the hitherto more favored genes but with the most extreme slowness even in terms of geologic time.[15]

Even if selection is relaxed to such a point that the selection coefficients of many of the genes are not much greater than mutation rates, the conditions are not favorable for a rapid evolution (figure 20). The amount of variability in the population may be great, maximum in fact, but if the distributions of gene frequencies are closely concentrated about single values, the situation approaches one of complete equilibrium and hence of complete cessation of evolution. At best an extremely slow, adaptive, and hence probably orthogenetic advance is to be expected from new mutations and from the effects of shifting conditions.

It should be added that a relatively rapid shift of gene frequencies can be brought about in this case by vigorous increase in the intensity of selection. The effects of unopposed selection of various sorts and in various relations of the genes has been studied exhaustively by HALDANE, with regard to the time required to bring about a shift of gene frequency of any required amount. The end result, however, is the situation previously discussed. The rapid advance has been at the expense of the store of variability of the species and ultimately puts the latter in a condition in which any further change must be exceedingly slow. Moreover, the advance is of an essentially reversible type. There has been a parallel movement of all of the equilibria affected and on cessation of the drastic selection, mutation pressure should (with extreme slowness) carry all equilibria back to their original positions. Practically, complete reversibility is not to be expected, and especially under changes in selection which are more complicated than can be described as alternately severe and relaxed. Nevertheless, the situation is distinctly unfavorable for a continuing evolutionary process.

Thus conditions are unfavorable for evolution both in very small and in very large, freely interbreeding, populations, and largely irrespective of severity of selection. We have next to consider the intermediate situa-

[15] This, nevertheless, seems to be the case which FISHER (1930) considers most favorable to evolution. The greatest difference between our conclusions seems to lie here. His theory is one of complete and direct control by natural selection while I attribute greatest immediate importance to the effects of incomplete isolation.

tion in which s is not much greater than u for many genes and the latter is not much greater than 1/4N. Such a case is illustrated in figure 19. The size of population is sufficient to prevent random fixation of genes, but insufficient to prevent random drifting of gene frequencies about their mean values, as determined by selection and mutation. It is to be supposed that the relations of the selection and mutation coefficients vary from factor to factor. The more indifferent ones drift about through a wide range of frequencies in the course of geologic time while those under more severe selection oscillate about positions close to complete fixation. In any case, all gene frequencies are continually changing even under uniform environmental conditions. But the selection coefficients themselves are in general to be considered functions of the entire array of gene frequencies and will therefore also be continually changing. The probability arrays of some genes will travel to the right and close up as their selection coefficients stiffen, while some of the genes which have been nearly fixed will come to be less severely selected and their probability arrays will shift to the left and open out or even move to the extreme left under displacement by another allelomorph. A continuous and essentially irreversible evolutionary process thus seems inevitable even under completely uniform conditions. The direction is largely random over short periods but adaptive in the long run. The less the variation of gene frequency about its mean value, the closer the approach to an adaptive orthogenesis. Complete separation of the species into large subspecies should be followed by rather slow more or less closely parallel evolutions, if the conditions are similar, or by adaptive radiation, under diverse conditions, while isolation of smaller groups would be followed by a relatively rapid but more largely nonadaptive radiation.

As to rate, since the process depends mainly on the value of 1/4N, assumed to be somewhat less than u (and s) the process cannot be as rapid as one due temporarily to either unopposed selection or unopposed mutation pressure. Hundreds of thousands of generations seem to be required at best for important nonadaptive evolutionary changes of the species as a whole; while adaptive advance, depending on the chance attainment of favorable combinations would be much slower. Even so the process is much the most rapid non-self-terminating one yet considered.

In reaching the tentative conclusion that the situation is most favorable for evolution in a population of a certain intermediate size, one important consideration has been omitted. This is the tendency toward subdivision into more or less completely isolated subgroups in widely distributed populations. Within each subgroup there is a distribution of gene fre-

quencies dependent largely on its own size (N_1), amount of crossing with the rest of the species (m_1), selection due to local conditions (s_1) and the mean gene frequency of migrants from the rest of the species (q_m). It may be assumed that $1/4N_1$ is so much larger than u that mutation pressure (and also the average selection coefficient, s, for the whole species) can be ignored.

$$y = Ce^{4N_1s_1q}q^{4N_1m_1q_m-1}(1 - q)^{4N_1m_1(1-q_m)-1}.$$

Gene frequency in each subgroup oscillates about a mean value, which is that of the whole species only if conditions of selection are uniform. Figure 21 represents various cases. The random variations of gene frequency have effects similar to those described above within each group. The result is a partly nonadaptive, partly adaptive radiation among the subgroups. Those in which the most successful types are reached presumably flourish and tend to overflow their boundaries while others decline, leading to changes in the mean gene frequency of the species as a whole. In this case, the rate of evolution should be much greater than in the previous cases. The coefficients $1/4N_1$ and s_1 may be relatively large and bring about rapid differentiation of subgroups, while the competition between subgroups will bring about rapid changes in the gene frequencies of the species as a whole. The direction of evolution of the species as a whole will be closely responsive to the prevailing conditions, orthogenetic as long as these are constant, but changing with sufficiently long continued environmental change.

A question which requires consideration is the effect of alternation of conditions, large and small size of population, severe and low selection. The effects of changes in the conditions of selection have already been touched upon. Persistence of small numbers or of severe selection for such periods of time as to bring about extensive fixation of factors compromises evolution for a long time following, there being no escape from fixation except by mutation pressure. Many thousands of generations may be required after restoration to large size and not too severe selection, before evolutionary plasticity is restored. Short time oscillations in population number or severity of selection, on the other hand, probably tend to speed up evolutionary change by causing minor changes in gene frequency.

Control of evolution

With regard to control of the process, it is evident that little is possible either within a small stock or a freely interbreeding large one. Even drastic

selection is of little effect in the former, and in the latter, while it may bring about a rapid immediate change in the particular respect selected, this must be at the expense of other characters, and in any case, soon leads to a condition in which further advance must wait on the occurrence of mutations more favorable than those fixed by the selection. The limitations in this case have been well brought out in a recent discussion by KEMP (1929). Maximum continuous progress in a homogeneous population requires an intensity of selection for each of the more indifferent genes not much greater than its mutation rate and also a certain size of population. Even so, the direction of advance is somewhat uncertain and the rate to be measured in geologic time.

If infrequency of mutation is the limiting factor here, it would seem that a considerable increase in the rate of evolution should be made possible by a speeding up of mutation, as by X-rays. There is a limit, however, imposed by the prevailingly injurious character of mutations. Even the most rigorous culling of individuals means in general, only a low selection coefficient (in absolute terms) for each of the presumably numerous unfixed genes, which are not in themselves lethal or sublethal in effect. Such culling would become insufficient to hold mutation pressure in check when the latter had increased beyond a certain point ($u > s$). Moreover, as the number of unfixed genes becomes greater under an increased mutation rate, the smaller becomes the separate gene selection coefficients, making it certain that mutation rate could not increase very much before the possibility of effective selection (in all respects at once) rather than infrequency of mutation would become the limiting factor. With respect to lethal mutations, it has already been noted that the observed natural time rate in Drosophila is such as would mean immediate extinction, if transferred to the human species. It is clear that an evolution in the direction of increased gene stability, rather than mutability, has been a necessary phase, in the evolution of the longer lived higher animals. This makes it unlikely that a general increase in mutation rate would increase the rate of evolutionary advance along adaptive lines.

The only practicable method of bringing about a rapid and non-self-terminating advance seems to be through subdivision of the population into isolated and hence differentiating small groups, among which selection may be practiced, but not to the extent of reduction to only one or two types (WRIGHT 1922a). The crossing of the superior types followed by another period of isolation, then by further crossing and so on *ad infinitum* presents a system by means of which an evolutionary advance through the field of possible combinations of the genes present in the original stock, and

arising by occasional mutation, should be relatively rapid and practically unlimited. The occasional use of means for increasing mutation rate within limited portions of the population should add further to the possibilities of this system.

Agreement with data of evolution

We come finally to the question as to how far the characteristics of evolution in nature can be accounted for on a Mendelian basis. A review of the data of evolution would go far beyond the scope of the present paper. It may be suggested, however, that the type of moving equilibrium to be expected, according to the present analysis, in a population comparable to natural species in numbers, state of subdivision, conditions of selection, individual adaptability, etc. agrees well with the apparent course of evolution in the majority of cases, even though heredity depend wholly on genes with properties like those observed in the laboratory. Adaptive orthogenetic advances for moderate periods of geologic time, a winding course in the long run, nonadaptive branching following isolation as the usual mode of origin of subspecies, species and perhaps even genera, adaptive branching giving rise occasionally to species which may originate new families, orders, etc.; apparent continuity as the rule, discontinuity the rare exception, are all in harmony with this interpretation.

The most serious difficulties are perhaps in apparent cases of nonadaptive orthogenesis on the one hand and extreme perfection of complicated adaptations on the other. In so far as extreme degeneration of organs is concerned, there is little difficulty—this is to be expected as a by-product of other evolutionary changes. Because of their multiple effects, there can be no really indifferent genes, whatever may be true of organs which have been reduced beyond a certain size. Zero as the value of a selection coefficient is merely a mathematical point between positive and negative values. It is common observation that mutations are more likely to reduce the development of an organ than to stimulate it. It follows that evolutionary change in general will have as a by product the gradual elimination of indifferent organs. Nonadaptive orthogenesis of a positive sort, increase of size of organs to a point which threatens the species, constitutes a more difficult problem, if a real phenomenon. Probably many of the cases cited are cases in which the line of evolution represents the most favorable immediately open to a species doomed by competition with a form of of radically different type or else cases in which selection based on individual advantage leads the species into a cul-de-sac. The nonadaptive differentiation of small subgroups and the great effectiveness of subsequent

selection between such groups as compared with that between individuals seem important factors in the origin of peculiar adaptations and the attainment of extreme perfection. It is recognized that there are specific cases which seem to offer great difficulty. This should not obscure the fact that the bulk of the data indicate a process of just the sort which must be occurring in any case to some extent as a statistical consequence of the known mechanism of heredity. The conclusion seems warranted that the enormous recent additions to knowledge of heredity have merely strengthened the general conception of the evolutionary process reached by DARWIN in his exhaustive analysis of the data available 70 years ago.

"Creative" and "emergent" evolution

The present discussion has dealt with the problem of evolution as one depending wholly on mechanism and chance. In recent years, there has been some tendency to revert to more or less mystical conceptions revolving about such phrases as "emergent evolution" and "creative evolution." The writer must confess to a certain sympathy with such viewpoints philosophically but feels that they can have no place in an attempt at scientific analysis of the problem. One may recognize that the only reality directly experienced is that of mind, including choice, that mechanism is merely a term for regular behavior, and that there can be no ultimate explanation in terms of mechanism—merely an analytic description. Such a description, however, is the essential task of science and because of these very considerations, objective and subjective terms cannot be used in the same description without danger of something like 100 percent duplication. Whatever incompleteness is involved in scientific analysis applies to the simplest problems of mechanics as well as to evolution. It is present in most aggravated form, perhaps, in the development and behavior of individual organisms, but even here there seems to be no necessary limit (short of quantum phenomena) to the extent to which mechanistic analysis may be carried. An organism appears to be a system, linked up in such a way, through chains of trigger mechanisms, that a high degree of freedom of behavior as a whole merely requires departures from regularity of behavior among the ultimate parts, of the order of infinitesimals raised to powers as high as the lengths of the above chains. This view implies considerable limitations in the synthetic phases of science, but in any case it seems to have reached the point of demonstration in the field of quantum physics that prediction can be expressed only in terms of probabilities, decreasing with the period of time. As to evolution, its entities, species and ecologic systems, are much less closely knit than individual organisms.

One may conceive of the process as involving freedom, most readily traceable in the factor called here individual adaptability. This, however, is a subjective interpretation and can have no place in the objective scientific analysis of the problem.

SUMMARY

The frequency of a given gene in a population may be modified by a number of conditions including recurrent mutation to and from it, migration, selection of various sorts and, far from least in importance, mere chance variation. Using q for gene frequency, v and u for mutation rates to and from the gene respectively, m for the exchange of population with neighboring groups with gene frequency q_m, s for the selective advantage of the gene over its combined allelomorphs and N for the effective number in the breeding stock (much smaller as a rule than the actual number of adult individuals) the most probable change in gene frequency per generation may be written:

$$\Delta q = v(1 - q) - uq - m(q - q_m) + sq(1 - q)$$

and the array of probabilities for the next generation as $[(1-q-\Delta q)a + (q+\Delta q)A]^{2N}$. The contribution of zygotic selection (reproductive rates of aa, Aa and AA as $1-s^1:1-hs^1:1$) is $\Delta q = s^1q(1-q)[1-q+h(2q-1)]$. In interpreting results it is necessary to recognize that the above coefficients are continually changing in value and especially that the selection coefficient of a particular gene is really a function not only of the relative frequencies and momentary selection coefficients of its different allelomorphs but also of the entire system of frequencies and selection coefficients of non-allelomorphs. Selection relates to the organism as a whole and its environment and not to genes as such. The mutation rate to a gene (v) can usually be treated as of negligible magnitude assuming the prevalence of multiple allelomorphs.

In a population so large that chance variation is negligible, gene frequency reaches equilibrium when $\Delta q = 0$. Among special cases is that of opposing mutation rates $\left(q = \frac{v}{u+v}\right)$, of selection against both homozygotes $\left(q = \frac{1-h}{1-2h}\right)$, of mutation against genic selection $\left(q = 1 - \frac{u}{s}\right)$, of mutation against zygotic selection $\left(q = 1 - \frac{u}{hs^1}\right.$ unless h approaches 0, when $\left. q = 1 - \sqrt{\frac{u}{s}}\right)$, of selection and migration $\left(q = 1 - \frac{m}{s}(1 - q_m)\right)$ or

$-\frac{mq_m}{s}$ if s is much greater than m, $q = q_m\left(1+\frac{s}{m}(1-q_m)\right)$ if s is much smaller than m, while the values $q = \sqrt{q_m}$ or $1-\sqrt{1-q_m}$ when $s = \pm m$ illustrate the intermediate case).

Gene frequency fluctuates about the equilibrium point in a distribution curve, the form of which depends on the relations between population number and the various pressures. The general formula in the case of a freely interbreeding group, assuming genic selection, is

$$y = Ce^{4Nsq}q^{4N[mq_m+v]-1}(1-q)^{4N[m(1-q_m)+u]-1}.$$

The correlation between relatives is affected by the form of the distribution of gene frequencies through FISHER's "dominance ratio." It appears that this is less than 0.20 in small populations under low selection but may even approach 1 in large populations under severe selection against recessives.

In a large population in which gene frequencies are always close to their equilibrium points, any change in conditions other than population number is followed by an approach toward the new equilibria at rates given by the Δq's. Great reduction in population number is followed by fixation and loss of genes, each at the rate 1/4N per generation, where N refers to the new population number. This applies either in a group of monoecious individuals with random fertilization or, approximately, in one equally divided between males and females (9.6 percent instead of 12.5 percent, however, under brother-sister mating, N = 2). More generally with an effective breeding stock of N_m males and N_f females, the rates of fixation and of loss are each approximately $(1/16N_m+1/16N_f)$ until mutation pressure at length brings equilibrium in a distribution approaching first the form $y = C(1-q)^{-1}$ with decay at rate u and ultimately $Cq^{-1}(1-q)^{-1}$. The converse process, great increase in the size of a long inbred population, is followed by a slow approach toward the new equilibrium at a rate dependent in the early stages on mutation pressure.

With respect to genes which are indifferent to selection, the mean frequency is always $q = v/(u+v)$. The variance of characters, dependent on such genes, is proportional (at equilibrium) to population number up to about $N = 1/4u$. Beyond this, there is approach of variance to a limiting value.

In the presence of selection (s considerably greater than 2u) the mean frequency at equilibrium varies between approximate fixation of the favored genes ($q = 1 - u/s$) in large populations and approximate, if not complete, fixation of mutant allelomorphs ($q = v/(u+v)$) in small popula-

tions, the rate of change from one state to the other being the mutation rate (u). A consequence is a slow but increasing tendency to decline in vigor in inbred stocks, to be distinguished from the relatively rapid but soon completed fixation process, described above as occurring at rate 1/2N. The variance of characters in this as in the preceding case, is approximately proportional to population number up to a certain point (N less than 1/4s) and above this rapidly approaches a limiting value. Variance is inversely proportional to the severity of selection in large populations unless the selection is very slight but in small populations is little affected by selection unless the latter is very severe (s greater than 1/4N).

Evolution as a process of cumulative change depends on a proper balance of the conditions, which, at each level of organization—gene, chromosome, cell, individual, local race—make for genetic homogeneity or genetic heterogeneity of the species. While the basic factor of change—the infrequent, fortuitous, usually more or less injurious gene mutations, in themselves, appear to furnish an inadequate basis for evolution, the mechanism of cell division, with its occasional aberrations, and of nuclear fusion (at fertilization) followed at some time by reduction make it possible for a relat vely small number of not too injurious mutations to provide an extensive field of actual variations. The type and rate of evolution in such a system depend on the balance among the evolutionary pressures considered here. In too small a population (1/4N much greater than u and s) there is nearly complete fixation, little variation, little effect of selection and thus a static condition modified occasionally by chance fixation of new mutations leading inevitably to degeneration and extinction. In too large a freely interbreeding population (1/4N much less than u and s) there is great variability but such a close approach to complete equilibrium of all gene frequencies that there is no evolution under static conditions. Change in conditions such as more severe selection, merely shifts all gene frequencies and for the most part reversibly, to new equilibrium points in which the population remains static as long as the new conditions persist. Such evolutionary change as occurs is an extremely slow adaptive process. In a population of intermediate size (1/4N of the order of u) there is continual random shifting of gene frequencies and a consequent shifting of selection coefficients which leads to a relatively rapid, continuing, irreversible, and largely fortuitous, but not degenerative series of changes, even under static conditions. The rate is rapid only in comparison with the preceding cases, however, being limited by mutation pressure and thus requiring periods of the order of 100,000 generations for important changes.

Finally in a large population, divided and subdivided into partially isolated local races of small size, there is a continually shifting differentiation among the latter (intensified by local differences in selection but occurring under uniform and static conditions) which inevitably brings about an indefinitely continuing, irreversible, adaptive, and much more rapid evolution of the species. Complete isolation in this case, and more slowly in the preceding, originates new species differing for the most part in nonadaptive respects but is capable of initiating an adaptive radiation as well as of parallel orthogenetic lines, in accordance with the conditions. It is suggested, in conclusion, that the differing statistical situations to be expected among natural species are adequate to account for the different sorts of evolutionary processes which have been described, and that, in particular, conditions in nature are often such as to bring about the state of poise among opposing tendencies on which an indefinitely continuing evolutionary process depends.

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Effect of active immunization using a novel GnRH vaccine on reproductive function in rats

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ABSTRACT

To investigate the effect of gonadotropin-releasing hormone 2-multiple antigen peptide (GnRH2-MAP) on reproductive function. In our study, 20-day-old male rats (n = 90) were randomly allocated to one of three treatment groups: GnRH2-MAP immunization, GnRH2 immunization, and non-immunized control groups. The immunized animals were administered three doses of GnRH2-MAP or GnRH2 vaccines from 0 to 6 weeks at 2-week intervals. The control group only received oil adjuvant. Blood and right testis samples were collected, and the left testis was weighed and its volume was measured at 0, 2, 4, 6, 8, 10 and 12 weeks after the first immunization. The serum antibody titer and testosterone concentration were determined by ELISA, and the right testis samples were collected for histological analysis. The results revealed that the serum of vaccinated rats elicited a significantly higher antibody titer and a lower T concentration compared with the control group two weeks after the first immunization ($P < 0.05$), but the highest antibody titer and lowest T concentration were found in animals treated with GnRH2-MAP ($P < 0.05$). The second immunization resulted in a significant decrease in testicular weight and volume ($P < 0.05$) in both immunized groups compared to the control, but these values were significantly lower in the GnRH2-MAP group than in the GnRH2 group. Furthermore, seminiferous tubules revealed more significant atrophy in the GnRH2-MAP group than in the GnRH2 group, and no sperm were observed in rats of the GnRH2-MAP group. Thus, GnRH2-MAP may be an effective antigen and a potential immunocastration vaccine with higher effectiveness.

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1. Introduction

Immunocastration is an alternative method to traditional surgical castration and has been successfully implemented for fertility control in animals [1]. The gonadotropin-releasing hormone 2 (GnRH) vaccine increases the serum level of anti-GnRH antibody to neutralize endogenous GnRH, a decapeptide that is synthesized from hypothalamic neurons and then acts on the anterior pituitary via the portal circulation. Exogenous GnRH can be produced as an immunological castration vaccine, because it can block testicular function by disturbing the hypothalamic-pituitary-gonadal (HPG) axis. Thus, most studies focus on developing vaccines to overcome the shortcomings of existing vaccines, such as low efficiency and safety and high cost [2,3].

There are two ways to enhance the immunogenicity of GnRH: coupling macromolecules to GnRH or changing the structure of GnRH. Existing GnRH vaccines can be divided into three categories [1,3,4], all of which have unavoidable shortcomings. Due to the weak immunogenicity of the GnRH monomer, recent studies have coupled various carrier substances or albumin fractions such as ovalbumin (OVA), keyhole limpet hemocyanin, and bovine serum albumin (BSA) [5–7], to GnRH and GnRH analogues in order to enhance the immunogenicity of the vaccine. Han et al. [8,9] found that linking the GnRH tandem dimer with OVA prior to immunizing male rats and rams can induce testicular atrophy, inhibit spermatogenesis, and reduce the level of testosterone (T), inhibin A, LH,

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and FSH. However, the low rate of GnRH-carrier protein linking inhibits its commercial application. Moreover, chemical conjugation did not result in consistent homogeneity across different batches [1]. Consequently, genetically engineered and nucleic acid vaccines have emerged as potential low-cost vaccines. Stewart et al. [10] used live recombinant vectors and for the first time attempted to produce Tra T-GnRH fusion protein and achieved good results. However, as it was a live carrier, its safety was questioned. Han et al. [11] used the human *GnRH* gene and hepatitis B surface antigen S (HBsAg-S) gene to build a recombinant plasmid in mice, pVAx-HbsAg-S-2GnRH, which was used to encode the target protein vaccine. This vaccine was found to significantly inhibit testicular function; however, the cellular uptake rate of DNA vaccines is low, and consequently protein expression is also low. However, as the effect of the recombinant vaccine on other animals has not yet been verified, it can only be used as a potential new type of attenuated vaccine. Zhang et al. [12] inserted the *OVA-GnRH* gene into a prokaryotic expression vector system to obtain recombinant OVA-GnRH protein; however, it required a high number of *OVA-GnRH* genes in t he inserted plasmid. In brief, genetically engineered vaccines are limited at present due to the low protein expression, high genetic requirements, and complex purification process. To simplify the purification procedure and to stabilize plasmid expression, foreign genes encoding the antigen of interest are cloned into an expression plasmid, as a result of which the foreign gene can directly express antigen in the host cells, in this case, the nucleic acid vaccine [13]. Studies have found that the transfection rate of eukaryotic plasmids is low, and is at a potential risk of being integrated into the host genome. Recent research has proved that chemically synthesized peptide vaccines are associated with high safety and ease of synthesis and purification and are therefore preferred over other type of vaccines [14]. However, the effects of immunization among vaccines were not uniform, and immunocastration could only be achieved after the conjugation of carrier protein. Therefore, developing a low-cost, safe, and efficient vaccine is the main solution for fertility control in commercially raised animals.

The multiple antigen peptide (MAP) was proposed by James et al., in 1988 [15]. Studies show that there are two types of MAPs. The first type has a branched structure formed by multiple antigen peptides linked by lysine; it usually consists of three or seven Lys residues that form the core of four-branched or eight-branched peptides [16]. The second has a linear skeleton of Gly-Lys [17], within which Lys is linked to multiple antigenic peptides to form branched peptides perpendicular to the linear backbone. At present, more studies focus on the first type of MAP, as the Lys residues in its core can randomly connect to other amino acids by forming peptide bonds to increase the molecular weight of the antigen. The advantage of this structure is that its radial form not only enhances the immunogenicity of the synthetic peptide but also prolongs its half-life in vivo without causing the body to produce antibodies against the Lys core structure. Huang et al. [18] used antigenic peptides derived from *Schistosoma mansoni* glutathione-*S*-transferase (Sm28GST) to synthesize a four-branched peptide with an MAP structure by solid-phase peptide synthesis. Immunization of mice with this vaccine effectively induced antibody responses without the need for any adjuvant. After the schistosome infection, the number of worms and eggs in the liver of the mice dropped significantly compared with the control group, and the results showed that the protective effect of MAP was much better than that of the single peptide. Feng et al. [19] found that epitope tetrameric peptide (MAP) had a purity of >95% when the 9-fluorenylmethoxycarbonyl (FMOC) was used to synthesize the lipopolysaccharide-binding protein by solid-phase synthesis. Thus, the MAP system can make up for the low connection rate defect of the carrier protein vaccine. Therefore, we speculated that synthetic GnRH2 with straight chains could connect to Lys due to its binding capacity to produce the GnRH2-MAP vaccine. Due to its radial structure and molecular weight, GnRH2-MAP can act as a better antigen with high immunogenicity. In particular, it can make up for the shortcomings of protein vaccines with high connection and purity rates.

The current study aimed to test the feasibility and reliability of the novel GnRH vaccine (GnRH2-MAP) in achieving immunocastration in male Sprague-Dawley (SD) rats. It is hoped that the proposed method will resolve these issues in immunocastration vaccines.

2. Materials and methods

2.1. Vaccine formulation

GnRH2 peptide [20] and GnRH2-MAP were synthesized (Shanghai Shanjing Ltd., China). GnRH2 was modified to the eight-branched peptide system of MAP to produce GnRH2-MAP [21]. The purity of GnRH2 and GnRH2-MAP was found to be 95% by high-performance liquid chromatography. Oil adjuvant (30 ml, MONTANIDE™; Franch SEPPIC Company) and saline (30 ml) were combined using a stirring rod, beakers, for autoclaving. After low speed centrifugation, GnRH2-MAP and GnRH2 lyophilized powders (100 μg each) were dissolved in 7.5 ml saline, and then mixed with 7.5 ml oil adjuvant with the magnetic stirrer (X85-2S, Shanghai Chijiiu Company) at 800 rpm for 5 min.

2.2. Animals

Ninety healthy male SD rats (20 days of age) were used in this experiment. The animals were reared in a controlled temperature (22 °C) and light (12:12 h light:dark cycle) environment. Food and tap water were provided ad libitum. The study was approved by the Animal Care and Use Committee of Anhui Agricultural University. All experimental procedures involving rats were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China; revised in June 2004).

2.3. Experimental design and immunization

The rats were randomly allocated into one of the following three groups with 30 rats per group: GnRH2-MAP, GnRH2, and control groups. The immunized animals in the GnRH2-MAP and GnRH2 groups were treated with the novel GnRH2-MAP vaccine (100 μg/dose) or GnRH2 (100 μg/dose), respectively. The control group was injected with 0.5 mL oil adjuvant alone. The primary immunization was carried out at 0 weeks followed by two booster doses at 2-week intervals. All rats were administered 0.5 mL of the vaccine by subcutaneous injections on the back.

2.4. Collection and processing of samples

The rats were decapitated at 9:00–11:00 h and the blood samples (1.5–2.0 mL) were obtained without anticoagulant. The samples were incubated at 4 °C for 3 h, and centrifuged for 15 min at 2000 rpm to collect the serum, which was used to determine the T concentration and antibody titer by enzyme-linked immunosorbent assay (ELISA). After each blood collection, the rats ($n = 5$) were sacrificed by decapitation under intraperitoneal injection of 2% pentobarbital sodium. Paired testes were removed, their weights and axis lengths were measured, and data were recorded immediately. Then, the right testes were directly immersed into 4% v/v

paraformaldehyde at 4 °C for 12–24 h for subsequent histological analysis.

2.5. Histological analysis of the testes

The right testis sample was removed from paraformaldehyde, and the tissue was cut into flakes (thickness, about 5 μm) into the embedding box to water for the night. Testicular tissue was dehydrated using an ascending ethanol series (80%, 95%, and 100%), cleaned in xylene, embeded in paraffin wax, and stored at 4 °C. Then, 5-μm sections were obtained using a microtome (Shenyang Longshou Electronic Instrument limited computer, LS-2055+), and stained with hematoxylin and eosin (H-E). All sections were photographed with a microscope (Motic, Hong Kong, China).

2.6. Detection of antibody titer and T concentration

The serum antibody titer and T concentration in serum were determined by ELISA 2 weeks after sampling. The ELISA kit for antibody detection (Lianshuo, Shanghai, China) had a sensitivity of 1.0 mIU/ml, and the T kit (Yuanye, Shanghai, China) had a sensitivity of 0.1 nmol/L. Both kits were allowed to equilibrate for 20 min at room temperature. Then, 50 μl of standard substance was added to standard wells, and 50 μL of serum (1:50 dilution) was added to each of the sample wells almost simultaneously. Then, 100 μL of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (IgG) solution was added to the standard and sample wells, and the plate was incubated for 1 h at 37 °C. After successive washing with PBS, 3,3,5,5-tetra-methyl-benzidene (TMB, A and B, 50 μL each) was added to react with the enzyme-antigen conjugate, and the mixture was incubated at 37 °C for 15 min. Finally, the reaction was terminated by adding 50 μL of H_2SO_4. The optical density was determined by measuring the absorbance at 450 nm using a microplate reader (BioTek, USA).

2.7. Measurements of the testicular volume, weight, and body weight

At each sampling timepoint, the animals (n = 5) were weighed immediately prior to death. After the rats were sacrificed, the abdominal skin was quickly cut to remove and weigh the left testis. The testicular volume was calculated after measuring the largest width (a) and length (b) of the testis according to formula $V = [4\pi (a/2)^2 (b/2)] /4$ [8].

2.8. Statistical analysis

Data were expressed as means ± standard error of the mean, and processed and analyzed using SPSS version 17.0. Data were analyzed by one-way analysis of variance. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Individual reaction differences

The comprehensive antibody titer, T concentration, testis and body weights, and histological analysis of the rat tissue samples revealed no significant differences (non-effective individuals) in these two immunized groups compared with the control group (Table 1). Only one of five rats in weeks 6 and 10 in the GnRH2-MAP group, respectively. In the GnRH2 group, invalid immune rats did not appear two weeks after the second immunization, but this number increased after the third immunization.

Table 1
Non-effective individuals in the GnRH2-MAP- and GnRH2-immunized groups.

Time (wks)	GnRH2-MAP (n = 5)	GnRH2 (n = 5)
2W	0	1
4W	0	0
6W	1	2
8W	0	1
10W	1	1
12W	0	2

The time of the first vaccination was set to 0 weeks. Rats were immunized three times from 0 to 6 weeks with 2-week intervals. There were no significant differences ($P > 0.05$) in the GnRH antibody titer, T concentration, testis volume and histological features in the non-responsive immunization group between these three groups at this time point.

3.2. Serum antibody titer

Fig. 1 shows the serum antibody titer in the immunized, control, and non-effective male rats for 12 weeks after the first immunization. In the immunized groups, the antibody titer began to significantly increase two weeks after the first immunization ($P < 0.05$). It appeared to decrease slightly after the third immunization, after which it peaked at four weeks after the third injection. After the eighth week, the antibody titer in the GnRH2-MAP group first declined then showed a second increase at 12 weeks, but the antibody titer in the GnRH2 group maintained a downward trend.

Overall, the antibody titer was higher in the GnRH2-MAP group than in the GnRH2 group, and the difference was significant ($P < 0.05$) at 2, 4, 8, and 12 weeks after the first immunization. Moreover, there was no significant difference ($P > 0.05$) between the non-effective and control rats, except at the second immunization.

3.3. Serum T concentration

The serum T concentration in the immunized, control, and non-effective rats were determined for 12 weeks after the first immunization (Fig. 2). There were significant differences ($P < 0.05$) from the second immunization onwards between the immunized and control groups, and the GnRH2-MAP group had an obviously lower T concentration than the GnRH2 group. Both immunized groups exhibited the lowest T concentration at week 12. In the GnRH2 group, T concentration was maintained at a low level but showed

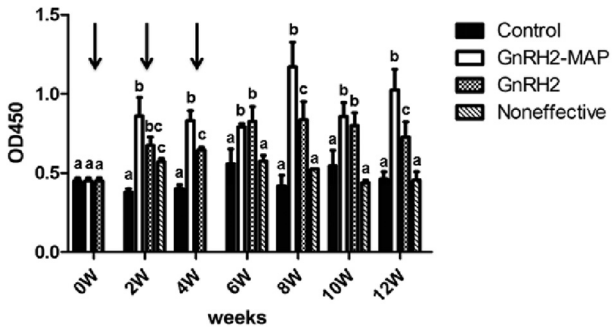


Fig. 1. High antibody titers in the serum of male rats immunized with GnRH2-MAP or GnRH from 0 to 12 weeks with 2-week intervals. Compared with the control group (n = 30), the GnRH vaccinated group (n = 30) showed a significantly high titer ($P < 0.05$) at two weeks after the first immunization, and the antibody titer was higher in the GnRH2-MAP group than in the GnRH2 group. Values are presented as mean ± standard deviation and show the absorbance at 450 nm for 1:500 dilution of sera. Arrows indicate the times of immunization. Significantly different from control * $P < 0.05$; ** $P < 0.01$.

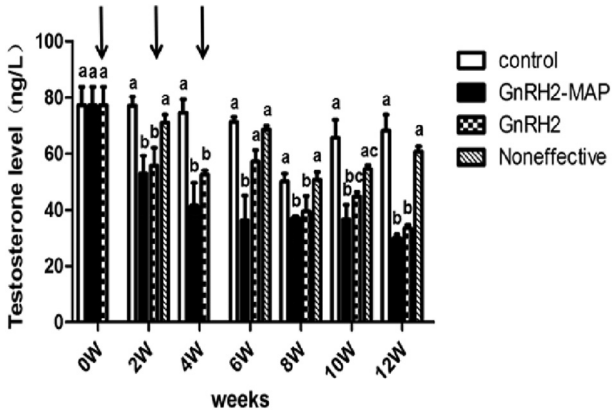


Fig. 2. Observation of the serum testosterone concentration in male SD rats immunized with GnRH2-MAP or GnRH at 2-week time intervals. In comparison with control group, the GnRH2-MAP (n = 30) and GnRH2 (n = 30) groups presented significantly lower testosterone concentrations ($P < 0.05$) from the second week after the first immunization. Values are presented as means ± standard deviation and show the absorbance at 450 nm for 1:500 dilution of sera. Arrows indicated the time of immunization. Significantly different from control * $P < 0.05$; ** $P < 0.01$.

increases at 2 and 6 weeks after the third immunization. At the same time, the T concentration between the immunized groups showed a significant difference ($P < 0.05$). Moreover, there was a slight but not obvious decrease in the non-effective group relative to the control group.

3.4. Body weight

After the first immunization, no significant difference was found in body weight between the GnRH2-MAP group, the GnRH2 group, the non-effective group and the control group ($P > 0.05$) (Table 2), which means that the vaccine does not affect the growth of rats.

3.5. Testicular weight and volume

Testicular weight was determined for 12 weeks from the first immunization (Table 3). The testis weight was significantly lower ($P < 0.05$) in the GnRH2-MAP group than in the control group, and this difference was significant at 4 and 8 weeks. Significant differences between the immunized groups only appeared at 6 and 10 weeks (Table 4). After the first immunization, the significant difference in testicular volume between groups was also reflected by the significant difference in testis weights, which can be seen in Fig. 3.

3.6. Histological observation of the testes

Fig. 4 shows the results of the histological analysis of the right

Table 2
Body weight (g) of male rats immunized with GnRH2-MAP and GnRH2 vaccine compared with the control group.

Time (weeks)	GnRH2-MAP (g)	GnRH2 (g)	Non-effective (g)	Control (g)
0	31.78 ± 0.224	31.78 ± 0.224	–	31.78 ± 0.224
2	118.9 ± 3.1	121.9 ± 5.8	122.5	125.6 ± 5.2
4	171.2 ± 10.6	158.6 ± 13.7	–	186.2 ± 4.1
6	415.5 ± 14.6	398.7 ± 27.1	441.7 ± 35.8	413.2 ± 7.8
8	342.8 ± 16.6	326.4 ± 24.3	356.5	361.4 ± 8.8
10	459.5 ± 22.6	473.1 ± 53.8	535.7 ± 32.7	512.7 ± 23.7
12	489.2 ± 18.5	498.9 ± 32.3	542.5 ± 44.6	510.8 ± 15.8

Table 3
Testicular weight of male rats immunized with the GnRH2-MAP and GnRH2 vaccine compared with the control group.

Time (weeks)	GnRH2-MAP (g)	GnRH2 (g)	Non-effective (g)	Control (g)
0	0.064 ± 0.224^{a}	0.064 ± 0.224^{a}	–	0.064 ± 0.224^{a}
2	0.283 ± 0.027^{b}	0.326 ± 0.073^{bc}	0.496 ± 0.032^{a}	0.43 ± 0.014^{ab}
4	0.766 ± 0.084^{b}	0.706 ± 0.114^{b}	–	1.208 ± 0.047^{a}
6	1.088 ± 0.103^{c}	1.44 ± 0.076^{b}	1.763 ± 0.038^{a}	1.688 ± 0.051^{ab}
8	1.52 ± 0.066^{c}	1.553 ± 0.087^{bc}	1.775^{ab}	1.904 ± 0.082^{a}
10	1.236 ± 0.275^{b}	1.843 ± 0.056^{a}	2.19 ± 0.048^{a}	1.91 ± 0.048^{a}
12	1.366 ± 0.282^{b}	1.698 ± 0.075^{ab}	2.00 ± 0.026^{a}	1.936 ± 0.039^{a}

The time of the first dose of vaccination was set to 0 weeks. Rats were immunized three times from 0 to 6 weeks with 2-week intervals. All data are presented as the mean ± standard deviation values. Different superscript letters in a row indicate significant differences between groups ($P < 0.05$). - indicated no data (because the number of non-effective rats was 0).

Table 4
Left testicular volume (cm^3) of male SD rats immunized with the GnRH2-MAP and GnRH2 vaccines compared with the control group.

Time (weeks)	GnRH2-MAP (n = 5)	GnRH2 (n = 5)	Non-effective (n = 5)	Control (n = 5)
0	0.051 ± 0.007^{a}	0.051 ± 0.007^{a}	–	0.051 ± 0.007^{a}
2	0.216 ± 0.053^{c}	0.237 ± 0.033^{bc}	0.343 ± 0.042^{ab}	0.296 ± 0.034^{ab}
4	0.416 ± 0.068^{b}	0.398 ± 0.066^{b}	–	0.758 ± 0.031^{a}
6	0.705 ± 0.098^{c}	1.14 ± 0.032^{b}	1.278 ± 0.038^{ab}	1.382 ± 0.086^{a}
8	0.887 ± 0.052^{c}	1.080 ± 0.076^{bc}	1.275^{ab}	1.381 ± 0.096^{a}
10	0.863 ± 0.191^{b}	1.401 ± 0.122^{a}	1.560 ± 0.057^{a}	1.513 ± 0.048^{a}
12	0.868 ± 0.163^{b}	1.168 ± 0.075^{ab}	1.383 ± 0.091^{a}	1.344 ± 0.032^{a}

The time of the first dose of vaccination was set to 0 weeks. Rats were immunized three times from 0 to 6 weeks with 2-week intervals. All data are presented as the mean ± standard deviation values. Different superscript letters in a row indicate significant differences between the two groups ($P < 0.05$). - indicated no data available (because the number of non-effective rats was 0).



Fig. 3. Testicular size in the GnRH2-MAP, GnRH2, and control groups. Testicular photographs of the immunized and control groups. Compared to the control group, the testes of rats in the GnRH2-MAP and GnRH2 groups were sharply atrophied, and the degree of atrophy was more significant in the GnRH2-MAP group ($P < 0.05$).

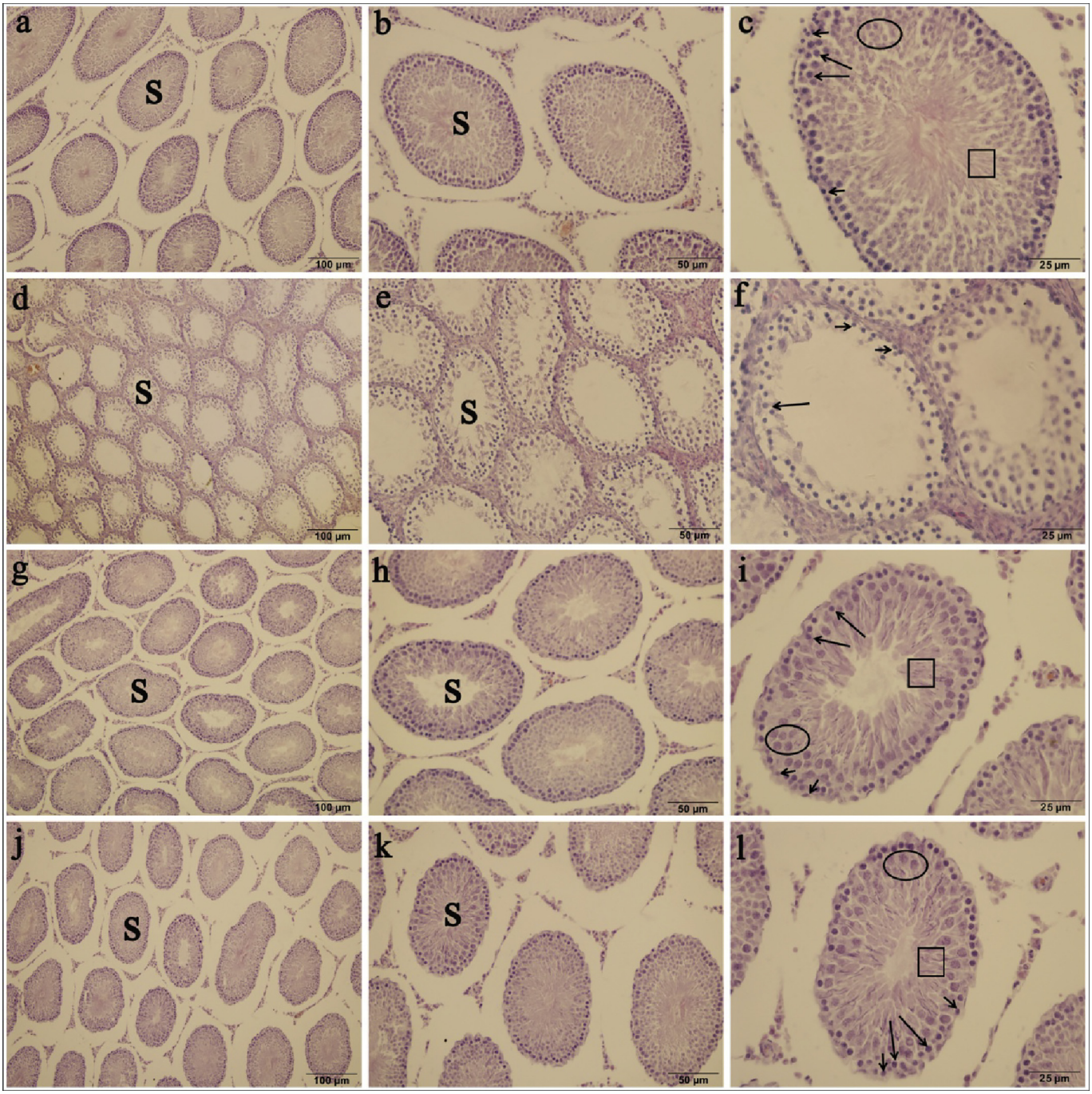


Fig. 4. Microscopic observation of tissue sections in the GnRH2-MAP, GnRH2, and control groups. Testicular tissue of rats in the control group (a, b, c), GnRH2-MAP group (d, e, f), GnRH2 group (g, h, i), and non-effective group (j, k, l). Seminiferous tubules (S) contain spermatogonia (short arrows), primary spermatocytes (long arrows), spermatid (ellipses), and spermatozoa (squares). Note: (a, d, g, and j) scale bar = 100 μm; (b, e, h and k) scale bar = 50 μm; (c, f, I, and l) scale bar = 25 μm. Testicular tissues were stained by hematoxylin and eosin.

testes. The seminiferous tubules in the control group (Fig. 4a, b, and c) were normal and exhibited a large number of spermatozoa in the lumen, with a close arrangement of spermatogonial cells, spermatocytes, and sperm cells. The number of spermatogonia, spermatocytes, and sperm cells in the germinal epithelium was significantly reduced in the GnRH2-MAP group, and there were almost no sperm in the lumen (Fig. 4d, e, and f). In the GnRH2 group, the seminiferous tubules of the testes were normal, with closely arranged spermatogonial cells, spermatocytes, and sperm cells. However, there were relatively fewer spermatozoa in the lumen (Fig. 4g, h, and i). There were no differences in testicular morphology between the non-effective (Fig. 4j, k, and l) and control (Fig. 4a, b, and c) groups; moreover, in the non-effective group, the seminiferous tubules were normal, with closely arranged spermatogonia, spermatocytes, and sperm cells and a large number of sperm in the lumen. Ten seminiferous tubules were randomly selected from each group to analyze the number of spermatogonia, primary spermatocytes, and spermatozoa. We found that the spermatogonia, primary spermatocytes, and sperm cells were significantly lower in the GnRH2-MAP and GnRH2 groups than in

Table 5
Number of spermatogonia, primary spermatocytes, sperm cells and spermatozoa in testicular seminiferous tubules in the GnRH2-MAP, GnRH, and control groups.

Group	GnRH2-MAP (n = 5)	GnRH2 (n = 5)	Non-effective (n = 5)	Control (n = 5)
Spermatogonia	54.30 ± 6.077^{a}	41.444 ± 6.333^{a}	88.80 ± 3.841^{b}	79.70 ± 2.951^{b}
Primary spermatocytes	57.90 ± 3.737^{a}	63.888 ± 6.886^{a}	88.10 ± 1.251^{b}	84.00 ± 2.304^{b}
Sperm cells	86.70 ± 3.422^{a}	88.90 ± 5.20^{a}	223.70 ± 15.56^{b}	207.70 ± 11.65^{b}
Sperm	0.00^{a}	31.698 ± 5.075^{b}	137.50 ± 5.335^{c}	122.00 ± 4.784^{c}

Rats were treated thrice from 0 to 6 weeks with 2-week intervals. All data are presented as the means ± standard deviation. Different superscript letters in the row indicate significant differences between the two groups ($P < 0.05$).

the control group ($P < 0.05$). No spermatozoa were present in the seminiferous tubules of GnRH2-MAP group, and there were significantly fewer spermatozoa in the GnRH2 group than in the control group ($P < 0.05$). There were no significant differences in the numbers of spermatozoa between the non-effective and control groups ($P > 0.05$; Table 5)

4. Discussion

The most commonly used immunocastration vaccine is the GnRH vaccine, which contains synthetic peptides. However, the restrictions on the commercial promotion of this vaccine could be attributed to its low binding rate, low purity, and the differences in quality between batches, which could not ensure a consistent castration effect [22]. Moreover, while some current vaccines are effective in some animals, the higher cost associated with these vaccines is one of the biggest factors limiting vaccine development [8]. Here, we aimed to overcome some of these shortcomings using GnRH2 linked to MAP, i.e., GnRH2-MAP. Three aspects of this novel vaccine enhance immunogencity. One, GnRH2 is synthesized by two GnRH monomers whose structure is modified by replacing the sixth position of Gly with D-type Lys [20]. Second, the amino terminal of one GnRH monomer is linked to the carboxyl terminal of another monomer, which has been reported to be an effective method to enhance vaccine immunogenicity [6]. Third, MAP has eight branches with a Lys core and four Lys terminals. Based on the special structure of MAP, peptides or proteins can be randomly linked to it to increase the molecular weight of the antigen. Increasing the molecular weight of the antigen is well known to induce a higher immunogencity. In our study, we found that the binding rate and the novel antigen purity were higher than those of the former vaccine; this could be attributed to the homology of MAP and endogenous GnRH [18]. Comprehensive analysis of antibody concentration, testosterone concentration, testicular parameters, and histological features revealed that the GnRH2-MAP vaccine may be an effective alternative vaccine for use in immunocastration. Individual differences following administration of the immunocastration vaccine is observed in most studies. Although we ensured consistency in breed, age, weight, genetic background, and health status; immunization program; and feeding environment, some experimental differences were shown significantly in some of these animals [23]. In our study, the testes of two rats in the GnRH2-MAP group developed normally, and their serum antibody and T levels did not differ from those in the control group; similarly, 9 such cases appeared in the GnRH2 group. After GnRH active immunization, the immune “escape” phenomenon was observed in rats [39], pigs [40] and some other animals. The testosterone concentration and testicle size decreased only slightly after immunization with GnRH, but the values were not significantly different from those of the control group. Some studies suggest that the weaker antibody response after GnRH immunization is responsible for the “escape reaction” in animals. Because of the effective antibody response after active GnRH immunization, the anti-GnRH antibodies produced are the key to successful castration. In the present study, testosterone decline occurred after active immunization in the GnRH2-MAP group, while the testosterone level in non-responsive rats was not significantly changed. Robbins et al. [41] immunized cats with a recombinant fusion protein of GnRH and found that the testosterone level in immunized cats was maintained at the same level as in the surgically castrated group when the serum GnRH antibody titers reached the threshold of 30% of the binding rate. This suggests that the serum anti-GnRH antibody level after GnRH active immunization needs to reach a certain threshold to inhibit reproductive function in animals. It is also possible that a small amount of gonadal hormones still exist in GnRH2-MAP rats. In addition, it may also be related to individual differences between rats with regard to the absorption and distribution of the vaccine. Thus, further studies are required to explore the cause of these individual differences.

In our study, GnRH2-MAP induced high anti-GnRH antibody titers in male rats from the first immunization to week 12, and most related studies have consistent results about antibody titer. Our study showed that the antibody peak appeared four weeks after the third immunization, but Balet et al. [24] found that the peak appeared two weeks after the second immunization in dairy cattle, which is consistent with the result of Janett et al. in bulls [25]. This difference may be because antigen sensitivity differs in different species or animals [26,27]. Moreover, different type of antigens are used in experiments, and there is a natural difference in the degree and effect of the immune antibody response [28,29]. The antibody concentration was maintained at a high concentration after the second dose of immunization until the end of the 12-week experiment. Similarly, the GnRH2 group also exhibited similar results, but the antibody titer of the novel vaccine was lower than that of the GnRH2 vaccine, causing its weak immunogenicity. Moreover, factors underlying the maintenance of a high antibody response time also need further study.

The reduction in the serum T concentration of immunized male SD rats reflects inhibition of testicular function. This result is also discussed in previous papers [1,30]. In this experiment, we found that the serum T concentration decreased again at 12 weeks after maintaining a low level after the third immunization, which was consistent with the secondary increase in the antibody concentrations at 12 weeks. We speculated that antigen was released slowly, causing enfolded by the oil adjuvant, which increased the amount of antigen release after three immunizations. When the antigen enters body several times, the corresponding antigen levels in the body will rise, resulting in the simultaneous production of a large number of memory cells. Furthermore, the serum T concentration decreased significantly after the first immunization, and maintained a low stable level after the third injection. This may be due to the decrease in the LH and FSH levels in the pituitary. Without stimulation from LH and FSH, testicular degradation occurs due to the low concentrations of T secreted by Leydig cells, and it may partly disrupt testicular secretion [25,31]. According to a recent study, in bulls immunized with Improvac W at two weeks

after the second immunization, the serum T concentration reached a low level at 22 weeks, which was maintained [25]. This result was further demonstrated in the same year, when Bopriva-immunized bulls also produced a T minimum peak two weeks after the second immunization [32]. In contrast, our T peak appeared in the last week, which may be due to species differences [23,33].

Active immunization using a GnRH vaccine causes the testes to shrink, indicating the castration effect of this antigen. Studies have indicated that the thickness of the spermatogenic epithelium, the cytoplasmic content of testicular stromal cells, and testicular weight were significantly related [34]. Previous studies have found a close association between testis weight and serum T concentration [35]. Contrary to these reports, we found that the T concentration decreased at 12 weeks after the first immunization, but testicular weight increased. Therefore, the relationship between serum T concentration and testicular weight seems to be unclear, and the differences between studies may be attributable to differences in species, immunogenicity of the vaccine, or other reasons. Our study suggested that testicular weight and volume changes are consistent, which was in accordance with previous study findings [36]. Our result showed that GnRH2-MAP caused testicular atrophy. The volume reduction is natural, and is based on the temporary reduction or disappearance of testicular materials. The phenomenon of testicular function returning to normal was detected in previous studies [34,35,37].

Histological observation of the testis revealed that sperm production was significantly affected. In the later period following immunization, no sperm could be detected in the lumens of seminiferous tubules. Although spermatogonial cells could be detected, they presented an irregular arrangement. Moreover, spermatids and sperms present in the lumen of seminiferous tubules were almost undetectable. This finding was consistent with the testis weight, as the reduced volume of the testis may be the primary reason for this observed decline in the number of sperm cells and spermatozoa. Similarly, a previous study in dogs administered GnRH-I also reported that spermatogenesis was inhibited, with no sperm detected in the epididymis [38].

In this study, we administered our novel vaccine GnRH2-MAP to male SD rats and studied its effects. However, due to several volatile factors such as species, vaccine dose, time intervals between vaccine doses, and types and doses of adjuvants used, further studies are required to determine the actual castration effect of this vaccine in rats.

5. Conclusion

In conclusion, active immunization usingGnRH2-MAP elicited a significantly high antibody titer, which interfered with the HPG axis, thereby inhibiting testicular function. This finding suggested that GnRH2-MAP can induce a significant castration effect. Therefore, GnRH2-MAP may be an alternative and commercial castration vaccine in animals. However, future experiments are warranted to identify the best dose, procedure of immunization, and the adjuvant types.

Declaration of interest

The authors declare that there is no conflict of interest.

The time of the first dose of vaccination was set to 0 weeks. Rats were immunized three times from 0 to 6 weeks with 2-week intervals. All data are presented as the mean ± standard deviation values. No significant differences were found between the groups ($P > 0.05$). - indicated no data available (because the number of non-effective rats was 0).

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WILDLIFE FERTILITY CONTROL: FACT & FANCY



A Publication of the Science and Conservation Center at ZooMontana

INTRODUCTION

One of the major efforts of the Science and Conservation Center at ZooMontana involves the humane control of wildlife populations by means of fertility control. To that end, the Science and Conservation Center was created in 1998, an independent non-profit organization that is the world's only dedicated facility for the development of wildlife contraceptives and methods of application. This Center produces and carries out quality control for a wildlife contraceptive vaccine, distributes the vaccine and is the repository for all records and data required by the Food and Drug Administration and Environmental Protection Agency. Additionally, the Center and its staff coordinate and in some cases carry out field-based application of contraception to wildlife populations.

The Center receives many inquiries from around the world regarding wildlife contraception, an arcane subject that can be complicated and confusing. These inquiries center about many different species, diverse challenges, and a variety of settings, and those inquiring have a wide breadth of understanding about the subject - from very little to quite a lot. To complicate matters, there is a great deal of controversy regarding this subject, some based in misunderstanding of facts and some based in social, cultural, political or economic objections to this approach to wildlife management. To facilitate the educational process, The Science and Conservation Center, has published this overview, with the intent of providing a realistic picture of the state of the art of wildlife fertility control. To be sure, the complexity of the subject greatly exceeds the possibility of a comprehensive discussion here, but this publication can acquaint the reader with the basics necessary to develop a realistic picture, and the bibliography can lead the interested reader to a wealth of more detailed information.



The Science and Conservation Center at ZooMontana is where contraceptive vaccine is produced.

WHY FERTILITY CONTROL?

At earlier times in history wildlife populations were controlled by two natural processes. These included mortality control and fertility control. When animal populations exceed the carrying capacity of their environment, animals die from starvation and disease as well as predation. At the same time, high densities among wildlife populations lead to a decrease in reproductive success; animals delay the age at which they will first breed, they produce fewer offspring, and juvenile mortality rates increase.

Urbanization and modern agricultural development led to the reduction of predators, and regulated hunting and trapping soon replaced predators as population control devices. Dwindling wildlife resources and loss of habitat led to the creation of reserves, parks, and special legislation that protect certain species from traditional lethal controls. Examples might include wild horses protected by the Wild Free-Roaming Horse and Burro Act or elk, or elephants living in national parks where large predators no longer exist, or even zoological gardens, where unregulated reproduction can lead to "surplus" animals and massive ethical problems associated with the disposition of these excess animals.

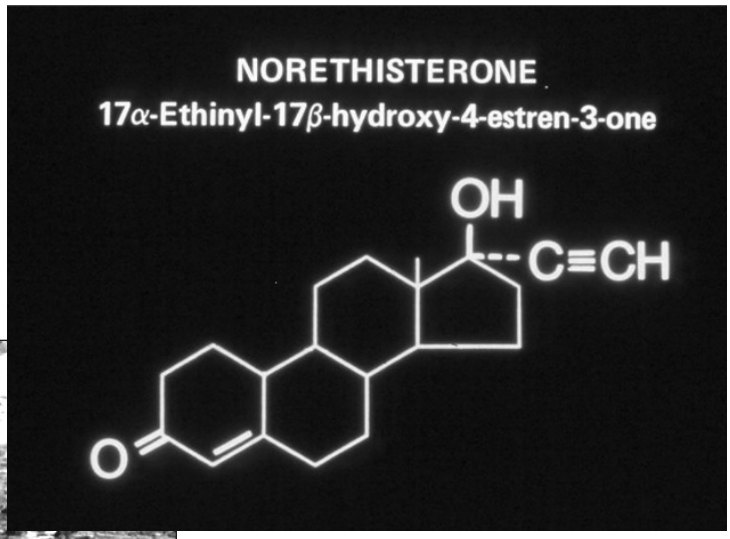
Humans have chosen to impose artificial human-induced mortality control on wild populations, through regulated hunting, trapping, and poisoning, and this is accepted as "normal" human activity. In many areas of the world, and with many species, traditional human-induced mortality control will continue to be the primary management tool. In recent history, however, increasing urbanization, the withdrawal of private lands from the public hunting domain, regulatory prohibitions on the use of poisons, legislation against trapping, low fur prices and, most important, changing public attitudes about lethal wildlife control methods have reduced the effectiveness of human-induced mortality control as a management tool for many species. Thus, we now face exploding populations of some adaptable or highly protected species but without adequate management tools with which to protect environment and animals alike.

The use of human-imposed fertility control however, is still often viewed as "bizarre" or "unnatural", and the reasons for this view are not understood. It may have something to do with the simplicity or relatively lower cost of mortality control, or something as simple as a cultural mindset. Regardless of the answer to this question, we are rapidly facing a point in time when safe, humane and publicly acceptable wildlife management paradigms are beginning to replace lethal methods. The public demands it and the animals we have displaced deserve it.

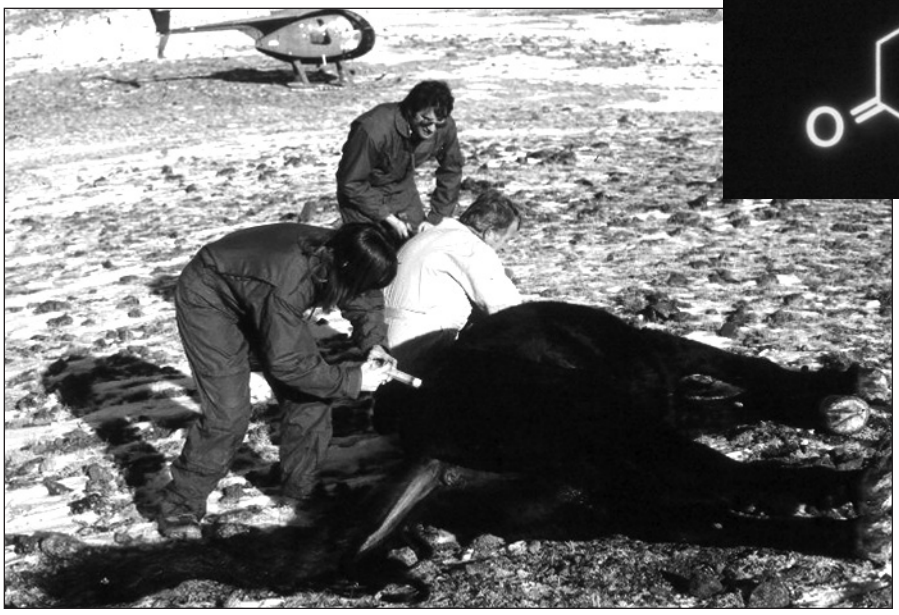
The scientific community is also responding to this need, and thus far, six international conferences on wildlife fertility control have been held around the world, and public acceptance has followed suit.

A BRIEF HISTORY OF WILDLIFE FERTILITY CONTROL

The concept of wildlife fertility control is not new and research has been carried out for many years. Most of this work was based on the fruits of human contraceptive research and therefore mirrored human contraceptive technologies. This in turn meant that until recently, the most common approach to wildlife contraception was through the use of steroid hormones, and particularly natural and synthetic estrogens, progestins, and androgens, similar to those found or used in human contraception. Zoos really led the way, administering these compounds to captive animals, where delivery was not an issue, but their use in free-roaming wildlife was another matter. These compounds often worked in a pharmacological sense but they fell far short of the standards that permit them to be used with free-roaming wildlife. Basically, they failed because (1) they had to be given in extremely large quantities, ruling out remote delivery, (2) they had to be administered too often, (3) they caused a variety of pathologies in treated animals, (4) the cost was relatively high, (5) they often had profound effects upon social behaviors, (6) they were often unsafe to administer to pregnant animals, and (7) they passed through the food chain to predators - human and otherwise - and scavengers. Because of these shortcomings, there was little hope that they would ever be publicly acceptable for use in free-roaming wildlife by regulatory agencies. For more extensive reading on the history of wildlife contraception we suggest Kirkpatrick and Turner 1991, (referenced in the Bibliography).



Steroids, similar to Northisterone, often change behaviors and they can pass through the food chain.



Steroid hormones have to be given in such large amounts that the animals hae to undergo stressful capture procedures, such as this wild stallion receiving a hormone injection.

CHARACTERISTICS OF THE IDEAL WILDLIFE CONTRACEPTIVE

The failure to achieve practical results with steroid hormones led to a re-examination of the problem. It became apparent by the early 1990s that research was proceeding without a "gold standard" by which to evaluate each new approach. Without such a standard evaluation of potential wildlife fertility control methods could be likened to a discussion of law without a constitution. Thus, a theoretical "standard" was created and included:

1. Contraceptive effectiveness of at least 90%.
2. The ability for remote delivery, with no handling of animals.
3. Reversibility of contraceptive effects (more important for some species than others).
4. Safety for use in pregnant animals.
5. Absence of significant health side effects, short- or long-term.
6. No passage of the contraceptive agent through the food chain.
7. Minimal effects upon individual and social behaviors.
8. Low cost.

While some of these standards are more or less arbitrary, they at least provided reasonable guidelines and any future discussions of new wildlife contraceptive agents or discussions of current agents should attend to these characteristics (see Kirkpatrick and Turner 1991).

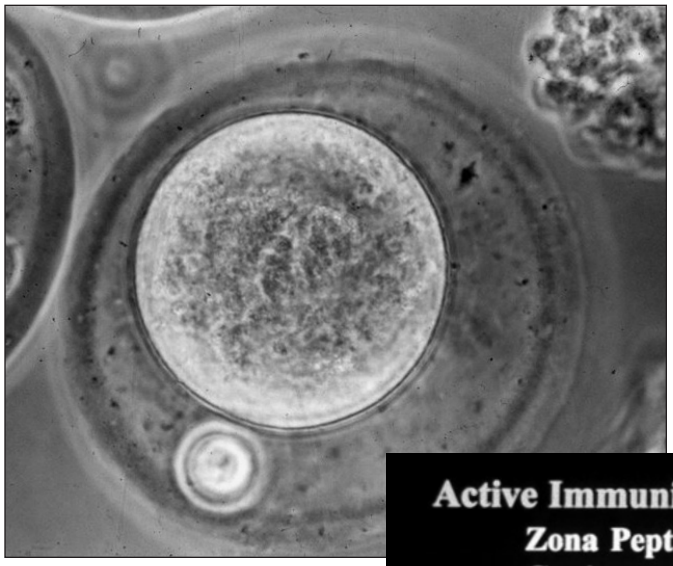
IMMUNOCONTRACEPTION

In 1988, a new approach to wildlife contraception was launched. Immunocontraception is based on the same principles as disease prevention, through vaccination. We vaccinate ourselves and other animals against diseases by injecting dead or attenuated (weakened) disease bacteria or viruses, or with molecules that are harmless but similar to the toxins that these disease organisms produce. Our immune systems produce antibodies that attack the material we injected and any similar organism or their toxins in our systems. An immunocontraceptive vaccine works in much the same way, only it causes the production of antibodies against some essential event or structure in the reproductive process. There are a variety of immunocontraceptive vaccines under development or already registered, including vaccines against brain reproductive hormones such as gonadotropin-releasing hormone (GnRH) and vaccines against the sperm and the egg, which in turn prevent fertilization. The advantages of vaccines are that they can be delivered remotely in very small doses, and because they are primarily protein, they are readily destroyed in digestion and cannot pass through the food chain. The vaccine which thus far has had the largest application to wildlife is the Porcine Zona Pellucida, or PZP vaccine.

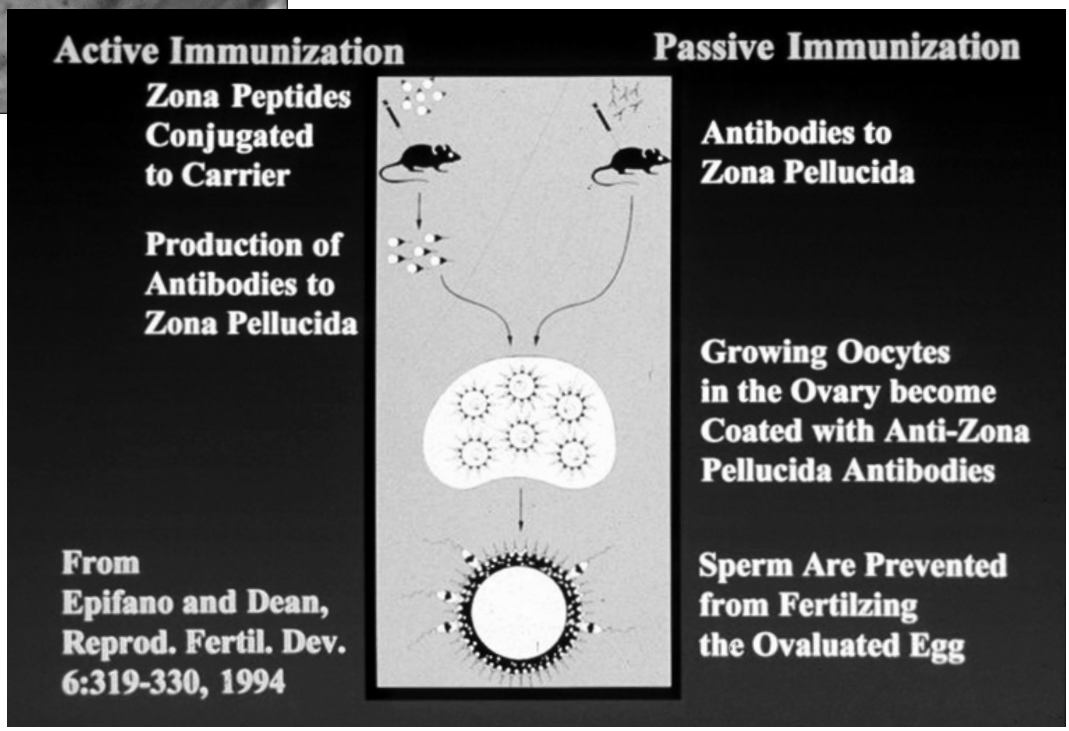
PORCINE ZONA PELLUCIDA VACCINE

What is PZP and How Does It Work?: A non-cellular membrane known as the zona pellucida (ZP) surrounds all mammalian eggs. The ZP consists of several glycoproteins (proteins with some carbohydrate attached), one of which, ZP3, is thought to be the sperm receptor (the molecule which permits attachment of the sperm to the egg during the process of fertilization). The PZP vaccine is derived from the ZP protein from pig eggs. When this vaccine is injected into the muscle of the target female animal, it stimulates her immune system to produce antibodies against the vaccine. These antibodies also attach to the sperm receptors on the ZP of the female's own eggs and distort their shape, thereby blocking fertilization (see Paterson and Aitkin 1990).

Thus far PZP has been a successful form of contraception in wildlife because (1) it has prevented pregnancy an average of 90% or greater of the time in treated animals, (2) it can be delivered remotely by small darts, (3) the contraceptive effects are reversible, (4) it is effective across many species, (5) there are no debilitating health side-effects even after long-term use, (6) it has almost no effects on social behaviors, (7) the vaccine cannot pass through the food chain, and (8) it is safe to give to pregnant animals (see Kirkpatrick et al. 2009).



A mammalian egg showing the dark non-cellular zona pelucida membrane, which is the site of sperm attachment.



The mechanism of action of the PSP contraceptive vaccine. The mechanism on the left-active immunization is the precise mechanism used in wildlife contraception.

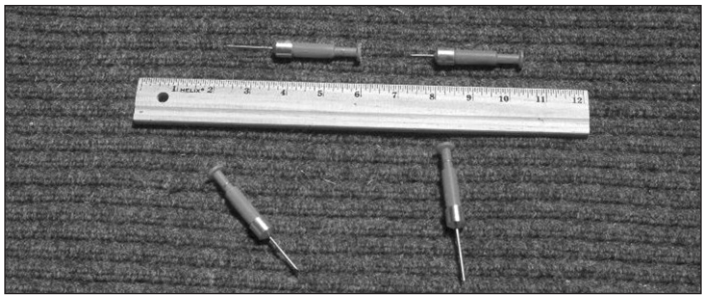
How is the Vaccine Delivered?: The PZP vaccine must be injected into the muscle of the target animal. This can be done by hand if the animal is restrained, or by dart, for remote delivery. There are many commercial dart systems available but the thick viscosity of the vaccine requires a large needle and a quick injection. Thus far, Pneu-Dart® systems (Williamsport, PA) work the best. The Pneu-Dart® 1.0 cc barbless darts can be fired from Pneu-Dart® capture guns or from several other commercial dart guns (Dan-Inject®, for example). The darts are disposable, and after hitting the animal in the rump or hip (the only acceptable location for darting) they inject by means of a small powder charge and pop out. Because of their bright colors the darts are usually retrieved in the field. Unrecovered darts cannot be discharged by stepping on them nor by any other kind of casual contact. Over a 20 year period on Fire Island, Assateague Island, and Cape Lookout National Seashores, and more than 2,000 treatments of deer and horses, 98% of darts have been recovered.

Normally, each animal is darted twice the first year, with the first injection being given up to a year before a booster, just preceding the breeding season (March for wild horses or September for deer). Thereafter, a single annual booster inoculation will maintain contraception (Kirkpatrick et al. 1991). The second inoculation of the first year requires (1) that you are able to recognize the individual animals, or (2) that you do the first inoculation with a special "marker dart" which leaves a dye mark on the animal at the same time it injects the vaccine, or (3) that animals are treated opportunistically and randomly, with the hope of eventually treating a large proportion of the total population over the course of several years.



An alternative strategy is to give only a single inoculation the first year, from which there will be little contraception, and then a single annual inoculation thereafter, from which there will be significant contraception (see McShea et al. 1997).

Different dart delivery systems for the PZP contraceptive vaccine. From top to bottom: Pneu-Dart® X-Caliber CO_2 Rifle, Dan-Inject CO_2 Rifle, Pneu-Dart® Cartridge Rifle, Dan-Inject® Pistol Grip Blowgun.



Pneu-Dart® disposable self-injecting darts of the type used for delivering the PZP vaccine.

APPLICATION OF PZP TO WILDLIFE

Wild Horses: The vaccine has been used successfully to manage the wild horse population of Assateague Island National Seashore under the authority of the National Park Service (NPS). This population has been treated since 1988 without health problems, and the population has decreased more than 30% since management-level application began in 1995. Wild horses are also being treated on Cape Lookout National Seashore for the NPS, on Carrot Island, for the Rachel Carson National Estuarine Reserve, and on Corolla Island, both in NC, the Pryor Mountain and Little Book Cliff National Wild Horse Ranges, MT and CO, respectively, the McCullough Peaks wild horse range in WY, on many areas of Nevada, Utah, Oregon for the Bureau of Land Management (BLM), on the Carson National Forest, for the National Forest Service, on several wild horse sanctuaries, and on the Navaho and Pima/Maricopa Indian Reservations in NM and AZ. In addition to controlling the horse population on Assateague Island, treatment has extended the lives and improved the health condition of older mares, by removing the stresses of pregnancy and lactation (see Kirkpatrick and Turner 2002; Kirkpatrick et al. 1990, 1991; Liu et al. 1989; Turner and Kirkpatrick 2002; 2003, 2007, 2008).

Zoo Animals: In order to prevent the production of "surplus" animals, zoo animals in more than 120 zoos have been successfully treated with PZP. Thus far PZP has shown contraceptive effects in more than 80 species. In all probability, PZP will prove to be a successful contraceptive for all ungulates (hoofed animals), but it also works in bears, pinnipeds (seals and sea lions) and elephants. We do note differences between species in the timing of the booster inoculations. Porcine Zona Pellucida vaccine is now a standard recommendation for many species by the Contraceptive Advisory Group of the Association of Zoos and Aquariums (AZA). The obvious advantage of the PZP vaccine for zoo animals is that it can be administered without the stresses of restraint (see Kirkpatrick et al. 1992b, 1995b; Deigert et al. 2003; Frank et al. 2005; Asa and Porton 2005).

Deer: The PZP vaccine was shown to block pregnancies in captive white-tailed deer as early as 1990. Since that time, numerous projects have been mounted with free-roaming deer in MD, NJ, NY, CT, WA, VA and OH. The two largest projects are on Fire Island National Seashore (FINS), NY, where approximately 215 deer are treated annually since 1993, and on the campus of the National Institute of Standards and Technology (NIST), in MD, where more than 150 deer have been treated since 1996. In both these programs the deer population has been decreased significantly. The studies and projects thus far have shown that contraception with PZP will often extend the breeding season of the female by one to two months, but that the energy cost is far less than that of getting pregnant and nursing. Although they will often follow the treated female deer, males do not carry on fights or expend additional energy beyond the normal breeding season (see McShea et al. 1997; Naugle et al. 2002; Turner et al. 1992, 1995; Rutberg and Naugle 2008).

African Elephants: In order to eliminate the need to legally kill elephants in African national parks to prevent rapid population increases, the PZP vaccine has been tested in African elephants in The Kruger National Park in South Africa. The vaccine was very successful and the results paralleled those of horse contraception. One of the most important findings was that the vaccine caused no changes in the social behaviors of family groups or harassment of cycling females by bulls. As a result of the Kruger Park studies, at least eleven game parks throughout South Africa are now using PZP to control elephant populations, rather than culling them. Growth rates have been reduced to almost zero. Demand for vaccine for elephant contraception grew so rapidly, that in 2003 the SCC trained an African team to make the vaccine in South Africa (Fayrer-Hosken et al. 2001; Delsink 2006, 2007).

Other Species: The PZP vaccine has also been used successfully in feral water buffalo inhabiting the U.S. Naval Base in Guam, feral burros in Virgin Islands National Park (Turner et al. 1996), Elk on Point Reyes National Seashore (CA) (Shideler et al. 2002), and more recently, bison on Catalina Island (CA).

Training: The SCC conducts training programs for personnel from NGOs and government agencies, for the proper use of PZP in wildlife. Examples of agencies participating in these training programs include the Bureau of Land Management, Biological Resources Division of USGA, US Fish and Wildlife Service, U.S. Navy, and the National Park Service.



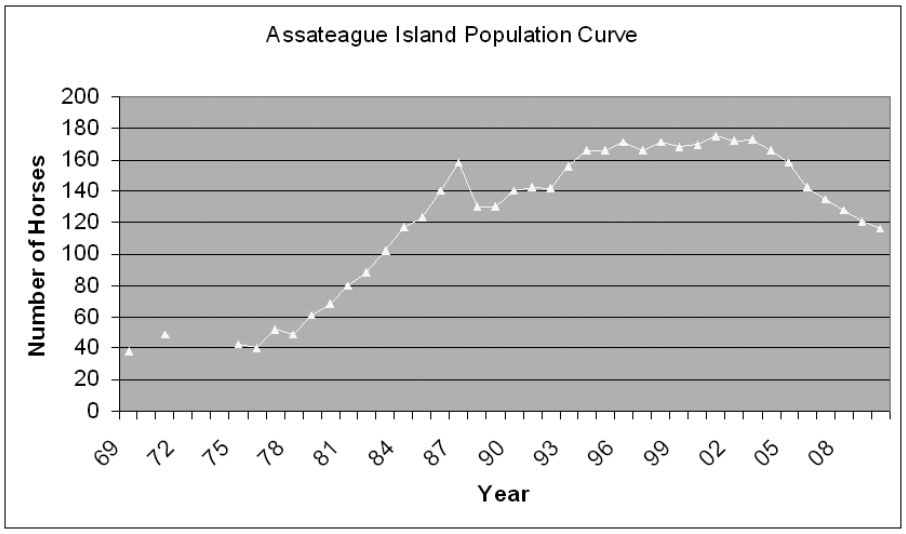
An African Elephant being darted with PZP Vaccine

REGULATORY ISSUES

One of the most poorly understood dimensions of wildlife contraception is the regulatory process. Depending upon the species in question, one or more agencies, local, state and federal may be involved, and the agencies involved have changed over time. A brief description of the process and regulations follows:

Federal Regulation. Since the mid-1990's, all SCC projects in which the vaccine crossed state lines have been reported to the U. S. Food and Drug Administration (FDA). These projects have been carried out under the authority of two separate Investigational New Animal Drug documents (INADs) issued by the FDA to The Humane Society of the United States (HSUS). One of these INAD's covers wild horses, the other covers deer and zoo animals. For each project, the SCC files a form with the FDA specifying how much vaccine is being shipped and what species are to be treated. The INAD also requires that a detailed research protocol be filed for each project, data from each project be gathered in a systematic way and filed, and data made available to the FDA when the need arises. These files are maintained at the Center.

In about 2005, however, FDA began to transfer authority over most applications of wildlife contraceptives (other than zoo animals, which remain with FDA) to the Environmental Protection Agency (EPA) under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). This includes wild horses, deer, geese, and other wildlife that is "capable of doing environmental damage." The EPA approval process follows that agency's registration requirements for pesticides. (The language of "environmental damage" and



Changes in Assateague Island wild horse population as a result of immunocontraception. (Management begins in 1995.)

"pesticide" is in many ways regrettable, but is forced by the language of FIFRA.) At this writing, EPA has registered two wildlife contraceptives, OvoControl® (a nicarbazine-based oral contraceptive for geese and pigeons) and GonaCon® (a GnRH-based injectable immunocontraceptive for deer). HSUS has also applied to EPA for registration of PZP for use on wild horses.

Under EPA regulations, users of registered contraceptives must follow the directions on the label, which may include requirements for certification of applicators, target species, and methods, times, and place of application. Unlike FDA-approved drugs, which may be prescribed by physicians or veterinarians for purposes other than those described on the label, there is no such thing as "off-label use" for EPA-registered contraceptives. In addition, an EPA-registered contraceptive must generally be registered in the states in which it is being applied.

Aside from meeting FDA requirements, the Animal Care Committee of each zoo must also provide permission to treat zoo animals. The federal Animal Welfare Act also requires that experimental applications of PZP to free-ranging wildlife be reviewed and authorized by the Institutional Animal Care and Use Committee of a university or responsible agency.

Wild Horses. Contraceptives can only be applied with the permission of and/or under the supervision of the legal owners or managers of the horses (including government agencies such as the NPS, BLM, or USFS, or sanctuary owners). As each new project is identified, The HSUS reviews the need for the project in the context of scientific and ethical issues and, if approved, issues permission to proceed. Applications of PZP to wild horses by federal agencies also require compliance with the National Environmental Policy Act (NEPA), which generally entails preparation of an Environmental Assessment (EA).

Deer. The first step in deer contraception is for the affected landowners and local legal authority (city, township, county, park board or some combination thereof) to agree to carry out a deer project. The second step is a review by The HSUS, to determine the scientific and ethical dimensions of the project (do these deer really need more management?). The third step is the preparation of a proposal to the state fish and wildlife agency, regardless of whether the deer in question reside on public or private property. The principal exception to state authority is wildlife residing on federal lands, which are not subject to state authority. However, federal managers generally try to be responsive to the concerns of state wildlife agencies.



If the contraceptive is already registered with the EPA and the state, no further permissions are needed. If not, the project should be carried out under an FDA INAD or under an EPA experimental use permit. Like the INAD, the EPA's experimental use permit is granted for use at specific sites under specific conditions.

An Assateague mare being targeted for a PZP inoculation. Effective ranges are up to 50 meters.

In addition to the regulations regarding the vaccine, the use of delivery equipment such as capture guns often requires additional state and local authorization and/or training. For example, the use of capture gun equipment within an NPS unit, even to deliver contraceptive drugs, requires special NPS certification. This includes documentation of prior experience with the delivery equipment, a certificate of completion of an NPS sponsored wildlife immobilization course or its equivalent, current CPR certification, passage of a qualifying test on the range, and a letter of certification from a superintendent of an NPS unit. Those contemplating projects with deer should also be sensitive to state laws and local ordinances regarding the discharge of firearms, which may apply to delivery equipment in some jurisdictions.

HOW MANY ANIMALS HAVE TO BE TREATED?

This is a very important and often-asked question, but usually it is asked without much understanding. The number of animals or percent of an animal population that needs to be treated is dependent upon many factors and there is no simple answer. For example, what is the goal? Is it the absence of all animals (impossible with contraception), or a 20% reduction or a 50% slowing of the growth rate or zero population growth? Even after the goal has been identified, data required to answer this question include at very least the reproductive rate, sex ratio, mortality rate, and immigration and emigration rate of the population. Equally important, these data on reproduction and mortality must be site-specific to the particular herd in question and not generalized data or data derived from other locations and populations. One of the most serious deficiencies noted at discussions of new projects is the lack of sound biological data and vague or no goals (Kirkpatrick and Turner 2008; Ballou et al. 2008).

Another consideration is the length of time necessary to achieve population goals. Contraception is not a good way to reduce population numbers rapidly. It takes time for animals to die off and, as seen earlier, one of the results of wildlife contraception may be increased longevity among treated animals. Wildlife contraception must be viewed as a long-term commitment. Also, humans should consider that most wildlife overpopulation problems were created over many years and that quick solutions are not the result of rational thought or fair to the wildlife.



A dart with a cut-away needle showing the small pellets containing PZP. This approach can deliver a year's worth of contraception with a single inoculation.

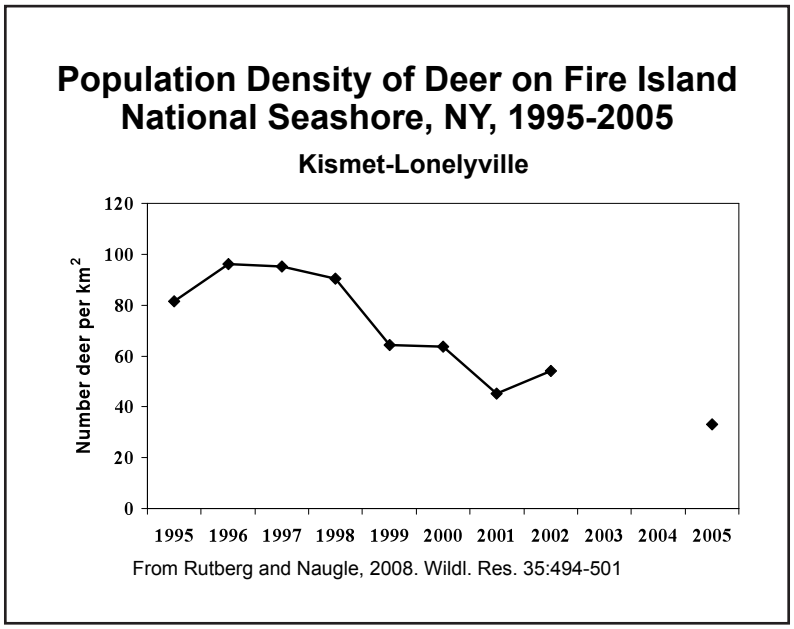
DIRECTIONS FOR FUTURE RESEARCH

Several areas of active research are underway by the PZP Wildlife Contraceptive Group, and these areas of research reflect current shortcomings of the PZP vaccine. These current research endeavors include the following:

A One-Inoculation Vaccine: Because of the need to inoculate animals twice the first year, and the difficulty of doing this with wild species, research is proceeding toward a "one-inoculation" vaccine. Such a vaccine would permit a single darting to cause one or more years of contraception. The approach under study incorporates the PZP into a non-toxic, biodegradable material, which can be formed into small pellets. The pellets can be designed to release the vaccine at predetermined times after injection (at one, three and nine months currently) much the way time-release cold pills work. Trials thus far have been encouraging and continued trials are underway (Turner et al.2001, 2008). Additional research is being conducted with a biodegradable polymer gel, in collaboration with the U. of Tennessee.

Pellet-delivery Darts: There is a need for darts that can deliver the long-acting pellets remotely. Current collaborative research with at least two proprietary companies are directed at developing a reliable dart for remote pellet delivery.

A Genetically Engineered or A Synthetic ZP Vaccine: The ability to produce large quantities of the PZP vaccine at low cost will permit the increased use of this vaccine in wildlife. Currently collaborative work with the Indian National Institute of Immunology is testing a recombinant form of PZP that might be useful as a booster inoculation. Native PZP will remain the only useful "primer" inoculation.



The decline in deer population on Fire Island National Seashore brought about by PZP contraception over several years.

Oral Contraceptives: It is intuitive that the ability to deliver contraceptives to wildlife in baits would be easier and more cost effective. However, for safety and ethical reasons, both the public and regulatory agencies are likely to demand that any oral contraceptives must be species-specific (that is, it must work in only one species for which it was intended). This will be very difficult with the PZP vaccine because of its protein nature.

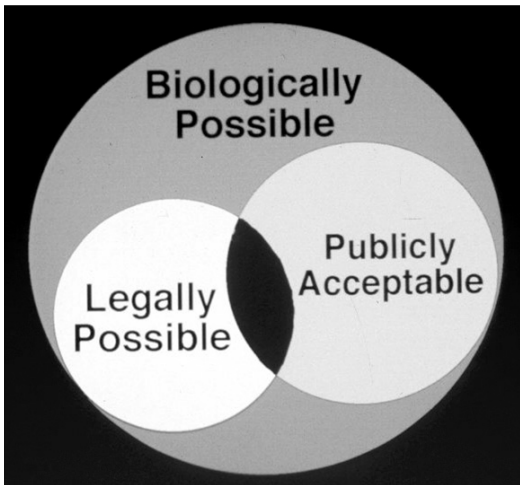
Biologically Modified-Vectored Contraceptives: Researchers working with the Australian government have sought to engineer the genes for PZP and similar contraceptive molecules into non-pathogenic viruses. These viruses could then be transmitted from animal to animal in wild populations. While there are some attractive features to this approach, the safety, environmental, and ethical issues associated with this technology has raised strong objections from the public and regulatory agencies in the U.S. and abroad, and it probably will never be used here.

Abortifacients: Research into compounds which will cause abortion in the recipient animals has largely been abandoned. This has already been shown to be feasible in deer, but most researchers are not pursuing this approach because of the social objections that attend this method of wildlife control.

ETHICAL AND SOCIAL ISSUES

The entire subject of wildlife contraception is attended by a variety of strongly held attitudes both favoring and objecting to this approach. Public discussions are seldom rational. For example, despite the obvious limitations to the available technology some advocates will make loud and unrealistic claims that wildlife contraception can one day completely replace public hunting. On the other side, and despite evidence to the contrary, opponents will express fears of harm after eating treated animals, or will object on the basis of high cost, or will insist that a problem that took 20 years to develop be solved in one year. Anyone seriously considering involving themselves or their community in wildlife contraception should first read Kirkpatrick and Turner 1995, 1997. At very least, do not expect unemotional and dispassionate discussions to occur when this topic is broached.

The most serious ethical considerations are when to manage and why, and the nature of the alternatives. Should wildlife populations be reduced by any method because they inconvenience humans? Should contraception be applied to seals because they are suspected of harming the economy of a fishing village? Should contraception be applied to wolves because they are eating caribou that sport hunters spend large amounts of money to shoot? Should contraception be applied to an endangered species? If contraception is not used, what will be the alternative management strategy? Who makes the decisions, and on what basis? These are serious questions involving the ethics of both science and wildlife management and they must be considered before application of this technology is applied to our wildlife resources (Kirkpatrick 2007).



The ability to control wildlife populations depends upon approaches that are collectively scientifically possible, legal and publicly acceptable.

WHO IS THE PZP CONTRACEPTIVE RESEARCH TEAM?

The entire PZP contraceptive effort involves many people, several institutions, and numerous funding agencies. At last count there were at least 12 individuals on this team, representing the Science and Conservation Center, the University of California - Davis, the Toledo University Medical College, University of Iowa, and The Humane Society of the U.S., as well as others. This team works together, bringing many disparate disciplines and talents together to solve the problems at hand.

WHO FUNDS PZP CONTRACEPTIVE RESEARCH AND APPLICATIONS?

Funding for applications of the vaccine to wildlife has been provided by many individual communities, agencies, and organizations, including but not limited to: The Humane Society of the U.S., Park Foundation, ASPCA, the Elinor Patterson Baker Trust, THAW Charitable Trust, Burket-Plack Foundation, the Bernice Barbour Foundation, Delta-Sonics, the PNC Corporation, the U. S. Navy, the National Park Service, the Bureau of Land Management, the Rachel Carson National Estuarine Reserve, the National Forest Service, the U. S. Department of Commerce, the National Institutes of Health, the Fire Island Community Association, >100 different zoos in North America, Europe and Australia, the South African National Parks Board, several Indian tribes, and several anonymous donors. This list is not all-inclusive but provides a picture of the breadth of support for this approach to wildlife management.

HOW IS THE PZP VACCINE OBTAINED?

Once all necessary authorizations and approvals have been obtained for the use of the vaccine, it is ordered from The Science and Conservation Center, 2100 South Shiloh Road, Billings, MT 59106; phone 406-652-9718; fax 406-652-9733; e-mail Kim Frank at sccpzp@hotmail.com. The vaccine is not commercially available and it is provided at 60% of the cost of production, or about $24/dose.



Cow bison being darted with PZP on Catalina Island.

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