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Comparison of Methods To Examine Diet of Feral Horses from Noninvasively Collected Fecal Samples[☆]

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ABSTRACT

Feral horses (*Equus ferus caballus*) have become abundant on public lands in the American West, particularly over the past 10 yr. In areas where they are overabundant, there is risk of habitat degradation. Most previous studies on diet and habitat use of feral horses were conducted more than 20 yr ago; rangelands have changed considerably in that time, so it is useful to revisit horse diets. We conducted a study to examine the diet of feral horses using noninvasive methods and subjectively compare diet analysis techniques. We collected feral horse fecal samples from a sagebrush/pinyon-juniper ecosystem in Colorado in May, August, and October 2014. We analyzed 30 fecal samples from each collection session by both microhistology and plant DNA barcoding. Both microhistology and plant DNA barcoding results indicated horse diet consisted primarily of graminoids (78.5% and 68.8%, respectively, both of which are in greater proportion than availability based on ecological site descriptions); however, the two methods differed in species composition of grasses. Similar to other studies, microhistological analyses underestimated the proportion of forbs in the diet compared with plant DNA barcoding analyses, which showed a surprisingly high contribution of forbs to the diet compared with previous studies. Our results suggest plant DNA barcoding analyses have great potential, although both methods have inherent biases.

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Introduction

The Wild Horse and Burro Act was passed in 1971, providing federal protection for feral horses on public lands. High population growth rates coupled with lack of predators has led to subsequent population increases, particularly over the past 10 yr (Bureau of Land Management data). This increase has led to impacts on the habitat and native wildlife in areas where horse densities are overabundant (Beever et al., 2008; Beever and Aldridge, 2011; Davies et al., 2014; Hall et al., 2016; Baur et al., 2017). The domestic horse (*Equus ferus caballus*) is not native to the United States, and vegetation in Great Basin rangelands where they are most numerous has evolved without the presence of heavy grazing pressure from large ungulates (Knapp, 1996). Equids are hindgut fermenters, and unlike ruminants, they can compensate for a low-quality diet by increasing consumption of lower-quality forage or bulk

(Schoenecker et al., 2016). In combination with their burgeoning population and presence year-round on the landscape, these physiological features mean that feral horses have potential to impose a stronger effect on their habitat than ruminants, despite ruminants currently representing the majority of livestock and wild large-bodied grazers on western rangelands. A recent meta-analysis highlighted the lack of recent data on feral horse diets in North America, with only one published paper in the peer-reviewed literature since the 1980s (Scasta et al., 2016). Climate change, drought, invasive species, and changes in land use patterns over the past 30 yr (Parmesan and Galbraith, 2004; Vias and Carruthers, 2005; Notaro et al., 2012) mean that the vegetation in areas where feral horses are most abundant have likely changed since previous studies were conducted 20 yr ago. There is a need to reexamine horse diets in light of changing ecosystems and development of new techniques to analyze samples.

Most studies of feral horse diets have used microhistological analyses of fecal matter (summarized in Scasta et al., 2016). This technique involves preparing a small amount of fecal sample on a slide and then determining the occurrence of individual plant species across up to 100 fields on the slide. Plant species are identified by their histological characteristics, such as cell size and shape and cuticle structure (Johnson et al., 1983). Microhistology is known to provide good precision of diets and is a commonly used tool for many wildlife species.

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However, it can have low accuracy due to differential digestion of plant species, particularly tending to underestimate abundance of forbs, which can be more completely digested (Holechek et al., 1982). Microhistology is also labor intensive, requiring extensive training for accurate determination of plant species. An alternative technique that is becoming more widespread is the use of plant DNA barcoding (Valentini et al., 2009). This involves amplifying chloroplast trnL from a genomic DNA sample to determine plant species or genera present in the diet. The relative abundance of chloroplasts is a representation of the relative protein content of that plant, rather than the biomass. Sequenced DNA is matched against a reference database to identify plant taxa (Czernik et al., 2013; Craine et al., 2015). As sequences can be collected from even tiny amounts of plant matter, perhaps more accurate estimates of plant species in a diet might be possible using this method.

Our objective was to evaluate the diet of feral horses in a pinyon-juniper/sagebrush ecosystem (i.e., dominated by pinyon pine *Pinus edulis* Engelm., juniper *Juniperus* spp. L., and sagebrush *Artemisia* spp. L.) using both the long-established method of microhistological analyses and the more recently developed plant DNA barcoding technique. By analyzing the same samples with both methods, we could evaluate differences in the results. We also sought to contribute to a more current understanding of feral horse diet; this study represents one of only two peer-reviewed publications to assess feral horse diet in 30 yr.

Methods

Study Area

We collected feral horse fecal samples at the Little Book Cliffs Wild Horse Herd Management Area (HMA), near Grand Junction, Colorado (UTM 12 722588, 4342571, WGS84 datum) in May, August, and October 2014. The HMA is managed by the Bureau of Land Management within the US Department of Interior. The area is semiarid (precipitation averaged 4.4 cm per month during the study period, mostly in August and September; King et al., 2018) and consists of mesa tops dissected by deep canyons, some of which have grass and water along the bottom. Water sources located throughout the HMA consist of man-made tanks or natural streams. There are four primary vegetation community types within the horse range:

1. Piñon-Juniper (dominated by piñon pine *Pinus edulis* Engelm. and juniper *Juniperus* spp. L.);
2. Mountain Shrub (main plant species are: Gambel oak *Quercus gambelii* Nutt., serviceberry *Amelanchier* spp. Medik., snowberry *Symphoricarpos* spp. Duham., bluegrass *Poa* spp. L., Wyoming sagebrush *Artemisia tridentata* Nutt.);
3. Salt-desert Shrub (consisting of plants such as shadscale and fourwing saltbush *Atriplex* spp. L., galleta grass *Pleuraphis jamesii* Torr., sandberg bluegrass *Poa secunda* J. Presl., salina wildrye *Leymus salinus* [M.E. Jones] Á. Löve, Indian ricegrass *Oryzopsis hymenoides* [Roem. & Schult.] Ricker ex Piper, needle and thread grass *Hesperostipa comata* [Trin. & Rupr.] Barkworth, cactus *Opuntia* spp. Mill., scarlet globemallow *Sphaeralcea coccinea* [Nutt.] Rydb.);
4. Sagebrush/Grassland (Wyoming sagebrush, rubber rabbitbrush *Ericameria nauseosa* [Pall. ex Pursh] G.L. Nesom & Baird, western wheatgrass *Pascopyrum smithii* [Rydb.] Á. Löve, squirreltail *Elymus elymoides* [Raf.] Swezey, globemallow *Sphaeralcea* spp. A. St.-Hil., Indian paintbrush *Castilleja* spp. Mutis ex L. f., lupine *Lupinus* spp. L., mulesear *Wyethia amplexicaulis* [Nutt.] Nutt., and other grasses).

Cheatgrass *Bromus tectorum* L. is found in all vegetation types.

Sampling Method

We sampled the area in spring (May), summer (August), and fall (October), following the same methods on each visit. The HMA was subjectively divided into sampling areas using ArcGIS. Each sampling area

covered a variety of vegetation types, generally including areas of piñon-juniper forest, sagebrush/grassland, and scrub. The aim of the sampling regime was to collect fecal samples from across as much of the HMA as possible, so sampling areas were not defined by vegetation type but instead to accommodate a random sampling scheme. Sampling areas avoided steep cliffs and areas inaccessible to horses and/or field staff. The order and choice of sampling areas to visit were randomized on each sampling occasion; thus while sampling areas across the whole HMA were sampled on each visit, the same areas were not always visited in each month. To collect fecal samples, we walked across a sampling area in a group of 3–6 people in parallel lines roughly 10–20 m apart, with each person covering a 3- to 4-m-wide belt transect, conducting as many sweeps as were necessary to cover the majority of the area. Not all sampling areas could be swept in their entirety due to cliffs or other topographical hazards that had not been visible in GIS. Within a sweep, each member of the team would look for dung piles in his or her belt. Samples were aged on a four-point scale based on color, appearance, and deterioration. We collected samples ranging from fresh to old but did not collect samples that were disintegrating; samples were therefore from 0 to about 6 months old, with most estimated to be < 3 months old (see Appendix; King et al., 2018). Samples of different ages were collected to maximize sample size. We collected and stored samples in a paper bag: two whole fecal boli from the same fecal pile were placed in the same bag. We avoided contamination of samples by using nitrile gloves or sterile tongue depressors to manipulate fecal boli. We placed paper bag samples in large cotton bags to air dry under shelter during the 10-d sampling session and then air dried them in a drying oven for 3 d at 40°C. We collected > 1 800 samples during the three collection periods.

Laboratory Methods

We randomly selected 90 samples (30 from each collection period: May, August, October) for analysis by microhistology and plant DNA barcoding. We excluded samples that had touched the ground to avoid contamination by vegetable matter that was not consumed, were moldy, were part of a stud pile (large dung pile marked by multiple stallions), or were small, indicating they were probably deposited by a foal.

We sent 90 dried subsamples (about a quarter of a fecal bolus) to the Wildlife Habitat and Nutrition Laboratory at Washington State University for microhistological analyses. At the laboratory, each sample was made into four microscope slides and each slide was viewed 25 times. Results were reported as percentage diet composition of each sample at the plant genus or species level using epidermal fragment cover. Where results were given separately for leaf and stem, they were combined to form a species total for analyses. Moss, seeds, and *Equisetum* sp. were removed from analyses as they were rare (found in 6, 2, and 1 samples, respectively); seeds were not identified to species by the laboratory. In some samples *Festuca scabrella* could not be identified from *Elymus* sp., so it was combined with *Elymus* sp. for analyses. The percentage composition of each plant genus or species was summed across all samples. Where the sum was < 20%, the genus/species was combined with other unknown or rare members of that plant type (graminoid, forb, or shrub). Overall percentage component of the diet was formed from the sum across all samples as a percentage of the total.

We sent 90 subsamples from the same fecal boli used for microhistology analysis rehydrated in vials of alcohol to Jonah Ventures laboratory (<https://jonahventures.com>) for plant DNA barcoding analyses. Sequences were demultiplexed by taking advantage of Golay barcodes via QIIME v1.9.1 (Caporaso et al., 2010). The following options were used to output raw unfiltered fastq files for both forward and reverse reads: `split_libraries_fastq.py -q 0 -max_bad_run_length 250 -min_per_read_length_fraction 0.0001 -sequence_max_n 250 -store_demultiplexed_fastq`. Forward and reverse read sequences were trimmed to 235 nucleotides via the `usearch8` option `-fastx_truncate`. Sequences were then merged using the `-fastq_mergepairs` option in

usearch8 (Edgar, 2010). The forward primer (5'-CGAATCGGTAGACGCTACG-3') and reverse primer (5'-CCATTGAGTCTCTGCACCTATC-3') were removed using cutadapt (Martin, 2011). From here forward, the trnL amplicons were processed via the UPARSE pipeline (Edgar, 2013) and taxonomy was assigned via the SINTAX protocol (http://www.drive5.com/usearch/manual/utax_user_train.html) available in usearch (v8.1.1861; Edgar, 2010). Specifically, sequences were quality trimmed to have a maximum expected number of errors per read of < 0.5 and operational taxonomic units (OTUs) were clustered at 99% similarity with de novo chimera checking enabled. To assign taxonomy to each OTU, an "in-house" SINTAX trnL reference database was constructed by downloading any annotated GenBank (Benson et al., 2005) records that contain the trnL gene. The amplicon region bounded by the trnL c & h primers (Taberlet et al., 2007) was extracted from the GenBank records using the UTAH protocol. All extracted amplicon regions were dereplicated to 100% sequence identity and any identical sequence across lineages collapsed to the lowest-common-ancestor. Closed-reference OTUs were generated by searching against the trnL reference database at 99% sequence similarity. To ensure increased specificity of trnL OTU assignment against the reference database, the -maxaccepts and -maxrejects usearch options were set to 0. Additional OTUs were generated when sequencing plant voucher specimens that were collected privately to supplement the set of species present in GenBank.

One sample did not produce enough amplifiable DNA, leaving 89 samples in the analyses. The percentage of a sample made up by an OTU represents the relative protein content of the plant species, rather than the biomass that they represent. Sixty percent of OTUs identified plants at the resolution of species. Twenty percent of OTUs contained multiple genera, so for analyses, and to provide comparison with microhistology results, we selected a representative genus that was most likely for the study area from these OTUs. Multiple OTUs of the same genus were combined for analyses. Moss was removed from the analyses (found in 9 samples). As with the microhistology data, we summed the percentage of each genus OTU across all samples. Where the sum was < 20%, the OTU was combined with other rare members of that plant type (graminoid, forb, or shrub). Overall percentage component of the diet was formed from the sum across all samples as a percentage of the total.

Data Analyses

We used the same analyses for both microhistology and plant DNA barcoding data; all analyses were conducted using R (R Core Team, 2017). We had more samples from midage fecal piles because they were more abundant in the landscape (Table S1; available online at <https://doi.org/10.1016/j.rama.2019.02.005>), so we first used a 1-way analysis of variance (ANOVA) to test any effect of fecal pile age on the number of species or OTUs. There was no effect of fecal pile age on the number of plant species for microhistology ($F_{3, 85} = 2.064, P = 0.111$) or OTUs for DNA plant barcoding samples ($F_{3, 85} = 0.918, P = 0.436$); thus, we continued analyses without using fecal pile age as a factor. We also confirmed that there was no interaction between month of collection and fecal pile age using a 2-way ANOVA (Table S2; available online at <https://doi.org/10.1016/j.rama.2019.02.005>).

Scientific names follow those provided by the respective laboratories in order to avoid making incorrect assumptions about plant species found by the different methods. We used ANOVA tests to examine the effect of collection period on number of species or OTUs, as well as on plant class, acknowledging that we are referring to season rather than month of collection due to the age of the fecal piles sampled. We explored differences in microhistology and plant DNA barcode results at a coarse scale (plant class, rather than plant species or OTU) examining the variance across pairs of subsamples from the same fecal pile with F tests. To examine variance within results of graminoid diet for each method across seasons, we used Bartlett tests. The microhistology

sample corresponding to the plant DNA barcode sample that did not amplify was removed.

To provide an indication of plant types potentially available to feral horses within the habitat, we used ecological site descriptions of the nine soil types found in the HMA (Soil Conservation Service, 1994, 1975a, 1975b, 1975c, 1975d, 1975e, 1975f, 1975g, 1975h). For each ecological site description, we took data of the percent species composition by weight, summarized by plant type (grasses, forbs, and shrubs), and then derived a mean value for the HMA as a whole.

Results

Microhistology

Out of 22 species or species groups (Table 1), there were an average of 11.5 per sample. The diet was mostly made up of graminoids (78.5%, including 0.89% *Carex* sp. L.), with 5.44% forbs and 16.07% shrubs/trees (see Table 1). The laboratory assumed that all *Bromus* spp. L. found in the samples was *Bromus tectorum*, which was found in all but five samples and made up 14.18% of the diet. Other grass species found at high abundance were *Pascopyrum* (*Agropyron*) *smithii* (16.11%), *Poa* spp. (14.55%), *Stipa* spp. L. (14.04%), and *Elymus/Festuca* (12.66%).

Fewer species/groups were present in spring diets than the other sampling periods ($F_{2, 86} = 5.967, P = 0.00374$). In the spring sampling period, graminoids made up a larger part of the diet, and forbs a lesser part, than the other sampling periods (graminoids: $F_{2, 86} = 3.958, P = 0.0227$; forbs: $F_{2, 86} = 3.358, P = 0.0394$; Fig. 1). Shrubs made up a greater proportion of the diet in the fall ($F_{2, 86} = 4.574, P = 0.013$).

Plant DNA Barcoding

After combining some OTUs that had the same genus, we had a total of 42 OTUs (see Table 1). There were an average of 28.06 OTUs per sample. Similar to the microhistology data, the plant DNA barcoding results were mostly made up of graminoids (68.76%, including 0.92% *Carex* sp.), but unlike the microhistology results there were more forbs (19.24%) than shrubs/trees (11.99%; see Table 1). The most abundant grass OTUs were *Trisetum* spp. Pers. (17.40%), *Poa* spp. (14.81%), and *Aegilops* spp. L. (13.57%), followed by *Bromus* spp. (8.86%). *Bromus tectorum* was detected to species in 2 of the 25 OTUs containing *Bromus* spp., making up 1.80% of the diet.

There was no difference in the number of OTUs across the sampling periods ($F_{2, 86} = 2.357, P = 0.101$). However, there was a difference in the representation of the different plant classes in different sampling seasons (graminoids: $F_{2, 86} = 3.565, P = 0.0326$; forbs: $F_{2, 86} = 5.096, P = 0.0081$; shrubs: $F_{2, 86} = 7.056, P = 0.00415$; see Fig. 1).

Comparison of Methods

Feral horse diets determined by microhistology had fewer species, and different species, than diets determined by plant DNA barcoding (see Table 1). Microhistology diets had fewer graminoid and forb species, with few species common between the two methods. Of particular note is the lack of concordance in the most abundant grass species in the diet; for instance, *Stipa* spp. made up 14.04% of the microhistology diet, but only *Hesperostipa* spp. was found in the plant DNA diet, comprising just 1.41%. There was no difference in the variance of graminoids between samples analyzed by microhistology and plant DNA barcoding (F test = 1.0768, num df = 88, denom df = 88, $P = 0.7295$). However, the microhistology results had a significantly lower variance of forbs ($F = 6.2086$, num df = 88, denom df = 88, $P = < 0.0001$) and a greater variance of shrubs/trees ($F = 0.488$, num df = 88, denom df = 88, $P = 0.0008979$) than the plant DNA results (see Fig. 1). Although there was no difference in the variance among seasons of graminoids in the microhistology diet (Bartlett's K-squared = 1.2527, df = 2, P value =

Table 1

Diet composition of feral horse fecal samples from Little Book Cliffs herd management area in Colorado by microhistological and plant DNA barcoding analyses. Percentage composition of each plant genus or species was summed across all samples and combined with other unknown or rare members of that plant type when the sum was < 20%. Overall percentage component of the diet presented here was formed from the sum across all samples as a percentage of the total. Results are sorted by highest percentage within each plant class. Plant names are given as reported by the laboratory.

Microhistology analyses		Plant DNA barcoding analyses	
Genus	Overall %	Genus	Overall %
Total graminoids:	78.50	Total graminoids:	68.76
<i>Pascopyrum (Agropyron) smithii</i>	16.11	<i>Trisetum</i>	17.40
<i>Poa</i>	14.55	<i>Poa</i>	14.81
<i>Bromus tectorum</i>	14.18	<i>Aegilops</i>	13.57
<i>Stipa</i>	14.04	<i>Bromus</i>	8.86
<i>Elymus/Festuca</i>	12.66	<i>Paspalum</i>	2.95
<i>Hilaria</i>	2.09	Other grasses	2.70
<i>Oryzopsis</i>	1.85	<i>Triticum</i>	1.56
Unknown or rare grass	1.65	<i>Hesperostipa</i>	1.41
<i>Carex</i>	0.89	<i>Calamagrostis</i>	1.22
<i>Sporobolus</i>	0.47	<i>Carex</i>	0.92
		<i>Simplicia</i>	0.85
		<i>Achnatherum</i>	0.79
		<i>Digitaria</i>	0.72
		<i>Elymus</i>	0.45
		<i>Phalaris</i>	0.34
		<i>Hordeum</i>	0.22
Total forbs:	5.44	Total forbs:	19.24
<i>Astragalus</i>	1.09	<i>Hedysarum</i>	4.37
Unknown forbs	1.58	Other forbs	3.22
<i>Lupinus</i>	1.53	<i>Astragalus</i>	2.09
<i>Mustard</i>	0.95	<i>Lappula</i>	1.09
<i>Sphaeralcea</i>	0.30	<i>Comandra</i>	1.06
		<i>Chenopodium</i>	0.97
		<i>Eurybia</i>	0.95
		<i>Aster</i>	0.91
		<i>Crepis</i>	0.71
		<i>Herrickia</i>	0.63
		<i>Polygonum</i>	0.53
		<i>Grayia</i>	0.48
		<i>Helianthus</i>	0.45
		<i>Erigeron</i>	0.32
		<i>Centaurea</i>	0.32
		<i>Nicotiana</i>	0.31
		<i>Solanoideae spp.</i>	0.31
		<i>Solidago</i>	0.31
		<i>Silene</i>	0.22
Total shrubs:	16.07	Total shrubs:	11.99
<i>Quercus</i>	5.15	<i>Quercus</i>	5.68
<i>Atriplex</i>	3.48	<i>Atriplex</i>	3.04
<i>Artemisia</i>	4.97	<i>Salix</i>	1.21
<i>Chrysothamnus</i>	0.93	<i>Pinus</i>	1.16
<i>Krascheninnikovia (Ceratoides)</i>	0.68	<i>Artemisia</i>	0.33
Unknown shrub	0.57	Other shrub/tree	0.31
<i>Ephedra</i>	0.29	<i>Thuja</i>	0.27

0.5345), there was in the plant DNA barcoding diet (K-squared = 8.8864, df = 2, P value = 0.01176; see Fig. 1).

We did not have fine-scale data on percent of each plant species across the landscape, so we relied on ecological site descriptions to determine the general proportion of vegetation classes in the study area. Ecological site descriptions estimated the species composition of Little Book Cliffs HMA consists of ~60% grasses, ~10% forbs, and ~30% shrubs, with trees found in some areas. Although we unfortunately do not have data to look at this in more detail, this implies that horses are consuming grasses in greater proportion than their availability, as well as shrubs less than their availability. DNA barcoding results imply that horses consume forbs about twice as much as their availability.

The other difference between the two methods was in terms of cost and turnaround time. As the only laboratory we could find in the United States that is still conducting microhistological analyses, the Wildlife Habitat and Nutrition Laboratory at Washington State University is

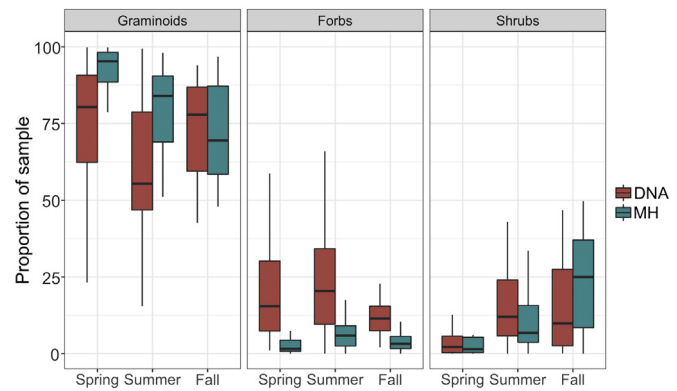


Figure 1. Proportion of graminoids, forbs, and shrubs in feral horse fecal samples from different seasons (spring, summer, and fall 2014) at Little Book Cliffs Herd Management Area, Colorado. DNA (dark red) represents results from plant DNA barcoding analyses, and MH (teal blue) represents results from microhistology analyses.

understandably backlogged. Wait times for return of samples are estimated at 1 yr or more, and cost is currently around \$200/sample for plant genus/species level of identification. The plant DNA barcoding analyses at Jonah Ventures laboratory cost around \$80/sample and results were returned in about 2 mo.

Discussion

Horse diets were found by both analytical methods to be mostly made up of graminoids (78.5% of microhistology diets, 68.8% of plant DNA barcoding diets). Other studies of spring-to-fall horse diets in North America ranged from 76% to 91% graminoids (summarized in Scasta et al., 2016 and in Schoenecker et al., 2016), so our results are at the low end of what was previously found. However, the individual grass species making up the diet differed between the two methods. Microhistological analyses found the main grass species to be *Pascopyrum (Agropyron) smithii*, *Poa* spp., *Bromus tectorum*, *Stipa* spp., and *Elymus/Festuca*. In the plant DNA barcoding analyses, *Trisetum* spp. and *Aegilops* spp. were the dominant grasses, although there was a similar proportion of *Poa* spp. in the diet in both methods. In other studies *Stipa* spp. made up to a quarter of horse diets (Krysl et al., 1984; Mcinnis and Vavra, 1987; Crane et al., 1997). Although it made up 14% of the microhistology diet, *Hesperostipa* spp. formed < 2% of the plant DNA barcoding diet. Studies of feral horse diets in Wyoming and Colorado (Hansen and Clark, 1977; Crane et al., 1997) reported that sedges (*Carex* spp.) constituted up to 76% of the diet. In both analytical techniques *Carex* spp. made up < 1% of feral horse diet in our Colorado study site, probably because there was little mesic habitat.

Bromus spp. is found throughout the study area, and this was represented by its ubiquity in our samples from both methods. Although other species of *Bromus* are present, the microhistology analyses assumed that the *Bromus* spp. detected was cheatgrass (*B. tectorum*). *Bromus* spp. was found in 25 OTUs and in all but 4 the plant DNA barcoding analyses identified the species; *B. tectorum* was only present in 2 OTUs, with the rest consisting of other *Bromus* species. Cheatgrass is an invasive species that has colonized the western United States (Mack, 1981). The nutritional quality of cheatgrass is high when it is green in the early spring but quickly declines as it cures in the summer (Young and Allen, 1997). As horses are potential dispersers of cheatgrass (King et al., 2019), it is important to quantify how much of this plant they are consuming.

Microhistological analyses are known to underestimate the proportion of forbs in the diet, as they tend to be digested more completely than other plant types (Shrestha and Wegge, 2006). Conversely, the proportion of shrubs/trees may be overestimated as these plants tend to be digested less completely. The two methods found a similar proportion of the diet made up by trees and shrubs, with representation of

Quercus sp. and *Atriplex* spp. as the main browse species being similar in both methods. Browse is typically rare in horse diets, with greater proportions consumed in winter when grass may be covered with snow or be less nutritious (around 3 – 11% of the diet across studies; Scasta et al., 2016; Schoenecker et al., 2016). Similarly, in our study more browse was seen in the fall diet.

Plant DNA barcoding found a higher proportion of forbs in the diet than any previous study: over twice as much as the mean 8% forbs found across other studies of feral horse diets based on microhistology (summarized in Scasta et al., 2016 and Schoenecker et al., 2016) and four times as much as in our microhistology results. Some discrepancy in forb intake results between the two methods was expected, as microhistology is known to underestimate forbs in the diet, and plant DNA barcoding could overestimate this due to the high protein content of forbs. Esophageal fistulas revealed greater consumption of forbs than found by microhistology of cattle fecal samples (Vavra et al., 1978), so it is possible that forbs have always been a more important food source for horses than realized from microhistological analyses. It could also represent a changing trend in horse diets as they respond to changes in plant communities from effects of climate change (Notaro et al., 2012).

Although we do not know the true diet of the feral horses at Little Book Cliffs HMA, the other most noticeable difference in the results between the two methods is the diversity of the diet. Plant DNA barcoding found almost twice as many genera as plant microhistology. If this was due to microhistology being unable to identify plants, we would expect it to have a higher proportion of unknown/rare species. But these were similar for each plant class between the two methods. It is likely that plant DNA barcoding was able to detect plant species from smaller fragments of plant than were discernable by microhistology. However, plant DNA barcoding results also contained some genera that are unlikely to be present in Little Book Cliffs. For instance, while *Thuja* spp. L. (a member of the cypress family) is present in North America, it is possible that it was misclassified from another member of the Pinales order such as pine or juniper. These misclassifications are likely to become less common as more species are added to GenBank, and this could have been corrected if we had a plant library from the study area. A study on small mammal diets recommended using both techniques to gain a better overview of diets, as both methods detected diet items that the other missed (Khanam et al., 2016). The difference in plants found by the two methods in our study confirm this.

Implications

Directly evaluating plant microhistology analyses and plant DNA barcoding results from the same samples has enabled us to see differences in results between the two methods. We concur with previous studies in finding a lower proportion of forbs in diets from microhistology relative to plant DNA barcoding and suggest that plant DNA barcoding results are more likely to perform better relative to forbs. Both methods found that most of the diet was made up of graminoids and that shrubs were more commonly consumed in the fall, similar to previous studies.

Both methods provided results to the level of genus, but DNA barcoding results identified more genera and more plants to the species level. The greater resolution of data provided by the plant DNA barcoding results is useful, and the accuracy of these results will only improve in the future. Other studies have obtained species-level results from plant DNA barcoding assisted by a reference database genotyped from local vegetation (Czernik et al., 2013; Bergmann et al., 2015). As more plant species are barcoded and the sequences become publicly available, this method will become more refined. In a comparison between the two methods on vole diets, in which all potential diet species had been genotyped (Soininen et al., 2009), DNA barcoding provided more detailed taxonomic resolution than microhistology, similar to our results. It is likely that plant DNA barcoding will become more mainstream in the future, but choice of technique will depend on the

research question to be answered. Studies that only require a broad overview of diet may choose to use microhistology, whereas those needing more detail might use plant DNA barcoding. In particular, plant DNA barcoding is likely to be more useful for diets consisting largely of forbs. Once a plant DNA barcode library has been compiled, it will potentially be possible for any genetics laboratory to run these analyses. By comparison, we found only one laboratory in the United States that is conducting microhistological analyses, likely because the technique is highly specialized and time consuming (Holechek et al., 1982), plus the pursuit of diet studies has waned in the past 10 – 15 yr, decreasing demand for laboratory services.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rama.2019.02.005>.

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