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ABSTRACT

Buchanan, Courtney E. *Foraging Ecology and Biotic Relationships with Gut Microbiota in Pronghorn and Free-Roaming Horses,* Ph.D., Department of Ecosystem Science and Management, August 2025

The aim of my dissertation was to explore the underlying biotic relationships between plants, herbivores, and the herbivore gut microbiome in two species, pronghorn (*Antilocapra americana*) and free roaming horses (*Equus caballus*). To understand these relationships, I used fecal DNA metabarcoding to elucidate community composition of gut bacteria in both species and to identify plants in the free-roaming horse diet. I also adapted fecal particle size protocols for a field-based approach in free-roaming horses to serve as a proxy for investigating diet digestibility. Much of this research aimed to describe how the diet and/or microbial communities changed in different seasons, study areas, and environments in these species. In free-roaming horses, I tested simplified fecal particle size methods to determine if this measure varied by season or study area. I also investigated the potential for using this measure as a biomarker by evaluating the relationship of fecal particle size with horse diet and body condition. I explored the potential use of microbial composition as a biomarker by exploring relationships between the microbial community and host health, body condition, and diet. Overall, microbiome composition had a spatial and seasonal component in both species, illustrating the ability of microbial communities to vary in rangeland herbivores across animals' ranges and lifetimes. I formatted my dissertation with four research chapters intended for journal submission (Chapters 2-5), an introduction in Chapter 1 of the general aims of the research and knowledge gaps addressed, and a conclusion of my findings in Chapter 6.

In Chapter 2, we investigated the bacterial composition of the gut microbiome in pronghorn, which had yet to be described in the literature. Pronghorn microbiome composition

was predominantly composed of the phyla Firmicutes and Bacteroidota, both commonly documented as the predominant phyla in ruminant animals. We noted small but significant differences in microbial communities relative to study area, capture period, and body fat measurements; however, these factors were only able to explain small amounts of microbial community variation. We also found that microbial composition differed in animals located north and south of Interstate 80, reinforcing that the pronghorn microbiome has an important spatial component. This chapter was published in 2024 in *PLOS One*.

Chapter 3 examined the dietary composition of free-roaming horses in 16 Bureau of Land Management (BLM) Herd Management Areas (HMAs). The proportion of graminoids in fecal material differed by HMA and ranged from 31.1 to 83.5% in summer and 11.0 to 82.6% in winter. Horses in most HMAs tended to eat more graminoids in summer as compared to winter, and winter diets tended to contain more plants in the families Asteraceae and Chenopodiaceae than in summer. Despite varying dietary composition across HMAs, average body condition in all HMAs was good, ranging from 4.6 to 5.2 in summer and 4.8 to 5.2 in winter, and most individual horses observed had body condition scores of 5 or better. Our results indicated that while horses are considered grazers, they are able to maintain healthy body condition while consuming a variety of plant types. This chapter has been accepted for publication in *Rangeland Ecology and Management*.

Chapter 4 investigated drivers of the gut microbial community in free roaming horses, using the same horses and HMAs studied in Chapter 3. Season, HMA, and ecoregion were all significant drivers of the horse gut microbial community at various scales. Season explained more variation of microbial composition within individual HMAs (7.0–18.1%), compared to a range-wide scale (about 3.5%) when using samples from individual horses, but explained about

15% of the variation at the range-wide scale when HMA average microbiomes were considered instead. We identified 158 bacterial families present during the winter, while only 128 of these families were observed during the summer. Ecoregion explained about 4.5% of the variation using individual horses and around 20% of microbial variation when using HMA average microbiomes, while HMA was more explanatory, explaining about 9% of the variation when evaluating individual horses. These findings were similar whether we considered factors independently from one another or evaluated the marginal effect in combined models. In addition, Mantel tests indicated the community composition of the microbial community co-varied with the composition of diet in free-roaming horses. Furthermore, nine of the ten bacterial families that contributed most to the overall dissimilarity in the microbial community were correlated with at least one plant family present in free-roaming horse diets. These bacterial families most often exhibited relationships with the graminoid families of Cyperaceae, Poaceae, and Juncaceae and the forb and shrub families of Asteraceae and Chenopodiaceae. Our findings indicate that microbial community composition was variable in different environments and seasons making it potentially an adaptive trait for free-roaming horses and other herbivores.

Chapter 5 explored field-based methods for conducting particle size analysis in free-roaming horses. We chose fecal particle size as a proxy for digestibility because previous researchers have correlated this measure with digestibility in herbivores. We adapted described fecal particle size protocols to allow for methods more amenable to those without expensive lab-based equipment, with a goal of providing a low cost, minimal lab alternative for researchers to measure a surrogate for diet digestibility in wildlife. We were able to use these methods to detect differences in fecal particle size in different HMAs and between seasons. We also investigated the relationship between fecal particle size and body condition and diet composition to explore

the potential use of fecal particle size as a herd health biomarker. While there were some patterns evident, our methods likely need further adjustment to account for high within-sample variation before this technique can be informative to explain herd health or diet composition.

Foraging Ecology and Biotic Relationships with Gut Microbiota in Pronghorn and Free-Roaming Horses

By

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locations within the HMA, sharing stories with me, and showing me around. Also, thanks to my friends Angie, Shelby, and Toni and my husband Steffen for being crazy enough to join me on trips find fecal samples.

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CHAPTER 1. Introduction

The ability of wildlife to adapt to novel environments is increasingly important in the face of anthropogenic change and climate fluctuations. Extensive research is undertaken on wildlife species worldwide; however, research often focuses on questions of presence or absence, movement, behaviors, resource selection, or timing of these or other variables. In many species, we have knowledge of the types of environments or resources these animals select but still lack understanding of the mechanisms for how animals adapt to utilize specific resources or environments. Modern technologies provide new ways to monitor individual responses to environmental stimuli, which can help to answer the questions of how and why animals do what they do (Hawkes et al. 2021). Understanding how species react chemically, metabolically, and physically to their environments is key as ecosystems face growing changes due to anthropogenic disturbance and climate variability. Greater understanding of wildlife physiology and adaptive mechanisms could help predict species' flexibility to adjust to changes. Likewise, a better understanding of the physiology underlying these measures is necessary before managers can successfully apply this knowledge to management decisions.

Many physiological traits exhibit plasticity in response to the environment (Killen et al. 2017). Some changes may signify conditions an animal is currently experiencing, while others represent accumulation of environmental impacts over longer time scales. For example, body condition represents the accumulated effects of diet, metabolic efficiency, and energetic costs and changes slowly over time. Microbiome composition has been linked to many animal health metrics, especially those involving diet, body condition, and digestion (e.g., Turnbaugh et al. 2008; Myer et al. 2015; Shabat et al. 2016), and can be thought of as a physiological trait, especially in herbivores that require microbial symbionts to assist with the digestive process.

Researchers have proposed gut microbiome plasticity as a mechanism to allow greater host adaptation capability in the face of changing climates (Alberdi et al. 2016). Others have discussed the importance of considering microbiome composition when performing reintroductions or translocations (Bahrndorff et al. 2016; Li et al. 2019; Trevelline et al. 2019; Yao et al. 2019) and mention probiotics as a possible management tool (McKenzie et al. 2018).

The research presented in my dissertation contributes to the understanding of two iconic herbivores in North America—pronghorn (*Antilocapra americana*) and free-roaming horses (*Equus caballus*). The overall aim of my research was to explore the biotic relationships that these species experience—specifically the inter-specific relationships between plants, herbivores, and the herbivore gut microbiome. These interactions potentially affect the adaptive potential of herbivores to novel environments and are likely related to the ability of herbivores to utilize diverse forages and maintain good body condition. Throughout the chapters, I endeavor to find linkages between host animal health, microbial composition, diet composition, and diet digestibility, and investigate how these measures are similar or different throughout environments or seasons. Studies investigating the relationships of microbiome, diet, or animal health in wild or free-roaming herbivores are becoming more common (e.g., Bergmann et al. 2015, Kartzinel et al. 2019, Li et al. 2019, Yao et al. 2019). However, my dissertation specifically adds to the literature on this topic by exploring these microbial relationships in pronghorn, a previously understudied species in microbiome literature. The literature regarding the gut microbiome composition of pronghorn is limited, and the previous literature on the topic is specific to anaerobic fungi (Liggenstoffer et al. 2010) and rumen protozoa (Dehority 1995). To my knowledge, no one had previously investigated the bacterial composition of the pronghorn microbiome. In addition to documenting the core bacteria present in the gut microbiota of

pronghorn, I investigated the relationships between microbial composition, capture period, location, and intrinsic measures such as disease status and body condition, novel topics for pronghorn.

While there is a large body of literature regarding microbial composition (Kobayashi et al. 2006; Antwis et al. 2018; Salem et al. 2018; Theelen et al. 2021; Zaitseva et al. 2023), diet (Hosten et al. 2007; Scasta et al. 2016; King and Schoenecker 2019), and sometimes even the relationships between these metrics in domestic (Willing et al. 2009; Hansen et al. 2015; Fernandes et al. 2021) and free-roaming (Kartzinel et al. 2019; Li et al. 2019) equids, my research is unique in the large geographic scale at which I evaluated relationships between these factors. Previous research on stabled and pastured domestic horses has revealed that season can affect the composition of the gut microbiome (Kobayashi et al. 2006; Salem et al. 2018; Fernandes et al. 2021; Theelen et al. 2021; Zaitseva et al. 2023). In addition, in a study of Przewalski's horses (*E. ferus przewalskii*) grazing on nature reserves, researchers found variation in microbial communities in animals of different herds or locations, which they hypothesized may be linked to different plant species in different environments (Li et al. 2019). Indeed, microbial composition and diet have been linked in domestic horses (Willing et al. 2009; Hansen et al. 2015; Fernandes et al. 2021). While many of these previous studies are limited in the number of animals or geographic scale of their research, I build upon these studies by investigating the composition of diet and microbiome in hundreds of free-roaming horses across a wide geographical extent of the western United States. In addition, free-roaming horses in my study were residing in and foraging on relatively wild rangelands whereas in many of these previous studies animals were fed hay or concentrate feeds or kept on pastures with limited forage diversity (but see Li et al. 2019, Zaitseva et al. 2023). I first explored the seasonality and

spatial variation of diet and microbial composition at herd level and range-wide scales. I also investigated links between diet, microbial composition, body condition, and fecal particle size, a proxy for diet digestibility. I considered the potential efficacy of physiological measurements such as fecal particle size or microbial composition as biomarkers by investigating relationships between these measures and animal health metrics such as diet composition or body condition.

Overall, I aimed to understand the physiologic relationships between herbivores, microbes, and forages to understand potential mechanisms pronghorn and free-roaming horses use to adapt to novel environments and investigate potential traits that could serve as informative biomarkers. Identifying differing microbial communities in pronghorn or free-roaming horses within distinct study areas or seasons could provide evidence to support the hypothesis that locally adapted microbial communities assist animals in adapting to diverse environments. These findings could offer support for future efforts to conduct research into fecal transplants or probiotics to increase wildlife fitness during relocations and translocations, or to bolster resident populations during stressful events such as disease outbreaks.

REFERENCES

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M.L., Gilbert, M.T.P., 2016. Do vertebrate gut metagenomes confer rapid ecological adaptation? Trends Ecol. Evol. 31, 689–699. https://doi.org/10.1016/j.tree.2016.06.008

Antwis, R.E., Lea, J.M.D., Unwin, B., Shultz, S., 2018. Gut microbiome composition is associated with spatial structuring and social interactions in semi-feral Welsh Mountain ponies. Microbiome 6, 207. https://doi.org/10.1186/s40168-018-0593-2

Bahrndorff, S., Alemu, T., Alemneh, T., Nielsen, J.L., 2016. The microbiome of animals: implications for conservation biology. Int. J. Genomics 2016, 5304028. https://doi.org/10.1155/2016/5304028

Bergmann, G.T., Craine, J.M., Robeson, M.S., Fierer, N., 2015. Seasonal shifts in diet and gut microbiota of the American bison (*Bison bison*). PLoS One 10, 1–14. https://doi.org/10.1371/journal.pone.0142409

Dehority, B.A., 1995. Rumen ciliates of the pronghorn antelope (*Antilocapra americana*), mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*) and elk (*Cervus canadensis*) in the northwestern United States. Archiv für Protistenkunde. 146, 29–36. http://doi.org/10.1016/S0003-9365(11)80252-6

Fernandes, K.A., Gee, E.K., Rogers, C.W., Kittelmann, S., Biggs, P.J., Bermingham, E.N., Bolwell, C.F., Thomas, D.G., 2021. Seasonal variation in the faecal microbiota of mature adult horses maintained on pasture in New Zealand. Animals 11, 1–21. https://doi.org/10.3390/ani11082300

Hansen, N.C.K., Avershina, E., Mydland, L.T., Næsset, J.A., Austbø, D., Moen, B., Måge, I., Rudi, K., 2015. High nutrient availability reduces the diversity and stability of the equine caecal microbiota. Microb. Ecol. Heal. Dis. 26, 1–8. https://doi.org/10.3402/mehd.v26.27216

Hawkes, L.A., Fahlman, A., Sato, K., 2021. Introduction to the theme issue: Measuring physiology in free-living animals. Phil. Trans. R. Soc. B. 376, 20200210. https://doi.org/10.1098/rstb.2020.0210

Hosten, P.E., H. Whitridge, H., Broyles, M., 2007. Diet overlap and social interactions among cattle, horses, deer and elk in the Cascade-Siskiyou National Monument, southwest Oregon. U.S. Department of the Interior, Bureau of Land Management, Medford District. http://soda.sou.edu/bioregion.html

Kartzinel, T.R., Hsing, J.C., Musili, P.M., Brown, B.R.P., Pringle, R.M., 2019. Covariation of diet and gut microbiome in African megafauna. Proc. Natl. Acad. Sci. U. S. A. 116, 23588–23593. https://doi.org/10.1073/pnas.1905666116

Killen, S.S., Marras, S., Nadler, L., Domenici, P., 2017. The role of physiological traits in assortment among and within fish shoals. Phil. Trans. R. Soc. B. 372, 20160233. https://doi.org/10.1098/rstb.2016.0233

King, S.R.B., Schoenecker, K.A., 2019. Comparison of methods to examine diet of feral horses from noninvasively collected fecal samples. Rangel. Ecol. Manag. 72, 661–666. https://doi.org/10.1016/j.rama.2019.02.005

Kobayashi, Y., Koike, S., Miyaji, M., Hata, H., Tanaka, K., 2006. Hindgut microbes, fermentation and their seasonal variations in Hokkaido native horses compared to light horses. Ecol. Res. 21, 285–291. https://doi.org/10.1007/s11284-005-0118-x

Li, Y., Zhang, K., Liu, Y., Li, K., Hu, D., Wronski, T., 2019. Community composition and diversity of intestinal microbiota in captive and reintroduced Przewalski's horse (*Equus ferus przewalskii*). Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.01821

Liggenstoffer, A.S., Youssef, N.H., Couger, M.B., Elshahed, M.S., 2010. Phylogenetic diversity and community structure of anaerobic gut fungi (phylum Neocallimastigomycota) in ruminant and non-ruminant herbivores. ISME J. 4, 1225–1235. http://doi.org/10.1038/ismej.2010.49

McKenzie, V.J., Kueneman, J.G., Harris, R.N., 2018. Probiotics as a tool for disease mitigation in wildlife: Insights from food production and medicine. Ann. N. Y. Acad. Sci. 1429, 18–30. https://doi.org/10.1111/nyas.13617

Myer, P.R., Smith, T.P.L., Wells, J.E., Kuehn, L.A., Freetly, H.C., 2015. Rumen microbiome from steers differing in feed efficiency. PLoS One 10. https://doi.org/10.1371/journal.pone.0129174

Salem, S.E., Maddox, T.W., Berg, A., Antczak, P., Ketley, J.M., Williams, N.J., Archer, D.C., 2018. Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period. Sci. Rep. 8, 1–10. https://doi.org/10.1038/s41598-018-26930-3

Scasta, J.D., Beck, J.L., Angwin, C.J., 2016. Meta-analysis of diet composition and potential conflict of wild horses with livestock and wild ungulates on western rangelands of North America. Rangel. Ecol. Manag. 69, 310–318. https://doi.org/10.1016/j.rama.2016.01.001

Shabat, S.K.., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M.E., White, B.A., Shterzer, N., Mizrahi, I., 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME J. 10, 2958–2972. https://doi.org/10.1038/ismej.2016.62

Theelen, M.J.P., Luiken, R.E.C., Wagenaar, J.A., Sloet van Oldruitenborgh-Oosterbaan, M.M., Rossen, J.W.A., Zomer, A.L., 2021. The equine faecal microbiota of healthy horses and ponies in the Netherlands: Impact of host and environmental factors. Animals 11, 1–17. https://doi.org/10.3390/ani11061762

Trevelline, B.K., Fontaine, S.S., Hartup, B.K., Kohl, K.D., 2019. Conservation biology needs a microbial renaissance: A call for the consideration of host-associated microbiota in wildlife management practices. Proc. R. Soc. B Biol. Sci. 286. https://doi.org/10.1098/rspb.2018.2448

Turnbaugh, P.J., Bäckhed, F., Fulton, L., Gordon, J.I., 2008. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe. 3, 213–223. https://doi.org/10.1016/j.chom.2008.02.015

Willing, B., Vörös, A., Roos, S., Jones, C., Jansson, A., Lindberg, J.E., 2009. Changes in faecal bacteria associated with concentrate and forage-only diets fed to horses in training. Equine Vet. J. 41, 908–914. https://doi.org/10.2746/042516409X447806

Yao, R., Xu, L., Hu, T., Chen, H., Qi, D., Gu, X., Yang, X., Yang, Z., Zhu, L., 2019. The "wildness" of the giant panda gut microbiome and its relevance to effective translocation. Glob. Ecol. Conserv. 18, e00644. https://doi.org/10.1016/j.gecco.2019.e00644

Zaitseva, S., Dagurova, O., Radnagurueva, A., Kozlova, A., Izotova, A., Krylova, A., Noskov, S., Begmatov, S., Patutina, E., Barkhutova, D.D., 2023. Fecal microbiota and diet composition of Buryatian horses grazing warm- and cold-season grass pastures. Microorganisms 11, 1–14. https://doi.org/10.3390/microorganisms11081947

CHAPTER 2. Relating gut microbiome composition and life history metrics for pronghorn (Antilocapra americana) in the Red Desert, Wyoming

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ABSTRACT

Host microbial communities (hereafter, the 'microbiome') are recognized as an important aspect of host health and are gaining attention as a useful biomarker to understand the ecology and demographics of wildlife populations. Several studies indicate that the microbiome may contribute to the adaptive capacity of animals to changing environments associated with increasing habitat fragmentation and rapid climate change. To this end, we investigated the gut microbiome of pronghorn (*Antilocapra americana*), an iconic species in an environment that is undergoing both climatic and anthropogenic change. The bacterial composition of the pronghorn gut microbiome has yet to be described in the literature, and thus our study provides important baseline information about this species. We used 16S rRNA amplicon sequencing of fecal samples to characterize the gut microbiome of pronghorn—a facultative sagebrush (*Artemisia spp.*) specialist in many regions where they occur in western North America. We collected fecal pellets from 159 captured female pronghorn from four herds in the Red Desert of Wyoming during winters of 2013 and 2014. We found small, but significant differences in diversity of the gut microbiome relative to study area, capture period, and body fat measurements. In addition, we found a difference in gut microbiome composition in pronghorn across two regions separated by Interstate 80. Results indicated that the fecal microbiome may be a potential biomarker for the

spatial ecology of free-ranging ungulates. The core gut microbiome of these animals—including bacteria in the phyla Firmicutes (now Bacillota) and Bacteroidota—remained relatively stable across populations and biological metrics. These findings provide a baseline for the gut microbiome of pronghorn that could potentially be used as a target in monitoring health and population structure of pronghorn relative to habitat fragmentation, climate change, and management practices.

INTRODUCTION

Species do not exist in isolation, but rather experience interactions with a myriad of other species, including microorganisms. In a growing number of publications, individuals are viewed as a holobiont—a combination of the host and associated microbes, rather than a standalone organism (reviewed in [1]). Holobionts possess a hologenome, which is the sum of the host and microbial genomes [1]. Understanding how organisms function as a holobiont underpins symbiotic relationships that contribute to host physiology and demographics. The composition of the microbiome can influence many bodily processes, including immunity (reviewed in [2, 3]) and reproduction (reviewed in [4]). Specifically, the gut microbiome- or the microorganisms living in the digestive tract of an animal host - can have a large influence on processes related to digestion and nutrient absorption. To illustrate, the importance of symbiotic microbial genomes for cellulose digestion in mammalian herbivores has long been known [5, 6]. Growing research has recently revealed other important health effects of microbial symbionts. For example, the gut microbiome has been linked to feed efficiency in livestock [7-9], and studies in both house mice (*Mus musculus*) and humans (*Homo sapiens*) have established links between gut microbiome composition and fat deposition, body condition, and metabolism [10-15]. Microbes also may grant host species the ability to degrade secondary metabolites in plant foods, as seen in greater

sage-grouse (*Centrocercus urophasianus* [16]), several insect species (see examples in [17]), and woodrats (*Neotoma lepida* [18]).

While host-microbiome relationships are known to influence host health in humans, livestock, and model organisms and can potentially influence the management of wildlife species worldwide [19], fewer studies have investigated the influence of the microbiome on the health of wild animals [20]. Wild animals are more difficult to study because they often inhabit remote locations, and study conditions are difficult to control relative to laboratory environments. However, wild animals have been shown to possess distinct gut microbiomes from their captive or domestic counterparts [21-25]. As native landscapes undergo increasing conversion, fragmentation, and rapid climate change, microbial plasticity may confer greater adaptive capacity among host animals to mitigate deleterious effects [19, 26-27]. Although common in humans and agricultural applications, probiotic treatments may also represent a management tool for wildlife species [19, 28]. Additionally, recent research has explored topical applications of microbes to fight infectious diseases in bats [29] and amphibians [30] and evaluated the potential to use other strains of bacteria as wildlife gut probiotics [31]. Studies of host-microbiome interactions in wildlife could prove informative, particularly in habitats undergoing a change in land use.

Rangelands in the Intermountain West—which are dominated by sagebrush (*Artemisia spp.*)—serve as an ideal location to study wild microbiomes and their effects on host health in a rapidly changing landscape. The sagebrush steppe ecosystem covers a large portion of terrestrial North America. However, it has been reduced to 56% of its historic extent due to anthropogenic development, conversion to cropland, invasion of non-native plants, and conifer encroachment [32, 33]. Sagebrush species along with other woody plants in this ecosystem (e.g., bitterbrush

[*Purshia tridentata*], rabbitbrush [*Chrysothamnus spp.*], greasewood [*Sarcobatus vermiculatus*], saltbushes [*Atriplex spp.*], and junipers [*Juniperus spp.*]) are defended with potentially toxic chemicals [34-42]. Greater and Gunnison sage-grouse (*Centrocercus spp.*), pygmy rabbits (*Brachylagus idahoensis*), and pronghorn (*Antilocapra americana*) represent the relatively few vertebrate herbivores able to consume large amounts of sagebrush. Sagebrush can comprise nearly 100% of the diet of pygmy rabbits and sage-grouse in winter [43, 44]. These species have known physiological adaptations that explain their tolerance to sagebrush toxins [44-47] including the gut microbiota in sage-grouse that may degrade toxins [16]. While sagebrush may also dominate the diets of pronghorn [48-51], they can also subsist on grasses and forbs [51, 52]. Pronghorn can also shift to entirely different chemically-defended shrubs (e.g., rabbitbrush) when habitat fragmentation and degradation alters shrub communities [53]. Unlike other sagebrush specialists, the ruminant digestion [54], migratory behavior [55- 57], and observed dietary plasticity [53] of pronghorn may result in unique microbial communities and adaptations.

As landscapes continue to change, there is a need to understand the relationships between hosts, their microbial symbionts, and the environment. Here, we studied the gut microbiome of pronghorn, an endemic and iconic big game species of the American West with considerable ecological and economic value that reside largely in the sagebrush steppe. While the species' population size is lower than historical levels (i.e., before westward expansion), there are around 900,000 pronghorn as of 2017, and recent population trends are stable or increasing [58, 59]. However, some areas have seen local declines in pronghorn populations [58, 60-62]. Several anthropogenic factors create disturbance that pronghorn avoid and sometimes increase mortality for pronghorn, including fencing [63-68], livestock agriculture [57], human development [69], roads [67-72], and energy development [64, 67, 72-74]. Environmental factors such as harsh

winters or climatic changes [61, 62, 75, 76], disease [77], and coyote (*Canis latrans*) predation of fawns [78] can also negatively affect pronghorn populations. Epizootic Hemorrhagic Disease (EHD) and Bluetongue Virus (BTV) are two hemorrhagic diseases of pronghorn and other ungulates that can cause large die off events of animals [77, 79, 80], with one outbreak of BTV killing an estimated 3,200 pronghorn [77]. Pronghorn with higher body condition scores have been shown to be more resilient to harsh winters [75], and populations in better overall health and body condition likely will be more resilient to multiple stressors. Body condition has been positively related to population growth in bighorn sheep (*Ovis canadensis* [81]) and mule deer (*Odocoileus hemionus* [82]). Because gut microbiome composition has been related to feed efficiency and various measures of production in livestock [7-9], it is possible that gut microbiome composition could serve as a potential bio-indicator in wildlife populations as well. Indeed, work in mule deer has shown relationships between specific bacteria taxa and health metrics relating to protein and fat storage [83].

With the exception of studies evaluating protozoa in the rumen [84] and gut anaerobic fungi [85], the pronghorn gut microbiome has not been studied. To our knowledge, our study is the first to characterize bacterial gut microbiome communities in pronghorn. Here, we use fecal samples to describe the gut microbiome community in pronghorn and provide novel, baseline information on the core bacterial gut microbiome that can be used in future studies to compare with pronghorn residing in different environments, during different seasons, or after experiencing predicted future climatic or anthropogenic changes. In addition to this objective, we explored the relationships between gut microbiome composition and environmental (location, time of year), life history (age, body mass, body condition), and health (disease status for EHD and BTV) metrics. Prior to our study, body condition was assessed and related to the survival of

individual pronghorn [75]; we build on this previous work by investigating potential relationships between gut microbiome and body condition, with the goal of identifying a potential mechanism that may influence pronghorn survival. Based on previous literature regarding the relationships between microbiome and body condition [10-15], immunity (reviewed in [2, 3]), or environment [83, 86], we predicted pronghorn with differing body condition, serum disease status, or location would have different gut microbiome composition. Because limited work has been done on the pronghorn gut microbiome, our analyses were exploratory and descriptive in nature, with the goal of providing information for more targeted future research endeavors.

METHODS

Study Area and Capture Methods:

Our study used legacy samples and data collected from live-captured pronghorn in 2013 and 2014 from four study areas in south-central Wyoming: Baggs, Bitter Creek, Continental Divide-Creston Junction (hereafter CDC), and Red Desert (Fig 1). The focus of this earlier study was to better understand how environmental and intrinsic factors and anthropogenic stressors affected pronghorn survival and seasonal habitat selection during daytime and nighttime by those pronghorn populations. The four study areas occurred within the Red Desert, an iconic landscape for pronghorn, where populations were declining in the face of environmental and anthropogenic changes including increasing energy infrastructure. Thus, the study areas from which we obtained legacy samples were selected to meet the objectives for this earlier research, which are further described in Reinking et al. 2018 and 2019 [67, 75]. The predominant vegetation community within these study areas was Wyoming big sagebrush (*A. tridentata wyomingensis*) with an herbaceous understory of perennial grasses and forbs. Low lying areas with alkaline or

saline soils were dominated by black greasewood (*S. vermiculatus*) and Gardner's saltbush (*Atriplex gardneri*). In contrast, higher elevation areas were dominated by mountain big sagebrush (*A. t. vaseyana*), mixed shrub communities, and stands of aspen (*Populus tremuloides*). Other wildlife species common in the area included American badgers (*Taxidea taxus*), common raven (*Corvus corax*), elk (*Cervus canadensis*), greater sage-grouse, (*C. urophasianus*), mule deer (*O. hemionus*), sage thrasher (*Oreoscoptes montanus*), and white-tailed jackrabbit (*Lepus townsendii*). The topography of the area included sand deserts, rolling hills, badlands, and buttes. Oil and natural gas extraction, livestock grazing, and big game hunting were major land uses. For further information about the study areas, see [67, 75].

We captured 167 adult female pronghorn in November 2013 (n = 116), February 2014 (n = 13), and November 2014 (n = 38) [67, 75]. We used 159 samples in our analyses (n = 111 from November 2013, n = 13 from February 2014, and n = 35 from November 2014). For more information about the number of animals captured from each area in each capture period, see Table A in S1 Appendix. We captured pronghorn using helicopter net-gunning following the procedures of Jacques et al. [87] to reduce stress and capture-related mortality. During capture, we weighed animals to the nearest 0.1 kg and estimated age in half-year increments based on tooth eruption and wear [88]. A previous study corrected estimated age for this same group of animals based on cementum annuli analysis from dead animals [75]. Here, we applied the same correction factor to our age estimates to test whether this corrected age metric produced different results from estimated age. In addition, we measured the thickness of subcutaneous fat (mm) directly cranial to the cranial process of the tuber ischium [89] via ultrasound and assigned a leanness score based on the depth of the indentation (in inches) between the sacrosciatic ligament and caudal vertebrae (hereafter referred to as "ss-ligament") [75]. We collected fecal samples

directly from animals by rectal palpation and froze samples in a chest freezer at -18°C for later use. We took blood samples from the jugular vein using an 18-gauge, 2.54-cm needle, and the resulting samples were tested for two common diseases in ungulates: (1) Epizootic Hemorrhagic Disease (EHD) and (2) Bluetongue Virus (BTV). Blood samples were analyzed by the Wyoming Game and Fish Department Wildlife Health Laboratory/Wyoming State Veterinary Laboratory (Laramie, WY) to determine disease antibody status for each individual pronghorn for BTV or EHD (M. Miller, University of Wyoming, personal communication). We captured pronghorn a single time for this study, so data represents animal health at a single point in time. For more details on capture protocols, see [67, 75]. Pronghorn capture, handling, and monitoring procedures were approved by the Wyoming Game and Fish Department (Chapter 33-923 Permit) and the University of Wyoming Institutional Animal Care and Use Committee (protocol 20131028JB00037).

Microbial Community Profiling:

Pronghorn fecal samples were sequenced for gut microbiome composition using 16S rRNA amplicon sequencing [90], which involves amplifying the bacterial 16S ribosomal RNA (rRNA) gene to determine which bacteria are present in the sample, and in what quantity. The DNA extraction, library preparation, and pooling were conducted by the Knight Lab in the Center for Microbiome Innovation at the University of California, San Diego (UCSD), and sequencing was conducted at the UCSD Institute for Genomic Medicine Genomics Facility using previously published methods [91] as described in [92]. Briefly, the Qiagen MagAttract PowerSoil DNA KF Kit was used for DNA extraction, in accordance with manufacturer protocols. The 515FB forward primer (5’ –GTGYCAGCMGCCGCGGTAA-3’) and the 806RB reverse primer (5’-GGACTACNVGGGTWTCTAAT-3’) were used to target the V4 region of the bacterial 16S

rRNA gene. Samples were amplified in triplicates with 25µL polymerase chain reaction (PCR) reactions and then pooled samples before running on an agarose gel. Amplicons were quantified using a Quant-iT PicoGreen dsDNA Assay Kit following manufacturer instructions and cleaned using MoBio UltraClean PCR Clean-Up Kit following manufacturer instructions [91,92]. The laboratory facility included eight extraction blanks for negative controls that contained no sample but were processed through their standard extraction and library preparation protocol. 16S libraries were sequenced on one lane of an Illumina MiSeq using 2 x 150 bp paired-end sequencing. Sequence data was stored under study number 12842 on the Qiita platform [93].

Data Analysis:

We performed data processing and initial analysis using QIIME2 v. 2021.4 [94]. We de-multiplexed FASTQ files, yielding 173 samples (including 8 blanks) and a total of 3,888,708 reads (mean read count ± SD =22,478 ± 8,047 including blanks or 23,239 ± 7,209 excluding blanks). We performed de-noising and de-replication steps using the Divisive Amplicon Denoising Algorithm (DADA2) [95] module within the QIIME2 pipeline. After visual quality score inspection, we trimmed reads before base pair 12 and after base pair 150 to maintain high quality (Phred Score >30, for >95% of reads). After filtering, the total read count was 2,035,077 (mean read count ± SD = 12,114 ± 4,082 excluding blanks). Read counts at various steps of the DADA2 pipeline can be found in the supporting information (Table B in S1 Appendix). We found 3,389 unique amplicon sequence variant (ASV) sequences from this dataset. We used SILVA databases (Silva 138 SSURef NR99 515F/806R region sequences Silva 138 SSURef NR99 515F/806R region taxonomy and Silva 128 SEPP reference database) [96] to assign taxonomy to the species level, using QIIME2.

We conducted downstream analysis using R v. 4.3.0. We imported QIIME2 readable files (*.qza) into R using the package 'qiime2R' (v. 0.99.6) [97]. We used the 'phyloseq' package (v.1.44.0) [98] to remove non-bacterial (14 ASVs), mitochondrial (10 ASVs), and chloroplast (24 ASVs) ASVs, yielding 3341 ASVs. We used the package 'decontam' (v.1.20.0) [99] to decontaminate the remaining reads using the blanks as negative controls. We then removed negative controls and samples with ambiguous metadata, yielding 159 samples and 3,065 ASVs. We generated a rarefaction curve (S1 Fig) to determine a rarefaction cutoff of 4,936 reads for alpha diversity- or diversity within a sample- analysis, thereby excluding four samples due to low read count from alpha diversity analysis. During rarefaction, an additional 273 ASVs were removed. We calculated alpha diversity metrics including Simpson's diversity index, Shannon's diversity index, and observed richness using the 'phyloseq' package. For our analyses, we set the statistical significance at alpha = 0.10. Our alpha level was higher than the conventional level of 0.05 that is often used, however, we adjusted to a higher alpha to better align with our more exploratory research objectives [100]. We felt that with these objectives in mind, accepting a higher false positive (Type I) error rate would allow us lower false negative (Type II) error [101] and thus the ability to find more potential patterns that could be explored in future studies. We compared alpha diversity metrics across the discrete pronghorn metrics (capture period, study area, and disease status) using Kruskal-Wallis tests, because assumptions of normality were not met for most comparisons. We made pairwise comparisons using a pairwise Wilcoxon test with a Bonferroni correction. To evaluate whether continuous life history metrics (age, body weight, body condition measures) were correlated with measures of alpha diversity, we performed Spearman's rank correlations between alpha diversity metrics and these continuous metadata metrics. Because some samples were missing capture data for certain metrics, we ran each

analysis on the maximum number of samples possible of the 155 rarified samples that contained complete information for the chosen metric. As later analyses showed the presence of interactions, we conducted additional alpha diversity analyses within subsets of our groups. A description of these analyses and results can be found in Appendix 2 (S2 Appendix). We also ran linear regression models to look at the effect of the combined pronghorn metrics on observed richness. Details of this analysis can be found in Appendix 2 (S2 Appendix).

For analysis of beta diversity—or the differences in the diversity of species between ecosystems in a similar area—we transformed the read counts of our non-rarefied data to relative abundance. We divided the number of reads for each taxon within a sample by the total number of reads for that sample. We also log-transformed our data to test whether trends were any stronger when relative abundance was represented on a logarithmic scale to account for dominance of a few ASVs. However, patterns that we found mirrored results on the original scale (Table H and I in S3 Appendix) and we chose to report our non-log transformed data for more intuitive interpretation. For more information on log-transformation and analysis see Appendix 3 (S3 Appendix). To visualize gut microbiome differences among pronghorn for each metric, we generated ordination plots using Principal Coordinate Analysis (PCoA) using the Bray Curtis dissimilarity metric and included all 159 samples. In addition, we used the envfit() function in the 'vegan' package (v. 2.6.4) [102], to investigate how continuous values of age, weight, and ss-ligament were related to explanatory PCoA axes. We performed permutational multivariate ANOVA (PERMANOVA) tests with 999 permutations using the adonis2 function [103] in the 'vegan' package, also using Bray Curtis as a distance metric.

As our study was somewhat exploratory in nature, we completed a two-phase analysis to better understand important patterns. We first performed PERMANOVA tests on pronghorn

metadata metrics individually to test for differences ($p < 0.100$), with continuous variables binned to allow for comparisons among groups. In the second phase, for each metric of interest, we chose one of the variables representing each pronghorn metric for input into a model, avoiding inputting multiple factors that represented the same metadata metric. Our combined PERMANOVA investigated the marginal effects of the different metrics and included study area; BTV status; EHD status; weight by 5-kg increments; age by young, middle age, and old groupings; and ss-ligament. We also performed the combined PERMANOVA with interactions between study area and the pronghorn intrinsic measures to investigate if the relationships between pronghorn gut microbiome and pronghorn age, disease status, body fat, weight, or age varied in different study locations. For more information on initial exploratory analysis, binning, and choice of factors for our second phase of analysis see Appendix 3 (S3 Appendix).

To ease the interpretation of the effects of the continuous pronghorn metrics of age, weight, and ss-ligament, we also performed a Redundancy Analysis (RDA). We centered and standardized the variables of age, weight, and ss-ligament using the decostand() function in the 'vegan' [102] package. We transformed the relative abundance of the microbial community using the Hellinger transformation to avoid issues of double zeros in community abundance data increasing the similarity between sites [104]. We performed RDA analysis both with the reduced set of continuous metrics of age, weight, and ss-ligament as well as the full set of metrics including discrete variables of study area, EHD status, and BTV status. After performing the RDA, we calculated the adjusted R^2 to correct for our number of explanatory variables using the RsquareAdj() function in the 'vegan' package [102] and determine the amount of variance explained.

RESULTS

Pronghorn Life History, Environmental, and Health Metrics

Our final data set included information from 159 pronghorn. Of these, 30 animals (18.87%) were positive for EHD and 21 animals (13.21%) were positive for BTV. We included 45 animals in Baggs, 45 in Red Desert, 22 in CDC, and 47 in Bitter Creek study areas. Pronghorn ages ranged from 1 to 12 years, while our corrected pronghorn ages ranged from 2.80 to 11.44 years. Pronghorn weights ranged from 39.66 to 59.46 kg. The measures for indentation of ss-ligament ranged from 0-3.81 cm (0-1.50 inches), with higher values indicating a greater depression above the sacrosciatic ligament (i.e., less body fat padding this area). Measurements for maximum rump fat thickness ranged from 0 to 7 mm. For more information about pronghorn metric values within each study area see Supplemental Table C (Table C in S1 Appendix)

Microbiome Composition

Bacterial composition of pronghorn fecal samples included 23 phyla, 35 classes, 83 orders, 143 families, 267 genera, and 308 species (Table 1). Composition of the bacterial community was dominated by the phyla Firmicutes (now Bacillota), followed by Bacteroidota across all study areas (Fig 2). Even at finer taxonomic levels (e.g., Family), 15 core bacterial groups dominated the community and represented approximately 95% of our assigned reads (Table 1). The composition of bacterial phyla, classes, orders, and families was similar across samples from different study areas (Figs 2-5). Common families included Bacteroidaceae, Christensenellaceae, Lachnospiraceae, Oscillospiraceae, and Ruminococcaceae (Fig 5). We observed this similar pattern of dominant bacterial groups when we grouped samples by other metrics (S2-4 Fig), albeit with variation among individuals (S5 Fig).

Alpha diversity

We found few statistical differences when assessing alpha diversity relative to pronghorn metrics. For our discrete metrics, we found differences ($p < 0.100$) in all alpha diversity metrics between the different capture periods (Table 2) with November 2013 samples having higher Shannon and Simpson indices compared to both November 2014 and February 2014 samples, and November 2013 and 2014 samples having higher observed richness than February 2014 samples (Table 3, Fig 6A). The study area did not affect this general pattern observed between capture periods (Fig 6B). For animals that were either north or south of Interstate 80 (I-80), we found Shannon's diversity index (mean ± SE: north = 4.334 ± 0.034, south = 4.412 ± 0.032) and observed richness measures (north = 186.281 ± 5.653, south = 201.099 ± 5.017) were different (Table 2), with animals south of the highway having higher alpha diversity (Table 3, Fig 6C). Simpson's diversity index did not differ between pronghorn north or south of I-80, while no diversity indices differed between animals in different study areas or with differing disease statuses (Table 2). We did not observe strong correlations with alpha diversity metrics and any of our continuous life history metrics of age, weight, and body condition (Table 4). The models used to evaluate the combined effects of pronghorn metrics on alpha diversity supported the results we found when looking at metrics singularly (S2 Appendix). Pronghorn north and south of I-80 showed overall similar relationships between alpha diversity metrics and pronghorn metrics with some slight differences (S2 Appendix).

Beta diversity

We found that the first two axes of PCoA using the Bray Curtis distance for ordinations accounted for 13.6% (7.6% and 6.0% respectively) of the variation among our samples (Fig 7). When we visualized with PCoA ordination plots, microbial communities appeared to group clearly when partitioned by study area (Fig 7A) in addition to whether a study area was north or

south of I-80 (Fig 7A; note shape beta dispersion). None of the continuous life history metrics of age, weight, or ss-ligament were related to our PCoA axes 1 and 2 (Fig 7B). While we observed some separation between the November 2014 group compared to November 2013 and February 2014 groups (Fig 7C), it is difficult to attribute this solely to a capture period effect, as the November 2014 group was dominated by individuals from the CDC study area, and there was only a single capture period at this location (Table A in S1 Appendix).

Our first phase of PERMANOVA analysis indicated differences based on the study area, location north or south of I-80, capture period and season, EHD status, and ss-ligament (Table H in S3 Appendix) when run as single metrics. This first stage of analysis also indicated that the best explanatory variable for location was study area versus whether the area was north or south of I-80, although both were highly significant ($p = 0.001$; Table H in S3 Appendix). For more information on the results of the first phase of PERMANOVA and our choice of metrics for our second phase of analysis see Appendix 3 (S3 Appendix).

In the second phase of analysis, where we combined the study area, ss-ligament, categorical age, binned weights, BTV, and EHD metrics together into a PERMANOVA testing for marginal effect of each variable, we found significant differences only for study area ($p = 0.001$; Table 5) with both EHD ($p = 0.823$) and ss-ligament ($p = 0.253$) no longer important when the marginal effects of other variables were accounted for (Table 5). When we added interactions between study area and each pronghorn intrinsic metric, we found the interaction between study area and ss-ligament ($p = 0.035$) and study area and age ($p = 0.077$) to be important ($p < 0.100$), while interactions between study area and either BTV ($p = 0.691$), EHD ($p = 0.643$), or weight ($p = 0.800$) were not significant (Table 5). This indicated that the effect of age or ss-ligament may differ across study areas, so we subset our data by study area to explore

this. Further PERMANOVAs within subsets of single study areas showed that within the Red Desert study area, there were differences in the microbiome of pronghorn of different ages ($p = 0.029$, Table M in S4 Appendix). However, we found no differences related to age within other study areas (Tables J, K, and L in S4 Appendix). Ss-ligament was not statistically related to the microbiome composition within any subset of a single study area (Tables J, K, L, and M in S4 Appendix). Results of analyses on subsets of single study areas are detailed in Appendix 4 (S4 Appendix).

When we completed RDA modeling including only continuous variables of age, weight, and ss-ligament, we did not produce a significant model ($p = 0.889$). However, when we added study area, EHD status, and BTV status, a significant model ($p = 0.001$) resulted with the variables for study area ($p = 0.001$) and EHD status ($p = 0.050$) being important. ($p < 0.100$). However, the effects were subtle with only 8.8% (3.1% adjusted) of the microbial community composition explained by our included pronghorn life metrics. Overall, the results from our RDA analysis support the results from the PERMANOVA, with the study area showing a significant effect but only explaining a small portion of the variation in the gut microbiome community.

DISCUSSION

To our knowledge, this is the first study of the bacterial gut microbiome of pronghorn. Thus, our first objective was to describe the gut microbial composition. We found that the core microbiome—or the set of microbes that are characteristic of a specific environment or host species—exhibited high consistency in pronghorn in the Red Desert. The phyla Firmicutes (now Bacillota) and Bacteroidota dominated pronghorn gut microbiomes across all study areas. Even at finer taxonomic scales (e.g., order, class, family, and genus), we saw a similar group of 10-15

bacterial ASVs constituting the majority (>95%) of the gut microbiome in these animals, with the composition similar when animals were grouped by various metrics. These results agree with other gut microbiome studies of North American wild and domestic herbivores, which demonstrate Firmicutes and Bacteroidota make up a large proportion of the gut microbiome [7, 8, 83, 105]. Previous studies have shown both of these phyla to be important in breaking down complex carbohydrates [106]. Bacteroidota tend to be more important for polysaccharide breakdown [107] as evidenced by their evolution of polysaccharide utilization loci [108], while various Firmicutes have shown to be integral to cellulose degradation [109]. As previous literature on the pronghorn bacterial gut microbiome is lacking, and pronghorn are not listed within the existing Animal Microbiome Database [110], our analysis is a foundational descriptor of the pronghorn gut microbiome as represented by fecal samples, providing a novel starting point for future research endeavors. In relation to our second objective, we found relationships between pronghorn gut microbiome composition and both study area and capture period, while relationships between gut microbiome composition and life history and health metrics had more subtle effects.

Similarities in gut microbial communities of animals within the same geographic area and differences between animals in different geographic areas may be attributed to interactions with conspecifics, similar habitats (plants available or environmental conditions), or both. Our finding of beta diversity differences between study areas is supported by both our PERMANOVA and RDA results and agrees with previous studies in wild mammals. For example, differences have been found in the gut microbiomes of mule deer in different seasons and geographic locations [83] and brown bears (*Ursus arctos*) living under different environmental conditions [86]. Additionally, studies in equines [111], baboons (*Papio cynocephalus*) [112], and humans [113]

have shown that an animal's gut and other microbiomes can be affected by social interactions (review in [114]). We also observed differences in alpha and beta diversity when our study animals were grouped into those individuals occurring north or south of I-80 (Table 2, Fig 7A, and Table H in S3 Appendix). Highways with higher traffic levels or non-wildlife-friendly fencing can become complete barriers to pronghorn [64], and I-80 has been identified as a largely impermeable barrier to pronghorn movement [115]. The inability of pronghorn to easily cross I-80 and acquire microbes from novel environments or conspecifics via social interaction may contribute to these different gut microbiome communities (i.e., microbiome dispersal limitation). Moeller et al. [116] found that the diversification of gut microbiome composition of various mammals was affected by the barrier effect that geographical distance presents to bacterial dispersal. In our case, I-80 could be acting as a similar barrier to microbial dispersal in pronghorn populations.

Pronghorn gut microbiome composition differed by capture period in many of our tests. While our sample representation from different seasons was not consistent due to the uneven sampling nature of the legacy study, capture period and thus season showed differences in gut microbiome composition throughout many of our analyses and warrants further investigation in future studies. Alpha diversity, as measured by Shannon's index, Simpson's index, and observed richness, was lower during February 2014 than either one or both November capture periods, depending on the chosen diversity measure of interest (Table 3, Fig 6A). This pattern was consistent across study areas, with observed richness trending lower in February compared to November in the Baggs, Bitter Creek, and Red Desert study areas (Fig 6B). Beta diversity also differed by capture period in our single factor PERMAONVAS (Table H is S3 Appendix), with November 2014 samples appearing distinct from the other two groups in ordination (Fig 7C).

However, because all animals in the CDC study area were captured in November 2014 (Table A in S1 Appendix), we cannot attribute this effect solely to the capture period. Our finding of potential seasonal effects on the gut microbiome is consistent with findings in mule deer in Utah, USA, which were shown to have different gut microbiomes in December as compared to March [83]. Although these researchers were unable to establish causal links, they suggested potential mechanisms that could explain relationships between select microbial taxa and winter physiologic changes of mule deer, such as fat or protein catabolism [83]. Pronghorn also experience similar changes across seasons including metabolization of fat stores [117], shifts in diet [49, 51, 52], or seasonal behavior changes such as changes in group size [48, 118]. Any of these seasonal shifts in pronghorn life history have the potential to influence gut microbiome composition, a possible explanation for the differences we observed in alpha and beta diversity between capture periods. Unfortunately, we were unable to include capture period, and therefore the effect of season, in our combined PERMANOVA and RDA models as some pronghorn metrics of interest were not recorded during February captures. The effect of season should be investigated in future studies of wild animal gut microbiomes to better understand how microbial composition changes across seasons in different species. Identifying which microbial taxa are involved in seasonal shifts may prove important to understanding the mechanisms for how host animals cope with nutrient scarcity in the winter or could inform research into selecting specific microbial taxa for use in probiotic applications or as animal health bio-indicators.

In addition to geography and season, we investigated relationships between gut microbiome composition and animal health markers such as body condition and disease status. Previous research has demonstrated links between body condition and gut microbiome composition including relationships with feed efficiency in livestock [7- 9] and links between gut

microbiome composition and fat deposition, body condition, and metabolism in both mice and humans [10-15]. Therefore, we predicted we would see differences in the gut microbiome of pronghorn with differing levels of body condition. Ss-ligament, a measure of body condition, was previously related to pronghorn survival in our study location [75]. Therefore, we chose this specific body condition metric in our analyses because a link between ss-ligament and gut microbial composition could show potential for a link between gut microbiome and survival in pronghorn. When testing metrics with individual PERMANOVAs, differences in gut microbiomes of animals with varying ss-ligament measures seemed to be supported (Table H in S3 Appendix). However, our combined PERMANOVA model suggests body condition, as indexed by ss-ligament, to have a more context-dependent relationship with gut microbiome composition, with the effect of ss-ligament not significant on its own in the combined model, but having a significant interaction with study area when interactions were added to the combined model (Table 5). In addition, measures of alpha diversity were not correlated with values for body condition, except for a subtle relationship within a subset of only animals south of I-80 (Table F in S2 Appendix). Therefore, while some of our analyses provide support for subtle gut microbiome differences in animals of varying body condition, our results do not conclusively show a clear link between gut microbiome composition and measures for body condition. However, the potentially complex trends we saw warrant further investigation into the relationship between gut microbiome and body condition. We saw some support for differences in animals with differing EHD status in our single metric PERMANOVAs and RDA, but we did not see differences in gut microbiome community related to disease status for BTV or EHD in our final PERMANOVA model. In addition, within our alpha diversity analyses we only saw alpha diversity differing by disease status in a subset of animals north of I-80 (Table D in S2

Appendix). These results suggest that the pronghorn gut microbiome in our study may be resilient across these disease exposures. Similar results regarding disease were found in gut microbiomes of moose in Minnesota, with pathogen exposure not predictive of gut microbial community. As in our study, pathogens in these moose were detected by serological evidence, showing evidence of previous exposure rather than current infection [105]. Similar to the moose study, we reason that the lack of consistent disease effect on the gut microbiome could be due to animals not having a current infection, or that the animal's gut microbiomes are resilient to pathogen exposure.

While we did find that gut microbial communities differed based on some of our study metrics, these metrics only explained a small amount of the variation in the pronghorn gut microbiome. This may be due to the consistency in the core bacteria that dominated the pronghorn gut microbiome. This consistency in gut microbiome with only subtle differences may be seen because we only evaluated a single species of host animal. A study in seven species of woodrats (*Neotoma* spp.) found that while diet, host phylogeny, and geography collectively explained 49% of the variation in the wild woodrat gut microbiome composition, host species had the greatest effect, explaining 35% of the variation [119]. Another possibility is that individual variation, known to explain large amounts of variation in the gut microbiome in other species, was not included in our study. A study of horses that collected multiple samples over time from the same animals found large variation among individuals, and individual animal ID accounted for about 50% of the variation in the samples [111]. Due to concerns about capture-related mortality during repeat captures [75], we only implemented a single capture for each individual, so we were unable to investigate this effect. However, when assessing the gut microbial community of individual animals (S5 Fig), we observed that some individuals

possessed gut microbiome compositions divergent from the general patterns. It is possible that within pronghorn, gut microbial differences are largely unique for individuals, and thus, we saw only subtle differences when comparing grouped animals. In order to properly account for this effect, we would need a study design that collects repeated samples from the same individual pronghorn. Diet has also been shown to have a large influence on gut microbial composition. This is evident when comparing animals with diverse diets and gut types [21], and in morphologically similar animals. A study of two similar woodrat species (*N. bryanti and N. lepida*) consuming different plants found that gut microbiome composition was associated with the common diet plants of each species [120]. Another study of woodrats found that diet richness was correlated with gut microbiome richness, and diet explained 16% of the variation in microbial composition [119]. We do not have data regarding the diet of pronghorn in our study, however, it is possible that because all captures occurred during the dormant season for plants, pronghorn may only have had limited forage availability, thus similar diets, leading to similarities in gut microbiomes. Therefore, since our study includes hosts of the same species, and we could not include the effect of diet or individuality—all factors that have been shown to be highly explanatory of gut microbiome composition—it is logical for our results to only show subtle effects.

CONCLUSIONS

The microbial community can change and adapt within an animals' lifetime; thus, microbiome changes could enable hosts to adapt more quickly to changing conditions [121]. It has been proposed that plasticity in the gut microbiome may lead to greater host adaptation capability for wildlife in the face of rapidly changing environments [26]. To our knowledge, our study was the first to investigate the bacterial composition of the pronghorn gut microbiome; thus, more data is

needed from pronghorn occupying a broader geographic area before conclusions can be made as to whether the core gut microbiome we observed in winter in the Red Desert of south-central Wyoming is similar across the range of pronghorn, or unique to this population. Our study found differing gut microbiome composition in various study areas; if future studies confirm pronghorn gut microbiomes continue to differentiate with increasing habitat differences or geographic distances, pronghorn may be exhibiting locally adapted gut microbiomes. On the other hand, if future studies find microbes in diverse populations are more constrained, it may show there is little plasticity potential in the gut microbiome composition of pronghorn, potentially due to similarity in host diet composition, host genetics, or both. Furthermore, our study shows that movement barriers, such as I-80, may limit a population's ability to exchange microbes with other populations or novel environments, potentially decreasing advantages conferred by microbiome plasticity.

In either scenario, understanding differences or similarities in pronghorn gut microbiomes in distinct habitats may have important implications as wildlife managers increasingly recognize the value of microbial tools for future management. Scientists are discussing the need to consider microbiome composition when reintroducing or translocating animals [19, 23, 27]. The potential value of probiotic treatments for wildlife species has been discussed [19, 28], with some strains of bacteria being evaluated as wildlife gut probiotics [31]. One study identified potential taxa that could serve as bio-indicators for mule deer health [83]. If effective, tools based on locally adapted or core gut microbes in any of these capacities, such as increasing translocation success, serving as a beneficial probiotic to increase herd health, or being used as a biomarker for management, will be valuable not only in pronghorn but also in more vulnerable species. We believe that understanding the gut microbiome composition in

pronghorn across their range will prove useful as we attempt to manage populations in a time of growing human disturbance and environmental change and our study provides a baseline understanding of the gut microbiome composition in these iconic herbivores.

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REFERENCES

1. Bordenstein SR, Theis KR. Host biology in light of the microbiome: Ten principles of holobionts and hologenomes. PLoS Biol. 2015; 13(8): e1002226. doi: 10.1371/journal.pbio.1002226

2. Cerf-Bensussan N, Gaboriau-Routhiau V. The immune system and the gut microbiota: Friends or foes? Nature Reviews Immunology. 2010; 10: 735–44. doi: 10.1038/nri2850

3. Levy M, Blacher E, Elinav E. Microbiome, metabolites and host immunity. Curr Opin Microbiol. 2017;35:8–15. doi: 10.1016/j.mib.2016.10.003

4. Comizzoli P, Power M. Reproductive microbiomes in wild animal species: A new dimension in conservation biology. Adv Exp Med Biol. 2019;1200:225–40. PMID: 31471799 doi: 10.1007/978-3-030-23633-5_8

5. Janis C. The evolutionary strategy of the Equidae and the origins of rumen and cecal digestion. Evolution (NY) 1976; 30(4): 757-774.

6. Van Soest, P.J. Nutritional Ecology of the Ruminant, 2nd ed. Ithaca, New York: Cornell University Press; 1994.

7. McCann JC, Wiley LM, Forbes TD, Rouquette FM, Tedeschi LO. Relationship between the rumen microbiome and residual feed intake-efficiency of Brahman bulls stocked on bermudagrass pastures. PLoS One. 2014;9(3):1–6. e91864. doi: 10.1371/journal.pone.0091864

8. Myer PR, Smith TPL, Wells JE, Kuehn LA, Freetly HC. Rumen microbiome from steers differing in feed efficiency. PLoS One. 2015;10(6). E0129174. doi: 10.1371/journal.pone.0129174

9. Shabat SK., Sasson G, Doron-Faigenboim A, Durman T, Yaacoby S, Berg Miller ME, et al. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME J. 2016;10(12):2958–2972. doi:10.1038/ismej.2016.62

10. Bäckhed F, Ding H, Wang T, Hooper L V., Gou YK, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A. 2004;101(44):15718–23. doi: 10.1073/pnas.0407076101

11. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A. 2005;102(31):11070–5. doi: 10.1073/pnas.0504978102

12. Ley, R.E., Turnbaugh, P.J., Klein, S., Gordon JI. Human gut microbes associated with obesity. Nature. 2006;444:1022–3. doi:10.1038/nature4441022a

13. Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. Diet-Induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe. 2008;3(4):213–23. doi: 10.1016/j.chom.2008.02.015

14. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature. 2009;457:480–4. doi: 10.1038/nature07540

15. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. Nature. 2013;500:541–6. doi: 10.1038/nature12506

16. Kohl KD, Connelly JW, Dearing MD, Forbey JS. Microbial detoxification in the gut of a specialist avian herbivore, the greater sage-grouse. FEMS Microbiol Lett. 2016;363(14):1–6. doi:10.1093/femsle/fnw144

17. Hammer TJ, Bowers MD. Gut microbes may facilitate insect herbivory of chemically defended plants. Oecologia. 2015;179(1):1–14. doi: 10.1007/s00442-015-3327-1

18. Kohl KD, Weiss RB, Cox J, Dale C, Dearing MD. Gut microbes of mammalian herbivores facilitate intake of plant toxins. Ecol Lett. 2014;17(10):1238–46. doi: 10.1111/ele.12329

19. Trevelline BK, Fontaine SS, Hartup BK, Kohl KD. Conservation biology needs a microbial renaissance: A call for the consideration of host-associated microbiota in wildlife management practices. Proc Biol Sci. 2019;286(1895). doi: 10.1098/rspb.2018.2448

20. Amato KR. Co-evolution in context: The importance of studying gut microbiomes in wild animals. Microbiome Sci Med. 2013;1(1):10–29. doi: 10.2478/micsm-2013-0002

21. McKenzie VJ, Song SJ, Delsuc F, Prest TL, Oliverio AM, Korpita TM, et al. The effects of captivity on the mammalian gut microbiome. Integr Comp Biol. 2017;57(4):690–704. doi: 10.1093/icb/icx090

22. Metcalf JL, Song SJ, Morton JT, Weiss S, Seguin-Orlando A, Joly F, et al. Evaluating the impact of domestication and captivity on the horse gut microbiome. Sci Rep. 2017;7(1):1–9. doi: 10.1038/s41598-017-15375-9

23. Li Y, Zhang K, Liu Y, Li K, Hu D, Wronski T. Community composition and diversity of intestinal microbiota in captive and reintroduced Przewalski's Horse (Equus ferus przewalskii). Front Microbiol. 2019;10(1821). doi: 10.3389/fmicb.2019.01821

24. Salgado-Flores A, Tveit AT, Wright AD, Pope PB, Sundset MA. Characterization of the cecum microbiome from wild and captive rock ptarmigans indigenous to Arctic Norway. PLoS One. 2019;14(3):1–21. doi: 10.1371/journal.pone.0213503

25. Yao R, Xu L, Hu T, Chen H, Qi D, Gu X, et al. The "wildness" of the giant panda gut microbiome and its relevance to effective translocation. Glob Ecol Conserv. 2019;18. doi: 10.1016/j.gecco.2019.e00644

26. Alberdi A, Aizpurua O, Bohmann K, Zepeda-Mendoza ML, Gilbert MTP. Do vertebrate gut metagenomes confer rapid ecological adaptation? Trends Ecol Evol. 2016;31(9):689–99. doi: 10.1016/j.tree.2016.06.008

27. Bahrndorff S, Alemu T, Alemneh T, Lund Nielsen J. The microbiome of animals: implications for conservation biology. Int J Genomics. 2016. doi: 10.1155/2016/5304028

28. Mckenzie VJ, Kueneman JG, Harris RN. Probiotics as a tool for disease mitigation in wildlife: Insights from food production and medicine. Ann N Y Acad Sci. 2018;1429:18–30. doi: 10.1111/nyas.13617

29. Cheng TL, Mayberry H, McGuire LP, Hoyt JR, Langwig KE, Nguyen H, et al. Efficacy of a probiotic bacterium to treat bats affected by the disease white-nose syndrome. J Appl Ecol. 2017;54(3):701–8. doi: 10.1111/1365-2664.12757

30. Bletz MC, Loudon AH, Becker MH, Bell SC, Woodhams DC, Minbiole KPC, et al. Mitigating amphibian chytridiomycosis with bioaugmentation: Characteristics of effective probiotics and strategies for their selection and use. Ecol Lett. 2013;16(6):807–20. doi: 10.1111/ele.12099

31. Stedman A, van Vliet AHM, Chambers MA, Gutierrez-Merino J. Gut commensal bacteria show beneficial properties as wildlife probiotics. Ann N Y Acad Sci. 2020;1467(1):112–32. doi: 10.1111/nyas.14302

32. Schroeder MA, Aldridge CL, Apa AD, Bohne JR, Braun CE, Bunnell SD, et al. Distribution of sage-grouse in North America. Condor. 2004;106(2):363–76. doi: 10.1650/7425

33. Davies KW, Boyd CS, Beck JL, Bates JD, Svejcar TJ, Gregg MA. Saving the sagebrush sea: An ecosystem conservation plan for big sagebrush plant communities. Biol Conserv. 2011;144(11):2573–84. doi: 10.1016/j.biocon.2011.07.016

34. Kelsey RG, Stephens JR, Shafizadeh F. The chemical constituents of sagebrush foliage and their isolation. J Range Manag. 1982;35(5):617-22. doi:10.2307/3898650

35. Adams RP, Hogge L. Chemosystematic studies of the Western North American junipers based on their volatile oils. Biochem Syst Ecol. 1983;11(3):189–93. doi: 10.1016/0305-1978(83)90052-2

36. Hegerhorst DF, Weber DJ, McArthur ED, Khan AJ. Chemical analysis and comparison of subspecies of Chrysothamnus nauseosus and other related species. Biochem Syst Ecol. 1987;15(2):201–8. doi: 10.1016/0305-1978(87)90020-2

37. Sanderson SC, Pendleton RL, McArthur ED, Harper KT. Saponin effect on small mammal forage preference in a planting of Atriplex canescens. In: Provenza FD, Flinders JT, McArthur ED, compilers. Proceedings - Symposium on plant-herbivore interactions; 1985 Aug 7-9; Snowbird, UT. Ogden, UT: USDA Forest Service, Intermountain Research Station; 1987 Jan. General technical report: INT-222. pp. 74-77.

38. Clausen TP, Provenza FD, Burritt EA, Reichardt PB, Bryant JP. Ecological implications of condensed tannin structure: A case study. J Chem Ecol. 1990;16(8):2381–92. doi: 10.1007/BF01017463

39. Meyer MW, Karasov WH. Chemical aspects of herbivory in arid and semiarid habitats. In: Palo RT, Robbins CT. Plant defenses against mammalian herbivory. Boca Raton: CRC Press; 1991. pp. 167- 187.

40. Cibils AF, Swift DM, McArthur ED. Plant-herbivore interactions in Atriplex: current state of knowledge. Ogden (UT): USDA Forest Service, Rocky Mountain Research Station; 1998 Aug. General Technical Report: RMRS-GTR-14.

41. Jaeger DM, Runyon JB, Richardson BA. Signals of speciation: volatile organic compounds resolve closely related sagebrush taxa, suggesting their importance in evolution. New Phytol. 2016;211(4):1393–401. doi: 10.1111/nph.13982

42. Hudon SF, Zaiats A, Roser A, Roopsind A, Barber C, Robb BC, et al. Unifying community detection across scales from genomes to landscapes. Oikos. 2021;130(6): 831–43. doi: 10.1111/oik.08393

43. Connelly JW, Schroeder MA, Sands AR, Braun CE. Guidelines to manage sage grouse populations and their habitats. Wildl Soc Bull. 2000;28(4): 967–85.

44. Shipley LA, Davis EM, Felicetti LA, McLean S, Forbey JS. Mechanisms for eliminating monoterpenes of sagebrush by specialist and generalist rabbits. J Chem Ecol. 2012;38(9): 1178–89. doi: 10.1007/s10886-012-0192-9

45. Kohl KD, Pitman E, Robb BC, Connelly JW, Dearing MD, Forbey JS. Monoterpenes as inhibitors of digestive enzymes and counter-adaptations in a specialist avian herbivore. J Comp Physiol B. 2015;185:425–34. doi: 10.1007/s00360-015-0890-z

46. Nobler JD, Camp MJ, Crowell MM, Shipley LA, Dadabay C, Rachlow JL, et al. Preferences of specialist and generalist mammalian herbivores for mixtures versus individual plant secondary metabolites. J Chem Ecol. 2019;45(1):74–85. doi: 10.1007/s10886-018-1030-5

47. Oh KP, Aldridge CL, Forbey JS, Dadabay CY, Oyler-Mccance SJ. Conservation genomics in the sagebrush sea: Population divergence, demographic history, and local adaptation in sage-grouse (Centrocercus spp.). Genome Biol Evol. 2019;11(7):2023–34. doi:10.1093/gbe/evz112

48. Bayless SR. Winter food habits, range use, and home range of antelope in Montana. J Wildl Manage. 1969;33(2): 538–51. doi: 10.2307/3799376

49. Ngugi KR, Powell J, Hinds FC, Olson RA. Range animal diet composition in southcentral Wyoming. J Range Manag. 1992;45(6): 542–5. Doi: 10.2307/4002568

50. Singer FJ, Norland JE. Niche relationships within a guild of ungulate species in Yellowstone National Park, Wyoming, following release from artificial controls. Can J Zool. 1994;72(8):1383–94. doi:10.1139/z94-183

51. Cain JW, Avery MM, Caldwell CA, Abbott LB, Holechek JL. Diet composition, quality and overlap of sympatric American pronghorn and gemsbok. Wildlife Biol. 2017; doi: 10.2981/wlb.00296

52. Schwartz CC, Nagy JG. Pronghorn diets relative to forage availability in northeastern Colorado. J Wildl Manage. 1976;40(3):469–78. doi: 10.2307/3799949

53. Boccadori SJ, White PJ, Garrott RA, Borkowski JJ, Davis TL. Yellowstone pronghorn alter resource selection after sagebrush decline. J Mammal. 2008;89(4):1031–40. doi: 10.1644/07-MAMM-A-173.1

54. O'Gara BW, Ball SK. Skeletal and visceral anatomy. In: O'Gara BW, Yoakum JD. Pronghorn ecology and management. Boulder: The University Press of Colorado; 2004. pp. 195-230.

55. Sawyer H, Lindzey F, McWhirter D. Mule deer and pronghorn migration in western Wyoming. Wildl Soc Bull. 2005;33(4):1266–73. doi: 10.2193/0091-7648(2005)33[1266:MDAPMI]2.0.CO;2

56. White PJ, Davis TL, Barnowe-Meyer KK, Crabtree RL, Garrott RA. Partial migration and philopatry of Yellowstone pronghorn. Biol Conserv. 2007;135(4):502–10. doi:10.1016/j.biocon.2006.10.041

57. Yoakum JD, Jones PF, Cancino J, Guenzel RJ, Seidler R, Munguia-Vega A, et al. Pronghorn management guides fifth edition: Twenty-fifth biennial pronghorn workshop; 2014; Santa Ana Pueblo, New Mexico. Western Association of Fish and Wildlife Agencies' Pronghorn Workshop and New Mexico Department of Game and Fish: 159 pp.

58. Schroeder C. Western state and province pronghorn status report, 2018. 2018 Proceedings of the Western States and Provinces Twenty-eighth Biennial Pronghorn Workshop. 2018; 29–35.

59. Jakes A. Pronghorn. In: Remington TE, Deibert PA, Hanser SE, Davis DM, Robb LA, Welty JL. Sagebrush conservation strategy–challenges to sagebrush conservation. U.S. Geological Survey; 2021.Open-File Report 2020–1125. doi:10.3133/ofr20201125.

60. Sievers JD, Jacques CN, Jenks JA, Roddy DE. Evaluating movements of pronghorns in Wind Cave National Park, South Dakota. Proc S Dak Acad Sci. 2007; 86: 191–9.

61. Christie KS, Jensen WF, Schmidt JH, Boyce MS. Long-term changes in pronghorn abundance index linked to climate and oil development in North Dakota. Biol Conserv. 2015;192:445–53. doi: 10.1016/j.biocon.2015.11.007

62. Gedir J V., Cain JW, Harris G, Turnbull TT. Effects of climate change on long-term population growth of pronghorn in an arid environment. Ecosphere. 2015;6(10) doi: 10.1890/ES15-00266.1

63. Harrington JL, Conover MR. Characteristics of ungulate behavior and mortality associated with wire fences. Wildl Soc Bull. 2006;34(5):1295–305. doi: 10.2193/0091-7648(2006)34[1295:coubam]2.0.co;2

64. Seidler RG, Long RA, Berger J, Bergen S, Beckmann JP. Identifying impediments to long-distance mammal migrations. Conserv Biol. 2015;29(1):99–109. doi: 10.1111/cobi.12376

65. Jones PF, Jakes AF, Eacker DR, Seward BC, Hebblewhite M, Martin BH. Evaluating responses by pronghorn to fence modifications across the Northern Great Plains. Wildl Soc Bull. 2018;42(2):225–36. doi: 10.1002/wsb.869

66. Jones PF, Jakes AF, Telander AC, Sawyer H, Martin BH, Hebblewhite M. Fences reduce habitat for a partially migratory ungulate in the northern sagebrush steppe. Ecosphere. 2019;10(7):e02782. doi:10.1002/ecs2.2782

67. Reinking AK, Smith KT, Mong TW, Read MJ, Beck JL. Across scales, pronghorn select sagebrush, avoid fences, and show negative responses to anthropogenic features in winter. Ecosphere. 2019;10(5). doi: 10.1002/ecs2.2722

68. Jones PF, Jakes AF, Vegter SE, Verhage MS. Is it the road or the fence? Influence of linear anthropogenic features on the movement and distribution of a partially migratory ungulate. Mov Ecol. 2022;10(37): doi: 10.1186/s40462-022-00336-3

69. Christie KS, Jensen WF, Boyce MS. Pronghorn resource selection and habitat fragmentation in North Dakota. J Wildl Manage. 2017;81(1):154–62. doi: 10.1002/jwmg.21147

70. Gavin SD, Komers PE. Do pronghorn (Antilocapra americana) perceive roads as a predation risk? Can J Zool. 2006; 84:1775-80. doi: 10.1139/Z06-175

71. Taylor KL, Beck JL, Huzurbazar S V. Factors influencing winter mortality risk for pronghorn exposed to wind energy development. Rangel Ecol Manag. 2016;69(2):108–16. doi: 10.1016/j.rama.2015.12.003

72. Jakes AF, DeCesare NJ, Jones PF, Cormack Gates C, Story SJ, Olimb SK, et al. Multi-scale habitat assessment of pronghorn migration routes. PLoS One. 2020;15(12): e0241042. doi: 10.1371/journal.pone.0241042

73. Beckmann JP, Murray K, Seidler RG, Berger J. Human-mediated shifts in animal habitat use: Sequential changes in pronghorn use of a natural gas field in Greater Yellowstone. Biol Conserv. 2012;147(1):222–33. doi: 10.1016/j.biocon.2012.01.003

74. Smith KT, Taylor KL, Albeke SE, Beck JL. Pronghorn winter resource selection before and after wind energy development in south-central Wyoming. Rangel Ecol Manag. 2020;73(2):227–33. doi: 10.1016/j.rama.2019.12.004

75. Reinking AK, Smith KT, Monteith KL, Mong TW, Read MJ, Beck JL. Intrinsic, environmental, and anthropogenic factors related to pronghorn summer mortality. J Wildl Manage. 2018;82(3):608–17. doi: 10.1002/jwmg.21414

76. Barrett MW. Distribution, behavior, and mortality of pronghorns during a severe winter in Alberta. J Wildl Manage. 1982;46(4):991–1002. doi:10.2307/3808232

77. Thorne ET, Williams ES, Spraker TR, Helms W, Segerstrom T. Bluetongue in free-ranging pronghorn antelope (Antilocapra americana) in Wyoming: 1976 and 1984. J Wildl Dis. 1988;24(1):113–9. doi: 10.7589/0090-3558-24.1.113

78. Dunbar MR, Velarde R, Gregg MA, Bray M. Health evaluation of a pronghorn antelope population in Oregon. J Wildl Dis. 1999;35(3):496–510. doi: 10.7589/0090-3558-35.3.496

79. Brodie SJ, Bardsley KD, Diem K, Mecham JO, Norelius SE, Wilson WC. Epizootic Hemorrhagic Disease: Analysis of tissues by amplification and in situ hybridization reveals widespread Orbivirus infection at low copy numbers. J Virol. 1998;72(5):3863–71. doi:10.1128/JVI.72.5.3863-3871.1998

80. Howerth E.W., Stallknecht D.E., Kirkland P.D. Bluetongue, epizootic haemorrhagic disease, and other orbivirus-related diseases. In: Williams E.S., Barker I.K., editors. Infectious Diseases of Wild Mammals. Iowa State University Press; Ames, IA, USA: 2001. pp. 77–97

81. Stephenson TR, German DW, Frances Cassirer E, Walsh DP, Blum ME, Cox M, et al. Linking population performance to nutritional condition in an alpine ungulate. J Mammal. 2020;101(5):1244–56. doi: 10.1093/jmammal/gyaa091

82. Monteith KL, Bleich VC, Stephenson TR, Pierce BM, Conner MM, Kie JG, et al. Life-history characteristics of mule deer: Effects of nutrition in a variable environment. Wildl. Monogr. 2014;186:1–56. doi: 10.1002/wmon.1011

83. Eddington HS, Carroll C, Larsen RT, McMillan BR, Chaston JM. Spatiotemporal variation in the fecal microbiota of mule deer is associated with proximate and future measures of host health. BMC Vet Res. 2021;17(1):1–8. doi: 10.1186/s12917-021-02972-0

84. Dehority BA. Rumen ciliates of the pronghorn antelope (Antilocapra americana), mule deer (Odocoileus hemionus), white-tailed deer (Odocoileus virginianus) and elk (Cervus canadensis) in the northwestern United States. Arch Protistenkunde. 1995;146(1):29–36. doi: 10.1016/S0003-9365(11)80252-6

85. Liggenstoffer AS, Youssef NH, Couger MB, Elshahed MS. Phylogenetic diversity and community structure of anaerobic gut fungi (phylum Neocallimastigomycota) in ruminant and non-ruminant herbivores. ISME J. 2010;4:1225–35. doi: 10.1038/ismej.2010.49

86. Trujillo SM, McKenney EA, Hilderbrand GV, Mangipane LS, Rogers MC, Joly K, et al. Intrinsic and extrinsic factors influence on an omnivore's gut microbiome. PLoS One. 2022;17(4):e0266698. doi: 10.1371/journal.pone.0266698

87. Jacques CN, Jenks JA, Deperno CS, Sievers JD, Grovenburg TW, Brinkman TJ, et al. Evaluating ungulate mortality associated with helicopter net-gun captures in the northern great plains. J Wildl Manage. 2009;73(8):1282–91. doi: 10.2193/2009-039

88. Lubinski PM. Estimating age and season of death of pronghorn antelope (Antilocapra americana ord) by means of tooth eruption and wear. Int J Osteoarchaeol. 2001;11(3):218–30. doi: 10.1002/oa.536

89. Stephenson TR, Bleich VC, Pierce BM, Mulcahy GP. Validation of mule deer body composition using in vivo and post-mortem indices of nutritional condition. Wildl Soc Bull. 2002;30(2):557–64. doi: 10.2307/3784516

90. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. J Clin Microbiol. 2007;45(9):2761–4. doi: 10.1128/JCM.01228-07

91. Caporaso JG, Ackermann G, Apprill A, Bauer M, Berg-Lyons D, Betley J et al. 2018. EMP 16S Illumina amplicon protocol V1. 2018. Available from: https://dx.doi.org/10.17504/protocols.io.nuudeww

92. Ul-Hasan S, Bowers RM, Figueroa-Montiel A, Licea-Navarro AF, Michael Beman J, Woyke T, et al. Community ecology across bacteria, archaea and microbial eukaryotes in the sediment and seawater of coastal Puerto Nuevo, Baja California. PLoS One. 2019;14(2): e0212355. doi: 10.1371/journal.pone.0212355

93. Gonzalez A, Navas-Molina JA, Kosciolek T, McDonald D, Vázquez-Baeza Y, Ackermann G, et al. Qiita: rapid, web-enabled microbiome meta-analysis. Nat Methods. 2018;15(10):796–8. doi: 10.1038/s41592-018-0141-9

94. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37(8):852–7. doi: 10.1038/s41587-019-0209-9

95. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581–3. doi: 10.1038/nmeth.3869

96. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 2013;41(D1):590–6. doi: 10.1093/nar/gks1219

97. Bisanz JE. Qiime2R: Importing QIIME2 artifacts and associated data into R sessions. 2018. v0.99.6. Accessed at https://github.com/jbisanz/qiime2R

98. McMurdie PJ, Holmes S. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 2013;8(4). e61217. doi: 10.1371/journal.pone.0061217

99. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 2018;6(1):1–14. doi: 10.1186/s40168-018-0605-2

100. McDonald JH. Handbook of Biological Statistics. 3rd ed. Baltimore: Sparky House Publishing; 2014.

101. Zar, JH. Biostatistical analysis. 5th ed. Upper Saddle River: Pearson Prentice Hall; 2010.

102. Oksanen AJ, Blanchet FG, Friendly M, Kindt R, Legendre P, Mcglinn D, et al. Package 'vegan' Community Ecology Package. Version 2.6-4. Published 2022-10-11. Available from: https://cran.r-project.org/web/packages/vegan/index.html

103. Mcardle BH, Anderson MJ. Fitting multivariate models to community data: A comment on distance-based redundancy analysis. Ecology. 2001;82(1):290–7. doi: 10.2307/2680104

104. Borcard D, Gillet F, Legendre P. Numerical Ecology with R. Use R! New York: Springer; 2011. doi: 10.1007/978-1-4419-7976-6.

105. Fountain-Jones NM, Clark NJ, Kinsley AC, Carstensen M, Forester J, Johnson TJ, et al. Microbial associations and spatial proximity predict North American moose (Alces alces) gastrointestinal community composition. J Anim Ecol. 2020;89(3):817–28. doi: 10.1111/1365-2656.13154

106. Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. Gut Microbes. 2012;3(4):289-306. doi: 10.4161/gmic.19897

107. Thomas F, Hehemann JH, Rebuffet E, Czjzek M, Michel G. Environmental and gut Bacteroidetes: The food connection. Front Microbiol. 2011;2(93):1–16. doi: 10.3389/fmicb.2011.00093

108. Seshadri R, Leahy SC, Attwood GT, Teh KH, Lambie SC, Cookson AL, et al. Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. Nat Biotechnol. 2018;36(4):359–67. doi: 10.1038/nbt.4110

109. Flint HJ, Bayer EA. Plant cell wall breakdown by anaerobic microorganisms from the mammalian digestive tract. Ann N Y Acad Sci. 2008; 1125:280–8. doi: 10.1196/annals.1419.022

110. Yang J, Park J, Jung Y, Chun J. AMDB: A database of animal gut microbial communities with manually curated metadata. Nucleic Acids Res. 2022;50(D1): D729–35. doi: 10.1093/nar/gkab1009

111. Antwis RE, Lea JMD, Unwin B, Shultz S. Gut microbiome composition is associated with spatial structuring and social interactions in semi-feral Welsh Mountain ponies. Microbiome. 2018;6(1):207. doi: 10.1186/s40168-018-0593-2

112. Tung J, Barreiro LB, Burns MB, Grenier JC, Lynch J, Grieneisen LE, et al. Social networks predict gut microbiome composition in wild baboons. Elife. 2015;4: e05224. doi: 10.7554/eLife.05224

113. Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D, et al. Cohabiting family members share microbiota with one another and with their dogs. Elife. 2013;2: e00458. doi: 10.7554/eLife.00458

114. Archie EA, Tung J. Social behavior and the microbiome. Curr Opin Behav Sci. 2015; 6:28–34. doi: 10.1016/j.cobeha.2015.07.008

115. Robb BS, Merkle JA, Sawyer H, Beck JL, Kauffman MJ. Nowhere to run: semi-permeable barriers affect pronghorn space use. J Wildl Manage. 2022;86(4): e22212. doi: 10.1002/jwmg.22212

116. Moeller AH, Suzuki TA, Lin D, Lacey EA, Wasser SK, Nachman MW. Dispersal limitation promotes the diversification of the mammalian gut microbiota. Proc Natl Acad Sci U S A. 2017;114(52):13768–73. doi: 10.1073/pnas.1700122114

117. Bear GD. Seasonal trends in fat levels of pronghorns, Antilocapra americana, in Colorado. J Mammal. 1971;52(3):583–9. doi: 10.2307/1378593

118. White PJ, Gower CN, Davis TL, Sheldon JW, White JR. Group dynamics of Yellowstone pronghorn. J Mammal. 2012;93(4):1129–38. doi: 10.1644/10-MAMM-A-257.1

119. Weinstein SB, Martinez-Mota R, Stapleton TE, Klure DM, Greenhalgh R, Orr TJ, et al. Microbiome stability and structure is governed by host phylogeny over diet and geography in woodrats (Neotoma spp.). Proc Natl Acad Sci U S A. 2021;118(47): e2108787118. doi: 10.1073/pnas.2108787118

120. Nielsen DP, Harrison JG, Byer NW, Faske TM, Parchman TL, Simison WB, et al. The gut microbiome reflects ancestry despite dietary shifts across a hybrid zone. Ecol Lett. 2023;26(1):63–75. doi: 10.1111/ele.14135

121. Zilber-Rosenberg I, Rosenberg E. Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. FEMS Microbiol Rev. 2008;32(5):723–35.doi: 10.1111/j.1574-6976.2008.00123.x

TABLES

Table 1. Taxonomic depth and breadth of read assignments.

	Total Members Identified	Percent reads assigned to depth	Top 10 (% of assigned reads)	Top 15 (% of assigned reads)	Top 20 (% of assigned reads)
Phylum	23	99.999%	99.995%	99.999%	100% (rounded)
Class	35	99.994%	99.906%	99.980%	99.998%
Order	83	98.245%	97.781%	99.251%	99.723%
Family	143	98.060%	87.324%	95.295%	97.431%
Genus	267	85.948%	75.053%	84.484%	88.366%
Species	308	54.486%	61.415%	71.381%	78.034%

The first two columns indicate the number of members identified at each taxonomic level as well as the percentage of total sample reads that were assigned to that taxonomic level. The following columns show the percentage of read counts composed by the top 10, 15, or 20 members of the community at a given taxonomic level, showing the presence of a core group of microbes. Note: when taxonomy was assigned not all amplicon sequence variants (ASVs) could be assigned down to each taxonomic level, so percentages in the final three columns are representative of the reads that could be assigned to that level.

Table 2. Statistical comparison of three alpha diversity metrics among discrete pronghorn metrics.

	n	Shannon		Simpson		Observed Richness	
		χ^2	p	χ^2	p	χ^2	p
Study Area	155	3.204	0.361	2.150	0.542	4.433	0.218
BTV	152	0.587	0.444	0.189	0.663	0.085	0.771
EHD	152	2.059	0.151	0.214	0.643	1.968	0.161
I-80	155	**2.868**	**0.090**	0.611	0.435	**3.648**	**0.056**
Capture period	155	**20.698**	**<0.001**	**16.391**	**< 0.001**	**12.441**	**0.002**

Comparisons of Shannon's diversity index, Simpson's diversity index, and observed richness in the microbial community across pronghorn metrics including study area, BTV and EHD status, location relative to I-80, and capture period. Observed richness represents amplicon sequence variant (ASV) richness. We report maximum sample sizes (n), available for each metric. To maintain consistency in comparisons, we conducted a non-parametric Kruskal-Wallis test ($\chi 2$) as normality assumptions were not met for all metrics. **Significant differences are bolded ($p < 0.100$).**

Table 3. Values for alpha diversity metrics for groups of pronghorn

	n	Shannon	Simpson	Observed Richness
Capture period	155			
November 2013	110	4.448 ± 0.025 (a)	0.971 ± 0.001 (a)	201.882 ± 4.492 (a)
February 2014	11	4.122 ± 0.079 (b)	0.964 ± 0.003 (b)	154.000 ± 10.308 (b)
November 2014	34	4.241 ± 0.051 (b)	0.959 ± 0.003 (b)	185.912 ± 7.499 (a)
I-80	155			
North	64	4.334± 0.034 (a)	0.968 ± 0.002 (a)	186.281 ± 5.653 (a)
South	91	4.412 ± 0.032 (b)	0.968 ± 0.002 (a)	201.099 ± 5.017 (b)

Values for the above alpha diversity metrics within pronghorn metrics that showed significant differences in Kruskal-Wallis tests. Values reported for mean (± SE) during each capture period and in areas north or south of I-80. Matching letters denote when values for different capture periods or locations relative to I-80 are not significantly different ($p > 0.1$) using a Wilcoxon rank sum test with Bonferroni correction.

Table 4. Spearman's rank correlations (r_s) between alpha diversity measures and continuous pronghorn life history metrics.

			Alpha Diversity Metric		
Pronghorn Metric	n		Observed Richness	Shannon Diversity	Simpson Diversity
Age	152	r_s	-0.027	0.015	0.045
		p	0.741	0.854	0.578
Corrected age	152	r_s	-0.027	0.015	0.045
		p	0.741	0.854	0.578
Body weight (kg)	150	r_s	-0.080	-0.135	-0.138
		p	0.329	**0.010**	**0.093**
Ss-ligament	144	r_s	-0.013	0.038	-0.007
		p	0.875	0.654	0.930
Max fat	144	r_s	-0.064	-0.131	-0.087
		p	0.447	0.116	0.298

Alpha diversity metrics of Shannon diversity index, Simpson diversity index, and observed richness and their correlations to age, corrected age, body weight, and body condition metrics. Note: although correlation of body weight and both Shannon and Simpson diversity index was significant at the alpha = 0.100 level the accompanying correlations were not strong. **Significant correlations are bolded (p < 0.100).** We conducted correlations on the 155 pronghorn included in the rarified dataset, ignoring missing observations for each metric of interest. Thus, sample size (n) reports the number of observations included for each correlation.

Table 5. Model fit statistics from PERMANOVA ran with multiple metrics and multiple metrics with interactions.

Model before interactions					
Term	F	df	R^2	% Variation attributed	P
Study area	**2.752**	**3**	**0.059**	**5.9%**	**0.001**
EHD	0.845	1	0.006	0.6%	0.823
SS-ligament	1.036	10	0.074	7.4%	0.253
BTV	1.004	1	0.007	0.7%	0.423
Age (young, middle, old)	1.121	2	0.016	1.6%	0.173
Weight (5 kg increments)	0.931	3	0.020	2.0%	0.761
Model with interactions					
Term	F	df	R^2	% Variation attributed	P
Study area	3.097	3	0.066	6.6%	0.001
EHD	1.166	1	0.008	0.8%	0.150
SS-ligament	1.057	10	0.075	7.5%	0.191
BTV	1.102	1	0.008	0.8%	0.225
Age (young, middle, old)	1.173	2	0.017	1.7%	0.097
Weight (5 kg increments)	0.943	3	0.020	2.0%	0.698
Study Area x EHD	0.955	3	0.020	2.0%	0.643
Study Area x SS-ligament	**1.088**	**18**	**0.138**	**13.8%**	**0.035**
Study Area x BTV	0.942	3	0.020	2.0%	0.691
Study Area x Age (young, middle, old)	**1.106**	**6**	**0.047**	**4.7%**	**0.077**

Study Area x Weight (5 kg increments)	0.950	9	0.060	6.0%	0.800

Test statistic (F), degrees of freedom (df), R^2, and p are reported for each metric. Factors significant at the $p < 0.100$ level are bolded. Sample size for combined PERMANOVAs was 134. When interactions were not present, the PERMANOVA was run to account for the marginal effect of each variable. When the model was run with interactions there were significant interactions between study area and ss-ligament and study area and age.

FIGURES

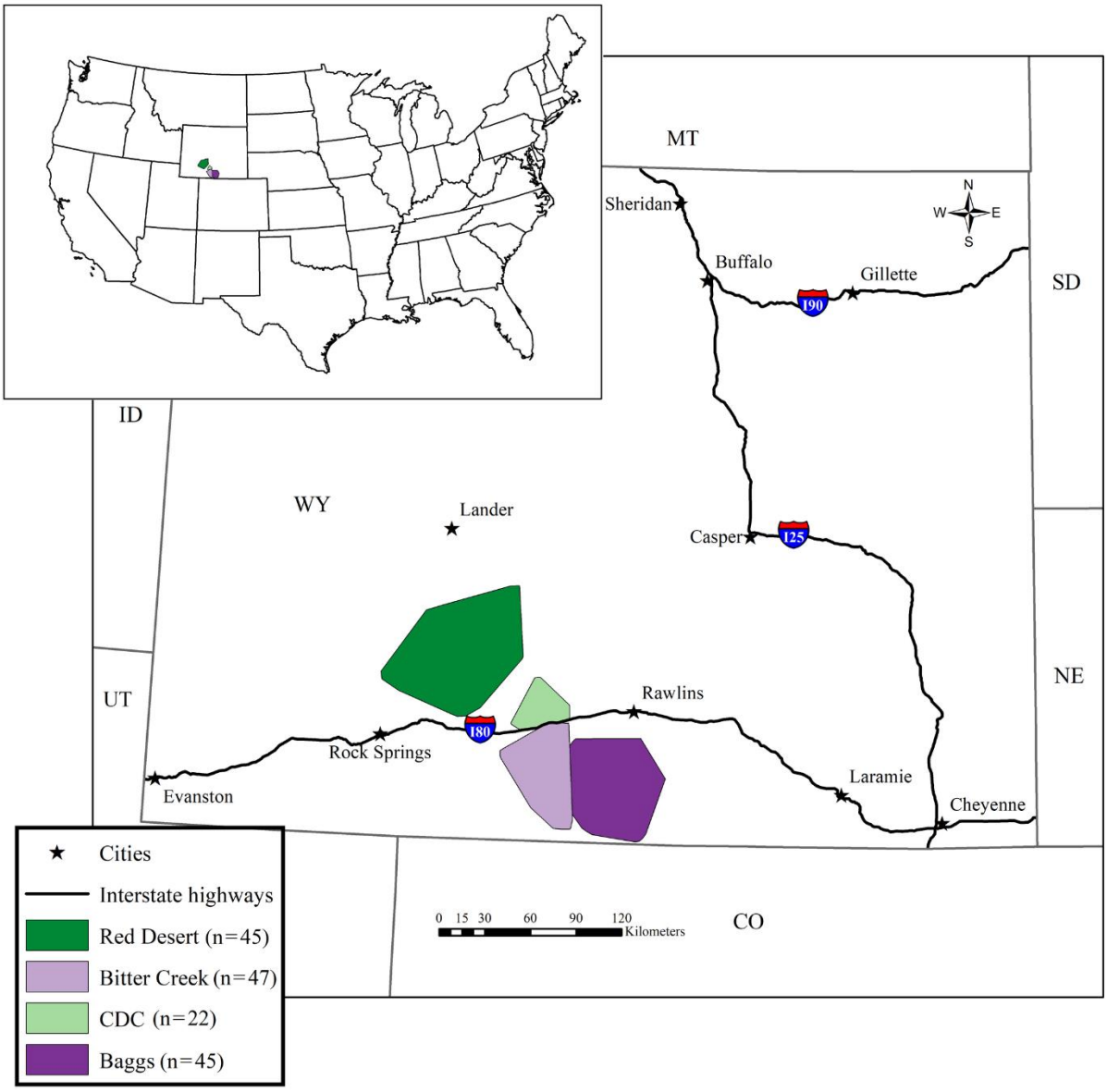


Figure 1. Map of study area locations.
Location of the Red Desert, Continental Divide-Creston (CDC), Baggs, and Bitter Creek study areas with pronghorn numbers in each study area listed. Study area boundaries were delineated using a 100% minimum convex polygon including the pronghorn locations recorded within each study area.

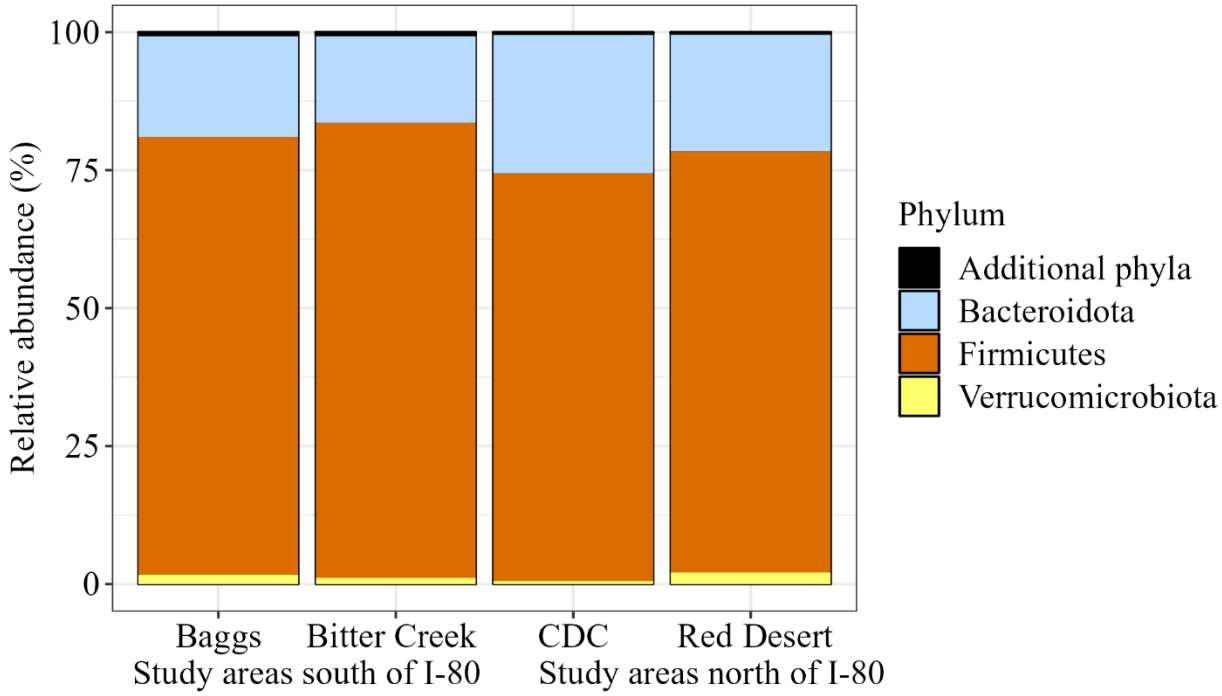


Figure 2. Pronghorn microbiome composition represented by phylum relative abundance. Phyla relative abundance is grouped and visualized by study area and location relative to Interstate 80 (I-80). The top 3 phyla depicted represent 99.382% of the assigned amplicon sequence variants (ASVs) present in our samples.

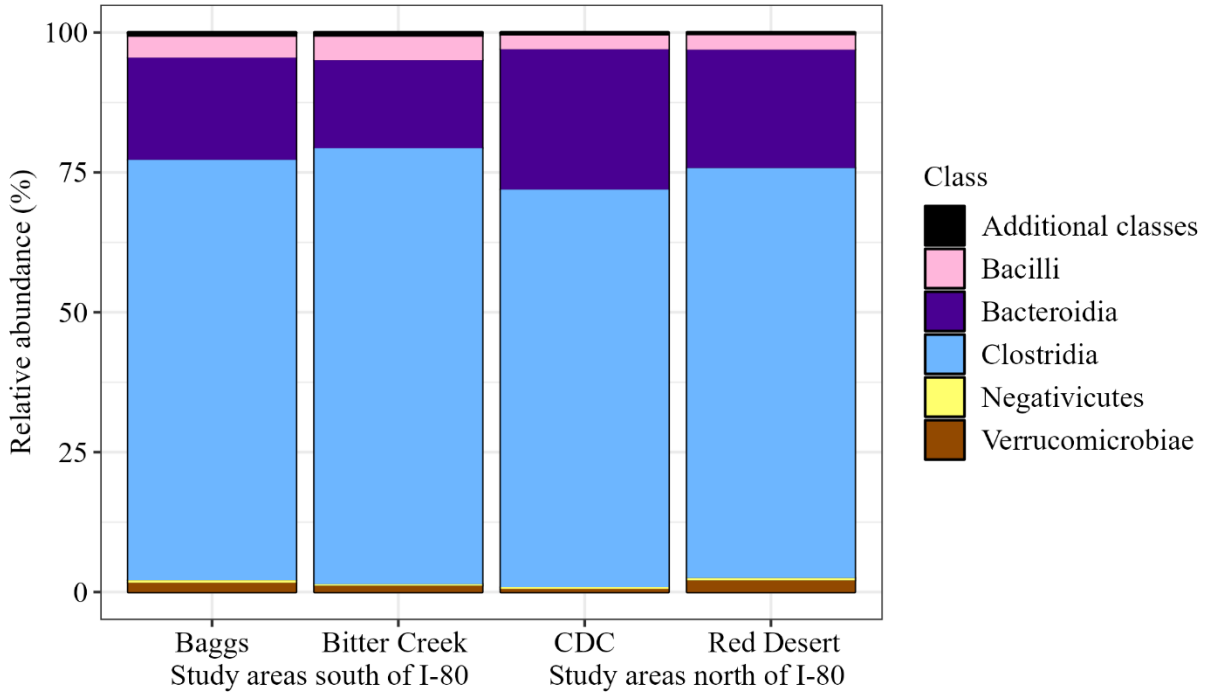


Figure 3. Pronghorn microbiome composition represented by class relative abundance.

Class relative abundance is grouped and visualized by study area and location relative to Interstate 80. The top 5 classes depicted represent 99.355% of the assigned amplicon sequence variants (ASVs) present in our samples.

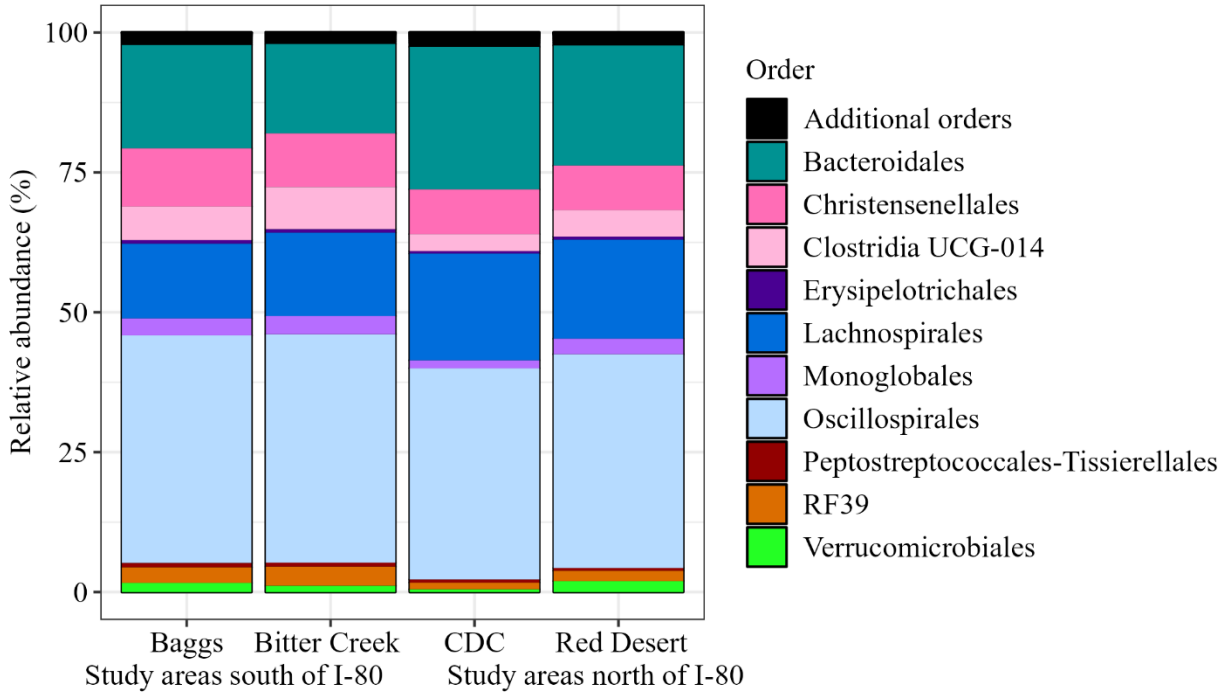


Figure 4. Pronghorn microbiome composition represented by order relative abundance. Order relative abundance is grouped and visualized by study area and location relative to Interstate 80 (I-80)**.** The top 10 orders depicted represent 97.781% of the assigned amplicon sequence variants (ASVs) present in our samples (Table 1).

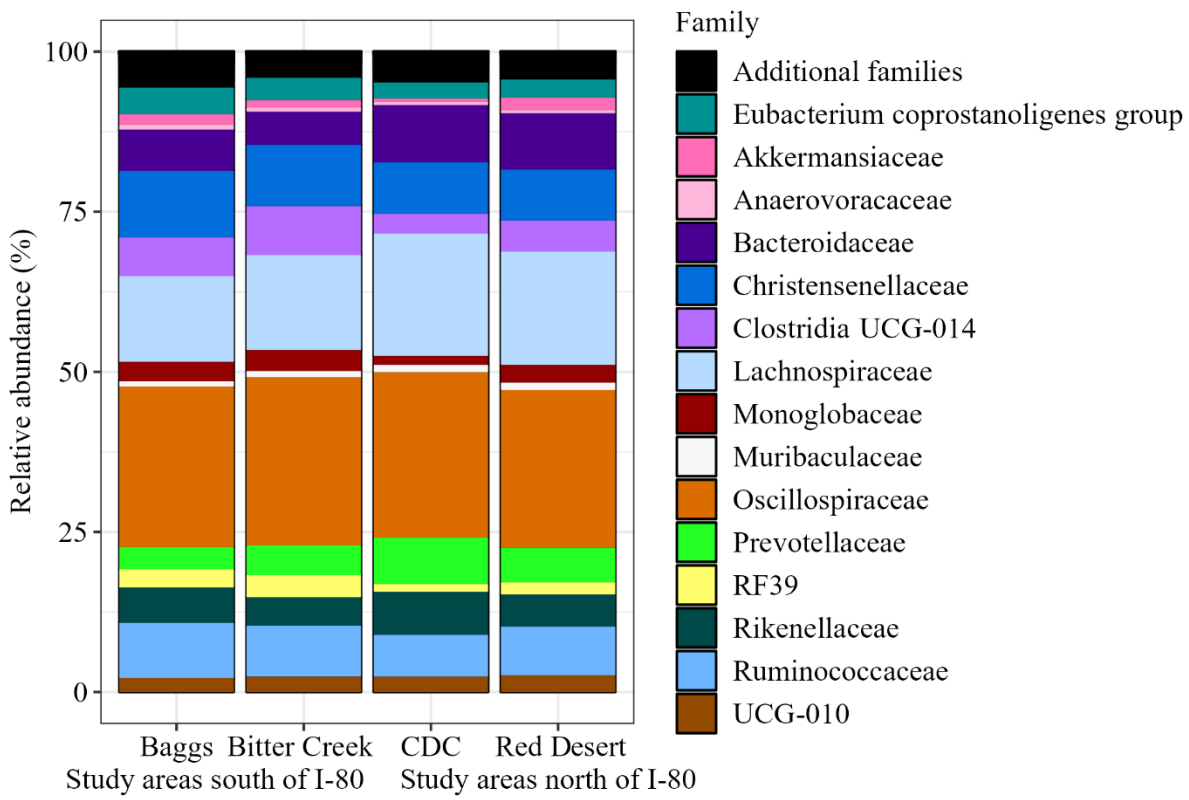


Figure 5. Pronghorn microbiome composition represented by family relative abundance. Family relative abundance is grouped and visualized by study area and location relative to Interstate 80**.** The top 15 families depicted represent 95.295% of the assigned amplicon sequence variants (ASVs) present (Table 1).

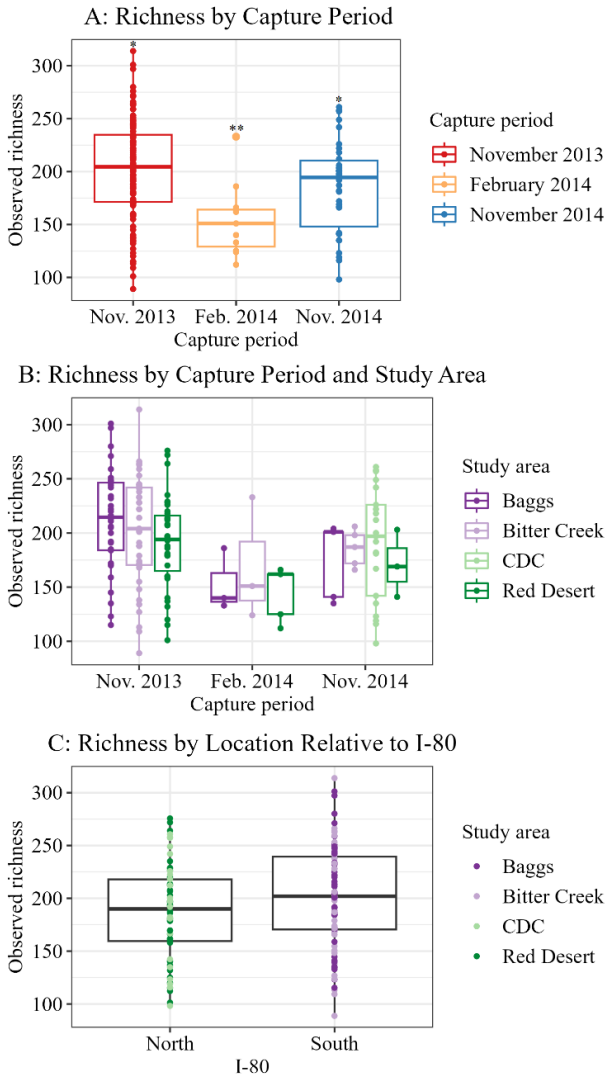


Figure 6. Visualization of alpha diversity differences amongst pronghorn study metrics.

A) Observed richness for pronghorn captured during 3 capture periods ($p = 0.002$) with animals captured in February 2014 having lower observed richness than animals captured during both November periods. Differing numbers of * indicate significant differences ($p < 0.100$). B) The same visualization, now showing that trend of lower alpha diversity in February 2014 was consistent across study areas. C) Animals south of Interstate 80 (I-80) exhibited higher observed richness than animals north of I-80 ($p = 0.070$), study areas within are depicted by different colors.

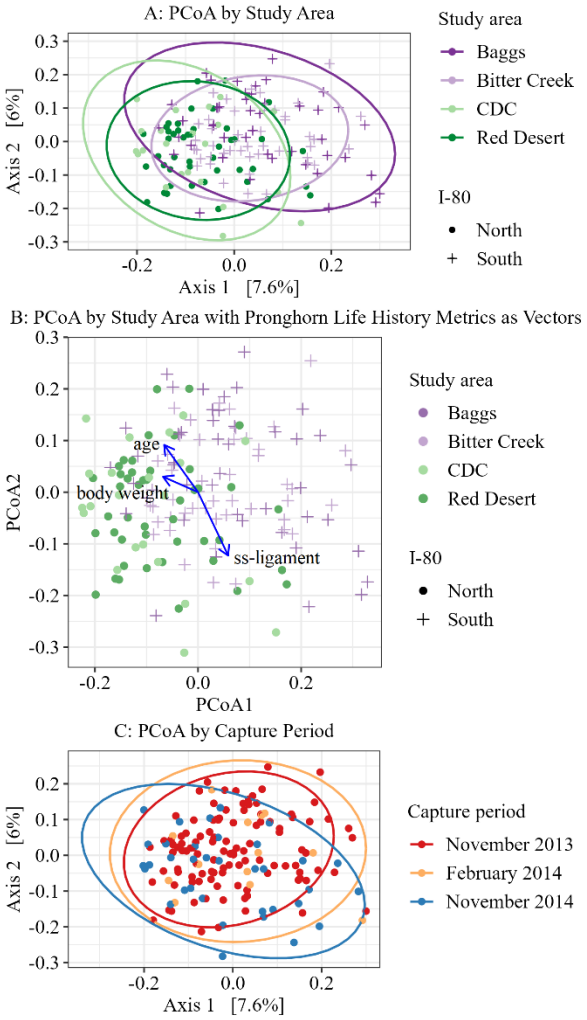
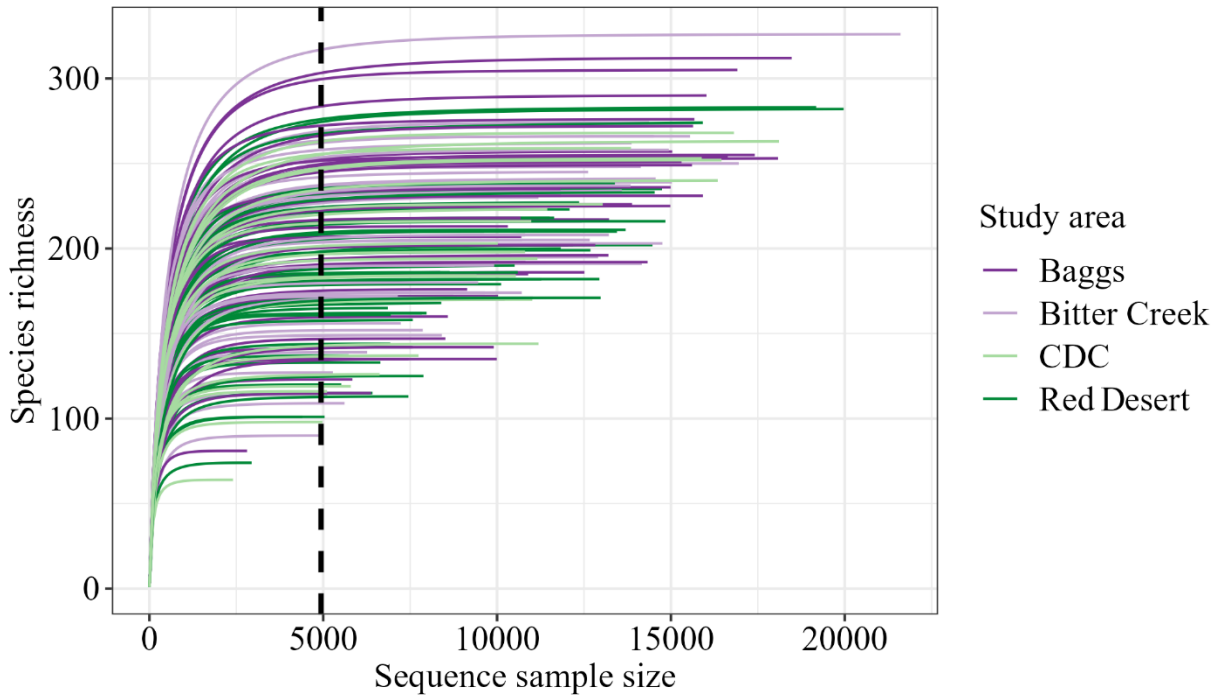


Figure 7. PCoA ordinations using Bray-Curtis Dissimilarity measure.

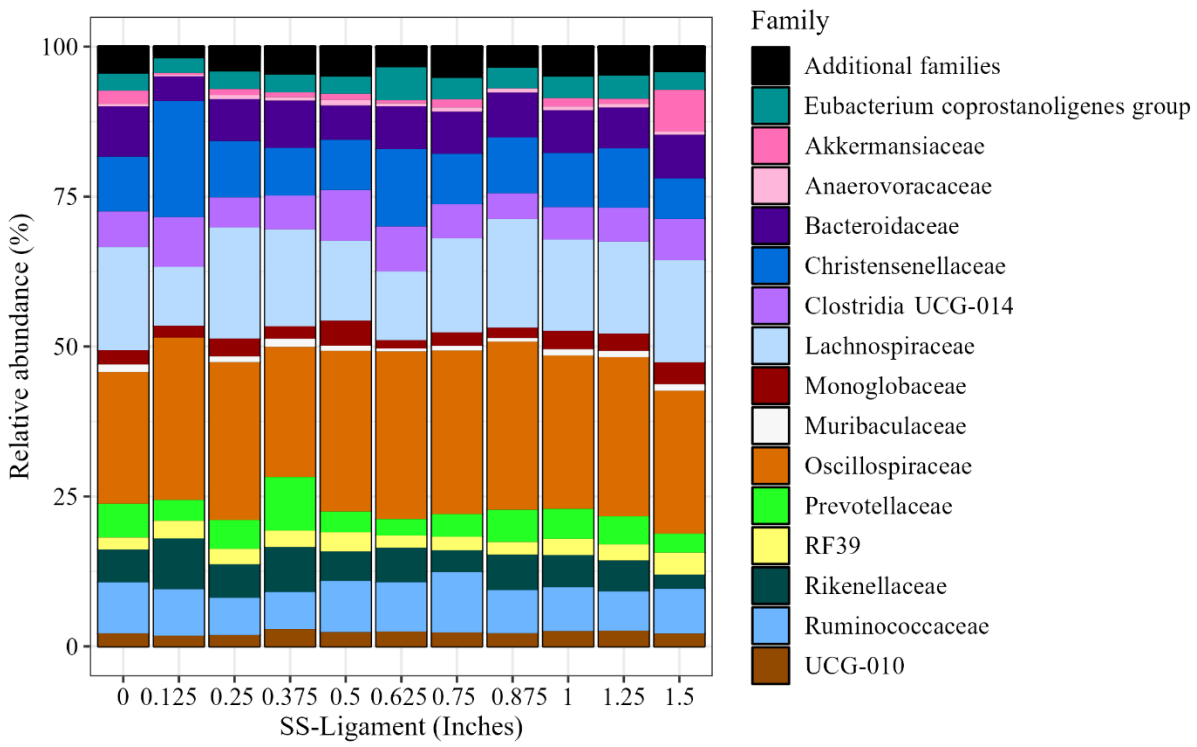
Data represent counts transformed to relative abundance without rare taxa removed and are grouped by (A) study area with ellipses of 95% CI, (B) study area with vectors representing continuous variables of explanatory pronghorn life history metrics of age, ss-ligament, and weight, and (C) capture period with ellipses of 95% CI. The first two axes explain 13.6% of the variation in the samples. Note in 7A that Baggs and Bitter Creek (south of Interstate 80, plus signs) are more similar and Continental Divide-Creston (CDC) and Red Desert (north of Interstate 80, circles) are more similar. 7B shows the addition of vectors representing age, weight, and ss-ligament, however these were not significantly related to PCoA axis 1 or 2.

SUPPORTING INFORMATION

S1 Table. Pronghorn metadata. This is a large data frame. To access the file, please see https://github.com/courtney-buchanan/Pronghorn_microbiome

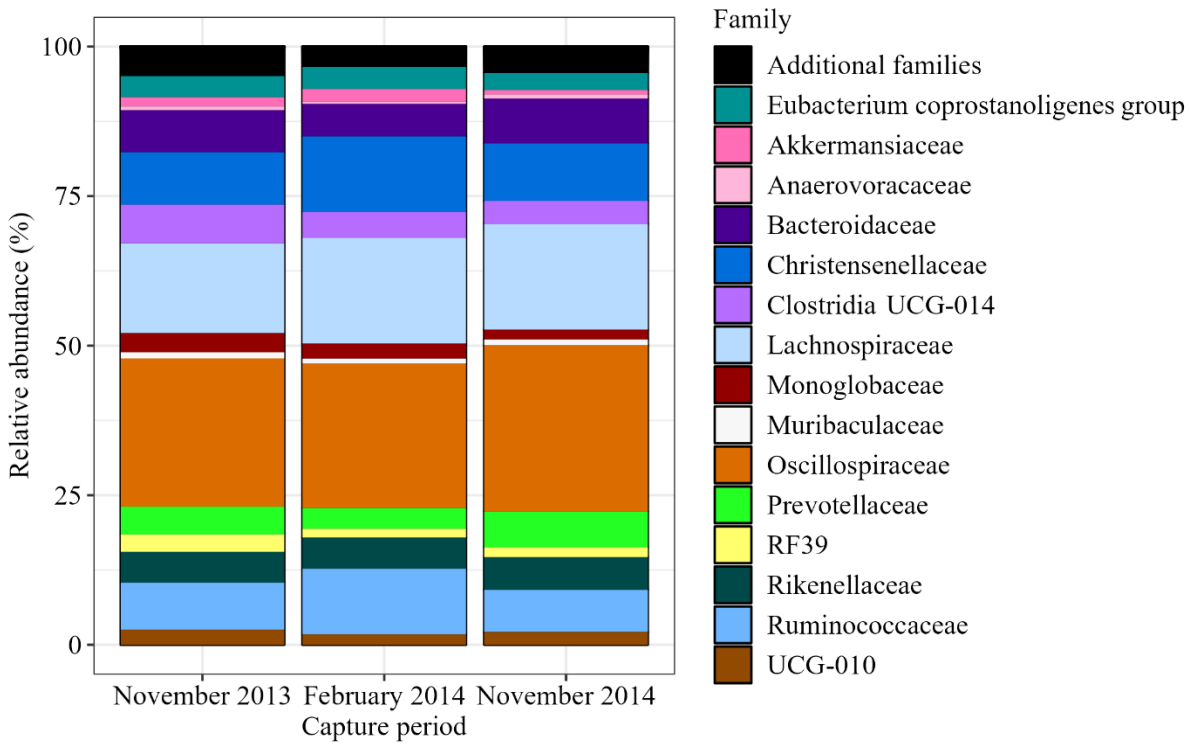


S1 Fig. Rarefaction curve.
Dotted line shows the chosen rarefaction point of 4936 reads per sample. Samples are color coded by study area, so we could be assured samples dropped in the rarefaction step were not all from the same location.



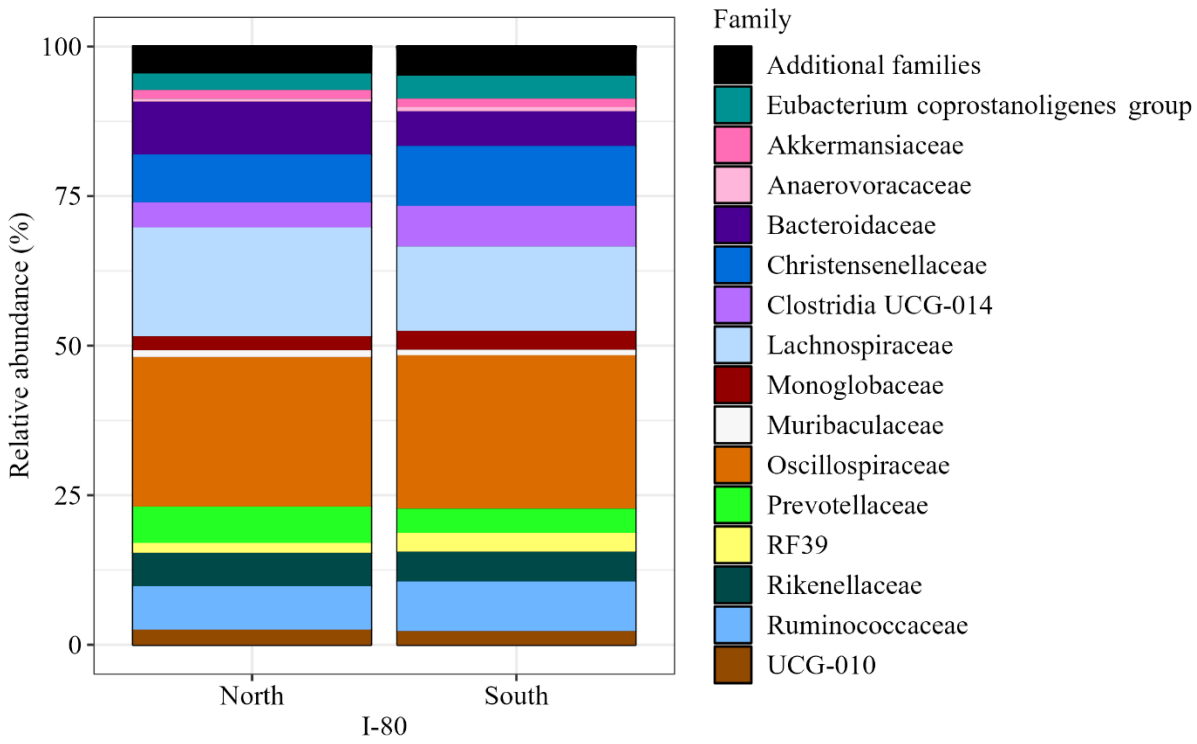
S2 Fig. Pronghorn microbiome composition represented by family relative abundance and grouped by body condition.

Family relative abundance is grouped and visualized by ss-ligament (measured in inches of depression)**.** Top 15 families depicted make up 95.295% of the assigned amplicon sequence variants (ASVs) present. Larger values for ss-ligament represent leaner animals.

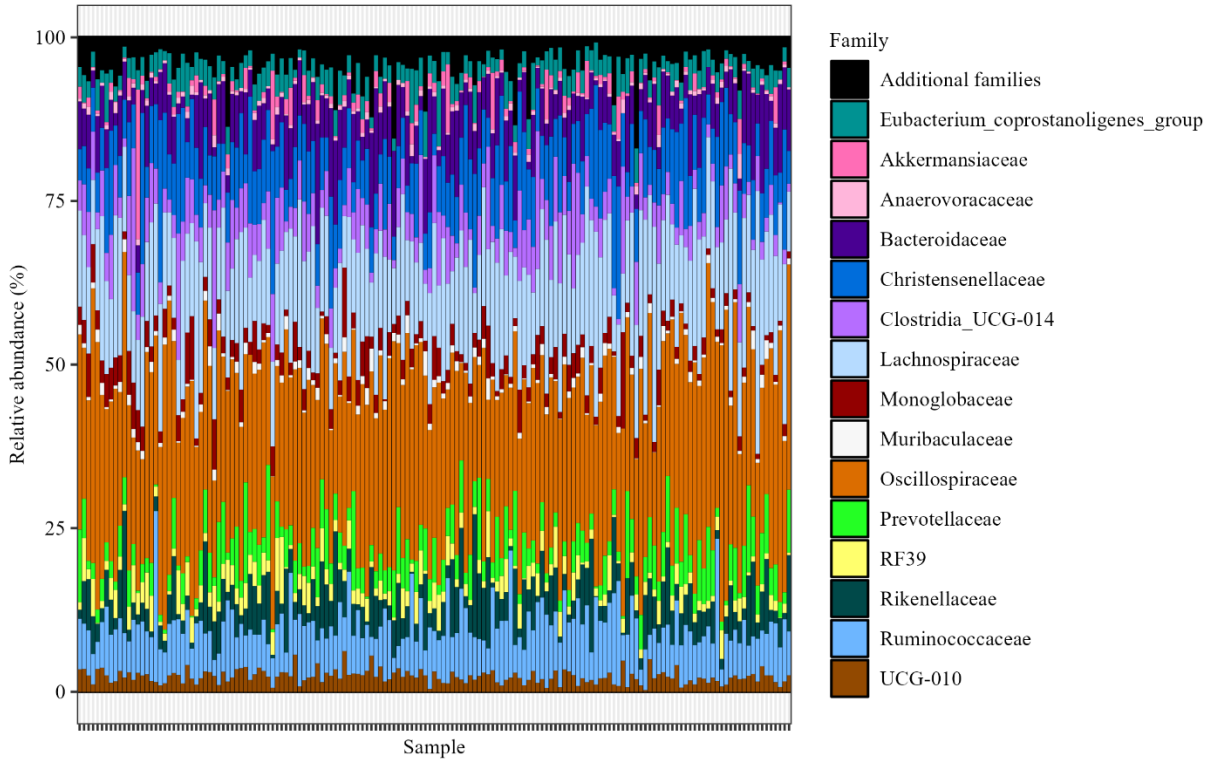


S3 Fig. Pronghorn microbiome composition represented by family relative abundance and grouped by capture period.

Family relative abundance is grouped and visualized by capture period**.** Top 15 families depicted make up 95.295% of the assigned amplicon sequence variants (ASVs) present.



S4 Fig. Pronghorn microbiome composition represented by family relative abundance and grouped by location relative to Interstate-80.
Family relative abundance is grouped and visualized by location relative to Interstate 80**.** Top 15 families depicted make up 95.295% of the assigned amplicon sequence variants (ASVs) present.



S5 Fig. Pronghorn microbiome composition for individual samples represented by family relative abundance.
Top 15 families present in the pronghorn microbiome for each individual animal's sample. The top 15 families depicted make up 95.295% of the assigned amplicon sequence variants (ASVs) present (Table 1).

S1 Appendix: Other Tables

Table A. Pronghorn captures by time period and study area.

	November 2013	February 2014	November 2014	Total
Baggs	36	4	5	45
Bitter Creek	39	3	5	47
CDC	0	0	22	22
Red Desert	36	6	3	45
Total	111	13	35	159

Capture data showing where and when the 159 animals that yielded fecal samples used in this study were captured.

Table B. Read count remaining after the various steps in the DADA 2 pipeline in QIIME process.

	Initial	After filter	De-noised	Merged	Non-Chimeric
Read count	3,888,708	3,326,145	3,225,577	2,349,642	2,035,077
Percentage	100%	85.5%	82.9%	60.4%	52.3%

Initial read counts are shown as well as read counts after filtering, de-noising, merging, and removing chimeras throughout the DADA2 pipeline.

Table C. Pronghorn metrics by study area.

	Baggs	Bitter Creek	CDC	Red Desert	Total of all areas
Number pronghorn (n)	45	47	22	45	159
Positive for EHD	11 (24.22%)	13 (27.66%)	2 (9.09%)	4 (8.89%)	30 (18.87%)
Positive for BTV	7(15.56%)	7 (14.89%)	1 (4.55%)	6 (13.33%)	21 (13.21%)
					Range for all areas
Age	4.54 years (± 1.54)	5.70 years (± 2.68)	6.05 years (± 2.60)	4.92 years (± 2.03)	1-12 years
Corrected Age	5.57 years (± 1.21)	6.49 years (± 2.11)	6.76 years (± 2.04)	5.88 years (± 1.59)	2.80 - 11.44 years
Weight	49.11 kg (± 3.87)	48.98 kg (± 3.83)	50.86 kg (± 4.68)	50.16 kg (± 3.62)	39.66 – 59.46 kg
SS-ligament	1.78 cm (± 0.93)	2.20 cm (± 0.88)	1.88 cm (± 1.05)	1.77 cm (± 1.15)	0- 3.81 cm depression
Maximum fat thickness	1.37 mm (±1.85)	0.80 mm (± 1.23)	1.14 mm (± 1.46)	1.10 mm (± 1.64)	0-7 mm

Top portion of the table shows the number of pronghorn captured and number positive for each disease in each study area, along with total positives among all captured animals. Lower portion of the table shows the average value for each metric (+/- SD) for each study area as well as the range of values among all animals captured.

S2 Appendix: Additional Alpha Diversity Analyses

To investigate potential differences the relationships of alpha diversity and pronghorn metrics in populations north and south of I-80, we repeated our alpha diversity analysis on subsets of the data for animals north and south of I-80.

As in our pronghorn population as a whole, we found that all three alpha diversity metrics differed ($p < 0.100$) between different capture periods in pronghorn populations both north and south of I-80 (Table D). North of I-80, the November 2013 captured animals displayed higher observed richness and Shannon's diversity index than February captured pronghorn, and a higher Simpson's diversity index than November 2014 captured animals (Table E). South of I-80, the pattern was similar with the November 2013 captured animals having higher observed richness than the February captured animals, a higher Simpson's index than November 2014 captured animals, and a higher Shannon's index than both the February and November 2014 groups (Table E). In addition, we found there was a difference in alpha diversity for animals of differing BTV status north of I-80 but not in animals south of I-80 (Table D). North of I-80, we found that animals that were negative for BTV had a higher alpha diversity, but only as measured by Shannon's diversity index (Table E).

Table D. Kruskal-Wallis tests of three alpha diversity metrics of pronghorn gut microbiome among discrete pronghorn metrics for animals north and south of I-80

North of I-80							
	n	Shannon		Simpson		Observed Richness	
		χ^2	p	χ^2	p	χ^2	p
Study Area	64	0.243	0.622	1.392	0.238	0.442	0.506
BTV	64	**3.624**	**0.057**	1.750	0.1859	2.569	0.109
EHD	64	0.013	0.908	0.026	0.872	0.030	0.863
Capture period	64	**5.992**	**0.050**	**5.290**	**0.071**	**5.146**	**0.076**
South of I-80							
		χ^2	p	χ^2	p	χ^2	p
Study Area	91	0.006	0.937	0.086	0.769	0.287	0.592
BTV	88	0.188	0.665	0.230	0.632	1.102	0.294
EHD	88	0.968	0.325	0.022	0.881	1.062	0.303
Capture period	91	**13.631**	**0.001**	**11.164**	**0.004**	**7.808**	**0.020**

Comparisons of Shannon's diversity index, Simpson's diversity index, and observed richness in the microbial community across pronghorn metrics including study area, BTV and EHD status, and capture period for animals north and south of I-80. Observed richness represents amplicon sequence variant (ASV) richness. We report maximum sample sizes (n), available for each metric. To maintain consistency in comparisons, we conducted non-parametric Kruskal-Wallis tests ($\chi 2$) as normality assumptions were not met for all metrics. Significant differences are bolded ($p < 0.100$).

Table E. Values for alpha diversity metrics for sub-groups of pronghorn

North of I-80				
	n	Shannon	Simpson	Observed Richness
Capture period	64			
November 2013	35	4.414 ± 0.034 (a)	0.972 ± 0.001 (a)	191.000 ± 7.141 (a)
February 2014	5	4.131 ± 0.146 (b)	0.964 ± 0.006 (ab)	145.000 ± 11.196 (b)
November 2014	24	4.257 ± 0.065 (ab)	0.962 ± 0.004 (b)	187.917 ± 10.113 (ab)
BTV	64			
Positive	7	4.313 ± 0.016(a)	-	-
Negative	57	4.507 ± 0.037 (b)	-	-
South of I-80				
	n	Shannon	Simpson	Observed Richness
Capture period	91			
November 2013	75	4.464 ± 0.034 (a)	0.971 ± 0.002 (a)	206.960 ± 5.618 (a)
February 2014	6	4.114 ± 0.094 (b)	0.965 ± 0.003 (ab)	161.167 ± 16.835 (b)
November 2014	10	4.203 ± 0.081 (b)	0.952 ± 0.007(b)	181.100 ± 8.367 (ab)

Values for the above alpha diversity metrics within pronghorn metrics for pronghorn north and south of I-80. Values are reported for mean (± SE) during each capture period and in animals positive and negative for BTV (north only) where they differ. Matching letters denote when values for different capture periods or BTV status are not significantly different ($p > 0.100$) using a Wilcoxon rank sum test with Bonferroni correction.

We also saw slight differences in the correlations between continuous pronghorn metrics and alpha diversity measures when looking at the north and south populations separately. In animals north of I-80 we saw no correlations between pronghorn metrics and alpha diversity measures (Table F). However, in animals south of I-80 we saw that as the measure for maximum fat had a negative relationship with both Shannon and Simpson's Diversity indices, however these correlations were not very strong (-0.222 and -0.204 respectively, Table F).

Overall, these relationships showed only slight differences between animals residing north or south of I-80 when compared to the relationships we saw for the population as a whole. This would lead us to believe that pronghorn on both sides of I-80 exhibit relationships between alpha diversity and capture period. In addition, our results hint at potential relationships that may differ in these two populations between the alpha diversity of the gut microbiome and disease status for BTV or body condition, as measured by maximum rump fat. It is possible that the composition of the gut microbiome experiences different relationships with animal health factors in different populations, a possibility that could be explored further in future, more pointed studies.

Table F. Spearman's rank correlations (r_s) between alpha diversity measures and continuous pronghorn life history metrics in animals north and south of I-80.

North of I-80					
			Alpha Diversity Metric		
Pronghorn Metric	n		Observed	Shannon	Simpson
Age	64	r_s	0.080	0.082	0.044
		p	0.529	0.519	0.732
Corrected age	64	r_s	0.080	0.082	0.044
		p	0.529	0.519	0.732
Body weight (kg)	60	r_s	-0.048	-0.126	-0.131
		p	0.714	0.338	0.317
Ss-ligament	59	r_s	-0.115	-0.122	-0.208
		p	0.384	0.358	0.113
Max fat	59	r_s	0.071	0.020	0.079
		p	0.594	0.879	0.551
South of I-80					
Pronghorn Metric	n		Observed	Shannon	Simpson
Age	88	r_s	-0.075	0.006	0.056
		p	0.490	0.955	0.601
Corrected age	88	r_s	-0.075	0.006	0.056
		p	0.490	0.955	0.601
Body weight (kg)	90	r_s	-0.050	-0.107	-0.097

		p	0.641	0.317	0.363
Ss-ligament	85	r_s	0.066	0.128	0.133
		p	0.546	0.244	0.226
Max fat	85	r_s	-0.179	**-0.222**	**-0.204**
		p	0.101	**0.041**	**0.061**

Alpha diversity metrics of Shannon diversity index, Simpson diversity index, and observed richness and their correlations to age, corrected age, body weight, and body condition metrics in pronghorn both north and south of I-80. **Significant correlations are bolded (p < 0.100).** We conducted correlations on the pronghorn included in the rarified dataset, ignoring missing observations for each metric of interest. Thus, sample size (n) reports the number of observations included for each correlation.

To look at the combined effects of our pronghorn variables on alpha diversity, we built a series of models to look at the effects of the variables together on alpha diversity- specifically observed richness. For categorical variables we created a series of dummy variables to represent the effect of each. We used November 2013 capture, EHD or BTV negative, south of I-80, and Baggs study area as our reference groups when creating dummy variables. We created multiple models because, due to confounded variables, we were unable to look at all variable simultaneously. To expand on this: study area and capture period are confounded due to all CDC animals being captured in November 2014. In addition, our models included only one of either the I-80 variable or study area variable at a time, as these represent similar measures. Due to the lack of observations for body condition during the February captures we created a model that left out the

ss-ligament measure so that we could include observations for all 3 capture periods in this model. The models included the following:

1) Including variables for age, body weight, ss-ligament, I-80 location, capture group (only the 2 November captures could be represented due to missing ss-ligament values in the February captures), BTV status, and EHD status.
2) Including variables for age, body weight, I-80 location, capture group (all 3 capture periods can be represented here), BTV status, and EHD status.
3) Including variables for age, body weight, ss-ligament, study area, BTV status, and EHD status.

We ran the three models for observed richness using linear regression as assumptions were met. We found that our second model was the only informative model (Table G) as Models 1 and 3 were not significant (Table G). As with our previous alpha diversity tests, in model 2 we saw that capture period had an effect. In this model, the variable for the February capture group had an effect on alpha diversity with animals captured in February 2014 compared to the first large 2013 November capture showing a decrease in observed richness (Table G). We believe capture effect was only observed in the second model, as this was the only one in which we were able to include all three capture groups. Overall this confirmed what we saw in Table 2 and 3 with our alpha diversity tests investigating single metrics individually- February 2014 pronghorn had lower observed richness values in those analyses as well. In our regression models we did not see an effect for other pronghorn metrics on our observed richness measure of alpha diversity. Unlike in our pairwise tests, we did not see an effect of I-80 on observed richness. Overall, the results generally confirmed the patterns we saw with our alpha diversity correlations and pairwise tests, and we did not explore this avenue further.

Table G. Models for Observed Richness

Observed Richness: Model 1				
Multiple R^2	**Adjusted R^2**	**F-statistic**	**p-value**	
0.045	-0.009	0.832	0.563	
Coefficients:				
	Estimate	**Standard Error**	**t-value**	**p-value**
Intercept	195.863	55.936	3.502	**<0.001**
SS-ligament	-3.173	11.097	-0.286	0.775
Age	-1.514	1.098	-0.794	0.429
Body Weight	0.417	1.118	0.373	0.709
I-80 (north)	-9.981	9.343	-1.068	0.287
November 2014 Capture	-12.106	10.714	-1.130	0.261
BTV (positive)	-4.073	12.415	-0.328	0.743
EHD (positive)	8.679	11.418	0.760	0.449
Observed Richness: Model 2				
Multiple R^2	**Adjusted R^2**	**F-statistic**	**p-value**	
0.111	0.065	2.419	**0.023**	
Coefficients:				
	Estimate	**Standard Error**	**t-value**	**p-value**
Intercept	200.499	50.669	3.957	<0.001
Age	-1.657	1.789	-0.926	0.356
Body Weight	0.292	1.035	0.283	0.778

I-80 (north)	-10.450	8.463	-1.235	0.219
February 2014 Capture	**-50.323**	**14.955**	**-3.365**	**<0.001**
November 2014 Capture	-11.549	10.344	-1.117	0.266
BTV (positive)	-5.528	11.638	-0.475	0.636
EHD (positive)	9.253	10.453	0.887	0.377
Observed Richness: Model 3				
Multiple R^2	**Adjusted R^2**	**F-statistic**	**p-value**	
0.036	-0.026	0.576	0.796	
Coefficients:				
	Estimate	**Standard Error**	**t-value**	**p-value**
Intercept	204.109	56.343	3.623	**<0.001**
SS-ligament	-1.689	11.337	-0.149	0.882
Age	-1.526	1.969	-0.775	0.440
Body Weight	0.243	1.119	0.217	0.828
Bitter Creek Study Area	-4.064	11.090	-0.366	0.715
CDC Study Area	-16.063	13.661	-1.176	0.242
Red Desert Study Area	-14.871	11.709	-1.270	0.206
BTV (positive)	-1.228	12.375	-0.099	0.921
EHD (positive)	6.640	11.401	0.582	0.561

S3 Appendix: Single metric PERMANOVAS

To better understand the biological relevance of microbiome differences in various groups of animals in our comparisons, we binned values for continuous metrics to observe where patterns occurred. Pronghorn weights ranged from 39.66 to 59.46 kg and we grouped animals by quartile (<46.77, 46.77-49.22, 49.26-51.96, and >51.96), by kg (40 = all animals below 41 kg, 41 = 41.00-41.99 kg, 42 = 42.00-42.99 kg etc.), and by 5-kg increments (<45 kg, 45-49.99 kg, 50-54.99 kg, and 55-60 kg). We grouped age by year (1 = 1 to 1.99, 2= 2-2.99, etc.), 2-year increments (0-2, 2.5-4, 4.5-6, 6.5-8, 8.5-10, >10), quartile within ages (<4, 4 -4.5, 5-6.5, and 7+), and into bins based on biologically relevant age stages of pronghorn: young = <4 years (before all teeth have erupted), middle = 4-7.5 years, and old = 8 years or older. A previous study corrected estimated age for this same group of animals based on cementum annuli analysis from dead animals [75]; we applied the same correction factor to test whether this metric was more relevant biologically and also grouped by the young/middle/old distinctions described above.

We first performed PERMANOVA tests on each pronghorn metric individually to test for significant differences ($p < 0.100$). We tested the significance of study area, location relative to a major interstate highway bisecting our study areas (I-80), ss-ligament, maximum rump fat thickness, all age metrics, disease status for EHD, disease status for BTV, all weight metrics, capture period, and capture season (February or November). We analyzed capture timing and study area metrics on the full set of 159 samples, while we analyzed a subset of 134 samples for pronghorn intrinsic measurements (disease status, age, weight, body condition) as some capture data was not recorded for all samples. Many of the pronghorn metrics were represented by multiple related variables (e.g., we binned weight and age in multiple ways), so we used these single metric PERMANOVAs to determine whether certain binning groups were more

biologically relevant to microbial communities (Table H). As none of the age or weight factors were significant in single metric PERMANOVAs (Table H), we chose the factor for each metric that we believed would have the strongest biological relevance in pronghorn. We chose to use ss-ligament measurements rather than maximum rump fat thickness, because previous work in pronghorn has shown this measurement to have biological relevance for survival [75]. While significant in our initial PERMAOVAs of single metrics (Table H), we chose not to include a capture period metric in our combined PERMANOVA analysis due to the fact that we captured all animals at the CDC study area during the same capture period, confounding this metric with the study area (Table A in S1 Appendix). In addition, certain pronghorn measurements were not collected during the February captures, meaning we needed to remove all samples from February captured pronghorn from combined PERMANOVA analysis to avoid errors with missing values. Location of the study area north or south of I-80 also was significant in single metric PERMANOVA (Table H), however we chose not to include this metric in combined PERMANOVAs as it was also confounded with study area.

We also log-transformed data to test whether trends were any stronger when relative abundance was represented on a logarithmic scale. We transformed relative abundance values (x) using the formula log (1 + x) to account for zero values. We conducted log transformations for taxa relative abundance to highlight effects of less common taxa, however patterns were not different from data held to the original scale (Table I). We thus chose to report data with non-log transformed relative abundance for more intuitive interpretation.

Table H. Results of single metric PERMANOVA tests.

	R^2	p
Study area	**0.060**	**0.001**
BTV	0.009	0.174
EHD	**0.011**	**0.019**
Age- young, mid, old	0.016	0.327
Age- (actual number)	0.155	0.226
Corrected Age- young, mid, old	0.014	0.757
Corrected Age (number)	0.155	0.226
Age- 1 year	0.068	0.410
Age- 2 years	0.038	0.410
Age- quartile	0.024	0.199
Weight-value (continuous)	0.719	0.288
Weight 1kg	0.136	0.489
Weight 5kg	0.024	0.254
Weight quartile	0.023	0.479
Ss-ligament	**0.083**	**0.045**
Max fat	0.048	0.910
I-80	**0.034**	**0.001**

Capture period	**0.029**	**0.001**
Capture season	**0.010**	**0.007**

Single metric PERMANOVAs comparing pronghorn metrics to the microbial community composition transformed to relative abundance. Significant ($p < 0.100$) effects are bolded. Note: capture period and study area metrics were conducted on the full set of 159 animals as data were available for all samples. Other metrics related to disease, age, weight and body condition were run on a subset of 134 animals as this metadata was not collected for all animals.

Table I. Results of log transformed single metric PERMANOVA tests.

Log-transformed relative abundance		
	R^2	p
Study area	**0.060**	**0.001**
BTV	0.009	0.164
EHD	**0.011**	**0.017**
Age- young, mid, old	0.016	0.321
Age- (actual number)	0.155	0.216
Corrected Age- young, mid, old	0.014	0.758
Corrected Age (number)	0.155	0.216
Age- 1 year	0.068	0.412
Age- 2 years	0.038	0.404
Age- quartile	0.024	0.193
Weight-value (continuous)	0.719	0.289
Weight 1kg	0.135	0.493
Weight 5kg	0.024	0.245
Weight quartile	0.023	0.475

Ss-ligament	**0.083**	**0.046**
Max fat	0.048	0.917
I-80	**0.034**	**0.001**
Capture period	**0.029**	**0.001**
Capture season	**0.010**	**0.007**

Log transformation was conducted to see if we could identify effects of less common taxa, however patterns were not different from the original scale so we chose to report on the original scale for more intuitive interpretation. Significant ($p < 0.100$) effects are bolded. Note: capture period and study area metrics were conducted on the full set of 159 animals as data were available for all samples. Other metrics related to disease, age, weight and body condition were run on a subset of 134 animals as this metadata was not collected for all animals.

S4 Appendix: PERMANOVAS in single study areas

After finding a significant interaction between study area and ss-ligament and study area and age we subset the data by study area to look at each area separately. Within each study area, we ran both single metric PERMAVOAS on age (young, middle aged, old) and ss-ligament to look at each variable separately. We also conducted combined PERMANOVAS to look at the marginal effects of BTV, EHD, weight, age, and ss-ligament. Within a single study area, the only factor that was significant was that of age ($p = 0.029$) within the Red Desert study area in single factor PERMANOVA (Table M).

Table J. PERMANOVAS within the Baggs Study Area, n = 36 animals

Single factor PERMAOVAS				
Term	F	df	R^2	P
Age (young, middle, old)	1.063	2	0.061	0.295
SS-ligament	1.079	9	0.272	0.135
Combined PERMANOVA				
Term	F	df	R^2	P
Age (young, middle, old)	1.099	2	0.060	0.224
BTV	0.977	1	0.027	0.515
EHD	1.229	1	0.034	0.120
SS-ligament	1.073	9	0.266	0.163
Weight (5 kg increments)	1.0055	3	0.083	0.455

Table K. PERMANOVAS within the Bitter Creek Study Area, n= 41 animals

Single factor PERMAOVAS				
Term	F	df	R^2	P
Age (young, middle, old)	1.011	2	0.051	0.451
SS-ligament	1.011	7	0.177	0.420
Combined PERMANOVA				
Term	F	df	R^2	P
Age (young, middle, old	1.052	2	0.052	0.307
BTV	1.0826	1	0.027	0.278
EHD	0.890	1	0.022	0.698
SS-ligament	0.984	7	0.171	0.529
Weight (5 kg increments)	0.984	3	0.073	0.528

Table L. PERMANOVAS within the CDC Study Area, n=22 animals

Single factor PERMAOVAS				
Term	F	df	R^2	P
Age (young, middle, old)	0.915	2	0.088	0.709
SS-ligament	1.098	6	0.305	0.168
Combined PERMANOVA				
Term	F	df	R^2	P
Age (young, middle, old)	1.149	2	0.107	0.171
BTV	0.837	1	0.039	0.770
EHD	1.124	1	0.053	0.242
SS-ligament	1.091	6	0.306	0.221
Weight (5 kg increments)	1.004	3	0.141	0.461

Table M. PERMANOVAS within the Red Desert Study Area, n = 35 animals

Single factor PERMAOVAS				
Term	F	df	R^2	P
Age (young, middle, old)	1.256	2	0.073	**0.029**
SS-ligament	1.103	6	0.191	0.109
Combined PERMANOVA				
Term	F	df	R^2	P
Age (young, middle, old)	1.143	2	0.066	0.128
BTV	1.112	1	0.032	0.249
EHD	0.922	1	0.027	0.642
SS-ligament	1.090	6	0.190	0.143
Weight (5 kg increments)	0.832	3	0.072	0.967

CHAPTER 3. Free-roaming horse diet and body condition differences across seasons and ecologically diverse herd management areas

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ABSTRACT

Increasing populations of free-roaming horses (*Equus caballus*) residing on federal lands pose management challenges across the American West, affecting rangeland health and co-occurring wildlife and livestock species. To better understand how free-roaming horses interact with rangeland ecosystems through herbivory, we used amplicon sequencing (P6 loop of chloroplast *trnL*) of horse fecal material to quantify plant composition of diets across a gradient of herbaceous availability in 16 Herd Management Areas (HMAs) managed by the Bureau of Land Management. These HMAs encompassed several ecosystems including the Colorado Plateau, Great Basin, Mojave Desert, and Wyoming Basin. We collected 1409 visual body condition scores (on a 1-to-9 scale) and 465 individual fecal collections in summer 2020 and winter 2020/2021 across HMAs. Because horses are considered grazers, we explored whether the dietary proportion of graminoids (i.e., grasses and grass-like plants) changed seasonally between and among HMAs. The proportion of graminoids in fecal material differed by HMA and ranged from 31.17 to 83.50% in summer and 11.00 to 82.60% in winter. Summer diets trended toward higher graminoid composition in most HMAs and many winter diets shifted to include non-graminoid plants in the Asteraceae and Chenopodiaceae families. Despite varying dietary graminoid composition, herd average body condition scores indicated most free-roaming horses

were in good condition. Across HMAs, herd average body condition scores in summer averaged 5.01 (minimum 4.59 and maximum 5.24) and averaged 4.98 in winter (minimum 4.72 and maximum 5.22). Understanding which plant groups form seasonal diets of free-roaming horses across different environments is important for managers balancing potential forage competition among free-roaming horses, wildlife, and livestock. Our results indicate that while free-roaming horses are considered grazers, they are also capable of subsisting and maintaining good body condition while consuming a variety of plants, with graminoids not always forming the majority of the diet.

KEY WORDS:

Equus caballus; diet composition; trnL; DNA metabarcoding; body condition

INTRODUCTION

Free-roaming horses (*Equus caballus*) are an introduced species to North American rangelands. These horses are not descended from prehistoric North American equids, which became extinct sometime between 10,000 and 12,500 years ago (Meltzer and Mead 1985; Grayson 1989; Guthrie 2003; Guthrie 2006). Instead, today's free-roaming horses in North America are descended from horses that evolved in Eurasia that were domesticated and later introduced by early European settlers and explorers starting in the 1500s and 1600s (Haines 1938a, 1938b; McKnight 1959). Once introduced, it took about 200 years for horses to be found across much of the western United States (Haines 1938b). The expansion of free-roaming horses across western North America was accelerated by the practice of ranchers turning out horses to nearby rangelands for later round up and use, Native American tribes trading horses, and by homesteaders, cavalry, and miners turning out their domestic horses to roam free when they were no longer needed (McKnight 1959). Over the years, free-roaming horses came to inhabit a

variety of ecosystems. Today, free-roaming horses are found in a range of environments across the Colorado Plateau, Great Basin, Mojave Desert, and Wyoming Basin including sagebrush (*Artemisia* L. *spp.*) steppe, pinyon-juniper (twoneedle pinyon [*Pinus edulis* Engelm] and singleleaf pinyon [*Pinus monophylla* Torr. & Frém.] -*Juniperus* L. spp.), salt desert, and timbered mountainous areas (BLM, undated). After passage of the Wild Free-Roaming Horses and Burros Act of 1971, areas that free-roaming horses and burros (*E. asinus*) resided in became designated as Herd Management Areas (HMAs) and Herd Areas on U. S. Department of Interior Bureau of Land Management (BLM) lands and Wild Horse and Burro Territories on U. S. Forest Service lands. Today, HMAs are managed for free-roaming horse use in addition to other multi-use goals while HAs are not specifically managed for free-roaming horse use. The 1971 Act prevented roundups by private citizens and began a new chapter in the history of free-roaming horse management. Free-roaming horse herds can increase approximately 20% annually (Garrott et al. 1991), with annual growth rates of this magnitude leading to populations doubling in four years and tripling in six (National Research Council, 2013). Fiscal Year 2025 estimated 53,797 horses and 19,333 burros roaming on BLM lands, whereas the range-wide appropriate management level (AML) is only a combined 25,556 horses and burros, representing an excess of >45,000 free-roaming equids in HMAs (BLM, 2025). As free-roaming horse numbers have increased, so has the need for greater understanding of the role these large herbivores serve in the ecosystems they inhabit. An increased understanding will assist practitioners to better manage the multiple use mandate of the BLM.

Continued population growth of free-roaming equids may cause rangeland degradation and competition with native wildlife and livestock. Free-roaming horses are associated with decreased vegetation diversity, species richness, shrub density, shrub and grass abundance, grass

height, percent cover of grass and shrubs, soil aggregate stability, greater soil penetration resistance, and increased bare ground (Beever and Brussard 2000; Beever and Herrick 2006; Beever et al. 2008; Davies et al. 2014; Hennig et al. 2021a). One concern of surging free-roaming horse populations is competition between horses and wildlife for resources such as water (Ostermann-Kelm et al. 2008; Perry et al. 2015; Hall et al. 2016; Gooch et al. 2017; Hennig et al. 2021b). Free-roaming horses have been shown to select similar seasonal habitat areas and resources as pronghorn (*Antilocapra americana*; Hennig et al. 2023, 2024), indicating the potential for shared resource competition. Free-roaming horses have affected species community composition of small mammals and reptiles: in a study occurring in the Great Basin, free-roaming horse occupied areas had less community completeness of small mammals and horse-removed sites had greater reptile species richness as well as greater abundance of most reptile species (Beever and Brussard 2004). Areas with grazing by free-roaming horses had decreased numbers of small animal burrows as compared to horse grazing exclosures (Beever and Brussard 2000). There is potential for free-roaming horses to impact greater sage-grouse (*Centrocercus urophasianus*) both directly and indirectly by altering habitat characteristics (Beever and Aldridge 2011; Hennig et al. 2021a) and disturbing lekking grouse (Muñoz et al. 2021). Higher free-roaming horse populations are predicted to have detrimental effects on sage-grouse populations as horse numbers increase where these species co-occur (Coates et al. 2021; Beck et al. 2024). In addition to interactions with wildlife, free-roaming horses have been reported to have high diet overlap with cattle (*Bos taurus*; Scasta et al. 2016) leading to the potential for competition with livestock. While our study focuses on North America, high activity of free-roaming horses has decreased environmental quality and had negative effects on the ecosystems in areas they inhabit worldwide (Eldridge et al. 2020).

The ability of free-roaming horses to thrive on what is often considered low quality forage can, at least in part, be attributed to the physiology of their digestive system. Horses are hindgut fermenters, with fermentation occurring in the cecum. Soluble protein and carbohydrates are absorbed in the small intestine before fermentation occurs (Janis 1976). Products of cellulose fermentation in the cecum are then absorbed in the enlarged colon (Janis 1976). Equids are not as efficient at breaking down plant cell walls as the foregut fermentation system present in ruminants (Hanley 1982) and digest their food less completely than ruminants (Duncan et al. 1990). However, by not digesting food as completely, equids are able to exhibit shorter forage retention time, enabling higher intake rates and nutrient extraction (Duncan et al. 1990), while also having the advantage of absorption of nutrients in the small intestine occurring before microbial actions. A study in France found that horses consumed 63% more forage than cattle grazing in a similar area, and as such were able to extract more total nutrients per day from grazing than cattle (Menard et al. 2002). It is suggested that in this way, hindgut fermenters can subsist on poorer quality forage than ruminants, which are limited by rumen fill and longer retention time (Janis 1976; Hanley 1982; Duncan et al. 1990). Free-roaming horses are traditionally thought to graze selectively on grasses and grass-like plants (hereafter "graminoids"). A meta-analysis of diet studies indicated that free-roaming horses across the western United States and Canada consume graminoids as a large proportion (mean ≥77%) of their diet across seasons (Scasta et al. 2016). However, in some environments, or during certain seasons, free-roaming horses have also been reported to eat large (>25%) proportions of woody browse (Krysl et al. 1984; Stephenson et al. 1985; Smith et al. 1998) or forbs (Hansen 1976, King and Schoenecker 2019).

Many of the previous diet studies of free-roaming horses in North America were conducted over 25 years ago, with only a few more recent diet studies in free-roaming horses (Hosten et al. 2007; King and Schoenecker 2019) and one in free-roaming burros (Esmaeili et al. 2023). Many of these previous diet studies in free-roaming horses used microhistology techniques, including the 12 studies summarized in the meta-analysis by Scasta et al. (2016). While this method has proven useful in quantifying horse diets (Morrison 2008) and has long been considered the "gold standard" for diet studies (Garnick et al. 2018), it does have some drawbacks. It has been demonstrated that the microhistology technique over-estimates graminoid composition and under-estimates the forb and some shrub component of diets because these plants are differentially more digestible than graminoids (Vavra and Holechek 1980; Holechek et al. 1982; Garnick et al. 2018). Consequently, researchers often conduct digestibility trials to obtain correction factors to account for differential digestibility (Pulliam and Nelson 1979; Smitman 1980). DNA metabarcoding is an emerging technology that uses DNA remaining in fecal material to characterize diet (Valentini et al. 2009), and may be less biased than microhistology against easily digestible forbs (King and Schoenecker 2019). Indeed, in recent studies, DNA barcoding has reported higher amounts of forbs or browse in American bison (*Bison bison*) diets (Bergmann et al. 2015; Craine et al. 2015; Leonard et al. 2017; Jorns et al. 2020; Hecker et al. 2021) and a study in free-roaming horses revealed higher forb content using DNA metabarcoding analysis as compared to microhistology within the study (King and Schoenecker 2019) and in comparison to previous microhistology studies (Scasta et al. 2016). Contrary to these findings of high forb consumption in species traditionally considered strict grazers, a study employing DNA metabarcoding found that two species of zebras (plains [*Equus quagga*] and Grévy's [*E. grevyi*]) exhibited diets composed of over 95% of grass, as expected in

a grazing species (Kartzinel et al. 2015). This same study of African megafauna found that the grass composition in diets based on DNA relative read abundance was positively correlated with results from stable-isotope analysis, and using relative read abundance was a reliable proxy for relative consumption of grass versus other plant families (Kartzinel et al. 2015). However, some critiques of the genomic technique have indicated discrepancies due to differential digestibility of plants still exists. A study using cattle fed known rations found that graminoids were both under- and over-estimated depending on what other plant functional groups they were paired with in diet formulations (Scasta et al. 2019). The genomic approach also has limitations when assessing resolutions deeper than family or genus level (Garnick et al. 2018). However, taxonomic resolution can be improved with local plant knowledge and as more robust reference databases are developed (Scasta et al. 2019).

Our study used DNA metabarcoding to investigate free-roaming horse diets across 16 BLM HMAs, largely covering the geographic extent in which free-roaming horses reside in the western United States. Many of the previous free-roaming horse diet studies only evaluated single, localized study areas (for example, see Hansen 1976; Salter and Hudson 1979; Hanley and Hanley 1982; Stephenson et al. 1985; McInnis and Vavra 1987; Sullivan 1988; Smith et al. 1998; Hosten et al. 2007; King and Schoenecker 2019). While these studies are informative, results from studies in different areas or seasons may not always be directly comparable due to differences in collection techniques or lab protocols. Our study is novel in that we visited multiple HMAs in summer and then repeated visits to these same areas in the winter. Because we used consistent field and lab methods with all fecal collections in our study, this makes our results more directly comparable across HMAs, environments, and seasons and provides a comprehensive estimate of range-wide diet composition of free-roaming horses. In addition, our

study provides current insight to the diets of free-roaming horses, as diets may have adjusted with changing rangelands and climates over the past 25+ years. We developed research objectives to guide our investigation of free-roaming horse diets within HMAs in seven western states (Fig. 1). First, we described the diet composition of free-roaming horses in each study HMA in summer and winter (Fig A1). Based on previous diet literature, we expected graminoids to dominate the diet composition of free-roaming horses (Scasta et al. 2016). Second, we evaluated the graminoid portion of the free-roaming horse diet across season and location. We quantified seasonal changes in the diet composition of graminoids within individual HMAs but also at the broader range-wide scale of all HMAs in our study. Third, we investigated whether the amount of graminoids in the diet was related to the amount of herbaceous cover or biomass available at the HMA scale. We predicted that free-roaming horses would have a greater composition of graminoids in their diet in areas with greater proportions of remote sensed herbaceous cover or amounts of biomass. Fourth, we investigated if the average amount of graminoids in free-roaming horse diets in an HMA was related to the overall average body condition of horses in an HMA, and whether seasonal differences in graminoid consumption were associated with seasonal differences in body condition at the HMA and range-wide scales.

METHODS

Study Areas

We selected study areas among all 177 Bureau of Land Management (BLM) Herd Management Areas (HMAs) to represent the geographical and ecosystem diversity across the western United States (Table 1, Fig. 1). We initially evaluated HMAs for selection based on information available on the BLM's Wild Horse and Burro Herd Management Areas Website (BLM, undated, https://www.blm.gov/programs/wild-horse-and-burro/herd-management/herd-

management-areas) and through discussions with BLM personnel. We considered ease of access by off-road vehicles or on foot in our selection of HMAs. We further consulted with BLM personnel to identify HMAs where free-roaming horses could be safely and reliably accessed during both summer and winter. We calculated herbaceous cover values for each HMA based on values from The Multi-Resolution Land Characteristics Consortium 2016 National Land Cover Database (NLCD) Shrubland Fractional Component cover data (Dewitz, 2019). We focused our stratification on herbaceous cover because the literature indicated free-roaming horses consume predominantly graminoids (Scasta et al. 2016) and we selected HMAs to ensure we represented the gradient of herbaceous availability across the range. We identified the United States Environmental Protection Agency (EPA) level III ecoregion (EPA 2024) that each HMA was in by overlaying shape files of HMAs with shapefiles of the ecoregions. For HMAs that included multiple ecoregions within their border, we assigned them to the ecoregion in which the majority of the HMA was located. This process yielded 16 HMAs across our herbaceous gradient and located within six different EPA Level III ecoregions. We used the 2016 NLCD data to design the study because 2016 was the most current year available when we were designing the study. However, because vegetation communities can change over time, we re-calculated herbaceous cover *post-hoc* for HMAs based on 2020 herbaceous cover values from the Rangeland Analysis Platform (RAP) (Robinson et al. 2019; Allred et al. 2021; Jones et al. 2021) so that cover information would have a more accurate temporal relationship with free-roaming horses during our study. We then stratified our HMAs into low (<20%), medium (20-35%), and high (>35%) herbaceous cover to maintain an even spread of five HMAs per cover category. Due to inclement weather conditions and impassable roads, we were unable to access the Sand Basins HMA in

Idaho in winter 2020/2021. Consequently, we only report summer data from this HMA and removed it from seasonal comparisons and correlations.

Field Methods

Fecal Sampling

At each HMA, we collected free-roaming horse fecal material from 15 distinct piles in summer 2020 (May–July) and winter 2020/2021 (late November to January) at each HMA. Due to the nature of free-roaming horses to move long distances daily in search of food and water (Hampson et al. 2010; Hennig et al. 2018), they may not have been foraging in the same area where fecal material was obtained. In each HMA, locations of frequent or probable free-roaming horse use (usually water sources) from which to begin searching for horses were identified from information shared by managers or maps. From these locations, we drove on accessible roads until free-roaming horses or fresh fecal material were visible. When possible, we collected fecal material from at least three spatially distinct areas in each HMA to achieve a representative diversity of free-roaming horses across a geographical gradient within an HMA. However, in some HMAs, collections from multiple areas were not possible due to uneven use by free-roaming horses across seasons or concentration of free-roaming horses in a few spots (e.g., one study area only had a single perennial water source). Multiple collection areas were also limited by large groups where most free-roaming horses were found in close proximity to one another at a single time point, lack of access to parts of the HMA, or inability to find fresh signs of use by free-roaming horses within selected sampling areas.

From each fresh fecal pile, we collected at least three fecal boluses, which we assumed to represent a single individual free-roaming horse. We obtained fecal material from the freshest piles, immediately after observed defecations if possible, and we assigned each fecal collection a

freshness index, as described in the supplementary methods in Appendix A. Although we did not observe each defecation, we only collected fecal boluses with moist interiors to ensure representativeness of the season and dates we were sampling. Horse fecal material has been reported to maintain good rates of DNA amplification for up to two months (King et al. 2018). To collect each fecal bolus we turned a sealable plastic freezer bag inside out without touching the bag interior. If we needed to further place material into the bag properly, we wore latex gloves to prevent contamination of fecal material. We collected the fecal material from the interior of piles to minimize potential environmental contamination and then returned plastic bags to proper orientation and sealed them without touching the interior of the bag. We stored individual bagged material on dry ice in the field and transferred frozen fecal material within two weeks to a -20°C freezer. Freezing was our preferred method to store fecal material because freezing has effectively preserved fecal DNA in other species (Vlčková et al. 2012; Czernik et al. 2013).

Body condition

In the field, we visualized and recorded body condition scores (BCS) for free-roaming horses based on the Henneke system. The Henneke method rates the animal's accumulation of subcutaneous adipose tissue on a scale from 1–9, with 1 being extremely emaciated and 9 being extremely fat (Henneke 1983). Measurements are observed at the lumbar spinous processes, ribs, tailhead, area behind the shoulder, neck and withers (Henneke et al. 1983) and the method calls for both visual observations and palpation. This method of body condition scoring has been correlated to ultrasonic measures of adipose tissue (Gentry et al. 2004). Because we could not palpate these horses, we applied this system on a visual-only basis while evaluating the same regions stated in the system. This method of visual body scoring has been previously applied in

other studies of free-roaming horses (Schoenecker et al. 2024). We observed free-roaming horses with a spotting scope (16-48x magnification), binoculars (8 x 42 magnification), or by eye when horses were close enough to perform observational measurements. In most HMAs (13/16), to achieve a larger sample size of BCS, we scored every observable free-roaming horse we found that was unobscured (not standing behind another animal, rocks, or vegetation) and within 400 m to distinguish features. We observed free-roaming horse groups for long periods when possible, to get multiple views and angles for a more accurate score of each animal. When large herds were present (>100 horses) we systematically scored every fifth horse in the herd to control sampling bias of only selecting some horses from the herd. We made observations of horse BCS to determine the herd average BCS within each HMA, thus we recorded BCS from more horses than we collected fecal samples from. In total, we collected 90 out of 465 fecal samples from free-roaming horses of known BCS. While a similar body condition scoring method using scores of 1-5 was repeatable between different observers (Carrol and Huntington 1988), our study had the added consistency of a single researcher making all BCS observations.

Lab Methods

Fecal Processing

We stored our fecal material in a -20°C freezer and later sub-sampled the fecal material from each discrete pile into individual 2 ml test tubes to be submitted for DNA extraction. As this fecal material was also being used for a gut microbiome study, we conducted sub-setting work under a hood and took additional steps to prevent contamination from the environment and from one horse to another. While working in the lab we kept fecal material on ice while not being directly handled to prevent thawing and returned material to the freezer when we finished each group of 10-15 individuals. We used metal corers driven into the bolus with a rubber mallet to

take an internal core from each frozen fecal bolus. We took cores from multiple boluses within each individual fecal collection to better represent each animal. Whenever possible we only used the inner portion of each core to avoid any external environmental contamination present on the surface of fecal boluses when collected. To minimize contamination further we used a new set of gloves and clean corer for each individual horse's fecal matter and we wiped surfaces with ethanol and RNase-AWAY ™ (Fisher Scientific) to remove any residual bacteria and DNA or RNA fragments. We autoclaved metal corers, subsample tubes, and other tools that could withstand autoclaving before each use to further ensure we minimized contamination. After adding 230–250 mg of fecal material to each tube under the hood, we stored sub-samples in a -20°C freezer.

DNA Extractions, Library Preparation, and Sequencing

We submitted fecal material to the Genome Technologies Lab at the University of Wyoming for DNA extraction and library preparation, in which samples are amplified for the region of interest and prepared for high-throughput sequencing. A QIAGEN DNeasyPowerSoil Kit was used for the extraction of fecal materials. Manufacturer's protocols were used for the extraction process, with tungsten carbide beads used for mechanical sample lysis. Samples were normalized to 10ng/µL prior to amplification and library preparation.

The P6 loop of the *trnL* locus was amplified using the *-g* (GGGCAATCCTGAGCCAA) and *-h* (CCATTGAGTCTCTGCACCTATC) primers described by Taberlet et al. (2007). Library preparation was conducted using custom designed one-step primers, including Illumina adaptors and unique oligo barcodes so that the indexes and barcodes were within the read. Two libraries were prepared for each individual fecal collection, to assess consistency in library preparation and sequencing. PCR conditions for library preparation included a 10 minute denaturation

(95°C) followed by 35 cycles of denaturation (95°C, 30s), annealing (55°C, 30s), and extension (72°C, 30s). This was followed by a final extension period (72°C, 9 minutes) and a hold at 4°C. PCR cleanup was conducted using the Axygen™ AxyPrep MAG PCR Clean-Up Kit following the manufacturer's protocol.

The University of Colorado conducted sequencing. Libraries were pooled with those of a 16S bacterial region of the same fecal collection (data not reported here) at a ratio of four to one (16S: *trnL*), to account for differences in amplicon length. Libraries were sequenced at 2x250 on a NovaSeq6000, with a 10% PhiX addition. For further protocol details, please see Appendix A.

Bioinformatics

We used the program STAND (Weinstein et al. 2021) to create a custom *trnL* database for fecal metabarcoding. We generated this database from plant chloroplast sequences and compiled it from all existing NCBI GenBank entries on 6 January 2023 using the search query "biomol_genomic[PROP] AND is_nuccore[filter] AND chloroplast[filter]" within the *download_data.py* function. Using the *build_databases.py* function, we trimmed and compiled the *trnL* p6 loop of all sequences into a FASTA file, with taxonomic classification for species belonging to "Viridiplantae."

We trimmed amplicon sequencing variants (ASVs) using DADA2 v. 1.22 and Cutadapt v. 2.6 for *trnL* -g and -h primers. Using DADA2, we filtered reads with parameters set for a minimum length of 10 base pairs, truncated reads when the quality score was less than two, and all reads with more than two errors were discarded. We dereplicated and merged reads using DADA2. Sequences were classified against the *trnL* database using the classify_sequences.py script within the STAND pipeline (Weinstein et al. 2021). Briefly, we aligned trimmed and filtered sample *trnL* sequences against our database using BLASTN 2.15. We did not assign

taxonomy if there was an E-value above 1, a percent identity less than 90, or an alignment length above 10% of the ASV length.

We read the results of the STAND pipeline and our metadata into Program R (Version 4.3.0; R Core Team, 2023) for further downstream analysis. We used the R package 'phyloseq' (McMurdie and Holmes 2013) to combine the taxonomy assignment table, fecal collection metadata, and the ASV table for analyses. The lab protocols used produced two sets of reads for each fecal collection, so we merged the reads for these two sets to create a single set of read counts for each horse. We subset reads to only those ASVs assigned to the plant kingdom "Viridiplantae", yielding 3201 unique ASVs. As our study was more focused on general diet trends and not as concerned with rare species present only in small amounts within diets we further filtered reads to exclude any ASV that was not present with at least 1% abundance in at least one horse. This also helped to remove potential erroneous reads present in low numbers due to DNA amplification and sequencing errors. This left us with 284 unique ASVs present in the diets of our free-roaming horses, which were assigned varying levels of taxonomic depth.

To inform which taxonomic level to analyze the data at, we checked the depth of taxonomic assignment of each ASV and noted that most ASVs (282 of 284, 99.30%) were assigned to at least a family level, representing 99.97% of our total reads (Table 2). We found fewer ASVs were assigned to a genus level (80.28% of ASVs and 69.32% of reads) and only 58 (20.42%) ASVs and 11.24% of total reads were assigned to a species level (Table 2).

Because DNA metabarcoding is still a developing technology for diet analysis of free-ranging animals, not all plants potentially present in wild herbivore diets have been sequenced and improved reference libraries have been called for (Scasta et al. 2019). Consequently, if a plant found in fecal DNA is not yet present in the database, a plant may be matched to a

taxonomically close relative that has been sequenced but may not occur in the region of study. Authors using this technology have suggested pairing DNA results with knowledge of the plant community or other sources of information about plant availability (Scasta et al. 2019). Therefore, from the remaining 284 ASVs, we crosschecked the deepest taxonomic assignment of each ASV (family, genus, or species) using the USDA plant database (USDA, NRCS), Rocky Mountain Herbarium (Rocky Mountain Herbarium, 2022), and iNaturalist (iNaturalist 2024). We conducted this step to gain a better understanding of the potential accuracy of taxonomic assignments of each ASV based on the location that the assigned plant family, species, or genus is known to occur. We describe this process further in Appendix A.

Based on our analyses of depth of taxonomic assignment and findings of our plant crosschecks, we chose to pool all ASVs at the family level for further analysis of diet composition. This assisted us to gain an overall understanding of the composition of each diet that was detailed enough to understand what the animals were eating, but not taxonomically specific enough to exclude important information. For example, if we had chosen to analyze at the genus level we would have excluded over 30% of our reads due to the lack of consistent taxonomic depth (Table 2). Using the family level allowed us enough taxonomic depth to explore our research questions, which mainly centered on the proportion of graminoids present in free-roaming horse diets. Furthermore, our decision to analyze at this taxonomic depth was supported by previous findings that relative read abundance was a suitable proxy of grass composition in the diet relative to other plant families (Kartzinel et al. 2015), and that this technology had some uncertainties at the genus and species level (Scasta et al. 2019). Two recent studies using DNA metabarcoding have achieved the goals of diet elucidation without deep taxonomic assignment by grouping results at the family level (Esmaeili et al. 2023) or even

morphological group level (Julian et al. 2024). While our crosschecks did reveal some reads were assigned to plants not previously documented in the western United States, this likely means a genetically similar but exotic relative was assigned as the closest taxonomic match because the true plant consumed has not been sequenced and added to the database yet. Alternatively, undocumented small populations of these exotic species may occur in the sagebrush steppe. Rather than excluding these reads entirely from analysis, we included them by assigning reads to the family level. With the current scope of genetic databases, we are confident that taxonomic assignments are accurate to the family level even if the species assignments are potentially questionable.

Because each fecal collection was only a small temporal sample of what an individual free-roaming horse was consuming for a given day, we decided to pool within each HMA and season to better represent the average diet throughout each HMA in each season. During our short collection period in each HMA, (one to three days) there was sometimes large variation in what each of the free-roaming horses within an HMA were consuming (Figs. A2–A3). The average rate of passage through the digestive system for horses eating a forage-based diet varies based on many factors but previous studies report it to generally fall between 24–48 hours (Pearson and Merritt 1991; Pearson et al. 2006; Clauss et al. 2014; Miyaji et al. 2014). Because our study design only allowed for a single collection time point in each HMA and season, we felt that averaging the 15 horse diets in an HMA together to get a "herd" average evened out the daily foraging variability of a single horse and gave a more accurate representation of what the average horse would consume over the course of a season in a specific HMA. To further account for our short sampling window, we also pooled the HMA average diet compositions to create a range-wide average diet profile for both summer and winter at the plant family level (Figure 2).

Statistical Methods

To address our second through fourth research objectives, which centered on graminoid consumption, we combined reads of our three grass-like families of interest: Cyperaceae (sedges), Juncaceae (rushes), and Poaceae (grasses). We used these values to calculate the average proportion of plants in the grass and grasslike families (hereafter "graminoids") for each HMA and season. To evaluate average diet composition of graminoids at the range-wide scale, we completed bootstrapped and traditional non-bootstrapped t-tests using each HMA's herd average dietary graminoid composition for our experimental unit, with our analysis paired by HMA. We then compared the seasonal graminoid diet composition for each HMA separately using the 30 individual fecal collections from each HMA (15 summer and 15 winter). Because our sampling design did not positively identify a specific horse associated with each fecal collection, we could not guarantee the independence between animals in summer and winter. Therefore, to improve the confidence in our results we used a weight of evidence approach and conducted multiple t-tests within each HMA to assess whether a change in the assumption of independence affected our findings. These included an independent bootstrapped t-test and bootstrapped t-tests paired for both minimum and maximum variation. By completing this analysis with the minimum and maximum variation, we found the upper and lower limits of p-values possible from our observations within each HMA. Therefore, even if we could not be 100% confident that fecal samples were not collected from the same horses in both seasons, we obtained a range of possible p-values, despite the independence assumptions. By comparing the results of these multiple tests, we were able to determine whether we had strong evidence for or against a seasonal difference within each HMA. These methods are detailed further in Appendix A. We also performed the traditional non-bootstrapped versions of these tests to assess whether

the assumption of normality affected our results. We analyzed data in Program R using both 't.test' from the 'stats' package (version 4.3.0; R Core Team 2023) for the traditional non-bootstrapped t-test and the 'boot.t.test' function from the 'Mkinfer' package (bootstrapped t-test; Efron and Tibshirani 1993) using the default setting of 9999 replicates for the bootstrapped t-test.

To investigate whether cover category or ecoregion of an HMA influenced whether there were differences in summer and winter graminoid composition we performed a two-factor ANOVA using herbaceous cover category (Low [0-20%], Medium [20-35%], and High [>35%]) and ecoregion (Central Basin and Range [CB], Colorado Plateau [CP], Mojave Basin and Range [MB], Northern Basin and Range [NB], Snake River Plain [SR], Wyoming Basin [WB]) as factors and the difference between summer and winter HMA average graminoid composition as our response variable. We were unable to fit the model with an interaction, because there was not an HMA from every ecoregion present within each cover class.

To explore the relationship between the composition of graminoids present in free-roaming horse diets and the herbaceous forage available to horses, we performed analyses using both the HMA average herbaceous availability as well as developing a herd average based on potential daily movement of free-roaming horses. First, we obtained 2020 Rangeland Analysis Platform (RAP) herbaceous cover and biomass spatial information. The RAP cover data provided estimates of cover of annual forbs and grasses, bare ground, perennial forbs and grasses, shrubs, and trees at 30-m resolution while the biomass information provided an estimate of annual forbs and grasses, perennial forbs and grasses, and total herbaceous biomass (a combination of the annual and perennial estimates; Robinson et al. 2019; Allred et al. 2021; Jones et al. 2021). To obtain the average amount of total (annual and perennial) herbaceous cover and biomass for each HMA, we extracted these values for each HMA boundary using the

terra package (Hijmans et al. 2024) in R. We also took the GPS point of each fecal collection location, created a 13.5-km diameter buffer around the point, and calculated the average herbaceous cover and biomass within that buffer also using the terra package. This buffer size represented the average daily movement of a free-roaming horse of 9 km (Hennig et al. 2018) multiplied by 1.5 for the average passage rate of 36 hours. The literature indicated average mean retention rates between 24 and 48 hours (Pearson and Merritt 1991; Pearson et al. 2006; Clauss et al. 2014; Miyaji et al. 2014). Thus, we represent a measure of the potential available forage to an individual free-roaming horse within the time frame that they would have consumed forage leading to the fecal matter we collected. We calculated this value for each individual fecal collection and averaged the 15 values within an HMA/season together to compute an average herbaceous cover and biomass value for each HMA based on free-roaming horse fecal collection locations and potential horse movement rather than the HMA boundary. We then conducted Spearman's rank correlation tests using 'cor.test' function in the 'stats' package (R Core Team 2023) with total herbaceous cover and herbaceous biomass values of both the HMA boundary average and the HMA average created from each animal's potential "foraging zone" correlated against the average graminoid composition of diets in each HMA and season. Due to questions of the normality assumption, we performed Spearman's rank correlations for these comparisons, however we also explored logit transformations of proportions, which we describe further in Appendix A.

We used our BCS observations within each HMA to conduct the independent bootstrapped t-test described above to test whether there was a difference between summer and winter BCS within each HMA. Due to uneven sample sizes in winter and summer BCS observations, we did not repeat these tests with the paired method we used for graminoid

composition in the diet. We also calculated an average BCS for each HMA or "herd". We conducted a paired bootstrapped two-sample t-test using the HMA average BCS as an experimental unit to test whether there was a seasonal change in body condition at the range-wide scale. We also used these HMA BCS averages to conduct a Spearman's rank correlation (see methods above) between herd average BCS and herd average graminoid consumption within each season. Finally, we conducted a Spearman's rank correlation to evaluate whether average herd graminoid composition in the summer was related to herd average BCS in the winter.

Throughout our analyses, we used an alpha value of 0.05 for all statistical analyses and deemed tests moderately significant when p-values were between 0.10 and 0.05. Although an alpha value of 0.10 is higher than often used, accepting a higher false positive (Type I) error rate allows lower false negative (Type II) error (Zar 2010). We also report p-values throughout to allow the reader transparency to draw conclusions based on our weight of evidence approach.

RESULTS

Grass (Poaceae) and grasslike (Cyperaceae and Juncaceae) plant families made up the largest component of diets of free-roaming horses in most HMAs in both seasons (Figs. 2, A1). The use of graminoid plants by free-roaming horses was so ubiquitous in our study that only two individual free-roaming horse diets did not contain graminoids (see Appendix B for more details). Excluding Sands Basin HMA, the average graminoid composition in the diets of free-roaming horses was 54.7% (SD = 18.2) across all HMAs and seasons. However, the amount of graminoids present varied across HMAs and seasons, with summer averaging 59.4% (SD = 18.0, range 31.1%–83.5%) and winter averaging 49.9% (SD = 17.7, range 11.0%–82.6%; Figs. 2, A1, Table 3). Several other plant families were large components of diets with the plant families of Asteraceae [composites], Brassicaceae [mustards], Chenopodiaceae [goosefoot family],

Fabaceae [legumes], and Polygonaceae [buckwheats] each making up over 25% of the herd average diet composition in at least one HMA and season (Fig. A1, Table A1). In particular, the Asteraceae and Chenopodiaceae families occurred in diets in most HMAs (Table 4, Fig. A1). These families were both more prevalent in winter than summer diets with range-wide average diet compositions of 5.70% (SD = 5.83) and 4.07% (SD = 8.39) in the summer and 15.05% (SD = 15.79) and 14.53% (SD = 15.23) in the winter for Asteraceae and Chenopodiaceae respectively (Table 4, Fig. 2). Both paired traditional and paired bootstrapped t-tests indicated that seasonal differences existed for the range-wide average diet composition of Asteraceae (bootstrap p = 0.010, traditional t = -2.447, df = 14, p = 0.028) and Chenopodiaceae (bootstrap p = 0.014, traditional t = -2.312, df = 14, p = 0.037). The families of Brassicaceae, Fabaceae, and Polygonaceae were locally important in certain HMAs, detailed in Appendix B, but were not as common across HMAs.

We found higher graminoid composition in the summer as compared to the winter at the range-wide scale and that many HMAs tended toward higher graminoid composition in the summer, though differences in individual HMAs were not always statistically significant (Table 3). At the range-wide scale, using each HMA average as the experimental unit, a paired bootstrapped t-test showed moderate support for higher summer graminoid composition (p = 0.050, Table 3) and the traditional, non-bootstrapped paired t-test also displayed evidence of this difference (p = 0.036, t = 2.317, df = 14, Table A2). Our weight of evidence approach using independent and paired bootstrapped t-tests within each HMA indicated in three HMAs (Frisco, UT; Palomino Buttes, OR; and Saylor Creek, ID) there was strong evidence for higher summer graminoid consumption. A further two HMAs displayed moderate support for higher graminoid composition in the summer (Little Book Cliffs, CO; Stinkingwater, OR), while one HMA

(Onaqui, UT) had moderate support for higher graminoid composition in the winter. Four HMAs (Adobe Town, WY; Pine Nut Mountains, NV; South Steens, OR; Twin Peaks, CA and NV) demonstrated a consistent lack of difference in summer and winter graminoid composition across our various tests. All HMAs with a consistent lack of seasonal difference displayed less than a 5 percentage point difference between summer and winter graminoid composition, thus we used this as the cutoff point where we interpreted graminoid composition as the same between seasons. Using this cutoff, nine of the 15 (60.00%) HMAs tended toward higher graminoid composition in the summer, while two (13.33%) tended toward higher winter graminoid composition. Additional interpretation of these results are reported in Appendix B. The overall results from our two-factor ANOVA test ($F_{7,7} = 1.936$, $p = 0.202$) indicated that neither cover category (low, medium, or high herbaceous cover) nor ecoregion (Central Basin and Range [CB], Colorado Plateau [CP], Mojave Basin and Range [MB], Northern Basin and Range [NB], Snake River Plain [SR], Wyoming Basin [WB]) was a significant predictor of difference in graminoid composition between summer and winter.

Using the total herbaceous cover and biomass values derived from the HMA boundary, we did not find a relationship between HMA cover or biomass and HMA average graminoid diet composition in either season. In the summer, there was no significant correlation between average dietary graminoid composition and either total herbaceous cover ($r_s = -0.379$, p = 0.165; Fig. 3A) or total herbaceous biomass ($r_s = -0.439$, p = 0.103; Fig. 3B). In the winter, there was no significant correlation between average dietary graminoid composition and either total herbaceous cover ($r_s = -0.279$, p = 0.314, Fig. 3E) or total herbaceous biomass ($r_s = -0.371$, p = 0.174; Fig. 3F). However, there was a negative trend for all Spearman's rank correlations calculated in both seasons, with summer exhibiting stronger trends (Fig. 3A, B, E, F). When we

used the 15 individual foraging buffers to calculate an average cover and biomass value for each HMA, our results were slightly different. As with using the HMA boundary derived values, we observed a negative trend in the relationship between both herbaceous cover and herbaceous biomass and HMA average graminoid composition (Fig. 3 C, D, G, H). These trends were not significant in the winter months for either total herbaceous cover (r_s = -0.268, p = 0.333, Fig. 3G) or biomass (r_s = -0.286, p = 0.301 Fig. 3H). However, in the summer months, the trend was moderately significant for a negative relationship between average dietary graminoid composition and both total herbaceous cover (r_s = -0.475, p = 0.076 Fig. 3C) and biomass (r_s = -0.479, p = 0.073 Fig. 3D).

We conducted 707 BCS observations in the summer (22–95 animals per HMA; mean 45.53 [SD = 22.06] horses [excluding Sands Basin]) and 702 observations in the winter (19–76 animals per HMA; mean 46.80 [SD =18.04] horses). Observations of individual free-roaming horses ranged from a BCS of 3 to 7 during both seasons. Herd average BCS were close to 5 in all HMAs, with an average BCS during the summer of 5.01 (SD = 0.16, range: 4.59–5.24) and an average BCS in winter of 4.98 (SD = 0.14, range: 4.72–5.22). Most free-roaming horses were in good body condition, with 1,323 of the 1,409 observations (93.90%) scored as ≥ 5 (Fig. 4).

Overall, summer and winter body conditions were similar at the HMA and range-wide level (Table 5). Only two HMAs in Oregon (Palomino Buttes and South Steens) had herd average body condition scores that differed ($p < 0.05$) between summer and winter and one HMA that straddles California and Nevada (Twin Peaks) displayed a moderate difference (p = 0.080; Table 5). We did not detect a difference in BCS between seasons at the range-wide scale when using the seasonal HMA herd averages as the experimental unit (p = 0.205; Table 5).

We found a negative relationship between the herd average dietary graminoid composition and herd average BCS in the summer months (r_s = -0.554, p = 0.035) with herds that had higher consumption of graminoids tending to have lower BCS scores (Fig. 5A). In the winter, we did not find a relationship between the herd average graminoid composition and herd average BCS (r_s = -0.118, p = 0.676; Fig. 5B). Finally, we did not find a relationship between the previous summer herd average graminoid composition and the subsequent winter herd average body condition (r_s = -0.068, p = 0.812; Fig. 5C).

DISCUSSION

Overall, as expected graminoids were the largest component of free-roaming horse diets in most study areas. However, the proportion of graminoids present in diet composition was lower than in many other studies of free-roaming horse diets. While a previous meta-analysis of microhistological diet studies that included free-roaming horses from 12 studies across western North America found graminoid composition of 88% of summer and 77% of winter diets (Scasta et al. 2016), our results indicated graminoid composition of diets averaged around 60% in the summer and 50% in the winter. We even found that graminoid composition was below 50% in both seasons in four of the HMAs we studied (McCullough Peaks, WY, Palomino Buttes, OR, Pine Nut Mountains, NV, Twin Peaks, CA/NV), and below 50% in the winter in four additional HMAs (Little Book Cliffs, CO, Red Rock, NV, Saylor Creek, ID, and Stinkingwater, OR). Our lower graminoid composition compared to previous studies may be attributed to the DNA metabarcoding technique used as compared to studies previously using microhistology. Microhistology may overestimate diet components such as grasses that are less digestible (Holechek et al. 1982). Indeed, a recent study of free-roaming horse diets found lower graminoid diet composition when using DNA metabarcoding (68.8% graminoids) as compared to

microhistological techniques (78.5% graminoids; King and Schoenecker 2019). Lower amounts of graminoids were also found in bison diets when using a similar DNA method as compared to other techniques (Craine et al. 2015). Even when using microhistology, diet components other than graminoids have sometimes been revealed to make up large parts (>25%) of horse diets in some or all seasons (Hansen 1976; Krysl et al. 1984; Stephenson et al. 1985; Smith et al. 1998). Two studies using microhistology that also found free-roaming horses eating less than 50% graminoids in winter seasons (Hansen 1976; Stephenson et al. 1985).

While free-roaming horses tended to have a higher graminoid diet composition in the summer compared to the winter at the range-wide scale and in many HMAs, dietary composition differed across the range with HMA herd averages ranging from a high of 83.50% graminoid composition in the summer to a low of 11.00% in the winter. A recent study of free-roaming burros managed on BLM HMAs also using DNA metabarcoding found varying amounts of graminoids in the diets of burros in two different HMA locations and ecosystems (Esmaeili et al. 2023), lending merit to the idea that location differences could explain the diversity of dietary graminoid composition across HMAs in our study. There was no distinct pattern of seasonal graminoid composition related to a specific ecoregion. The five HMAs demonstrating support for higher summer graminoid consumption occurred in four different ecoregions. Within an ecoregion, there were often different patterns of graminoid composition between HMAs. For example, within the Central Basin and Range ecoregion, free-roaming horses from Frisco (UT) consumed more graminoids in the summer, Pine Nut Mountain (NV) horses had no seasonal difference, and Onaqui (UT) horses had moderate evidence toward higher winter graminoid composition. The same was true when we evaluated high (>35%), medium (20-35%), and low (0-20%) herbaceous cover categories used to stratify HMAs. HMAs that demonstrated support

for a higher summer graminoid composition occurred across all three cover categories and HMAs with a lack of seasonal difference occurred in both high and low cover categories. Thus, while there was an overall general pattern toward higher summer graminoid composition across the range, dietary strategy may be more tied to individual HMAs rather than ecoregions or overall herbaceous cover availability. This was supported by our ANOVA model findings as well, where we found that differences in summer and winter graminoid composition were not explained by cover category or ecoregion.

The decrease in graminoid composition in winter diets in many HMAs was countered by an increase in plants from the Asteraceae and Chenopodiaceae families. This was evident when comparing the average seasonal diet at the range-wide scale (Fig. 2), but this tendency was also perceptible at the HMA scale with most HMA average diet compositions exhibiting higher amounts of one or both of these families in winter (Table 4, Fig. A1). Other plant families including Brassicaceae, Fabaceae, and Polygonaceae made up large portions (>25%) of HMA average diet composition in specific HMAs. However, these families were not found as consistently in free-roaming horse diets across the range, meaning their use as forage plants may only be locally important in specific HMAs or seasons. Other studies have revealed that free-roaming horses can consume plants different from what would be expected for a strict grazer. For example, Smith et al. (1998) reported free-roaming horse diets in New Mexico consisting of 28% honey mesquite (*Prosopis glandulosa* Torr.) during the warm/wet season of July to November. During an earlier study in a similar area of New Mexico, Hansen (1976) found that winter free-roaming horse diets consisted of 42% Russian thistle (*Salsola kali* in publication now *Salsola tragus* L.) while summer and fall diets contained 28 and 22% of mesquite, respectively. Free-roaming horses in another area of New Mexico consumed winter diets with 43% shrub

composition including both the Asteraceae and Chenopodiaceae families with winterfat (*Eurotia lanata* [Pursh] Moq. in publication, now *Krascheninnikovia lanata* [*Pursh*] *A. Meeuse & Smit*) being the most used species (19%) followed by fringed sagewort (*Artemisia frigida*, 8%; Stephenson et al. 1985). Horses in the Red Desert of Wyoming consumed shrubs in the Asteraceae and Chenopodiaceae families during both summer (28% of diets) and winter (39% of diets) including big sagebrush (*Artemisia tridentata*), fourwing saltbush (*Atriplex canescens* [Pursh] Nutt.), horsebrush (*Tetradymia canescens* D.C.), and winterfat by (Krysl et al. 1984).

Because we only visited each HMA during a short sampling window (generally one to three days), some dietary differences between HMAs in the summer may have been due to plant phenological differences. When possible, we mitigated phenology by visiting more southern HMAs earlier in the summer when plants were actively growing and then later sampling HMAs farther north where plant growth occurs later in the season. In the winter months, our sampling period occurred during the dormant season, so time of sampling should not have affected forage selection by free-roaming horses due to plant phenology. There is potential that snow cover may have affected availability of forage, as snow cover was only present during our collection period in half of our HMAs (Adobe Town, WY, Conger, UT, Frisco, UT, Green Mountain, WY, Onaqui, UT, Palomino Buttes, OR, South Steens, OR, and Stinkingwater, OR). However, free-roaming horses are adept at pawing through snow up to 60 cm to reach forage as well as "plowing" shallow snow with their muzzles (Salter and Hudson 1979). In addition, we experienced a mild and low snowfall start to winter 2020/2021 resulting in snow cover that was often not uniformly covering all topographical aspects of the HMAs and where present was never deeper than several centimeters. With the ability to paw through the snow to access forage, and the shallow snowpack, it is unlikely that the snow cover variation across HMAs affected

which forage types were available to free-roaming horses. Due to our short sampling window, our information may not fully represent what free-roaming horses consume in a given HMA over the course of a season. We did our best to address this by visiting multiple locations within an HMA when possible to collect fecal material representative of where free-roaming horses may have foraged across the geographical extent of HMAs. However, to properly inform management decisions, future studies in specific HMAs should verify seasonal herd average dietary composition by collecting during multiple sampling windows within each season to obtain a more representative diet composition.

There is the possibility that our findings may be biased towards plants that exhibit multiple copies of the gene region targeted, plants with shorter sequences, or other sequence biases due to our DNA metabarcoding technique (Garnick et al. 2018). With the limitations of this technique, readers should be aware that while we report the percentage contribution of certain plant families to diet composition, our results might not necessarily directly represent the absolute amount of certain plant families within the free-roaming horse diet. Rather, if using this type of data for management purposes, conclusions should be drawn based on the relative amounts compared within and between HMAs and seasons. Indeed, another study employing DNA metabarcoding in yearling cattle interpreted diet composition results as a comparison between two grazing groups rather than interpreting it as the absolute diet contribution (Jorns et al. 2023). However, previous studies have indicated that the relative read abundance of grasses using this technique could provide reliable quantitative information about grass consumption (Kartzinel et al. 2015).

We evaluated the relationship between the proportion of graminoids in the diet and the amount of herbaceous cover and biomass present at the HMA scale. While neither average cover

nor biomass for each HMA boundary was significantly correlated with HMA average graminoid diet composition, we did see a negative trend in this relationship. Interestingly, when we adjusted our HMA average to be the average of the 15 "foraging buffers" within each HMA, we saw moderate support for a negative relationship of graminoid composition with both herbaceous cover and biomass in the summer. We initially predicted that free-roaming horses occurring in HMAs with higher herbaceous cover or biomass would also have higher graminoid diet composition, so the trends in the opposite direction were surprising. A possible explanation is that free-roaming horses in areas of high herbaceous cover and biomass had more availability of forbs and were consuming these plants instead of graminoids. However, the fact that our data resolution drops off at the family taxonomic level prevents us from confirming this because many plant families, such as Asteraceae, include both forbs and shrubs. In addition, spatial data predicts herbaceous cover of forbs and graminoids; if we were able to look specifically at just graminoid cover, we may see a stronger relationship between graminoid availability and consumption. The potential movement of a free-roaming horse during a day creates a large buffer for each animal, and while our HMA average based on foraging buffers is likely more realistic than using the HMA boundary, a buffer based on potential movement may still not be representative of the true area free-roaming horses were using. Even when potential available herbaceous cover was similar between individual free-roaming horses within an HMA, graminoid composition varied widely in the diets of free-roaming horses (Fig. A4). These results indicate that diets can vary among individuals in a similar environment. To explain this variation, future diet studies that could pair GPS locations of free-roaming horse use throughout the period leading up to fecal collection with diet composition from fecal material could provide an even more realistic view of whether the cover and biomass available in used areas relates to diet

composition. In addition, future researchers could investigate age, sex, or reproductive stages such as pregnancy or lactation as drivers of dietary variation.

Despite variable diet strategies, free-roaming horses in all HMAs on average were in good body condition in summer and winter. Across all our body condition score (BCS) observations (1,409) we found that only 14 (0.1%) observed free-roaming horses had a body condition score of 3 or below, and most scored at an ideal body condition of 5. Our scores were consistent with another study that reported BCS of free-roaming horses using the same Henneke scale in the western USA. In that study, most horses had BCS of 4 or 5 and an average BCS of 4.7 (Schoenecker et al. 2024). When testing differences between herd average summer and winter BCS, we only observed support for seasonal difference in BCS in two HMAs (Palomino Buttes, OR and South Steens, OR), and moderate support in one HMA (Twin Peaks, CA/NV) with winter scores being lower than in summer in each HMA. Interestingly, all three of these HMAs were in the Northern Basin and Range ecoregion. While we detected a statistical difference in BCS in these HMAs, we question whether this amounts to a biological difference. Even in the HMA where we saw the largest decrease in BCS between summer and winter (South Steens, OR), this only amounted to a 0.25 difference between summer and winter averages. Results indicate that in most HMAs, free-roaming horses maintained condition throughout seasons. However, as our observations were conducted during the earlier part of winter (late November to January) this observation may be biased high compared to recording this information later in winter. Accumulation of fat stores during the summer and subsequent use of these stores throughout winter is a strategy used by many ungulates (Arnold et al. 2020) including other free-roaming equids in seasonally cold climates, such as the Przewalski's horse (*E. f. przewalskii;* Kuntz et al. 2006). It is possible that we observed few seasonal differences in

body condition because we sampled in early winter during a year with a relatively mild snowpack, before much of this seasonal fat loss would occur. It would be more informative in future studies to observe body condition in late winter or early spring to document the effects of a full winter season on body condition.

We found that free-roaming horses maintained good body condition across varied landscapes. Within the HMAs where we recorded body condition, we found free-roaming horses maintained good body condition across different seasons, ecoregions, and herbaceous cover and biomass availability. HMA average body condition scores remained near 5 despite differences in HMA average dietary composition of graminoids and other plant families. In the summer months, HMAs that averaged higher graminoid composition in their diets also tended to have lower body condition. As grasses are considered selected forage for horses, we found this result to be counter-intuitive. A recent study of domestic sheep (*Ovis aries*) winter diets found that monocot (graminoid) dominated diets were nutrient limiting and did not meet requirements for important production stages while dicot-dominated (shrub and forb) diets contained more important macro and micro minerals (Julian et al. 2024). We could be detecting a similar outcome in free-roaming horses during the summer months, with herds that consume graminoid-dominated diets not meeting certain macro and micronutrient requirements, resulting in lower body condition. It is also possible that our analysis using the coarse taxa level of graminoids does not capture the varying nutrient quality of different graminoid species in different HMAs. Indeed, protein content among rangeland and pasture grasses was shown to be higher in C3 (cool season) grasses as compared to C4 (warm season) grasses during the growing season (Barbehenn et al. 2004) and many graminoids common across western rangelands also had varying nutrient composition in winter, with crude protein values ranging between 2.5% and 8.4% (Julian et al.

2024). Free-roaming horse herds with lower graminoid (monocot) composition diets by default consume a higher proportion of dicots, which we hypothesize could indicate their diets contained more nutrients leading to the higher body condition we observed. In a review of diet selection, Provenza et al. (2003) contend that rangeland herbivores with access to a variety of forage alternatives performed better than when consuming a single forage type, due to a multitude of factors including forage nutrient availability, potentially toxic secondary metabolites, individual animal physiology differences, palatability, and availability of medicinal herbs. A study in bison found that although food items varied, the macronutrients provided by varying diets stayed similar throughout different diets, allowing bison to meet their needs using different plants (Hecker et al. 2021). Free-roaming horses could simply be using different forage plants to meet a similar macronutrient balance across seasons and HMAs, or could be intentionally selecting different forages to meet different nutritional needs. Exploring this would require future studies to determine the nutrient values of many native forage plants and pair this with diet composition data. Although horses are considered grazers, our study provides evidence that they can maintain healthy body condition and potentially achieve higher condition while consuming a variety of plants. This adaptability of horses to use different forage plants could offer one reason why populations of free-roaming horses are increasing across federal lands in the western United States.

IMPLICATIONS

Across the HMAs we sampled, free-roaming horses used a diverse array of plant families, with graminoids not always forming the majority of the diet. These patterns are complex and differ by HMA rather than grouping by ecoregion or overall herbaceous cover availability. Free-roaming horse herds in individual HMAs may have developed specific diet selection strategies over time,

or this could simply reflect plant availability in the specific areas or seasons we conducted our study. Either way, these animals appear highly adaptable and have demonstrated remarkable ability to maintain body condition throughout different ecoregions, seasons, herbaceous availability, and while consuming varied diets. Because low body condition scores are often what trigger management intervention, such as emergency gathers, it is important to note that free-roaming horses seem well adapted to thrive in a diverse array of ecosystems across their range. Our findings indicate that free-roaming horses can employ varying diet strategies in different HMAs and seasons, which should be considered when managers make decisions regarding overlapping distributions of free-roaming horses and other herbivores that may compete for available forage. A meta-analysis of diet studies across western North America indicated high overlap in graminoid consumption for cattle and free-roaming horses (Scasta et al. 2016). Grazing permits for cattle on federal lands are often granted in the summer months in the western United States. Forage competition with cattle may increase if these permits occur in areas where free-roaming horses exhibit high graminoid consumption in the summer. In HMAs where these herbivores overlap, more rangeland planning may be needed to account for increased demand and potential conflict in graminoid forage use. We also observed that free-roaming horse diets in winter often contained large proportions of plants in the Asteraceae and Chenopodiaceae families. Both families include plants that provide winter forage for wildlife including greater sage-grouse (Connelly et al. 2000), mule deer (*Odocoileus hemionus*; Sullivan 1988; Singer and Norland 1994), pronghorn (Bayless 1969; Stephenson et al. 1985; McInnis and Vavra 1987; Singer and Norland 1994), and elk (*Cervus canadensis*; Stephenson et al. 1985). Additional reliance on these plants by free-roaming horses in the winter may create competition with wildlife species. While studies in individual locations and a meta-analysis of western North

America have indicated low potential for direct dietary competition with free-roaming horses and wild ungulates that consume woody species such as mule deer (Hubbard and Hansen 1976; Hansen 1977; Hosten et al 2007; Scasta et al. 2016) and pronghorn (Olsen and Hansen 1977; McInnis and Vavra 1987; Smith et al. 1998; Scasta et al. 2016) our findings indicate there may be more potential for dietary overlap and competition during winter. Understanding what free-roaming horses are eating and at what time of the year in specific HMAs can help with locally specific rangeland management planning to help mitigate free-roaming horse conflict and balance multiple uses.

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DATA STATEMENT

In the interest of reproducible research, the R code, input files, and metadata files for this research are available at https://github.com/courtney-buchanan/Horse_diet/. Please contact the authors for any additional information.

REFERENCES

Allred, B.W., Bestelmeyer, B.T., Boyd, C.S., Brown, C., Davies, K.W., Duniway, M.C., Ellsworth, L.M., Erickson, T.A., Fuhlendorf, S.D., Griffiths, T. V., Jansen, V., Jones, M.O., Karl, J., Knight, A., Maestas, J.D., Maynard, J.J., McCord, S.E., Naugle, D.E., Starns, H.D., Twidwell, D., Uden, D.R., 2021. Improving Landsat predictions of rangeland fractional cover with multitask learning and uncertainty. Methods Ecol. Evol. 12, 841–849. https://doi.org/10.1111/2041-210X.13564

Arnold, W., 2020. Review: Seasonal differences in the physiology of wild northern ruminants. Animal 14, S124–S132. https://doi.org/10.1017/S1751731119003240

Barbehenn, R.V., Chen, Z., Karowe, D.N., Spickard, A., 2004. C3 grasses have higher nutritional quality than C4 grasses under ambient and elevated atmospheric CO2. Glob. Change Biol. 10. 1565 - 1575. http://doi.org/10.1111/j.1365-2486.2004.00833.x.

Bayless, S., 1969. Winter food habits, range use, and home range of antelope in Montana. J Wildl Manage. 33, 538–51. https://doi.org/10.2307/3799376

Beck, J.L., Milligan, M.C., Smith, K.T., Street, P.A., Pratt, A.C., Kirol, C.P., Wanner, C.P., Hennig, J.D., Dinkins, J.B., Scasta, J.D., Coates, P.S., 2024. Free-roaming horses exceeding appropriate management levels affect multiple vital rates in greater sage-grouse. J. Wildl. Manage. 88, e22669. https://doi.org/10.1002/jwmg.22669

Beever, E.A., Aldridge, C.L., 2011. Influences of free-roaming equids on sagebrush ecosystems, with a focus on Greater Sage-Grouse, in: Knick, S.T., Connelly, J.W. (Eds.), Greater Sage-Grouse: ecology and conservation of a landscape species and its habitats. Studies in Avian Biology (vol. 38), University of California Press, Berkeley, CA pp. 272–290. https://doi.org/10.1525/california/9780520267114.003.0015

Beever, E.A., Brussard, P.F., 2000. Examining ecological consequences of feral horse grazing using exclosures. West. North Am. Nat. 60, 236–254.

Beever, E.A., Brussard, P.F., 2004. Community- and landscape-level responses of reptiles and small mammals to feral-horse grazing in the Great Basin. J. Arid Environ. 59, 271–297. https://doi.org/10.1016/j.jaridenv.2003.12.008

Beever, E.A., Herrick, J.E., 2006. Effects of feral horses in Great Basin landscapes on soils and ants: Direct and indirect mechanisms. J. Arid Environ. 66, 96–112. https://doi.org/10.1016/j.jaridenv.2005.11.006

Beever, E.A., Tausch, R.J., Thogmartin, W.E., 2008. Multi-scale responses of vegetation to removal of horse grazing from Great Basin (USA) mountain ranges. Plant Ecol. 196, 163–184. https://doi.org/10.1007/s11258-007-9342-5

Bergmann, G.T., Craine, J.M., Robeson, M.S., Fierer, N., 2015. Seasonal shifts in diet and gut microbiota of the American bison (Bison bison). PLoS One 10, 1–14. https://doi.org/10.1371/journal.pone.0142409

Bureau of Land Management, 2019. North Hills Wild Horse Gather https://www.blm.gov/programs/wild-horse-and-burro/utah/2019-north-hills (accessed: 22 October 2020)

Bureau of Land Management, 2020. Herd Area and Herd Management Area Statistics 2020. https://www.blm.gov/sites/blm.gov/files/wildhorse_2020_HAHMA_Stats.pdf

Bureau of Land Management, undated. Herd Management Areas https://www.blm.gov/programs/wild-horse-and-burro/herd-management/herd-management-areas/ (accessed: 26 October 2020)

Bureau of Land Management, 2025. Herd Area and Herd Management Area Statistics 2025. https://www.blm.gov/sites/default/files/docs/2025-03/2025_Wild_Horse_and_Burro_Population_Estimates.pdf (accessed: 6 July 2025)

Carroll, C.L., Huntington, P.J., 1988. Body condition scoring and weight estimation of horses. Equine Vet. J. 20, 41-45. https://doi.org/10.1111/j.2042-3306.1988.tb01451.x

Clauss, M., Schiele, K., Ortmann, S., Fritz, J., Codron, D., Hummel, J., Kienzle, E., 2014. The effect of very low food intake on digestive physiology and forage digestibility in horses. J. Anim. Physiol. Anim. Nutr. (Berl). 98, 107–118. https://doi.org/10.1111/jpn.12053

Coates, P.S., O'Neil, S.T., Muñoz, D.A., Dwight, I.A., Tull, J.C., 2021. Sage-grouse population dynamics are adversely affected by overabundant feral horses. J. Wildl. Manage. 85, 1132–1149. https://doi.org/10.1002/jwmg.22089

Connelly, J.W., Schroeder, M.A., Sands, A.R., Braun, C.E., 2000. Guidelines to manage sage grouse populations and their habitats. Wildl. Soc. Bull. 28, 967–985.

Craine, J.M., Towne, E.G., Miller, M., Fierer, N., 2015. Climatic warming and the future of bison as grazers. Sci. Rep. 5, 1–8. https://doi.org/10.1038/srep16738

Czernik, M., Taberlet, P., Świsłocka, M., Czajkowska, M., Duda, N., Ratkiewicz, M., 2013. Fast and efficient DNA-based method for winter diet analysis from stools of three cervids: Moose, red deer, and roe deer. Acta Theriol. (Warsz). 58, 379–386. https://doi.org/10.1007/s13364-013-0146-9

Davies, K.W., Collins, G., Boyd, C.S., 2014. Effects of feral free-roaming horses on semi-arid rangeland ecosystems: An example from the sagebrush steppe. Ecosphere 5. https://doi.org/10.1890/ES14-00171.1

Dewitz, J., 2019, National Land Cover Database (NLCD) 2016 Products (ver. 3.0, November 2023): U.S. Geological Survey data release, https://doi.org/10.5066/P96HHBIE.

Duncan, P., Foose, T.J., Gordon, I.J., Gakahu, C.G., Lloyd, M., 1990. Comparative nutrient extraction from forages by grazing bovids and equids: a test of the nutritional model of equid/bovid competition and coexistence. Oecologia 84, 411–418. https://doi.org/10.1007/BF00329768

Efron, B., Tibshirani, R.J., 1993. An Introduction to the Bootstrap. Chapman and Hall/CRC. https://rdrr.io/cran/MKinfer/man/boot.t.test.html

Eldridge, D.J., Ding, J., Travers, S.K, 2020. Feral horse activity reduces environmental quality in ecosystems globally. Biol. Conserv. 241, 108367. https://doi.org/10.1016/j.biocon.2019.108367

Environmental Protection Agency, 2013. Level III and IV ecoregions of the continental United States: Corvallis, Oregon, U.S. EPA National Health and Environmental Effects research Laboratory. Accessed: 18 April 2024. Available from: https://www.epa.gov/eco-research/level-iii-and-iv-ecoregions-continental-united-states

Esmaeili, S., King, S.R.B., Schoenecker, K.A., 2023. Browsers or grazers? New insights into feral burro diet using a non-invasive sampling and plant DNA metabarcoding approach. Animals 13, 1–17. https://doi.org/10.3390/ani13162683

Garnick, S., Barboza, P.S., Walker, J.W., 2018. Assessment of animal-based methods used for estimating and monitoring rangeland herbivore diet composition. Rangel. Ecol. Manag. 71, 449–457. https://doi.org/10.1016/j.rama.2018.03.003

Garrott, R.A., Siniff, D.B., Eberhardt, L.L., 1991. Growth rates of feral horse populations. J. Wildl. Manage. 55, 641–648. https://www.jstor.org/stable/3809513

Gentry, L.R., Thompson, D.L., Gentry, G.T., Del Vecchio, R.P., Davis, K.A., Del Vecchio, P.M., 2004. The relationship between body condition score and ultrasonic fat measurements in mares of high versus low body condition. J Equine Vet. Sci. 24, 198-203. https://doi.org/10.1016/j.jevs.2004.04.009

Gooch, A.M.J., Petersen, S.L., Collins, G.H., Smith, T.S., McMillan, B.R., Eggett, D.L., 2017. The impact of feral horses on pronghorn behavior at water sources. J. Arid Environ. 138, 38–43. https://doi.org/10.1016/j.jaridenv.2016.11.012

Grayson, D.K., 1989. The chronology of North American late pleistocene extinctions. J. Archaeol. Sci. 16, 153–165. https://doi.org/10.1016/0305-4403(89)90063-0

Guthrie, R.D., 2003. Rapid body size decline in Alaskan Pleistocene horses before extinction. Nature 426, 169–171. https://doi.org/10.1038/nature02098

Guthrie, R.D., 2006. New carbon dates link climatic change with human colonization and Pleistocene extinctions. Nature 441, 207–209. https://doi.org/10.1038/nature04604

Haines, F., 1938a.The northward spread of horses among the plains indians. American Anthropologist 40, 429–437. www.jstor.org/stable/662040.

Haines, F., 1938b. Where did the Plains Indians get their horses? American Anthropologist 40, 112–117. www.jstor.org/stable/661795.

Hall, L.K., Larsen, R.T., Westover, M.D., Day, C.C., Knight, R.N., McMillan, B.R., 2016. Influence of exotic horses on the use of water by communities of native wildlife in a semi-arid environment. J. Arid Environ. 127, 100–105. https://doi.org/10.1016/j.jaridenv.2015.11.008

Hampson, B.A., Laat, M.A.D.E., Mills, P.C., Pollitt, C.C., 2010. Distances travelled by feral horses in ‘outback’ Australia. Equine Vet. J. 42, 582–586. https://doi.org/10.1111/j.2042-3306.2010.00203.x

Hanley, T.A., 1982. The nutritional basis for food selection by ungulates. J. Range Manag. 35, 146–151.

Hanley, T.A., Hanley, K.A., 1982. Food resource partitioning by sympatric ungulates on Great Basin rangeland. J. Range Manag. 35, 152-158. https://doi.org/10.2307/3898380

Hansen, R.M., 1976. Foods of free-roaming horses in southern New Mexico. J. Range Manag. 29, 347. https://doi.org/10.2307/3897105

Hansen, R.M., Clark, R.C., Lawhorn, W., 1977. Foods of wild horses, deer, and cattle in the Douglas Mountain Area, Colorado. J. Range Manag. 30, 116. https://doi.org/10.2307/3897750

Hecker, L.J., Coogan, S.C.P., Nielsen, S.E., Edwards, M.A., 2021. Latitudinal and seasonal plasticity in American bison *Bison bison* diets. Mamm. Rev. 51, 193–206. https://doi.org/10.1111/mam.12229

Henneke, D.R., Potter, G.D., Kreider, J.L., Yeates, B.F., 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15, 371–372. https://doi.org/10.1111/j.2042-3306.1983.tb01826.x

Hennig, J.D., Beck, J.L., Scasta, J.D., 2018. Spatial ecology observations from feral horses equipped with global positioning system transmitters. Human-Wildlife Interact. 12, 75–84. https://doi.org/10.26077/z9cn-4h37

Hennig, J.D., Beck, J.L., Duchardt, C.J., Derek Scasta, J., 2021a. Variation in sage-grouse habitat quality metrics across a gradient of feral horse use. J. Arid Environ. 192, 104550. https://doi.org/10.1016/j.jaridenv.2021.104550

Hennig, J.D., Beck, J.L., Gray, C.J., Scasta, J.D., 2021b. Temporal overlap among feral horses, cattle, and native ungulates at water sources. J. Wildl. Manage. 85, 1084–1090. https://doi.org/10.1002/jwmg.21959

Hennig, J.D., Scasta, J.D., Pratt, A.C., Wanner, C.P., Beck, J.L., 2023. Habitat selection and space use overlap between feral horses, pronghorn, and greater sage-grouse in cold arid steppe. J. Wildl. Manage. 87, e22329. https://doi.org/10.1002/jwmg.22329

Hennig, J.D., Beck, J.L., Scasta, J.D., 2024. Feral horses and pronghorn: a test of the forage maturation hypothesis in an arid shrubland. Anim. Behav. 210, 55–61. https://doi.org/10.1016/j.anbehav.2024.01.015

Hijmans, R.J., Bivand, R., Cordano, E., Dyba, K., Pebesma, E., Sumner, M.D., 2024. Terra: spatial data analysis. R package version 1.7-83, https://cran.r-project.org/web/packages/terra/index.html

Holechek, J.L., Vavra, M., Pieper, R.D., 1982. Botanical Composition Determination of Range Herbivore Diets: A Review. J. Range Manag. 35, 309-315. https://doi.org/10.2307/3898308

Hosten, P.E., H. Whitridge, H., Broyles, M., 2007. Diet overlap and social interactions among cattle, horses, deer and elk in the Cascade-Siskiyou National Monument, southwest Oregon. U.S. Department of the Interior, Bureau of Land Management, Medford District. http://soda.sou.edu/bioregion.html

Hubbard, R.E., Hansen, R.M., 1976. Diets of wild horses, cattle, and mule deer in the Piceance Basin, Colorado. J. Range Manag. 29, 389-392. https://doi.org/10.2307/3897147

iNaturalist. Available from https://www.inaturalist.org. Accessed 3 September 2024.

Janis, C., 1976. The evolutionary strategy of the Equidae and the origins of rumen and cecal digestion. Evolution. 30, 757-774. https://doi.org/10.2307/2407816

Jones, M.O., Robinson, N.P., Naugle, D.E., Maestas, J.D., Reeves, M.C., Lankston, R.W., Allred, B.W., 2021 . Annual and 16-day rangeland production estimates for the western United States. Rangel. Ecol. Manag. 77, 112-117. https://doi.org/10.1016/j.rama.2021.04.003

Jorns, T., Craine, J., Towne, E.G., Knox, M., 2020. Climate structures bison dietary quality and composition at the continental scale. Environ. DNA 2, 77–90. https://doi.org/10.1002/edn3.47

Jorns, T. R., Scasta, J.D., Derner, J. D., Augustine, D. J., Porensky, L. M., Raynor, E. J., 2023. Adaptive multi-paddock grazing management reduces diet quality of yearling cattle in shortgrass steppe. Rangeland J. 45, 160-172. https://doi.org/10.1071/rj23047

Julian, A.A.M., Scasta, J.D., Stewart, W.C., 2024. Sheep winter diets parameterized with fecal DNA metabarcoding and forage sampling informs mineral nutrition management. Rangel. Ecol. Manag. 94, 168–177. https://doi.org/10.1016/j.rama.2024.03.003

Kartzinel, T.R., Chen, P.A., Coverdale, T.C., Erickson, D.L., Kress, W.J., Kuzmina, M.L., Rubenstein, D.I., Wang, W., Pringle, R.M., 2015. DNA metabarcoding illuminates dietary niche partitioning by African large herbivores. Proc. Natl. Acad. Sci. U.S.A. 112, 8019-8024. https://doi.org/10.1073/pnas.1503283112

King, S.R.B., Schoenecker, K.A., Fike, J.A., Oyler-McCance, S.J., 2018. Long-term persistence of horse fecal DNA in the environment makes equids particularly good candidates for noninvasive sampling. Ecol. Evol. 8, 4053–4064. https://doi.org/10.1002/ece3.3956

King, S.R.B., Schoenecker, K.A., 2019. Comparison of methods to examine diet of feral horses from noninvasively collected fecal samples. Rangel. Ecol. Manag. 72, 661–666. https://doi.org/10.1016/j.rama.2019.02.005

Krysl, L.J., Sowell, B.F., Hubbert, M.E., Plumb, G.E., Jewett, T.K., Smith, M.A., Waggoner, J.W., 1984. Horses and cattle grazing in the Wyoming Red Desert, I. Food habits and dietary overlap. J. Range Manag. 37, 72–76. https://doi.org/10.2307/3899149

Kuntz, R., Kubalek, C., Ruf, T., Tataruch, F., Arnold, W., 2006. Seasonal adjustment of energy budget in a large wild mammal, the Przewalski horse (Equus ferus przewalskii) I. Energy intake. J. Exp. Biol. 209, 4557–4565. https://doi.org/10.1242/jeb.02535

Leonard, J.L., Perkins, L.B., Lammers, D.J., Jenks, J.A., 2017. Are bison intermediate feeders? Unveiling summer diet selection at the northern fringe of historical distribution. Rangel. Ecol. Manag. 70, 405–410. https://doi.org/10.1016/j.rama.2017.01.005

McInnis, M.L., Vavra, M., 1987. Dietary relationships among feral horses, cattle, and pronghorn in southeastern Oregon. J. Range Manag. 40, 60 -66. https://doi.org/10.2307/3899363

McKnight, T.L., 1959. The feral horse in Anglo-America. Georg. Rev. 49, 506–525. https://www.jstor.org/stable/212210

McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217

Meltzer, D.J., Mead, J.I., 1985. Dating late Pleistocene extinctions: theoretical issues, analytical bias, and substantive results. Environments and Extinctions: Man in Late Glacial North America 145–173.

Menard, C., Duncan, P., Fleurance, G., Georges, J.Y., Lila, M., 2002. Comparative foraging and nutrition of horses and cattle in European wetlands. J. Appl. Ecol. 39, 120–133. https://doi.org/10.1046/j.1365-2664.2002.00693.x

Miyaji, M., Ueda, K., Hata, H., Kondo, S., 2014. Effect of grass hay intake on fiber digestion and digesta retention time in the hindgut of horses. J. Anim. Sci. 92, 1574–1581. https://doi.org/10.2527/jas.2013-6676

Morrison, J.I., 2008. Using microhistological techniques to predict botanical composition of horse diets on cool-season grass pasture [MS thesis]. University of Kentucky, Lexington, KY, USA. Available at: https://uknowledge.uky.edu/gradschool_theses/504/

Muñoz, D.A., Coates, P.S., Ricca, M.A., 2021. Free-roaming horses disrupt greater sage-grouse lekking activity in the Great Basin. J. Arid Environ.184, 104304. https://doi.org/10.1016/j.jaridenv.2020.104304

National Research Council, 2013. Using science to improve the BLM wild horse and burro program: A way forward. The National Academies Press, Washington D.C. pp: 436. https://doi.org/10.17226/13511

Olsen, F.W., Hansen, R.M., 1977. Food relations of wild free-roaming horses to livestock and big game, Red Desert, Wyoming. J. Range Manag. 30, 17-20. https://doi.org/10.2307/3897326

Ostermann-Kelm, S., Atwill, E.R., Rubin, E.S., Jorgensen, M.C., Boyce, W.M., 2008. Interactions between feral horses and desert bighorn sheep at water. J. Mammal. 89, 459–466. https://doi.org/10.1644/07-mamm-a-075r1.1

Pearson, R.A., Archibald, R.F., Muirhead, R.H., 2006. A comparison of the effect of forage type and level of feeding on the digestibility and gastrointestinal mean retention time of dry forages given to cattle, sheep, ponies and donkeys. Br. J. Nutr. 95, 88–98. https://doi.org/10.1079/bjn20051617

Pearson, R.A., Merritt, J.B., 1991. Intake, digestion and gastrointestinal transit time in resting donkeys and ponies and exercised donkeys given ad libitum hay and straw diets. Equine Vet. J. 23, 339–343. https://doi.org/10.1111/j.2042-3306.1991.tb03734.x

Perry, N.D., Morey, P., Miguel, G.S., 2015. Dominance of a natural water source by feral horses. Southwest. Nat. 60, 390–393. https://doi.org/10.1894/0038-4909-60.4.390

Provenza, F.D., Villalba, J.J., Dziba, L., Atwood, S.B., Banner, R.E., 2003. Linking herbivore experience, varied diets, and plant biochemical diversity. Small Rumin. Res. 49. 257-274. 10.1016/S0921-4488(03)00143-3.

Pulliam, D. E., Jr., AND J. R. Nelson. 1979. Determination of digestibility coefficients for quantification of fecal analysis with elk. In: M. S. Boyce and L. D. Hayden-Wing [eds.]. North American elk: Ecology, behavior, and management. Laramie, WY: University of Wyoming. 240–246

R Core Team (2023). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Robinson, N.P., Jones, M.O., Moreno, A., Erickson, T.A., Naugle, D.E., and Allred, B.W., 2019. Rangeland productivity partitioned to sub-pixel plant functional types. Remote Sens. 11, 1427. https://doi.org/10.3390/rs11121427

Rocky Mountain Herbarium 2008. RM Herbarium Specimen Database. Last updated 7 February 2022. Accessed 3 September 2024. Available at https://rmh.uwyo.edu/data/search.php

Salter, R.E., Hudson, R.J., 1979. Feeding ecology of feral horses in western Alberta. J. Range Manag. 32, 221-225. https://doi.org/10.2307/3897127

Scasta, J.D., Beck, J.L., Angwin, C.J., 2016. Meta-analysis of diet composition and potential conflict of wild horses with livestock and wild ungulates on western rangelands of North America. Rangel. Ecol. Manag. 69, 310–318. https://doi.org/10.1016/j.rama.2016.01.001

Scasta, J.D., Jorns, T., Derner, J.D., Lake, S., Augustine, D.J., Windh, J.L., Smith, T.L., 2019. Validation of DNA metabarcoding of fecal samples using cattle fed known rations. Anim. Feed Sci. Technol. 255, 114219. https://doi.org/10.1016/j.anifeedsci.2019.114219

Schoenecker, K.A., King, S.R.B., Hennig, J.D., Cole, M.J., Scasta, J.D., Beck, J.L., 2024. Effects of telemetry collars on two free-roaming feral equid species. PLoS One 19, 1–25. https://doi.org/10.1371/journal.pone.0303312

Singer, F.J., Norland, J.E., 1994. Niche relationships within a guild of ungulate species in Yellowstone National Park, Wyoming, following release from artificial controls. Can. J. Zool. 72, 1383–1394. https://doi.org/10.1139/z94-183

Smith, C., Valdez, R., Holechek, J.L., Zwank, P.J., Cardenas, M., 1998. Diets of native and non-native ungulates in southcentral New Mexico. Southwestern Nat. 43, 163–169. https://www.jstor.org/stable/30055352

Smitman, G. E. 1980. An examination of laboratory techniques for quantification of elk fecal analysis [thesis]. Pullman, WA: Washington State University. 58 p.

Stephenson, T.E., Holechek, J.L., Kuykendall, C.B., 1985. Diets of four wild ungulates on winter range in northcentral New Mexico. Southwest. Nat. 30, 437–441.

Sullivan, M.G., 1988. Distribution, habitat use, and food habits of reintroduced elk in Theodore Roosevelt National Park, North Dakota [MS thesis]. Montana State University, Bozeman, MT, USA.

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T., Corthier, G., Brochmann, C., Willerslev, E., 2007. Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. Nucleic Acids Res. 35, e14. https://doi.org/10.1093/nar/gkl938

USDA-NRCS. The PLANTS Database. Available at: http://plants.usda.gov. Accessed 09/27/2024.

U.S. Department of the Interior, Bureau of Land Management and U.S. Department of Agriculture, U.S Forest Service, 2018. North Hills wild horse herd management area plan and gather plan. 1–108 Available at: https://eplanning.blm.gov/public_projects/nepa/100793/150749/184965/North_Hills_HMAP_Gather_EA_Public_Comment_Draft_9July2018.pdf

United States Forest Service, 2014. US Forest Service Wild Horse and Burro Territories. https://www.fs.usda.gov/wild-horse-

burro/documents/territories/USFSWildHorseBurroTerritories2014.pdf (accessed 23 October 2020).

Valentini, A., Pompanon, F., Taberlet, P., 2009. DNA barcoding for ecologists. Trends Ecol. Evol. 24, 110–117. https://doi.org/10.1016/j.tree.2008.09.011

Vavra, M., Holechek, J.L., 1980. Factors influencing microhistological analysis of herbivore diets. J. Range Manag. 33, 371–374.

Vlčková, K., Mrázek, J., Kopečný, J., Petrželková, K.J., 2012. Evaluation of different storage methods to characterize the fecal bacterial communities of captive western lowland gorillas (Gorilla gorilla gorilla). J. Microbiol. Methods 91, 45–51. https://doi.org/10.1016/j.mimet.2012.07.015

Weinstein, S.B., Martinez-Mota, R., Stapleton, T.E., Klure, D.M., Greenhalgh, R., Orr, T.J., Dale, C., Kohl, K.D., Denise Dearing, M., 2021. Microbiome stability and structure is governed by host phylogeny over diet and geography in woodrats (Neotoma spp.). Proc. Natl. Acad. Sci. U. S. A. 118, e2108787118. https://doi.org/10.1073/pnas.2108787118

Zar, J.H., 2010. Biostatistical analysis, fifth ed. Pearson Prentice Hall, Upper Saddle River, New Jersey.

TABLES

Table 1

Characteristics of selected Bureau of Land Management–Herd Management Areas (HMAs) in the western United States used to collect diet and body condition scores from free-roaming horses in summer 2020 and winter 2020/2021. Data on HMA area, appropriate management level (AML), and population estimates were sourced from the 2020 BLM Herd Area and HMA statistics (BLM 2020). Percent AML was calculated by dividing the 2020 estimated population by the high end of AML.

HMA	State	RAP[1] cover category	RAP[1] herbaceous cover %	AML (low)	AML (high)	2020 Population	% AML	Km^2	EPA III ecoregion[2]
Little Book Cliffs	CO	Low	12.67	90	150	151	101%	146	CP
Pine Nut Mountains	NV	Low	19.93	118	179	232	130%	422	CB
Red Rock	NV	Low	19.61	16	27	50	185%	655	MB
Frisco	UT	Low	18.55	30	60	208	347%	244	CB
Adobe Town	WY	Low	17.28	610	800	1,114	139%	1,938	WB
Palomino Buttes	OR	Med	33.36	32	64	277	433%	300	NB
Conger	UT	Med	20.63	40	80	247	309%	692	CB
North Hills[3]	UT	Med	20.72	42	63	104	165%	343	CB
Green Mountain	WY	Med	33.99	170	300	328	109%	472	WB
McCullough Peaks	WY	Med	25.77	70	140	167	119%	487	WB
Twin Peaks	CA/NV	High	38.32	448	758	3,877	511%	3,068	NB
Sand Basin	ID	High	67.14	33	64	36	56%	47	NB
Saylor Creek	ID	High	50.55	40	50	137	274%	412	SR

South Steens	OR	High	37.82	159	304	979	322%	544	NB
Stinkingwater	OR	High	48.17	40	80	372	465%	373	NB
Onaqui	UT	High	36.73	121	210	412	196%	972	CB

[1] Herbaceous cover and cover category based on Rangeland Analysis Platform (RAP) spatial information were calculated by methods described above from summer 2020 cover data.

[2] Abbreviations for United States Environmental Protection Agency (EPA) ecoregions are as follows: Central Basin and Range (CB), Colorado Plateau (CP), Mojave Basin and Range (MB), Northern Basin and Range (NB), Snake River Plain (SR), Wyoming Basin (WB).

[3] North Hills HMA is managed in conjunction with the USFS North Hills Wild Horse Territory (WHT) as a Joint Management Area (JMA) so totals were combined. AML for the Forest Service WHT of 18–27 (USFS 2014) was added to the BLM AML of 24–36 horses. Acreage for the total JMA was retrieved from the Gather Plan for the latest roundup that occurred (U.S DOI BLM and USDA USFS 2018). The 2020 population was estimated as follows: According to the BLM gather report, before the gather of the JMA in December 2019 the estimated population was 317. 213 were removed and zero returned to the range (BLM 2019) which left an estimated 104 free-roaming horses on the range going into 2020.

Table 2

Taxonomic depth of read assignments of plant DNA metabarcoding of fecal material collected from free-roaming horses across 16 Herd Management Areas (HMAs) in summer 2020 and winter 2020/2021.

	Number of distinct taxa identified	Number of ASVs[1] assigned to taxonomic depth (of 284 total)	Percentage of reads assigned to taxonomic depth
Phylum	1	284 (100%)	100%
Class (Subphylum)	1	284 (100%)	100%
Order	31	283 (99.65%)	99.98%
Family	50	282 (99.30%)	99.97%
Genus	120	228 (80.28%)	69.32%
Species	46	58 (20.42%)	11.24%

[1] Amplicon Sequence Variants

Table 3

Average graminoid composition for each Herd Management Area (HMA) and season. Underlined percentages indicate whether summer or winter diets had a higher graminoid composition when the difference between seasons was at least 5 percentage points. Seasonal differences (percentage points) are listed as negative when winter value was higher than summer. **Bolded p-values** indicate differences at the alpha = 0.05 level while ***bold italicized p-values*** indicate differences at the alpha = 0.10 level. Abbreviations for United States Environmental Protection Agency (EPA) ecoregions are as follows: Central Basin and Range (CB), Colorado Plateau (CP), Mojave Basin and Range (MB), Northern Basin and Range (NB), Snake River Plain (SR), and Wyoming Basin (WB).

HMA	Herbaceous cover category	EPA Level III ecoregion	Graminoid composition Summer (%)	Graminoid composition Winter (%)	Seasonal difference percentage points	Independent bootstrap	Bootstrap minimum variation	Bootstrap maximum variation
Clear Support for Seasonal Difference								
Frisco	Low	CB	**83.50%**	53.43%	30.07%	**<0.001**	**<0.001**	**0.016**
Palomino Buttes	Medium	NB	**35.45%**	11.00%	24.45%	**0.006**	**<0.001**	**0.019**
Saylor Creek	High	SR	**68.87%**	36.89%	31.98%	**<0.001**	**<0.001**	**0.030**
No Support for Seasonal Difference								
Adobe Town	Low	WB	76.75%	74.90%	1.85%	0.851	0.654	0.875
Pine Nut Mountains	Low	CB	33.99%	38.44%	-4.45%	0.617	0.132	0.721
Twin Peaks	High	NB	39.66%	39.82%	-0.16%	0.976	0.972	0.981
South Steens	High	NB	53.29%	57.22%	-3.93%	0.621	0.101	0.691
Mixed Support for Seasonal Difference – Leaning Toward Difference								
Little Book Cliffs	Low	CP	**74.92%**	46.96%	27.96%	**0.027**	**<0.001**	0.121
Onaqui	High	CB	58.20%	**82.60%**	-24.4%	**0.009**	**<0.001**	***0.084***
Stinkingwater	High	NB	**56.14%**	39.49%	16.65%	***0.075***	**0.001**	0.216

Mixed Support for Seasonal Difference- Leaning Against Difference								
Red Rock	Low	MB	**53.96%**	45.82%	8.14%	0.415	**0.006**	0.563
Conger	Medium	CB	**76.06%**	61.20%	14.86%	0.193	**0.019**	0.293
Green Mountain	Medium	WB	**69.42%**	53.96%	15.46%	0.142	**<0.001**	0.320
McCullough Peaks	Medium	WB	31.07%	**38.43%**	-7.36%	0.530	**0.028**	0.6173
North Hills	Medium	CB	**80.33%**	67.94%	12.39%	0.194	**<0.001**	0.309
Range-wide (HMA as experimental unit)								
			59.44%	49.87%	9.57%	***Paired: 0.050***		

Table 4

Range-wide and Herd Management Area (HMA) mean composition of Asteraceae and Chenopodiaceae families in both summer and winter (± SD). HMA averages are based on n = 15 observations in each season and range-wide averages are based on n = 15 HMAs in the summer and the winter with Sands Basin excluded from the summer average. HMAs are organized in alphabetical order within each herbaceous cover category. Abbreviations for United States Environmental Protection Agency (EPA) ecoregions are as follows: Central Basin and Range (CB), Colorado Plateau (CP), Mojave Basin and Range (MB), Northern Basin and Range (NB), Snake River Plain (SR), and Wyoming Basin (WB).

HMA	Herbaceous Cover Category	EPA ecoregion III	Summer Asteraceae	Winter Asteraceae	Summer Chenopodiaceae	Winter Chenopodiaceae
Adobe Town	Low	WB	0.53 (± 1.13) %	0.27 (± 0.78) %	1.65 (± 2.02) %	8.25 (± 18.80) %
Frisco	Low	CB	0.53 (± 1.54) %	0.19 (± 0.72) %	1.15 (± 3.35) %	23.71 (± 22.09) %
Little Book Cliffs	Low	CP	0.73 (± 1.85) %	17.11 (± 18.77) %	0.00 (± 0.00) %	3.08 (± 9.98) %
Pine Nut Mountains	Low	CB	1.39 (± 4.65) %	10.97 (± 9.20) %	3.28 (± 7.44) %	6.12 (± 6.19) %
Red Rock	Low	MB	8.08 (± 10.19) %	21.54 (± 12.96) %	6.49 (± 13.68) %	17.71 (± 15.18) %
Conger	Medium	CB	0.48 (±1.39) %	0.97 (± 2.22) %	5.64 (± 9.94) %	34.41 (± 40.70) %
Green Mountain	Medium	WB	0.68 (± 2.35) %	34.72 (± 27.08) %	0.16 (± 0.63) %	0.33 (± 1.29) %
McCullough Peaks	Medium	WB	2.45 (± 7.23) %	11.32 (± 10.04) %	0.54 (± 1.26) %	22.10 (± 24.10) %
North Hills	Medium	CB	1.18 (± 3.25) %	4.13 (± 8.28) %	1.49 (± 2.39) %	19.73 (± 20.57) %
Palomino Buttes	Medium	NB	15.98 (± 31.99) %	9.82 (± 13.28) %	1.21 (± 2.94) %	0.00 (± 0.00) %
Onaqui	High	CB	5.32 (± 6.81) %	2.49 (± 3.12) %	1.08 (± 3.10) %	13.33 (± 21.60) %
Sands Basin	High	NB	11.07 (± 10.28) %	-	1.48 (± 3.86) %	-

Saylor Creek	High	SR	13.78 (± 9.48) %	1.46 (± 2.14) %	1.20 (± 2.32) %	55.36 (± 30.64) %
South Steens	High	NB	14.05 (± 10.94) %	26.92 (± 17.22) %	3.59 (± 5.73) %	3.50 (± 12.41) %
Stinkingwater	High	NB	12.44 (± 8.62) %	54.42 (± 33.52) %	0.00 (± 0.00) %	0.00 (± 0.00) %
Twin Peaks	High	NB	7.86 (± 10.67) %	29.45 (± 29.37) %	33.55 (± 28.38) %	10.35 (±13.78) %
Range Average			5.70 (± 5.83) %	15.05 (± 15.79) %	4.07% (± 8.39) %	14.53% (± 15.23) %

Table 5

Herd average body condition scores (BCS) for free-roaming horses in summer and winter at each Herd Management Area (HMA) including the number of observations and p-values from bootstrapped t-tests. **Bolded p-values** indicate differences at the alpha = 0.05 level while ***bold italicized p-values*** indicate differences at the alpha = 0.10 level. Abbreviations for United States Environmental Protection Agency (EPA) ecoregions are Central Basin and Range (CB), Colorado Plateau (CP), Mojave Basin and Range (MB), Northern Basin and Range (NB), Snake River Plain (SR), and Wyoming Basin (WB).

HMA	Herbaceous Cover Category	EPA ecoregion III	Summer BCS	Number of observations	Winter BCS	Number of observations	Bootstrapped t-test p-value
Adobe Town	Low	WB	5.06	48	5.17	47	0.137
Frisco	Low	CB	4.93	27	4.84	19	0.475
Little Book Cliffs	Low	CP	4.96	27	5.03	58	0.251
Pine Nut Mountains	Low	CB	4.93	45	4.79	29	0.176
Red Rock	Low	MB	4.86	29	4.92	26	0.698
Conger	Medium	CB	4.95	38	4.97	34	0.734
Green Mountain	Medium	WB	5.07	28	5.12	59	0.625
McCullough Peaks	Medium	WB	5.24	75	5.22	46	0.838
North Hills	Medium	CB	4.59	22	4.72	32	0.461
Palomino Buttes	Medium	NB	5.15	48	4.93	40	**0.003**
Onaqui	High	CB	5.04	95	4.96	73	0.264
Sands Basin	High	NB	5.08	24	-	-	-
Saylor Creek	High	SR	5.03	35	5.02	49	0.932

South Steens	High	NB	5.13	83	4.88	74	**<0.001**
Stinkingwater	High	NB	5.19	47	5.10	40	0.273
Twin Peaks	High	NB	5.08	36	5.00	76	***0.080***
Range Scale (HMA as experimental unit)			5.01	707	4.98	702	Paired: 0.205

FIGURES

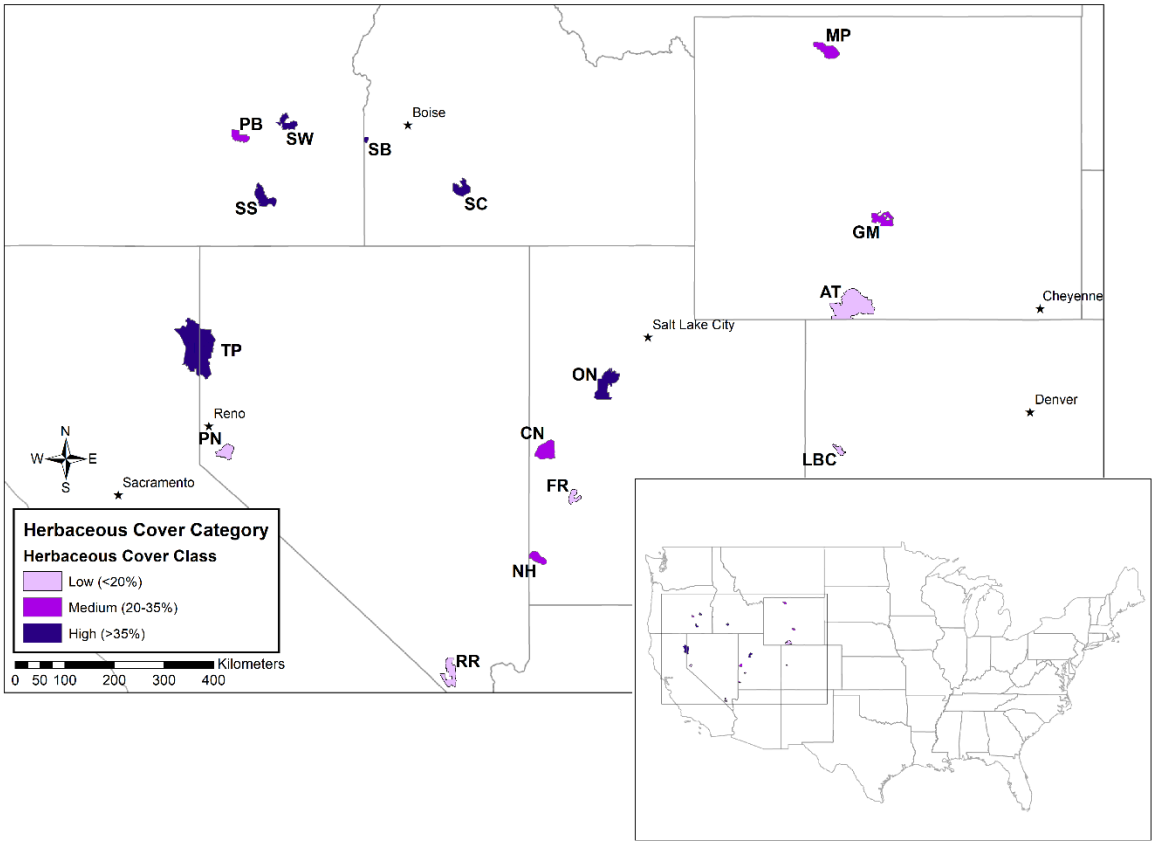

Figure 1. Bureau of Land Management–Herd Management Areas (HMAs) where sampling was conducted in summer 2020 and winter 2020/2021. Shading represents average herbaceous cover of HMAs based on 2020 RAP cover stratification of low (0-20%), medium (20-35%), and high (>35%) herbaceous cover. HMAs are labeled in black and are coded as follows: AT = Adobe Town, CN = Conger, FR = Frisco, GM = Green Mountain, LBC = Little Book Cliffs, MP = McCullough Peaks, NH = North Hills, ON = Onaqui Mountain, PB = Palomino Buttes, PN = Pine Nut Mountains, RR = Red Rock, SB = Sands Basin, SC = Saylor Creek, SS = South Steens, SW = Stinkingwater, and TP = Twin Peaks.

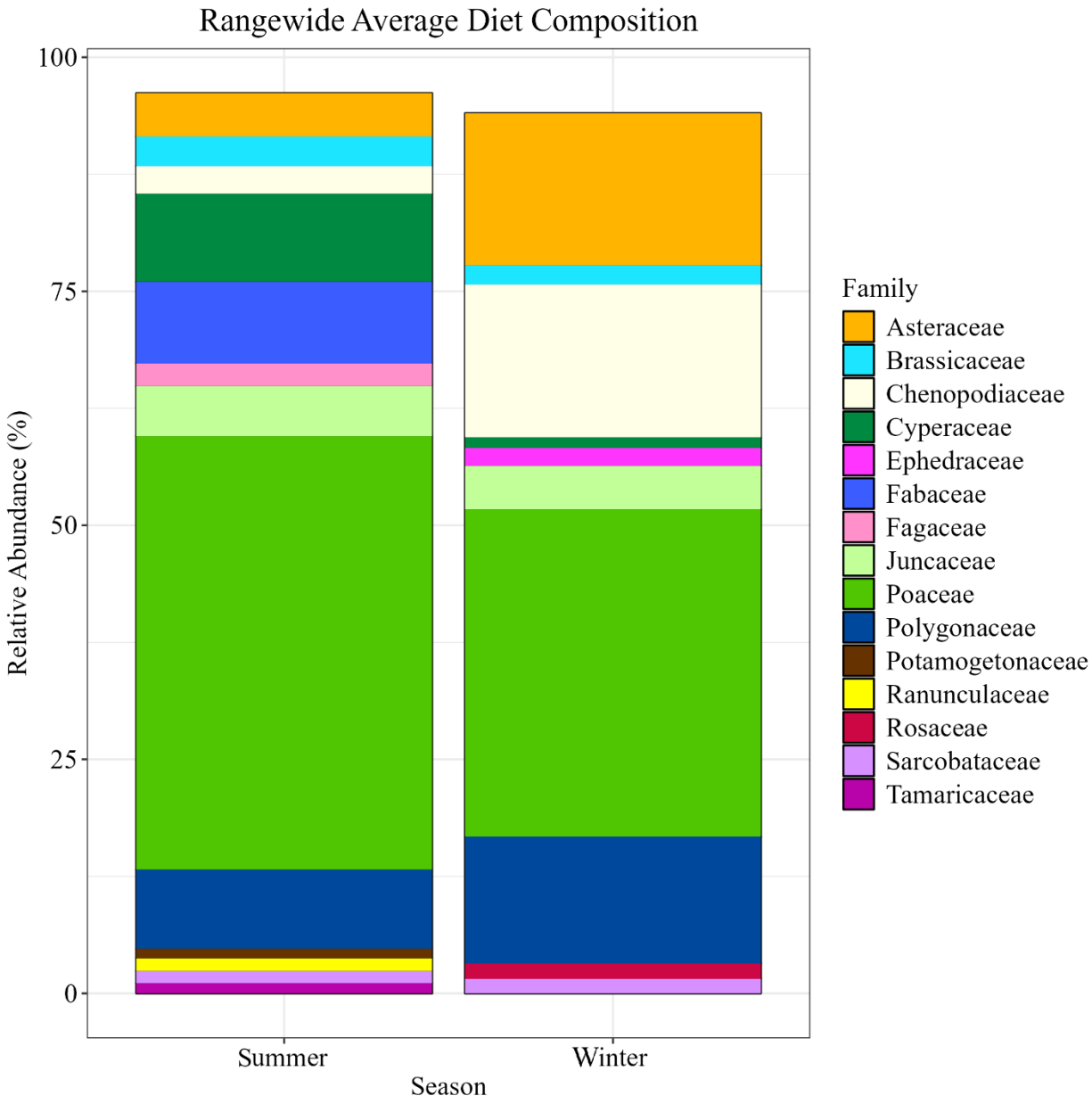

Figure 2. Average diet composition of free-roaming horses in each season grouped by family across the western USA. Plant families that did not represent at least 1% of the average diet at the range wide scale within at least one season were removed to simplify the visual and results in bars not reaching 100%. The three graminoid families are all depicted in shades of green- Poaceae (lime green), Cyperaceae (dark green), and Juncaceae (light green).

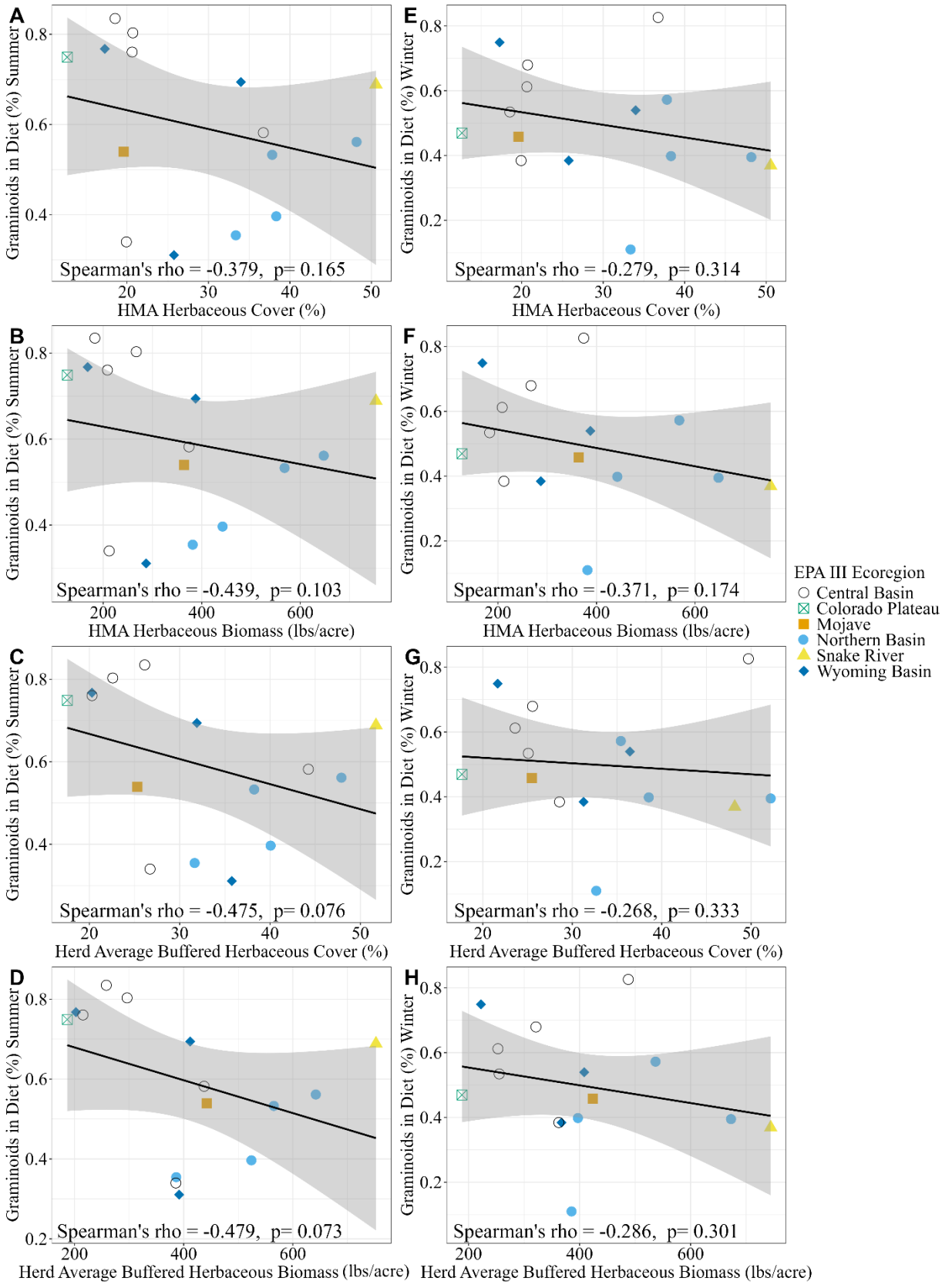
A
Graminoids in Diet (%) Summer
0.8
0.6
0.4
Spearman's rho = -0.379, p= 0.165
20
30
40
50
HMA Herbaceous Cover (%)
E
Graminoids in Diet (%) Winter
0.8
0.6
0.4
0.2
Spearman's rho = -0.279, p= 0.314
20
30
40
50
HMA Herbaceous Cover (%)
B
Graminoids in Diet (%) Summer
0.8
0.6
0.4
Spearman's rho = -0.439, p= 0.103
200
400
600
HMA Herbaceous Biomass (lbs/acre)
F
Graminoids in Diet (%) Winter
0.8
0.6
0.4
0.2
Spearman's rho = -0.371, p= 0.174
200
400
600
HMA Herbaceous Biomass (lbs/acre)
C
Graminoids in Diet (%) Summer
0.8
0.6
0.4
Spearman's rho = -0.475, p= 0.076
20
30
40
50
Herd Average Buffered Herbaceous Cover (%)
G
Graminoids in Diet (%) Winter
0.8
0.6
0.4
0.2
Spearman's rho = -0.268, p= 0.333
20
30
40
50
Herd Average Buffered Herbaceous Cover (%)
D
Graminoids in Diet (%) Summer
0.8
0.6
0.4
0.2
Spearman's rho = -0.479, p= 0.073
200
400
600
Herd Average Buffered Herbaceous Biomass (lbs/acre)
H
Graminoids in Diet (%) Winter
0.8
0.6
0.4
0.2
Spearman's rho = -0.286, p= 0.301
200
400
600
Herd Average Buffered Herbaceous Biomass (lbs/acre)
EPA III Ecoregion
Central Basin
Colorado Plateau
Mojave
Northern Basin
Snake River
Wyoming Basin

Figure 3. Scatterplots on the upper half of the figure (A, B, E, F) depict cover and biomass values using the Herd Management Area (HMA) average found by overlying the HMA boundary with Rangeland Analysis Platform (RAP) data. Scatterplots depict the correlation of HMA herbaceous cover (A, E) or biomass (B, F) with the HMA boundary average composition of graminoids in diets across all HMAs in both summer (A, B) and winter (E, F). Note that none of these was a significant correlation; however all had a negative trend. The lower four scatterplots (C,D,G,H) depict average cover and biomass for each HMA calculated by taking a buffer around each fecal collection point to represent the horse's potential foraging area and then averaging the cover or biomass across the 15 horses in each HMA. Graphs are displayed for both cover (C, G) and biomass (D, H) in the summer (C, D) and winter (G, H). Spearman's rho and p-values for each correlation are included in the bottom of each scatterplot. Colors and shapes are added to depict the United States Environmental Protection Agency (EPA) ecoregion III of each study area.

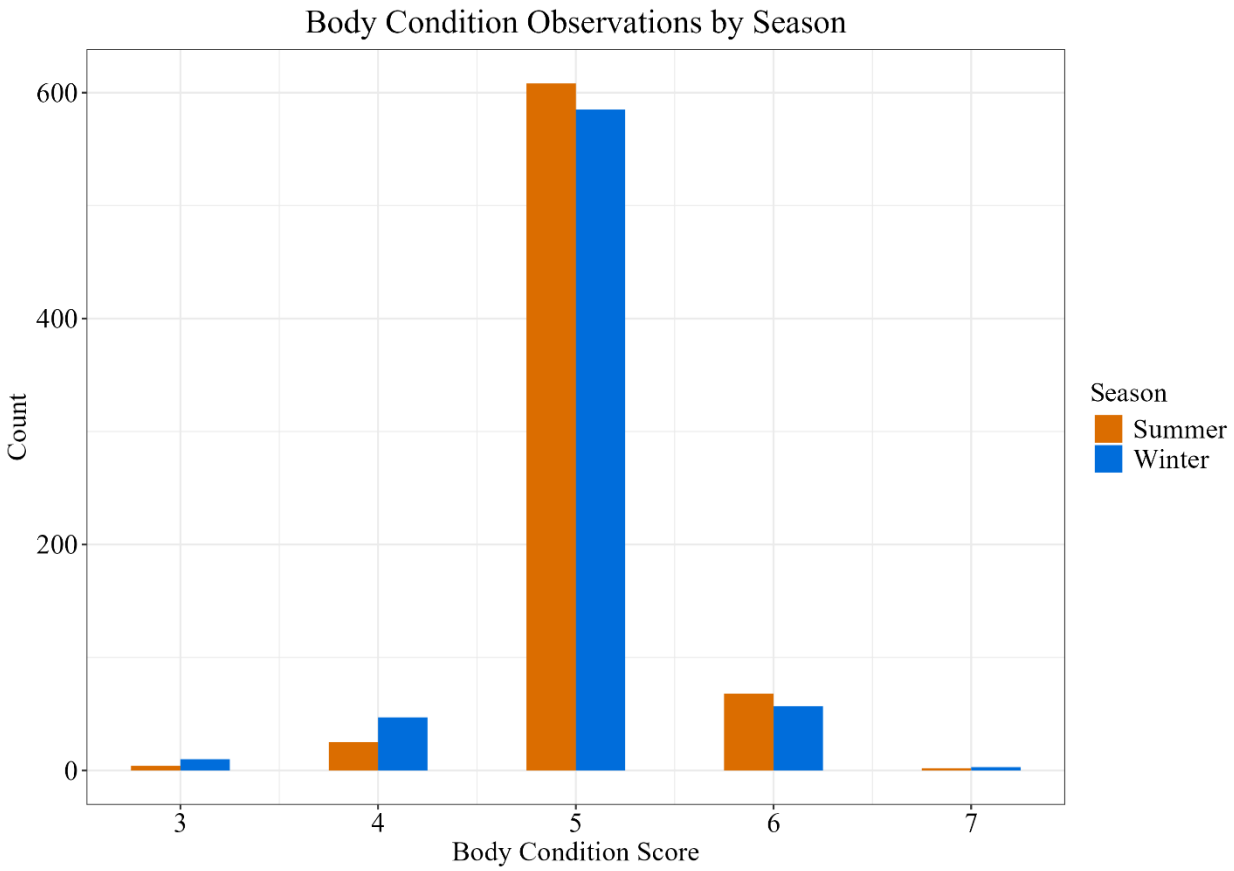

Figure 4. Distribution of Body Condition Scores (BCS) for free-roaming horses by season across all Herd Management Areas (HMAs). Summer 2020 is depicted in orange and winter 2020/2021 is depicted in blue.

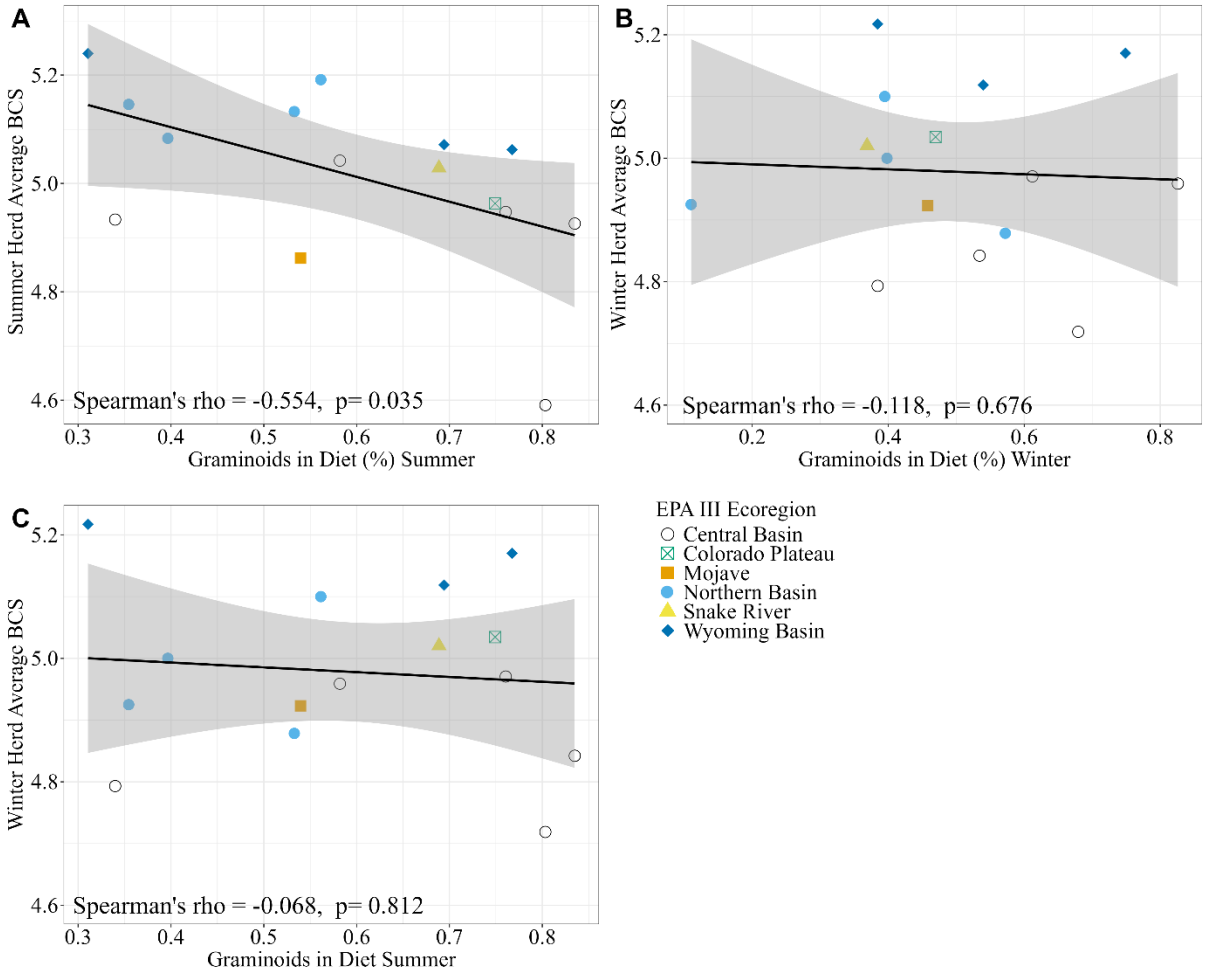

Figure 5. Correlation of the Herd Management Area (HMA) average composition of dietary graminoids vs. HMA average body condition score (BCS) in A) summer 2020, B) winter 2020/2021, and C) winter 2020/2021 body condition scores compared to summer 2020 graminoid diet composition. Spearman's rho and p-values for each correlation are included in the bottom left of each scatterplot. Colors and shapes were added to depict the United States Environmental Protection Agency (EPA) III ecoregion of each study area.

APPENDIX A – SUPPLEMENTARY METHODS

Fecal freshness indices

Although we did not directly observe each defecation before collection, we only collected fecal material that appeared fresh enough to represent the periods that we were sampling. Fresh samples were not crucial to identifying plant diet composition, because previous research has indicated that horse fecal material maintains good rates of DNA amplification for up to two months (King et al. 2018). However, the fecal samples we collected were also being used for a parallel study of microbiome, so we needed to have fresh fecal samples for that study, because microbial communities only remain stable for a short window after defecation. We conducted observations of domestic horse fecal material appearance after defecation and developed a minimum criterion for freshness of fecal samples for collection, which is the equivalent of a fecal sample assigned as a 5 below. When it was necessary to handle fecal material to assign a score accurately, we tested fecal freshness on a fecal bolus on the outside of the fecal pile before collecting un-contaminated fecal material from the interior of the fecal pile. Our freshness indices were as follows:

1) We observed the individual horse defecate and collected a sample immediately after horse had moved far enough to allow us to collect the fecal material safely.
2) The outer surface of the fecal material was still moist, and the outer and inner portion of the fecal bolus were similar color. Horses were in the immediate vicinity and likely defecated the sample, but we did not directly observe the defecation. The fecal material appeared similar to a sample rated as a 1, however the actual defecation was not observed.

3) The outer surface of the fecal bolus was slightly darker than the inner, but the bolus was still slightly moist on the outside. It was easy to penetrate the fecal sample's outer surface with the tip of a pencil.
4) The outer surface of the fecal material had dried, however it was still soft or had a thin, softer crust developing but was still easy to penetrate the outer surface of the fecal material with the tip of a pencil. The inner portion of the fecal material was still moist.
5) The outer surface of the fecal material was dry and dark colored. The outer part of the material had become firm and formed a slight crust, but we were still able to penetrate it without much difficulty with the tip of a pencil. The inside of the fecal material was still moist.

Additional laboratory methods

For further detail of protocols used by the genome technologies lab at the University of Wyoming, please see:

https://microcollaborative.atlassian.net/wiki/spaces/MICLAB/pages/1357119625/TRNL+herbivorous+diet+study+TRNL1

https://microcollaborative.atlassian.net/wiki/spaces/MICLAB/pages/1796931617/TRNL+16S+Library+Prep

Crosschecks of taxonomic assignments

Due to the large geographic scale of our study, we crosschecked plant occurrence at a state level. We first cross-checked the deepest assigned taxonomy (family, genus, or species) of each ASV with the USDA plant database (USDA, NRCS) to confirm whether that family, genus, or species was known to occur in each of the seven states where we conducted our research (CA, CO, ID, NV, OR, UT, WY). If a plant was not shown to occur in all seven states in the USDA plant

database, we explored whether that plant was found in those missing states in herbarium specimens databased in the Rocky Mountain Herbarium (Rocky Mountain Herbarium 2022), or in observations in iNaturalist (iNaturalist, 2024). Finally, if our three sources indicated a plant was known to occur in some but not all our seven states, we crosschecked which free-roaming horse diets contained this plant, and whether the horse resided in a state where the plant was not known to occur.

We found that the assigned taxonomy for 24 of our 284 ASVs (8.45%) matched with plants that were not documented by all three of our sources in any of our seven study states, and often not in the United States. However, these ASVs made up only 2.93% of total reads. We found an additional 12 ASVs (4.23% of ASVs and 4.94% of reads) that were associated with plants that occurred in at least one of the states included in the HMAs we sampled, but were found in free-roaming horse diets from a state in which none of our three sources documented that plant occurring. An additional nine ASVs (3.17% of ASVs and 5.12% of reads) were detected in free-roaming horse diets in states that matched reported plant occurrence in iNaturalist, but not the USDA plant database or Rocky Mountain Herbarium.

While these crosschecks revealed that 7.87% of our reads were assigned to plants that should theoretically not have been available to the free-roaming horse consuming it, this likely occurred because the actual dietary plant was not included in our reference database yet, and a taxonomically similar relative was assigned as the closest match. Alternatively, there is the possibility that small populations of these plants, often exotic species, do occur in the sagebrush steppe, but in low enough levels that they have not been documented. Therefore, rather than excluding these reads from analysis, we were able to include them by assigning reads to the family level.

Weight of evidence approach with various t-tests

Our first t-test analysis was conducted assuming independence, or that 15 random fecal collections were derived from 15 different random free-roaming horses in the summer and winter season within each HMA. For this test, we used a two-sample independent t-test to compare the summer and winter graminoid composition of each HMA. To evaluate the effect of the assumption of independence, we paired the 15 summer and 15 winter graminoid composition values in each HMA for maximum and minimum variation in the difference between summer and winter graminoid composition. To pair for minimum variation, we sorted both summer and winter values in ascending order, paired observations, and calculated the difference between the paired summer and winter values. To find the maximum variation, we sorted summer values in ascending order while sorting winter values in descending order before pairing and calculating the difference. By completing this analysis with the minimum and maximum variation, we found the upper and lower limits of p-values possible from our observations within each HMA. Therefore, even if we could not be fully confident that fecal samples did not originate from the some of the same horses in both seasons, we obtained a range of possible p-values, despite the independence assumption.

Logit transformations

We chose to perform Spearman's rank correlations throughout our analysis due to the smaller sample size of 15 HMAs per season and questions of normality of the data in its original form. However, to investigate these relationships further we also explored logit transformations of dietary graminoid composition and herbaceous cover and performed simple linear regressions for the same comparisons. Applying logit transformations and simple linear regression did not

improve model fit in comparisons of graminoid composition with herbaceous cover and biomass or herd average body condition, thus we only report the Spearman's Rank correlation results.

APPENDIX B – SUPPLEMENTARY RESULTS

Additional dietary graminoid composition information

Our various versions of the bootstrapped t-test indicated that three HMAs (Frisco, UT; Palomino Buttes OR; Saylor Creek, ID) displayed a higher graminoid composition in summer compared to winter, as tests all indicated a seasonal difference ($p < 0.05$) despite whether samples were considered independent or were paired for minimum or maximum variation (Table 3). We found moderate evidence for two additional HMAs (Little Book Cliffs, CO; Stinkingwater, OR) having higher graminoid composition in the summer than winter. In Little Book Cliffs, the independent test and minimum variation paired test both indicated a seasonal difference ($p < 0.05$) while the paired test for maximum variation did not provide evidence for a seasonal difference ($p = 0.121$). For Stinkingwater, the independent test indicated moderate support for a seasonal difference ($p = 0.075$), the minimum variation test indicated support for difference ($p < 0.05$), while the maximum variation test did not provide evidence for a seasonal difference ($p = 0.216$). We also found moderate evidence for one HMA (Onaqui, UT) having lower graminoid composition in the summer than winter with the independent test and minimum variation paired test both indicating a seasonal difference ($p < 0.05$) while the paired test for maximum variation indicated moderate support for a difference ($p = 0.084$). Four HMAs (Adobe Town, WY; Pine Nut Mountains, NV; South Steens, OR; Twin Peaks, CA and NV) had a consistent lack of difference in summer and winter graminoid composition across the three tests ($p > 0.05$), with these HMAs displaying less than 5-percentage points difference in graminoid composition between seasons. An additional five HMAs (Conger, UT; Green Mountain, WY; McCullough Peaks, WY; North

Hills, UT; Red Rock, NV) only had evidence for a difference between summer and winter graminoid composition with the bootstrap t-test paired for minimum variation, not providing very strong support for a difference in seasonal graminoid composition. The patterns were similar when we conducted the traditional non-bootstrapped t-tests, with the same three HMAs displaying clear seasonal differences and the same three HMAs displaying moderate support for difference between summer and winter graminoid composition, indicating the assumption of normality did not affect results (Table A2).

Dietary composition of additional plant families information

HMA average diet compositions indicated that other plant families might be locally important forage for free-roaming horses in certain HMAs and seasons. Polygonaceae composed 78.05% of HMA average winter diets and 20.12% of summer diets in Palomino Buttes (OR, Table A1, Fig A1) and was present in the diets of all 15 horses sampled in the winter and five of the 15 horses in the summer (Table A3). Polygonaceae also composed 22.69% of winter and 40.79% of summer diets in the Pine Nut Mountains (NV, Table A1, Fig A1) and was present in the diets of 12 and 11 horses sampled in the winter and summer respectively (Table A3). In McCullough Peaks (WY) average free-roaming horse diets consisted of 37.19% Fabaceae plants in the summer (Table A1, Fig A1) with 13 of the 15 horses sampled consuming this plant family (Table A3). However Fabaceae was barely present (0.40%, Table A1) in the HMA average winter diet composition, with only three horses sampled having this family present in their diet composition, all in amounts under 5% (Table A3). Brassicaceae plants were an important component of free roaming horse diets in Sands Basin (ID), comprising 34.93% of the herd average diet in the summer (Table A1, Fig A1) and present in the diets of nine of the 15 horses sampled. While it is possible that we happened to visit HMAs during a short time window when plant phenology

caused horses to use these plant families with abnormally high regularity, we would need additional collection windows during each season to confirm this. With our current information available, we feel it is important to note that it is possible for horses to consume these families as important diet components.

The diet composition of two horses did not include any graminoids. These included a summer fecal sample from Sand Basin HMA in Idaho and a winter fecal sample from Twin Peaks HMA in CA/NV. A diet analysis from fecal material only represents a single time point, so while this is interesting, this does not necessarily reflect each horses' diet as a whole over time. The plant families present in the Sand Basin sample included Brassicaceae (95.41%) and Nyctaginaceae (4.59%). The plant families present in the Twin Peaks sample included Asteraceae (83.27%) and Chenopodiaceae (16.73%).

The diet composition for individual free-roaming horses (15 summer and 15 winter) within two different HMAs is depicted in Figures A2 and A3. This illustrates that while there was always some variation in individual diet composition, in some seasons and HMAs such as Little Book Cliffs in the winter (Fig. A2), there was more individual variation in both the amount of graminoids and families present in the diet composition. Meanwhile diet composition in some HMAs was more consistent in both graminoid composition and plant families present among the individual horses, such as Adobe Town in the summer (Fig. A3). Figures A2 and A3 depict the plant families that make up at least 1% of at least one diet for each HMA, with composition then normalized to 100%. While there were 50 plant families across the dataset that made up at least 1% of at least one horse's diet composition, not all were present in each HMA. Colors representing family composition are consistent between Supplemental Figures A1, A2, and A3

and within Figure 2 in the main paper (e.g., Poaceae is always depicted in lime green, Asteraceae is always gold colored, etc.).

Supplemental Tables

Table A1

Herd Management Area (HMA) average diet composition in summer and winter of the 50 plant families that occurred with at least 1% abundance in at least one horse diet. HMA averages are based on n = 15 observations in each season. HMAs are organized in alphabetical order and abbreviated as follows: AT = Adobe Town, CN = Conger, FR = Frisco, GM = Green Mountain, LBC = Little Book Cliffs, MP = McCullough Peaks, NH = North Hills, ON = Onaqui Mountain, PB = Palomino Buttes, PN = Pine Nut Mountains, RR = Red Rock, SB = Sands Basin, SC = Saylor Creek, SS = South Steens, SW = Stinkingwater, and TP = Twin Peaks. Note this is a large table, to access the file, please see https://github.com/courtney-buchanan/Horse_diet

Table A2

Results for the parametric versions of t-tests to evaluate whether the assumption of normality affected results of seasonal graminoid differences. **Bolded p-values** indicate differences at the alpha = 0.05 level while ***bold italicized p-values*** indicate differences at the alpha = 0.10 level.

HMA	Herbaceous cover category	EPA Ecoregion III	Two sample t-test p value (independent)	Two sample t-test p value (paired min variation)	Two sample t-test p value (paired max variation)
Clear Support for Seasonal Difference					
Frisco	Low	CB	**<0.001**	**<0.001**	**0.008**
Palomino Buttes	Medium	NB	**0.008**	**<0.001**	**0.031**
Saylor Creek	High	SR	**<0.001**	**<0.001**	**0.009**
No Support for Seasonal Difference					
Adobe Town	Low	WB	0.857	0.732	0.885
Twin Peaks	High	NB	0.990	0.952	0.993
South Steens	High	NB	0.611	0.129	0.712
Mixed Support for Seasonal Difference- Leaning Toward Difference					
Little Book Cliffs	Low	CP	**0.024**	**<0.001**	0.100
Onaqui	High	CB	**0.009**	**<0.001**	***0.053***
Stinkingwater	High	NB	***0.077***	**<0.001**	0.200
Mixed Support for Seasonal Difference- Leaning Against Difference					
Pine Nut Mountains	Low	CB	0.624	***0.080***	0.721
Red Rock	Low	MB	0.416	**<0.001**	0.566
Conger	Medium	CB	0.193	**0.031**	0.313
Green Mountain	Medium	WB	0.141	**<0.001**	0.297
McCullough Peaks	Medium	WB	0.529	***0.062***	0.638
North Hills	Medium	CB	0.191	**0.002**	0.305
Range wide (HMA as experimental unit)					
			Paired: 0.036		

Table A3

Diet composition of the 50 plant families that occurred with at least 1% abundance in at least one horse diet, reported by each fecal sample collection. Fecal collections are named according to HMA and are abbreviated as follows: AT = Adobe Town, CN = Conger, FR = Frisco, GM = Green Mountain, LBC = Little Book Cliffs, MP = McCullough Peaks, NH = North Hills, ON = Onaqui Mountain, PB = Palomino Buttes, PN = Pine Nut Mountains, RR = Red Rock, SB = Sands Basin, SC = Saylor Creek, SS = South Steens, SW = Stinkingwater, and TP = Twin Peaks. Names beginning with a "W" indicate winter collections. Note this is a large table, to access the file, please see https://github.com/courtney-buchanan/Horse_diet

Supplemental Figures

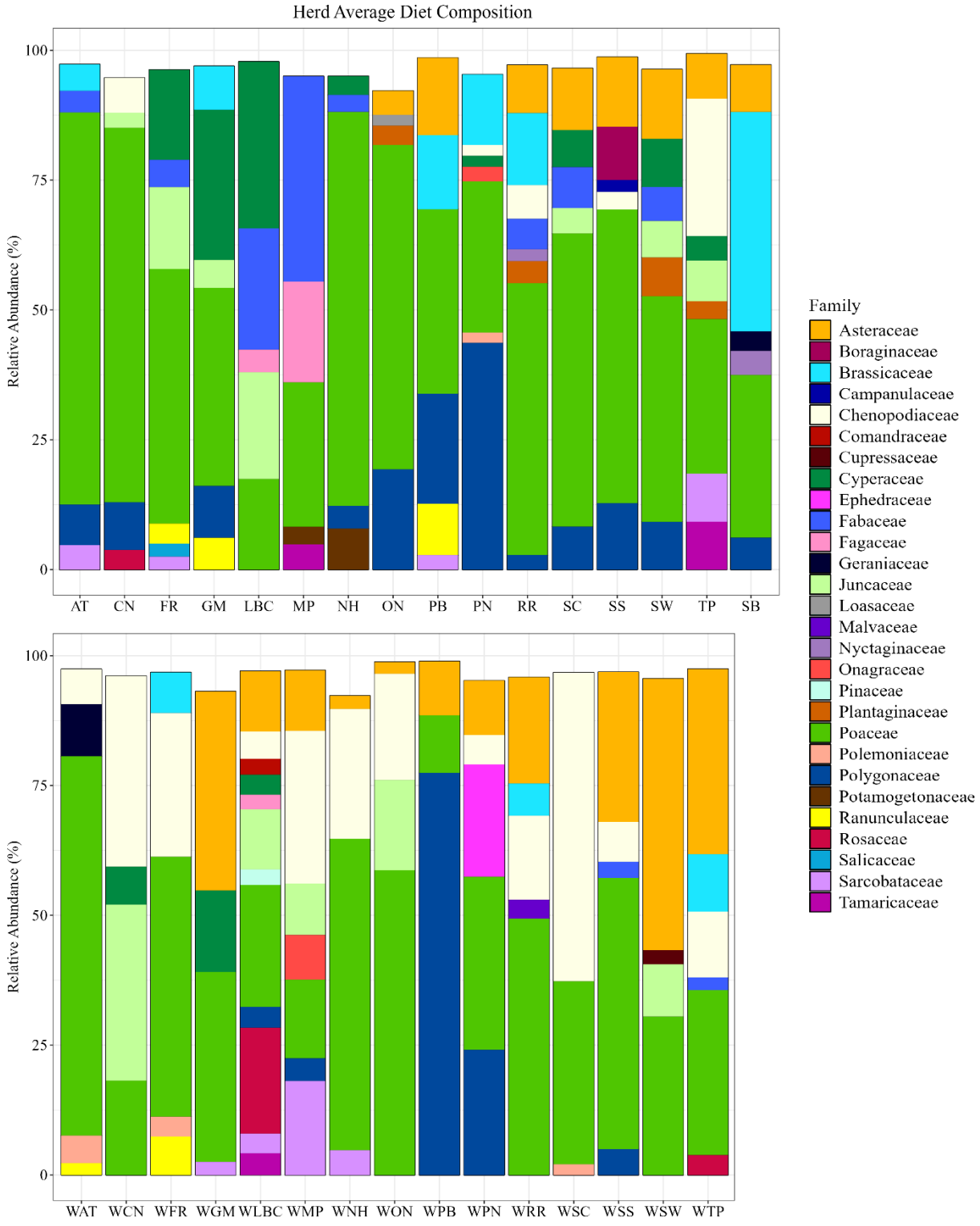

Figure A1. Average diet composition by HMA and season for 16 BLM HMAs managed for free-roaming horses, summer 2020 (upper panel) and winter 2020/2021 (lower panel). Stacked bar charts represent the average composition of the diet of each HMA in each season grouped by family. Plant families that did not represent at least 2% of the average diet of at least one herd were removed to simplify the visual; therefore, bars do not reach 100%. Sands Basin, ID (SB) was only visited during the summer months and only appears in the upper panel. The three graminoid families are all depicted in shades of green- Poaceae (lime green), Cyperaceae (dark green), and Juncaceae (light green).

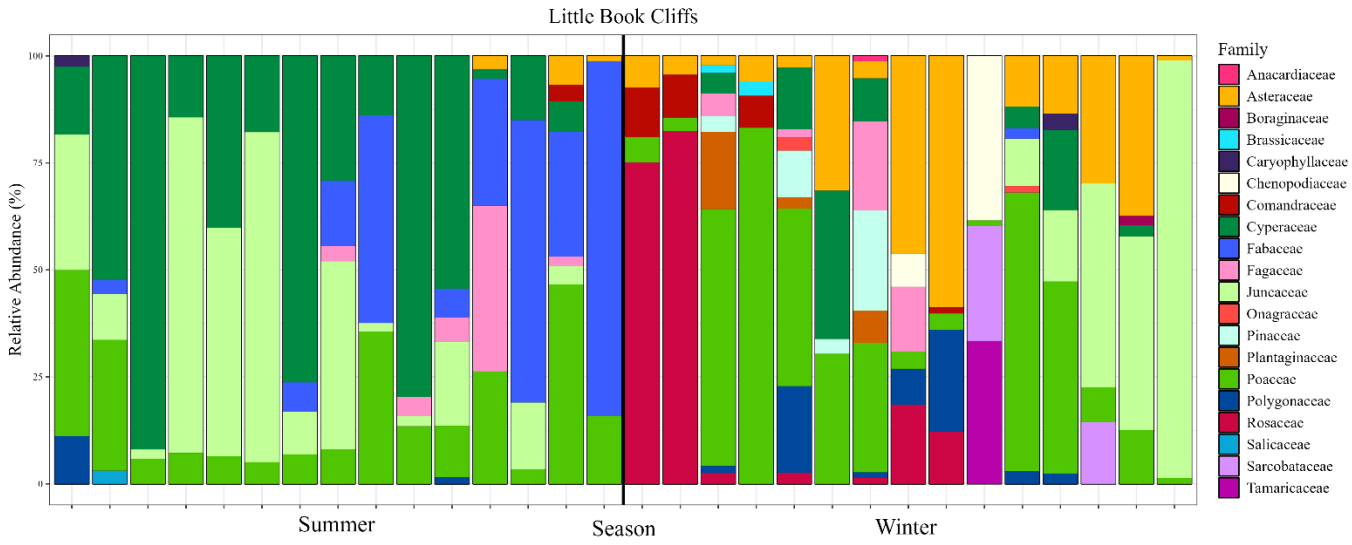

Figure A2: Diet composition for free-roaming horses in the Little Book Cliffs HMA in Colorado depicting individual horse diet composition in summer 2020 and winter 2020/2021. The stacked bar chart reports the composition of the diet of each horse grouped by family, and the black vertical line separates summer and winter. The three graminoid families are all depicted in shades of green- Poaceae (lime green), Cyperaceae (dark green), and Juncaceae (light green).

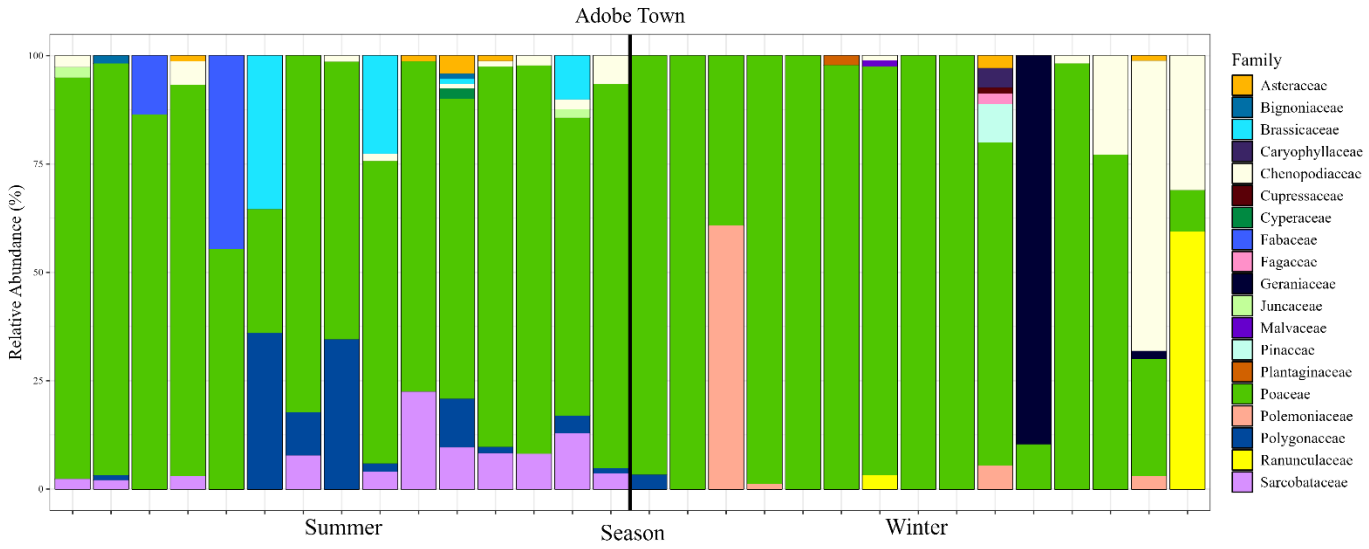

Figure A3: Diet composition for free-roaming horses in the Adobe Town HMA in Wyoming depicting individual horse diet composition in summer 2020 and winter 2020/2021. The stacked bar chart reports the composition of the diet of each horse grouped by family, and the black vertical line separates summer and winter. The three graminoid families are all depicted in shades of green- Poaceae (lime green), Cyperaceae (dark green), and Juncaceae (light green).

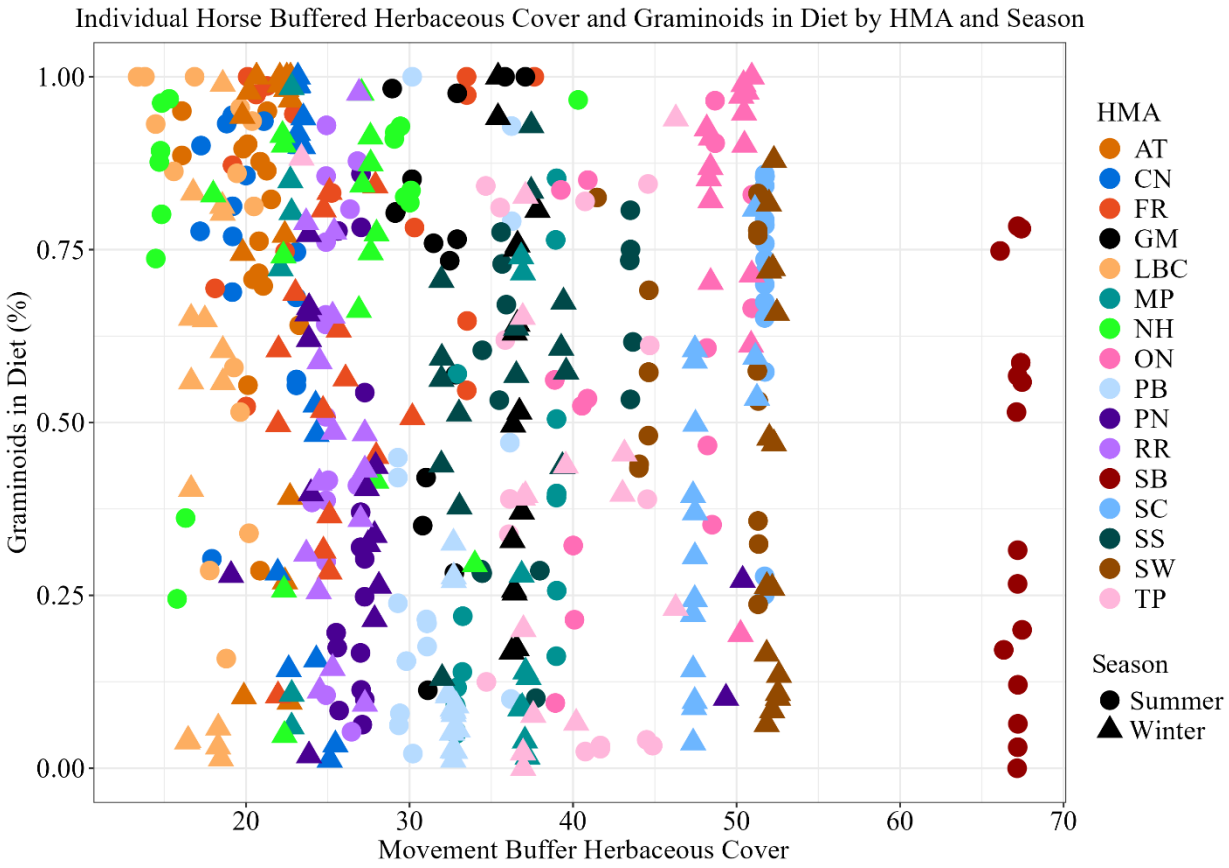

Figure A4. Scatterplot for individual free-roaming horse movement buffered (13.5- km diameter) proportion of available herbaceous cover and proportion graminoids present in the diet. Circles represent summer fecal collections while triangles represent winter collections. Points are color coded by HMA. Note that even within a single HMA horses exhibited varying amounts of dietary graminoid composition when similar proportions of herbaceous cover were available.

CHAPTER 4. Drivers of bacterial gut microbiome composition in free-roaming horses across western North America

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ABSTRACT

Gut microbial communities may serve important functional roles for wild herbivores. Indeed, in recent years, increasing research into gut microbiome composition in wild and free-roaming species indicates that flexible microbial pools could allow greater plasticity for animals to adapt to changing conditions. However, the factors that affect and drive bacterial gut microbiome composition remain poorly characterized in wild and free-roaming species. We investigated the bacterial gut microbiome of free-roaming horses in the western United States to determine whether the bacterial community varied with season, environment, or diet. We collected fecal samples in summer 2020 and winter 2020/2021 from 16 Bureau of Land Management Herd Management Areas (HMAs) across differing environments including the Colorado Plateau, Great Basin, Mojave Desert, and Wyoming Basin. We found that season, environment, and diet were all significant drivers of variation in gut microbial community composition. Seasonality explained about 3% of microbial community variation at the range-wide scale; however, seasonality explained 7–18% of variation within a single HMA. We identified 158 bacterial families present in free-roaming horse microbiomes during the winter, while only observing 128 of these families during the summer. Environment, as measured by HMA or ecoregion was more explanatory than season, with HMA explaining 9% and ecoregion explaining about 4% of microbial community variation at the range-wide scale. Both season and environment explained greater variation when we averaged individual horse microbiomes within each HMA to create a "herd average" microbiome composition. Mantel tests indicated that diets that were more

dissimilar were associated with increasingly dissimilar microbial communities. Of the ten bacterial families that contributed the most to microbial community dissimilarity, nine were correlated with at least one plant family present in the diets of free-roaming horses. We found that the graminoid families of Cyperaceae, Poaceae, and Juncaceae and the forb and shrub families of Asteraceae and Chenopodiaceae most commonly displayed relationships with these microbial families. Free-roaming horses in the western United States maintain body condition and high rates of reproduction on what are often considered poor quality rangelands. It is possible that one contributor to their success may be that flexible gut microbiomes assist these animals in adapting to a wide range of environmental conditions. Studies such as ours that highlight the potential benefits of microbial plasticity to free-roaming animals elucidate the promise for research into microbial tools such as probiotics or microbial augmentations in wildlife management, which could prove useful to threatened species worldwide.

INTRODUCTION

Free-roaming herbivores interact with other herbivores, predators, parasites, plant species, and microorganisms, all of which can affect their survival and fitness. While many of these interactions have been the interest of scientists for generations, one area that has been recently gaining attention is the relationship between animals and the microbial communities that make up their "gut microbiomes"—the microbial organisms residing in animals' digestive tracts. The benefits of microbial symbionts to break down forage in mammalian herbivores including many domestic livestock species have long been noted (Janis 1976; Hanley 1982; Van Soest 1982). However, more recent studies have shown additional benefits to microbial communities such as links between gut microbiome composition and feed efficiency in livestock (McCann et al. 2014; Myer et al. 2015; Shabat et al. 2016; Li et al. 2019a), and the ability for some mammalian

herbivores such as desert woodrats (*Neotoma lepida*) to degrade plant secondary metabolites (Kohl et al. 2014).

While many studies have investigated relationships between gut microbes and their hosts in domestic livestock or laboratory animals, fewer have explored the complexity of microbial communities of wild or free-roaming animals in natural habitats (Amato 2013). There is foundational knowledge regarding gut microbiomes of diverse animals, including those from zoo settings (Ley et al. 2008; Muegge et al. 2011; McKenzie et al. 2017); however, wild animals have been shown to have differing microbiomes from their conspecifics located in captive environments (McKenzie et al. 2017; Metcalf et al. 2017; Chong et al. 2019; Li et al. 2019b; Salgado-Flores et al. 2019; Yao et al. 2019). In recent years more microbiome studies of wild or free-roaming herbivore species have been conducted (Bergmann et al. 2015; Antwis et al. 2018; Kartzinel et al. 2019; Li et al. 2019; Salgado-Flores et al. 2019; Yao et al. 2019; Fountain-Jones et al. 2020; Eddington et al. 2021; Weinstein et al. 2021; Nielsen et al. 2023; Buchanan et al. 2024), adding further insights into the role microbiome plays in herbivore biology and ecology.

Gut microbial communities have the potential to be influential in wildlife management (Trevelline et al. 2019). Some authors have highlighted the potential for microbial tools such as probiotics treatments in wildlife species to prevent infectious diseases or improve animal health (McKenzie et al. 2018; Trevelline et al. 2019). Other scientists have reported specific bacterial strains for use in preventing infectious diseases such as bovine tuberculosis (Stedman et al. 2020), white nose-syndrome (Cheng et al. 2017), or chytridiomycosis (Bletz et al. 2013). Some researchers have suggested that microbial plasticity may offer an adaptive advantage to host animals (Alberdi et al. 2016; Bahrndorff et al. 2016; Trevelline et al. 2019). Others have also noted the potential importance of microbial communities to translocation success, and proposed

modifications to translocation efforts such as providing probiotic inoculum to animals destined for reintroduction (McKenzie et al. 2018), allowing longer adjustment time to increase the microbiome "wildness" (Yao et al. 2019), or performing microbial community monitoring post-translocation (Chong et al. 2019). Understanding how the composition of gut microbial communities change across landscape scales—as well as factors that drive and shape microbial composition in free-roaming herbivores—will be important if management tools such as microbial transfers or probiotics in endangered, elusive, or sensitive wildlife species continue to be explored.

Countless factors can affect composition of the gut microbiome community. Social networks have been shown to influence microbial composition in wild baboons (*Papio spp.*, Tung et al. 2015), humans (*Homo sapiens*) and their dogs (*Canis lupus familiaris*, Song et al. 2013), and in semi-feral equids (Antwis et al. 2018). Studies have shown an effect of season in free-ranging brown bears (*Ursus arctos*, Sommer et al. 2016), mule deer (*Odocoileus hemionus*, Eddington et al. 2021), American bison (*Bison*, Bergmann et al. 2015), pastured horses (*Equus caballus*; Kobayashi et al. 2006; Salem et al. 2018; Zaitseva et al. 2023), and stabled horses (Theelen et al. 2021). While some of these microbial changes could be caused by changing seasonal diet or metabolism in some species, an experiment in laboratory mice exposed to cold temperatures revealed a microbiome shift in cold exposed mice (Chevalier et al. 2015), which provides support for the impact of ambient seasonal temperature on microbial communities. Environment has been shown to be influential, with variation in microbial communities observed in animals of different herds or locations in Przewalski's horses (*E. ferus przewalskii*, Li et al. 2019b), mule deer (Eddington et al. 2021), pronghorn (*Antilocapra americana*, Buchanan et al. 2024), and brown bears (Trujillo et al. 2022). Finally, diet has been identified as an important

driver of microbial composition. There is substantial literature indicating that dietary differences explain variation in microbial community composition. This includes studies at broad scales evaluating feeding niches such as omnivores, carnivores, and herbivores (Ley et al. 2008; Muegge et al. 2011; McKenzie et al. 2017) or when comparing different herbivore species (Henderson et al. 2015; Kartzinel et al. 2019). There are also interspecific examples within the same genera such as woodrats (Weinstein et al. 2021; Nielsen et al. 2023), or within a single species such as American bison (Bergman et al. 2015), brown bears (Trujillo et al. 2022), various African megafauna (Kartzinel et al. 2019), Przewalski's horses (Li et al. 2019b), and domestic horses (Willing et al. 2009; Hansen et al. 2015; Fernandes et al. 2021).

Free-roaming horses have become a management challenge across the western United States. They are a non-native species, descended from horses introduced to North America by European explorers and settlers starting in the 1500s and 1600s (Haines 1938a; 1938b; McKnight 1959). Free-roaming horses spread with the continued colonization across the western United States and through trade with Native American tribes (McKnight 1959). Today, free-roaming horses are present in many western ecosystems including the Colorado Plateau, Great Basin, Mojave Desert, and Wyoming Basin. Populations in most western states are currently above their set appropriate management levels (BLM 2025); this trend is poised to continue, as free-roaming horse populations can increase at rates of 20% per year (Garrott et al. 1991). Large numbers of free-roaming horses can have negative effects on the ecosystems they inhabit (Beever and Brussard 2000; Beever and Herrick 2006; Beever et al. 2008; Davies et al. 2014; Davies and Boyd 2019; Hennig et al. 2021a). Another concern as horse numbers increase is potential for impacts on native wildlife species through direct effects such as competition at watering locations (Ostermann-Kelm et al. 2008; Perry et al. 2015; Hall et al. 2016; Gooch et al.

2017; Hennig et al. 2021b) or disturbing lekking sage grouse (Muñoz et al. 2021) or through indirect effects such as altering habitat (Beever and Aldridge 2011; Hennig et al. 2021a). Despite inhabiting sometimes harsh conditions, a previous study found that free-roaming horses across the range appeared to be in relatively good condition in summer and winter (Buchanan et al. *in press*). One reason horses are believed to succeed while grazing poor quality forage is their digestive system, which allows horses to leverage low quality, abundant forage by quickly passing ingesta through their digestive tract (Janis 1976; Hanley 1982). Hindgut fermenters, such as horses, digest food less completely compared to ruminants, allowing horses to maintain a quicker ingesta passage rate compared to many other herbivores (Duncan et al. 1990). In ruminants, forage intake is limited because of the necessity for ingesta to be broken into small particles to pass from the rumen to the remainder of the digestive tract (Hanley 1982). Conversely, horses do not have this particle size limitation and can more quickly pass ingesta through their digestive tract, enabling a "quantity over quality" strategy able to take advantage of abundant low-quality forage (Janis 1976, Hanley 1982). As an example, in a previous study horses consumed 63% more (on a per body weight basis) than cattle, allowing them to consume more digestible nutrients per day although the cattle digested food more completely (Menard et al. 2002). However, it is still unknown how these animals are able to adapt to differing available forage, ecosystems, and conditions across the western United States, especially because modern horses did not evolve in North America.

Here, we investigate the relationship between free-roaming horses and their gut microbiomes to understand whether gut microbial composition may be a potential adaptive trait that allows these animals to succeed. Our overall objective was to investigate whether microbial variation is present in free-roaming horses in different seasons or environments. If apparent,

variable microbial community composition could be an adaptive trait that allows horses to succeed, despite often inhabiting areas considered poor quality foraging habitats for large grazing animals. We first investigated whether microbiome composition varied by season. We hypothesized that because microbiome composition differed between season in other North American species (Bergmann et al. 2015; Sommer et al. 2016; Eddington et al. 2021) and in pastured or free-roaming horses in other parts of the world (Kobayashi et al. 2006; Salem et al. 2018; Zaitseva et al. 2023), we would measure a seasonal difference in free-roaming horse microbiomes at both the range-wide scale and when focusing on individual HMAs. Second, we investigated whether the equine microbiome varied in different ecosystems by comparing the microbial composition in different HMAs and ecoregions. Conspecifics occupying different habitats may have access to different regional microbes and could acquire distinct microbial communities (Amato et al. 2013). Indeed, literature from other North American herbivores (Eddington et al. 2019, Buchanan et al. 2024) and Przewalski's horses (Li et al. 2019b) has demonstrated variation in microbial composition between study areas. Thus, we hypothesized differences in microbial composition would be detected in different HMAs and ecoregions. Finally, we evaluated whether microbiome composition in free-roaming horses varied with diet composition. Relationships between diet and microbial composition have been noted in many herbivores (Bergman et al. 2015; Henderson et al. 2015; Kartzinel et al. 2019) including in the Przewalski's horse (Li et al. 2019b), an endangered equid species that has been successfully reintroduced into native habitat in China and Mongolia. Studies in domestic horses have also documented microbial changes with dietary changes (Willing et al. 2009, Hansen et al. 2015; Fernandes et al. 2021). Therefore, we hypothesized we would find a link between diet and

microbiome composition and that free-roaming horses in our study with more similar diets would have more similar microbial communities as well.

METHODS

Study Areas

We chose study areas to represent the ecological and geographic range of free-roaming horses across Bureau of Land Management (BLM) Herd Management Areas (HMAs). We used information from the BLM's Wild Horse and Burro Herd Management Areas Website (BLM, undated, https://www.blm.gov/programs/wild-horse-and-burro/herd-management/herd-management-areas) to initially select HMAs and refined choices based on discussions with BLM personnel. We selected 16 HMAs where local knowledge indicated that we could safely access horses in summer and winter seasons on foot or with four-wheel drive vehicles. Additionally, we chose HMAs to cover a representative gradient of herbaceous availability. Once HMAs were selected, we identified the United States Environmental Protection Agency (EPA) level III ecoregion (EPA 2024) of each of our selected HMAs by overlaying HMA and ecoregion shape files. Our study HMAs included six distinct EPA III Ecoregions including the Central Basin and Range, Colorado Plateau, Mojave Basin and Range, Northern Basin and Range, Snake River Plain, and Wyoming Basin. We were unable to access one HMA (Sands Basin, ID) in the winter due to unfavorable weather and impassible roads. For more information on selected HMAs, see the study area description, Table 1, and Figure 1 in Buchanan et al. (*in press*).

Field Methods

Within each HMA, we collected free-roaming horse fecal material from 15 separate fecal piles during summer 2020 (May–July) and winter 2020/2021 (late November to January). We relied on local knowledge from BLM personnel or wild horse groups to identify locations frequented

by free-roaming horses and additionally used maps to identify probable watering locations to begin searching for free-roaming horses. Starting at these locations, we then drove along roads until we detected horses or fresh fecal material (for description of our definitions of freshness of fecal material please see Appendix A of Buchanan et al. *in press*). Whenever possible, we collected fecal material from at least three geographically separate areas of an HMA to represent free-roaming horse use across the geographic gradient of the HMA. This was not possible in all HMAs or seasons due to lack of safe access to portions of the HMA, failure to find fresh signs of horse use within sections of an HMA, or uneven distribution of free-roaming horses throughout an HMA. In some HMAs, most horses were observed near one another during a single temporal point, while other HMAs only had a single perennial water source, making it impossible to find fresh fecal material in other areas of these HMAs during our sampling window.

To ensure fecal material had not been exposed to environmental factors for prolonged periods of time, we obtained fecal material from fresh piles with moist interiors, and when possible, collected fecal samples immediately after observed defecations. A previous study in domestic horses indicated that microbial communities remained stable for 6 hours and started to change by 12 hours post defecation (Theelen et al. 2021). Based on the criteria we had for collecting fecal material, we feel that collections likely fell within this stable period, however, we still assigned each fecal collection a freshness index to investigate the effect of fecal freshness on microbial communities (for more information about fecal freshness indices see Appendix A in Buchanan et al. *in press*). Our main concern in requiring fresh samples was for microbial community preservation, because plant DNA in horse fecal material was reported to remain stable for up to two months when exposed to the environment (King et al. 2018). We collected at least three fecal boluses from each pile to achieve a representative sample of an

individual free-roaming horse and gathered fecal matter from the interior of piles to minimize environmental contamination. For each fecal collection, we turned a sealable plastic freezer bag inside out without touching the bag interior and collected the sample into the bag. We wore latex gloves to prevent contamination of fecal material if we needed to place material into the bag. After collecting fecal material, we returned the plastic bags to proper orientation, sealed them, and stored fecal material on dry ice while in the field. We transferred frozen fecal material to a -20°C freezer within two weeks. We chose to freeze fecal material for preservation until laboratory analyses because freezing has been shown to effectively preserve fecal DNA in other species (Vlčková et al. 2012; Czernik et al. 2013).

Sample Preparation

We kept fecal material stored in a -20°C freezer and sub-sampled fecal material for DNA extraction and sequencing after all field collections were made. We carried out all sub-setting procedures under a hood to prevent contamination from the laboratory or technicians. During this process, we removed small groups of fecal samples from the freezer and kept them on ice to prevent thawing. We used a rubber mallet to drive a metal corer to obtain an interior sample for three fecal boluses from each animal. We then used autoclaved tools to deposit the interior of each core into a 2-ml test tube. We avoided placing portions of the surface of a fecal bolus into the test tube to minimize environmental microbial contamination. To prevent any further contamination, we used a clean autoclaved corer and new set of gloves for each individual horse's fecal matter, and we wiped surfaces with ethanol and RNase AWAY to remove any residual bacteria and DNA or RNA fragments. We added 230-250 mg of fecal material to each tube and stored sub-samples in a -20°C freezer awaiting laboratory analysis.

DNA Extractions, Library Preparation, and Sequencing

We submitted fecal material to the Genome Technologies Lab at the University of Wyoming for DNA extraction and library preparation to characterize both the plant and microbial communities found in free-roaming horse fecal material. A single DNA extraction was made for both microbial and plant assays using a QIAGEN DNeasyPowerSoil Kit following manufacturer's protocols. Prior to amplification and library preparation, samples were normalized to 10ng/µL.

Library preparation was conducted using custom designed one-step primers, including Illumina adaptors and unique oligo barcodes so indexes and barcodes were within the reads. For both plant and microbial assessments, two libraries were prepared for each individual fecal collection to assess consistency in library preparation and sequencing. For plants, the P6 loop of the *trnL* locus was amplified using the *-g* (GGGCAATCCTGAGCCAA) and *-h* (CCATTGAGTCTCTGCACCTATC) primers described by Taberlet et al. (2007). PCR conditions for library preparation included a 10-minute denaturation (95°C) followed by 35 cycles of denaturation (95°C, 30s), annealing (55°C, 30s), and extension (72°C, 30s). This was followed by a final extension period (72°C, 9 minutes) and a hold at 4°C. For microbial analysis, the variable 4 (V4) region of the 16S rRNA intron was amplified using the 515f (GTGYCAGCMGCCGCGGTAA) and the 806r (GGACTACHVGGGTWTCTAAT) primers described in Walters et al. (2016). The 16S PCR cycling conditions encompassed a 3-minute denaturation at 95°C followed by 35 cycles of denaturation (98°C, 30s), annealing (62°C, 30s), and extension (72°C, 30s). There was a final extension period of 5 minutes at 72°C followed by a hold at 4°C. PCR cleanup was conducted using the Axygen™ AxyPrep MAG PCR Clean-Up Kit following the manufacturer's protocol.

Sequencing was conducted by the University of Colorado. Libraries were pooled at a ratio of four to one (16S: *trnL*), to account for amplicon length differences. Libraries were sequenced at

2x250 on a NovaSeq6000, with a 10% PhiX addition. Please see Appendix A in Buchanan et al. (*in press*) for further information about protocols.

Bio-informatics

We used the QIIME2 (Bolyen et al. 2019) pipeline for initial bioinformatics of microbial communities. Our demultiplexing steps yielded an initial 548,176,831 reads with a mean read count of 515,204 per sample. We used the DADA2 program (Callahan et al. 2016) within QIIME2 to complete filtering and de-noising. After examining quality scores, we truncated forward and reverse reads at 240 base pairs to allow overlap for merging. We report read counts at various steps of the DADA2 pipeline in the Appendix (Table A1). We identified 325,501 unique amplicon sequence variants (ASV), with each ASV representing a unique genetic sequence recognized in samples during the sequencing process. We assigned taxonomy to the species level using Silva databases (Silva 138 SSURef NR99 515F/806R region sequences Silva 138 SSURef NR99 515F/806R region taxonomy and Silva 128 SEPP reference database; Quast et al. 2013).

We conducted downstream analysis in Program R (Version 4.3.0; R Core Team, 2023) and read QIIME2 outputs into R using the package 'qiime2R' (Bisanz 2018). We used the R package 'phyloseq' (McMurdie and Holmes 2013) to combine taxonomy, collection metadata, and the ASV table for analyses. Our initial laboratory protocols produced two sets of reads for each horse, so after checking for consistency of patterns across the duplicate samples we merged the reads to create a single set of reads for each horse. We initially began with 325,501 distinct ASVs. We subset these to include only reads assigned to the kingdom bacteria to remove archaea or any ASVs with an unassigned Kingdom, resulting in 313,253 ASVs remaining. We then removed mitochondrial (1444 ASVs) and chloroplast (1071 ASVs) reads yielding 310,738

unique ASVs. We then transformed read counts to relative abundance by dividing the number of reads for each taxon within each horse by the total reads for that horse. Because our goals were more focused on assessing patterns driving microbial communities rather than identifying rare taxa, we filtered to exclude reads that had a relative abundance of less than 0.001%. After filtering, 16,896 ASVs remained. By eliminating rarer low abundance ASVs, we were also able to exclude ASVs that represented potential erroneous reads or sequencing errors. For information about the bio-informatics and data processing steps for the plant community composition please see Buchanan et al. (*in press*).

Statistical Analysis

We assessed the taxonomic depth of coverage by determining the percentage of reads assigned to each taxonomic level (Table A2), and visualized community composition using stacked bar charts (Figure A1, A2). We also quantified the number of different bacterial families present in different seasons, ecoregions, and HMAs. To visualize the effects of season, ecoregion, or HMA on free-roaming horse microbiome composition, we created ordination plots using Principal Coordinate Analysis (PCoA) and Bray–Curtis dissimilarity measures.

To assess differences in microbial communities among HMAs, ecoregions, and seasons we conducted permutational multivariate ANOVA (PERMANOVA) tests with 999 permutations using the adonis2 function (McArdle and Anderson 2001; Anderson 2001) in the 'vegan' package. We also used Bray–Curtis as a distance metric when performing PERMANOVA tests. We investigated season, ecoregion, and HMA as potential drivers of microbial community composition at various scales relevant to free-roaming horse management using a series of PERMANOVA tests.

To investigate the amount of variance explained by the factors of season, ecoregion, and HMA at the range-wide scale we first performed PERMANOVA tests using each single factor on the full set of 465 fecal collections: 15 fecal collections per each of 15 HMAs for each season (summer and winter), plus the 15 from Sands Basin HMA in Idaho, which was only visited in summer 2020. We used the pairwise.adonis function (Arbizu 2020) with Bonferroni correction to assess which ecoregions or HMAs were different from one another. Next, to investigate the marginal effect of these factors when considered in combination, we created combined PERMANOVA models that included either season and HMA or season and ecoregion. We also completed another set of models that included the interactions of these factors. Because HMA and ecoregion were confounded with one another, we did not include both ecoregion and HMA in models together. To investigate the more localized effect of season within a single HMA, we subset to one HMA at a time (15 summer and 15 winter fecal collections) and performed a PERMANOVA test for season using only each 30-horse HMA subset. Finally, because individual animal identity has been shown to explain high amounts of variation in the microbial community, up to 50% in one study (Antwis et al. 2018), we created a "herd average" microbial composition for each HMA and season to investigate the effects of ecoregion and season at the "herd average" scale once individual animal variation was removed. Finally, we used SIMPER analysis (Clarke 1993) in the vegan package (Oksanen et al. 2022) to explore which microbial families contributed the most to the overall dissimilarity in microbial communities between free-roaming horses and specifically to seasonal differentiation in microbial communities at the range-wide scale.

Initial data analysis indicated free-roaming horse diet composition included seasonal patterns that mirrored the seasonal variation we observed in the microbial community (Table A3,

Figure A3). To assess this potential relationship between free-roaming horse diet and microbiome, we first performed a Mantel test using the mantel function in the vegan package (Oksanen et al. 2022) to evaluate the covariance among the microbial community and diet composition of each horse. We created a distance matrix from the relative abundance ASV tables for both the microbial community and diet composition among all 465 horses using Bray Curtis as our distance metric. To assess the effect of taxonomic depth on potential diet-microbe relationships, we completed Mantel tests at the ASV and family levels. At the ASV level, we created distance matrices that included all 16,896 bacterial ASVs and 284 plant ASVs after we filtered rare taxa. For the family-level Mantel tests, we reduced taxonomic depth to a simplified community composition that included the 158 bacterial and 50 plant families and created distance matrices from these simplified community profiles. For more information about the data processing steps leading to the plant composition ASV table, please see Buchanan et al. (*in press*). To further understand links between diet and gut microbial composition, we investigated the relationship between family level diet composition and the ten bacterial families that SIMPER analysis indicated had the largest contributions to differences in bacterial communities. We used rcorr in the Hmisc package (Harrell 2023) to conduct correlations using the default setting of Pearson's correlations between these select bacterial families and the 50 plant families identified in free-roaming horse diets, using the full set of 465 horses. We adjusted p-values for multiple comparisons with the false discovery rate method (Benjamini and Hochberg 1995) using the p.adjust function.

RESULTS

Our filtered microbiome dataset included 21 phyla, 47 classes, 100 orders, 158 families, 287 genera, and 382 species. Most reads were assigned to a depth of family level (96.67% of reads)

while fewer were assigned to the genus (80.87%) or species (52.30%) levels (Table A2). We summarized the bacterial community within each HMA and season combination in stacked bar charts by both phyla and family (Figure A1 and A2). The family chart represents only those families (30 in total) that occurred with at least 1% relative abundance in each HMA (Figure A2). We found all 158 identified families in winter, while only 128 were present in summer. The number of families present in free-roaming horse microbiomes from different ecoregions averaged 123.50 and varied from a high of 148 families in the Wyoming Basin ecoregion to a low of 107 families in the Mojave Basin and Range ecoregion. However, we must note that the Mojave ecoregion only contained one HMA and thus it is not surprising for there to be less diversity of microbial families present than in ecoregions containing multiple HMAs. The average number of families present in a single HMA averaged 112.87 (excluding Sands Basin) with a high of 138 in Green Mountain (WY) and a low of 99 in Adobe Town (WY, Table A4).

When assessing drivers of microbial community composition at the range-wide scale using all 465 horse fecal collections, season, ecoregion, and HMA were all significant as a single predictor ($p = 0.001$) but only explained 3.5%, 4.4%, and 9.5% of microbial variation, respectively (Table 1). After averaging the 15 free-roaming horses in each HMA and season to get a "herd average" microbiome for each HMA and season, we found that both season ($p = 0.001$) and ecoregion ($p = 0.044$) were significant predictors of microbiome composition and explained 15.3% and 20.3% of the microbial variation, respectively (Table 1). HMA was no longer a significant predictor ($p = 0.887$, Table 1), likely because there were only two data points (one per season) for each HMA when analyzing data at this scale. Ordinations clearly indicated separation between seasons (Figure 1) but visual patterns were less clear between ecoregions (Figure 2) or HMAs.

Overall, our multifactor PERMANOVA models supported the trends detected with single factor PERMANOVAS. When we combined season and ecoregion in PERMANOVAs at the range-wide scale using all individual horses, both factors were significant ($p = 0.001$) when considering their marginal effects and their interaction was also significant (Table 2). The same was true when considering the marginal effects of season and HMA as well as their interaction (Table 2). When accounting for the marginal effect of season and HMA, HMA ($R^2 = 0.093$) was more explanatory of microbial community variation than season ($R^2 = 0.033$), however when the model was run including an interaction, their interaction ($R^2 = 0.070$) also accounted for some of the variation. When the marginal effect of season ($R^2 = 0.036$) and ecoregion ($R^2 = 0.045$) were accounted for, both factors explained similar amounts of variation. A similar amount of variation was explained by their interaction ($R^2 = 0.031$).

When evaluating these drivers at finer scales, these factors were able to explain more of the variation in microbial communities. When investigating the effect of season at the HMA scale using only the 30 horses in each HMA subset at a time, differences in summer and winter microbial composition were evident in all HMAs ($p = 0.001$, Table 3). Different HMAs had R^2 values ranging from 0.070 to 0.181, meaning season explained between 7.0 to 18.1% of the variation in the microbial community within a single HMA (Table 3) which is two to five times more explanatory power than when evaluating season at the range-wide scale. When we conducted multifactor models on the "herd average" microbial compositions, season and ecoregion were both significant ($p = 0.001$ and 0.002 respectively) and explained 15.8 and 20.8% of the variation, respectively, when evaluating each of these factors individually (Table 2). When we included their interaction, both factors were still significant ($p = 0.001$), explained 15.3 and 20.8% of the variation, and the interaction was significant ($p = 0.033$), explaining a further

16.2% of the variation (Table 2). We did not include HMA in these models, as it was not significant on its own as a factor at the "herd average" scale.

SIMPER analysis indicated that ten bacterial families explained the majority (0.595) of the average overall dissimilarity in microbial composition (Table A5). Of the ten families that contributed the most to differences between microbial communities, seven (Acidaminococcaceae, Anaerovoracaceae, Lachnospiraceae, Oscillospiraceae, Rikenellaceae, Saccharimonadaceae, and Spirochaetaceae) had significantly different abundances between summer and winter samples. Bacteria in the families Anaerovoracaceae, Lachnospiraceae, and Saccharimonadaceae were more prevalent in summer gut microbiomes while Acidaminococcaceae, Oscillospiraceae, Rikenellaceae, and Spirochaetaceae were more associated with winter microbiomes (Table A6; Figure A4). An additional 58 families also differed significantly ($p < 0.05$; 48 families) or were moderately significant ($p = 0.5$ to 0.10; 10 families) between the two seasons, but did not have as strong a contribution to overall seasonal differences (Table A6).

Our exploratory data analysis and visualizations (Table A3, Figure A3) revealed seasonal patterns in free-roaming horse diet composition that were similar to the microbial community, supporting a relationship between diet and microbial community composition. Mantel tests indicated that there was a positive correlation between the distance matrices of microbial and plant (diet) communities at the ASV level. As the diet communities of free-roaming horses became more dissimilar, so did the microbial communities. While the correlation was significant ($p = 0.001$), it was not strong ($r = 0.101$). However, it is still important to note this important relationship among diet and microbiome exists—essentially the two communities co-varied and horses with more similar diet composition also tended to have more similar microbiomes. When

we reduced both the plant community and microbial community to the family level, the correlation was marginally significant ($p = 0.093$) and less explanatory ($r = 0.037$). Interestingly, when we reduced the microbial community to a family level but considered the plant community at an ASV level, results were significant ($p = 0.001$); however, the correlation was still not as strong ($r = 0.074$) as when both communities were considered at the ASV level. Therefore, it is likely that functional relationships between diet and microbial communities were strongest at deeper taxonomic levels such as species level interactions, especially when considering the plants free-roaming horses were consuming.

Although relationships between microbial and plant taxa more strongly co-varied at the ASV level, we chose to evaluate specific taxa relationships at the Family level, due to the large numbers of bacterial ASVs as well as uncertainty of taxa assignment at deeper taxonomic levels in the diet data (see Buchanan et al. *in press* for more explanation of this). We found that nine (Acidaminococcaceae, Anaerovoracaceae, Lachnospiraceae, Oscillospiraceae, P-251-05, Prevotellaceae, Rikenellaceae, Saccharimonadaceae, and Spirochaetaceae) of the ten bacterial families that contributed most to microbial community differences were significantly correlated with at least one plant family in the diets of free-roaming horses (Table A7). The microbial families that exhibited relationships with the most plant families were Lachnospiraceae and Rikenellaceae (Table A7). Plant families that were significantly correlated with one or more of the ten selected bacterial families included Asteraceae, Berberidaceae, Brassicaceae, Cucurbitaceae, Chenopodiaceae, Cupressaceae, Cyperaceae, Ephedraceae, Fabaceae, Juncaceae, Juncaginaceae, Nyctaginaceae, Poaceae, Polygonaceae, Potamogetonaceae, Solanaceae, Tamaricaceae, and Zygophyllaceae. When evaluating the relationships between plant families and these selected bacterial families, we found that when bacterial families exhibited a

relationship with both graminoid (grass and grass-like plants [Cyperaceae, Juncaceae, and Poaceae families]) and forb or shrub families the relationships tended to exhibit opposite patterns in direction of correlation with graminoid families as compared to forb or shrub families. Bacterial families that exhibited negative relationships with graminoid families included Acidaminococcaceae, Oscillospiraceae, Prevotellaceae, and Rikenellaceae, while those exhibiting positive relationships included Anaerovoracaceae, Lachnospiraceae, and Saccharimonadaceae (Table A7). For more detailed results and interpretations of these correlations, see the Appendix and Table A7.

DISCUSSION

In support of our hypotheses, we found that season, HMA, ecoregion, and diet were all significant drivers of free-roaming horse gut microbiomes. These factors had a significant effect on microbial composition whether considered alone or in combination, and across HMA and range-wide scales. These results indicate that, although not always explanatory of a large amount of variation, these factors are important drivers of free-roaming horse microbial composition. The literature also provides evidence of the gut microbiome of horses and other equids being affected by season (Kobayashi et al. 2006; Salem et al. 2018; Theelen et al. 2021; Zaitseva et al. 2023), environment (Li et al. 2019b), or diet (Willing et al. 2009; Hansen et al. 2015; Li et al. 2019b; Kartzinel et al. 2019; Fernandes et al. 2021). One reason that we potentially did not explain large amounts of variation, especially in the models including all individual horses, is the large amount of individual variation in microbial composition that has been reported in other free-roaming equids (Antwis et al. 2018).

The effect of season was apparent at all scales but was more evident within a single HMA than at the range-wide scale, explaining 7 to 18% of the microbial variation within individual

HMAs but only around 3% of the microbial variation at the range-wide scale. Similarly, season explained a small but significant portion of variation in a study of domestic horses in the Netherlands that found it to account for 2.8% of the beta diversity variation in microbial communities (Theelen et al. 2021). Likewise, for mule deer in Utah, microbial communities were significantly different between December and March, but this only explained a small portion (R^2 = 0.02) of the variation in microbial communities (Eddington et al. 2022). We may have observed that more variation in the microbial community was explained by season at the HMA scale because environmental variation was minimized to the conditions of a single HMA. Certain microbial taxa may respond differently to seasonal fluctuations within variable conditions of different HMAs depending on the environmental conditions. Indeed, this was the case with seasonal dietary changes in free-roaming horses, the seasonal effect on dietary graminoid composition varied by HMA (Buchanan et al. *in press*).

Environment explained about three times more microbial community variation as compared to season at the range-wide scale. However, a significant interaction between these factors indicates that the effects of season and HMA are not mutually exclusive. The same was true when analyzing the marginal effect of ecoregion and season. Environment or location has been shown to be a driver of microbial composition in other species. Eddington et al. (2021) found an interaction of season and herd location in mule deer. Buchanan et al. (2024) revealed that study area explained about 6% of the variation in pronghorn gut microbiome and Trujillo et al. (2022) found that beta diversity among brown bears in Alaska was driven by location among three different parks, while season did not account for differences in beta diversity. In another equid species, Przewalski's horses, there were significant differences in microbial composition between two different nature reserves in China (Li et al. 2019b).

We could not model the marginal effect of HMA and ecoregion together, as these metrics were confounded, but when analyzed using separate PERMANOVA tests, HMA was more explanatory than ecoregion. There are multiple potential reasons for this. First, horses within an HMA are more likely to be closely related to one another than to horses within other HMAs. Evolutionary history can be a strong driver of microbial composition in wildlife, as shown among closely related species such as woodrats (Weinstein et al. 2021) or in sympatric species such as African megafauna (Kartzinel et al. 2019). Genetics can also drive microbial composition within a single species; for example, Li and colleagues found that some features of cattle rumen microbiomes were heritable (Li et al. 2019a) and Linnenbrink et al. found that in wild house mice (*Mus musculus*) the genetic clusters mice belonged to influenced microbial communities (Linnenbrink et al. 2013). Specifically in horses, variation in microbial composition between different breeds has been reported (Zhao et al. 2015; Wen et al. 2022). Because free-roaming horse herds have roots in different founding stock ranging from the original horses brought to North American by Spanish conquistadors to horses turned out or escaping various human endeavors such as ranching, mining, draft, or saddle mounts (McKnight 1959), free-roaming horses in different HMAs often exhibit different breed types. Though we did not investigate any associations between genetic relatedness of horses and their microbiomes, this is something that could be investigated in future studies. In addition, social structure can affect microbial transmission in horses, with horses that interact more closely having more similar microbiomes (Antwis et al. 2018). Because animals within the same HMA are more likely to interact, this could further explain why HMA was able to explain more of the microbial variation than ecoregion. The environmental conditions within an HMA are also likely to be more similar than those across an entire ecoregion, potentially leading to more similar microbial communities

within an HMA. Finally, there are also dispersal limitations to microbes: spatial location among groups of mammals has been shown to account for variation in microbial communities independent of dietary or phylogenetic differences (Moeller et al 2017). Geography was also the strongest predictor of microbial variation in wild mice populations in France and Germany (Linnenbrink et al. 2013) and spatial proximity of individual moose (*Alces alces*) in Minnesota was important in shaping their gut microbial communities (Fountain-Jones et al. 2020). Therefore, horses within an HMA being located in closer spatial proximity to one another compared to all horses within an ecoregion is another possible explanation for the stronger effect of HMA.

In our study, free-roaming horse diet co-varied with microbial community, suggesting a relationship between dietary composition and the gut microbial community. This was not surprising, as many aforementioned studies have indicated this link exists (but see Theelen et al. 2021, where researchers found no effect of diet on microbiome in horses). While significant, the relationship of diet and microbiome in our study only explained about 10% of the variation in the horse gut microbial community. This is similar to studies in other free-roaming species. For example, in closely related woodrat species, diet was a significant predictor of the microbial community explaining 16% of microbial variation (Weinstein et al. 2021). In African megafauna, correlations between microbial and diet dissimilarities were significant in 14 of 17 species studied with correlation values (*r*) ranging from 0.133 to 0.583 (Kartzinel et al. 2019). In this study, two equid species, donkeys (*E. asinus*) and Plain's zebras (*E. burchellii*), exhibited significant dietary and microbial correlations while in another equid, Grévy's zebra *(E. grevyi*), the relationship between dietary and microbial dissimilarity was not significant (Kartzinel et al. 2019). However, in studies of wild or free-ranging animals the effect of season, diet, and location

are often difficult to tease apart. In a study of pasture-raised horses in New Zealand, diet, season, and month all had significant effects on microbial communities, and authors suggest the temporal effects of season could be driven by changes in nutrient composition of pastures (Fernandes et al. 2021). While we found that diet composition co-varied with microbial composition, it is likely that the effects of season, location, and diet are all related.

Furthermore, we found that many of the specific bacterial families that contributed most to community dissimilarity in the free-roaming horse microbiome were correlated with particular plant families. This association is not surprising, as it is well known that many herbivores form relationships with bacteria to assist with degrading plant materials. Specifically, we identified Lachnospiraceae and Rikenellaceae as the microbial families associated with the greatest number of plant families. Lachnospiraceae are involved with fermenting plant complex polysaccharides and can break down fiber into short chain fatty acids (Zaplana et al. 2024). Members of the Rikenellaceae family have been identified as having a role in digestion of crude fiber (Huang et al. 2021) and the abundance of this bacterial family in the digestive tract has been shown to be affected by diet differences in cattle (Zened et al. 2013). These two families, in addition to others, may exhibit relationships with diet composition in free-roaming horses for multiple reasons. It is possible that these microbes proliferate in the gut due to a functional response to specific nutritional components of dietary constituents of select plant taxa. It is also possible that these bacteria are naturally present on the surface microbiomes of select plants, thus as the animal consumes these plants the relative abundance of these microbes passively increases. Moreover, recent research has indicated that the composition of an animal's gut microbiome may serve as a driver for diet selection (Trevelline and Kohl 2022). Better understanding of the cause and effect of these relationships would be a topic for future investigation with more specific

experimental trials, because with our observational design we were only able to identify correlation and not causation.

One limitation of our study is the lack of positive identification of all free-roaming horses that fecal material was collected from, leading to the possibility that the same animal was sampled in the summer and winter. However, to complete our study at this large scale, we made the assumption that our opportunistically collected samples represent a random independent sample of the free-roaming horse population in each HMA. We feel our sampling design of covering multiple areas within each HMA, coupled with the fact most HMAs had populations numbering in the hundreds and sometimes thousands of horses during our sampling periods (BLM 2020) reduced the chance that double sampling occurred. While we were able to suggest a method to address this mathematically during data analysis in a previous study that investigated the dietary proportion of graminoids and other forage plants from the same fecal collections (Buchanan et al. *in press*), addressing this limitation in high multidimensional space becomes challenging without reducing the dimensionality of the data. We decided that the loss of information from reducing dimensionality was a higher cost than accepting this limitation to our collection methods. Future studies could address this limitation by positively identifying every free-roaming horse as fecal material is collected to ensure there is no overlap in animals sampled. However, in some HMAs horses lack distinctive coloring or markings, making collections from individual animals infeasible without management interventions. Researchers could positively identify horses through tracking collared animals or applying an identifying feature such as a conspicuous brand or colored ear tag. While identification methods like this are commonly used in many wildlife species, free-roaming horses are a chimera of sorts that are simultaneously viewed as livestock, pets, or wildlife by different communities and interest groups (Hennig et al.

2023), making management decisions troublesome due to social and political factors (Scasta et al. 2018; Davies and Boyd 2019; Hennig et al. 2023). Indeed, collaring free-roaming horses in the United States is a contentious topic, and due to animal welfare concerns has only been completed in limited instances in recent decades (Hennig et al. 2020; Schoenecker et al. 2020; Schoenecker et al. 2024). Applying collars or other identifiers to horses would necessitate a gather, increasing costs, and requiring another activity that often receives public pushback (Scasta et al. 2018). Another option would be ensuring animal identities are unique through additional genetic tests of fecal material, although this would increase costs significantly. Opportunistic fecal sampling is not unique to our study. Other microbiome studies in free-roaming animals have described using opportunistic collections of fecal material for microbial study (Kartzinel et al. 2019, Perofsky et al. 2019). Overall, we contend that our assumption that samples represent a random independent sample of each HMA was realistic given the scope of our study; however, we point this limitation out in hopes that future researchers can address similar sampling issues.

In long-lived animals such as horses, the capacity of animal genetics to quickly adapt to changing conditions is limited. It has been suggested that microbial plasticity may allow for adaptation to changing conditions or climates because microbes have rapid generational times. Therefore, the genetic profile of an animal's microbial community can change within their lifetime, offering additional adaptive capacity. Various authors have previously proposed the idea of the microbiome as an adaptive trait to the host animal (Amato 2013; Alberdi et al. 2016; Bahrndorff et al. 2016; Trevelline et al. 2019). Indeed, by following individuals through the reintroduction process or in comparing captive raised animals to their post-reintroduction counterparts, researchers have shown that pandas (*Ailuropoda melanoleuca* ,Yao et al. 2019),

Przewalski's horses (Li et al. 2019b), and Tasmanian devils (*Sarcophilus harrisii*, Chong et al. 2019) all exhibited altered, more "wild-type" microbiomes after translocation as compared to captivity. For wild brown bears, microbial communities varied with changing diets throughout different seasons and habitats, likely helping bears adjust to changing resources throughout the year (Trujillo et al. 2022). In free-roaming horses, we observed variation in microbial communities within different HMAs, ecoregions, and seasons, and detected co-variation of diet and microbial communities indicating that the free-roaming horse microbiome is flexible and adaptive to differing conditions and dietary components. Free-roaming horses in North America have been shown to maintain desirable body condition across environments and seasons while consuming a variety of diets (Buchanan et al. *in press*) so it is possible that adaptive microbial communities could be a contributor that assists free-roaming horses to be successful in a variety of environments in the western United States.

IMPLICATIONS

Many species for which captive to wild relocations or wild-to-wild translocations are needed have experienced genetic bottlenecks in which a loss of genetic diversity is experienced due to a large population decline. While these species may not exhibit broad genetic diversity within the animal population to allow for adaptive capacity, it is possible that flexibility in the microbial community could help overcome some of these hurdles. Indeed, in Przewalski's horses, a species that once was reduced to 31 individuals, reintroduced animals in two reserves were shown to have differing microbiomes from conspecifics in the breeding center, revealing that these animals' microbiomes can adapt from a captive setting and diet to a wild one (Li et al. 2019b). Our findings demonstrate that the community composition of gut bacteria in free-roaming horses varied with season, ecoregion, HMA, and diet; therefore, it is possible that the ability of the

microbial community to adapt to environmental and seasonal changes and novel dietary resources is helping these animals to inhabit varying ecological conditions across the range. With studies including ours demonstrating variation of microbial communities in free-roaming animals, and the fact that many species worldwide are experiencing the effects of habitat loss, changing environments, or anthropogenic effects, the need for further research into the applications of microbial tools in wildlife management is more pressing than ever.

REFERENCES

Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M.L., Gilbert, M.T.P., 2016. Do vertebrate gut metagenomes confer rapid ecological adaptation? Trends Ecol. Evol. 31, 689–699. https://doi.org/10.1016/j.tree.2016.06.008

Amato, K.R., 2013. Co-evolution in context: The importance of studying gut microbiomes in wild animals. Microbiome Sci. Med. 1, 10–29. https://doi.org/10.2478/micsm-2013-0002

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26, 32–46. https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x

Antwis, R.E., Lea, J.M.D., Unwin, B., Shultz, S., 2018. Gut microbiome composition is associated with spatial structuring and social interactions in semi-feral Welsh Mountain ponies. Microbiome 6, 207. https://doi.org/10.1186/s40168-018-0593-2

Arbizu, P.M., 2020. pairwiseAdonis: Pairwise multilevel comparison using adonis. R package version 0.4. https://github.com/pmartinezarbizu/pairwiseAdonis

Archie, E.A., Tung, J., 2015. Social behavior and the microbiome. Curr. Opin. Behav. Sci. 6, 28–34. https://doi.org/10.1016/j.cobeha.2015.07.008

Bahrndorff, S., Alemu, T., Alemneh, T., Nielsen, J.L., 2016. The microbiome of animals: implications for conservation biology. Int. J. Genomics 2016, 5304028. https://doi.org/10.1155/2016/5304028

Beever, E.A., Brussard, P.F., 2000. Examining ecological consequences of feral horse grazing using exclosures. West. North Am. Nat. 60, 236–254. https://pubs.usgs.gov/publication/70170444

Beever, E.A., Herrick, J.E., 2006. Effects of feral horses in Great Basin landscapes on soils and ants: Direct and indirect mechanisms. J. Arid Environ. 66, 96–112. https://doi.org/10.1016/j.jaridenv.2005.11.006

Beever, E.A., Tausch, R.J., Thogmartin, W.E., 2008. Multi-scale responses of vegetation to removal of horse grazing from Great Basin (USA) mountain ranges. Plant Ecol. 196, 163–184. https://doi.org/10.1007/s11258-007-9342-5

Beever, E.A., Aldridge, C.L., 2011. Influences of free-roaming equids on sagebrush ecosystems, with a focus on Greater Sage-Grouse, in: Knick, S.T., Connelly, J.W. (Eds.), Greater Sage-Grouse: ecology and conservation of a landscape species and its habitats. Studies in Avian Biology (vol. 38), University of California Press, Berkeley, CA pp. 272–290. https://doi.org/10.1525/california/9780520267114.003.0015

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B. 57, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bergmann, G.T., Craine, J.M., Robeson, M.S., Fierer, N., 2015. Seasonal shifts in diet and gut microbiota of the American bison (*Bison bison*). PLoS One 10, 1–14. https://doi.org/10.1371/journal.pone.0142409

Bisanz JE. Qiime2R: Tutorial: Integrating QIIME2 and R for data visualization and analysis using qiime2R (v0.99.6) 2018. v0.99.6. Accessed at https://github.com/jbisanz/qiime2R

Bletz, M.C., Loudon, A.H., Becker, M.H., Bell, S.C., Woodhams, D.C., Minbiole, K.P.C., Harris, R.N., 2013. Mitigating amphibian chytridiomycosis with bioaugmentation: Characteristics of effective probiotics and strategies for their selection and use. Ecol. Lett. 16, 807–820. https://doi.org/10.1111/ele.12099

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K. Bin, Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A. V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R.,

Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9

Buchanan, C.E., Galla, S.J., Muscarella, M.E., Forbey, J.S., Reinking, A.K., Beck, J.L., 2024. Relating gut microbiome composition and life history metrics for pronghorn (*Antilocapra americana*) in the Red Desert, Wyoming. PLoS One 19, e0306722. https://doi.org/10.1371/journal.pone.0306722

Buchanan, C.E., Galla, S.J., Studyvin J., Lautenbach, J.D., Scasta, J.D., Randolph G.D., Forbey, J.S., Beck, J.L. Free-roaming horse diet and body condition differences across seasons and ecologically diverse herd management areas. Rangel. Ecol. Manag. *In press*.

Bureau of Land Management, 2020. Herd Area and Herd Management Area Statistics 2020. https://www.blm.gov/sites/blm.gov/files/wildhorse_2020_HAHMA_Stats.pdf

Bureau of Land Management, 2025. Herd Area and Herd Management Area Statistics 2025. https://www.blm.gov/sites/default/files/docs/2025-03/2025_Wild_Horse_and_Burro_Population_Estimates.pdf (accessed: 3 April 2025)

Bureau of Land Management, undated. Herd Management Areas https://www.blm.gov/programs/wild-horse-and-burro/herd-management/herd-management-areas/ (accessed: 26 October 2020)

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869Callahan et al 2016

Cheng, T.L., Mayberry, H., McGuire, L.P., Hoyt, J.R., Langwig, K.E., Nguyen, H., Parise, K.L., Foster, J.T., Willis, C.K.R., Kilpatrick, A.M., Frick, W.F., 2017. Efficacy of a probiotic bacterium to treat bats affected by the disease white-nose syndrome. J. Appl. Ecol. 54, 701–708. https://doi.org/10.1111/1365-2664.12757

Chevalier, C., Stojanović, O., Colin, D.J., Suarez-Zamorano, N., Tarallo, V., Veyrat-Durebex, C., Rigo, D., Fabbiano, S., Stevanović, A., Hagemann, S., Montet, X., Seimbille, Y., Zamboni, N., Hapfelmeier, S., Trajkovski, M., 2015. Gut microbiota orchestrates energy homeostasis during cold. Cell 163, 1360–1374. https://doi.org/10.1016/j.cell.2015.11.004

Chong, R., Grueber, C.E., Fox, S., Wise, P., Barrs, V.R., Hogg, C.J., Belov, K., 2019. Looking like the locals - gut microbiome changes post-release in an endangered species. Anim. Microbiome 1, 1–10. https://doi.org/10.1186/s42523-019-0012-4

Clarke, K.R. 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18, 117–143. https://doi.org/10.1111/j.1442-9993.1993.tb00438.x

Czernik, M., Taberlet, P., Świsłocka, M., Czajkowska, M., Duda, N., Ratkiewicz, M., 2013. Fast and efficient DNA-based method for winter diet analysis from stools of three cervids: Moose, red deer, and roe deer. Acta Theriol. (Warsz). 58, 379–386. https://doi.org/10.1007/s13364-013-0146-9

Davies, K.W., Collins, G., Boyd, C.S., 2014. Effects of feral free-roaming horses on semi-arid rangeland ecosystems: An example from the sagebrush steppe. Ecosphere 5. https://doi.org/10.1890/ES14-00171.1

Davies, K.W., Boyd, C.S., 2019. Ecological effects of free-roaming horses in North American rangelands. Bioscience 69, 558–565. https://doi.org/10.1093/biosci/biz060

Duncan, P., Foose, T.J., Gordon, I.J., Gakahu, C.G., Lloyd, M., 1990. Comparative nutrient extraction from forages by grazing bovids and equids: a test of the nutritional model of equid/bovid competition and coexistence. Oecologia 84, 411–418. https://doi.org/10.1007/BF00329768

Eddington, H.S., Carroll, C., Larsen, R.T., McMillan, B.R., Chaston, J.M., 2021. Spatiotemporal variation in the fecal microbiota of mule deer is associated with proximate and future measures of host health. BMC Vet. Res. 17, 1–8. https://doi.org/10.1186/s12917-021-02972-0

Environmental Protection Agency, 2024. Level III and IV ecoregions of the continental United States: Corvallis, Oregon, U.S. EPA National Health and Environmental Effects research Laboratory. Accessed: 18 April 2024. Available from: https://www.epa.gov/eco-research/level-iii-and-iv-ecoregions-continental-united-states

Fernandes, K.A., Gee, E.K., Rogers, C.W., Kittelmann, S., Biggs, P.J., Bermingham, E.N., Bolwell, C.F., Thomas, D.G., 2021. Seasonal variation in the faecal microbiota of mature adult horses maintained on pasture in New Zealand. Animals 11, 1–21. https://doi.org/10.3390/ani11082300

Fountain-Jones, N.M., Clark, N.J., Kinsley, A.C., Carstensen, M., Forester, J., Johnson, T.J., Miller, E.A., Moore, S., Wolf, T.M., Craft, M.E., 2020. Microbial associations and spatial proximity predict North American moose (*Alces alces*) gastrointestinal community composition. J. Anim. Ecol. 89, 817–828. https://doi.org/10.1111/1365-2656.13154

Garrott, R.A., Siniff, D.B., Eberhardt, L.L., 1991. Growth rates of feral horse populations. J. Wildl. Manage. 55, 641–648. https://www.jstor.org/stable/3809513

Gooch, A.M.J., Petersen, S.L., Collins, G.H., Smith, T.S., McMillan, B.R., Eggett, D.L., 2017. The impact of feral horses on pronghorn behavior at water sources. J. Arid Environ. 138, 38–43. https://doi.org/10.1016/j.jaridenv.2016.11.012

Haines, F., 1938a.The northward spread of horses among the plains indians. American Anthropologist 40, 429–437. www.jstor.org/stable/662040.

Haines, F., 1938b. Where did the Plains Indians get their horses? American Anthropologist 40, 112–117. www.jstor.org/stable/661795.

Hall, L.K., Larsen, R.T., Westover, M.D., Day, C.C., Knight, R.N., McMillan, B.R., 2016. Influence of exotic horses on the use of water by communities of native wildlife in a semi-arid environment. J. Arid Environ. 127, 100–105. https://doi.org/10.1016/j.jaridenv.2015.11.008

Hanley, T.A., 1982. The nutritional basis for food selection by ungulates. J. Range Manag. 35, 146–151. https://www.jstor.org/stable/3898379?origin=crossref&seq=1

Hansen, N.C.K., Avershina, E., Mydland, L.T., Næsset, J.A., Austbø, D., Moen, B., Måge, I., Rudi, K., 2015. High nutrient availability reduces the diversity and stability of the equine caecal microbiota. Microb. Ecol. Heal. Dis. 26, 1–8. https://doi.org/10.3402/mehd.v26.27216

Harrell, F.E., 2023. Hmisc: Harrell Miscellaneous. R package version 5.1-1, https:// https://cran.r-project.org/web/packages/Hmisc/index.html

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Janssen, P.H., Abecia, L., Angarita, E., Aravena, P., Arenas, G.N., Ariza, C., Attwood, G.T., Avila, J.M., Avila-Stagno, J., Bannink, A., Barahona, R., Batistotti, M., Bertelsen, M.F., Brown-Kav, A., Carvajal, A.M., Cersosimo, L., Chaves, A.V., Church, J., Clipson, N., Cobos-Peralta, M.A., Cookson, A.L., Cravero, S., Carballo, O.C., Crosley, K., Cruz, G., Cucchi, M.C., De La Barra, R., De Menezes, A.B., Detmann, E., Dieho, K., Dijkstra, J., Dos Reis, W.L.S., Dugan, M.E.R., Ebrahimi, S.H., Eythórsdóttir, E., Fon, F.N., Fraga, M., Franco, F., Friedeman, C., Fukuma, N., Gagić, D., Gangnat, I., Grilli, D.J., Guan, L.L., Miri, V.H., Hernandez-Sanabria, E., Gomez, A.X.I., Isah, O.A., Ishaq, S., Jami, E., Jelincic, J., Kantanen, J., Kelly, W.J., Kim, S.H., Klieve, A., Kobayashi, Y., Koike, S., Kopecny, J., Kristensen, T.N., Krizsan, S.J., LaChance, H., Lachman, M., Lamberson, W.R., Lambie, S., Lassen, J., Leahy, S.C., Lee, S.S., Leiber, F., Lewis, E., Lin, B., Lira, R., Lund, P., Macipe, E., Mamuad, L.L., Mantovani, H.C., Marcoppido, G.A., Márquez, C., Martin, C., Martinez, G., Martinez, M.E., Mayorga, O.L., McAllister, T.A., McSweeney, C., Mestre, L., Minnee, E., Mitsumori, M., Mizrahi, I., Molina, I., Muenger, A., Munoz, C., Murovec, B., Newbold, J., Nsereko, V., O'Donovan, M., Okunade, S., O'Neill, B., Ospina, S., Ouwerkerk, D., Parra, D., Pereira, L.G.R., Pinares-Patino, C., Pope, P.B., Poulsen, M., Rodehutscord, M., Rodriguez, T., Saito, K., Sales, F., Sauer, C., Shingfield, K., Shoji, N.,

Simunek, J., Stojanović-Radić, Z., Stres, B., Sun, X., Swartz, J., Tan, Z.L., Tapio, I., Taxis, T.M., Tomkins, N., Ungerfeld, E., Valizadeh, R., Van Adrichem, P., Van Hamme, J., Van Hoven, W., Waghorn, G., Wallace, R.J., Wang, M., Waters, S.M., Keogh, K., Witzig, M., Wright, A.D.G., Yamano, H., Yan, T., Yanez-Ruiz, D.R., Yeoman, C.J., Zambrano, R., Zeitz, J., Zhou, M., Zhou, H.W., Zou, C.X., Zunino, P., 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Sci. Rep. 5. https://doi.org/10.1038/srep14567

Hennig, J.D., Scasta, J.D., Beck, J.L., Schoenecker, K.A., King, S.R.B., 2020. Systematic review of equids and telemetry collars: Implications for deployment and reporting. Wildl. Res. 47, 361–371. https://doi.org/10.1071/WR19229

Hennig, J.D., Beck, J.L., Duchardt, C.J., Derek Scasta, J., 2021a. Variation in sage-grouse habitat quality metrics across a gradient of feral horse use. J. Arid Environ. 192, 104550. https://doi.org/10.1016/j.jaridenv.2021.104550

Hennig, J.D., Beck, J.L., Gray, C.J., Scasta, J.D., 2021b. Temporal overlap among feral horses, cattle, and native ungulates at water sources. J. Wildl. Manage. 85, 1084–1090. https://doi.org/10.1002/jwmg.21959

Hennig, J.D., Duchardt, C.J., Esmaeili, S., Fuhlendorf, S.D., Beck, J.L., Francisco, T.I., Scasta, J.D., 2023. A crossroads in the rearview mirror: the state of United States feral equid management in 2023. Biosci. 73, 404–407. https://doi.org/10.1093/biosci/biad033

Huang, C., Ge, F., Yao, X., Guo, X., Bao, P., Ma, X., Wu, X., Chu, M., Yan, P., Liang, C., 2021. Microbiome and metabolomics reveal the effects of different feeding systems on the growth and

ruminal development of yaks. Front. Microbiol. 12:682989. https://doi.org/10.3389/fmicb.2021.682989

Janis, C., 1976. The evolutionary strategy of the Equidae and the origins of rumen and cecal digestion. Evolution. 30, 757-774. https://doi.org/10.2307/2407816

Kartzinel, T.R., Hsing, J.C., Musili, P.M., Brown, B.R.P., Pringle, R.M., 2019. Covariation of diet and gut microbiome in African megafauna. Proc. Natl. Acad. Sci. U. S. A. 116, 23588–23593. https://doi.org/10.1073/pnas.1905666116

King, S.R.B., Schoenecker, K.A., Fike, J.A., Oyler-McCance, S.J., 2018. Long-term persistence of horse fecal DNA in the environment makes equids particularly good candidates for noninvasive sampling. Ecol. Evol. 8, 4053–4064. https://doi.org/10.1002/ece3.3956

Kobayashi, Y., Koike, S., Miyaji, M., Hata, H., Tanaka, K., 2006. Hindgut microbes, fermentation and their seasonal variations in Hokkaido native horses compared to light horses. Ecol. Res. 21, 285–291. https://doi.org/10.1007/s11284-005-0118-x

Kohl, K.D., Weiss, R.B., Cox, J., Dale, C., Dearing, M.D., 2014. Gut microbes of mammalian herbivores facilitate intake of plant toxins. Ecol. Lett. 17, 1238–1246. https://doi.org/10.1111/ele.12329

Ley, R.E., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R., Bircher, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R., Gordon, J.I., 2008. Evolution of mammals and their gut microbes. Science 320, 1647–1651. https://doi.org/10.1126/science.1155725

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., Fitzsimmons, C., Plastow, G., Guan, L.L., 2019a. Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. Microbiome 7, 1–17. https://doi.org/10.1186/s40168-019-0699-1

Li, Y., Zhang, K., Liu, Y., Li, K., Hu, D., Wronski, T., 2019b. Community composition and diversity of intestinal microbiota in captive and reintroduced Przewalski's horse (*Equus ferus przewalskii*). Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.01821

Linnenbrink, M., Wang, J., Hardouin, E.A., Künzel, S., Metzler, D., Baines, J.F., 2013. The role of biogeography in shaping diversity of the intestinal microbiota in house mice. Mol. Ecol. 22, 1904–1916. https://doi.org/10.1111/mec.12206

McArdle, B.H., Anderson, M.J., 2016. Fitting multivariate models to community data: A comment on distance-based redundancy analysis. Ecology 82, 290–297. https://doi.org/10.2307/2680104

McCann, J.C., Wiley, L.M., Forbes, T.D., Rouquette, F.M., Tedeschi, L.O., 2014. Relationship between the rumen microbiome and residual feed intake-efficiency of brahman bulls stocked on bermudagrass pastures. PLoS One 9, 1–6. https://doi.org/10.1371/journal.pone.0091864

McKenzie, V.J., Song, S.J., Delsuc, F., Prest, T.L., Oliverio, A.M., Korpita, T.M., Alexiev, A., Amato, K.R., Metcalf, J.L., Kowalewski, M., Avenant, N.L., Link, A., Di Fiore, A., Seguin-Orlando, A., Feh, C., Orlando, L., Mendelson, J.R., Sanders, J., Knight, R., 2017. The effects of captivity on the mammalian gut microbiome. Integr. Comp. Biol. 57, 690–704. https://doi.org/10.1093/icb/icx090

McKenzie, V.J., Kueneman, J.G., Harris, R.N., 2018. Probiotics as a tool for disease mitigation in wildlife: Insights from food production and medicine. Ann. N. Y. Acad. Sci. 1429, 18–30. https://doi.org/10.1111/nyas.13617

McKnight, T.L., 1959. The feral horse in Anglo-America. Georg. Rev. 49, 506–525. https://www.jstor.org/stable/212210

McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217

Menard, C., Duncan, P., Fleurance, G., Georges, J.Y., Lila, M., 2002. Comparative foraging and nutrition of horses and cattle in European wetlands. J. Appl. Ecol. 39, 120–133. https://doi.org/10.1046/j.1365-2664.2002.00693.x

Metcalf, J.L., Song, S.J., Morton, J.T., Weiss, S., Seguin-Orlando, A., Joly, F., Feh, C., Taberlet, P., Coissac, E., Amir, A., Willerslev, E., Knight, R., McKenzie, V., Orlando, L., 2017. Evaluating the impact of domestication and captivity on the horse gut microbiome. Sci. Reps. 7, 1–9. https://doi.org/10.1038/s41598-017-15375-9

Moeller, A.H., Suzuki, T.A., Lin, D., Lacey, E.A., Wasser, S.K., Nachman, M.W., 2017. Dispersal limitation promotes the diversification of the mammalian gut microbiota. Proc. Natl. Acad. Sci. U. S. A. 114, 13768–13773. https://doi.org/10.1073/pnas.1700122114

Muegge, B.D., Kuczynski, J., Knights, D., Clemente, J.C., González, A., Fontana, L., Henrissat, B., Knight, R., Gordon, J.I., 2011. Diet drives convergence in gut microbiome functions across

mammalian phylogeny and within humans. Science 332, 970–974. https://doi.org/10.1126/science.1198719

Muñoz, D.A., Coates, P.S., Ricca, M.A., 2021. Free-roaming horses disrupt greater sage-grouse lekking activity in the Great Basin. J. Arid Environ.184, 104304. https://doi.org/10.1016/j.jaridenv.2020.104304

Myer, P.R., Smith, T.P.L., Wells, J.E., Kuehn, L.A., Freetly, H.C., 2015. Rumen microbiome from steers differing in feed efficiency. PLoS One 10. https://doi.org/10.1371/journal.pone.0129174

Nielsen, D.P., Harrison, J.G., Byer, N.W., Faske, T.M., Parchman, T.L., Simison, W.B., Matocq, M.D., 2023. The gut microbiome reflects ancestry despite dietary shifts across a hybrid zone. Ecol. Lett. 26, 63–75. https://doi.org/10.1111/ele.14135

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H.B.A., FitzJohn, R., Friendly, M., Furneaux, B, Hannigan, G., Hill, M.O., Lahti, L., McGlinn, D., Ouellette, M., Ribeiro Cunha, E., Smith, T., Stier, A., Ter Braak, C.J.F., Weedon, J., 2022. Vegan: Community Ecology Package. R package version 2.6-4, https://cran.r-project.org/web/packages/vegan/vegan.pdf.

Ostermann-Kelm, S., Atwill, E.R., Rubin, E.S., Jorgensen, M.C., Boyce, W.M., 2008. Interactions between feral horses and desert bighorn sheep at water. J. Mammal. 89, 459–466. https://doi.org/10.1644/07-mamm-a-075r1.1

Perofsky, A.C., Lewis, R.J., Meyers, L.A., 2019. Terrestriality and bacterial transfer: a comparative study of gut microbiomes in sympatric Malagasy mammals. ISME J. 13, 50–63. https://doi.org/10.1038/s41396-018-0251-5

Perry, N.D., Morey, P., Miguel, G.S., 2015. Dominance of a natural water source by feral horses. Southwest. Nat. 60, 390–393. https://doi.org/10.1894/0038-4909-60.4.390

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 41, 590–596. https://doi.org/10.1093/nar/gks1219

R Core Team (2023). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Salem, S.E., Maddox, T.W., Berg, A., Antczak, P., Ketley, J.M., Williams, N.J., Archer, D.C., 2018. Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period. Sci. Rep. 8, 1–10. https://doi.org/10.1038/s41598-018-26930-3

Salgado-Flores, A., Tveit, A.T., Wright, A.D., Pope, P.B., Sundset, M.A., 2019. Characterization of the cecum microbiome from wild and captive rock ptarmigans indigenous to Arctic Norway. PloS One, 14, e0213503. https://doi.org/10.1371/journal.pone.0213503

Scasta, J.D, Hennig, J.D, Beck, J.L, 2018. Framing contemporary U.S. wild horse and burro management processes in a dynamic ecological, sociological, and political environment. Human-Wildlife Interact. 12, 31–45. https://doi.org/10.26077/2fhw-fz24

Schoenecker, K.A., King, S.R.B., Collins, G.H., 2020. Evaluation of the impacts of radio-marking devices on feral horses and burros in a captive setting. Human-Wildlife Interact. 14, 73–86. https://doi.org/10.26077/127m-4x33

Schoenecker, K.A., King, S.R.B., Hennig, J.D., Cole, M.J., Scasta, J.D., Beck, J.L., 2024. Effects of telemetry collars on two free-roaming feral equid species. PLoS One 19, e0303312. https://doi.org/10.1371/journal.pone.0303312

Shabat, S.K., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M.E., White, B.A., Shterzer, N., Mizrahi, I., 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME J. 10, 2958–2972. https://doi.org/10.1038/ismej.2016.62

Sommer, F., Ståhlman, M., Ilkayeva, O., Arnemo, J.M., Kindberg, J., Josefsson, J., Newgard, C.B., Fröbert, O., Bäckhed, F., 2016. The gut microbiota modulates energy metabolism in the hibernating brown bear *Ursus arctos*. Cell Rep. 14, 1655–1661. https://doi.org/10.1016/j.celrep.2016.01.026

Song, S.J., Lauber, C., Costello, E.K., Lozupone, C.A., Humphrey, G., Berg-Lyons, D., Gregory Caporaso, J., Knights, D., Clemente, J.C., Nakielny, S., Gordon, J.I., Fierer, N., Knight, R., 2013. Cohabiting family members share microbiota with one another and with their dogs. Elife 2013, 1–22. https://doi.org/10.7554/eLife.00458

Stedman, A., van Vliet, A.H.M., A. Chambers, M., Gutierrez-Merino, J., 2020. Gut commensal bacteria show beneficial properties as wildlife probiotics. Ann. N. Y. Acad. Sci. 1467, 112–132. https://doi.org/10.1111/nyas.14302

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T., Corthier, G., Brochmann, C., Willerslev, E., 2007. Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. Nucleic Acids Res. 35, e14. https://doi.org/10.1093/nar/gkl938

Theelen, M.J.P., Luiken, R.E.C., Wagenaar, J.A., Sloet van Oldruitenborgh-Oosterbaan, M.M., Rossen, J.W.A., Zomer, A.L., 2021. The equine faecal microbiota of healthy horses and ponies in the Netherlands: Impact of host and environmental factors. Animals 11, 1–17. https://doi.org/10.3390/ani11061762

Trevelline, B.K., Fontaine, S.S., Hartup, B.K., Kohl, K.D., 2019. Conservation biology needs a microbial renaissance: A call for the consideration of host-associated microbiota in wildlife management practices. Proc. R. Soc. B Biol. Sci. 286. https://doi.org/10.1098/rspb.2018.2448

Trevelline, B.K, Kohl, K.D., 2022. The gut microbiome influences host diet selection behavior, Proc. Natl. Acad. Sci. U.S.A. 119, e2117537119. https://doi.org/10.1073/pnas.2117537119

Trujillo, S.M., McKenney, E.A., Hilderbrand, G. V., Mangipane, L.S., Rogers, M.C., Joly, K., Gustine, D.D., Erlenbach, J.A., Mangipane, B.A., Lafferty, D.J.R., 2022. Intrinsic and extrinsic factors influence on an omnivore's gut microbiome. PLoS One 17, 1–19. https://doi.org/10.1371/journal.pone.0266698

Tung, J., Barreiro, L.B., Burns, M.B., Grenier, J.C., Lynch, J., Grieneisen, L.E., Altmann, J., Alberts, S.C., Blekhman, R., Archie, E.A., 2015. Social networks predict gut microbiome composition in wild baboons. Elife 2015, 1–18. https://doi.org/10.7554/eLife.05224

Van Soest, P.J., 1994. Nutritional Ecology of the Ruminant, 2nd ed. Ithaca, New York: Cornell University Press.

Vlčková, K., Mrázek, J., Kopečný, J., Petrželková, K.J., 2012. Evaluation of different storage methods to characterize the fecal bacterial communities of captive western lowland gorillas (*Gorilla gorilla gorilla*). J. Microbiol. Methods 91, 45–51. https://doi.org/10.1016/j.mimet.2012.07.015

Walters, W., Hyde, E.R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., Gilbert, J.A., Jansson, J.K., Caporaso, J.G., Fuhrman, J.A., Apprill, A., Knight, R., 2016. Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. mSystems 1, e00009-15. https://doi.org/10.1128/msystems.00009-15

Wen, X., Luo, S., Lv, D., Jia, C., Zhou, X., Zhai, Q., Xi, L., Yang, C., 2022. Variations in the fecal microbiota and their functions of Thoroughbred, Mongolian, and Hybrid horses. Front. Vet. Sci. 9. https://doi.org/10.3389/fvets.2022.920080

Weinstein, S.B., Martinez-Mota, R., Stapleton, T.E., Klure, D.M., Greenhalgh, R., Orr, T.J., Dale, C., Kohl, K.D., Denise Dearing, M., 2021. Microbiome stability and structure is governed by host phylogeny over diet and geography in woodrats (*Neotoma spp*.). Proc. Natl. Acad. Sci. U. S. A. 118. https://doi.org/10.1073/pnas.2108787118

Willing, B., Vörös, A., Roos, S., Jones, C., Jansson, A., Lindberg, J.E., 2009. Changes in faecal bacteria associated with concentrate and forage-only diets fed to horses in training. Equine Vet. J. 41, 908–914. https://doi.org/10.2746/042516409X447806

Yao, R., Xu, L., Hu, T., Chen, H., Qi, D., Gu, X., Yang, X., Yang, Z., Zhu, L., 2019. The "wildness" of the giant panda gut microbiome and its relevance to effective translocation. Glob. Ecol. Conserv. 18, e00644. https://doi.org/10.1016/j.gecco.2019.e00644

Zaplana, T., Miele, S., Tolonen, A.C., 2024. *Lachnospiraceae* are emerging industrial biocatalysts and biotherapeutics. Front Bioeng. Biotechnol. 11:1324396. https://doi.org/10.3389/fbioe.2023.1324396.

Zaitseva, S., Dagurova, O., Radnagurueva, A., Kozlova, A., Izotova, A., Krylova, A., Noskov, S., Begmatov, S., Patutina, E., Barkhutova, D.D., 2023. Fecal microbiota and diet composition of Buryatian horses grazing warm- and cold-season grass pastures. Microorganisms 11, 1–14. https://doi.org/10.3390/microorganisms11081947

Zened, A., Combes, S., Cauquil, L., Mariette, J., Klopp, C., Bouchez, O., Troegeler-Meynadier, A., Enjalbert, F., 2013. Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. FEMS Microbiol. Ecol. 83, 504–514. https://doi.org/10.1111/1574-6941.12011

Zhao, Y., Li, B., Bai, D., Huang, J., Shiraigo, W., Yang, L., Zhao, Q., Ren, X., Wu, J., Bao, W., Dugarjaviin, M., 2016. Comparison of fecal microbiota of Mongolian and Thoroughbred horses by high-throughput sequencing of the V4 region of the 16S rRNA gene. Asian-Australas J. Anim. Sci. 29, 1345-1352. https://doi.org/10.5713/ajas.15.0587.

TABLES

Table 1. Single factor PERMAVOAS evaluating the effect of season, ecoregion, and HMA on microbial community variation. The top panel evaluates the effects at range scale with all 465 individuals included as the experimental unit. The bottom panel shows the "herd average" scale including 16 HMA average microbial communities in the summer and 15 HMAs in the winter. Factors with significant p-values are indicated in **bold.**

Using all 465 fecal samples				
	F	df (total)	R^2	p
Season	**16.733**	**464**	**0.035**	**0.001**
HMA	**3.134**	**464**	**0.095**	**0.001**
EPA Ecoregion	**4.219**	**464**	**0.044**	**0.001**
Using HMA average microbial composition				
Season	**5.242**	**30**	**0.153**	**0.001**
Ecoregion	**1.2722**	**30**	**0.203**	**0.044**
HMA	0.883	30	0.469	0.887

Table 2. PERMANOVA multifactor models evaluating the effects of season, ecoregion, and HMA on the variation of microbial communities. Multifactor models without interactions evaluated the marginal effect of included factors. Results are shown for the range-wide scale models incorporating all individual samples and for "herd average" microbial composition models.

All 465 Samples				
Models before interactions				
Term	F	df	R^2	p
Season	17.187	1	0.033	0.001
HMA	3.198	15	0.093	0.001
Residual		448		
Total		464		
Season	17.942	1	0.036	0.001
Ecoregion	4.490	5	0.045	0.001
Residual		458		
Total		464		
Models with interactions				
Term	F	df	R^2	p
Season	18.881	1	0.035	0.001
HMA	3.368	15	0.093	0.001
Season x HMA	2.708	14	0.070	0.001
Residual		434		
Total		464		
Season	17.780	1	0.035	0.001
Ecoregion	4.598	5	0.045	0.001
Season x Ecoregion	3.198	5	0.031	0.001
Residual		453		
Total		464		
HMA "Herd Average" Models				
Model before interactions				
Term	F	df	R^2	p
Season	5.941	1	0.158	0.001
Ecoregion	1.562	5	0.208	0.002
Residual		24		
Total		30		
Model with interactions				
Term	F	df	R^2	p
Season	6.092	1	0.153	0.001
Ecoregion	1.655	5	0.208	0.001
Season x Ecoregion	1.286	5	0.162	0.033
Residual		19		
Total		30		

Table 3. Results of PERMANOVA tests on the effect of season on microbial community within each HMA. All comparisons were made with 15 summer and 15 winter fecal collections and have 29 degrees of freedom.

HMA	R^2	F	p
Adobe Town	0.148	4.855	0.001
Conger	0.070	2.102	0.001
Frisco	0.143	4.663	0.001
Green Mountain	0.111	3.503	0.001
Little Book Cliffs	0.074	2.227	0.001
McCullough Peaks	0.088	2.688	0.001
North Hills	0.095	2.934	0.001
Onaqui	0.072	2.175	0.001
Palomino Buttes	0.163	5.469	0.001
Pine Nuts	0.115	3.637	0.001
Red Rock	0.155	5.130	0.001
Saylor Creek	0.147	4.833	0.001
South Steens	0.181	6.201	0.001
Stinkingwater	0.145	4.756	0.001
Twin Peaks	0.076	2.309	0.001

FIGURES

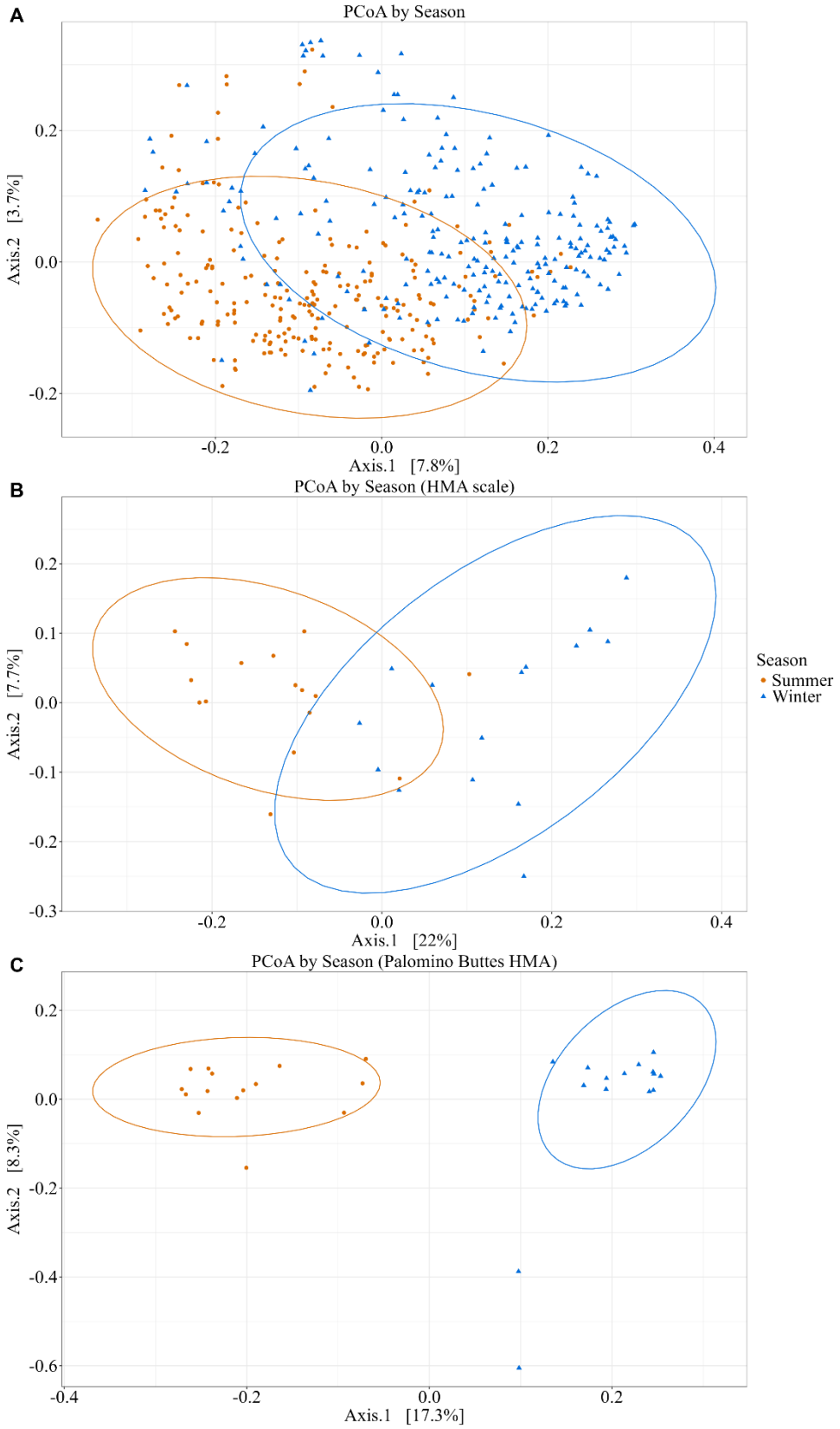
A
PCoA by Season
Axis.2 [3.7%]
Axis.1 [7.8%]
B
PCoA by Season (HMA scale)
Axis.2 [7.7%]
Axis.1 [22%]
Season
Summer
Winter
C
PCoA by Season (Palomino Buttes HMA)
Axis.2 [8.3%]
Axis.1 [17.3%]

Figure 1. PCoA ordinations using Bray–Curtis dissimilarity measure of seasonal free-roaming horse microbiome community composition at various scales. Data represents the microbial community transformed to relative abundance with rare taxa removed and ellipses delineating 95% confidence intervals. A) Separation of microbial communities by season when considering all individual horses at the range-wide scale. B) Separation of microbial communities by season at the range-wide scale when considering the summer and winter average microbial composition of each HMA as the experimental unit. C) Example from one HMA of the separation of microbial communities by season at the HMA scale in Palomino Buttes HMA, OR.

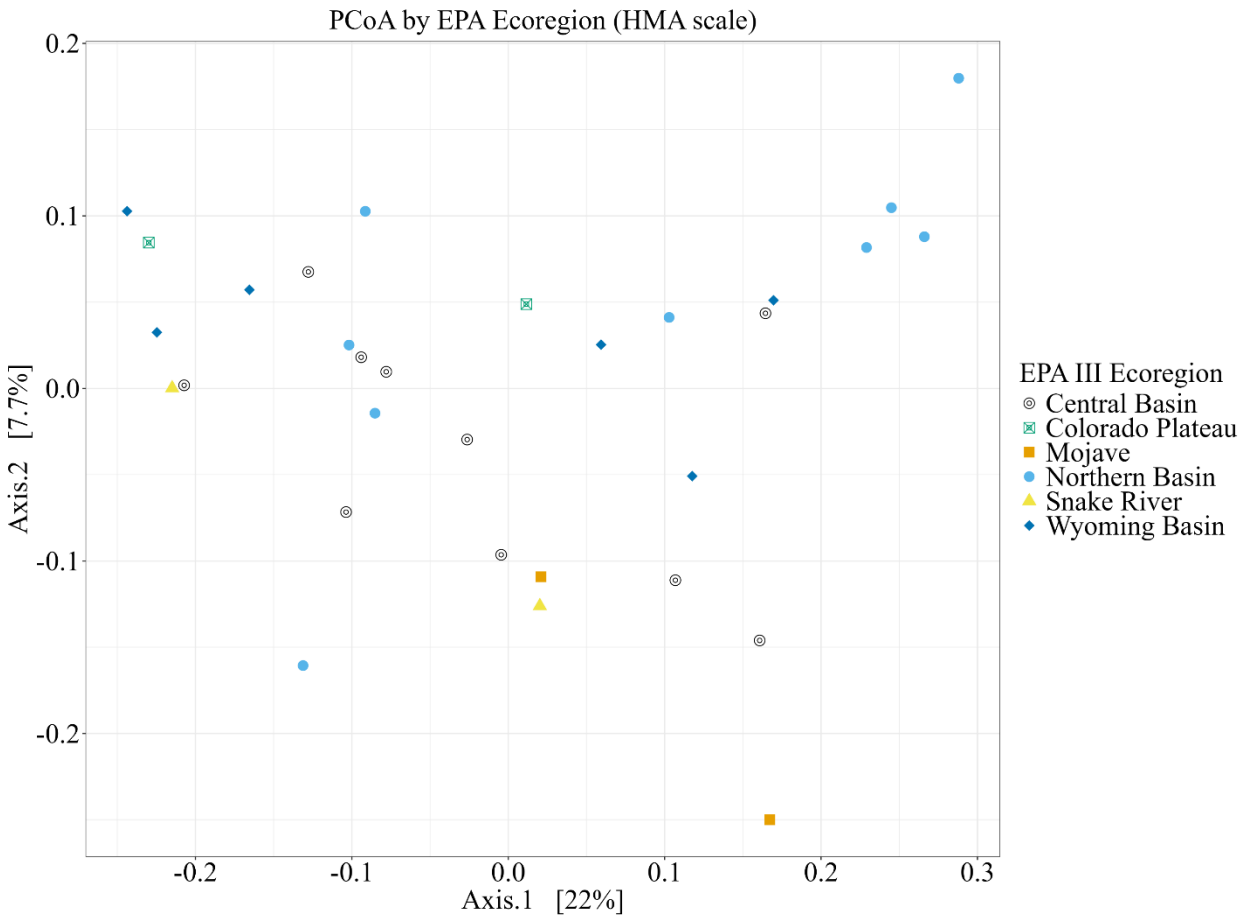

Figure 2. PCoA ordinations using Bray–Curtis dissimilarity measure of free-roaming horse microbiome community composition by ecoregion. Data represents microbial community transformed to relative abundance with rare taxa removed. Separation of microbial communities by ecoregion is depicted at the range-wide scale with each data point representing the summer (2020) or winter (2020/2021) average microbial composition of each HMA as the experimental unit. Shapes and colors represent the ecoregion of each HMA.

APPENDIX

Table A1. Read count remaining throughout the various steps in the DADA 2 pipeline. The initial mean read counts are shown as well as mean read counts after filtering, de-noising, merging, and removing chimeras.

	Initial	After filter	De-noised	Merged	Non-Chimeric
Total read count	548,176,831	474,775,056	465,602,318	413,653,483	121,920,006
Mean read count	515,204	446,217	437,596	388,772	114,586
Percentage	100%	86.61%	84.94%	75.46%	22.24%

Table A2: Taxonomic depth and breadth of read assignments.

	Total Members Identified	Percent reads assigned to depth
Phylum	21	99.734%
Class	47	99.531%
Order	100	98.702%
Family	158	96.673%
Genus	287	80.872%
Species	382	52.300%

Table A3. PERMAVOAS evaluating the effect of season on diet community variation. The upper rows evaluate the effect of season on diet composition within each HMA. All HMA level comparisons were made with 15 summer and 15 winter fecal collections. The next test evaluates the range-wide scale with all 465 individuals as the experimental units. The last test analyzes the "herd average" scale including 16 HMA average diet compositions in the summer and 15 HMAs in the winter. Factors with significant p-values are indicated in **bold.**

HMA	F	df	R^2	p
Adobe Town	14.147	29	0.336	**0.001**
Conger	10.816	29	0.279	**0.001**
Frisco	6.332	29	0.184	**0.001**
Green Mountain	4.968	29	0.151	**0.001**
Little Book Cliffs	4.645	29	0.142	**0.001**
McCullough Peaks	7.556	29	0.213	**0.001**
North Hills	5.660	29	0.168	**0.001**
Onaqui	3.911	29	0.123	**0.001**
Palomino Buttes	16.678	29	0.373	**0.001**
Pine Nuts	11.133	29	0.284	**0.001**
Red Rock	6.173	29	0.181	**0.001**
Saylor Creek	24.314	29	0.465	**0.001**
South Steens	10.786	29	0.278	**0.001**
Stinkingwater	5.967	29	0.176	**0.001**
Twin Peaks	4.850	29	0.148	**0.001**
Range-wide: Using all fecal samples	11.225	464	0.024	**0.001**
Range-wide: Using HMA average	2.022	30	0.065	**0.002**

Table A4. Number of microbial families identified by season, United States Environmental Protection Agency (EPA) level III ecoregion, and Herd Management Area (HMA).

	Number of families identified
Season	
Summer	128
Winter	158
EPA III Ecoregion	
Central Basin and Range	125
Colorado Plateau	110
Mojave Basin and Range	107
Northern Basin and Range	134
Snake River Plain	117
Wyoming Basin	148
HMA	
Adobe Town	99
Conger	102
Frisco	107
Green Mountain	138
Little Book Cliffs	110
McCullough Peaks	109
North Hills	110
Onaqui	103
Palomino Buttes	125
Pine Nuts	119
Red Rock	107
Sands Basin	108
Saylor Creek	117
South Steens	105
Stinkingwater	113
Twin Peaks	129

Table A5. Results of SIMPER analysis showing the contribution of each microbial family to overall differences between samples. The table reports the average contribution (Average) and standard deviation of the contributions (SD) of each bacterial family, and the cumulative sum of these contributions (Cumulative). Bacterial families are listed in order from highest to lowest contribution to overall microbial community differences.

Family	Average	SD	Cumulative
Lachnospiraceae	0.03697	0.03043	0.127
p-251-o5	0.02552	0.02264	0.214
Acidaminococcaceae	0.02132	0.02399	0.287
Prevotellaceae	0.02037	0.01577	0.357
Rikenellaceae	0.01908	0.01709	0.422
Spirochaetaceae	0.01156	0.01058	0.462
Saccharimonadaceae	0.01049	0.01092	0.497
Oscillospiraceae	0.01047	0.00927	0.533
WCHB1-41	0.00935	0.00867	0.565
Anaerovoracaceae	0.00852	0.00775	0.595
Ruminococcaceae	0.00753	0.00698	0.620
Christensenellaceae	0.00748	0.00602	0.646
Clostridiaceae	0.00738	0.01504	0.671
Eggerthellaceae	0.00669	0.00669	0.694
Coriobacteriales_Incertae_Sedis	0.00668	0.00990	0.717
UCG-010	0.00570	0.00429	0.736
Clostridia_UCG-014	0.00470	0.00418	0.752
Hungateiclostridiaceae	0.00457	0.00402	0.768
F082	0.00449	0.00390	0.783
[Eubacterium]_coprostanoligenes_group	0.00428	0.00384	0.798
Lactobacillaceae	0.00376	0.00682	0.811
Muribaculaceae	0.00361	0.00460	0.823
Erysipelotrichaceae	0.00346	0.00343	0.835
Fibrobacteraceae	0.00307	0.00320	0.846
Bacteroidales_UCG-001	0.00277	0.00292	0.855
Selenomonadaceae	0.00234	0.00235	0.863
Planococcaceae	0.00229	0.01358	0.871
Streptococcaceae	0.00225	0.01090	0.879
Akkermansiaceae	0.00213	0.00276	0.886
Defluviitaleaceae	0.00179	0.00160	0.892
RF39	0.00164	0.00156	0.898
Eubacteriaceae	0.00162	0.00152	0.903
Monoglobaceae	0.00153	0.00182	0.909
Gastranaerophilales	0.00144	0.00129	0.913

Synergistaceae	0.00135	0.00150	0.918
Bacteroidales_BS11_gut_group	0.00124	0.00216	0.922
Bacteroidales_RF16_group	0.00107	0.00100	0.926
Paludibacteraceae	0.00106	0.00152	0.930
Absconditabacteriales_(SR1)	0.00099	0.00090	0.933
Saccharimonadales	0.00099	0.00161	0.936
uncultured6	0.00099	0.00094	0.940
Pirellulaceae	0.00098	0.00182	0.943
uncultured7	0.00090	0.00112	0.946
Xanthobacteraceae	0.00084	0.00261	0.949
Marinifilaceae	0.00080	0.00192	0.952
Desulfovibrionaceae	0.00078	0.00087	0.955
Pseudomonadaceae	0.00076	0.00143	0.957
Peptococcaceae	0.00074	0.00079	0.960
Bacteroidaceae	0.00074	0.00071	0.962
Coriobacteriales	0.00073	0.00065	0.965
Atopobiaceae	0.00065	0.00065	0.967
Anaerofustaceae	0.00053	0.00058	0.969
[Clostridium]_methylpentosum_group	0.00052	0.00067	0.971
Peptostreptococcaceae	0.00046	0.00137	0.972
Bradymonadales	0.00036	0.00036	0.973
Micrococcaceae	0.00034	0.00090	0.975
Oligosphaeraceae	0.00033	0.00033	0.976
Chthoniobacteraceae	0.00032	0.00179	0.977
Mycobacteriaceae	0.00032	0.00125	0.978
Comamonadaceae	0.00029	0.00202	0.979
WD2101_soil_group	0.00025	0.00246	0.980
WD260	0.00024	0.00086	0.981
Isosphaeraceae	0.00024	0.00190	0.981
Propionibacteriaceae	0.00022	0.00113	0.982
Solibacteraceae	0.00022	0.00281	0.983
Subgroup_2	0.00021	0.00061	0.984
Halomonadaceae	0.00018	0.00121	0.984
Erysipelatoclostridiaceae	0.00017	0.00019	0.985
Enterobacteriaceae	0.00017	0.00119	0.985
Peptostreptococcales-Tissierellales	0.00016	0.00019	0.986
uncultured3	0.00015	0.00015	0.986
KD4-96	0.00014	0.00124	0.987
Bacillaceae	0.00012	0.00037	0.987
Campylobacteraceae	0.00011	0.00016	0.988
Gracilibacteraceae	0.00010	0.00015	0.988
Sphingobacteriaceae	0.00010	0.00088	0.988
gir-aah93h0	0.00010	0.00017	0.989
Ethanoligenenaceae	0.00009	0.00014	0.989

uncultured9	0.00009	0.00069	0.989
Burkholderiaceae	0.00008	0.00052	0.990
Butyricicoccaceae	0.00008	0.00012	0.990
Syntrophomonadaceae	0.00008	0.00010	0.990
Reyranellaceae	0.00008	0.00061	0.990
Gitt-GS-136	0.00008	0.00094	0.991
Xiphinematobacteraceae	0.00008	0.00055	0.991
uncultured2	0.00008	0.00115	0.991
Oxalobacteraceae	0.00008	0.00096	0.992
Acidobacteriaceae_(Subgroup_1)	0.00008	0.00050	0.992
uncultured1	0.00007	0.00015	0.992
Acetobacteraceae	0.00007	0.00100	0.992
vadinHA49	0.00007	0.00009	0.993
Moraxellaceae	0.00006	0.00052	0.993
Veillonellaceae	0.00006	0.00011	0.993
uncultured8	0.00006	0.00045	0.993
Gemmatimonadaceae	0.00006	0.00071	0.993
MB-A2-108	0.00006	0.00058	0.994
Chthonomonadales	0.00005	0.00009	0.994
Chitinophagaceae	0.00005	0.00055	0.994
MVP-15	0.00005	0.00012	0.994
Methylacidiphilaceae	0.00005	0.00046	0.994
Vicinamibacteraceae	0.00005	0.00054	0.994
WPS-2	0.00005	0.00059	0.995
Flavobacteriaceae	0.00005	0.00039	0.995
Bacteroidetes_BD2-2	0.00005	0.00010	0.995
Methylophilaceae	0.00005	0.00046	0.995
Weeksellaceae	0.00005	0.00047	0.995
Actinomycetaceae	0.00005	0.00035	0.995
Dysgonomonadaceae	0.00004	0.00011	0.996
uncultured4	0.00004	0.00008	0.996
Micromonosporaceae	0.00004	0.00059	0.996
67-14	0.00004	0.00056	0.996
Micropepsaceae	0.00004	0.00062	0.996
LWQ8	0.00004	0.00062	0.996
Rokubacteriales	0.00004	0.00049	0.996
Staphylococcaceae	0.00004	0.00032	0.997
Pyrinomonadaceae	0.00004	0.00058	0.997
Microbacteriaceae	0.00004	0.00010	0.997
COB_P4-1_termite_group	0.00004	0.00008	0.997
Longimicrobiaceae	0.00004	0.00056	0.997
SC-I-84	0.00004	0.00054	0.997
Rhodanobacteraceae	0.00003	0.00049	0.997
Bryobacteraceae	0.00003	0.00053	0.997

Diplorickettsiaceae	0.00003	0.00051	0.998
Oscillospirales	0.00003	0.00023	0.998
Pseudonocardiaceae	0.00003	0.00044	0.998
RCP2-54	0.00003	0.00040	0.998
IMCC26256	0.00003	0.00044	0.998
OM190	0.00003	0.00044	0.998
A21b	0.00003	0.00016	0.998
Anaeromyxobacteraceae	0.00002	0.00038	0.998
Pedosphaeraceae	0.00002	0.00023	0.998
Geminicoccaceae	0.00002	0.00035	0.998
Subgroup_5	0.00002	0.00035	0.998
Rhizobiales_Incertae_Sedis	0.00002	0.00034	0.999
Geobacteraceae	0.00002	0.00034	0.999
Solirubrobacteraceae	0.00002	0.00011	0.999
Sutterellaceae	0.00002	0.00005	0.999
Anaerolineaceae	0.00002	0.00031	0.999
uncultured5	0.00002	0.00006	0.999
Spirosomaceae	0.00002	0.00010	0.999
0319-7L14	0.00002	0.00010	0.999
Marinilabiliaceae	0.00002	0.00030	0.999
Thermicanaceae	0.00002	0.00030	0.999
AD3	0.00002	0.00019	0.999
Thermomonosporaceae	0.00002	0.00023	0.999
A0839	0.00002	0.00028	0.999
Gemmataceae	0.00002	0.00028	0.999
Gemellaceae	0.00002	0.00016	0.999
Streptomycetaceae	0.00002	0.00027	1.000
TK10	0.00002	0.00027	1.000
TRA3-20	0.00002	0.00027	1.000
Nocardioidaceae	0.00002	0.00025	1.000
Unknown_Family	0.00002	0.00008	1.000
Frankiaceae	0.00002	0.00026	1.000
Corynebacteriaceae	0.00002	0.00024	1.000
vadinBE97	0.00002	0.00005	1.000
M2PB4-65_termite_group	0.00002	0.00005	1.000
Caulobacteraceae	0.00001	0.00022	1.000

Table A6. Results of SIMPER analysis showing seasonal differences. The table reports the average contribution (Average) and standard deviation of the contributions (SD) of each bacterial family to the overall dissimilarity between seasons (0.3082), the percentage this represents of the overall dissimilarity (Percentage) and the cumulative sum of these contributions (Cumulative). Also reported are the average abundance of each family during the summer (Summer) and winter (Winter) seasons, and the p-value for the summer and winter comparison. Significant p-values (< 0.05) are in **bold** and marginally significant p-values are in ***bold italics***. Bacterial families are listed in order from highest to lowest contribution to microbial community differences.

Family	Average	SD	Percentage	Cumulative	Summer	Winter	p
Lachnospiraceae	0.04005	0.03173	0.12993	0.130	0.17186	0.13833	**0.001**
p-251-o5	0.02552	0.02257	0.08278	0.213	0.07130	0.07403	0.483
Acidaminococcaceae	0.02295	0.02384	0.07447	0.287	0.02569	0.04335	**0.001**
Rikenellaceae	0.02178	0.01749	0.07065	0.358	0.02471	0.04746	**0.001**
Prevotellaceae	0.02037	0.01570	0.06608	0.424	0.06584	0.06644	0.371
Oscillospiraceae	0.01169	0.00896	0.03792	0.462	0.03216	0.04240	**0.001**
Spirochaetaceae	0.01162	0.01054	0.03770	0.500	0.02397	0.02659	**0.028**
Saccharimonadaceae	0.01155	0.01137	0.03746	0.537	0.02090	0.00910	**0.001**
WCHB1-41	0.00934	0.00867	0.03030	0.567	0.02979	0.02879	0.650
Anaerovoracaceae	0.00892	0.00790	0.02894	0.596	0.02931	0.02344	**0.001**
Christensenellaceae	0.00820	0.00611	0.02662	0.623	0.02096	0.02850	**0.001**
Ruminococcaceae	0.00756	0.00689	0.02452	0.647	0.02341	0.02458	**0.049**
Clostridiaceae	0.00745	0.01503	0.02419	0.672	0.00726	0.00614	**0.045**
Eggerthellaceae	0.00740	0.00680	0.02401	0.696	0.01746	0.01072	**0.001**
Coriobacteriales_Incertae_Sedis	0.00696	0.00999	0.02257	0.718	0.01197	0.00656	**0.001**
UCG-010	0.00665	0.00446	0.02156	0.740	0.00894	0.01646	**0.001**
F082	0.00483	0.00398	0.01567	0.755	0.00660	0.01045	**0.001**
Clostridia_UCG-014	0.00476	0.00420	0.01544	0.771	0.01006	0.00842	**0.001**
Hungateiclostridiaceae	0.00474	0.00406	0.01537	0.786	0.01350	0.01078	**0.001**
[Eubacterium]_coprostanoligenes_group	0.00452	0.00397	0.01468	0.801	0.01488	0.01130	**0.001**
Lactobacillaceae	0.00400	0.00692	0.01298	0.814	0.00576	0.00155	**0.001**
Muribaculaceae	0.00381	0.00465	0.01235	0.826	0.00774	0.00475	**0.001**
Erysipelotrichaceae	0.00380	0.00354	0.01233	0.839	0.00732	0.00364	**0.001**
Fibrobacteraceae	0.00325	0.00326	0.01054	0.849	0.00359	0.00596	**0.001**
Bacteroidales_UCG-001	0.00293	0.00298	0.00950	0.859	0.00355	0.00567	**0.001**
Selenomonadaceae	0.00235	0.00238	0.00762	0.866	0.00586	0.00488	***0.077***
Streptococcaceae	0.00231	0.01101	0.00751	0.874	0.00227	0.00219	**0.001**
Planococcaceae	0.00226	0.01346	0.00732	0.881	0.00258	0.00069	0.839

Akkermansiaceae	0.00225	0.00286	0.00731	0.888	0.00430	0.00206	**0.001**
Defluviitaleaceae	0.00181	0.00157	0.00588	0.894	0.00473	0.00504	**0.011**
RF39	0.00179	0.00165	0.00582	0.900	0.00345	0.00160	**0.001**
Eubacteriaceae	0.00162	0.00153	0.00527	0.905	0.00347	0.00325	0.192
Monoglobaceae	0.00160	0.00183	0.00519	0.910	0.00287	0.00197	**0.001**
Gastranaerophilales	0.00150	0.00131	0.00486	0.915	0.00221	0.00315	**0.001**
Bacteroidales_BS11_gut_group	0.00139	0.00225	0.00452	0.920	0.00053	0.00221	**0.001**
Synergistaceae	0.00137	0.00152	0.00445	0.924	0.00274	0.00202	**0.001**
Bacteroidales_RF16_group	0.00110	0.00100	0.00357	0.928	0.00125	0.00177	**0.001**
Paludibacteraceae	0.00108	0.00152	0.00350	0.931	0.00117	0.00156	**0.001**
Saccharimonadales	0.00102	0.00161	0.00330	0.935	0.00137	0.00082	**0.001**
Pirellulaceae	0.00100	0.00184	0.00324	0.938	0.00149	0.00207	**0.001**
Absconditabacteriales_(SR1)	0.00099	0.00090	0.00323	0.941	0.00169	0.00142	0.290
uncultured6	0.00099	0.00094	0.00322	0.944	0.00199	0.00167	**0.012**
uncultured7	0.00091	0.00113	0.00296	0.947	0.00151	0.00096	**0.001**
Marinifilaceae	0.00088	0.00197	0.00287	0.950	0.00024	0.00127	**0.001**
Xanthobacteraceae	0.00085	0.00264	0.00275	0.953	0.00050	0.00067	0.111
Pseudomonadaceae	0.00084	0.00145	0.00273	0.956	0.00113	0.00015	**0.001**
Bacteroidaceae	0.00084	0.00074	0.00273	0.958	0.00055	0.00146	**0.001**
Peptococcaceae	0.00083	0.00082	0.00269	0.961	0.00111	0.00197	**0.001**
Desulfovibrionaceae	0.00077	0.00087	0.00251	0.964	0.00147	0.00134	0.880
Coriobacteriales	0.00073	0.00065	0.00236	0.966	0.00139	0.00122	0.370
Atopobiaceae	0.00065	0.00065	0.00211	0.968	0.00111	0.00126	***0.078***
Anaerofustaceae	0.00053	0.00058	0.00173	0.970	0.00099	0.00090	0.944
[Clostridium]_methylpentosum_group	0.00052	0.00067	0.00169	0.971	0.00062	0.00044	0.221
Peptostreptococcaceae	0.00046	0.00138	0.00150	0.973	0.00031	0.00040	0.287
Bradymonadales	0.00037	0.00036	0.00121	0.974	0.00032	0.00052	**0.001**
Oligosphaeraceae	0.00035	0.00034	0.00114	0.975	0.00026	0.00054	**0.001**
Micrococcaceae	0.00035	0.00090	0.00112	0.976	0.00018	0.00038	**0.014**
Chthoniobacteraceae	0.00033	0.00182	0.00107	0.978	0.00012	0.00036	***0.062***
Mycobacteriaceae	0.00033	0.00127	0.00106	0.979	0.00014	0.00036	**0.048**
Comamonadaceae	0.00029	0.00201	0.00093	0.980	0.00019	0.00021	0.526
WD2101_soil_group	0.00026	0.00249	0.00084	0.980	0.00002	0.00040	0.214
WD260	0.00025	0.00087	0.00080	0.981	0.00013	0.00020	***0.080***
Isosphaeraceae	0.00025	0.00192	0.00080	0.982	0.00005	0.00034	0.191
Propionibacteriaceae	0.00023	0.00115	0.00073	0.983	0.00004	0.00031	**0.001**
Solibacteraceae	0.00022	0.00285	0.00073	0.983	0.00001	0.00035	0.226
Subgroup_2	0.00021	0.00062	0.00067	0.984	0.00012	0.00016	0.115
Halomonadaceae	0.00018	0.00122	0.00059	0.985	0.00003	0.00027	***0.060***
Erysipelatoclostridiaceae	0.00017	0.00019	0.00056	0.985	0.00021	0.00015	0.149
Enterobacteriaceae	0.00017	0.00120	0.00055	0.986	0.00007	0.00020	**0.016**
Peptostreptococcales-Tissierellales	0.00017	0.00019	0.00054	0.986	0.00022	0.00009	**0.001**
uncultured3	0.00015	0.00015	0.00048	0.987	0.00015	0.00017	0.205
KD4-96	0.00014	0.00126	0.00046	0.987	0.00001	0.00021	0.166

Bacillaceae	0.00012	0.00037	0.00040	0.988	0.00011	0.00008	***0.093***
Campylobacteraceae	0.00011	0.00016	0.00036	0.988	0.00012	0.00008	0.754
Gracilibacteraceae	0.00011	0.00015	0.00036	0.988	0.00002	0.00015	**0.001**
Sphingobacteriaceae	0.00010	0.00089	0.00032	0.989	0.00000	0.00016	0.105
gir-aah93h0	0.00010	0.00017	0.00031	0.989	0.00007	0.00009	0.119
Ethanoligenenaceae	0.00009	0.00014	0.00029	0.989	0.00006	0.00010	**0.014**
uncultured9	0.00009	0.00070	0.00029	0.990	0.00002	0.00012	***0.054***
Burkholderiaceae	0.00009	0.00052	0.00028	0.990	0.00004	0.00009	0.219
Butyricicoccaceae	0.00009	0.00012	0.00028	0.990	0.00005	0.00010	**0.001**
Reyranellaceae	0.00008	0.00061	0.00027	0.990	0.00002	0.00011	***0.096***
Syntrophomonadaceae	0.00008	0.00010	0.00027	0.991	0.00007	0.00009	0.114
Gitt-GS-136	0.00008	0.00095	0.00026	0.991	0.00003	0.00010	0.391
Xiphinematobacteraceae	0.00008	0.00056	0.00026	0.991	0.00002	0.00010	0.128
uncultured2	0.00008	0.00117	0.00025	0.991	0.00000	0.00013	0.374
Oxalobacteraceae	0.00008	0.00097	0.00025	0.992	0.00000	0.00013	0.213
Acidobacteriaceae_(Subgroup_1)	0.00008	0.00051	0.00025	0.992	0.00002	0.00009	0.109
uncultured1	0.00008	0.00015	0.00024	0.992	0.00010	0.00002	**0.002**
Acetobacteraceae	0.00007	0.00102	0.00024	0.992	0.00000	0.00011	0.396
vadinHA49	0.00007	0.00010	0.00024	0.993	0.00004	0.00009	**0.001**
Moraxellaceae	0.00006	0.00053	0.00021	0.993	0.00004	0.00005	0.492
uncultured8	0.00006	0.00046	0.00021	0.993	0.00002	0.00007	0.190
Gemmatimonadaceae	0.00006	0.00072	0.00021	0.993	0.00000	0.00010	0.160
Veillonellaceae	0.00006	0.00011	0.00020	0.994	0.00006	0.00004	0.603
MB-A2-108	0.00006	0.00059	0.00020	0.994	0.00001	0.00008	0.139
Chthonomonadales	0.00005	0.00009	0.00017	0.994	0.00006	0.00003	**0.007**
Chitinophagaceae	0.00005	0.00055	0.00017	0.994	0.00001	0.00007	0.262
MVP-15	0.00005	0.00012	0.00017	0.994	0.00002	0.00006	**0.006**
Methylacidiphilaceae	0.00005	0.00047	0.00017	0.994	0.00001	0.00007	0.115
Vicinamibacteraceae	0.00005	0.00055	0.00016	0.995	0.00000	0.00008	0.199
WPS-2	0.00005	0.00059	0.00016	0.995	0.00000	0.00007	0.121
Dysgonomonadaceae	0.00005	0.00012	0.00016	0.995	0.00001	0.00006	**0.001**
Flavobacteriaceae	0.00005	0.00040	0.00016	0.995	0.00001	0.00005	0.101
Methylophilaceae	0.00005	0.00047	0.00015	0.995	0.00000	0.00007	0.106
Bacteroidetes_BD2-2	0.00005	0.00011	0.00015	0.995	0.00003	0.00004	***0.072***
Actinomycetaceae	0.00005	0.00036	0.00015	0.995	0.00002	0.00005	0.300
Weeksellaceae	0.00005	0.00047	0.00015	0.996	0.00003	0.00004	0.393
Micromonosporaceae	0.00004	0.00060	0.00014	0.996	0.00001	0.00007	0.438
uncultured4	0.00004	0.00008	0.00014	0.996	0.00004	0.00003	0.855
67-14	0.00004	0.00056	0.00014	0.996	0.00000	0.00007	0.124
Micropepsaceae	0.00004	0.00063	0.00014	0.996	0.00000	0.00007	0.363
LWQ8	0.00004	0.00063	0.00014	0.996	0.00000	0.00007	0.363
Rokubacteriales	0.00004	0.00049	0.00013	0.996	0.00000	0.00006	0.150
Staphylococcaceae	0.00004	0.00033	0.00013	0.997	0.00001	0.00005	0.242
Pyrinomonadaceae	0.00004	0.00058	0.00013	0.997	0.00000	0.00006	0.390

Microbacteriaceae	0.00004	0.00010	0.00012	0.997	0.00003	0.00002	0.846
COB_P4-1_termite_group	0.00004	0.00008	0.00012	0.997	0.00002	0.00004	**0.001**
Longimicrobiaceae	0.00004	0.00056	0.00012	0.997	0.00000	0.00006	0.370
SC-I-84	0.00004	0.00055	0.00012	0.997	0.00000	0.00006	0.360
Rhodanobacteraceae	0.00004	0.00049	0.00012	0.997	0.00000	0.00006	0.284
Bryobacteraceae	0.00004	0.00053	0.00012	0.997	0.00000	0.00006	0.359
Diplorickettsiaceae	0.00003	0.00052	0.00011	0.998	0.00000	0.00006	0.358
Oscillospirales	0.00003	0.00023	0.00011	0.998	0.00000	0.00005	**0.001**
Pseudonocardiaceae	0.00003	0.00045	0.00010	0.998	0.00000	0.00005	0.350
RCP2-54	0.00003	0.00040	0.00010	0.998	0.00000	0.00005	***0.093***
IMCC26256	0.00003	0.00044	0.00010	0.998	0.00000	0.00005	0.350
OM190	0.00003	0.00044	0.00010	0.998	0.00000	0.00005	0.350
A21b	0.00003	0.00016	0.00008	0.998	0.00001	0.00002	0.288
Anaeromyxobacteraceae	0.00003	0.00038	0.00008	0.998	0.00000	0.00004	0.337
Pedosphaeraceae	0.00002	0.00023	0.00008	0.998	0.00000	0.00003	0.193
Geminicoccaceae	0.00002	0.00035	0.00008	0.998	0.00000	0.00004	0.334
Subgroup_5	0.00002	0.00035	0.00008	0.998	0.00000	0.00004	0.334
Rhizobiales_Incertae_Sedis	0.00002	0.00034	0.00007	0.999	0.00000	0.00004	0.333
Geobacteraceae	0.00002	0.00034	0.00007	0.999	0.00000	0.00004	0.333
Solirubrobacteraceae	0.00002	0.00011	0.00007	0.999	0.00002	0.00001	0.758
Anaerolineaceae	0.00002	0.00032	0.00007	0.999	0.00000	0.00004	0.288
Sutterellaceae	0.00002	0.00005	0.00007	0.999	0.00002	0.00002	0.651
uncultured5	0.00002	0.00006	0.00006	0.999	0.00003	0.00000	0.180
Marinilabiliaceae	0.00002	0.00030	0.00006	0.999	0.00000	0.00003	0.329
Thermicanaceae	0.00002	0.00030	0.00006	0.999	0.00000	0.00003	0.335
AD3	0.00002	0.00019	0.00006	0.999	0.00000	0.00002	0.258
0319-7L14	0.00002	0.00010	0.00006	0.999	0.00001	0.00001	0.187
Spirosomaceae	0.00002	0.00010	0.00006	0.999	0.00002	0.00001	0.679
Thermomonosporaceae	0.00002	0.00024	0.00006	0.999	0.00000	0.00002	0.175
A0839	0.00002	0.00028	0.00006	0.999	0.00000	0.00003	0.326
Gemmataceae	0.00002	0.00028	0.00006	0.999	0.00000	0.00003	0.326
Streptomycetaceae	0.00002	0.00028	0.00006	0.999	0.00000	0.00003	0.326
TK10	0.00002	0.00028	0.00006	1.000	0.00000	0.00003	0.326
TRA3-20	0.00002	0.00028	0.00006	1.000	0.00000	0.00003	0.326
Gemellaceae	0.00002	0.00016	0.00006	1.000	0.00001	0.00002	0.449
Nocardioidaceae	0.00002	0.00025	0.00006	1.000	0.00000	0.00002	0.217
Frankiaceae	0.00002	0.00026	0.00006	1.000	0.00000	0.00003	0.321
Unknown_Family	0.00002	0.00008	0.00006	1.000	0.00001	0.00001	0.244
M2PB4-65_termite_group	0.00002	0.00005	0.00005	1.000	0.00000	0.00002	**0.001**
Corynebacteriaceae	0.00002	0.00025	0.00005	1.000	0.00000	0.00002	0.346
vadinBE97	0.00002	0.00005	0.00005	1.000	0.00001	0.00001	0.549
Caulobacteraceae	0.00001	0.00022	0.00005	1.000	0.00000	0.00002	0.324

Detailed Summary of Plant and Microbial Family Correlation Results

The two bacterial families associated with the most plant families were Lachnospiraceae and Rikenellaceae. Lachnospiraceae was positively correlated with the graminoid families Juncaceae (rushes) and Poaceae (grasses) while negatively correlated with the shrub and forb families Asteraceae (sunflower family), Berberidaceae (barberry family), Chenopodiaceae (goosefoot family), Ephedraceae (Ephedra family), and Solanaceae (nightshades). There was moderate evidence for a negative relationship with Cupressaceae (junipers and redwoods) and Polygonaceae (buckwheats; Table A7). Rikenellaceae was negatively correlated with the graminoid families Juncaceae and Poaceae, had a moderate negative relationship with Cyperaceae (sedges), and was positively correlated with the shrub and forb families Asteraceae, Chenopodiaceae, Cucurbitaceae (cucumber family), Cupressaceae, Tamaricaceae (tamarisk family), and Zygophyllaceae (caltrop family; Table A7). Acidaminococcaceae was positively correlated with the family Chenopodiaceae and negatively correlated with the family Cyperaceae (Table A7). Anaerovoracaceae was negatively correlated with the Asteraceae family while being positively correlated with the graminoid families Juncaceae and Poaceae. There was also moderate evidence for a positive relationship with the Juncaginaceae (arrowgrass) family (Table A7). Oscillospiraceae was positively correlated with the families of Asteraceae, Chenopodiaceae, and Cucurbitaceae and negatively correlated with the Cyperaceae family and the Fabaceae (legume) family (Table A7). Prevotellaceae was positively correlated with the family Brassicaceae (mustards) and the Potamogetonaceae (pondweed) family. There was a moderately significant negative correlation with the Poaceae family (Table A7). The family P-251-05, a family of uncultured bacterial in the Bacteroidales order, was positively correlated with the plant families Brassicaceae and Chenopodiaceae and had a moderately significant positive relationship

with Nyctaginaceae (the four-o-clock family; Table A7). The family Saccharimonadaceae was negatively correlated with the Asteraceae and Chenopodiaceae families and positively correlated with the Poaceae family (Table A7). The Spirochaetaceae family was positively correlated with the Asteraceae and Tamaricaceae families (Table A7). The microbial family of WCHB1-41 was not correlated with any of the plant families (Table A7).

Table A7. Correlations between each of the ten bacterial families explaining the most community dissimilarity and the 50 plant families present in the diets of free-roaming horses. The table shows the correlation value (r), p-value for each correlation, and the adjusted p-value calculated based on the false discovery rate. The table has been broken into two parts, each with five bacterial families to fit a standard page size.

Part 1:

	Lachnospiraceae			p.251.o5			Acidaminococcaceae			Prevotellaceae			Rikenellaceae		
Family	R	p	adj. p	r	p	adj. p	r	p	adj. p	r	p	adj. p	r	p	adj. p
Amaryllidaceae	-0.096	0.038	0.173	-0.050	0.282	0.671	-0.023	0.618	0.944	-0.045	0.338	0.768	0.017	0.719	0.817
Amblystegiaceae	-0.019	0.682	0.851	0.041	0.383	0.672	-0.003	0.943	0.982	0.062	0.183	0.677	0.069	0.139	0.452
Anacardiaceae	0.006	0.900	0.940	-0.005	0.916	0.942	0.000	0.994	0.994	0.028	0.551	0.955	-0.031	0.502	0.661
Apiaceae	-0.069	0.136	0.400	-0.043	0.351	0.672	0.008	0.867	0.982	-0.023	0.619	0.955	0.040	0.390	0.591
Asparagaceae	-0.016	0.735	0.855	-0.030	0.515	0.715	-0.040	0.392	0.944	-0.064	0.167	0.677	-0.054	0.244	0.554
Asteraceae	**-0.274**	**0.000**	**0.000**	-0.032	0.487	0.715	0.115	0.013	0.215	-0.029	0.538	0.955	**0.294**	**0.000**	**0.000**
Berberidaceae	**-0.135**	**0.003**	**0.029**	-0.085	0.067	0.469	-0.013	0.783	0.982	-0.103	0.027	0.266	-0.045	0.333	0.582
Bignoniaceae	0.005	0.921	0.940	0.024	0.609	0.802	0.019	0.680	0.944	0.106	0.022	0.266	0.085	0.068	0.307
Boraginaceae	-0.022	0.630	0.829	-0.065	0.160	0.617	-0.027	0.558	0.944	0.002	0.974	0.985	-0.009	0.851	0.887
Brassicaceae	0.013	0.779	0.885	**0.236**	**0.000**	**0.000**	0.041	0.373	0.944	**0.195**	**0.000**	**0.001**	0.035	0.455	0.649
Bryaceae	-0.019	0.676	0.851	0.006	0.902	0.942	-0.011	0.810	0.982	0.030	0.522	0.955	0.051	0.269	0.582
Campanulaceae	-0.023	0.627	0.829	-0.036	0.445	0.709	0.001	0.981	0.994	-0.001	0.985	0.985	0.019	0.682	0.792
Caprifoliaceae	-0.096	0.038	0.173	-0.071	0.128	0.583	-0.026	0.571	0.944	-0.045	0.338	0.768	0.096	0.038	0.191
Caryophyllaceae	0.064	0.167	0.463	-0.013	0.778	0.926	-0.034	0.470	0.944	-0.019	0.681	0.955	-0.078	0.094	0.361
Chenopodiaceae	**-0.166**	**0.000**	**0.004**	**0.143**	**0.002**	**0.049**	**0.155**	**0.001**	**0.026**	0.043	0.360	0.782	**0.143**	**0.002**	**0.032**
Comandraceae	-0.041	0.378	0.674	0.016	0.729	0.926	-0.020	0.667	0.944	0.012	0.802	0.955	0.059	0.201	0.554
Cucurbitaceae	0.030	0.516	0.801	-0.008	0.857	0.942	0.027	0.562	0.944	-0.027	0.561	0.955	-0.044	0.349	0.582
Cupressaceae	***-0.122***	***0.008***	***0.052***	-0.083	0.075	0.469	-0.010	0.828	0.982	-0.055	0.239	0.677	**0.127**	**0.006**	**0.044**
Cyperaceae	0.057	0.220	0.494	-0.092	0.047	0.406	**-0.152**	**0.001**	**0.026**	0.053	0.250	0.677	***-0.115***	***0.013***	***0.081***
Ephedraceae	**-0.127**	**0.006**	**0.045**	-0.047	0.312	0.672	-0.004	0.924	0.982	0.006	0.902	0.981	-0.013	0.787	0.872

Fabaceae	0.042	0.370	0.674	-0.051	0.277	0.671	-0.110	0.017	0.216	-0.053	0.257	0.677	-0.103	0.026	0.145
Fagaceae	0.027	0.561	0.801	-0.031	0.502	0.715	-0.080	0.086	0.579	0.004	0.938	0.985	-0.045	0.336	0.582
Geraniaceae	-0.040	0.394	0.679	0.013	0.773	0.926	-0.030	0.523	0.944	0.016	0.733	0.955	0.038	0.410	0.603
Hydrophyllaceae	0.042	0.362	0.674	0.071	0.128	0.583	0.012	0.793	0.982	0.082	0.078	0.504	-0.031	0.500	0.661
Juncaceae	**0.195**	**0.000**	**0.000**	-0.079	0.090	0.499	-0.078	0.093	0.579	-0.020	0.668	0.955	**-0.166**	**0.000**	**0.008**
Juncaginaceae	0.016	0.731	0.855	-0.052	0.263	0.671	-0.042	0.366	0.944	-0.059	0.205	0.677	-0.041	0.374	0.591
Krameriaceae	-0.010	0.825	0.916	-0.045	0.337	0.672	-0.035	0.454	0.944	-0.081	0.081	0.504	-0.029	0.526	0.675
Linaceae	-0.005	0.917	0.940	-0.052	0.265	0.671	-0.037	0.426	0.944	-0.086	0.063	0.504	-0.049	0.288	0.582
Loasaceae	0.029	0.530	0.801	-0.036	0.444	0.709	-0.014	0.766	0.982	0.067	0.151	0.677	0.000	0.996	0.996
Malvaceae	-0.059	0.201	0.494	0.060	0.195	0.652	0.072	0.119	0.659	0.009	0.841	0.955	0.068	0.145	0.452
Nyctaginaceae	0.003	0.948	0.948	***0.131***	***0.005***	***0.080***	0.004	0.930	0.982	0.060	0.200	0.677	-0.021	0.655	0.780
Onagraceae	0.085	0.067	0.258	-0.040	0.390	0.672	-0.029	0.530	0.944	0.015	0.741	0.955	-0.046	0.318	0.582
Orobanchaceae	-0.081	0.080	0.285	0.041	0.379	0.672	0.028	0.547	0.944	0.014	0.756	0.955	0.047	0.316	0.582
Phrymaceae	-0.026	0.581	0.807	0.091	0.049	0.406	-0.027	0.562	0.944	0.072	0.119	0.664	0.057	0.222	0.554
Pinaceae	-0.033	0.472	0.786	-0.046	0.318	0.672	-0.020	0.675	0.944	0.025	0.586	0.955	0.007	0.883	0.901
Plantaginaceae	-0.056	0.225	0.494	-0.053	0.256	0.671	0.015	0.749	0.982	-0.054	0.243	0.677	-0.009	0.838	0.887
Poaceae	**0.257**	**0.000**	**0.000**	-0.035	0.453	0.709	-0.021	0.657	0.944	***-0.133***	***0.004***	***0.067***	**-0.137**	**0.003**	**0.032**
Polemoniaceae	-0.027	0.559	0.801	-0.013	0.772	0.926	0.099	0.033	0.327	0.012	0.793	0.955	0.055	0.233	0.554
Polygonaceae	***-0.117***	***0.011***	***0.063***	-0.061	0.189	0.652	0.007	0.882	0.982	0.001	0.978	0.985	-0.044	0.345	0.582
Potamogetonaceae	0.074	0.112	0.372	0.001	0.979	0.979	-0.033	0.475	0.944	**0.154**	**0.001**	**0.021**	0.024	0.607	0.759
Ranunculaceae	0.056	0.227	0.494	0.114	0.014	0.176	0.063	0.177	0.887	0.017	0.711	0.955	-0.021	0.651	0.780
Rosaceae	-0.018	0.698	0.851	0.007	0.877	0.942	-0.037	0.422	0.944	0.010	0.829	0.955	0.063	0.172	0.506
Salicaceae	-0.031	0.511	0.801	-0.043	0.351	0.672	-0.030	0.524	0.944	-0.014	0.769	0.955	-0.012	0.802	0.872
Sarcobataceae	-0.044	0.342	0.674	0.032	0.493	0.715	-0.038	0.414	0.944	0.014	0.764	0.955	0.075	0.105	0.374
Scrophulariaceae	0.009	0.852	0.926	-0.029	0.530	0.717	0.085	0.068	0.568	-0.007	0.874	0.971	-0.033	0.484	0.661
Solanaceae	**-0.142**	**0.002**	**0.022**	-0.068	0.142	0.591	-0.005	0.914	0.982	-0.065	0.161	0.677	-0.040	0.389	0.591
Stachyuraceae	-0.048	0.302	0.630	-0.008	0.866	0.942	0.035	0.451	0.944	0.032	0.487	0.955	0.054	0.243	0.554
Tamaricaceae	-0.091	0.050	0.210	-0.005	0.923	0.942	-0.025	0.593	0.944	-0.020	0.663	0.955	**0.140**	**0.003**	**0.032**
Urticaceae	-0.057	0.223	0.494	-0.052	0.265	0.671	-0.038	0.410	0.944	-0.046	0.322	0.768	0.079	0.087	0.361
Zygophyllaceae	-0.071	0.128	0.400	0.008	0.871	0.942	0.036	0.440	0.944	-0.011	0.816	0.955	**0.131**	**0.005**	**0.040**

Table A7. Part 2:

	Spirochaetaceae			Saccharimonadaceae			Oscillospiraceae			WCHB1.41			Anaerovoracaceae		
Family	R	p	adj. p	r	p	adj. p	r	p	adj. p	r	p	adj. p	r	p	adj. p
Amaryllidaceae	-0.016	0.723	0.853	-0.037	0.426	0.710	-0.031	0.505	0.838	-0.025	0.593	0.899	-0.058	0.213	0.599
Amblystegiaceae	0.062	0.180	0.728	-0.053	0.252	0.704	0.004	0.932	0.932	-0.012	0.790	0.932	-0.045	0.332	0.616
Anacardiaceae	0.007	0.887	0.887	-0.010	0.830	0.883	-0.035	0.454	0.838	-0.006	0.893	0.932	-0.019	0.676	0.843
Apiaceae	-0.042	0.364	0.728	-0.029	0.533	0.740	0.016	0.726	0.887	-0.005	0.918	0.932	-0.071	0.124	0.477
Asparagaceae	-0.044	0.342	0.728	0.101	0.029	0.244	-0.053	0.253	0.602	-0.066	0.157	0.804	0.115	0.013	0.128
Asteraceae	**0.257**	**0.000**	**0.000**	**-0.233**	**0.000**	**0.000**	**0.153**	**0.001**	**0.016**	0.039	0.397	0.806	**-0.224**	**0.000**	**0.000**
Berberidaceae	-0.069	0.140	0.699	-0.047	0.307	0.704	-0.112	0.015	0.123	-0.107	0.021	0.699	-0.108	0.020	0.129
Bignoniaceae	0.015	0.749	0.853	-0.056	0.229	0.704	0.026	0.570	0.838	-0.006	0.894	0.932	-0.016	0.728	0.843
Boraginaceae	0.085	0.069	0.490	-0.041	0.375	0.704	0.057	0.223	0.586	0.080	0.085	0.804	0.029	0.535	0.818
Brassicaceae	0.014	0.757	0.853	0.010	0.828	0.883	-0.091	0.049	0.243	-0.072	0.121	0.804	-0.107	0.021	0.129
Bryaceae	0.065	0.161	0.728	-0.039	0.403	0.704	0.013	0.787	0.887	0.005	0.917	0.932	-0.028	0.540	0.818
Campanulaceae	0.099	0.032	0.322	-0.041	0.372	0.704	0.013	0.781	0.887	0.044	0.341	0.806	-0.024	0.612	0.827
Caprifoliaceae	0.029	0.534	0.802	-0.072	0.119	0.539	0.041	0.382	0.764	-0.004	0.931	0.932	-0.074	0.112	0.468
Caryophyllaceae	-0.059	0.207	0.728	0.112	0.016	0.158	-0.064	0.165	0.516	-0.015	0.752	0.932	0.058	0.208	0.599
Chenopodiaceae	-0.044	0.344	0.728	**-0.138**	**0.003**	**0.048**	**0.199**	**0.000**	**0.001**	-0.064	0.169	0.804	-0.093	0.045	0.227
Comandraceae	0.028	0.545	0.802	-0.011	0.811	0.883	-0.017	0.719	0.887	0.041	0.379	0.806	-0.018	0.693	0.843
Cucurbitaceae	-0.053	0.256	0.728	0.026	0.583	0.750	**0.142**	**0.002**	**0.027**	0.046	0.321	0.806	0.052	0.259	0.599
Cupressaceae	0.008	0.872	0.887	-0.088	0.059	0.342	0.077	0.098	0.385	-0.022	0.637	0.932	-0.094	0.043	0.227
Cyperaceae	-0.036	0.434	0.749	0.087	0.062	0.342	**-0.171**	**0.000**	**0.005**	-0.028	0.541	0.879	0.059	0.201	0.599
Ephedraceae	-0.071	0.124	0.699	0.001	0.990	0.990	0.028	0.541	0.838	-0.005	0.908	0.932	0.052	0.264	0.599
Fabaceae	-0.090	0.053	0.443	0.114	0.014	0.158	**-0.135**	**0.004**	**0.036**	0.026	0.581	0.899	0.024	0.599	0.827
Fagaceae	-0.048	0.302	0.728	0.080	0.083	0.417	-0.096	0.038	0.212	0.028	0.545	0.879	-0.024	0.611	0.827
Geraniaceae	-0.048	0.301	0.728	-0.005	0.909	0.927	0.014	0.771	0.887	0.046	0.325	0.806	0.007	0.876	0.912
Hydrophyllaceae	0.039	0.405	0.749	-0.026	0.572	0.750	-0.009	0.847	0.902	-0.045	0.329	0.806	-0.004	0.928	0.947
Juncaceae	-0.048	0.305	0.728	0.025	0.585	0.750	-0.110	0.017	0.123	-0.051	0.275	0.806	**0.158**	**0.001**	**0.016**
Juncaginaceae	-0.052	0.266	0.728	0.066	0.154	0.594	0.020	0.666	0.887	0.093	0.045	0.749	***0.125***	***0.007***	***0.084***
Krameriaceae	-0.035	0.453	0.755	0.095	0.040	0.288	-0.034	0.460	0.838	-0.020	0.665	0.932	0.024	0.604	0.827
Linaceae	-0.043	0.351	0.728	-0.015	0.741	0.846	-0.055	0.234	0.586	-0.032	0.490	0.845	0.037	0.424	0.731

Loasaceae	-0.022	0.636	0.852	-0.015	0.745	0.846	-0.048	0.307	0.666	-0.036	0.443	0.845	-0.013	0.784	0.852
Malvaceae	0.049	0.290	0.728	-0.020	0.663	0.789	0.016	0.736	0.887	-0.044	0.347	0.806	-0.044	0.349	0.623
Nyctaginaceae	-0.020	0.659	0.852	0.042	0.361	0.704	-0.072	0.123	0.440	-0.045	0.334	0.806	-0.052	0.267	0.599
Onagraceae	-0.014	0.768	0.853	-0.035	0.454	0.716	-0.018	0.696	0.887	-0.039	0.403	0.806	0.018	0.695	0.843
Orobanchaceae	-0.018	0.699	0.852	-0.040	0.393	0.704	0.015	0.747	0.887	-0.063	0.177	0.804	-0.049	0.291	0.599
Phrymaceae	-0.018	0.691	0.852	-0.030	0.513	0.740	-0.018	0.704	0.887	0.034	0.465	0.845	-0.022	0.638	0.839
Pinaceae	0.010	0.829	0.886	-0.038	0.408	0.704	-0.028	0.552	0.838	-0.004	0.932	0.932	-0.053	0.250	0.599
Plantaginaceae	-0.031	0.510	0.802	0.049	0.292	0.704	0.031	0.510	0.838	0.014	0.758	0.932	0.015	0.742	0.843
Poaceae	-0.105	0.023	0.291	**0.180**	**0.000**	**0.002**	0.065	0.159	0.516	0.070	0.129	0.804	**0.138**	**0.003**	**0.047**
Polemoniaceae	-0.019	0.686	0.852	-0.022	0.637	0.777	0.089	0.055	0.252	-0.059	0.205	0.806	-0.009	0.844	0.897
Polygonaceae	0.053	0.252	0.728	-0.045	0.329	0.704	-0.076	0.100	0.385	-0.010	0.827	0.932	0.053	0.251	0.599
Potamogetonaceae	-0.040	0.385	0.740	-0.030	0.521	0.740	-0.059	0.201	0.557	-0.069	0.138	0.804	-0.001	0.980	0.980
Ranunculaceae	-0.037	0.423	0.749	0.023	0.621	0.777	-0.060	0.197	0.557	0.050	0.278	0.806	-0.030	0.520	0.818
Rosaceae	0.008	0.864	0.887	-0.006	0.895	0.927	0.006	0.894	0.912	0.005	0.912	0.932	-0.014	0.767	0.852
Salicaceae	-0.027	0.561	0.802	0.031	0.499	0.740	-0.027	0.558	0.838	-0.039	0.398	0.806	0.050	0.278	0.599
Sarcobataceae	0.124	0.007	0.124	-0.050	0.285	0.704	0.043	0.353	0.735	0.069	0.138	0.804	-0.076	0.100	0.454
Scrophulariaceae	0.022	0.643	0.852	-0.043	0.354	0.704	0.007	0.874	0.910	0.053	0.258	0.806	0.016	0.730	0.843
Solanaceae	-0.046	0.327	0.728	-0.067	0.150	0.594	-0.108	0.020	0.123	-0.102	0.028	0.699	-0.107	0.020	0.129
Stachyuraceae	0.028	0.550	0.802	-0.034	0.458	0.716	0.011	0.809	0.887	0.008	0.863	0.932	-0.036	0.439	0.732
Tamaricaceae	**0.166**	**0.000**	**0.008**	-0.060	0.195	0.697	0.051	0.273	0.620	0.005	0.909	0.932	-0.067	0.149	0.531
Urticaceae	-0.010	0.833	0.886	-0.049	0.288	0.704	0.027	0.562	0.838	0.006	0.899	0.932	-0.046	0.325	0.616
Zygophyllaceae	0.069	0.136	0.699	-0.039	0.404	0.704	0.011	0.816	0.887	0.032	0.489	0.845	-0.048	0.299	0.599

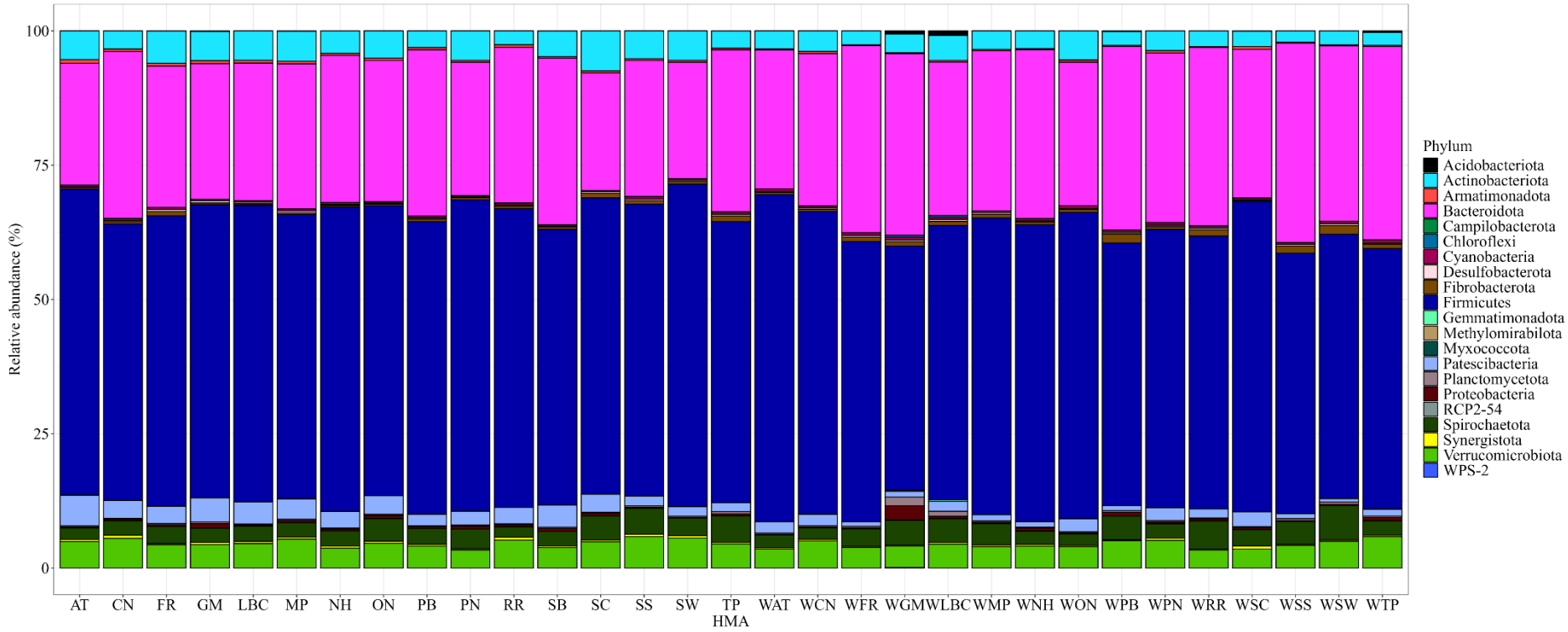

Figure A1. Free-roaming horse microbiome composition represented by phylum relative abundance. Phyla relative abundance was averaged and visualized by HMA and season, HMA labels starting with a "W" represent winter. All 21 phyla identified in the gut microbiomes of free-roaming horses after removing rare taxa (ASVs [Amplicon sequence variants] present in < 0.001% mean relative abundance) are depicted. Almost all reads (99.73%) were assigned to the Phyla level of taxonomic depth.

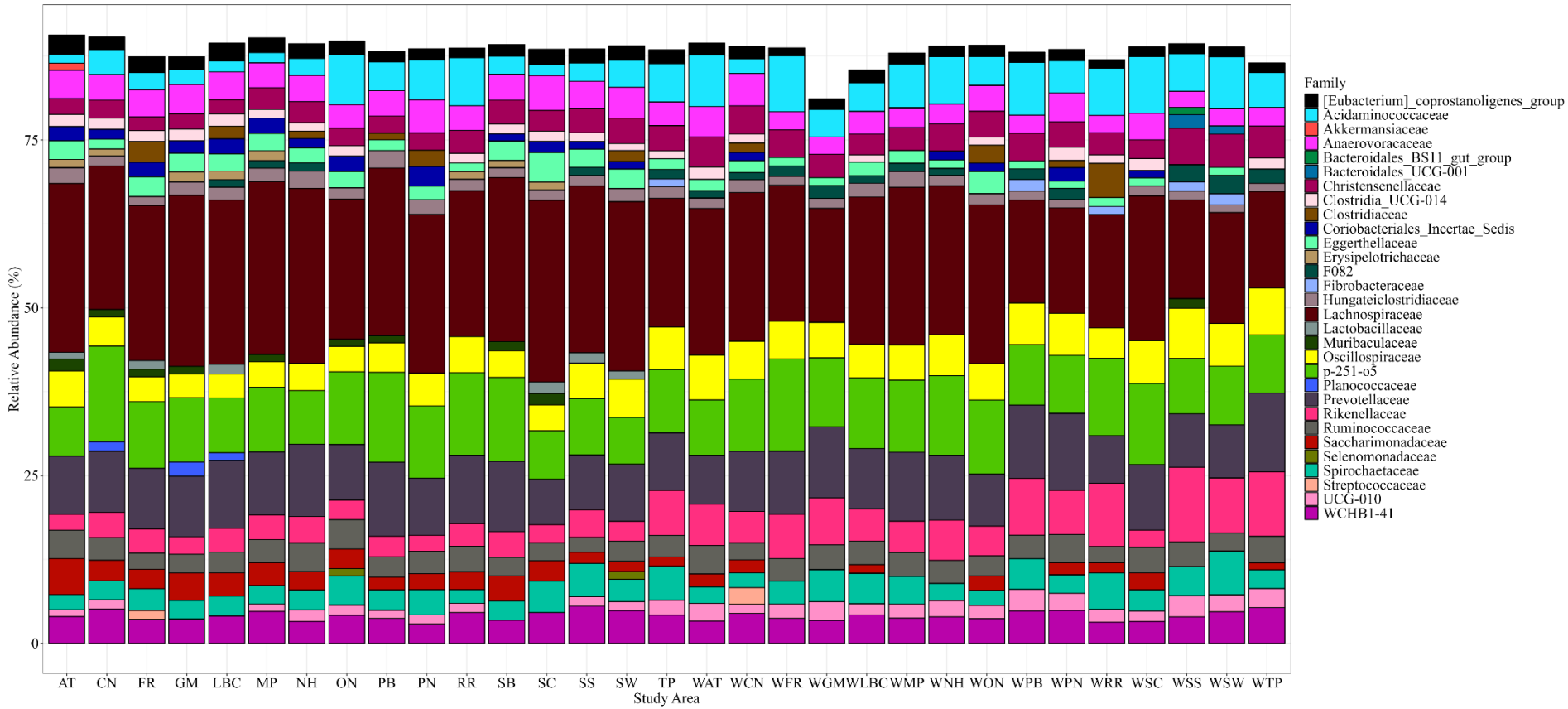

Figure A2. Free-roaming horse microbiome composition represented by family relative abundance. Family relative abundance was averaged and visualized by HMA and season. For simplified visualization, the stacked bar chart represents bacterial families that made up at least 1% of the relative abundance in each HMA, so bars will not total to 100%. This cutoff means that 30 of the 158 families identified in free-roaming horse gut microbiomes after removal of rare taxa (ASVs [Amplicon sequence variants] present in < 0.001% mean relative abundance) are represented. Almost all reads (96.67%) were assigned to the Family level of taxonomic depth.

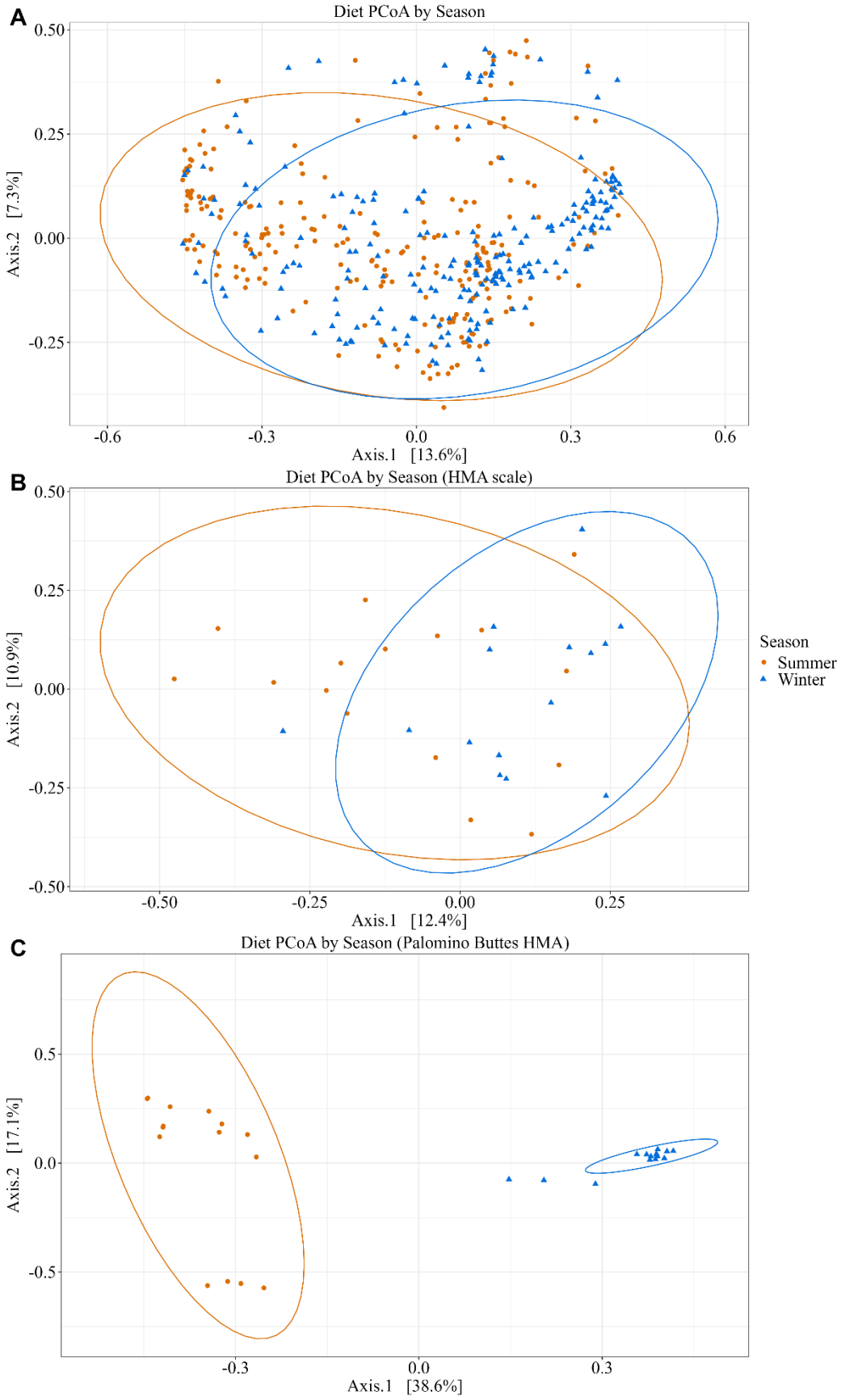
A
Diet PCoA by Season
0.50
0.25
0.00
-0.25
Axis.2 [7.3%]
-0.6
-0.3
0.0
0.3
0.6
Axis.1 [13.6%]
B
Diet PCoA by Season (HMA scale)
0.50
0.25
0.00
-0.25
-0.50
Axis.2 [10.9%]
-0.50
-0.25
0.00
0.25
Axis.1 [12.4%]
Season
Summer
Winter
C
Diet PCoA by Season (Palomino Buttes HMA)
0.5
0.0
-0.5
Axis.2 [17.1%]
-0.3
0.0
0.3
Axis.1 [38.6%]

Figure A3. PCoA ordinations using Bray–Curtis dissimilarity measure of seasonal free-roaming horse diet composition at various scales. Data represent diet composition transformed to relative abundance with rare taxa removed; ellipses show 95% confidence interval. A) Separation of diet by season when considering all individual horses at the range-wide scale. B) Separation of diet by season at the range-wide scale when considering the summer and winter average diet composition of each HMA as the experimental unit. C) Example from one HMA (Palomino Buttes HMA, OR) depicting the separation of diet composition by season at the HMA scale.

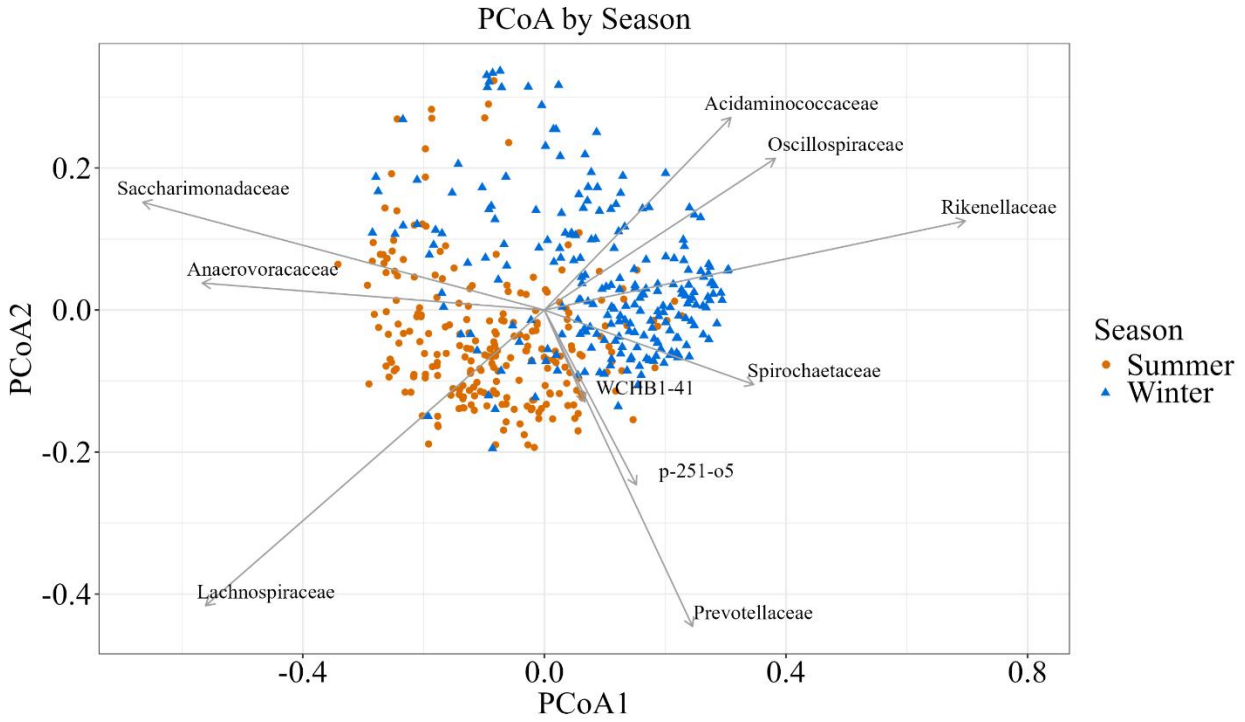

Figure A4. PCoA ordination using Bray–Curtis dissimilarity measure of seasonal free-roaming horse microbiome community composition. Data represents microbial community transformed to relative abundance with rare taxa removed. The ordination depicts all individual horses at the range-wide scale with summer in orange circles and winter in blue triangles. Vectors represent the ten bacterial families that had the greatest contributions to bacterial community dissimilarity.

CHAPTER 5. Field-based methods for fecal particle size analysis and its application in free-roaming horses

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ABSTRACT

Various methods have been used to evaluate diet composition, digestibility, and other aspects of nutrition for grazing herbivores, many of which require specialized feeding trials or expensive lab equipment and processes. Specifically, fecal particle size analysis uses either a wet or dry method to distribute fiber particles from fecal samples among descending sized sieves. In our research, we investigated employing "field-based" versions of wet-sieve fecal particle size analysis to see whether we could observe differences in the fecal particle size of free-roaming horses (*Equus caballus*) residing in different environments and during different seasons. Our research uses methods more amenable to those without expensive lab-based equipment, with a goal of allowing future researchers to perform particle size analysis in a field-based setting as a proxy for digestibility. Using free-roaming horse fecal samples, we investigated the repeatability of this measure in sub-samples of the same original fecal sample. We observed patterns in the distribution of particle weight across the different sized sieves and found the proportion of fecal material in our top sieve was highly correlated with mean particle size. We then developed a simplified one-sieve method using only the largest top sieve from the initial method, while collecting the remaining material in a filter. We found significant differences between some Herd Management Areas (HMAs) and a moderate difference between summer and winter season using these methods. We also detected an interaction between HMA and season, indicating horses from different HMAs exhibited differing patterns in particle size shifts from summer to winter. Our study indicates the scale of interest is critical for understanding these nutritional metrics;

where in the case of horses, we found directional changes in seasonal mean particle size were herd-specific rather than uniform across the range. We also tested the potential of using our measures as herd health biomarkers by investigating relationships between both the traditional particle size protocol and our simplified method with body condition of free-roaming horses and graminoid diet composition. While we found some patterns, results from these investigations were not definitive and our methods likely need further refinement to account for high intra-sample variation before they can prove informative for horse health and diet.

INTRODUCTION

Ungulate herbivores have evolved to subsist on plant material through various digestive strategies (Hanley and Hanley 1982) and these differences in digestive anatomy change how herbivores process forage. Ruminants such as cattle (*Bos taurus*), sheep (*Ovis aries*), and North America's wild ungulate species tend to have a slower passage time and more completely digest their food while hindgut fermenters such as horses (*Equus caballus*) tend to digest food less completely while maintaining a quicker passage rate (Duncan et al. 1990). In ruminants, ingesta must be broken into small particles to pass from the rumen to the rest of the digestive tract, limiting forage intake (Hanley 1982). Hind gut fermenters are not limited by ingesta particle size, and are able to quickly pass food and take advantage of abundant low-quality forage (Janis 1976, Hanley 1982). A study in France found that while cattle digested food more completely, horses ate 63% more (on a per body weight basis) than cattle, allowing them to consume more digestible nutrients per day (Menard et al. 2002). Consuming adequate forage to meet the energetic demands is important in both livestock production and for the long-term survival of wild herbivore populations.

Evaluating digestibility of feed is another way to understand how herbivores meet their energy needs. *In vivo* digestibility measures animal feed inputs and fecal outputs in controlled trials and because the interaction of animals and feed is fully represented, is considered the gold standard in understanding digestibility (Kitessa et al. 1999). *In vivo* tests are often not possible due to intensive time and cost demands or ethical concerns, so various laboratory techniques have been developed to predict *in vitro* digestibility from feed by simulating one or multiple steps of digestion (Boisen and Eggum 1991; Kitessa et al. 1999, Chernukha et al. 2021). However, these *in vitro* assays cannot fully replicate *in vivo* digestion. Methods include incubating samples in rumen fluid, single or multi-enzyme approaches, near infrared reflectance spectroscopy of feedstuffs, measuring pH drop, measuring gas production during fermentation, or combinations of these methods (Boisen and Eggum 1991; Kitessa et al. 1999; Chernukha et al. 2021). Accurate measures of feed intake and fecal outputs in free-roaming herbivores are difficult, making *in vivo* digestibility trials impractical. However, *in vitro* methods are often based around correlations with *in vivo* assessments (Boisen and Eggum 1991), making them difficult to apply accurately in under-studied wild species. A further consideration is that *in vivo* trials on captive animals, while capturing the full effects of the digestive process, cannot replicate the diverse conditions that free-roaming species encounter. Therefore, finding non-invasive ways to represent *in vivo* digestibility from animals in the field is especially important to study the digestive physiology of wild or free-roaming animals.

Particle size distribution of digesta can help elucidate digestive physiology of animals. This method can potentially overcome the challenges of applying digestibility trials to free-roaming animals, as it can be determined from fecal samples alone. Particle size analysis uses sets of sieves with decreasing mesh sizes to study the distribution of fiber particles using the

weight of material remaining on each sieve. A wet, compared to a dry-sieving procedure is better suited to evaluate particle size in feces and digesta contents (Uden and Van Soest 1982). Although results from sieve analysis can be interpreted in many ways including frequency plots or cumulative curves, Fritz et al. (2012) describe a process for calculating the discrete mean measure of particle size and recommend this as a standard way to summarize results of sieve analysis. This method uses the proportion of particle mass remaining on each sieve and the largest measured particle on the largest sieve to calculate the mean particle size and is robust when using different sieve sets, meaning it can provide comparable results between studies using sieves with different mesh sizes or numbers of sieves (Fritz et al. 2012).

Other studies have related fecal mean particle size to important digestive and physiological measures such as chewing efficiency, feed intake, retention time, and fiber digestibility (Clauss et al. 2009, 2014, 2015, Miyaji et al. 2011). A study of over 20 species, including ruminants, camelids, equids, and other hindgut fermenters found a negative correlation between fecal particle size and fiber digestibility when considering all the species together, though this was not significant when controlling for evolutionary history (Clauss et al. 2009). When ruminant and non-ruminant captive herbivores were fed a consistent grass hay diet, an increase in particle size lead to a decrease in fiber digestibility (Clauss et al. 2015). However, when studying ponies fed differing quality hay at various intake levels researchers found that lower quality hay yielded smaller mean particle size than higher quality hay when fed *ad libitum* (Clauss et al. 2014). Similarly, a study in Japan found that horses on a higher quality diet excreted more large fecal particles than horses on a lower quality hay diet (Miyaji et al. 2011). A more recent study in horses found no difference in particle size distribution in normal and reduced lignin alfalfa hays (*Medicago sativa*; higher quality, less fibrous; Grev et al. 2021). It

appears that across a variety of herbivore species larger particles are associated with lower digestibility; however, specifically in horses, larger particles may be associated with higher quality diets.

We chose to focus on horses because of the wealth of existing fecal particle size literature regarding this species (Miyaji et al. 2011; Clauss et al. 2014; Grev et al. 2021) and our access to fecal samples from free-roaming horses inhabiting Bureau of Land Management (BLM) Herd Management Areas (HMAs) in a variety of environments, including the Great Basin, Colorado Plateau, Mojave Desert, and Wyoming Basin. Free-roaming horse management poses a challenge on federal lands in the western United States (Danvir 2018; Davies and Boyd 2019; Scasta et al. 2018; Hennig et al. 2023) and worldwide (Eldridge et al. 2020). Large numbers of free-roaming horses have been shown to cause ecosystem degradation (Beever and Brussard 2000; Beever and Herrick 2006; Beever et al. 2008; Davies et al. 2014; Davies and Boyd 2019; Hennig et al. 2021) and population numbers are currently above their set appropriate management levels in most western states (BLM 2024). Populations are moderated by actions such as administering birth control or through roundups. These actions can be contentious as different stakeholders have conflicting positions on the value of these animals and what proper management entails (Scasta et al. 2018; Davies and Boyd 2019; Hennig et al. 2023). Management actions such as emergency gathers can be triggered by animal health concerns such as low body condition brought about by conditions such as limited forage, drought, or fire. Understanding diet quality through a metric such as fecal particle size could prove useful for managing free-roaming horses and contribute to future decisions regarding which lands would be most suitable to meet goals for free-roaming horse use versus other herbivores including wildlife species or domestic livestock such as cattle and sheep.

Our overarching goal was to design and validate a process for a "field-based" version of the mean particle size analysis described by Fritz et al. (2012). Previous studies relating particle size to digestive physiology (Clauss et al. 2009, 2014, 2015; Miyaji et al. 2011; Grev et al. 2021) were conducted on captive animals and methods involved sensitive laboratory techniques and equipment including vibrating sieve shakers or agitators, desiccators, and controlled flow rates of rinse water (Clauss et al. 2009, 2014, 2015; Miyaji et al. 2011; Grev et al. 2021). In addition, these previous studies fed restricted research diets (Miyaji et al. 2011; Grev et al. 2021; Clauss et al. 2014) to look at the specific effects of forage limitation or quality on fecal particle size, or fed consistent diets to a variety of herbivores to look at differences in species or digestive type (Clauss et al. 2015). While this research has built a substantial base of knowledge surrounding the effects of animal species, feed amount, or feed quality on fecal particle size, the conditions experienced by captive animals differ greatly from their wild or free-roaming counterparts. Free-roaming animals experience changing conditions that can lead to changing types, amounts, and quality of forage. They also experience exposure to pathogens, parasites, and even toxic plants that are oftentimes controlled in their captive counterparts, all which can impact their abilities to obtain proper nutrition from their environments. Here, we use fecal particle size to gain a picture of the digestive process in free-roaming herbivores exposed to these variable conditions.

Simultaneously, we envisioned making the process more suitable to researchers in field-based settings lacking the precision of expensive lab-based equipment. We hypothesized that due to the spatial, environmental, and temporal variations free-roaming horses experience between different HMAs and seasons, our "field-based" approach would still be able to detect differences in fecal particle size. Previous literature about equids indicates we would expect larger fecal particle size with higher quality diets (Miyaji et al. 2011, Clauss et al. 2014). Therefore, using

fecal particle size as a proxy for diet quality, we would expect summer fecal samples to have larger particle sizes compared to winter, as forage is actively growing and would exhibit higher protein and lower fiber than in the winter. However, Clauss et al. (2014) found a non-significant pattern of increasing particle size with decreasing food intake level, so a competing hypothesis would be that particle size could shift higher in seasons or HMAs that have more limitation on forage availability. We also evaluated the relationship between fecal particle size, horse body condition, and the amount of dietary graminoids (grasses and grass-like plants). Because higher quality diets have been associated with larger particle size in horses (Miyaji et al. 2011, Clauss et al 2014), we would expect to find higher body condition associated with larger particle size. Clear relationships of body condition or diet composition to fecal particle size may indicate potential for this technique as a non-invasive biomarker for herd health that could prove useful for management decisions.

We used a three-stage analysis process to validate our methods: In Stage 1, we conducted a small pilot study to assess repeatability of the mean particle size measure among replicates of a sample using a tower of 5 sieves using a modified "field based" version of the traditional mean particle size method described by Fritz et al. (2012). In Stage 2, we used the same method on a larger group of samples and evaluated differences in mean particle size among samples, seasons, and HMAs. In Stage 3, we used a simplified retained proportion method using the proportion of weight on the top sieve as a proxy for mean particle size and compared differences in this measure among seasons and HMAs. Finally, we tested associations between horse metrics and fecal particle size measures.

METHODS

Study Areas

We collected fecal samples from free-roaming horses from 16 Herd Management Areas (HMAs) across the western United States. We selected HMAs from among the 177 BLM HMAs available at that time to represent the geographical scope of free-roaming horse use across the western United States. This process led to the selection of HMAs across a gradient of herbaceous cover availability. Additionally, HMAs were selected based on ease of access by off-road vehicles or access on foot. Our chosen HMAs represented environments in the Great Basin, Colorado Plateau, Mojave Desert, and Wyoming Basin and were located in six distinct Environmental Protection Agency (EPA) level III ecoregions. We consulted with BLM personnel to identify HMAs where horses could be safely accessed during summer and winter field seasons. For further information about study areas please see Table 1 in Buchanan et al. (*in press*).

Sample Collection

In each HMA we collected 15 separate samples of fecal material in summer 2020 (May–August) and winter 2020/2021 (Late November to February) for a total of 465 individual fecal collections. Whenever possible, we collected fecal samples from at least three areas in each HMA to ensure we sampled a representative diversity of horses throughout the geographical gradient of an HMA. We collected fecal material immediately after observed defecations when possible and when we did not directly observe defecations, we collected fecal samples with moist interiors to ensure samples were representative of the current sampling season. From each fecal pile, we obtained fecal material from the interior to minimize environmental contamination and collected at least three fecal boluses to get a representative sample for each defecation. For each collection, we turned a clean plastic bag inside out to collect the sample without touching the bag's interior and then returned bags to proper orientation and sealed them. We stored

bagged fecal material on dry ice while in the field and samples were transferred as quickly as possible (≤2 weeks) to a -20°C freezer.

While in the field, we also made observations of body condition scores of free-roaming horses in each HMA. Body condition was assigned according to the Henneke system, which rates a horse's accumulation of subcutaneous adipose tissue on a 1-9 scale, with 1 representing an extremely emaciated animal and 9 representing an extremely fat animal (Henneke et al. 1983). This method involves observations of fat accumulation at the lumbar spinous processes, ribs, tailhead, neck, withers, and area behind the shoulder. While the Henneke method calls for observations based on visual assessment and palpation, we were only able to make visual observations. However, this method has previously been applied on a visual only basis in free-roaming horses (Schoenecker et al. 2024). We observed body condition scores by eye when horses were close (≤15 m), or through binoculars (8 x 42 magnification) or spotting scope if animals were farther away (16–48 magnification). We assigned body condition scores to horses that were within 400 m and unobscured (not standing behind another animal, rocks, or vegetation) and spent additional time observing when possible, to view animals from different angles and obtain a more accurate score based on all body regions of interest. In most of the HMAs we scored every observable free-roaming horse to achieve a larger sample size, however in some HMAs large herds (>100 horses) were present and we systematically scored every fifth horse in those instances. Body condition score observations, while potentially subjective, were consistent between HMAs as a single researcher made all observations.

Sieving

We used a selection of our horse fecal collections to test our various "field-based" particle size sieve methods. We subsampled frozen fecal material from each selected fecal collection to ~ 5g

wet weight and dried at 60 °C in a drying oven for 48 hours. From the dried fecal material from each horse, hereafter referred to as a "sample," we created replicates of ~400 mg dry weight, hereafter referred to as "subsamples," and placed each of these ~400 mg subsamples into a 50-ml plastic tube. Prior to sieving, we added 50 ml of water to each tube, shook by hand, and allowed the subsample to dissociate for at least 12 hours.

For our traditional mean particle size method (hereafter "traditional"), we assembled a stacked set of sieves using a Geotech sand shaker mechanical sieve field analysis kit (part number 11450022) and selected a descending size order of sieves 187 OPN (opening size in thousandths of an inch), 72 OPN, 26 OPN, 09 OPN, and 041 OPN (4.75 mm, 2.29 mm, 1.01 mm, 0.58 mm, 0.10 mm). The metal sieves were stacked in order between plastic stackable compartments to contain the water during the wet sieving process. We shook each tube containing a fecal subsample by hand again prior to sieving to re-suspend fecal particles and then poured wet fecal material through a funnel over the sieve set. We then used 100 ml of water to rinse any remaining particles from the 50 ml container and the funnel. We next used tweezers to gently break up any remaining large clumps on the top sieve without splitting any large particles. Then we dispensed 200 ml of water through the shower attachment of a HydraPak® water bladder to rinse any remaining small particles through the top sieve. We controlled the flow rate of rinse water by consistent use of the same shower attachment of a HydraPak® water bladder to dispense a consistent volume of rinse water. Once all water had drained from the sieve stack, we separated out the metal sieves from the plastic apparatus and placed the sieves into pre-weighed weigh tins. Each tin containing a sieve with wet sample was dried at 60 °C for 24 hours and then allowed to acclimate to ambient lab humidity for 1 hour. We then weighed weigh tins (~2.5g) containing sieves and measured the length (mm) of the largest particle on the top sieve using

calipers. We then subtracted the weight of the metal sieve and weigh tin from the total weight to calculate the weight of fecal material on each sieve for each sub-sample. From there we calculated the proportion of total weight on each sieve and then inputted those values into the formula from Fritz et al. 2012 (below) to determine traditional mean particle size (hereafter "MPS") of each subsample.

$$d\text{MEAN} = \sum_{i=1}^{n} p(i) * \frac{S(i+1) + S(i)}{2}$$

Where $p(i)$ = the proportion of particle mass on each sieve, $S(i)$ = the sieve mesh size, $S(i + 1)$ = the mesh size of the next largest sieve, S(1) = minimum sieve size, and S(n) = maximum sieve size.

For our simplified retained proportion particle size method (hereafter "simplified") we used the same Geotech shaker set but only used the 187 OPN (4.75 mm) sieve. We positioned the sieve tower so that any flow-through from the top sieve would be caught in a pre-weighed paper coffee filter held in a mesh strainer to allow water to slowly drain but capture fecal particles. As in the traditional method, we took soaked subsamples in 50 ml tubes and shook them by hand prior to sieving to re-suspend particles. We then poured the soaked subsample through a funnel onto the sieve tower and rinsed as previously described. Once the water had drained from the sieve and filter, we placed each sieve and filter into pre-weighed weigh tins and dried at 60 °C for 24 hours and then allowed them to rest one hour to acclimate to average lab humidity before being weighed. We subtracted the weight of the sieve or filter and weigh tin from the total weight leaving the weight of fecal material on the top sieve and in the filter. From there we calculated the retained proportion of weight on the 187 OPN sieve (hereafter "RP"), and this measure was used to represent mean particle size in subsequent analyses. See Appendix A for photos of the procedures for both methods.

Statistical Analysis

Because we were validating new methods, we completed our analysis in a 3- stage process with a combination of the traditional and simplified sieving methods described previously. All statistical analyses were conducted in Program R (Version 4.3.0; R Core Team, 2023).

For Stage 1, we ran a small pilot study of four subsamples of nine samples, each from a different HMA to evaluate repeatability of the traditional method of calculating mean particle size (MPS) across replicates of a single sample from fecal samples sourced from a variety of environments. We measured the variation in replicates of the same sample by evaluating the coefficient of variation (CV), calculated as the (standard deviation/mean) *100. We also investigated what aspects of the particle size calculation drove the variation in MPS in horses by comparing the CV of the largest particle measurement and the proportion of fecal material weight on the largest sieve within replicates of a single sample. We also visualized the pattern of distribution of particles across the various sieve sizes in subsample replicates of each sample.

In Stage 2, we used the traditional method again to measure MPS for five subsample replicates of five samples from three HMAs in both summer and winter. This yielded a total of 30 distinct samples analyzed and 150 total subsample MPS measures (five subsample replicates of each sample). Before averaging subsample MPS replicates to obtain a single MPS value for each sample, we used our five subsample replicate MPS values of each sample as the experimental unit to investigate if we could find a difference in MPS between individual samples using a Kruskal-Wallis test. We used Dunn's *post hoc* tests with Bonferroni corrections to test pairwise comparisons. Next, due to the high variation inherent in our data, we averaged the MPS value for the five subsample replicates of each sample together to get one MPS value per sample for comparative analysis between seasons and HMAs. We then used Kruskal-Wallis tests to

examine differences between different HMAs and seasons. When a significant result was found we used Dunn's *post hoc* tests to determine which pairs were different using Bonferroni corrections for multiple comparisons.

For Stage 3, we investigated differences in HMA and season further with a larger sample size including more HMAs. However, in line with our goals of developing an efficient field-based method, we wanted a method to maximize efficiency. Therefore, we investigated the relationships of different components of the MPS calculation including retained proportion of weight on the top sieve (RP), largest particle, and overall MPS to simplify the sieving procedure, and potentially reduce variation in our samples, while maintaining enough sensitivity to detect changes. We conducted Pearson's correlations (r) to investigate the relationship between the largest particle on the top sieve, RP, and MPS.

This led us to our simplified method using one sieve (size 187 OPN, 4.75 mm) and a paper coffee filter, to allow us to investigate MPS in a greater number of samples using RP as a proxy for MPS. In this third stage of analysis, we ran three subsample replicates of six samples from eight different HMAs in both summer and winter seasons. We then averaged the three subsample replicate values for RP together to get one RP value for each individual sample. This yielded 98 distinct samples analyzed (an extra sample was inadvertently run in two HMAs) and 294 total subsample RP measures (three subsample replicates of each sample). We used Kruskal-Wallis tests to investigate differences in RP between seasons and HMAs. We used Dunn's *post-hoc* tests with Bonferroni corrections to check pairwise comparisons when Kruskal-Wallis results indicated differences existed.

Lastly, we investigated whether the fecal particle size measures in our study were relevant to overall horse health or nutrition by comparing both MPS and RP to body condition

scores (BCS) and diet composition of horses. We investigated whether fecal particle size measures gained from using our traditional or simplified methods could be related to these nutritional and health measures in horses at an individual or herd level to examine the relative value of MPS or RP as a potential biomarker of use in free-roaming horse management.

We chose to evaluate the relationship between BCS and fecal particle size with only the larger dataset using the simplified RP method. We based our decision on lack of sample size and variability in both herd average BCS values and samples from horses of known body condition for the dataset using the traditional MPS method. Of the fecal samples we evaluated using the traditional MPS method, only 18 were from horses of a known body condition and 14 of these 18 horses had a BCS of 5. In addition, HMA herd averages were close to 5 across different HMAs (for more detail see results in Buchanan et al. *in press*). Using the dataset of RP values, we conducted Spearman's rank (r_s) correlations using 51 (of 98) samples with a known body condition score to investigate the relationship of each sample's average PROP across three subsample replicates to the corresponding horse's BCS. In addition, we separated our data into just summer (n = 19) or just winter (n = 32) and used Spearman's rank (r_s) correlations to evaluate the relationship between RP and HMA at the individual scale between seasons. Next, we averaged all the individual sample values for RP for each HMA and computed Spearman's rank (r_s) correlations between the HMA average RP and the average BCS of all horses observed in that HMA. We first ran correlations with the six summer, and six winter RP values averaged together to form one average RP for each HMA correlated against all horse BCS observations in each HMA (n =8) averaged together. We also conducted a correlation with summer and winter observations averaged separately so both a winter and summer BCS and RP for each HMA were included in the correlation (n = 16). Finally, we separated the data and conducted Spearman's

rank (r_s) correlations with summer (n = 8) and winter (n = 8) HMA averages for BCS and RP in separate correlations.

Finally, we compared whether the MPS when using the traditional method or RP when using the simplified method was related to the percentage of graminoids found in the diets of free-roaming horses using Spearman's rank (r_s) correlations. As previously described with body condition, we conducted these correlations for individual horses including both seasons together in a correlation (n = 30 MPS, n = 98 RP), and for summer (n = 15 MPS, n = 49 RP) and winter (n = 15 MPS, n = 49 RP) seasons separately. In addition, we investigated seasonal differences in RP in subsets of horses on the extreme ends of graminoid dietary composition. We conducted Kruskal-Wallis tests to determine whether summer and winter values were different in either high graminoid (horses consuming ≥ 75 % graminoids) or low graminoid (horses consuming ≤ 25% graminoids) horses. We did not perform these tests for MPS or for herd averages due to smaller sample sizes. Next, we investigated the relationship between the percentage of graminoids in the diet and MPS or RP at the herd level, using each HMA's average diet composition of graminoids with herd average RP. As with the body condition correlations, we ran these correlations using both seasons combined into one average per HMA (n = 8), using both season averages in the same correlation (n = 16), and with separate correlations of summer (n = 8) or winter (n = 8) averages. Values for the percentage of graminoids in free-roaming horse diets were obtained from DNA metabarcoding of fecal material for diet composition. Briefly, we submitted fecal material for each sample to the Genome Technologies Lab at the University of Wyoming for DNA extraction and library preparation. The p6 loop of the *trnL* locus was amplified and outputs from this process were sequenced at the University of Colorado Genomics Core. We used the STAND program (Weinstein et al. 2021) to classify read sequences against

NCBI GenBank entries for plant chloroplast sequence. We read results of the stand pipeline into Program R (Version 4.3.0; R Core Team, 2023), where we filtered reads to exclude any rare amplicon sequence variants (ASVs) that were not present with at least 1% abundance in at least one horse. We pooled ASVs at the family level, converted reads to relative abundance, and then combined the reads for the three grass and grass-like families (Poaceae, Juncaceae, and Cyperaceae) to obtain the composition of graminoids present in the fecal material from each horse. For further details on the protocols for obtaining graminoid composition, please see Buchanan et al. (*in press*)

RESULTS

Stage 1: Pilot Study using the traditional method

In our initial pilot study, we found mean particle size (MPS) of subsample replicates ranged from 2.56 to 8.26 mm, and when subsample replicates were averaged together, we found average MPS values for each sample ranged from 3.76 to 7.25 mm. We found that there was high variation in the MPS for replicates of an individual sample (Figure 1). Coefficients of variation among four subsample replicates of a sample ranged from 7.30 to 47.42 with an average among the nine samples of 24.00. We also saw high variation in the measure of largest particle (mm) among sample repeats (Figure 2), with CV ranging from 7.22 to 66.27 for an individual sample and an average CV of 21.82 across the nine samples. The CV values for proportion of weight on the top sieve for repeats of the same sample ranged from 13.32 to 45.92 with an average CV for all nine samples of 32.12. The largest particle size and proportion of fecal material weight on the top sieve are both key parts of the MPS calculation we used (outlined in Fritz et al. 2012), so it is logical for high variation in these measures to translate into high variation in MPS. We did notice that the pattern of particle size distribution varied among samples, and this often stayed

consistent across subsample replicates (Figure 3). This showed potential for differences existing among HMA locations and prompted continuation to a larger run of data with multiple samples per HMA despite the observed high variation.

Stage 2: Comparing MPS using the traditional method

In our larger test of samples with the traditional method, values (mm) for MPS among subsample replicates ranged between 2.32 and 11.39. When the five subsample replicates were averaged to get a single value for each sample, MPS values ranged from 2.97 to 8.06. We again saw high levels of variation within the five subsample replicates of our 30 individual samples, with CV values (mm) of samples ranging from 6.47 to 48.58 with an average CV of 19.96. We noted a significant difference among individual samples ($\chi^2 = 92.347$, $p < 0.001$; Figure 4) and *post hoc* analysis indicated 12 pairwise comparisons of individual samples were different ($p < 0.05$).

Differences were not as clear when samples were averaged at the herd HMA level. There were moderate differences in MPS between HMAs ($\chi^2 = 5.515$, $p = 0.063$) with the McCullough Peaks, Wyoming HMA showing moderate difference from the Pine Nut Mountains, Nevada HMA ($z = 2.159$, $p = 0.093$) in *post hoc* comparisons. There was no difference in MPS between seasons ($\chi^2 = 0.073$, $p = 0.788$), or when each combination of season and HMA was considered separately (Figure 5; $\chi^2 = 8.778$, $p = 0.118$). In addition, when we subset data to each individual HMA to evaluate the effect of season within an HMA, we did not find a seasonal difference in McCullough Peaks ($\chi^2 = 0.535$, $p = 0.465$, Table 1) or Pine Nut Mountains ($\chi^2 = 0.884$, $p = 0.347$, Table 1) and detected only a moderate seasonal difference in the South Steens, Oregon HMA ($\chi^2 = 3.153$, $p = 0.076$, Table 1). Also of note, there was not a consistent pattern of increase or decrease in MPS from summer to winter in our three HMAs (Figure 5), with samples from South Steens tending toward smaller particle size in winter while McCullough Peaks and Pine Nut

Mountains tended toward larger particle size in the winter. When we ran an ANOVA including both HMA and season as factors with an interaction between HMA and season we found a moderate significance for the interaction of HMA and season ($p = 0.065$), further pointing toward the possibility that MPS was affected by season differently in distinct HMAs.

When evaluating the variation and relationships in different components of the MPS calculation we found that there was again high variation in measures of largest particle and the proportion of weight on the top sieve. Among runs of subsample replicates, measures of largest particle ranged from 10.41 to 58.47 mm and once the five subsamples were averaged to one value for each sample, values ranged from 14.84 to 30.71 mm. The CV for each sample's largest particle ranged from 8.20 to 50.75 with an average of 24.92. The retained proportion of weight on the top sieve (RP) ranged from 0.03 to 0.60 among subsample replicates and ranged from 0.11 to 0.51 for sample averages of the five replicates. RP varied among replicates with a CV of 14.59 to 72.50 for individual samples, and an average of 31.00. RP was strongly correlated with mean particle size for subsample replicates ($r = 0.864$, $p < 0.001$; Figure 6) and sample averages ($r = 0.957$, $p < 0.001$; Figure 6). The correlation with MPS and largest particle was not as strong ($r = 0.567$ $p < 0.001$). Therefore, we chose to base our simplified method on the proportion of weight captured on the top sieve rather than the largest particle although there was more variation in the RP measure.

Stage 3: Comparing RP using the simplified method

When using the simplified sieve method we found among subsample replicates the proportion of weight on the top sieve (RP) ranged from zero to 0.66 and once the three subsample replicates were averaged for each sample values ranged from 0.001 to 0.62. Using proportion of weight on the top sieve as a proxy of mean particle size (MPS) we once again saw large variation in

subsample replicates. The CV for individual samples ranged from 4.99 to 173.21 with an average of 43.27.

Despite this large variation, we found significant differences between some HMAs (χ^2 = 34.521, p <0.001, Figure 7A). Through our *post hoc* analysis, we found Saylor Creek, Idaho and North Hills, Utah HMAs to be different from one another (z = -3.209, p = 0.037) and Little Book Cliffs, Colorado to be different from the North Hills HMA (z = 4.408, p < 0.001), the Onaqui, Utah HMA (z = 4.214, p < 0.001), and the Red Rock, Nevada HMA (z = 3.829, p = 0.004). We also found a difference between summer and winter (χ^2 = 3.944, p= 0.047, Figure 7B) with summer values being higher on average. There was also an important interaction between season and HMA (Figure 8) with the interaction p-value significant in an ANOVA including season, HMA and the interaction between season and HMA (p = 0.001 for the interaction term). As we saw in our run with fewer HMAs using the traditional MPS protocol, samples from different HMAs exhibited differing patterns in particle size shift from summer to winter. The areas of McCullough Peaks and Pine Nut Mountains tended toward higher particle size in the winter while Little Book Cliffs, Stinkingwater (Oregon), Saylor Creek, Red Rock, North Hills, and to a lesser degree Onaqui tended toward higher particle size in the summer. When we subset data to a single HMA at a time and tested for seasonal differences we saw a difference (p < 0.05) in Little Book Cliffs, North Hills, Pine Nut Mountains, Saylor Creek, and Stinkingwater, a moderate difference (p = 0.086) in Red Rock, and no difference in McCullough Peaks or Onaqui (Table 1).

Comparisons of particle size with horse metrics

We did not find strong evidence for a relationship between individual or herd average body condition score (BCS) and RP values (Appendix B). Individual horse MPS was not correlated with the percentage of graminoids in individual horses' diets (r_s = 0.055, p= 0.774, Table 2).

There was also no support for this relationship when investigating just summer or just winter samples from individuals (Table 2). When using RP as a proxy for particle size in individual horses, RP was not significantly correlated with the percentage of graminoids in horse diet (p = 0.696, Table 2). However, we found support for a relationship between dietary graminoids and RP when summer and winter samples from individual horses were considered in separate correlations. In the summer there was a positive correlation between percentage graminoids in diet and RP (r_s = 0.318, p= 0.026, Table 2, Figure 9) while in the winter this relationship was negatively correlated (r_s = -0.298, p= 0.038, Table 2, Figure 9). In addition, we found that individual horses with a high amount of graminoids in their diets (≥ 75 %) had higher values for RP in the summer compared to winter (χ^2 = 11.413, p = 0.001) while horses with low dietary graminoids (≤ 25%) did not exhibit a seasonal difference in RP (χ^2 = 0. 444, p = 0.505; Table 3). Correlations of herd average dietary graminoids and herd average RP did not produce any support for relationships at the herd level (Table 2).

DISCUSSION

Our comparisons provide much new knowledge about an efficient field-based method that could be used as a proxy for *in vivo* dietary digestibility or to compare with other measures of nutrition in rangeland herbivores. Despite high variation in both mean particle size (MPS) and retained proportion of weight in the top sieve (RP) in free-roaming horse fecal samples, we were still able to detect significant differences among individual horses, HMAs, or seasons using both our traditional and our simplified protocol. Also of note, values for MPS tended to be higher than in previous studies in horses and other equid species. Previous studies have found MPS ranging from 0.65 mm to 1.96 mm in captive equids and 0.73 to 2.97 mm specifically in horses and ponies (Clauss et al. 2009; Fritz et al. 2009; Miyaji et al. 2011, Clauss et al. 2014; Clauss et al.

2015; Grev et al. 2021). This could be due to differences in methodology such as larger volumes of rinse water (Clauss et al. 2014; Clauss et al. 2015), using dried rather than wet feces (Grev et al. 2021), or using sieve shakers (Miyaji et al. 2011; Clauss et al. 2014; Clauss et al. 2015; Grev et al. 2021). These previous studies were based on domestic or captive zoo animals that were fed more controlled diets, often consisting of hay, while study animals in our research were free-ranging and consuming varied diets available on the range, another factor that may have contributed to the larger MPS we observed in our study.

Using the traditional method, we did not find any significant differences in MPS between season or HMA, although some moderate differences were identified. However, when the added efficiency of the simplified method allowed us to run a greater number of samples in additional HMAs, more differences became evident. When using our simplified method we found seasonal differences in RP, a proxy for fecal particle size, in five of eight HMAs, and a moderate difference in one other HMA. We also found average RP was higher in the summer at the range-wide scale, when samples from all HMAs were averaged together. Contrary to the general tendency, one HMA (Pine Nut Mountains) had higher RP values in the winter. A potential explanation for this may be food limitation in the winter, as horses in a previous study were found to shift toward higher particle size when on restricted diets (Clauss et al. 2014), however without information about seasonal total forage consumption we cannot know for sure. However, most HMAs exhibited higher particle size in the summer, which supports our hypothesis that horses in the summer would consume higher quality forage leading to larger particle size in the summer. This is opposite to the pattern observed between forage digestibility and particle size in herbivores in general (Clauss et al. 2009, 2015) and seems counter intuitive, but is consistent with the fecal particle size literature in horses (Miyaji et al. 2011; Clauss et al. 2014). Horses

have higher chewing intensity when consuming higher fiber, lower quality forage (Janis et al. 2010), which may explain this phenomenon. It is possible that increased chewing on fibrous feeds may increase surface area of particles and allow for more nutrient absorption, or simply that more fibrous forage needs to be chewed more to be swallowed. Horses are less limited than ruminants by the need to reduce particle size to allow digestive passage and can be successful on low quality forage through their high intake, fast throughput, and low efficiency digestive strategy (Janis 1976). Due to the faster digestive throughput in horses, particle size may not be drastically reduced after chewing, regardless of digestibility, leading to smaller particle size on feed that is more fibrous.

Our attempts to relate RP to body condition scores did not produce strong results at the HMA or individual level. The absence of robust results relating fecal particle size to body condition could be due to lack of variation in body condition scores, with the majority of horses of known body condition (40 out of 51) scoring a BCS of 5, and herd average BSCs all close to 5. A mismatch in temporal scale could also affect our results. We measured fecal particle size, interpreted as a proxy for diet digestibility, from fecal samples that represent one discrete point in time. However, it would take the compounded effects of consistent high or low diet digestibility over a longer time scale to have a meaningful effect on body condition score.

We did find support for the relationship of RP to the amount of graminoids in the diet of free-roaming horses at the individual scale. In the winter, we found that higher dietary graminoid components were associated with lower values for RP while in the summer, higher graminoid dietary component was related to higher RP. This oppositional seasonal effect could be explained by differences in seasonal diet composition—we found in a parallel study that summer diets tended to have more graminoids overall whereas winter diets often included more plants in the

Asteraceae and Chenopodiaceae families (Buchanan et al. *in press*). Alternatively, as graminoids still tended to make up the largest component of diets in most of these horses (Buchanan et al. *in press*) and in previous studies (Scasta et al. 2016), the opposing relationship could relate to the seasonal changes in forage quality of grasses specifically. The protein content of rangeland grasses tends to decrease with maturity while the cellulose content (fiber) tends to increase with maturity (Kamstra 1973). Therefore, during the summer growing season, horses eating higher proportions of graminoids could be eating lower fiber diets, which previous literature has shown to be linked to higher MPS (Miyaji et al. 2011; Clauss et al. 2014). By winter, grasses have matured and contain more fiber, meaning horses eating more graminoids could have lower quality diets, possibly explaining smaller fecal particle size. Indeed, our findings specifically within the group of horses with high (≥ 75%) dietary graminoids also supports this notion. Horses with graminoid-dominated diets had higher RP in the summer (indicating larger particle size and potentially higher quality forage) when compared to horses consuming graminoid dominated diets in the winter, reflecting the seasonal phenology of graminoid forage quality. This seasonal shift in RP was not present when considering only individual horses consuming low graminoid (≤ 25%) diets, possibly indicating there was not this shift in diet quality for horses consuming other forage types. Also of interest, in three of the four HMAs where we had strong evidence for higher RP in the summer, we also observed in a parallel study (Buchanan et al. *in press*) that there was strong or moderate support for seasonal differences in graminoid diet composition with summer diets containing higher amounts of graminoids. Further studies are needed to understand the mechanisms behind this contrasting seasonal relationship of particle size and dietary graminoid consumption in free-roaming horses.

Our study also demonstrates the importance of evaluating findings at the proper scale of interest. Directional changes in seasonal fecal particle size, whether statistically or moderately significant, were HMA-specific, with some HMAs exhibiting higher MPS or RP values in the winter and others displaying higher summer values. These differences could be due to environmental differences in HMAs such as forage type or quality, overall forage availability, or elevation. Differences in the horses within each HMA including relatedness or herd genetics could also influence forage choices, digestibility, and subsequent particle size. Indeed, digestibility can be affected by many factors such as animal genetics, environment, or management (Boisen and Eggum 1991). Therefore, if fecal particle size were to be used as a herd health biometric, management decisions based on this metric may be more effective at smaller scales versus applying across-the-board generalizations at a range-wide scale. Also of note, differences in individual samples were often stronger than differences between herds (HMAs). In Stage 2, using the traditional method we were able to detect differences in MPS between individual samples, both in different HMAs as well as between animals in the same HMA. These differences also occurred in Stage 3 but were not significant once multiple comparisons were made, likely due to the larger number of samples and comparisons to correct for. Therefore, there is potential that fecal particle size metrics may be even more valuable to understand differences in diet digestibility between individual animals in addition to digestibility at the herd level.

Despite high within-sample variability, our methods offer some promise as an efficient field ready measure that may help understand diet digestibility in grazing herbivores. Using these methods, we were able to detect individual, HMA, and seasonal differences in fecal particle size and relate RP to dietary graminoid composition in individual horses. With that said, we also recommend that future researchers employing this technique make efforts to reduce the

variability between replicates of the same sample, or account for variability by running more replicates per fecal sample. Lower variation would make differences between individuals, seasons, or HMAs more effective and efficient to detect. It is possible that this is the reason previous studies have used more sensitive lab techniques, and applying a field-based technique may inherently cause more variation among replicates. However, using a larger amount of fecal material could potentially reduce replicate variability, as a more representative sample of each animal's defecation could be used. Horses produce large amounts of fecal material, with one study finding a mean fecal output of 4.6 kg of feces per 100 kg of body weight per day for pastured horses with a fecal dry matter content of 18.7% (Williams et al. 2014). Therefore, an average 450 kg horse would produce about 20 kg or just under 4 kg of dry fecal matter per day. Our methods using only 400 mg of dried fecal material per subsample may not have been large enough to be truly representative, leading to the large within-sample variation we observed. We chose to use 400 mg for our methods after some preliminary testing indicated that larger amounts of fecal material tended to clog our sieve set, however future research could accommodate larger amounts of fecal material by having larger sieve sets. Indeed, other studies have used 50 g of dried or 100 g of wet fecal material to evaluate fecal particle size distribution in horses (Miyaji et al. 2011; Grev et al. 2021). This consideration may be more important in horses as compared to a smaller animal that produces less total fecal material. Conversely, the equine digestive strategy of low efficiency, high intake, and quicker rate of passage (Janis 1976; Hanley 1982; Duncan et al. 1990) could mean that horses have inherently variable fecal particle size compared to other species. When using this method in the future, researchers should make considerations for species of interest, type of digestive system, and amount of fecal material used relative to the amount produced by the animal.

IMPLICATIONS

Horses can thrive on poor quality forage, while ruminants have a threshold of dietary fiber past which they cannot sustain themselves (Janis 1976). This dietary advantage may contribute to free-roaming horse success in low quality forage environments that are unable to sustain other grazing species. Free-roaming horse populations have increased dramatically across the western United States and can grow 20% per year (Garrott et al. 1991), therefore understanding factors contributing to their continued population growth is important to managers. While many methods to investigate diet composition, quality, or digestibility require intensive studies of captive animals, collection of fecal samples is possible without disturbing, capturing, or stressing animals. The non-invasive methods using fecal samples we used are amenable to a species such as free-roaming horses whose management is constantly scrutinized by the public. If proof of concept for these methods are established in other species, this technique could also prove useful for understanding dietary digestibility or quality in other free-roaming wildlife species, especially during a sensitive time such as winter when they are often already metabolically stressed. If diet digestibility could be monitored non-invasively through a method such as ours, it would allow managers to track diet quality changes and know when animals are in danger of enduring physiological stress due to poor nutrition, with fecal particle size a potential signal of early dietary stress. Identifying timing of diet quality changes could inform managers when wildlife protection areas need to be closed to the public to reduce animal stress or when supplemental feeding programs are needed during seasons of low forage quantity or quality or after events such as fires or droughts limit forage and cause nutritional stress. While our methods need further refinement to be useful in a management setting, they offer promise to help understand animal dietary digestibility in grazing herbivores such as free-roaming horses.

REFERENCES

Beever, E.A., Brussard, P.F., 2000. Examining ecological consequences of feral horse grazing using exclosures. West. North Am. Nat. 60, 236–254.

Beever, E.A., Herrick, J.E., 2006. Effects of feral horses in Great Basin landscapes on soils and ants: Direct and indirect mechanisms. J. Arid Environ. 66, 96–112. https://doi.org/10.1016/j.jaridenv.2005.11.006

Beever, E.A., Tausch, R.J., Thogmartin, W.E., 2008. Multi-scale responses of vegetation to removal of horse grazing from Great Basin (USA) mountain ranges. Plant Ecol. 196, 163–184. https://doi.org/10.1007/s11258-007-9342-5

Boisen, S., Eggum, B.O., 1991. Critical evaluation of in vitro methods for estimating digestibility in simple-stomach animals. Nutr. Res. Rev. 4, 141–162. https://doi.org/10.1079/nrr19910012

Buchanan, C.E., Galla, S.J., Studyvin J., Lautenbach, J.D., Scasta, J.D., Randolph G.D., Forbey, J.S., Beck, J.L. Free-roaming horse diet and body condition differences across seasons and ecologically diverse herd management areas. Rangel. Ecol. Manag. *In press.*

Bureau of Land Management, 2024. Herd Area and Herd Management Area Statistics 2024. https://www.blm.gov/sites/default/files/docs/2024-03/2024_HMA-HA_PopStats_2-29-2024_COMBINED_Clean_FINAL_web.pdf (accessed: 27 September 2024)

Chernukha, I.M., Meliashchenia, A. V., Kaltovich, I. V., Vasilevskaya, E.R., Aryzina, M.A., Smaliak, T.M., Senchenko, T. V., Fedulova, L. V., 2021. Evolution of methods for *in vitro*

product digestibility analysis: a systematic review. Theory Pract. Meat Process. 6, 300–310. https://doi.org/10.21323/2414-438X-2021-6-4-300-310

Clauss, M., Nunn, C., Fritz, J., Hummel, J., 2009. Evidence for a tradeoff between retention time and chewing efficiency in large mammalian herbivores. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 154, 376–382. https://doi.org/10.1016/j.cbpa.2009.07.016

Clauss, M., Schiele, K., Ortmann, S., Fritz, J., Codron, D., Hummel, J., Kienzle, E., 2014. The effect of very low food intake on digestive physiology and forage digestibility in horses. J. Anim. Physiol. Anim. Nutr. (Berl). 98, 107–118. https://doi.org/10.1111/jpn.12053

Clauss, M., Steuer, P., Erlinghagen-Lückerath, K., Kaandorp, J., Fritz, J., Südekum, K.H., Hummel, J., 2015. Faecal particle size: Digestive physiology meets herbivore diversity. Comp. Biochem. Physiol., Part A. 179, 182–191. https://doi.org/10.1016/j.cbpa.2014.10.006

Danvir, R.E., 2018. Multiple-use management of western U.S. rangelands: Wild horses, wildlife, and livestock. Human-Wildlife Interact. 12, 5–17. https://doi.org/10.26077/cz0b-6261

Davies, K.W., Collins, G., Boyd, C.S., 2014. Effects of feral free-roaming horses on semi-arid rangeland ecosystems: An example from the sagebrush steppe. Ecosphere 5. https://doi.org/10.1890/ES14-00171.1

Davies, K.W., Boyd, C.S., 2019. Ecological effects of free-roaming horses in North American rangelands. Biosci. 69, 558–565. https://doi.org/10.1093/biosci/biz060

Duncan, P., Foose, T.J., Gordon, I.J., Gakahu, C.G., Lloyd, M., 1990. Comparative nutrient extraction from forages by grazing bovids and equids: a test of the nutritional model of

equid/bovid competition and coexistence. Oecologia 84, 411–418. https://doi.org/10.1007/BF00329768

Eldridge, D.J., Ding, J., Travers, S.K, 2020. Feral horse activity reduces environmental quality in ecosystems globally. Biol. Conserv. 241, 108367. https://doi.org/10.1016/j.biocon.2019.108367

Fritz, J., Streich, W.J., Schwarm, A., Clauss, M., 2012. Condensing results of wet sieving analyses into a single data: A comparison of methods for particle size description. J. Anim. Physiol. Anim. Nutr. 96, 783–797. https://doi.org/10.1111/j.1439-0396.2011.01183.x

Fritz, J., Hummel, J., Kienzle, E., Arnold, C., Nunn, C., Clauss, M., 2009. Comparative chewing efficiency in mammalian herbivores. Oikos 118, 1623– 1632. https://doi.org/10.1111/j.1600-0706.2009.17807.x

Garrott, R.A., Siniff, D.B., Eberhardt, L.L., 1991. Growth rates of feral horse populations. J. Wildl. Manage. 55, 641–648. https://www.jstor.org/stable/3809513

Grev, A.M., Hathaway, M.R., Sheaffer, C.C., Wells, M.S., Reiter, A.S., Martinson, K.L., 2021. Apparent digestibility, fecal particle size, and mean retention time of reduced lignin alfalfa hay fed to horses. J. Anim. Sci. 99, 1–10. https://doi.org/10.1093/jas/skab158

Hanley, T.A., 1982. The nutritional basis for food selection by ungulates. J. Range Manag. 35, 146–151.

Hanley, T.A., Hanley, K.A., 1982. Food resource partitioning by sympatric ungulates on Great Basin rangeland. J. Range Manag. 35, 152-158. https://doi.org/10.2307/3898380

Henneke, D.R., Potter, G.D., Kreider, J.L., Yeates, B.F., 1983. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet. J. 15, 371–372. https://doi.org/10.1111/j.2042-3306.1983.tb01826.x

Hennig, J.D., Beck, J.L., Duchardt, C.J., Derek Scasta, J., 2021. Variation in sage-grouse habitat quality metrics across a gradient of feral horse use. J. Arid Environ. 192, 104550. https://doi.org/10.1016/j.jaridenv.2021.104550

Hennig, J.D., Duchardt, C.J., Esmaeili, S., Fuhlendorf, S.D., Beck, J.L., Francisco, T.I., Scasta, J.D., 2023. A crossroads in the rearview mirror: the state of United States feral equid management in 2023. Biosci. 73, 404–407. https://doi.org/10.1093/biosci/biad033

Janis, C., 1976. The evolutionary strategy of the Equidae and the origins of rumen and cecal digestion. Evolution. 30, 757-774. https://doi.org/10.2307/2407816

Janis, C.M., Constable, E.C., Houpt, K.A., Streich, W.J., Clauss, M., 2010. Comparative ingestive mastication in domestic horses and cattle: A pilot investigation. J. Anim. Physiol. Anim. Nutr. 94, e402-e409. https://doi.org/10.1111/j.1439-0396.2010.01030.x

Kamstra, L.D., 1973. Seasonal changes in quality of some important range grasses. J. Range Manag. 26, 289-291. https://doi.org/10.2307/3896580

Miyaji, M., Ueda, K., Hata, H., Kondo, S., 2011. Effects of quality and physical form of hay on mean retention time of digesta and total tract digestibility in horses. Anim. Feed Sci. Technol. 165, 61–67. https://doi.org/10.1016/j.anifeedsci.2011.02.003

Kitessa, S., Flinn, P.C., Irish, G.G., 1999. Comparison of methods used to predict the in vivo digestibility of feeds in ruminants. Aust. J. Agric. Res. 50, 825-842. https://doi.org/10.1071/AR98169

Menard, C., Duncan, P., Fleurance, G., Georges, J.Y., Lila, M., 2002. Comparative foraging and nutrition of horses and cattle in European wetlands. J. Appl. Ecol. 39, 120–133. https://doi.org/10.1046/j.1365-2664.2002.00693.x

R Core Team. 2023. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Scasta, J.D., Beck, J.L., Angwin, C.J., 2016. Meta-analysis of diet composition and potential conflict of wild horses with livestock and wild ungulates on western rangelands of North America. Rangel. Ecol. Manag. 69, 310–318. https://doi.org/10.1016/j.rama.2016.01.001

Scasta, J.D, Hennig, J.D, Beck, J.L, 2018. Framing contemporary U.S. wild horse and burro management processes in a dynamic ecological, sociological, and political environment. Human-Wildlife Interact. 12, 31–45. https://doi.org/10.26077/2fhw-fz24

Schoenecker, K.A., King, S.R.B., Hennig, J.D., Cole, M.J., Scasta, J.D., Beck, J.L., 2024. Effects of telemetry collars on two free-roaming feral equid species. PLoS One 19, 1–25. https://doi.org/10.1371/journal.pone.0303312

Uden, P., Van Soest, P.J., 1982. The determination of digesta particle size in some herbivores. Anim. Feed Sci. Technol. 7, 35–44. https://doi.org/10.1016/0377-8401(82)90034-7

Weinstein, S.B., Martinez-Mota, R., Stapleton, T.E., Klure, D.M., Greenhalgh, R., Orr, T.J., Dale, C., Kohl, K.D., Denise Dearing, M., 2021. Microbiome stability and structure is governed by host phylogeny over diet and geography in woodrats (*Neotoma* spp.). Proc. Natl. Acad. Sci. U. S. A. 118, e2108787118. https://doi.org/10.1073/pnas.2108787118

Williams, S., Horner, J., Orton, E., Green, M., McMullen, S., Mobasheri, A., Freeman, S.L., 2015. Water intake, faecal output and intestinal motility in horses moved from pasture to a stabled management regime with controlled exercise. Equine Vet. J. 47, 96–100. https://doi.org/10.1111/evj.12238

TABLES

Table 1: The upper table reports Kruskal-Wallis (χ^2) tests comparing summer and winter values for the mean particle size ([MPS]; mm) from horse fecal samples using the traditional method. Each HMA had fecal samples from five horses in the summer and five in the winter. Summer and winter means are reported with the standard deviation shown in parentheses. The bottom table reports Kruskal-Wallis tests comparing summer and winter values for retained proportion of fecal material on the top sieve (RP) using the simplified method in our analyses. Each HMA had fecal samples from six horses in the winter and six horses in the summer. HMAs with a significant seasonal difference are shown in **bold** and moderately significant ($p > 0.05$, but < 0.10) differences are shown in ***bold italics***.

HMA	Summer mean	Winter mean	Kruskal-Wallis χ^2	p-value
Stage 2 Traditional method (MPS)				
MP	5.19 (0.90)	5.98 (1.58)	0.535	0.465
PN	4.18 (0.88)	4.67 (0.79)	0.884	0.347
SS	***5.44 (1.52)***	***3.88 (0.96)***	***3.153***	***0.076***
Stage 3 Simplified method (RP)				
LBC	0.29 (0.04)	0.22 (0.04)	**5.026**	**0.025**
MP	0.17 (0.04)	0.20 (0.09)	0.231	0.631
NH	0.14 (0.06)	0.05 (0.03)	**7.410**	**0.006**
ON*	0.11 (0.08)	0.10 (0.04)	0.020	0.886
PN	0.09 (0.03)	0.24 (0.12)	**7.410**	**0.006**
RR*	0.19 (0.20)	0.08 (0.08)	***2.939***	***0.086***
SC	0.25 (0.07)	0.17 (0.09)	**4.333**	**0.037**
SW	0.27 (0.11)	0.11 (0.05)	**5.769**	**0.016**

*Although we designed the Stage 3 study to have six samples per HMA per season we inadvertently ran an extra summer sample in the Red Rock (RR) HMA and winter sample in the Onaqui (ON) HMA leading us to have seven rather than six total individuals in these HMAs and seasons.

Table 2: Spearman's rank correlations (r_s) between values for proportion graminoids in horse diet and fecal particle size measures of mean particle size >MPS) and retained proportion of weight on the top sieve (RP). Significant correlations are shown in **bold** and moderately significant (p >0.05, but <0.10) differences are shown in ***bold italics***

Comparison	Samples (n)	Spearman's (r_s)	p-value
Traditional sieve method			
Individual horse MPS and percent graminoids (both seasons)	30	0.055	0.774
Individual horse MPS and percent graminoids (summer only)	15	-0.118	0.676
Individual horse MPS and percent graminoids (winter only)	15	0.132	0.639
Simplified sieve method			
Herd average graminoids to RP (seasons combined)	8	-0.333	0.428
Herd average graminoids to RP (seasons separate)	16	0.059	0.831
Herd average graminoids to RP (summer only)	8	0.310	0.462
Herd average graminoids to RP (winter only)	8	-0.524	0.197
Individual horse RP and percent graminoids (both seasons) *	98	0.040	0.696
Individual horse RP and percent graminoids (summer only) *	49	**0.318**	**0.026**
Individual horse RP and percent graminoids (winter only) *	49	**-0.298**	**0.038**

*Although we designed the study to have 6 samples per HMA per season we inadvertently ran an extra summer sample in the Red Rock (RR) HMA and winter sample in the Onaqui (ON) HMA leading us to have 98 rather than 96 total individuals in these analyses.

Table 3: Kruskal-Wallis (χ^2) tests comparing summer 2020 and winter 2020/2021 means of the retained proportion of fecal material on the top sieve (RP) for individual horses that consumed high (≥ 75%) and low (≤ 25%) graminoid diets. Significant seasonal differences ($p < 0.05$) are shown in **bold** and the number of horses in each season and diet category (n) are reported.

	Summer mean RP	Summer SD	Summer n	Winter mean RP	Winter SD	Winter n	Kruskal-Wallis χ^2	p-value
High graminoid	0.22	0.09	19	0.09	0.05	11	11.413	**0.001**
Low graminoid	0.17	0.17	11	0.15	0.08	13	0.444	0.505

FIGURES

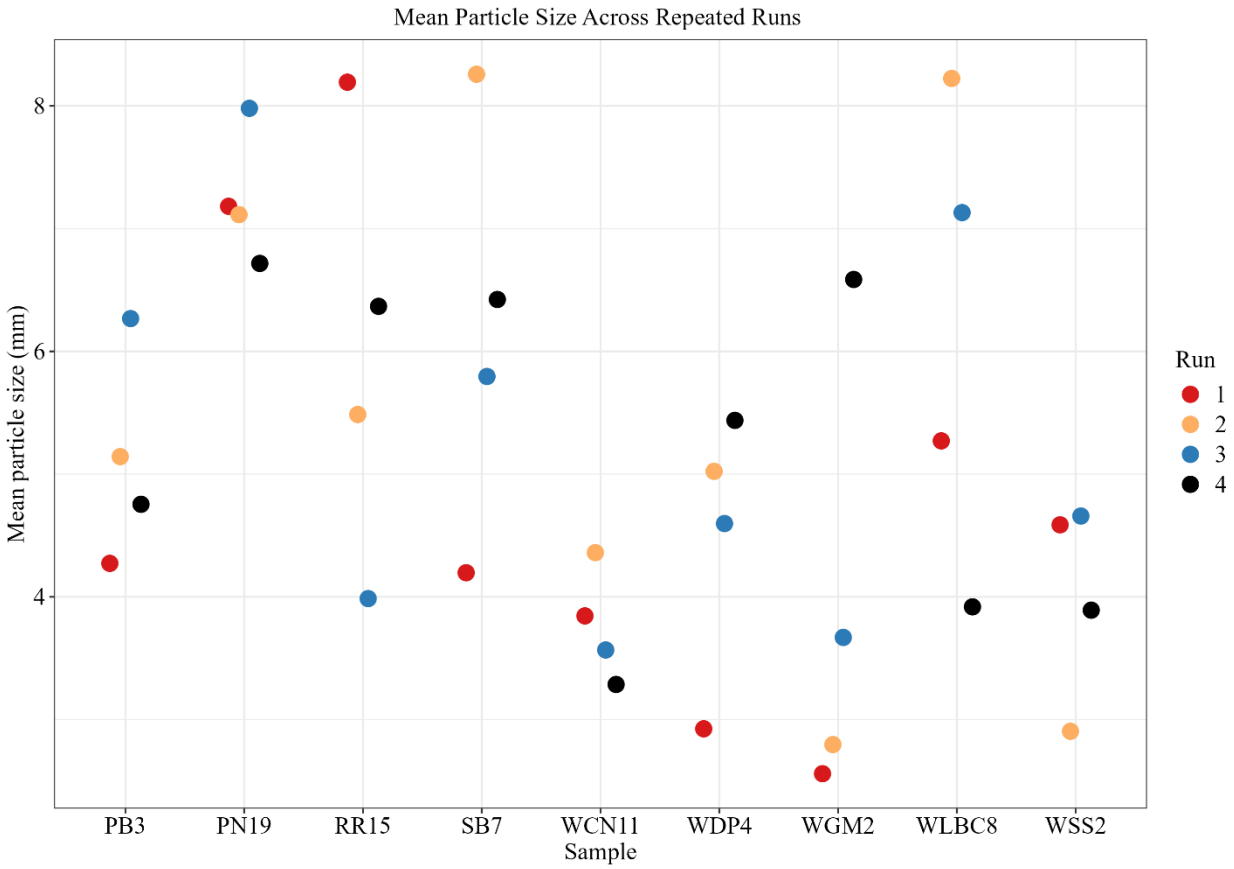

Figure 1. Values for mean particle size (mm) for nine samples in the Stage 1 analysis. Mean particle size is shown along the y-axis and each sample is plotted along the x-axis with each of the four replicates shown in a different color. Note there was often large variation in mean particle size for replicate runs of the same sample. Sample names refer to the HMA the sample was collected in and abbreviations are as follows: CN = Conger, Utah, GM = Green Mountain, Wyoming, LBC = Little Book Cliffs, Colorado, PB = Palomino Buttes, Oregon, PN = Pine Nut Mountains, Nevada, RR = Red Rock, Nevada, SB = Sand Basin, Idaho, and SS = South Steens, Oregon. Sample names starting with a "W" indicate winter collections. One sample from a domestic horse (WDP) was also included in this Stage 1 pilot analysis.

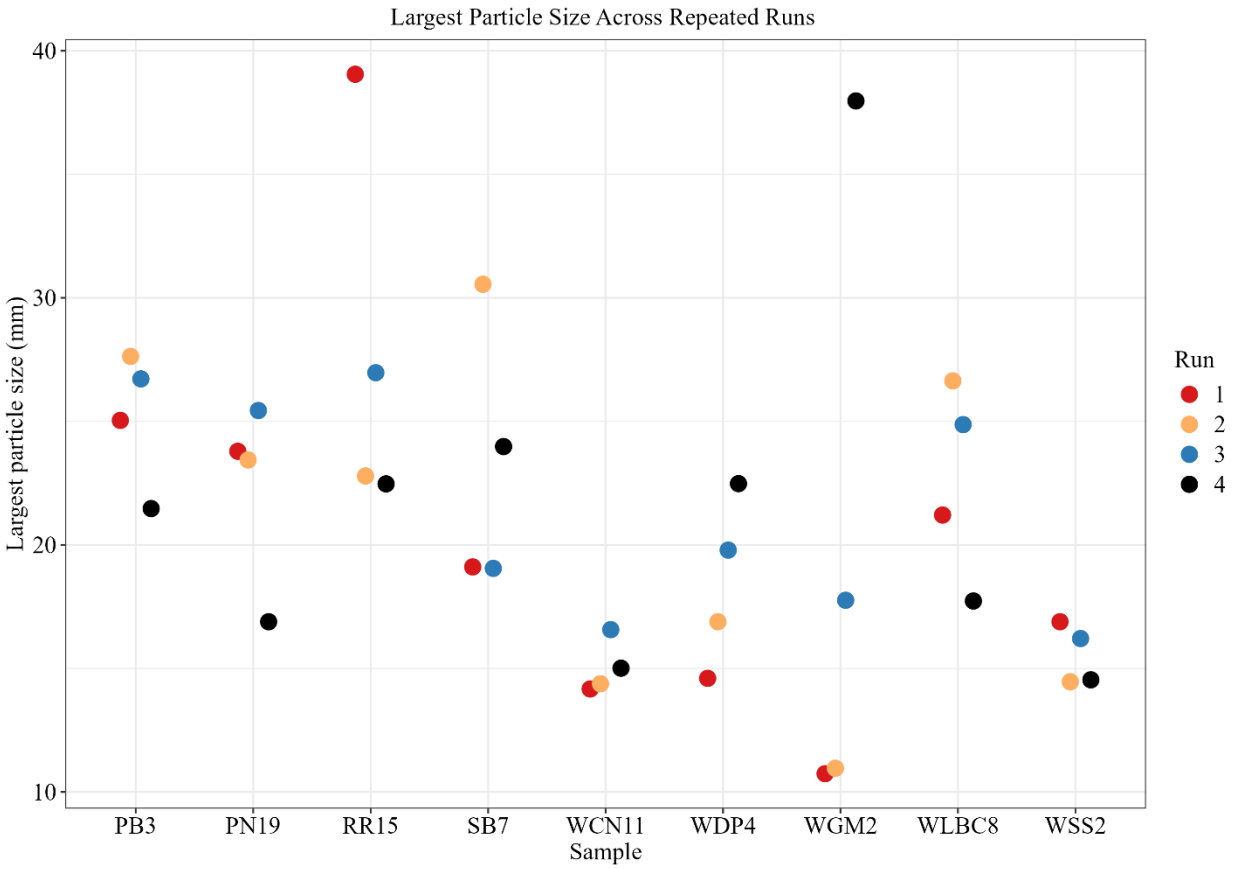

Figure 2: Values for largest measured particle (mm) for nine samples in Stage 1 analysis. Largest measured particle (a key measure for the calculation of mean particle size) is shown along the y-axis and each sample is shown along the x-axis with each of the four replicates shown in a different color. Note the similarity in many patterns in Figure 1 and Figure 2, showing the large influence that the largest particle had on mean particle size. Sample names refer to the HMA the sample was collected in and abbreviations are as follows: CN = Conger, Utah, GM = Green Mountain, Wyoming, LBC = Little Book Cliffs, Colorado, PB = Palomino Buttes, Oregon, PN = Pine Nut Mountains, Nevada, RR = Red Rock, Nevada, SB = Sand Basin, Idaho, and SS = South Steens, Oregon. Sample names starting with a "W" indicate winter collections. One sample from a domestic horse (WDP) was also included in this Stage 1 pilot analysis.

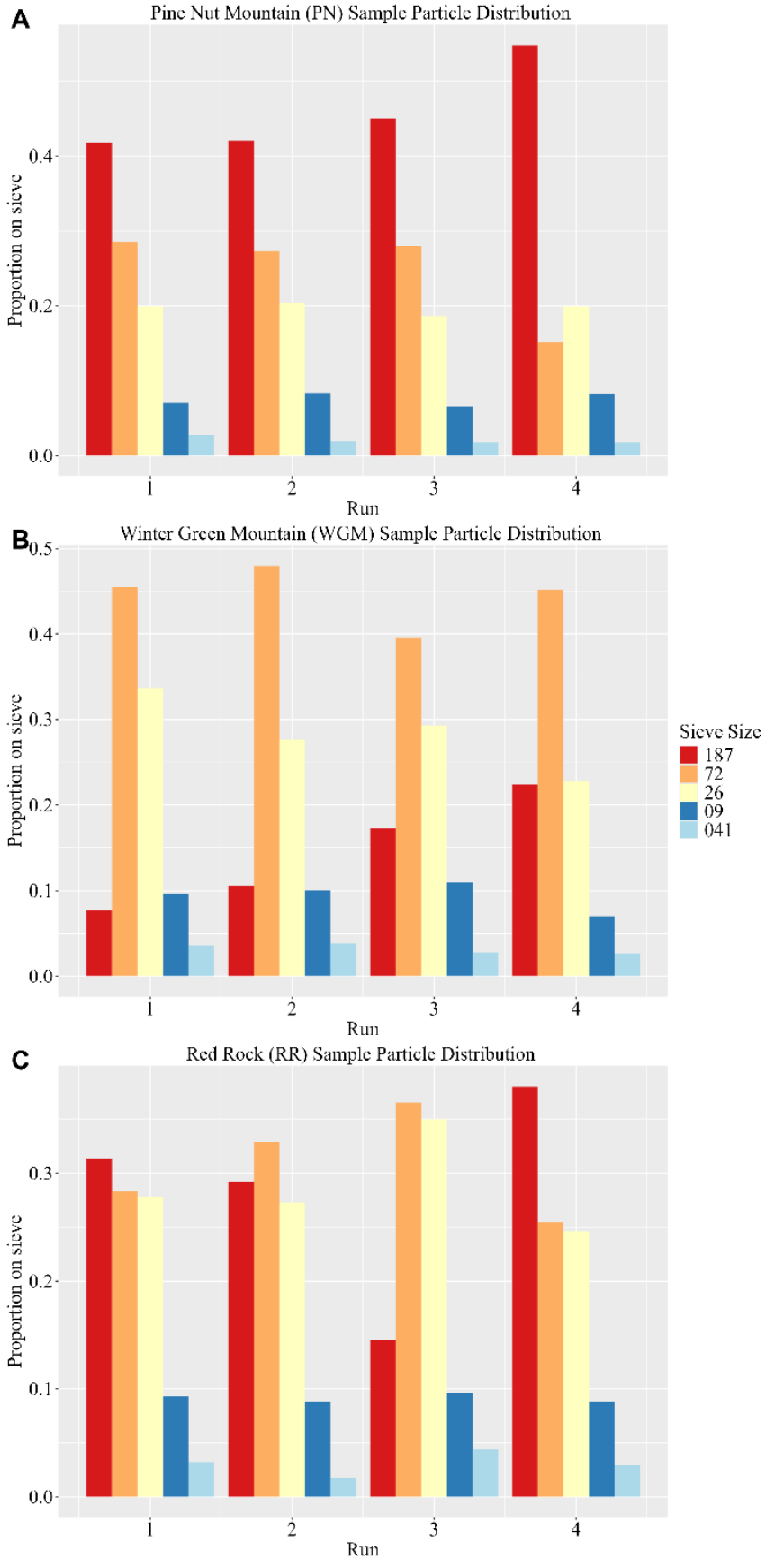
A
Pine Nut Mountain (PN) Sample Particle Distribution
Proportion on sieve
0.0
0.2
0.4
1
2
3
4
Run
B
Winter Green Mountain (WGM) Sample Particle Distribution
Proportion on sieve
0.0
0.1
0.2
0.3
0.4
0.5
1
2
3
4
Run
Sieve Size
187
72
26
09
041
C
Red Rock (RR) Sample Particle Distribution
Proportion on sieve
0.0
0.1
0.2
0.3
1
2
3
4
Run

Figure 3: Examples of different patterns observed in the distribution of particle size by proportion of weight on each sieve in three of nine samples run in the Stage 1 analysis. The y-axis shows the proportion of particles (by weight) on each size sieve and the x-axis shows each run color coded by the five sizes (OPN) of sieves in decreasing order (187-red, 72-orange, 26-yellow, 09-dark blue, 041-light blue). Note that the Pine Nut Mountains (PN) sample in panel A shows a higher proportion of particle weight on sieve 1, the Green Mountain winter (WGM) sample in panel B shows the highest proportion on sieve 2, and the Red Rock (RR) sample in panel C shows a more even spread across sieves 1–3.

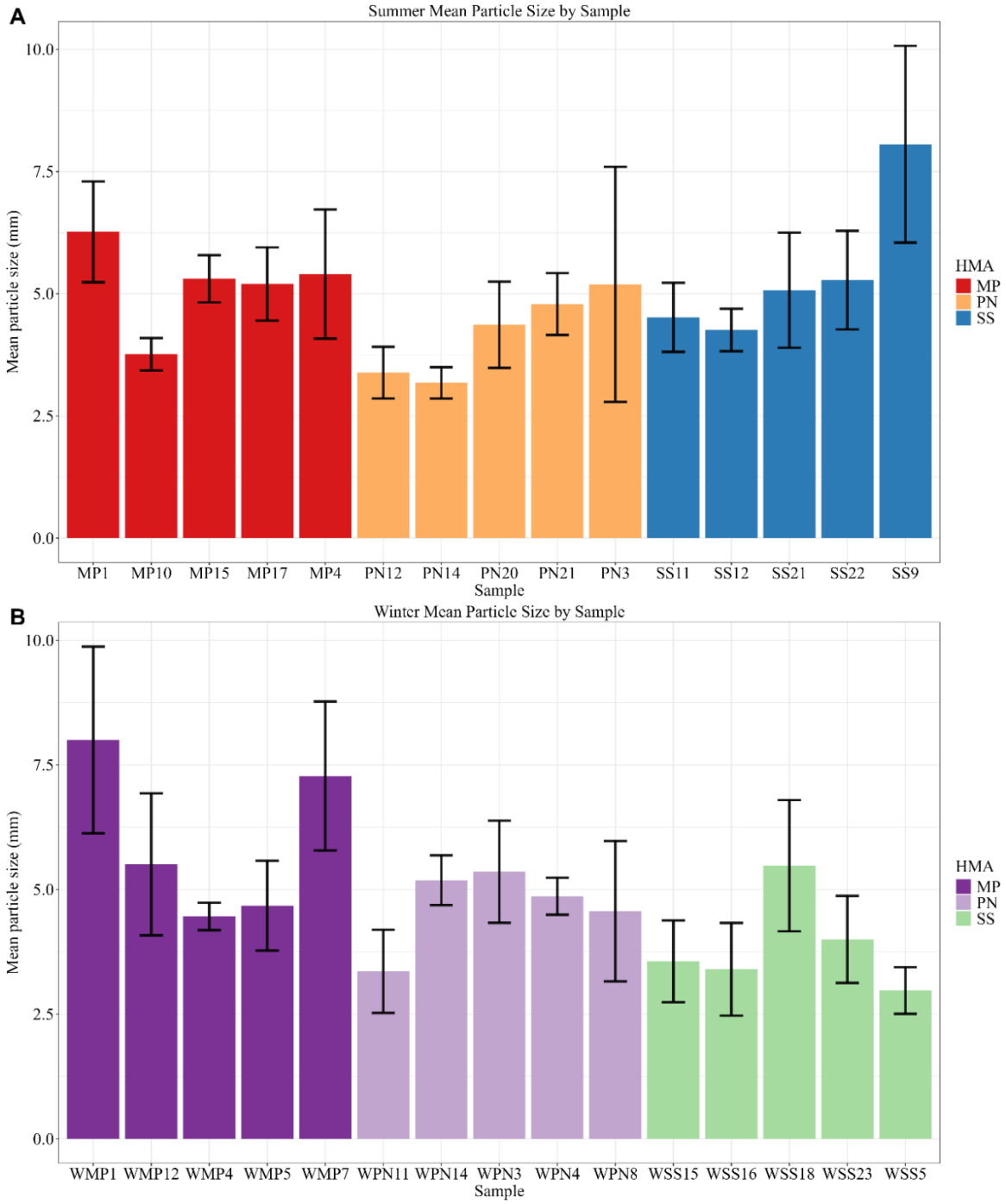

Figure 4: Average mean particle size ([MPS]; mm) among 30 individual horse samples each consisting of five replicated sub-samples. There were five individual horse samples from each of three HMAs (MP = McCullough Peaks, Wyoming, PN = Pine Nut Mountains, Nevada; SS = South Steens, Oregon) analyzed in Stage 2. Summer (A) and winter (B) samples shown separately. Mean (± 95% CI) particle size (mm) for each horse sample (average of 5 repeats) is

shown. Some samples show high variation among runs (e.g., PN3, SS9) while others show little variation (e.g., PN14, WMP4). Differences occur between mean values for individual samples both between HMAs (for example SS9 and PN 14) and within HMAs (for example SS9 and WSS5).

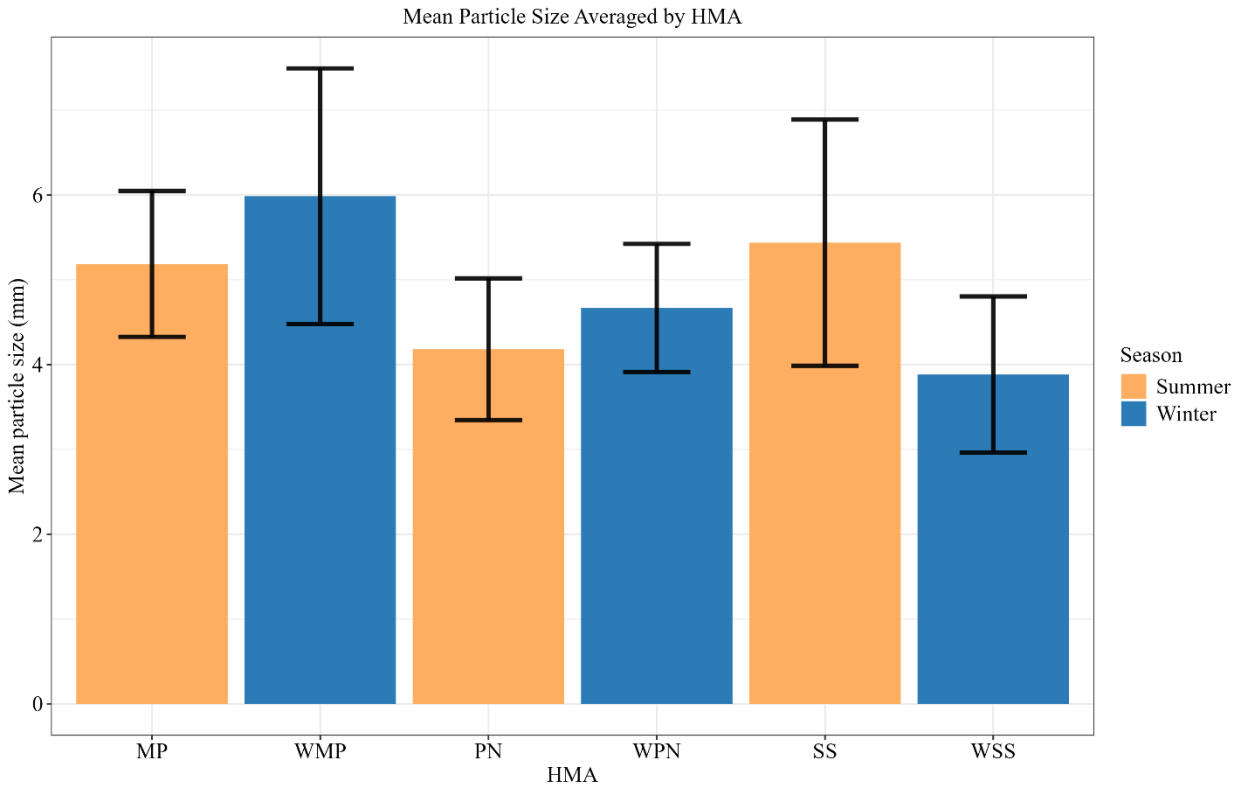

Figure 5: Comparisons of the herd average mean particle size (MPS; ± 95% CI, *n* = 5 individuals per HMA in summer 2020 and winter 2020/2021) within each Bureau of Land Management–Herd Management Area (HMA) and season for samples analyzed in Stage 2. Differences in summer 2020 and winter 2020/2021 were not statistically significant in the McCullough Peaks (MP), Wyoming or Pine Nut Mountains (PN), Nevada HMAs but were borderline significant in the South Steens (SS), Oregon HMA (p = 0.076). There were differences in the seasonal pattern of MPS within each HMA with SS showing a tendency towards a decrease from summer to winter while MP and PN show a tendency towards an increase.

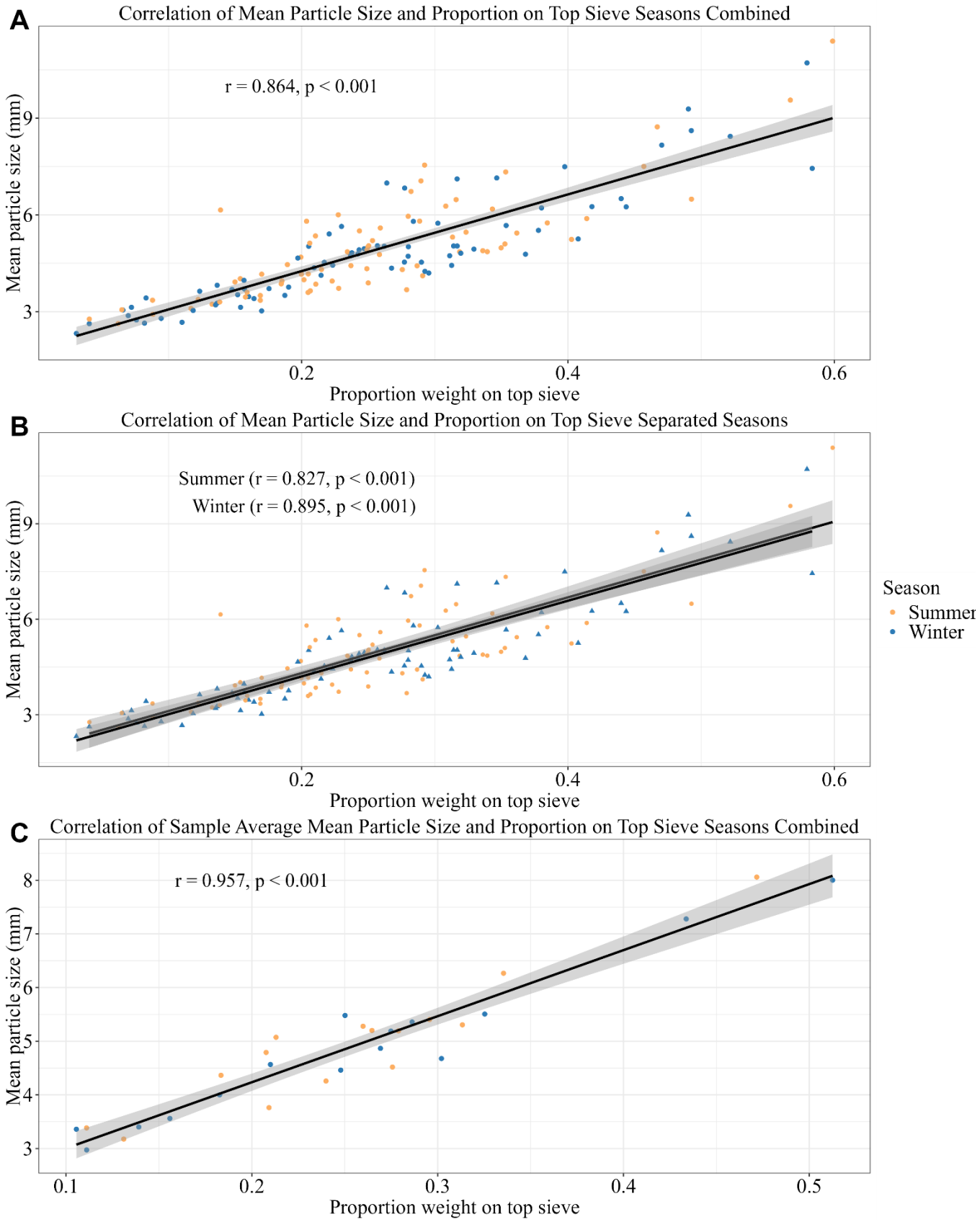
A
Correlation of Mean Particle Size and Proportion on Top Sieve Seasons Combined
r = 0.864, p < 0.001
Mean particle size (mm)
Proportion weight on top sieve
B
Correlation of Mean Particle Size and Proportion on Top Sieve Separated Seasons
Summer (r = 0.827, p < 0.001)
Winter (r = 0.895, p < 0.001)
Mean particle size (mm)
Proportion weight on top sieve
Season
Summer
Winter
C
Correlation of Sample Average Mean Particle Size and Proportion on Top Sieve Seasons Combined
r = 0.957, p < 0.001
Mean particle size (mm)
Proportion weight on top sieve

Figure 6: Correlation (*r*) of the proportion of weight on the top sieve (RP) and mean particle size ([MPS]; mm) for all subsamples (A and B) and for sample averages (C) in Stage 2 of analysis. Note correlations shown in A and B were run including each of five subsample replicates before averaging to get one value for each sample. Correlations include summer and winter subsamples in a single correlation (A) as well as for each season individually (B). The correlation for each sample's average MPS and average RP, when including both seasons (C), was even stronger (r = 0.957).

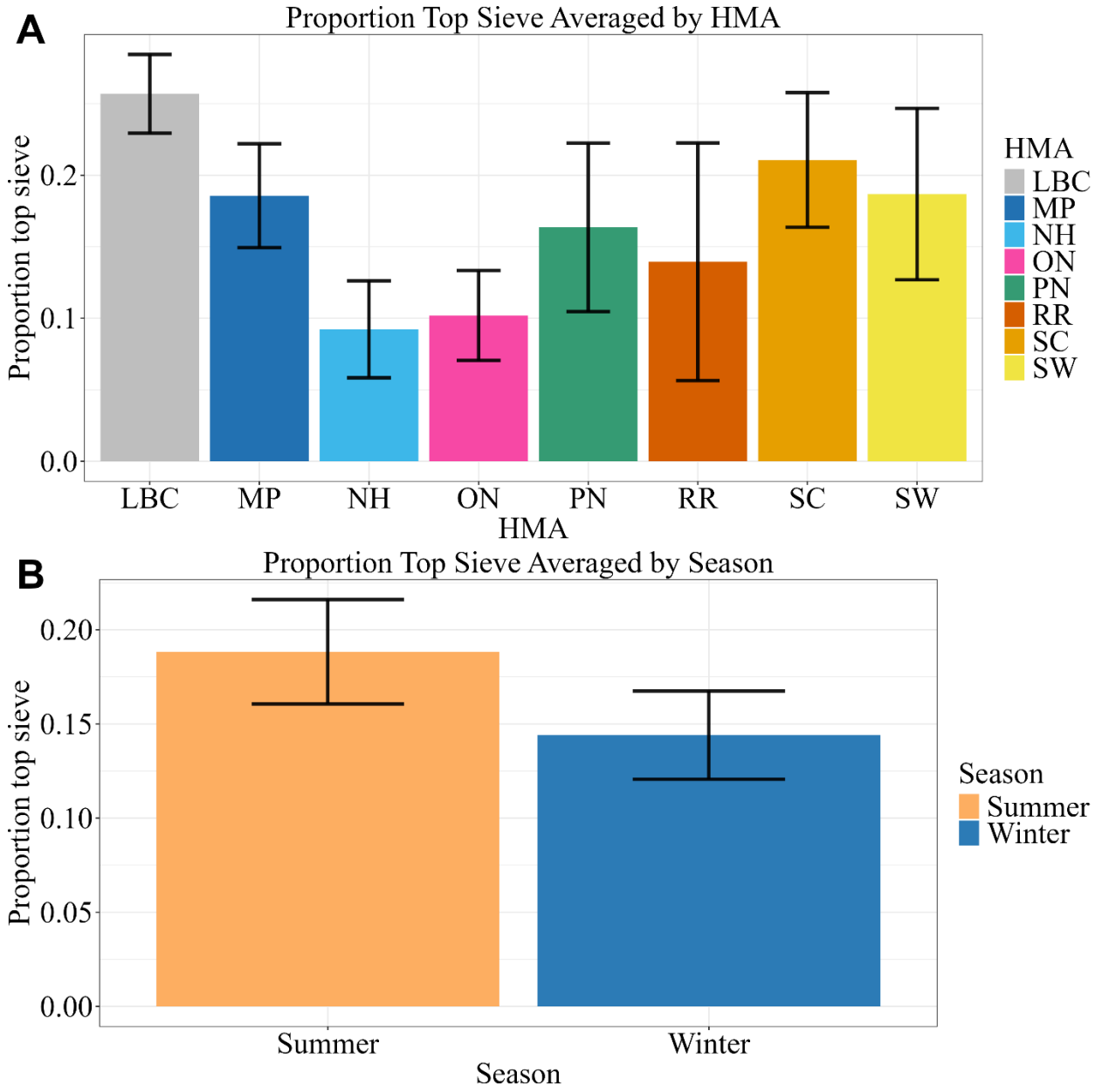

Figure 7: Proportion of weight on top sieve (RP ± 95% CI) by Bureau of Land Management Herd Management Area (HMA) (A) and season (B) for samples in Stage 3. There was a significant difference in RP by HMA ($p < 0.001$) and by season ($p = 0.047$). HMA abbreviations are as follows: LBC = Little Book Cliffs, Colorado; MP = McCullough Peaks, Wyoming; NH = North Hills, Utah; ON = Onaqui, Utah; PN = Pine Nut Mountains, Nevada; RR = Red Rock, Nevada; SC = Saylor Creek, Idaho; and SW = Stinkingwater, Oregon.

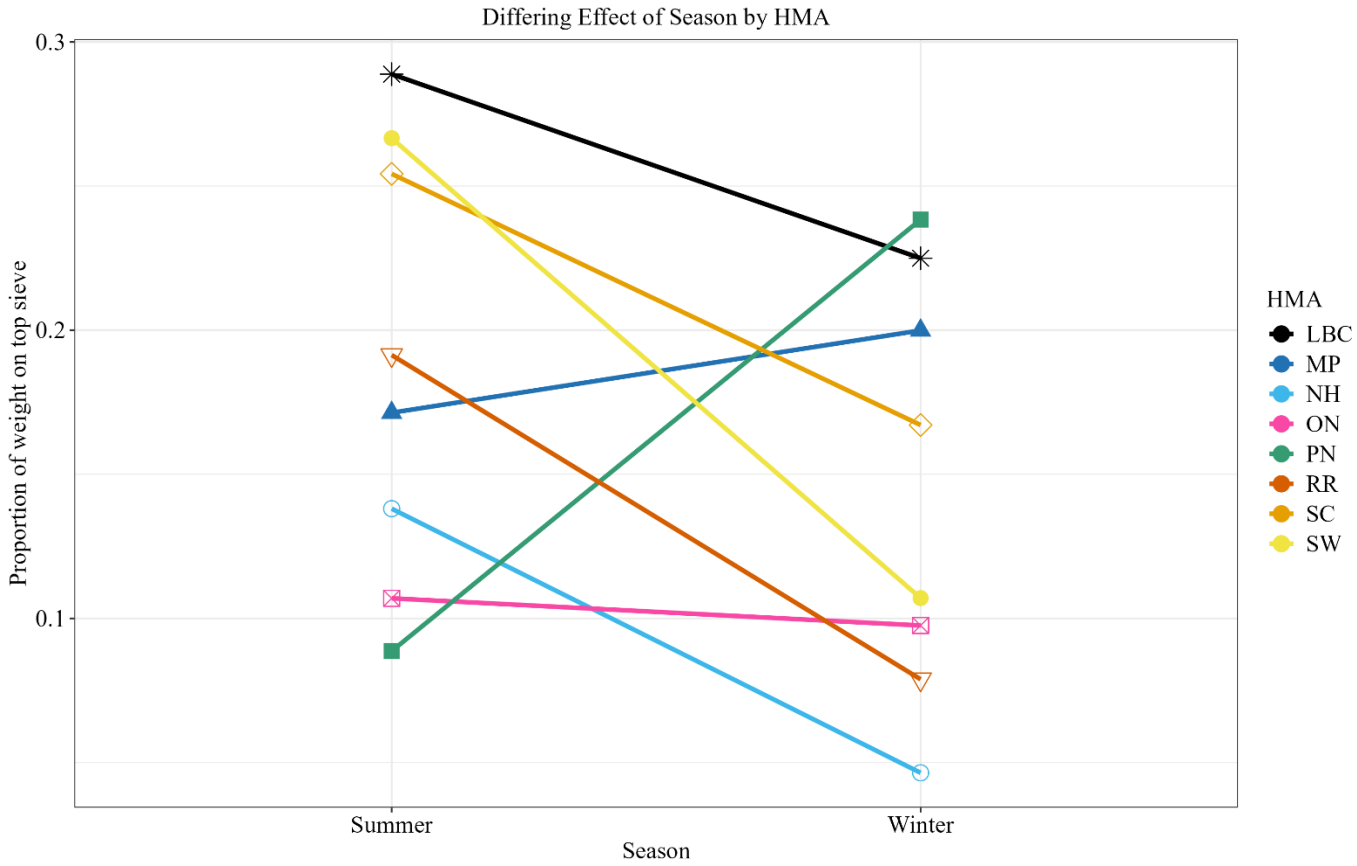

Figure 8: Interaction plot of season and Bureau of Land Management–Herd Management Area (HMA) on proportion of weight on top sieve (RP) for samples run in stage 3 analysis. Note that five HMAs (LBC, SC, RR, NH, SW) saw a decrease in RP (representing a decrease in particle size) from summer 2020 to winter 2020/2021 and three HMAs (PN ON, MP) saw an increase from summer to winter (representing an increase in particle size). Significant differences between seasons within a single study area were noted in LBC, NH, PN, SC, and SW ($p < 0.05$) by performing a Kruskal-Wallis test, see Table 1 for exact p-values and test statistics. HMA abbreviations are as follows: LBC = Little Book Cliffs, Colorado; MP = McCullough Peaks, Wyoming; NH = North Hills, Utah; ON = Onaqui, Utah; PN = Pine Nut Mountains, Nevada; RR = Red Rock, Nevada; SC = Saylor Creek, Idaho; and SW = Stinkingwater, Oregon.

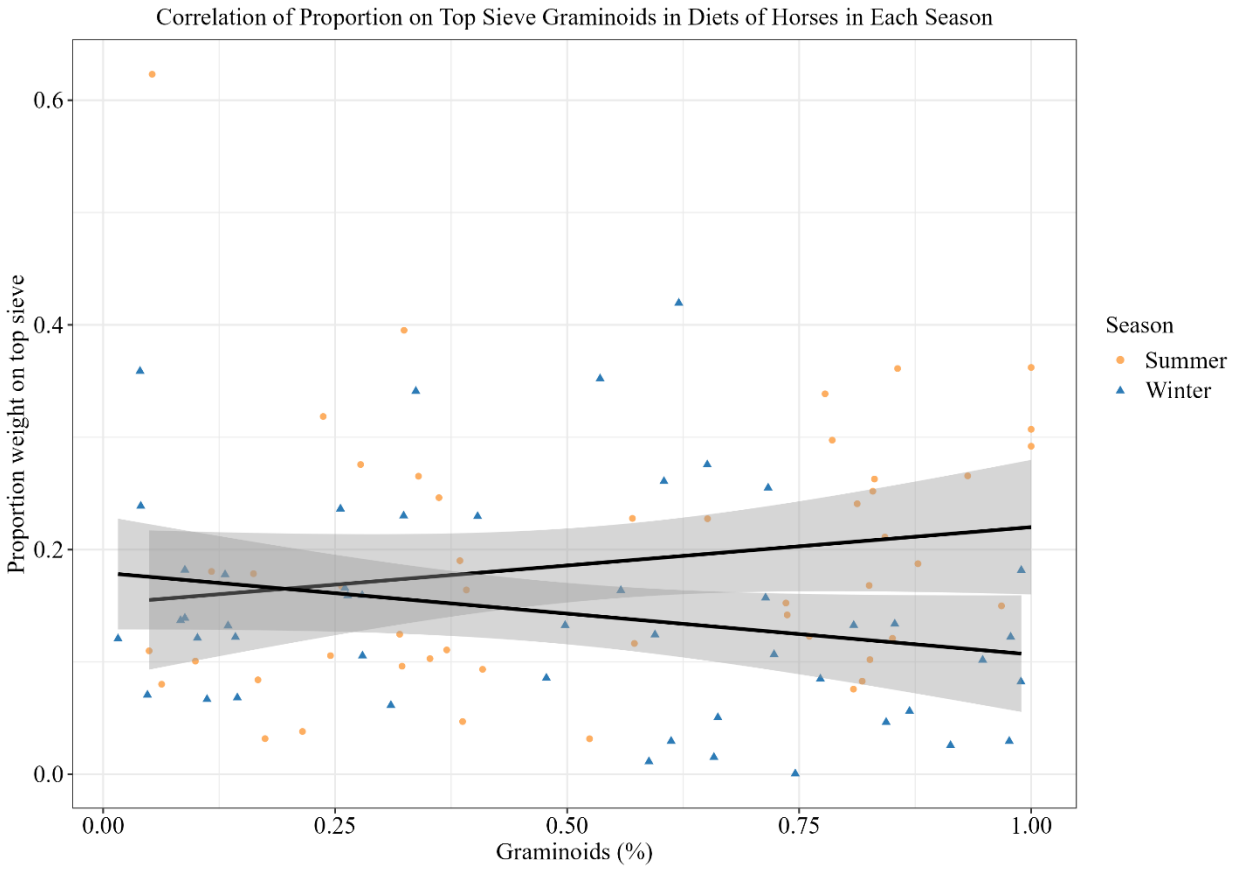

Figure 9: Spearman's rank correlations (r_s) of proportion of graminoids in horse diets with proportion of weight on the top sieve (RP) as a proxy for mean particle size (MPS) in summer 2020 (orange circles) and winter 2020/2021 (blue triangles). There was a positive relationship in the summer (r_s = 0.318, p = 0.026) while there was a negative relationship in these metrics in the winter (r_s = -0.298, p = 0.038).

APPENDIX A

Figure A1: View of the sieve set up during the rinsing phase of the traditional method.

Figure A2: Example of a completed sieve stack using the traditional method after rinsing and before removing the metal sieves to be dried.

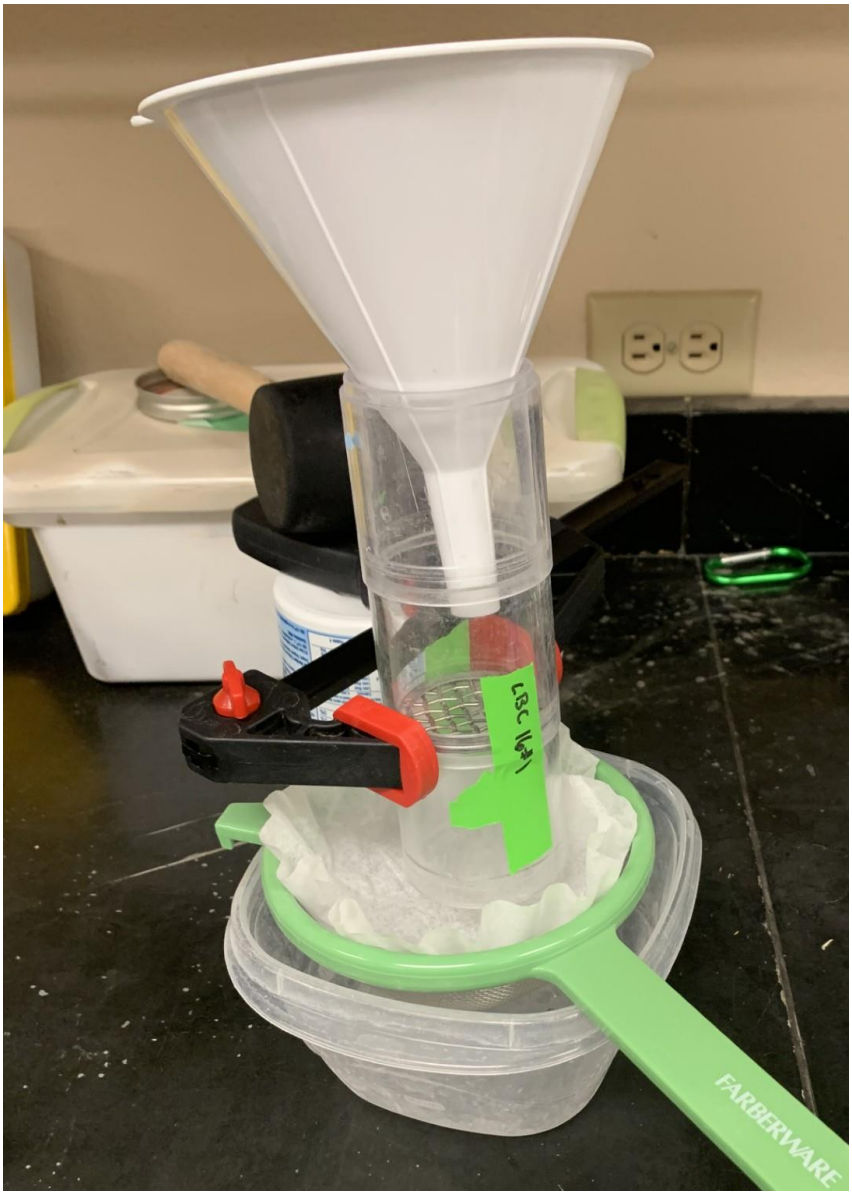

Figure A3: View of the setup of the simplified method before a sample is run through the sieve set-up.

Figure A4: View of the simplified method after a sample has been run through the set-up and before rinsing.

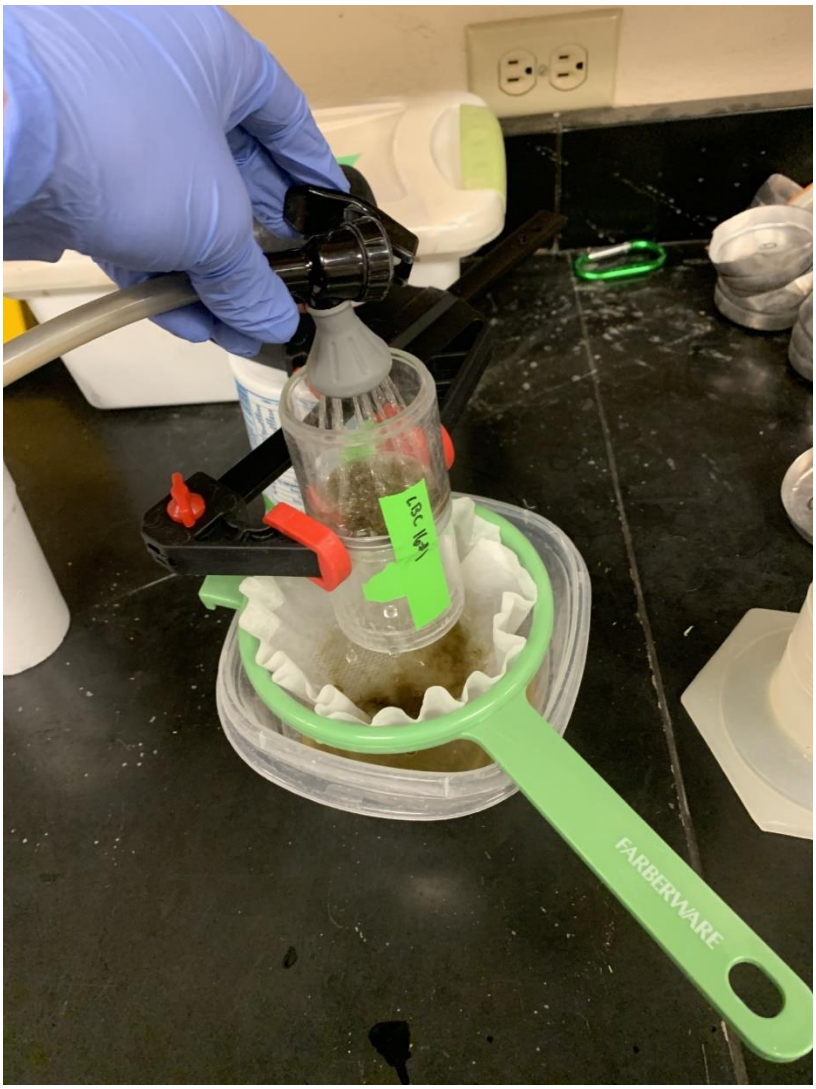

Figure A5: View of the simplified method during the rinsing phase.

APPENDIX B

When using our larger data set with proportion of weight on top sieve (RP) acting as a proxy for MPS we found moderate support for a negative relationship between RP and BCS (r_s = -0.267, p=0.058; Table A1) of individual horses with a known BCS. However, once again there was little variability in body condition scores with only 11/51 of the horses in this analysis having a BCS other than 5. This pattern remained negative when the data were subset to only summer or winter samples, however correlations did not show statistical support for this relationship in either season (p > 0.100, Table A1). When we averaged both RP and BCS at a herd level, we found a positive tendency regardless of whether seasonal averages were included together or separately (Table A1), although statistical support for a relationship was not shown through our correlations.

Across seasons, we found a positive, though not significant, pattern in the relationship of RP and BCS at the HMA level—horses with increasing RP, a proxy for increasing particle size, tended toward higher body condition. However, at the individual scale, we saw moderate support (p = 0.058) for a negative relationship between RP and BCS when samples from both seasons were considered together. Because previous studies in equids have shown diets with lower fiber content and higher quality led to higher particle size (Miyaji et al. 2011; Clauss et al. 2014), results at the individual scale are the opposite of what we would expect to find, since we would expect individuals with higher quality diet to be in higher body condition. However, our HMA level results demonstrating a tendency for a positive relationship agree more with our expectations.

One explanation for why we did not find strong relationships between measures body condition and fecal particle size could be the lag time between the diet horses are consuming and its effect

on body condition. Measures of fecal particle size offer a snapshot of horse diet for one day, while body condition changes slowly over time and represents the accumulated effects of diet, metabolic efficiency, and energetic costs. Therefore, this lack of convincing findings could be due to a mismatch in temporal scale. Another explanation for our absence of conclusive results could be due to lack of variability in observed body condition scores since, as described previously, most horses were observed to have a body condition score of 5. This made our comparisons of individual horses difficult due to the small number of horses scoring a BCS other than 5. In addition, the resulting lack of strong variability in herd average BCS combined with smaller sample sizes in HMA average comparisons also made patterns difficult to identify at the herd level.

Table A1: Spearman's rank correlations (r_s) between values for body condition scores (BCS) and retained proportion of weight on the top sieve (RP). Significant correlations are shown in **bold** and moderately significant (p >0.05, but <0.10) differences are shown in ***bold italics.***

Comparison	Number of samples (n)	Spearman's r_s	p-value
Herd average BCS to RP (seasons combined)	8	0.599	0.117
Herd average BCS to RP (seasons separate)	16	0.330	0.212
Herd average BCS to RP (summer only)	8	0.214	0.619
Herd average BCS to RP (winter only)	8	0.381	0.360
Individual horse BCS to PROP (both seasons)	***51***	***-0.267***	***0.058***
Individual horse BCS to RP (summer only)	19	-0.361	0.129
Individual horse BCS to RP (winter only)	32	-0.200	0.273

CHAPTER 6. Conclusion

There are five important take-away points I would like to highlight that resonate across my chapters. These points include 1) the importance of geographic scale in detecting differences in free-roaming horse (*Equus caballus*) metrics, 2) the capability of free-roaming horses, often considered true grazers, to subsist on a variety of forage species in summer and winter, all while maintaining good body condition, 3) the relationship of gut microbiome composition and body condition was weaker than we had expected in both pronghorn and horses, 4) there was a clear link between diet and microbial composition in free-roaming horses, and 5) the microbial community varied by season and location in both of our study species- pronghorn and horses.

First, the importance of the geographic scale being considered in seasonal comparisons across the free-roaming horse chapters. Both diet and particle size had general patterns at the range-wide scale with more dietary graminoids in summer compared to winter and larger fecal particle size in summer compared to winter. However, not all Bureau of Land Management Herd Management Areas (HMAs) followed these general patterns. While summer diets for horses in many HMAs included a higher proportion of graminoids, others displayed no statistical seasonal difference, and one HMA had evidence of higher graminoids in winter diets. Similarly, the majority of HMAs had higher particle size in the summer; however, one had statistically higher particle size in the winter. While this may just represent natural variation in the data, it is important to consider that the seasonal response of horses may differ in various HMAs. I also noted the importance of scale in horse microbial composition, with the effects of season on microbial composition explaining up to 5 times more variation at the HMA scale as compared to the range-wide scale. It is important to consider the effect of scale when making management decisions. While range-wide norms appear evident, responses between HMAs may differ or the

average response across the range may not be true in all HMAs and managers may need to consider specifics at the herd level.

The second important take away from my dissertation was that free-roaming horses across the range subsisted on a variety of diet strategies, which was surprising to find for a species generally considered to be a grazing herbivore. While graminoids often made up the majority of diets this was not always the case, and the proportions of graminoids varied by HMA. Other important plant families included Asteraceae, Brassicaceae, Chenopodiaceae, Fabaceae, and Polygonaceae, which all made up at least 25% of the diet composition in at least one HMA. Notably, Asteraceae or Chenopodiaceae plants made up large portions of the winter diets in many HMAs. Despite varying dietary strategies, horses in all HMAs maintained healthy body condition with the average body condition across all HMAs ranging between 4.59 and 5.24 in the summer and 4.72 and 5.22 in the winter. In addition, the majority of observed free-roaming horses had desirable body condition, with less than 1% of observed horses across all HMAs scoring a 3 or below (i.e., poor body condition). It appears that free-roaming horses can maintain desirable body condition while consuming a variety of forage plants.

Third, while we found some subtle signals relating microbiome composition to body condition these were not as strong as we had expected to find. There is much literature linking microbial composition to fat storage or body condition, although most studies were in laboratory or domestic settings. One study in wild mule deer (*Odocoileus hemionus*) identified links between fat and protein deposition and specific microbial taxa, revealing these taxa as potential health bio-indicators in mule deer (Eddington et al. 2021). We expected to add to the evidence for this linkage in free-roaming species by revealing similar relationships in pronghorn (*Antilocapra americana*) or free-roaming horses with microbial taxa that could serve as potential

biomarkers. However, relationships between pronghorn body condition and microbial composition were subtle and not as clear as we had hoped. In addition, the high proportion of free-roaming horses scoring a BCS of 5 made evaluating this relationship difficult in horses. Over 70% of the known horses (n = 90) we collected fecal samples from had a BCS of 5 (n = 68), with few horses scoring either 3 (n = 1) or 7 (n = 2). This did not provide a suitably wide distribution of animals across the scope of condition scores to evaluate links between BCS and microbial composition. To investigate this metric further, it would be helpful for researchers to consider multiple samples over time from individual animals to reduce or control for between-animal variation to tease apart these subtle relationships.

Fourth, there was a clear link between diet and microbial composition in free-roaming horses. Both diet and microbial composition revealed clear seasonal patterns and Mantel tests confirmed co-variation of dissimilarities between these communities. In addition, we identified the ten bacterial families that contributed most (0.595) to the overall and seasonal microbial community dissimilarity and found that the abundance of nine of these families was correlated with the abundance of at least one plant family. The plant families that most commonly displayed relationships with these ten bacterial families included the graminoid families of Cyperaceae, Poaceae, and Juncaceae and the forb and shrub families of Asteraceae and Chenopodiaceae. We did not investigate this metric in pronghorn because we did not sequence diet data, however this would be interesting for future research.

Fifth, microbial composition varied spatially and seasonally in both species that we studied. Both HMA and ecoregion were significant explanatory factors of microbial variation in free-roaming horses, indicating horse microbiome was different in various environments. In pronghorn, microbial composition varied between different study areas and based on whether

populations were north or south of Interstate 80. Microbial composition varied by season in free-roaming horses, and by capture period in pronghorn, although we could not completely separate this from the effect of study area due to study design. Our findings align with results of other studies in North American ungulates that have also found microbial variation associated with environmental factors such as location or season. Microbial composition in mule deer varied between different study sites as well as between December and March collections (Eddington et al. 2021). American bison (*Bison bison*) also exhibited shifts in microbial composition over the course of the growing season (Bergmann et al. 2015). When pairing conclusions in our research with findings from other herbivore studies in North America and beyond, it appears that gut microbes do indeed vary across environments and seasons. This supports the potential of the microbiome as an adaptive trait that allows animals to better adjust to variable environments; however, whether this effect is indeed adaptive to the host rather than just a response of bacteria to differing conditions remains to be proven conclusively in free-roaming species.

Our findings have different applications when thinking about native species, such as pronghorn, compared to free-roaming horses, an introduced species whose management often centers on overpopulation issues (Scasta et al. 2018). Scientists have mentioned the potential importance of considering microbial composition while performing reintroductions or translocations (Bahrndorff et al. 2016; Trevelline et al. 2019; Li et al. 2019). Our research indicates locally adapted microbes could be evident in pronghorn and free roaming horses, while researchers have also revealed this phenomenon in brown bears (*Ursus arctos*; Trujillo et al. 2022) and moose (*Alces alces*; Fountain-Jones et al. 2020). This recurring pattern has potential important applications to other North American ungulates. Management tools based on locally

adapted gut microbes could prove useful if administered to naïve animals during translocation, potentially allowing for greater adaptation capability and increasing translocation success.

Our research serves as a baseline for understanding the bacterial composition of the gut microbiome in pronghorn and which intrinsic or environmental factors are related to microbial variation. Future research in pronghorn or other native ungulates could build upon this by investigating specific bacterial taxa that could serve as biomarkers or probiotics that managers could administer to increase herd health or resilience. Already, bacterial taxa that could potentially serve as biomarkers have been identified in mule deer (Eddington et al. 2021). In addition, if we applied the diet composition or fecal particle size methods we investigated in free-roaming horses to native species such as pronghorn, managers could monitor microbial composition, diet composition, or diet quality through fecal particle size non-invasively, without disturbing, capturing, or stressing animals. These methods could allow managers to track diet quality changes or changes in animal physiology over time. Identifying timing of diet composition or quality changes or changes in microbial composition could inform managers specifically when wildlife become metabolically stressed during winter. This could help signal from a physiologic standpoint when areas need to be closed to the public to reduce animal stress or when interventions such as supplemental feeding programs are needed.

Our results in free-roaming horses serve as a case study for the relationships between forage plants, herbivores, and the gut microbiome. Understanding these relationships can shed light on these interactions, which may have important implications for animal health and adaptability to diverse environments. As discussed above, these applications can lead to management tools that could be helpful to species that are struggling. Because free-roaming horse numbers in most areas are above population targets (BLM 2025), use of any of these

management tools likely would not be needed or justified for horse management. However, our study does advance the knowledge of free-roaming horse life history, biology, and ecology in the western United States, which is important to consider when making management decisions. Horses are considered grazing animals and this contention was supported when a meta-analysis of 12 previous studies in free-roaming horses revealed that on average, free-roaming horse diets contain high amounts of graminoids in spring, summer, fall, and winter (Scasta et al. 2016). While we found graminoids formed the majority of horse diets in most HMAs, this was not always the case and other plant families were important, including plants in the Asteraceae and Chenopodiaceae families. Many shrubs in these families are important winter browse for native ungulates, and horse use of these resources means there is potential for competition. Furthermore, body condition scores of free-roaming horses were healthy across diet composition, seasons, HMAs, and environments, indicating these animals thrive across variable conditions of their range. This may be explained by their digestive anatomy and physiology that equips them to subsist in good body condition while selecting a variety of plants in different environments.

Some have pointed to the equid digestive system as a reason for their success — their "quantity over quality" strategy means that they can subsist on poor quality forage, as long as it is abundant. Hind gut fermenters, such as horses, are able to quickly move digesta through their digestive tracts and take advantage of abundant low-quality forage because, unlike ruminants, they are not limited by particle size of ingested material (Janis 1976; Hanley 1982; Duncan et al. 1990). In one study of horses and cattle, researchers found that although cattle digested feed more completely, horses had 63% higher intake, leading horses to obtain more digestible nutrients per day (Menard et al. 2002). This feeding strategy can allow horses to maintain body condition even on low quality forage. Because research has demonstrated that mares in better

condition have more breeding success, (Henneke et al. 1984; Morley and Murray 2014) this digestive strategy can ultimately lead to demographic success at the population level.

Others hypothesize that success and high population growth in free-roaming horses could be due to their domestic roots. Humans selected horses and other livestock for high reproductive potential, and due to these genetics, unlike many native ungulates, free-roaming horses may prioritize reproduction over survival (Grange et al. 2009; National Research Council 2013). In contrast to wild ungulates, which will decrease reproductive efforts as resources become limited, a study of feral horses found that during times of scarcity mares lost body condition while continuing to reproduce, sometimes to their own detriment (Grange et al. 2009). These high rates of fecundity in a once domestic species can lead to rapid growth in populations, until resources become limited and ultimately adult female survival is affected. This may offer one explanation as to why free-roaming horse populations in the western United States continue to grow even when conditions are marginal. While digestive strategy and high fecundity may explain the success of free-roaming horse populations, our research points to a further mechanism that could elucidate how these animals succeed, by having a flexible gut microbiome. We found that microbial composition co-varied with diet composition, potentially indicating that as horses consume different forages, their microbial composition adjusts to coincide with novel plants. This could allow free-roaming horses the capability to adapt to a wide variety of plants across their range. Heightened ability to adapt to novel forages would allow horses to maintain better body condition, potentially leading to higher population growth because mares in better condition are more reproductively efficient (Henneke et al. 1984; Morley and Murray 2014). It is likely a mixture of these factors that lead to the success of free-roaming horses in North

America; however our research indicates that both dietary and microbial flexibility could be contributing factors.

Based on some challenges we experienced throughout the data analysis process, we have developed recommendations for researchers designing similar studies in the future. First, creating balance in study metrics is important when possible. The fecal samples used for our pronghorn research were legacy samples from a previous study and therefore were not collected in a manner that always aligned with our study questions. We faced challenges in our analyses due to uneven representation of capture period and study area. In addition, biological samples and information for pronghorn in one study area were only collected during one capture period, which confounded the effect of study area and capture period with one another. In future research, even sampling across study areas and capture periods, such as we did when designing our horse study would be ideal.

Second, is the question of sample independence and animal identity. This was not an issue in the pronghorn dataset as each fecal sample was from a pronghorn that researchers individually captured, identified, and collared. With our free-roaming horse fieldwork, we made the assumption that our opportunistic fecal collections represented random independent samples, however as discussed in Chapters 3 and 4, there is the chance that we re-sampled the same horses within the same HMA, violating the assumption of independence. While we were able to use a weight of evidence approach when evaluating the proportion of graminoids in the diet, doing this in the high multi-dimensional space of microbial communities was not possible. In future studies, including methods for animal identification to guarantee there is no double sampling would be ideal. Identifying individual animals would also allow researchers to collect more individualized data for each animal, allowing further questions to be explored at the individual

animal level such as comparisons between individual animal body condition, diet, or microbial composition. The lack of individual animal identification for every fecal collection meant we could only reliably make these comparisons at the herd or range-wide level in our research.

This leads us to a third recommendation, collecting multiple time-point samples from each individual animal. While this was beyond the scope of our research and would have been logistically infeasible across the spatial extent of our study, it would assist scientists in comparing metrics over time and allow exploration of temporal individual animal variation in microbial or diet composition. Re-sampling all individuals in both seasons or across other time scales would also be another way to address the issue of independence, by specifically accounting for repeat samples for individuals. In a microbial study, this would also allow individual animal identity to be used as a predictor for explaining microbial variation, as this has explained large amounts of variation in previous studies. Repeat sampling would also allow the opportunity to explore how diet, microbial composition, or other animal metrics such as body condition change temporally and how the relationships between these factors vary over time. In addition, with more individualized information researchers could better explore what constitutes individual variation tied to specific traits, characteristics, or environmental conditions versus the natural random variation within a population.

Overall, we found evidence for variability of microbial composition based on environment and season in both free roaming horses and pronghorn. We also found that free-roaming horse diets included variable amounts of graminoids in different seasons; horses used a variety of plant families across their range; and diet composition co-varied with microbial composition in free-roaming horses, highlighting the flexibility of these herbivores. Our research adds to existing literature that documents microbial composition variability across seasons,

environments, or diet composition in North American herbivores and supports the contention that microbial composition could potentially be an adaptive trait helping animals adjust to novel conditions. Our findings that document these relationships between herbivores, forage, and gut microbes not only add important information to the current knowledge of the ecology of pronghorn and free-roaming horses but also have potential implications for future researchers hoping to apply microbial tools in herbivore species.

REFERENCES

Bahrndorff, S., Alemu, T., Alemneh, T., Nielsen, J.L., 2016. The microbiome of animals: implications for conservation biology. Int. J. Genomics 2016, 5304028. https://doi.org/10.1155/2016/5304028

Bergmann, G.T., Craine, J.M., Robeson, M.S., Fierer, N., 2015. Seasonal shifts in diet and gut microbiota of the American bison (Bison bison). PLoS One 10, 1–14. https://doi.org/10.1371/journal.pone.0142409

Bureau of Land Management, 2025. Herd Area and Herd Management Area Statistics 2025. https://www.blm.gov/sites/default/files/docs/2025-03/2025_Wild_Horse_and_Burro_Population_Estimates.pdf (accessed: 3 April 2025)

Duncan, P., Foose, T.J., Gordon, I.J., Gakahu, C.G., Lloyd, M., 1990. Comparative nutrient extraction from forages by grazing bovids and equids: a test of the nutritional model of equid/bovid competition and coexistence. Oecologia 84, 411–418. https://doi.org/10.1007/BF00329768

Eddington, H.S., Carroll, C., Larsen, R.T., McMillan, B.R., Chaston, J.M., 2021. Spatiotemporal variation in the fecal microbiota of mule deer is associated with proximate and future measures of host health. BMC Vet. Res. 17, 1–8. https://doi.org/10.1186/s12917-021-02972-0

Fountain-Jones, N.M., Clark, N.J., Kinsley, A.C., Carstensen, M., Forester, J., Johnson, T.J., Miller, E.A., Moore, S., Wolf, T.M., Craft, M.E., 2020. Microbial associations and spatial proximity predict North American moose (*Alces alces*) gastrointestinal community composition. J. Anim. Ecol. 89, 817–828. https://doi.org/10.1111/1365-2656.13154

Grange, S., Duncan, P., Gaillard, J., 2009. Poor horse traders: large mammals trade survival for reproduction during the process of feralization. Proc. R. Soc. B. 276, 1911-1919. https://doi.org/10.1098/rspb.2008.1828

Hanley, T.A., 1982. The nutritional basis for food selection by ungulates. J. Range Manag. 35, 146–151. https://www.jstor.org/stable/3898379?origin=crossref&seq=1

Henneke, D.R., Potter, G.D., Kreider, J.L., 1984. Body condition during pregnancy and lactation and reproductive efficiency of mares. Theriogenology 21, 897–909. https://doi.org/10.1016/0093-691X(84)90383-2

Janis, C., 1976. The evolutionary strategy of the Equidae and the origins of rumen and cecal digestion. Evolution. 30, 757-774. https://doi.org/10.2307/2407816

Li, Y., Zhang, K., Liu, Y., Li, K., Hu, D., Wronski, T., 2019. Community composition and diversity of intestinal microbiota in captive and reintroduced Przewalski's horse (*Equus ferus przewalskii*). Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.01821

Menard, C., Duncan, P., Fleurance, G., Georges, J.Y., Lila, M., 2002. Comparative foraging and nutrition of horses and cattle in European wetlands. J. Appl. Ecol. 39, 120–133. https://doi.org/10.1046/j.1365-2664.2002.00693.x

Morley, S.A., Murray, J., 2014. Effects of body condition score on the reproductive physiology of the broodmare: A review. J. Equine Vet. Sci. 34, 842–853. https://doi.org/10.1016/j.jevs.2014.04.001

National Research Council, 2013. Using science to improve the BLM wild horse and burro program: A way forward. The National Academies Press, Washington D.C. pp: 436. https://doi.org/10.17226/13511

Scasta, J.D., Beck, J.L., Angwin, C.J., 2016. Meta-analysis of diet composition and potential conflict of wild horses with livestock and wild ungulates on western rangelands of North America. Rangel. Ecol. Manag. 69, 310–318. https://doi.org/10.1016/j.rama.2016.01.001

Scasta, J.D. Hennig, J.D., Beck, J.L. 2018. Framing contemporary U.S. wild horse and burro management processes in a dynamic ecological, sociological, and political environment. Hum.-Wildl. Interact.12, 31–45. http://doi.org/10.26077/2fhw-fz24

Trevelline, B.K., Fontaine, S.S., Hartup, B.K., Kohl, K.D., 2019. Conservation biology needs a microbial renaissance: A call for the consideration of host-associated microbiota in wildlife management practices. Proc. R. Soc. B Biol. Sci. 286. https://doi.org/10.1098/rspb.2018.2448

Trujillo, S.M., McKenney, E.A., Hilderbrand, G. V., Mangipane, L.S., Rogers, M.C., Joly, K., Gustine, D.D., Erlenbach, J.A., Mangipane, B.A., Lafferty, D.J.R., 2022. Intrinsic and extrinsic factors influence on an omnivore's gut microbiome. PLoS One 17, 1–19. https://doi.org/10.1371/journal.pone.0266698