Evaluating the winter diet of a reintroduced herd of elk in the Cumberland Mountains, Tennessee, using next-generation sequencing techniques

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ABSTRACT

A distinct subspecies of elk (*Cervus canadensis*), the North American elk (*C. canadensis canadensis*), once inhabited portions of the southeastern United States, including Tennessee, until their extirpation in the mid 1800s. From 2000 to 2008, 201 Manitoban elk were reintroduced on the North Cumberland Wildlife Management Area (NCWMA). A year-long food habits study using histological analysis of plant material from feces was completed for the NCWMA elk from 2003 to 2004 and has since aided managers in their landscape planning. Since then, more elk have been released onto the area, food plots have been established throughout the NCWMA, and the population has had approximately 20 years to establish itself on the landscape. Thus, a reevaluation of dietary habits is warranted. We collected 357 groups of fecal pellets from 65 set openings within the 79,318 ha NCWMA weekly from February to April of 2019 for a winter fecal diet analysis using next-generation sequencing techniques, also referred to as metabarcoding. Metabarcoding is a non-invasive methodology that has proven to be more effective in identifying herbivore diets than previously used methods. We conducted DNA extractions, a two-step polymerase chain reaction protocol, and completed library preparation of the samples using the Illumina MiSeq sequencing protocol to isolate the plant DNA from the other genetic material in the scat. A bioinformatical analysis was then conducted to determine what plants were identified from sequencing. Statistical analyses performed include calculating proportions for the genera detected from sequencing, determining if specific plants were used differently by males and females from specific genetic groups on the NCWMA, investigating alpha and beta diversity of

sample sequences, and assessing the use of forage classes by elk during the winter of 2019. The results from this study will further inform managers of the dietary habits of the reintroduced NCWMA herd and assist them in future habitat management.

TABLE OF CONTENTS

1. Literature Review 1
General Elk Food Habits and Digestion 1
Elk Food Habits – Western North America 2
Elk Food Habits – Eastern North America 4
General Winter Diet of Elk 6
Ungulate Food Habit Differences by Sex 8
Previous Methods Used for Identifying Herbivore Diets 11
Use of Next-Generation Sequencing to Identify Food Habits 13
2. Introduction 16
Background 16
3. Objectives and Hypotheses 20
4. Methods 21
Study Area 21
Scat Collection 22
Fecal Metabarcoding Analysis 23
Statistical Analyses 27
5. Results 31
Taxonomic Analyses 31
Sex-Genetic Group Food Habits Analysis 33
Diversity Analyses 34
Overall and Seasonal Forage Class Analysis 35
6. Discussion 36
Elk Food Habits 36
Next-Generation Sequencing 40
Future Directions and Management Implications 42
Literature Cited 44
Appendices 58
Appendix A: Tables 59
Appendix B: Figures 79
Vita 88

LIST OF TABLES

Table 1.1. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Table output from rarefaction curve (Figure 1.2) displaying the number of samples (N), the mean number of sequences per sample for all 319 sequenced samples (Mean), the standard deviation (Std. Dev.), standard error (Std. Error), and the lower (Low CL) and upper (High CL) 95% confidence limits for the mean..... 60
Table 1.2. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA, from February 2019 through April 2019. Proportion of total sequences belonging to genera detected from sequencing and their corresponding family sequence percentages..... 61
Table 1.3. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Proportion of total sequences belonging to the 6 kingdoms (Animalia, Chromista, Fungi, Incertae sedis [unclassified], Plantae, and Protozoa) detected from sequencing..... 70
Table 1.4. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Summary of all pairwise comparisons (conducted using an ANOVA; post hoc pairwise comparisons calculated using Tukey's test) of genera detected from analysis with a significant difference found in the mean relative abundance (MRA) for the combined treatment groups (groups). Also included in this table is the statistical category for each comparison, the groups compared (contrast), standardized difference (SD), p-value (*p*), and statement of significance (Sig.)..... 71
Table 1.5. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Statistical comparisons of observed features (amplicon sequence variants [ASVs]) conducted for combined treatment groups using Kruskal-Wallis pairwise comparisons to investigate alpha (or within-sample group) diversity of sequences detected within each group. H = test statistic, *p* = p-value, and *q* = adjusted p-value. 73
Table 1.6. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Statistical comparisons of Shannon diversity indices of OTUs conducted for combined treatment groups using Kruskal-Wallis pairwise comparisons to

investigate alpha (or within-sample group) diversity of sequences detected within each group. H = test statistic, *p* = p-value, and *q* = adjusted p-value. 74
Table 1.7. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Statistical comparisons of beta (community) diversity analyzed using weighted UniFrac distance matrices used in a principal coordinate analysis. Pairwise analysis of similarities (ANOSIM) was utilized to determine if there were any significant differences in between-sample diversity between treatment groups. Included in this table are: The treatments groups being compared (Group 1, Group 2), sample size of the combined groups (N), permutations performed for that comparison, ANOSIM test statistic (R), p-value (*p*), and adjusted p-value (*q*)..... 75
Table 1.8. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Proportion of total sequences belonging to four major forage classes (forb, graminoid, legume, and woody plant) and any other classes detected from sequencing..... 76
Table 1.9. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Proportion of total Plantae sequences belonging to four major forage classes (forb, graminoid, legume, and woody plant) and any other classes detected from sequencing for the period of sample collection before spring green-up (2/14/19 – 3/18/19) and after (3/19/19 – 4/25/19)..... 77
Table 1.10. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Results from the Fisher's exact test run for forage classes who had rounded proportions >1% during both before spring green-up (SGU; 2/14/19 – 3/18/19) and after (3/19/19 – 4/25/19; forb, woody plant, graminoid, and legume) against all other forage classes' proportions to test for a difference in individual forage class sequence detection before and after spring green-up. The Bonferroni corrected p-value used for significance comparison in this analysis was 0.0125. Also included in this table is the p-value for the individual forage class comparison (*p*) and if *p* was significant (Sig.)..... 78

LIST OF FIGURES

Figure 1.1. The study area with the 65 scat collection sites within the North Cumberland Wildlife Management Area (NCWMA; 79,318-ha) in the Elk Restoration Zone (ERZ) in the Cumberland Mountains, Tennessee, USA. Elk food habits evaluated using a next-generation sequencing protocol with feces collected from NCWMA from February through April 2019. 80
Figure 1.2. Rarefaction curve displaying number of genera detected (y-axis) versus the number of sequences per sample found (x-axis). Statistical output in Table 1.1. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. 81
Figure 1.3. Proportion of sequences belonging to families with relative percentages >1.000% found from bioinformatical analysis. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. 82
Figure 1.4. Proportion of sequences belonging to genera with relative percentages >1.000% found from bioinformatical analysis. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. 83
Figure 1.5. Boxplot displaying statistical comparisons of observed features (amplicon sequence variant; ASVs; y-axis) conducted for combined treatment groups (x-axis) using Kruskal-Wallis pairwise comparisons to investigate alpha (or within-sample group) diversity of sequences detected within each group (F-EINPN n = 14; F-EINPS n = 44; M-EINPN n = 4; M-EINPS n = 14). Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. 84
Figure 1.6. Boxplot displaying distribution of Shannon diversity indices (y-axis) conducted for combined treatment groups (x-axis) using Kruskal-Wallis pairwise comparisons to investigate alpha (or within-sample group) diversity of sequences detected within each group (F-EINPN n = 14; F-EINPS n = 44; M-EINPN n = 4; M-EINPS n = 14). Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019..... 85
Figure 1.7. Principal coordinate analysis plot of weighted UniFrac data measuring beta (community) diversity of sequences detected between combined treatment groups with colors specified for each group: F-EINPN (red), F-EINPS (blue), M-EINPN (orange), and M-EINPS (green). Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North

Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. 86
Figure 1.8. Proportion of total sequences belonging to four major forage classes (forb, graminoid, legume, and woody plant) and any other classes detected from sequencing. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019..... 87

1. LITERATURE REVIEW

General Elk Food Habits and Digestion

To interpret the ecology and behavior of animals, it is imperative to understand their food habits (Cook 2002). Knowledge of food habits assists managers in evaluating habitat preference, recognizing potential for interspecific competition, and planning for managing habitats (Cook 2002). Elk (*Cervus canadensis*) are an important cervid species recognized for their recreational viewing and value as a game species. They are generally considered grazers for their ability to consume grasses. However, elk may be more accurately described as intermediate feeders, able to consume a wide variety of not only leafy, herbaceous vegetation such as grasses and forbs, but also woody plants and shrubs (Hofmann 1989, Mower and Smith 1989, Jenkins and Starkey 1991, Kirchhoff and Larsen 1998, Cook 2002, Geist 2002, Anderson et al. 2005, Christianson and Creel 2005, Schneider et al. 2006, Christianson and Creel 2009, Whittaker 2011). Although elk in the eastern part of North America were largely extirpated following European settlement, herds have been reintroduced, and their food habits studied, in Virginia (Baldwin and Patton 1938), Manitoba (Blood 1966), Michigan (Buss 1967, Spiegel et al. 1963, Moran 1973), Ontario (Jost et al. 1999), Kentucky (Schneider et al. 2006), North Carolina (Murrow 2007), Pennsylvania (Heffernan 2009), Tennessee (Lupardus et al. 2011), and Missouri (Murphy 1963, Smith et al. 2019).

The ruminant digestive system used by elk allows them to opportunistically feed on an array of vegetation throughout the year (Hofmann 1989). Ruminants use a multi-chambered “stomach”, which includes a rumen that acts as a fermentation vat, reticulum,

omasum, and abomasum (National Research Council 2007). Elk take up food through their mouths, where it is mixed with saliva and sent down the esophagus. After food is ingested, it travels to the rumen. The rumen is used for temporary food storage and conducts fermentation via its microbial population. Food stored in the rumen after initial consumption is sent back up the esophagus with help from the reticulum to the mouth for further chewing. The process is referred to as ruminating or cud-chewing (National Research Council 2007). Due to their close proximity, the rumen and reticulum are often referred to together. In intermediate feeders such as elk, the reticulum-rumen increases or decreases its volume based on forage availability, which often correlates with seasons. In the winter, when forage opportunities are low and elk tend to rely heavily on grasses, the two compartments decrease their volume; however, in the summer and spring when elk tend to have the most diverse forage opportunities, volume increases (National Resource Council 2007). Upon leaving the rumen, digesta travels into the omasum where water and minerals are removed before the material enters the abomasum (National Research Council 2007). The abomasum of ruminants is similar to stomachs possessed by nonruminant animals. This chamber is responsible for secreting pepsin-HCl, an important enzyme for digesting food before it can continue into the small intestine for further break down and eventual excretion (National Research Council 2007).

Elk Food Habits – Western North America

Food habits of elk in the western United States and Canada are well-documented, displaying similar seasonal patterns throughout the region (Blood 1966, Kufeld 1973,

Hobbs et al. 1981, Collins and Urness 1983, Leslie et al. 1984, Jenkins and Wright 1988, Sullivan 1988, Mower and Smith 1989, Jenkins and Starkey 1991, Kichhoff and Larsen 1998, Christianson and Creel 2005, Sandoval et al. 2005). Shrubs and forb species tend to dominate the summer diet while browse species become of particular importance in the late summer and autumn (Blood 1966, Kufeld 1973, Collins and Urness 1983, Sullivan 1988, Jenkins and Starkey 1991). Grass is an important aspect of the elk diet particularly throughout the winter and also the late spring (Blood 1966, Kufeld 1973, Hobbs et al. 1981, Leslie et al. 1984, Sullivan 1988, Jenkins and Starkey 1991, Kirchhoff and Larsen 1998, Christianson and Creel 2005, Sandoval et al. 2005). In harsher winters, elk may also consume a small amount of lichen, ferns, and more commonly conifers when snow covers grasses and other graminoids (Leslie et al. 1984, Jenkins and Wright 1988, Jenkins and Starkey 1991, Kirchhoff and Larsen 1998, Cook 2002, Sandoval et al. 2005).

Commonly consumed forbs by elk in the western United States include Pacific aster (*Aster chilensis*), decumbent goldenrod (*Solidago decumbens*), peavine (*Lathyrus* spp.), dandelion (*Taraxacum officinale*), fireweed (*Epilobium angustifolium*), coltsfoot (*Petasites sagittatus*), and huckleberry (*Gaylussacia* spp.; Blood 1966, Collins and Urness 1983, Jenkins and Starkey 1991, Kirchhoff and Larsen 1998). Some of the frequently consumed browse species include rose (*Rosa acicularis*), winterfat (*Eurotia lanata*), chokecherry (*Prunus virginiana*), salal (*Gaultheria shallon*), yucca (*Yucca glauca*), alder (*Alnus rubra*), and species of oak (*Quercus* spp.; Leslie et al. 1984, Sullivan 1988, Jenkins and Starkey 1991, Kichhoff and Larsen 1998). Graminoids documented regularly as western elk feed are bluegrass (*Poa* spp.), western wheatgrass

(*Agropyron smithii*), orchard grass (*Dactylis glomerata*), bluejoint reedgrass (*Calamagrostis canadensis*), and sedges (*Carex* spp.; Hobbs et al. 1981, Sullivan 1988, Jenkins and Starkey 1991, Sandoval et al. 2005). Among conifers that western elk feed on are western redcedar (*Thuja plicata*), western hemlock (*Tsuga heterophylla*), and ponderosa pine (*Pinus ponderosa*; Jenkins and Starkey 1991, Kirchhoff and Larsen 1998, Sandoval et al. 2005). Two of the most common ferns reported in western elk diets are deer fern (*Blechnum spicant*) and swordfern (*Polystichum munitum*; Leslie et al. 1984, Jenkins and Starkey 1991, Kirchhoff and Larsen 1998). Although a small portion (only 2%), for a particular herd in Etolin Island, Alaska, lichen such as witch's hair (*Alectoria sarmentosa*), lungwort (*Lobaria* spp.), and Methuselah's beard lichen (*Usnea longissimi*) were occasionally consumed by elk as well (Kirchhoff and Larsen 1998).

Elk Food Habits – Eastern North America

While not as extensively studied as the food habits of elk in western North America, the food habits of reintroduced elk in eastern parts of the U.S. and Canada have been in studied in Virginia (Baldwin and Patton 1938), Missouri (Murphy 1963), Michigan (Spiegel et al. 1963, Buss 1967), Ontario (Jost et al. 1999), Kentucky (Schneider et al. 2006, Whittaker 2011), North Carolina (Murrow 2007), Pennsylvania (Heffernan 2009), and Tennessee (Lupardus et al. 2011). Similar to that of elk in the western U.S., the winter diet of eastern elk is dominated by grasses, sedges, and some woody browse; elk in Tennessee and Pennsylvania also consumed ferns during winter months (Buss 1967, Schneider 2006, Murrow 2007, Heffernan 2009, Lupardus et al.

2011). During spring, grass remains an important aspect of elk diet, with forbs and woody browse also constituting a large portion (Devlin and Tzilkowski 1986, Schneider et al. 2006, Murrow 2007, Heffernan 2009, Lupardus et al. 2011). Forbs become the primary food source in the summer, followed by legumes, woody plants, and to a lesser degree graminoids (Merrill 1993, Schneider et al. 2006, Lupardus et al. 2011). In the fall, diet preference shifts to primarily woody plants (including acorns) and grasses (Merrill 1993, Schneider et al. 2006, Heffernan 2009, Lupardus et al. 2011). In Missouri, acorns and grass comprised the largest portions of the fall diet for 15 elk, with at least 4 species of *Quercus* making up 50% of the total rumen volume, and various grasses totaling 37% of rumen volume (Murphy 1963). Murphy (1963) also reported forbs such as coralberry (*Symphoriacarpos orbiculatus*), Korean lespedeza (*Lespedeza stipulacea*), and aster (*Aster* sp.) to be of particular importance in the fall diet of this Missouri herd.

Commonly identified forbs consumed by elk in the east are coralberry, Korean lespedeza, aster, galax (*Galax urceolata*), wintergreen (*Gaultheria procumbens*), juneberry (*Amelanchier canadensis*), jewelweed (*Impatiens* spp.), red clover (*Trifolium pretense*), fireweed, and Lespedeza (*Lespedeza cuneata*; Baldwin and Patton 1938, Murphy 1963, Spiegel et al. 1963, Buss 1967, Merrill 1993, Jost et al. 2009, Schneider et al. 2006, Lupardus et al. 2011). Important graminoids for eastern elk include small crabgrass (*Digitaria ischaemum*), crabgrass (*D. sanguinalis*), tall fescue (*Festuca arunidinacea*), big bluestem (*Andropogon gerardii*), little bluestem (*Schizachyrium scopariu*), orchard grass, rushes (*Juncus* spp.), sedges, field corn (*Zea mays*), and brome (*Bromus* spp.; Murphy 1963, Jost et al. 1999, Schneider 2006, Lupardus et al. 2011).

Some of the important browse plants for elk in the east include staghorn sumac (*Rhus typhina*), basswood (*Tilia americana*), cherries (*Prunus* spp.), northern white cedar (*Thuja occidentalis*), hemlock (*Tsuga* spp.), autumn olive (*Elaegnus* spp.), eastern redcedar (*Juniperus virginiana*), oaks, pines, maples (*Acer* spp.), flowering dogwood (*Cornus florida*), New Jersey tea (*Ceanothus americanus*), sourwood (*Oxydendrum arboretum*), blueberry (*Vaccinium* spp.), greenbrier (*Smilax* spp.), willow (*Silax* spp.), and black locust (*Robinia pseudoacacia*; Baldwin and Patton 1938, Spiegel et al. 1963, Buss 1967, Merrill 1993, Jost et al. 1999, Schneider et al. 2006, Lupardus et al. 2011). In Tennessee, Christmas fern (*Polystichum acrostichoides* Schott) was also utilized by elk in the winter (Lupardus et al. 2011).

General Winter Diet of Elk

Even though elk are intermediate feeders, the often harsh conditions associated with winter can limit their food availability (Cook 2002). When plants first begin growth in the spring, they contain mainly soluble, digestible, and nutritional products. However, as plants mature and stems become a more a prominent feature than their leaves, generally undigestible structural components like lignin accumulate in the stem of the plant. These structural components decrease the nutritive value of the plant, with the lowest point during winter (Leslie et al. 1984, Cook 2002). Elk are able to survive during the winter by relying on their storage of fat gained during summer and by consuming available plants, usually graminoids and woody plant materials (Blood 1966, Buss 1967, Leslie et al. 1984, Jenkins and Wright 1988, Sullivan 1988, Jenkins and Starkey 1991,

Jost et al. 1999, Cook 2002, Christianson and Creel 2005, Sandoval et al. 2005, Schneider et al. 2006, Heffernan 2009, Lupardus et al. 2011). Winter adaptability is facilitated by large body size and the efficient ruminant digestive system which decreases reticulum-rumen size during harsh conditions, allowing elk to subsist off of fibrous, less nutritious vegetation (Buss 1967, Holechek 1984, Christianson and Creel 2005, National Research Council 2007). In areas with high amounts of snow, elk may select plants which protrude through, or are unaffected by, snow cover (Buss 1967, Jenkins and Wright 1988, Jost et al. 1999, Cook 2002, Sandoval et al. 2005). In Michigan elk were observed bark-stripping small, young trees from November into April when the first snow fell (Moran 1973). This herd stripped bark the most from red maple (*Acer rubrum*), juneberry, basswood, cherries, striped maple (*Acer pensylvanicum*), witchhazel (*Hamamelis* spp.), staghorn sumac, and aspens (*Populus* spp.).

Elk in North America largely depend on a diet of grasses and woody plants during the winter. Some of the graminoids commonly consumed during this time are western wheatgrass, thread-leaved sedge, perennial ryegrass (*Lolium perenne*), tall fescue, Kentucky bluegrass (*Poa pratensis* L.), big bluestem, wheat, and orchard grass (Sullivan 1988, Sandoval et al. 2005, Schneider et al. 2006, Heffernan 2009, Lupardus et al. 2011) Furthermore, examples of woody browse ingested by wintering elk include: hemlock, winterfat, yucca, salal, huckleberry, trailing blackberry, western redcedar, autumn olive, and maples (Leslie et al. 1984, Sullivan 1988, Jenkins and Starkey 1991, Kirchhoff and Larsen 1998, Sandoval et al. 2005). Other plants important to the winter diet of elk

include swordfern, deer fern, and Christmas fern (Leslie et al. 1984, Jenkins and Starkey 1991, Lupardus et al. 2011).

Ungulate Food Habit Differences by Sex

Studies have examined differentiated feeding habits between sexes in many species of ungulates; examples include the red deer (*Cervus elaphus*; Clutton-Brock et al. 1982), white-tailed deer (*Odocoileus virginianus*; Beier 1987, McCullough et al. 1989), Nubian ibex (*Capra ibex nubiana*; Gross et al. 1996), alpine ibex (*Capra ibex ibex*; Villaret et al. 1997), and elk (Long et al. 2009) and have come to inconsistent conclusions as to whether or not males and females forage differently and what might cause these patterns or lack thereof (Main et al. 1996). One such study is that of Gross et al. 1996 which investigated forage digestion and passage rates among male, female, and lactating female Nubian ibex when all three groups were fed an identical diet of grass and alfalfa hay. Their initial hypothesis was that males would utilize longer forage retention times and have a more complete digestion of feed than either group of females due to the larger rumino-reticular volume in male ungulates and their general tendency to retain digesta longer (Gross et al. 1996). They found that while the male ibex did retain both types of forage longer than non-lactating females, this did not equate to greater fiber digestion, and that all three groups digested both the grass and alfalfa equally well. However, they also noted that lactating females increased both intake and retention time compared to non-lactating females by increasing their gut fill (Gross et al. 1996). The authors asserted that females were able to achieve greater digestion rates by masticating

their feed more rigorously than male ibex did. This study is one example of how ruminants (including elk) might be able to combat foraging issues inflicted by sex-specific diet and reproductive requirements, especially in females (Gross et al. 1996).

Some studies have found that ungulates differ their food habits by sex at different times of the year as their nutritional requirements shift (Clutton-Brock et al. 1982, Beier 1987, McCullough et al. 1989, Main et al. 1996, Villaret et al. 1997). Clutton-Brock et al. (1982) reported that male red deer will often inhabit lower quality areas and select more fibrous foods throughout the year than females. They found that this pattern was most pronounced during spring, summer, and winter. During the warmer months, females are giving birth and lactating, which requires a higher quality, more protein-rich diet. The conclusion that female ungulates have a higher quality diet was supported by a similar study with white-tailed deer, where they measured fecal nitrogen levels for both males and females; fecal nitrogen had a positive correlation with dietary protein, diet digestibility, and gross energy intake and therefore served as a reliable indicator of diet quality (Beier 1987). This study found that fecal nitrogen levels were highest for females in the spring, the lowest during winter, and were consistently higher in females versus males throughout the entirety of the study period, but especially during December and January. Beier (1987) proposed two hypotheses that might explain this finding: 1) there was spatial overlap between the sexes, but they selected forage disparately, or 2) there was spatial separation between the sexes which gave way to different feeding patterns due to dissimilar plant availability. Either hypothesis could have been employed to explain their findings and those in other ungulate sex-differentiated foraging

investigations (Beier 1987). Other studies have explained the sexual segregation seen in ungulates in relation to herbivory habits with similar hypotheses (McCullough et al. 1989, Main et al. 1996). Main et al. (1996) states that ungulate sexual segregation is most pronounced during periods when requirements influencing reproductive success differ most between sexes. For most ungulates this would be during the spring and summer when females are giving birth, lactating, and raising offspring while males acquire energy for the rut and during winter when males are attempting to recover physical condition lost during the rut (Main et al. 1996). Main et al. (1996) proposed and investigated three hypotheses to explain this: 1) the reproductive-strategy hypothesis (explains that separation is due to ecological factors influencing reproductive success, energetics, and security), 2) the sexual dimorphism-body size hypothesis (the contrasting body sizes in male and female ungulates give way to different dietary requirements), and 3) the social-factors hypothesis (social and behavioral mechanisms are at play for both sexes and expose them to different vegetation). The sexual dimorphism-body size hypothesis hinges on the idea that male ungulates use poorer habitat than females. It asserts that male ungulates' larger rumino-reticular volume and greater metabolic requirements might lead them to feed on more abundant, high-fiber forages and retain digesta longer to improve digestion efficiency via urea recycling through ruminal microbes (Main et al. 1996; Long et al. 2009). Meanwhile, smaller-bodied females might selectively feed on lower-fiber, higher quality forages to satisfy the nutritional needs associated with reproductive processes like gestation and lactation (Main et al. 1996). They were unable to find evidence supporting either the sexual dimorphism-body size hypothesis or the social-

factors hypothesis. Instead, they found that most ungulate habitat use studies which did see marked differences between males and females fell more in line with the reproductive-strategy hypothesis. Males will have as good or better diets than females as females are likely to select habitats better suited for preventing predation for them and their offspring while males can utilize areas with high-quality forage at all times of the year excluding the rut (Main et al. 1996).

Previous Methods Used for Identifying Herbivore Diets

Historically, three methods have primarily been used to evaluate the food habits of elk in North America: histological examination of fecal samples, analysis of rumen contents, and observation of feeding (Baldwin and Patton 1938, Murphy 1963, Spiegel et al. 1963, Blood 1966, Buss 1967, Hobbs et al. 1981, Collins and Urness 1983, Hobbs et al. 1983, Leslie et al. 1984, Devlin and Tzilkowski 1986, Jenkins and Wright 1988, Sullivan 1988, Mower and Smith 1989, Jenkins and Starkey 1991, Kirchhoff and Larsen 1998, Jost et al. 1999, Sandoval et al. 2005, Schneider et al. 2006, Murrow 2007, Heffernan 2009, Lupardus et al. 2011, Nanney et al. 2018). Each of these methods have advantages and disadvantages and may be utilized individually or concurrently within a study.

The histological examination of feces is one of the most commonly used methods in food habit studies (Baldwin and Patton 1938, McInnis et al. 1983, Leslie et al. 1984, Devlin and Tzilkowski 1986, Jenkins and Wright 1988, Sullivan 1988, Mower and Smith 1989, Jenkins and Sarkey 1991, Merrill 1993, Kirchhoff and Larsen 1998, Sandoval et al.

2005, Schneider et al. 2006, Murrow 2007, Heffernan 2009, Lupardus et al. 2011). Unlike rumen analysis, it is non-invasive to the target species and poses no threat to endangered and scarce populations (Anthony and Smith 1974). As described by Storr (1961), this methodology first requires the researcher to create reference slides of stained epidermal material from plants that are likely to be consumed by the target species. To prepare the fecal samples, the scat must first be dried and ground, after which it is boiled and stirred to allow the plant fragments to separate from the fecal matter (Storr 1961, Anthony and Smith 1974). From this material, several subsamples are placed onto microscope slides, where consumed and digested plants are compared to the plant reference slides (Anthony and Smith 1974). McInnis (1983) reported that fecal analysis tends to report a higher presence of graminoids than forbs compared to other methods and is time consuming (Anthony and Smith 1974). Differential rates of digestibility among plants may contribute to presence and identification of plant cells in the feces (McInnis 1983).

Although used less frequently than fecal analysis, the examination of rumen content has been used historically to determine large herbivore diets (Baldwin and Patton 1938, Murphy 1963, Blood 1966, Buss 1967, Anthony and Smith 1974, McInnis et al. 1983, Jost et al. 1999). Generally, rumen analysis involves taking a sample of the rumen content of a dead animal, preserving the contents, and isolating the plant material by washing the contents through a screen which are identified via microscopy (Murphy 1963, Blood 1966, Anthony and Smith 1974). Disadvantages of rumen content analysis include requiring the death of the individual being studied, necessity of a large sample,

and possible overestimation of graminoids due to the high digestibility of forbs compared to grasses (Anthony and Smith 1974, Smith and Shandruck 1979, McInnis et al. 1983).

Another commonly used method for determining food habits of herbivores is through direct feeding observation and habitat use (Baldwin and Patton 1938, Spiegel et al. 1963, Smith and Shandruck 1979, Hobbs et al. 1981, Collins and Urness 1983, Hobbs et al. 1983, McInnis et al. 1983, Jost et al. 1999, Nanney et al. 2018). These studies involve first-hand observation of an individual eating plant material and may rely on the researcher's ability to identify consumed (or partially consumed) plants within a study area (Smith and Shandruck 1979, McInnis 1983). This technique is often the least invasive but may fail to represent the full diet of an herbivore (McInnis 1983). Observations may fail to recognize light use of some plants of limited use and "invisible utilization", where herbivores fully remove a plant from the ground, leaving no trace upon consumption (Laylock et al. 1972, McInnis et al. 1983).

Use of Next-Generation Sequencing to Identify Food Habits

Using genetic analysis and metabarcoding techniques to identify the food habits of animals from their scat has become popular for diet analyses in recent years, and includes food habit studies for herbivorous species such as the alpine chamois (*Rupicapra rupicapra*; Raye et al. 2011); moose (*Alces alces*), roe deer (*Capreolus capreolus*), and red deer (Czernik et al. 2013); lowland tapir (*Tapirus terrestris*; Hibert et al. 2013); red-headed wood pigeon (*Columba janthina nitens*; Ando et al. 2013); collared and brown lemmings (*Dicrostonyx groenlandicus* and *Lemmus trimucronatus*, respectively; Soininen

et al. 2015); walia ibex (*Capra walie*; Gebremedhin et al. 2016); several species of large African herbivores (Kartzinel et al. 2015); bison (*Bison bison*; Leonard et al. 2017); the Pacific pocket mouse (*Perognathus longimembris pacificus*; Iwanowicz et al. 2016); the lesser white-fronted goose (*Anser erythropus*; Ando et al. 2018); and species of Mongolian sheep (*Ovis ammon*; Guo et al. 2018; Alberdi et al. 2019, McInnes et al. 2017, Moorhouse-Gann et al. 2018, Pompanon et al. 2012, Valentini et al. 2009b). This method has proven to be ever-improving, cost-effective, non-invasive, and has shown to be more accurate in determining food habits than histologically examining herbivore feces (Valentini et al. 2009a, b; Raye et al. 2011; Pompanon et al. 2012; Kress et al. 2015; Ando et al. 2018). Next-generation sequencing has also been utilized to study resource and niche partitioning between groups of organisms within their habitat; this was done in one study for seven abundant large African herbivore species wherein researchers looked at diet breadth, composition, and overlap using DNA metabarcoding (Kartzinel et al. 2015, Crisol-Martínez et al. 2016). This process involves extracting DNA from a collected sample, amplifying the genetic material from the sample via polymerase chain reaction (PCR) with specific "barcode" primers that best correspond with the taxa targeted for identification, high-throughput (next-generation) sequencing, and comparing the results with an established DNA barcode reference database (e.g. National Center for Biotechnology Information (NCBI), GenBank) that contains barcode information for the taxa being investigated (Haarsma et al. 2016).

Although next-generation sequencing is a relatively new technique in herbivore dietary analysis, the primers and targeted regions used have changed in relevance and

popularity overtime (Hollingsworth et al. 2011, Kress et al. 2015, Cheng et al. 2016, Alberdi et al. 2019). In earlier studies, a *trn*L approach was used where the plastid sequence of the P6 loop of the chloroplast *trn*L (UAA) intron was targeted via PCR (Soininen et al. 2009; Valentini et al. 2009a, b; Raye et al. 2011; Ando et al. 2013; Czernik et al. 2013; Hibert et al. 2013; Gebremedhin et al. 2015; Soininen et al. 2015; Leonard et al. 2017; Ando et al. 2018). This method facilitated the amplification of particularly degraded DNA with short sequences commonly associated with feces (Deagle et al. 2006, Valentini et al. 2009b). However, as the technique has been improved, different regions of the plant genome such as those from the plastid genome (e.g. *matK*, *rbcL*, *trnH-psbA*) and the nuclear internal transcribed spacer (ITS) region (including the ITS2 region) have been used both independently and concurrently to study food habits of herbivores due to their increased efficacy in identifying plants compared to older metabarcoding approaches (Kress et al. 2015, Cheng et al. 2016).

2. INTRODUCTION

Background

Tennessee Elk History and Reintroduction

Historically, the North American elk subspecies, *Cervus canadensis canadensis*, existed in the eastern United States, including Tennessee. However, overharvest and habitat destruction following European settlement led to their eventual extinction in Tennessee, with the last reported elk sightings being that of two that were shot in 1849 at Reelfoot Lake and in 1865 in Obion County (O'Gara and Dundas 2002).

In the late 1990's the Tennessee Wildlife Resources Agency (TWRA) set out to determine if a reintroduced population of elk would be successful in Tennessee based on criteria from Wathen et al. (1997). The area had to have: 1) 200,000+ available acres, 2) significant public land holdings, 3) significant open land acreage, or the potential to develop open areas, 4) potential to offer opportunities for public hunting, and 5) be an area with minimal crop depredation (TWRA 2018). A protocol by TWRA (2000) established an elk restoration zone (ERZ) around the North Cumberland Mountain Wildlife Management Area (NCWMA). The 271,145-hectare ERZ covers portions of Scott, Campbell, Morgan, Claiborne, and Anderson counties (TWRA 2018). The ERZ has a low human population, generally low amount of acreage dedicated to agriculture, and good hunting and viewing opportunities, all of which fit the criteria offered by Wathen et al. (1997) for elk restoration.

From 2000 to 2008, 201 Manitoban elk (*Cervus canadensis manitobensis*), were released into the NCWMA (TWRA 2018). The Manitoban elk subspecies is considered

to be closely related to the extinct eastern elk subspecies (TWRA 2018). Elk were brought in from Elk Island National Park (EINP) in Alberta, Canada (TWRA 2018). The first 50 elk were released on December 19, 2000; another EINP group of 36 were released in 2001, and 50 more in 2002 (TWRA 2018). In 2003, a group of 31 elk were released in the NCWMA from Land Between the Lakes (LBL), Kentucky; these elk had also originated from EINP. The last elk release took place in 2008 when 34 individuals from LBL were released into the ERZ (TWRA 2018).

Based on the populations of elk in other eastern states, TWRA biologists hypothesized that the ERZ could sustain a population of up to 2,000 elk (TWRA 2018). A population viability analysis was completed for the NCWMA elk from 2000 to 2004 (Kindall et al. 2011). This study identified the mean annual survival of the herd to be 80% but reported that the herd was still at risk of decline (Kindall et al. 2011). It was hoped that mortality risk would decrease if the herd was able to develop a resistance to meningeal worm (*Parelaphostrongylus tenuis*), a reduction in poaching occurred, and improvements were made to the habitat (Kindall et al. 2011). Lupardus et al. (2011) conducted a study from 2003 to 2004 using histological analysis of feces and rumen content analysis methods to determine the food habits of NCWMA elk. Since this initial food habits study, more elk were released into the area, food plots and forest clearings were established throughout the NCWMA, and the population has been established on the landscape for about 20 years. Therefore, a follow-up diet study was deemed essential by TWRA to aid in making future management decisions.

Previous/Current Elk Genetic Analyses

Prior to our research, an analysis was completed to investigate population structure following translocation of elk on the NCWMA (Muller et al. 2018). The elk on the NCWMA came from EINP which was divided into northern and southern portions by a major highway and the two areas are surrounded by fencing. The northern section was larger than the southern area (135.8-km^2 versus 58.2-km^2) and has been fenced off since 1907, although additions were added to it in both 1922 and 1947 (Muller et al. 2018). The smaller southern enclosure was used as an isolation area to maintain a population of wood bison (*Bison bison athabascae*) after 1965 (Griffiths 1979). The geographic barriers prevented intermixing between elk from the two areas, and it was this long-term separation of populations that acted as a catalyst for this original elk-genetics study; it was hypothesized that matrilineal associations from the source population would persist and that genetic groups would move similarly following translocation (Muller et al. 2018). This hypothesis was investigated through hair and blood samples taken from 167 elk at the time of handling prior to translocation. Samples were sent to Wildlife Genetics International in Nelson, British Columbia, Canada (WGI) where they analyzed the samples using 16 microsatellite markers commonly used in farmed elk. The samples were put through extractions, DNA purification, PCR, and PCR product visualization. Following lab protocols, results were put through the software programs CERVUS (version 3.0.3; Kalinowski et al. 2007) to evaluate heterozygosity and STRUCTURE (version 2.3.4; Pritchard et al. 2000, 2010) to determine if elk sampled originated from distinct populations. The individual methods and specifications utilized for these two

programs are described in Muller et al. 2018. This analysis identified two clusters of elk, which were determined to be those from EINP-South (EINPS) and EINP-North (EINPN). Moreover, results from this study revealed continued geographic and familial segregation in elk from both areas after 11+ years from release, supporting their hypothesis of persistent genetic structuring in elk following translocation despite the ability for these two groups to mix easily on the NCWMA (Muller et al. 2018).

The methodology used by Muller et al. (2018) was repeated for another ongoing elk research project investigating the genetic and familial structure for this population using the same 16 microsatellites with the exception of one which was not analyzed for all samples due to its poor performance during analysis in otherwise successful samples (E. Watson, University of Tennessee, unpublished data). However, this study utilized not only blood and hair samples, but also scat collected according to the methodology discussed in this manuscript. From this analysis, 171 of the 378 samples gathered were assigned to 94 elk that were successfully genotyped. This analysis also identified 179 samples coming from a male (56) or female (123), and 78 were successfully assigned to either ENIPN (18) or EINPS (60). This information was utilized during this research's analyses to investigate food habit differences that might exist between males and females originating from both areas in EINP on the NCWMA (E. Watson, University of Tennessee, unpublished data).

3. OBJECTIVES AND HYPOTHESES

I evaluated the winter diet of elk in the NCWMA located in the Cumberland Mountains in Tennessee using next-generation sequencing techniques to identify plant material from collected fecal samples of individually identified elk. I expected these methods would provide a more comprehensive list of plants used than previous diet study approaches and wanted to evaluate food habits by sex and genetic group. Specifically, the objectives and hypotheses for this study were:

Objective 1. To use metabarcoding techniques by isolating plant DNA from collected scat on the landscape with individual elk identification to facilitate the identification of plants consumed.

Hypothesis 1. The winter diet will primarily consist of graminoids and woody browse material.

Objective 2. To identify differences in food habits between genetic groups and sexes on the NCWMA during winter.

Hypothesis 2. There will be differences in food habits between genetic groups and males and females.

4. METHODS

Study Area

We conducted the study within the NCWMA (79,318-ha) within the 271,145-ha ERZ that spans through Scott, Campbell, Morgan, Claiborne, and Anderson counties (TWRA 2018). Smalley (1984) reported that the elevation range for the NCWMA ranges between 1,300 to 2,600 feet, and mean slopes are 40% to 60% (actual overall slope range is between 10% to 100%; Smalley 1984). The NCWMA is made up of 86% deciduous forest, 12% openings from reclaimed coal strip mines and fields, and 1% cropland (TWRA 2000). Cabrera (1969) described the NCMWA as a mixed-mesophytic forest which included sugar maple (*Acer saccharum*), yellow-poplar (*Liriodendron tulipifera*), basswood, and buckeye (*Aesculus flava*) as major north-facing cove communities. Sugar maple, northern red oak (*Quercus rubra*), yellow-poplar, and black locust communities constituted the north and west facing ridges and coves (Cabrera 1969). On the west and southwest facing coves and ridges, chestnut oak (*Quercus montana*) and black locust communities were found (Cabrera 1969). Once an area used for strip, bench, and deep coal mining, the WMA has been left with shelves and benches, some of which (approximately 300 ha) have been converted to wildlife openings (TWRA 2018). These repurposed openings tended to contain tall fescue and Lespedeza (TWRA 2018). Reclaimed fields were often planted with cool season vegetation such as wheat (*Triticum* spp.), clovers (*Trifolium* spp.), turnips (*Brassica* spp.), and alfalfa (*Medicago* L. spp.; TWRA 2018). Annual warm season vegetation such as soybeans (*Glycine* spp.), cowpeas (*Vigna* spp.), sunflowers (*Helianthus* spp.), and corn (*Zea* L. spp.) were planted in these

fields (TWRA 2018). To ensure prime elk foraging, TWRA utilized prescribed burning, herbicide treatment, mowing, and replanting on 2-3 year and 3-5 year cycles on these openings (TWRA 2018).

Scat Collection

We collected scat from the Hatfield Knob elk viewing area and 7 different "Elk Hunt Zones" on the NCWMA. Within these collection areas, 65 openings were selected for sampling based on their history of elk use and geographical representation of the majority of the NCWMA (Figure 1.1). Fields varied in size, vegetative make-up, and elevation. To perform an accurate elk winter diet analysis, we collected scat every week during the late winter and early spring months of 2019 (February through April). We collected between 10-15 pellets from piles of scat that were determined to be "fresh". We based freshness of scat on factors such as color, moisture, smell, and luster (Kirchhoff and Larsen 1998, Murrow 2007, Lupardus et al. 2011). We only collected scat during dry periods, as precipitation has been shown to destroy genetic material on feces (Brinkman et al. 2010). After a rain event, we allotted a waiting period of at least 1 day before further collection to allow the elk to re-enter, feed from, and defecate in the fields. We completed transects of up to 3.4 km (measured with GPS unit as 2.1 miles) using all-terrain vehicles (ATV) through the fields, with the collector stopping when a suitable sample was found within 1 meter in any direction of the ATV. Using gloves, we picked up pellets using an inside-out turned plastic bag, labelling them with an individual sample number (S#), date, location, and field number (as applicable) of the area they were found.

We recorded a GPS point at every suitable sample (labelled with the same descriptors as the collected pellets) using a Garmin eTrex 20x unit (Garmin Ltd., Kansas, USA). We recorded dew point, relative humidity, and temperature range of the day of collection using a Kestrel 3000 unit (Nielsen-Kellerman Co., Pennsylvania, USA). We also recorded weather conditions of the night before and a description of the pile and its surroundings. We immediately placed the collected pellets in a cooler for transportation back to the laboratory. In the laboratory, samples were placed in a -20°C freezer in an effort to preserve the integrity of the DNA in the pellets until the time of genetic analysis. During this collection season scat was also collected directly from 26 elk that were collared during a corresponding study.

Fecal Metabarcoding Analysis

We dried the scat by placing 5 frozen fecal pellets per sample into a 50 mL tube filled to approximately 12.5 mL with silica beads. Upon placement into the silica, the tube was placed immediately back into the freezer until the sample was completely dry. We cut 4 of the 5 dried fecal pellets from each sample for extraction, ensuring an extra pellet was available from each sample for any future analyses. We used Excelta High Precision (Three Star) Single Edge Carbon Steel Razor Blades (Excelta 1762) to cut into the pellets, exposing the inner portion of the scat where the plant material was located. The material was separated from the rest of the pellet, weighed, and used for analysis. We cleaned razors thoroughly between pellets with 95% ethanol and a new razor was used for each group of 4 pellets. The starting weight of each dried sample for DNA extractions

was between 0.15 and 0.18 g. We completed DNA extractions using the Qiagen QIAmp PowerFecal DNA Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. This protocol involved incubation at 65°C, the lysis of the plant material using garnet beads and a horizontal vortex adapter, cleaning of the genetic material using the kit-provided reagents, and elution of the final product, which yielded approximately 100 μL of solution. We stored final products in a freezer at -20°C. For the first 61 samples, each pellet from the group of 4 was treated as an individual sample throughout the entire protocol. However, after the elution step we used 25 μl of final product pooled from each of the 4 pellets from one group into one tube to be sent away for sequencing. For the remaining 296 collection samples approximately 0.04 g of dried material was taken from each of the 4 pellets and placed into one tube to be treated as a single sample during the extraction process. This was done to save time and resources while still ensuring the vegetative make-up of the sample was properly represented in the sample. The same methodology was carried out for the samples acquired from collaring, using between 0.15 g and 0.20 g of scat at the start of the extraction process.

We put these extraction samples through an initial PCR following Illumina's 16S Metagenomic Sequencing Library Preparation protocol, Part # 15044223 Rev. B [hereto after cited as (Illumina)]. In this PCR we used Kapa Hifi master mix taq (Roche) with the forward primer, ITS2-2For (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG; Chen et al. 2010), and the reverse primer, ITS2-3Rev (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG; Chiou et al. 2007), to amplify the ITS2 region of the internal transcribed spacer of the nuclear ribosomal DNA

given its proven efficacy in identifying plants in previous studies (Chiou et al. 2007, Chen et al. 2010, Yao et al. 2010, Garcia-Robledo et al. 2013, Sickel et al. 2015, Cheng et al 2016, Iwanowicz et al. 2016, Guo et al. 2018, Moorhouse-Gann et al. 2018). To allow the addition of the indexes in the two-step PCR process, Illumina-specific adapters were added to both the forward and reverse primers [(ITS2-2For: -ATGCGATACTTGGTGTGAAT) (ITS2-3Rev: -GACGCTTCTCCAGACTACAAT); (Illumina)]. The PCR conditions consisted of an initial denaturing step of 3 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 62.5°C, and 30 seconds at 72°C, with a final extension of 5 minutes at 72°C. We confirmed the amplification of the product using a 2% agarose gel following the initial PCR. After amplification verification, we purified the PCR product using Agencourt Ampure XP beads (Beckman Coulter) to eliminate any remaining primers and primer-dimers. This process involves binding the PCR product to the magnetic beads, the separation of the PCR product on the magnetic beads from contaminants, washing the product with ethanol, separating the product from the beads, and transferring to a new tube. Following bead clean-up, we completed an index PCR using Nextera XT Version 2 indexes (Illumina). This protocol attaches individual eight-base nucleotide sequences to each sample, allowing all samples to be pooled and demultiplexed after sequencing as well as attaching the flow cell primers, which aid in the attachment of the product to the flow cell loaded on the Illumina MiSeq. The index PCR involves a similar methodology as the initial PCR of denaturing, annealing, and elongation of the sample, but goes through only 8 cycles of priming. The final step in library preparation involved another round of magnetic bead

clean-up (as described earlier) to purify the final product before it was quantified. We measured the 260/280 and 260/230 ratios concentrations of amplified DNA (ng/µL) in samples using a Nanodrop spectrophotometer. We then multiplexed differing amounts of product from each sample into one pool, such that the amount loaded from each was approximately equal. For the final step in the pooling and quantification of our sample, we diluted the amplicon product to a final concentration of 4 pM following Illumina's specifications, combined the sample with 20% PhiX control (Illumina), and loaded it onto a Version 3 flow cell reading 275 bases paired-end on the Illumina MiSeq at the University of Tennessee's Genomics Core Facility.

To determine what plants were identified from sequencing, we sent our samples to the MRDNA Molecular Research lab in Shallowater, TX, where they performed bioinformatics using a custom pipeline. They removed the primer sequences and those sequences with a length of less than 150 bp (base pairs). Remaining sequences were quality filtered with a maximum expected error threshold of 1.0 and dereplicated. Dereplicated or unique sequences were denoised. Any unique sequences found via sequencing or PCR point errors were also removed, as were chimeras – producing denoised sequences of zero-radius operational taxonomic units (zOTUs). To classify these final zOTUs taxonomically, BLASTn was used against a curated sequence database derived from NCBI (NCBI Resource Coordinators 2018). Identified non-plant sequences were kept to look at the relative percentages of all organisms found. Final zOTUs were anthologized into taxonomic-level count (actual number of sequences) and percentage (relative percentage of sequences) files. Count files contain the actual number of

sequences whereas the percentage files include the relative percentage of sequences within each sample for each family and genus identified during analysis.

Statistical Analyses

Taxonomic, Treatment Group Food Habits, and Diversity Analyses

The MRDNA group performed accompanying statistical analyses using XLstat, NCSS 2007, "R", and NCSS 2010 (Addinsoft 2019, Hintze 2007, R Core Team 2017). Based on the results from bioinformatics we evaluated total sequences to genera found using a rarefaction curve in SAS (version 9.4, SAS Institute Inc. 2013) with a general linear model and a quadratic term. Analyses performed the MRDNA group were conducted on four assigned combined-sample groups: females from EINPN (F-EINPN), females from EINPS (F-EINPS), males from EINPN (M-EINPN), and males from EINPS (M-EINPS). To determine if specific genera use differed between combined groups, comparisons were made using an ANOVA and post hoc pairwise comparison using Tukey's test for rarified genera data whose relative abundance was > 0.1%. To compare species richness and evenness of plant genera found in each sample, two measurements of alpha diversity were estimated for each combined sample group and compared against each other. For the first alpha diversity analysis, statistical comparisons of observable features (amplicon sequences variant [ASVs]) were made using Kruskal-Wallis pairwise comparisons. The second alpha diversity analysis used a Shannon Diversity index analysis with OTUs also using Kruskal-Wallis pairwise comparisons. The observed features analysis is a gauge of alpha diversity that measures richness, or number of

genera from ASVs, for a sample group. The Shannon diversity indices analysis is also a measurement of alpha diversity which assesses not only richness, but also the evenness of the genera for the sample group from OTUs. For this study these analyses were conducted for each combined sample group; the pairwise comparison evaluates the diversity measurement of one sample group to another, producing an H test statistic and a p-value. A high H test statistic and p-value <0.05 indicates that the alpha diversity of the genera found in the samples of the two groups being compared are significantly different. To determine community diversity of plant genera between combined treatment groups, beta diversity was measured using weighted UniFrac distance matrices. Weighted UniFrac analyses sum the phylogenetic branch lengths of sequences from the studied communities and account for abundance of OTUs (Chang et al. 2011). From these matrices, a principal coordinate analysis (PCoA) plot was utilized to visualize the data, and a pairwise analysis of similarities (ANOSIM) was used to detect community plant genera differences. The ANOSIM calculates the ratio statistic "R"; an R calculation close to 1.0 implies that the groups being compared are dissimilar, while an R value closer to 0.0 indicates a similar diversity of samples between the compared groups. Alpha and beta diversity analyses were conducted as described in previous studies (Dowd et al. 2008a, Dowd et al. 2008b, Edgar 2010, Eren et al. 2011, Swanson et al. 2011) using Qiime 2 (Bolyen et al. 2018) wherein samples were rarefied to 1,000 sequences and significance was assigned for those analyses with a p-value less than 0.05.

Overall and Seasonal Forage Class Analyses

To assess forage class consumption differences, we performed statistical analyses using XLStat (Addinsoft 2019). The initial forage classes used during analysis were the same as those used by Lupardus et al. (2011): woody plant, graminoid, forb, legume, fern, and other. For this analysis we classified all genera detected within the Plantae kingdom into one of these five forage categories and calculated the proportion of all plant sequences found for that class. To determine if forage class consumption changed through the sampling period, we calculated the proportions of these forage classes before and after spring green-up (SGU) for all samples put through sequencing and bioinformatics, not just those put through bioinformatical statistics, that were collected during the 2019 field season. Based on field observation, we determined green up to occur in mid-March, and thus classified samples under "Before SGU" (BSGU) if they were collected between the start of sampling (2 February 2019) up until 14 March 2019. We classified all samples collected between 18 March 2019 to the end of the sampling period (25 April 2019) under "After SGU" (ASGU). To investigate whether individual forage class consumption differed before and after SGU, we performed a Fisher's exact test (Proc FREQ; SAS Institute Inc. 2018) on the rounded proportions of forage classes whose sequence detection was >1% both before and after SGU (forb, woody plant, graminoid, and legume) and compared them against the total proportion of all other forage classes. The p-values calculated for the forage classes were two-sided and compared against a Bonferroni corrected p-value. The Bonferroni corrected p-value was calculated by dividing 0.05 by the number of categories and this adjusted value was used

for significance comparison in all four analyses (Bonferroni's corrected p-value = 0.05/4 = 0.0125).

5. RESULTS

Taxonomic Analyses

During collection we gathered 357 samples from designated plots from 14 February 2019 to 25 April 2019 and took 31 samples from collared elk at the time of capture. We extracted all 357 field samples and 21 of the collared samples (n = 378). We put all 378 of these samples through the two-step PCR protocol. Following PCR those samples that were unsuccessful during genotyping for an elk genetics analysis using the same scat (K. Kurth, University of Tennessee, unpublished data) and failed to amplify during plant analysis were not put through next-generation sequencing; samples sequenced include 270 samples that amplified successfully for plant DNA and 49 remaining samples that successfully genotyped during elk analysis, but failed plant PCR (n = 319). Of those samples sequenced, 298 came from field collection and 21 from collared elk. The elk genetics analysis identified 94 unique individuals (23 male and 71 female) with some replication of sampling of the same individual over time. With replicates from the same individuals, we had 179 samples (56 male and 123 female). For the unique individuals, we did not include those elk with mixed genetics or ones immigrating from Kentucky for the 78 assigned to either EINPN (18) or EINPS (60). We bioinformatically analyzed all 319 samples that were put through next-generation sequencing. For the taxonomic proportion calculations, combined treatment group genera comparison, diversity analyses, and the forage class analysis only the first occurrence of those samples with both sex and geographic identification were utilized to avoid

pseudoreplication (using the same elk multiple times; n = 78). However, we used all 319 taxonomically analyzed samples for the SGU analysis.

The bioinformatical analysis detected 5,101,718 sequences that were then clustered and mapped to zOTUs. The mean read per sample was 15,992.85 sequences and the total number of sequences per sample for all 319 samples sequenced ranged from 11 to 46,941 sequences with a standard deviation of 9,657.25, a standard error of 540.70, and a confidence limit of 14,929.04 to 17,056.65 sequences (Table 1.1; Figure 1.2). We detected 382 genera assigned to 204 families from the 78 samples analyzed for all taxa (Table 1.2). These families were assigned to six kingdoms (Table 1.3). The majority of all sequences (98.2%) came from Plantae in 88 families. The family detected most often was Poaceae, with sequences assigned to 35 genera representing 18.9% of all sequences detected. The main genera represented in Poaceae were *Festuca* (8.5%), *Poa* (3.3%), *Alopecurus* (2.5%), and *Dichanthelium* (1.3%). Ericaceae was the second most detected family (17.4%) with one genus, *Vaccinuium*, making up 15.2% of all sequences found for all samples analyzed. Other families detected frequently include Rosaceae (13.3%), Fabaceae (10.7%), Eleagnaceae (5.2%), Aceraceae (4.1%), Aquafoliaceae (3.7%), Betulaceae (3.0%), Caryophyllaceae (2.9%), Asteraceae (2.7%), Plantaginaceae (2.6%), Juglandaceae (2.3%), Fagaceae (2.1%), and Brassicaceae (1.5%); these 12 families plus Poaceae and Ericaceae made up 90.4% of all sequences. All other detected families and their subsequent genera constituted less than 1.0% of all sequences respectively (Figure 1.3; Table 1.2). Genera with more than 1.0% of all sequences are reported in Figure 1.4 and Table 1.2.

Approximately 26.0% of all families (n = 53) came from the Animalia kingdom, representing 0.3% of all sequences detected. Less than 0.1% of all sequences came from Protozoa or were unassigned. Unclassified kingdoms (Incertae sedis) were represented by two families while four fell under Protozoa. Chromista had eight families and comprised 0.9% of all sequences. Fungi represented 0.6% of all sequences with 49 families.

Sex-Genetic Group Food Habits Analysis

The results from the ANOVA and Tukey's test post hoc pairwise comparisons analysis identified six genera with differences between males and females from EINPN and EINPS: *Rubus*, *Quercus*, *Rhus*, *Phleum*, *Oenothera*, and *Briza* (Table 1.4). *Rubus* constituted 4.5% of all sequences. The mean relative abundance (MRA) for Rubus from M-EINPN (14.609) was higher than M-EINPS (2.857) and F-EINPS (3.475). *Quercus* comprised 2.1% of all genera sequences detected. For *Quercus* sequence detection M-EINPN had a higher MRA (7.662) than M-EIPNS (0.856) and F-EINPS (1.397). *Rhus* was detected in 0.5% of all sequences. For *Rhus*, F-EINPN (MRA = 1.990) contained more sequences when compared to F-EINPS (0.018). *Phleum* comprised 0.4% of all sequences. For *Phleum* sequence detection M-EINPN had a higher MRA (6.599) than M-EIPNS (0.089), F-EINPN (0.085), and F-EINPS (0.102). *Oenothera* comprised 0.2% of all sequences. *Oenothera* was detected more in F-EINPN (MRA = 0.924) samples than both M-EINPS (0.000) and F-EINPS (0.025). *Briza* was reported in 0.1% of all sequences and is not native to the Southeastern United States. *Briza* sequences were

found in differing amounts between M-EINPN (MRA = 1.337) and M-EINPS (0.188), F-EINPN (0.054) and F-EINPS (0.046).

Diversity Analyses

The results from the statistical comparison of observed features (ASVs) alpha diversity analysis determined that the M-EINPN group had a significantly greater taxonomic diversity within its samples than the other three sample groups: M-EINPS, F-EINPN, and F-EINPS (Table 1.5 Figure 1.5). Conversely, the Shannon diversity indices analysis which accounts for genera evenness as well as richness found that only a significant difference of sample group alpha diversity of genera was present between M-EINPN and M-EINPS (H = 4.667; p-value = 0.031; Table 1.6; Figure 1.6). Based on the results of the ANOSIM beta diversity analysis, there appears to be no phylogenetic assemblage of plant sequences among any one of the combined treatment groups that is significantly different (dissimilar) from the other combined treatment groups. All reported p-values were greater than 0.05 and all calculated R values remained closer to 0.0 than 1.0, indicating similarity of genera sequences detected between sample groups (Table 1.7). These results are further supported in the weighted UniFrac PCoA plot, as there appear to no assemblages of clusters representing the sample sequences from each of the treatment groups (Figure 1.6).

Overall and Seasonal Forage Class Analysis

Using the same categories as Lupardus et al. 2011, we calculated the proportions of forage classes for the 78 samples put through bioinformatics and statistics based on their percentage of total sequences from the Plantae kingdom (Table 1.8; Figure 1.8). We classified 79.2% of all matter detected during this study as a woody plant (58.9%) or a graminoid (20.3%). Forbs constituted 16.2% of sequences, followed by legumes (4.4%). All other types of forage made up the smallest percentage of sequences (2.0%).

For samples collected both before SGU and after, woody plants constituted the majority of sequences (BSGU = 58.7%; ASGU = 52.4%; Table 1.9). The proportion of forb sequences more than doubled from before SGU (11.9%) to after (28.9%), while graminoid sequence detection decreased (BSGU = 23.9%; ASGU = 15.6%). Legumes were detected in similar amounts during both periods (5.5% versus 3.1%). All other Plantae sequences were consumed in similarly negligible amounts during both periods as well (BSGU and ASGU = 0.0%; Table 1.9). These results are reported in Table 1.9. Based on the results from the Fisher's exact test performed on all four forage class' proportions before SGU and after against all other forage classes' combined proportions before and after SGU, only forb sequence detection was significantly different before SGU and after, where forb detection increased after SGU (p-value = 0.0029; Table 1.10). All other calculated p-values for the remaining three forage classes (woody plant, graminoid, and legume) were greater than the Bonferroni corrected p-value (Bonferroni p-value = 0.0125) and thus were not significantly different before SGU and after.

6. DISCUSSION

Elk Food Habits

This study investigated the food habits of a reintroduced elk population using next-generation sequencing; this modern method has proven to be cost-effective, non-invasive, has shown to be more accurate in determining food habits than histological methods, and is ever-improving (Valentini et al. 2009a, b; Raye et al. 2011; Pompanon et al. 2012; Kress et al. 2015; Ando et al. 2018). Next-generation sequencing has revolutionized diet studies for many species, including herbivores, and it has been suggested that metabarcoding is more effective at identifying herbivore food habits from feces than methods used previously (Valentini et al. 2009a, b; Raye et al. 2011; Pompanon et al. 2012; Kress et al. 2015; Ando et al. 2018). Metabarcoding allows researchers to find and classify vegetative matter which endures digestion by isolating and amplifying the genetic material from the sample (McInnis 1983, Pompanon et al. 2012).

We hypothesized that elk on the NCWMA would consume primarily graminoids and woody plants during the winter collection period. Our results supported this, as woody plants constituted 58.9% and graminoids 20.3% of all sequences detected. Woody plants dominated both before and after spring green-up and graminoids remained an important forage as well. These results are similar to those of Lupardus (et al. 2011) who evaluated food habits using histological methods for plant identification of NCWMA elk shortly after their release into Tennessee during a year-long study accompanied with vegetation sampling. However, since the conclusion of that study more elk have been

released into the area, the population has been established on the landscape for almost two decades, and food plots and forest clearings have been established throughout the NCWMA.

Lupardus et al. (2011) found graminoids and woody plants constituted the majority of plants seen through histological methods during the winter sampling period. Grasses were 65.9% of all plant material found. However, histological examination of fecal samples and rumen content is prone to overestimating proportions of graminoid material since it easily persists through the digestive system. Lupardus et al. (2011) also found high elk use of ferns (12% of the diet composition). We did not detect fern sequences in any of the samples analyzed. Important winter food items listed by Lupardus et al. 2011 for their study included tall fescue, Christmas fern, big bluestem, little bluestem, barnyard grass (*Echinochloa grusgalli*), wheat, orchard grass, and *Rubus* to a lesser degree. In this study, fescue and *Rubus* remained prominent winter genera, comprising 8.4% and 4.5% of all sequences respectively. However, we did not detect any of the other species listed by Lupardus et al. (2011) with the exception of orchard grass which constituted less than 0.001% of all sequences.

The small number of genera that differed for the treatment groups indicated vegetation on the NCWMA was used similarly by both sexes of elk from both genetic groups. Out of 382 genera, only six were detected differently. Four of those six genera are common on the NCWMA, one is planted, and one was likely incorrectly identified during bioinformatics. This conclusion was further supported by the results from the diversity analyses. The alpha diversity of observed features indicated a greater diversity

of genera detected for the M-EINPN group than the other three groups. Moreover, when accounting for not only genera richness, but also evenness, the Shannon diversity index only found a significant difference in plant genera sequence detection between M-EINPN and M-EINPS. The beta diversity also supported this conclusion. There was no phylogenetic assemblage of plant sequences among any sex-genetic groups that was dissimilar from the others. Moreover, the weighted UniFrac PCoA plot also showed no groupings of clusters for any of the combined treatment groups, supplementing our conclusion that the four combined sex-genetic groups consumed similar vegetation in approximately proportionate amounts.

We had disparate sample sizes of the combined treatment groups. Varying sample size might have led to the detection of differences in the six genera between combined treatment groups. Specifically, the M-EINPN group had a much smaller sample size (n = 4) than F-EINPN (n = 14), M-EINPS (n = 14), and especially F-EINPS (n = 44) which constituted over half of all bioinformatically analyzed samples. Four of the six genera specified by the Tukey's post hoc analysis (*Briza*, *Phleum*, *Quercus*, and *Rhus*), for M-EINPN had a higher MRA than the other three groups; it is possible that the contrast between group samples sizes led to this conclusion. Movement data from one of the collared M-EINPN males (K. Kurth, University of Tennessee, unpublished data) and location of the remaining three genotyped M-EINPN scat samples showed that these four males utilized the same smaller area of the NCWMA possibly contributing to the bias in genera diversity and MRAs.

Overall, our results indicated that there was little variability in the overall food habits of elk on the NCWMA in either sex from either genetic group, although there were differences between individual animals. Elk are intermediate feeders, capable of consuming a wide array of vegetation at all times of the year based on the seasonal plant availability of their habitat (Hofmann 1989, Mower and Smith 1989, Jenkins and Starkey 1991, Kirchhoff and Larsen 1998, Cook 2002, Geist 2002, Anderson et al. 2005, Christianson and Creel 2005, Schneider et al. 2006, Christianson and Creel 2009, Whittaker 2011). The winters faced by elk in the Southeastern United States are milder than those experienced by those in colder, higher elevation areas. Elk on the NCWMA specifically have access to vegetation all year long, which might have contributed to the similarity of plant sequences found between males and females, as neither had to venture to find vegetation that fulfilled any potential sex-based seasonal dietary needs (Clutton-Brock et al. 1982, Beier 1987, McCullough et al. 1989, Main et al. 1996, Villaret et al. 1997). Our results indicated that the food habits of the genetic populations were similar, and likely did not contribute to their continued segregation. Instead, it is likely that some other genetic or familial factor is responsible for the enduring isolation of these two genetic groups on the NCWMA.

Forb sequence detection significantly increased from before to after spring green-up. Winter often forces elk to rely on more fibrous, less digestible and nutritious vegetation as plants reach their maximum maturity and lowest nutritive value gaining more structural, undigestible components during winter (Leslie et al. 1984, Cook 2002). However, when plants begin new growth cycles in the spring, they produce anatomical

structures and compounds that contain more soluble, digestible, and nutritious products. Our results showed that elk might consume plants with greater solubility and nutritive value like forbs as they become available in the late winter and early spring; however, they still appeared to rely heavily on spring growth of woody plant species as well.

Next-Generation Sequencing

Despite the advantages in cost and time management, accuracy of sequence identification, and promise of technique development provided by NGS protocols they are still relatively new methodologies and have room for improvement (Valentini et al. 2009a, b; Raye et al. 2011; Pompanon et al. 2012; Kress et al. 2015; Ando et al. 2018). For example, some NGS studies have found that their protocols were prone to "by-catch", or the identification of matter in close proximity to the sample but not actually representative of the sample (Pompanon et al. 2012, Edwards et al. 2018). We sought to combat this by cutting fecal pellets in half, exposing the inner portion of the scat where digested plant matter resides, and only using this material. However, as stated by Pompanon et al. (2012), there is still a possible issue of the identification of genetic material originating from organisms consumed through secondary (unintentional) predation and herbivory. This issue persisted during this research as evidenced by the identification of several families and genera of animals, fungi, and microorganisms atypical to the diet of elk including those of reptiles, invertebrates, amphibians, and mammals. However, it is of note that these types of sequences represented only 2% of all those detected. Moreover, the taxonomic identification of samples can often be muddied

by the presence of short sequences that lack adequate information to be correctly identified; this issue can be dealt with by choosing and applying careful techniques and quality thresholds during the various steps in NGS protocols (Pompanon et al. 2012). This study took measures to prevent the human contamination of samples, excluding samples that failed to amplify during PCR from sequencing, and imposing quality control parameters during bioinformatics like removing sequences with <150 bp, quality filtering sequences via the implementation of a maximum expected error threshold of 1.0, denoising remaining sequences, and removing unique and chimeric sequences.

The primers we selected have shown efficacy in identifying plants in previous herbivory studies but have also been reported to detect genetic material from other kingdoms as well (Chiou et al. 2007, Chen et al. 2010, Yao et al. 2010, Garcia-Robledo et al. 2013, Sickel et al. 2015, Cheng et al 2016, Iwanowicz et al. 2016, Guo et al. 2018, Moorhouse-Gann et al. 2018). This was also the case in this study, as 56.9% of families (n = 116) representing 1.8% of the sequences detected came from kingdoms other than Plantae. The majority of these families belonged to Animalia (n = 53; 0.3% of sequences) and Fungi (n = 49; 0.6% of sequences). The remainder of the non-plant sequences came from Chromista, Protozoa, and Incertae sedis (unclassified). Moreover, several of these families reported belong to taxa not native to the Southeastern United States and were thus likely incorrectly represented due to sample degradation or the short length of the sequence of origin.

Future Directions and Management Implications

This study was able to show that next-generation sequencing techniques can be used to effectively investigate vegetation consumption in elk by extracting and identifying plant genetic material from inside scat samples and can also be used to examine resource and niche partitioning between groups of organisms within their habitat as we attempted to do with both sexes within the distinct genetic populations of elk on the NCWMA. While these findings shed light on the winter food habits of NCWMA elk, they are not an indication of seasonal forage preference. To better investigate the diet of NCWMA elk using these or complementary methods, a year-long study using similar protocols could be conducted in combination with fecal nitrogen assessment of samples and vegetation sampling within the areas scat is to be collected. Doing so would give an indication of forage availability compounded with the actual plant sequences detected and would thus paint a more precise picture of the seasonal food habits of elk in Tennessee. Also, the addition of a fecal nitrogen analysis could provide insights to managers on the general nutrition levels of NCWMA elk, as it is has served as a reliable indicator of dietary protein, diet digestibility, and gross energy intake and could be examined to investigate seasonal nutritional shifts of the herd as a whole or between sexes or genetic groups in previous research (Beier 1987).

Studies that utilize next-generation sequencing often involve the creation of a library of sequences one expects to find that serves as a reference for researchers during the taxonomic identification of their samples' genetic material. This allows for a more a precise taxonomic interpretation of sequences found and would be useful during a year-

long food habits and vegetative availability study for this area to give land managers a more accurate idea of the vegetation consumed by elk, especially for important genera that are likely to have multiple species represented across the landscape like *Quercus*, *Rubus*, *Rhus*, *Acer*, *Lespedeza*, and *Trifolium*. Creating a reference library would also be useful to correctly identify sequences whose family or genera might have been incorrectly identified due a lack of representation in the DNA barcode reference database selected for bioinformatical analysis (Pompanon et al. 2012).

From this study land managers at the NCWMA can gain a better understanding of the forage classes used by elk during winter and early spring, as this research builds upon the findings from Lupardus et al. 2011. Managers should remember that elk are intermediate feeders, capable of utilizing an array of plants available to them at specific times of the year (Hofmann 1989, Mower and Smith 1989, Jenkins and Starkey 1991, Kirchhoff and Larsen 1998, Cook 2002, Geist 2002, Anderson et al. 2005, Christianson and Creel 2005, Schneider et al. 2006, Christianson and Creel 2009, Whittaker 2011). Elk on the NCWMA specifically have access to forage year-round, including the winter. Despite a potential shift in consumption to forbs following spring green-up, our results show that elk on the NCWMA consumed mainly woody plants and grasses during the winter of 2019. Managers should take these findings into account during future habitat planning.

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APPENDICES

Appendix A: Tables

Table 1.1. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Table output from rarefaction curve (Figure 1.2) displaying the number of samples (N), the mean number of sequences per sample for all 319 sequenced samples (Mean), the standard deviation (Std. Dev.), standard error (Std. Error), and the lower (Low CL) and upper (High CL) 95% confidence limits for the mean.

N	Mean	Std. Dev.	Std. Error	Lower CL	High CL
319	15992.85	9657.25	540.702	14929.04	17056.65

Table 2.2. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA, from February 2019 through April 2019. Proportion of total sequences belonging to genera detected from sequencing and their corresponding family sequence percentages.

Family	Proportion of all Family Sequences	Genus (within Family)	Proportion of all Genera Sequences
Poaceae	**18.915**	*Festuca*	8.446
		Poa	3.290
		Alopecurus	2.487
		Dichanthelium	1.303
		Phleum	0.429
		Muhlenbergia	0.416
		Lolium	0.345
		Torreyochloa	0.288
		Scolochloa	0.286
		Avena	0.279
		Digitaria	0.249
		Aegilops	0.243
		Polypogon	0.234
		Briza	0.143
		Anthoxanthum	0.100
		Holcus	0.095
		Imperata	0.086
		Bromus	0.077
		Melinis	0.026
		Zea	0.024
		Pascopyrum	0.017
		Oryza	0.017
		Elymus	0.009
		Paspalidium	0.007
		Paspalum	0.005
		Glyceria	0.004
		Dichanthium	0.003
		Panicum	0.003

Table 1.2 continued

		Tridens	0.002
		Other Poaceae Genera (<0.001%)	0.002
Ericaceae	**17.436**	*Vaccinium*	15.216
		Kalmia	1.253
		Rhododendron	0.311
		Epigaea	0.297
		Gaylussacia	0.185
		Oxydendrum	0.170
		Chimaphila	0.004
Rosaceae	**13.282**	*Rosa*	6.358
		Rubus	4.500
		Geum	1.532
		Potentilla	0.460
		Prunus	0.270
		Agrimonia	0.123
		Drymocallis	0.035
		Sanguisorba	0.003
		Sorbus	0.001
Fabaceae	**10.655**	*Robinia*	5.793
		Trifolium	2.213
		Medicago	1.513
		Lespedeza	0.422
		Cercis	0.250
		Amphicarpaea	0.210
		Lotus	0.068
		Desmodium	0.051
		Vicia	0.046
		Indigofera	0.024
		Uraria	0.021
		Centrosema	0.017
		Vigna	0.013
		Wisteria	0.006
		Arachis	0.004
		Chamaecrista	0.002
		Phaseolus	0.001
		Other Fabaceae Genera (<0.001%)	0.001

Table 1.2 continued

Eleagnaceae	**5.186**	*Elaeagnus*	5.186
Aceraceae	**4.134**	*Acer*	4.134
Aquafoliaceae	**3.651**	*Ilex*	3.651
Betulaceae	**3.030**	*Corylus*	2.558
		Betula	0.358
		Alnus	0.115
Caryophyllaceae	**2.867**	*Stellaria*	2.256
		Cerastium	0.611
		Dianthus	0.001
Asteraceae	**2.674**	*Solidago*	0.993
		Symphyotrichum	0.693
		Erigeron	0.256
		Iva	0.188
		Packera	0.135
		Canadanthus	0.099
		Eupatorium	0.090
		Helianthus	0.036
		Vernonia	0.034
		Lactuca	0.030
		Taraxacum	0.026
		Ageratina	0.020
		Nabalus	0.018
		Tanacetum	0.018
		Bidens	0.009
		Corethrogyne	0.008
		Achillea	0.005
		Sigesbeckia	0.004
		Cirsium	0.003
		Aster	0.002
		Cichorium	0.001
		Arnica	0.001
		Other Asteraceae Genera (<0.001%)	0.005
Plantaginaceae	**2.615**	*Plantago*	2.450
		Veronica	0.165
Juglandaceae	**2.299**	*Carya*	2.299
Fagaceae	**2.111**	*Quercus*	2.068

Table 1.2 continued

		Fagus	0.043
Brassicaceae	**1.453**	*Cardamine*	1.256
		Brassica	0.173
		Raphanus	0.012
		Rorippa	0.007
		Boechera	0.003
		Arabidopsis	0.001
Symbiodiniaceae	**0.858**	*Symbiodinium*	0.858
Oleaceae	**0.852**	*Ligustrum*	0.821
		Fraxinus	0.031
Cucurbitaceae	**0.791**	*Citrullus*	0.783
		Cayaponia	0.007
		Other Cucurbitaceae Genera (<0.001%)	<0.001
Saxifragaceae	**0.755**	*Tiarella*	0.719
		Heuchera	0.033
		Tellima	0.004
Pinaceae	**0.718**	*Pinus*	0.674
		Tsuga	0.042
		Abies	0.002
Juncaceae	**0.631**	*Juncus*	0.623
		Luzula	0.008
Anacardiaceae	**0.598**	*Toxicodendron*	0.128
		Rhus	0.470
Diapensiaceae	**0.479**	*Diapensia*	0.479
Cyperaceae	**0.379**	*Carex*	0.375
		Eleocharis	0.002
		Scirpus	0.001
Salicaceae	**0.260**	*Salix*	0.259
		Populus	0.001
Amaryllidaceae	**0.213**	*Allium*	0.213
Violaceae	**0.208**	*Viola*	0.199
		Viola	0.009
Geraniaceae	**0.206**	*Geranium*	0.206
Pythiaceae	**0.192**	*Phytophthora*	0.192
Onagraceae	**0.191**	*Oenothera*	0.187
		Circaea	0.005

Table 1.2 continued

Hydrangeaceae	**0.186**	*Hydrangea*	0.186
Malvaceae	**0.175**	*Tilia*	0.090
		Sida	0.085
Hamamelidaceae	**0.148**	*Hamamelis*	0.148
Schizophyllaceae	**0.142**	*Schizophyllum*	0.142
Apiaceae	**0.140**	*Daucus*	0.089
		Osmorhiza	0.039
		Zizia	0.005
		Erigenia	0.003
		Angelica	0.003
		Anthriscus	0.001
		Other Apiaceae Genera (<0.001%)	<0.001
Moraceae	**0.109**	*Morus*	0.107
		Ficus	0.002
Trichocomaceae	**0.106**	*Aspergillus*	0.057
		Neosartorya	0.045
		Talaromyces	0.004
Hypericaceae	**0.102**	*Hypericum*	0.102
Convolvulaceae	**0.074**	*Ipomoea*	0.074
		Calystegia	0.001
Plectosphaerellaceae	**0.065**	*Verticillium*	0.065
Aristolochiaceae	**0.064**	*Asarum*	0.064
Saccharomycetaceae	**0.063**	*Lachancea*	0.041
		Saccharomyces	0.014
		Komagataella	0.005
		Candida	0.001
		Kazachstania	0.001
Potamogetonaceae	**0.062**	*Potamogeton*	0.062
Lamiaceae	**0.054**	*Glechoma*	0.036
		Prunella	0.016
		Salvia	0.001
		Lamium	0.001
Incertae sedis	**0.053**	*Sporobolomyces*	0.018
		Chloroidium	0.012
		Trichomitus	0.011
		Ambrosiozyma	0.009

Table 1.2 continued

		Heliocosma	0.002
		Ramichloridium	0.001
		Mesozoanthus	0.001
Trichogrammatidae	**0.046**	*Trichogramma*	0.046
Dyakiidae	**0.044**	*Everettia*	0.044
Boraginaceae	**0.044**	*Phacelia*	0.044
Rubiaceae	**0.042**	*Houstonia*	0.039
		Mitrasacmopsis	0.001
		Crusea	0.001
		Other Rubiaceae Genera (<0.001%)	<0.001
Arecaceae	**0.040**	*Sabal*	0.040
Hominidae	**0.037**	*Homo*	0.037
Tricholomataceae	**0.033**	*Collybia*	0.033
Oenosandridae	**0.031**	*Discophlebia*	0.031
Primulaceae	**0.030**	*Lysimachia*	0.030
Steccherinaceae	**0.028**	*Antrodiella*	0.028
Nyssaceae	**0.023**	*Nyssa*	0.023
Cupressaceae	**0.022**	*Austrocedrus*	0.013
		Juniperus	0.008
		Calocedrus	0.001
Arthrodermataceae	**0.020**	*Arthroderma*	0.017
		Trichophyton	0.002
Polygonaceae	**0.020**	*Rumex*	0.017
		Fallopia	0.002
Orchidaceae	**0.018**	*Cephalanthera*	0.018
Muridae	**0.017**	*Rattus*	0.009
		Mus	0.008
Balsaminaceae	**0.016**	*Impatiens*	0.016
Pipidae	**0.015**	*Xenopus*	0.015
Verbenaceae	**0.013**	*Verbena*	0.013
Sebacinaceae	**0.012**	*Piriformospora*	0.012
Leptosphaeriaceae	**0.011**	*Coniothyrium*	0.011
Poritidae	**0.010**	*Stylaraea*	0.010
Phanerochaetaceae	**0.010**	*Phanerochaete*	0.009
		Pseudolagarobasidium	0.001
Cornaceae	**0.009**	*Cornus*	0.009

Table 1.2 continued

Myricaceae	**0.009**	*Morella*	0.005
		Comptonia	0.004
Ajellomycetaceae	**0.008**	*Paracoccidioides*	0.008
Caprifoliaceae	**0.008**	*Lonicera*	0.008
Didiniidae	**0.008**	*Didinium*	0.008
Steinernematidae	**0.007**	*Steinernema*	0.007
Ranunculaceae	**0.007**	*Hepatica*	0.003
		Anemone	0.003
		Ranunculus	0.001
Malasseziaceae	**0.007**	*Malassezia*	0.007
Hyaloscyphaceae	**0.006**	*Incrucipulum*	0.006
Blephariceridae	**0.006**	*Liponeura*	0.006
Rutaceae	**0.006**	*Citrus*	0.006
Tuberaceae	**0.006**	*Tuber*	0.006
Adoxaceae	**0.006**	*Sambucus*	0.006
Isotrichidae	**0.006**	*Isotricha*	0.006
Harrimaniidae	**0.005**	*Saccoglossus*	0.005
Celastraceae	**0.005**	*Euonymus*	0.003
		Celastrus	0.002
Carabidae	**0.004**	*Cicindela*	0.003
		Opisthius	0.001
		Other Carabidae Genera (<0.001%)	<0.001
Corticiaceae	**0.004**	*Waitea*	0.004
Peltulaceae	**0.004**	*Peltula*	0.004
Cichlidae	**0.004**	*Oreochromis*	0.004
Cercopithecidae	**0.004**	*Macaca*	0.004
Amaranthaceae	**0.004**	*Chenopodium*	0.004
Crassulaceae	**0.004**	*Sedum*	0.004
Scrophulariaceae	**0.004**	*Scrophularia*	0.004
Euphorbiaceae	**0.004**	*Acalypha*	0.004
Orobanchaceae	**0.003**	*Pedicularis*	0.003
Nadidae	**0.003**	*Limnodrilus*	0.003
Metschnikowiaceae	**0.003**	*Clavispora*	0.001
		Metschnikowia	0.001
Dothideaceae	**0.003**	*Aureobasidium*	0.003
Callitrichidae	**0.003**	*Callithrix*	0.003

Table 1.2 continued

Vitaceae	**0.003**	*Cyphostemma*	0.003
Caviidae	**0.002**	*Cavia*	0.002
Oxalidaceae	**0.002**	*Oxalis*	0.002
Psathyrellaceae	**0.002**	*Psathyrella*	0.002
Equidae	**0.002**	*Equus*	0.002
Xylariaceae	**0.002**	*Halorosellinia*	0.001
		Biscogniauxia	0.001
Nepticluidae	**0.002**	*Ectoedemia*	0.002
Ascarididae	**0.002**	*Ascaris*	0.002
Taeniidae	**0.002**	*Taenia*	0.002
Omphalotaceae	**0.002**	*Gymnopus*	0.002
Goniodomataceae	**0.002**	*Gambierdiscus*	0.002
Rhizophydiaceae	**0.002**	*Rhizophydium*	0.002
Chlamydomonadaceae	**0.001**	*Vitreochlamys*	0.001
		Other Chlamydomonadaceae Genera (<0.001%)	<0.001
Schistosomatidae	**0.001**	*Schistosoma*	0.001
Cricetidae	**0.001**	*Mesocricetus*	0.001
		Melanocarpus	0.001
Phytolaccaceae	**0.001**	*Phytolacca*	0.001
Sarcoscyphaceae	**0.001**	*Cookeina*	0.001
Acarosporaceae	**0.001**	*Sarcogyne*	0.001
Boletaceae	**0.001**	*Strobilomyces*	0.001
		Other Boletaceae Genera (<0.001%)	<0.001
Plutellidae	**0.001**	*Atemelia*	0.001
Galagidae	**0.001**	*Otolemur*	0.001
Culicidae	**0.001**	*Armigeres*	0.001
		Other Culicidae Genera (<0.001%)	<0.001
Stronglyocentrotidae	**0.001**	*Strongylocentrotus*	0.001
Polyporaceae	**0.001**	*Ganoderma*	0.001
Ostreidae	**0.001**	*Crassostrea*	0.001
Peyssonneliaceae	**0.001**	*Sonderopelta*	0.001
		Other Peyssonneliaceae Genera (<0.001%)	<0.001
Pteromalidae	**0.001**	*Nasonia*	0.001

Table 1.2 continued

Hymenochaetaceae	**0.001**	*Phellinidium*		0.001
Callidulidae	**0.001**	*Griveaudia*		0.001
Exobasidiaceae	**0.001**	*Exobasidium*		0.001
Asparagaceae	**0.001**	*Asparagus*		0.001
Suidae	**0.001**	*Sus*		0.001
Inocybaceae	**0.001**	*Inocybe*		0.001
Cistaceae	**0.001**	*Lechea*		0.001
Helotiaceae	**0.001**	*Cudoniella*		0.001
Sclerotiniaceae	**0.001**	*Sclerotinia*		0.001
Coenagrionidae	**0.001**	*Nesobasis*		0.001
Altingiaceae	**0.001**	*Liquidambar*		0.001
Hydropsychidae	**0.001**	*Polymorphanisus*		0.001
Diaporthaceae	**0.001**	*Stenocarpella*		0.001
Lejeuneaceae	**0.001**	Other Lejeuneaceae Genera (<0.001%)	<0.001	
Marasmiaceae	**0.001**	*Clitocybula*		0.001
Sympoventuriaceae	**0.001**	*Ochroconis*		0.001
Diplogasteridae	**0.001**	*Acrostichus*		0.001
		Micoletzkya	<0.001	
Aphididae	**0.001**	*Uroleucon*		0.001
		Other Aphididae Genera (<0.001%)	<0.001	
Tineidae	**0.001**	*Xystrologa*		0.001
Meruliaceae	**0.001**	*Phlebia*		0.001
Cladoniaceae	**0.001**	*Cladonia*		0.001
Lauraceae	**0.001**	*Litsea*		0.001
Cochliopodidae	**0.001**	*Cochliopodium*		0.001
Stilbosporaceae	**0.001**	*Stilbospora*		0.001
Syrphidae	**0.001**	*Rhingia*		0.001
Hydrodictyaceae	**0.001**	*Pediastrum*		0.001
Musaceae	**0.001**	*Ensete*		0.001
All Other Families (<0.001%)	**0.008**	All Other Genera (0.001%)		0.011

Table 3.3. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Proportion of total sequences belonging to the 6 kingdoms (Animalia, Chromista, Fungi, Incertae sedis [unclassified], Plantae, and Protozoa) detected from sequencing.

Kingdom	Proportion of all Sequences
Animalia	0.272
Chromista	0.874
Fungi	0.581
Incertae sedis	0.000
Plantae	98.261
Protozoa	0.012
Total	100.000

Table 4.4. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Summary of all pairwise comparisons (conducted using an ANOVA; post hoc pairwise comparisons calculated using Tukey's test) of genera detected from analysis with a significant difference found in the mean relative abundance (MRA) for the combined treatment groups (groups). Also included in this table is the statistical category for each comparison, the groups compared (contrast), standardized difference (SD), p-value (*p*), and statement of significance (Sig.).

Genus/ Group	MRA	Statistical Categories	Contrast	SD	*p*	Sig.
Rubus						
M-EINPN	14.609	A	M-EINPN vs M-EINPS	3.151	0.012	Yes
F-EINPN	6.71	AB	M-EINPN vs F-EINPS	3.196	0.011	Yes
F-EINPS	3.475	B	M-EINPN vs F-EINPN	2.088	0.166	No
M-EINPS	2.857	B	F-EINPN vs M-EINPS	1.578	0.397	No
			F-EINPN vs F-EINPS	1.58	0.396	No
			F-EINPS vs M-EINPS	0.317	0.989	No
Quercus						
M-EINPN	7.662	A	M-EINPN vs M-EINPS	3.279	0.008	Yes
F-EINPN	3.966	AB	M-EINPN vs F-EINPS	3.231	0.01	Yes
F-EINPS	1.397	B	M-EINPN vs F-EINPN	1.756	0.303	No
M-EINPS	0.856	B	F-EINPN vs M-EINPS	2.289	0.11	No
			F-EINPN vs F-EINPS	2.256	0.118	No
			F-EINPS vs M-EINPS	0.499	0.959	No
Rhus						
F-EINPN	1.99	A	F-EINPN vs F-EINPS	2.816	0.031	Yes
M-EINPN	1.74	AB	F-EINPN vs M-EINPS	2.305	0.106	No
M-EINPS	0.065	AB	F-EINPN vs M-EINPN	0.193	0.997	No
F-EINPS	0.018	B	M-EINPN vs F-EINPS	1.445	0.476	No
			M-EINPN vs M-EINPS	1.313	0.558	No
			M-EINPS vs F-EINPS	0.071	1	No
Phleum						
M-EINPN	6.599	A	M-EINPN vs F-EINPN	4.3	0	Yes
F-EINPS	0.102	B	M-EINPN vs M-EINPS	4.358	0	Yes

Table 1.4 continued

M-EINPS	0.089	B	M-EINPN vs F-EINPS	4.656	< 0.0001	Yes
F-EINPN	0.085	B	F-EINPS vs F-EINPN	0.021	1	No
			F-EINPS vs M-EINPS	0.017	1	No
			M-EINPS vs F-EINPN	0.004	1	No
Oenothera						
F-EINPN	0.924	A	F-EINPN vs M-EINPS	3.36	0.007	Yes
M-EINPN	0.139	AB	F-EINPN vs F-EINPS	3.9	0.001	Yes
F-EINPS	0.025	B	F-EINPN vs M-EINPN	1.843	0.262	No
M-EINPS	0	B	M-EINPN vs M-EINPS	0.33	0.988	No
			M-EINPN vs F-EINPS	0.29	0.991	No
			F-EINPS vs M-EINPS	0.112	0.999	No
Briza						
M-EINPN	1.337	A	M-EINPN vs F-EINPS	4.144	0.001	Yes
M-EINPS	0.188	B	M-EINPN vs F-EINPN	3.794	0.002	Yes
F-EINPN	0.054	B	M-EINPN vs M-EINPS	3.446	0.005	Yes
F-EINPS	0.046	B	M-EINPS vs F-EINPS	0.815	0.847	No
			M-EINPS vs F-EINPN	0.613	0.928	No
			F-EINPN vs F-EINPS	0.044	1	No

Table 5.5. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Statistical comparisons of observed features (amplicon sequence variants [ASVs]) conducted for combined treatment groups using Kruskal-Wallis pairwise comparisons to investigate alpha (or within-sample group) diversity of sequences detected within each group. H = test statistic, *p* = p-value, and *q* = adjusted p-value.

Group 1	**Group 2**	**H**	***p***	***q***
F-EINPN	F-EINPS	0.099	0.753	0.775
F-EINPN	M-EINPN	5.120	0.024	0.047
F-EINPN	M-EINPS	0.082	0.775	0.775
F-EINPS	M-EINPN	7.483	0.006	0.037
F-EINPS	M-EINPS	0.152	0.697	0.775
M-EINPN	M-EINPS	6.095	0.013	0.041

Table 6.6. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Statistical comparisons of Shannon diversity indices of OTUs conducted for combined treatment groups using Kruskal-Wallis pairwise comparisons to investigate alpha (or within-sample group) diversity of sequences detected within each group. H = test statistic, *p* = p-value, and *q* = adjusted p-value.

Group 1	**Group 2**	**H**	***p***	***q***
F-EINPN	F-EINPS	0.318	0.574	0.574
F-EINPN	M-EINPN	0.500	0.480	0.574
F-EINPN	M-EINPS	1.127	0.289	0.509
F-EINPS	M-EINPN	3.120	0.077	0.232
F-EINPS	M-EINPS	0.914	0.339	0.509
M-EINPN	M-EINPS	4.667	0.031	0.185

Table 7.7. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Statistical comparisons of beta (community) diversity analyzed using weighted UniFrac distance matrices used in a principal coordinate analysis. Pairwise analysis of similarities (ANOSIM) was utilized to determine if there were any significant differences in between-sample diversity between treatment groups. Included in this table are: The treatments groups being compared (Group 1, Group 2), sample size of the combined groups (N), permutations performed for that comparison, ANOSIM test statistic (R), p-value (*p*), and adjusted p-value (*q*).

Group 1	**Group 2**	**N**	**Permutations**	**R**	***p***	***q***
F-EINPN	F-EINPS	38	999	0.041	0.303	0.570
F-EINPN	M-EINPN	14	999	-0.080	0.723	0.868
F-EINPN	M-EINPS	19	999	0.053	0.202	0.570
F-EINPS	M-EINPN	32	999	0.022	0.380	0.570
F-EINPS	M-EINPS	37	999	0.150	0.076	0.456
M-EINPN	M-EINPS	13	999	-0.253	0.962	0.962

Table 8.8. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Proportion of total sequences belonging to four major forage classes (forb, graminoid, legume, and woody plant) and any other classes detected from sequencing.

Forage Class	Proportion of Plantae Sequences
Forb	16.238
Graminoid	20.280
Legume	4.433
Woody Plant	58.846
Other	0.202
Total	100.000

Table 9.9. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Proportion of total Plantae sequences belonging to four major forage classes (forb, graminoid, legume, and woody plant) and any other classes detected from sequencing for the period of sample collection before spring green-up (2/14/19 – 3/18/19) and after (3/19/19 – 4/25/19).

Before SGU (2/14/19 - 3/14/19)	**Forage Class**	**Proportion of all Sequences**
	Forb	11.860
	Graminoid	23.941
	Legume	5.522
	Woody Plant	58.665
	Other	0.013
After SGU (3/18/19 - 4/25/19)	**Forage Class**	**Proportion of all Sequences**
	Forb	28.905
	Graminoid	15.599
	Legume	3.065
	Woody Plant	52.404
	Other	0.027

Table 10.10. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019. Results from the Fisher's exact test run for forage classes who had rounded proportions >1% during both before spring green-up (SGU; 2/14/19 – 3/18/19) and after (3/19/19 – 4/25/19; forb, woody plant, graminoid, and legume) against all other forage classes' proportions to test for a difference in individual forage class sequence detection before and after spring green-up. The Bonferroni corrected p-value used for significance comparison in this analysis was 0.0125. Also included in this table is the p-value for the individual forage class comparison (*p*) and if *p* was significant (Sig.).

Forage Class	**Before SGU Prop. of Seq.**	**After SGU Prop. of Seq.**	***p***	**Sig.**
Forb	12	29		
All Other	89	71		
			0.0029	Yes
Woody Plant	59	52		
All Other	42	48		
			0.3962	No
Graminoid	24	16		
All Other	77	84		
			0.2162	No
Legume	6	3		
All Other	95	97		
			0.4978	No

Appendix B: Figures

See Attachment

Figure 1.1. The study area with the 65 scat collection sites within the North Cumberland Wildlife Management Area (NCWMA; 79,318-ha) in the Elk Restoration Zone (ERZ) in the Cumberland Mountains, Tennessee, USA. Elk food habits evaluated using a next-generation sequencing protocol with feces collected from NCWMA from February through April 2019.

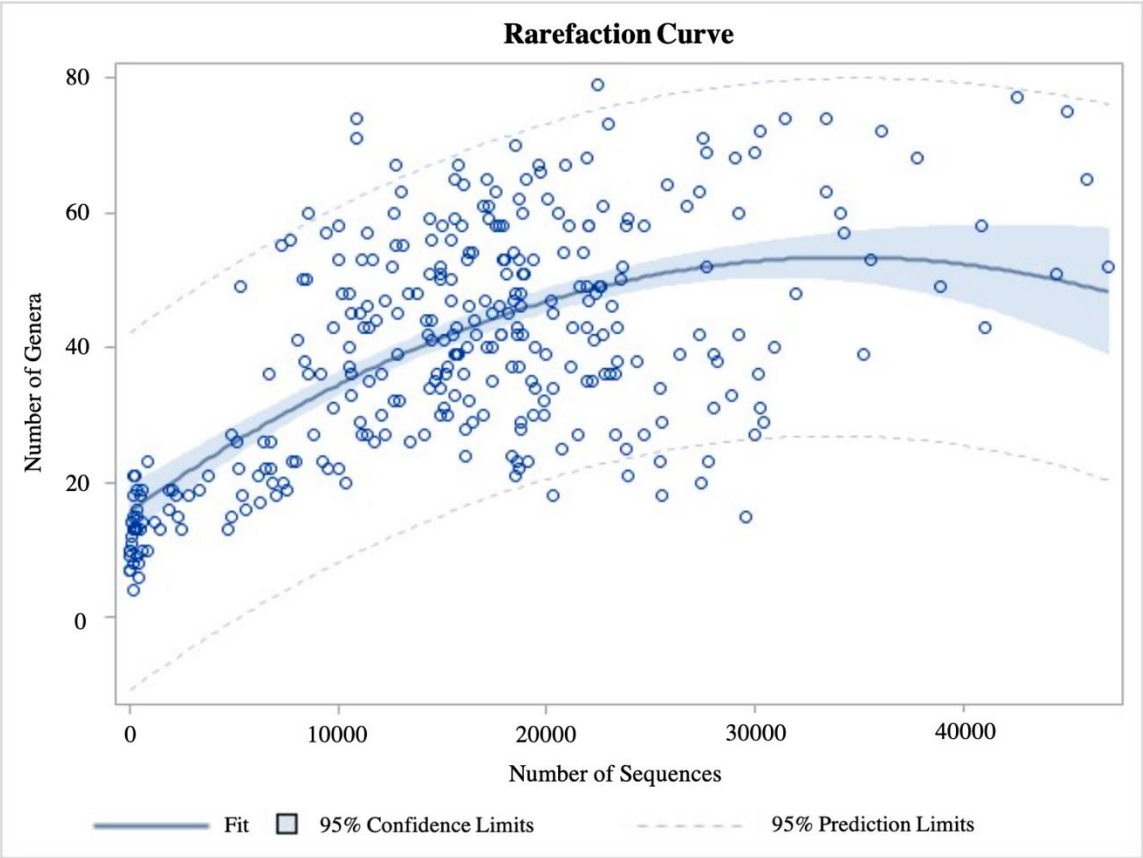

Figure 2.2. Rarefaction curve displaying number of genera detected (y-axis) versus the number of sequences per sample found (x-axis). Statistical output in Table 1.1. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019.

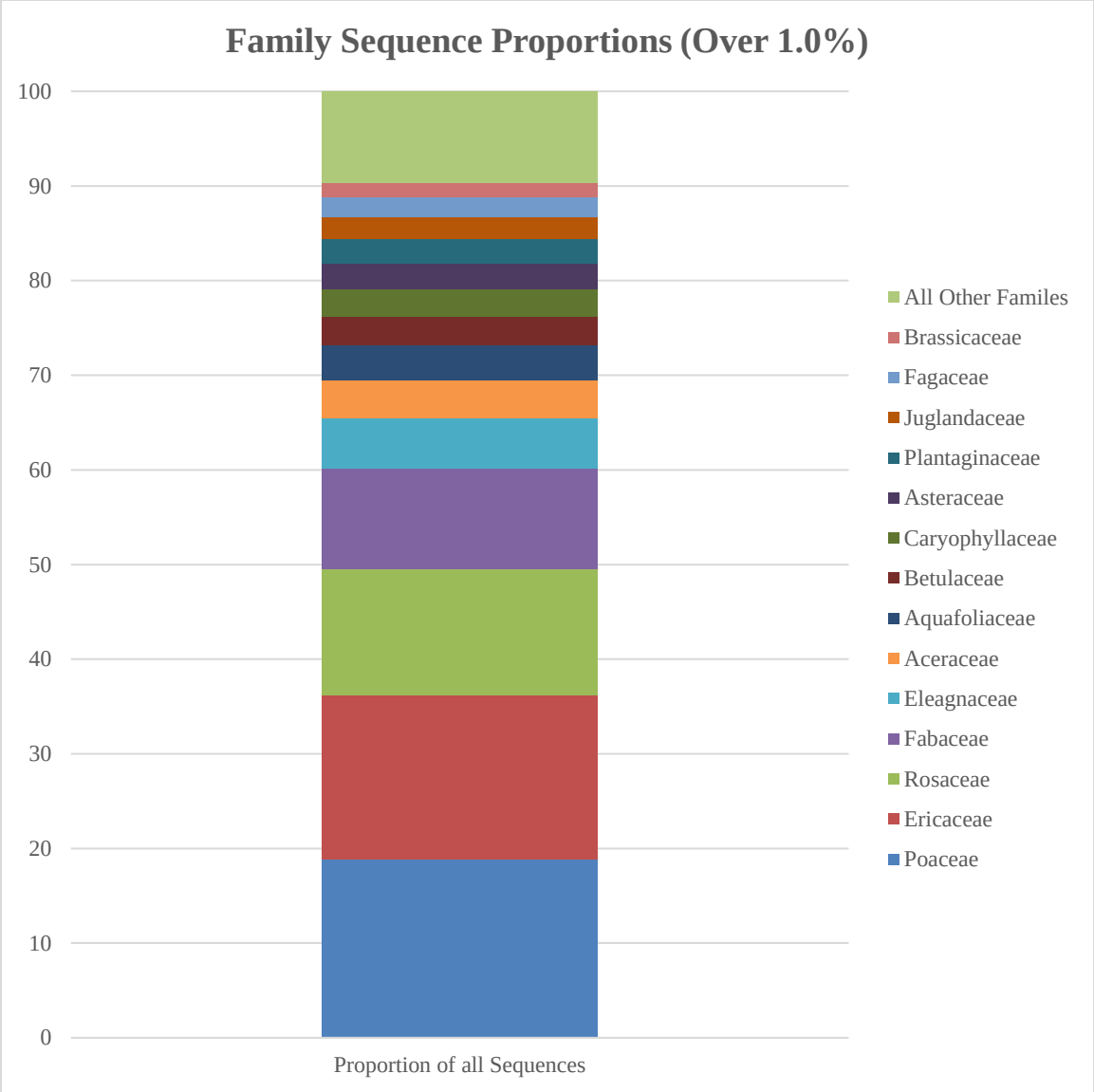

Figure 3.3. Proportion of sequences belonging to families with relative percentages >1.000% found from bioinformatical analysis. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019.

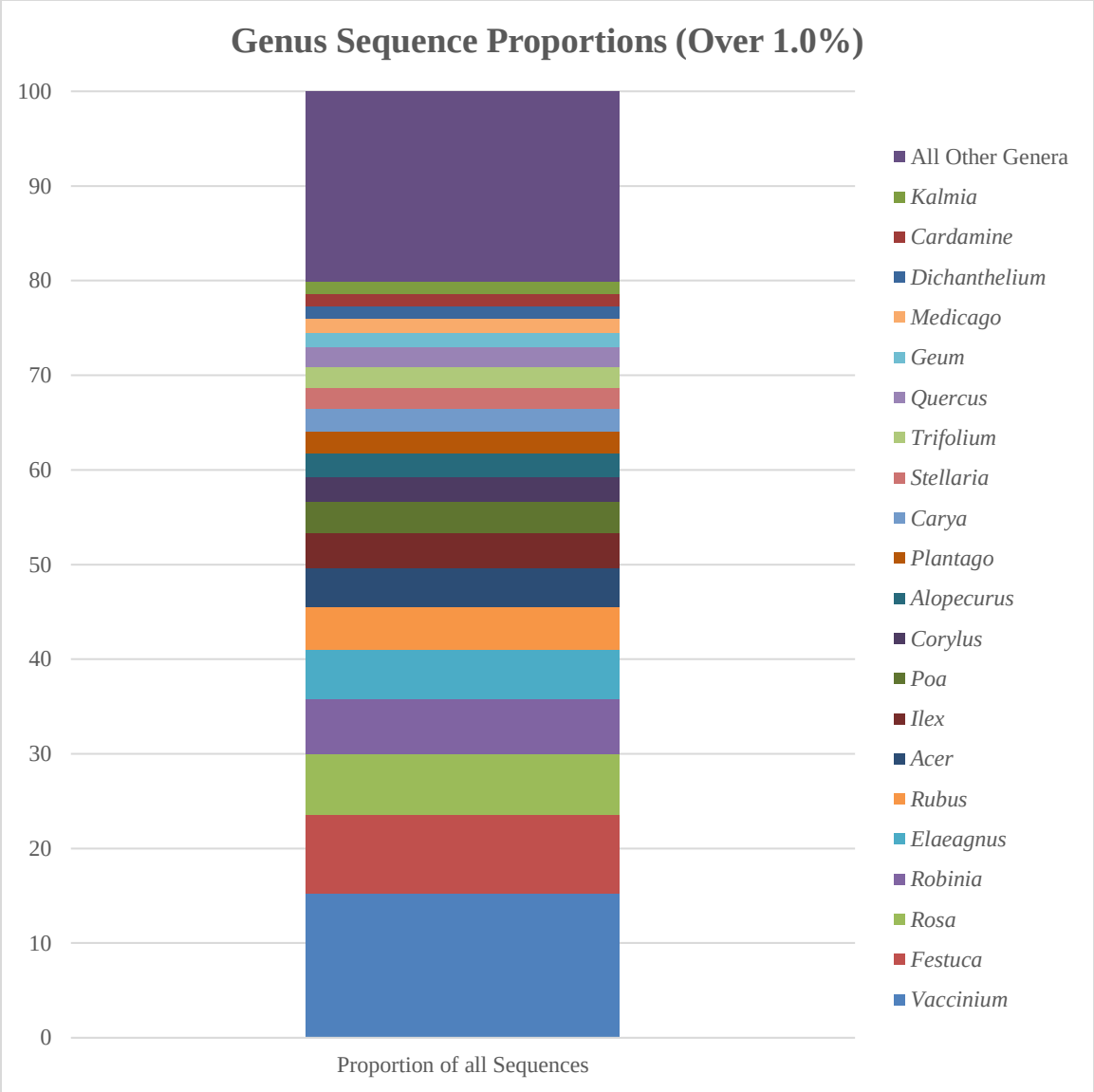

Figure 4.4. Proportion of sequences belonging to genera with relative percentages >1.000% found from bioinformatical analysis. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019.

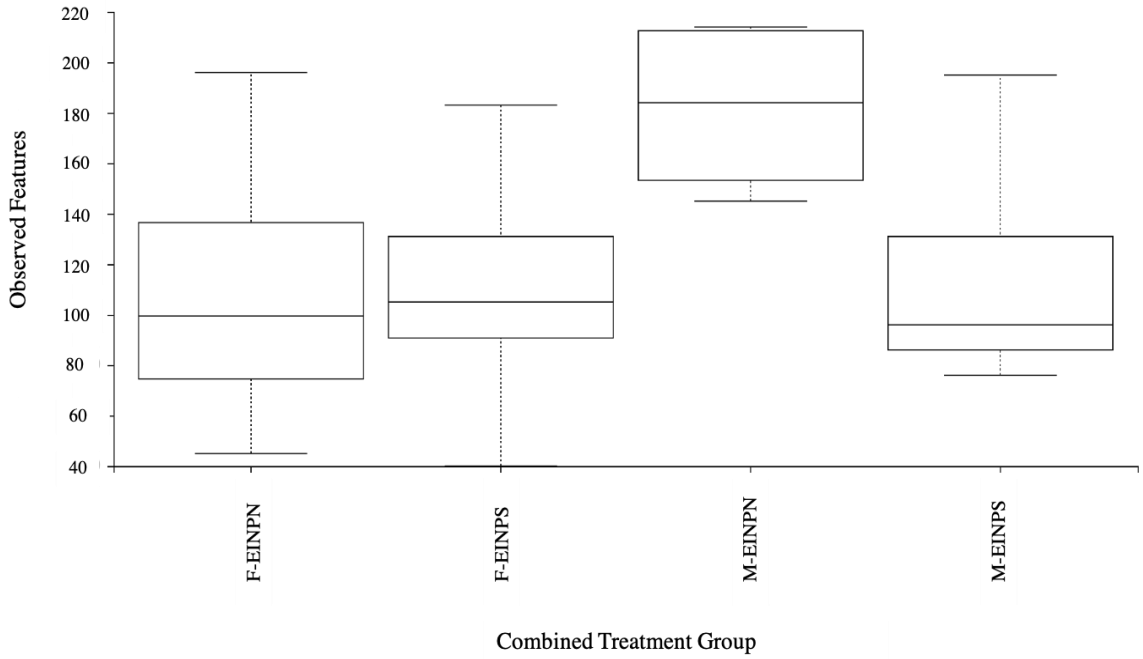

Figure 5.5. Boxplot displaying statistical comparisons of observed features (amplicon sequence variant; ASVs; y-axis) conducted for combined treatment groups (x-axis) using Kruskal-Wallis pairwise comparisons to investigate alpha (or within-sample group) diversity of sequences detected within each group (F-EINPN n = 14; F-EINPS n = 44; M-EINPN n = 4; M-EINPS n = 14). Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019.

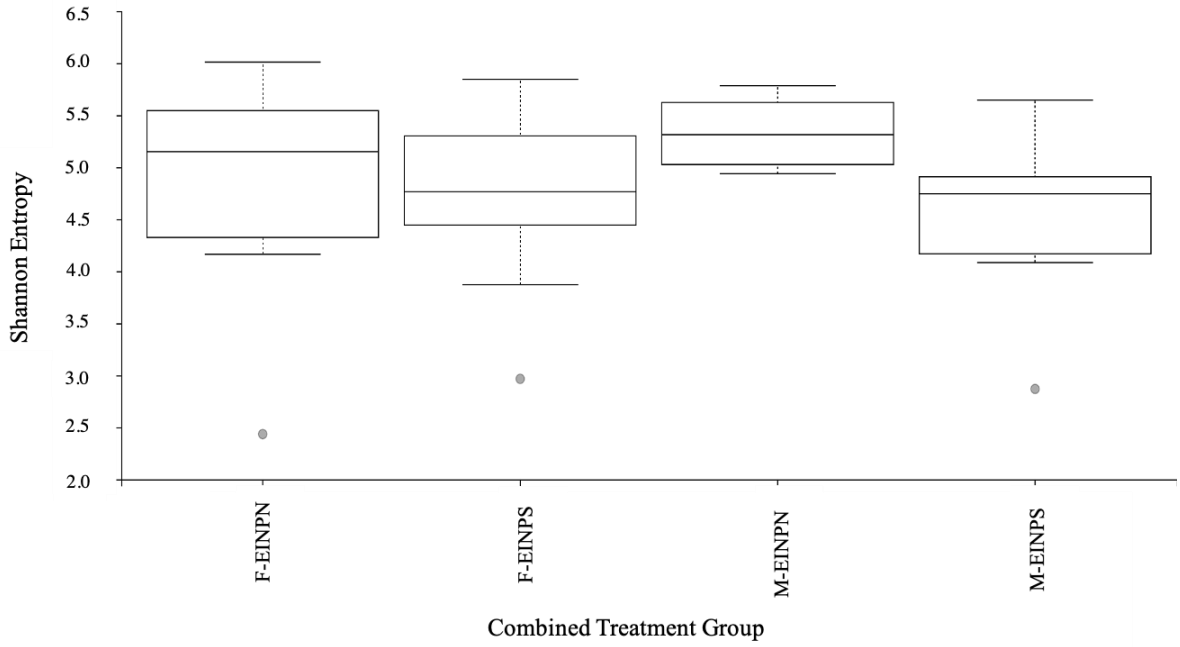

Figure 6.6. Boxplot displaying distribution of Shannon diversity indices (y-axis) conducted for combined treatment groups (x-axis) using Kruskal-Wallis pairwise comparisons to investigate alpha (or within-sample group) diversity of sequences detected within each group (F-EINPN n = 14; F-EINPS n = 44; M-EINPN n = 4; M-EINPS n = 14). Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019.

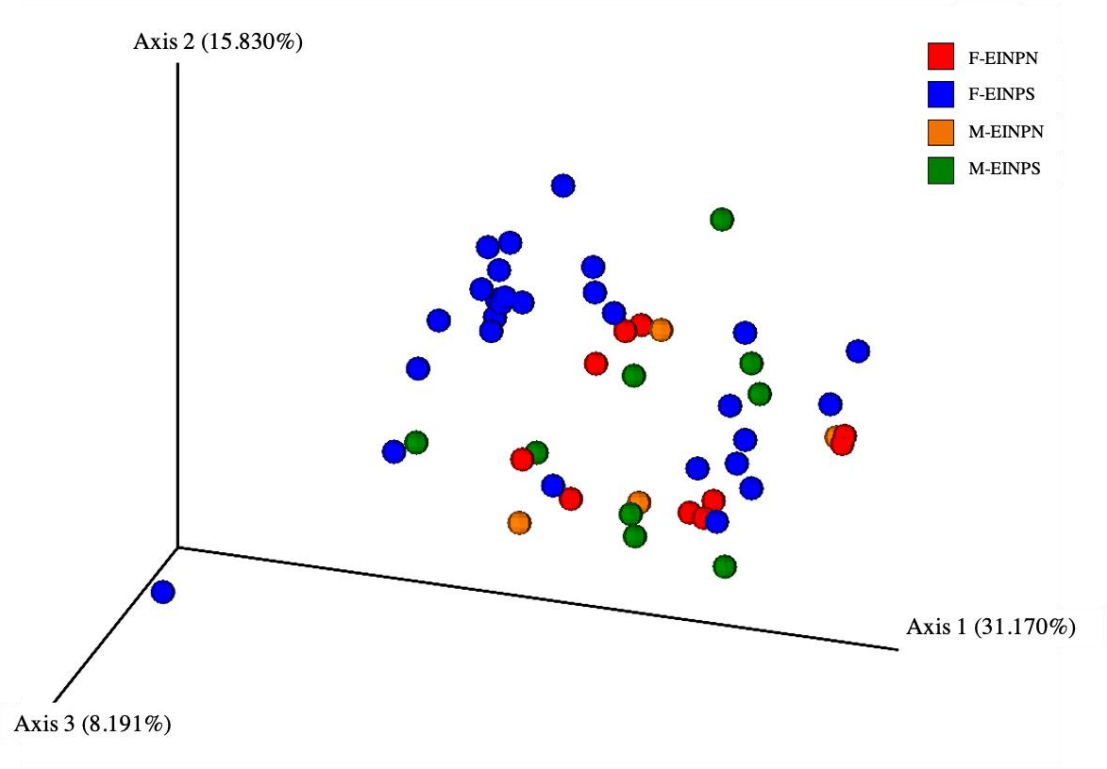

Figure 7.7. Principal coordinate analysis plot of weighted UniFrac data measuring beta (community) diversity of sequences detected between combined treatment groups with colors specified for each group: F-EINPN (red), F-EINPS (blue), M-EINPN (orange), and M-EINPS (green). Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019.

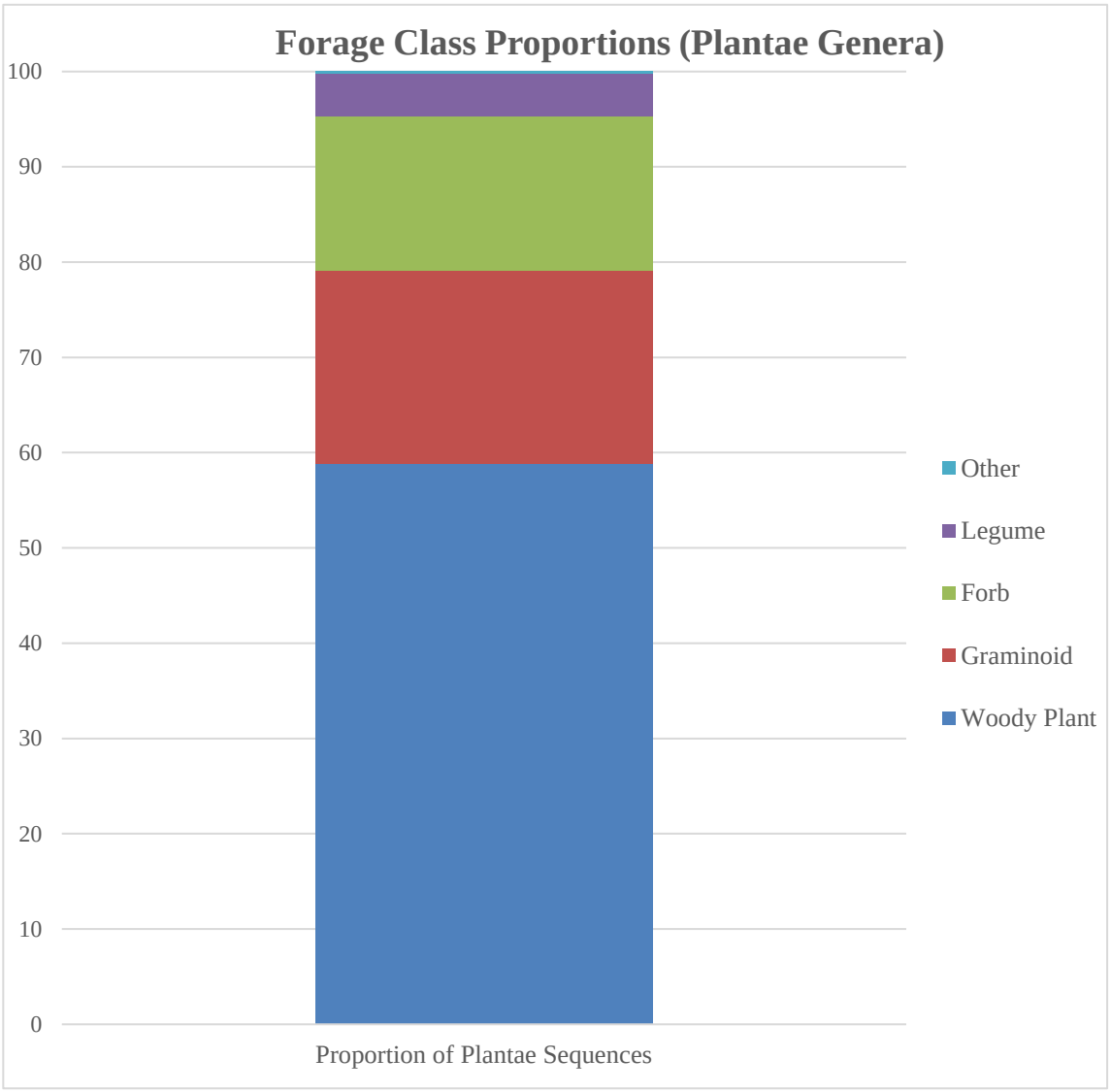

Figure 8.8. Proportion of total sequences belonging to four major forage classes (forb, graminoid, legume, and woody plant) and any other classes detected from sequencing. Reintroduced elk food habits found from a next-generation sequencing protocol used with feces collected from the North Cumberland Wildlife Management Area (79,318-ha), Tennessee, USA from February 2019 through April 2019.

VITA

Dailee Metts is originally from Cookeville, Tennessee, but started at the University of Tennessee in 2014. During that time, she worked in various labs studying plant biology and genetics and conducted an undergraduate research project studying seed viability in *Schoenoplectus americanus*. She graduated with a Bachelor's of Science in Ecology and Evolutionary Biology in 2018. In 2019 she started her graduate program as a Master's student in the Forestry, Wildlife & Fisheries Department under the advisement of Dr. Lisa Muller studying the food habits of a reintroduced population of elk in Tennessee using next-generation sequencing.

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