FROM MICROBES TO MANAGEMENT: SPATIAL AND TEMPORAL VARIATIONS IN AVAILABLE VEGETATION AFFECT THE GUT MICROBIOTA IN A POPULATION OF FREE-RANGING GENERALIST HERBIVORES

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DEDICATION

Dedicated to my grandmother Esperanza Martínez who has been the largest supporter of my academic endeavors and whose sacrifices have allowed me opportunities in the pursuit of education that she was not afforded.

Tú eras la primera Chingona.

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ABSTRACT

The effects of extrinsic environmental factors that shape ecological systems are not only seen at the macroscopic level, but additionally influence and govern the host-associated microbial communities of their mammalian hosts. These microbial communities are susceptible to the fluctuation of abiotic and biotic factors which affect their host organisms. The surge in the research of microbiota–communities of archaea, bacteria, fungi, and viruses residing in various environmental systems–has shown that these communities can profoundly influence animal health. As such, monitoring microbiota has allowed for a new approach to study animal health and physiology. This is of particular benefit in the conservation of wildlife who face foraging restrictions, climate fluctuations, infectious disease, and habitat disturbances such as deforestation, pollution, and urbanization. Because gut microbes are influenced by external stressors and can predict internal physiological condition of the host, they may serve as biomarkers for both animal health and severity of environmental threats on species survival by mitigating their effects on the animal.

One naturally occurring 'chess match' in wildlife systems involves mammalian herbivores and their plant food sources. Plants have developed a suite of secondary metabolites that are potentially toxic to herbivores when ingested. Herbivores must therefore make dietary choices that minimize the potentially harmful effects of plant secondary metabolites (PSMs) but also maximize the uptake of available nutrients. To do this, herbivores have developed physiologic mechanisms to tolerate PSM ingestion (Kohl

et al., 2014). Beyond their own mechanisms, Kohl et al. demonstrated that gut microbes are also crucial in allowing herbivores to consume toxic plants. While previous studies have highlighted the role of gut microbiota in plant digestion and toxin tolerance for the herbivore host, these studies have been limited to controlled, captive systems. Therefore, we used the large, wild vertebrate herbivore, moose (Alces alces) on Isle Royale National Park, Michigan as a case study to investigate if host-associated microbiota can vary by regional and temporal habitat differences and if diet variation can shift microbial communities in this large, free-range mammalian herbivore. This research contributes to the understanding of the impacts of spatial and temporal environmental variation on host-associated microbiota and the role of diet in shaping microbial communities as an initial step in unraveling identity relationships between host condition and external environmental variables.

As the complexities of wildlife conservation change and evolve, so do the methods of management. Multifaceted approaches are required to monitor populations and increasing evidence suggests that metagenomic analysis offers valuable insight into the health and nutrition of wildlife. It is, therefore, beneficial for the next generation of biological researchers to be taught bioinformatics and particularly metagenomic analysis, as skills gained in this field can be of value for those in the business of wildlife conservation. Specifically, the use of metrics of individual and community bacterial diversity can allow the gut microbiome to serve as a biomarker for animal health status which is of particular value for monitoring difficult-to-manage wildlife species who face foraging restrictions, climate fluctuations, infectious disease, and habitat disturbances.

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LIST OF ABBREVIATIONS

LURE Lab Based Undergraduate Research Experiences

CURE Course Based Undergraduate Research Experiences

GA:C Glucuronic Acid to Creatinine

UN:C Urea Nitrogen to Creatinine

PSM Plant Secondary Metabolite

ASV Amplicon Sequence Variant

PCoA Principal Coordinate Analysis

GTA Graduate Teaching Assistants

CHAPTER ONE: CHARACTERIZING THE METAGENOMIC RELATIONSHIP WITH NUTRITIONAL CONDITION OF A FREE-RANGING MAMMALIAN HERBIVORE

Abstract

Intestinal microbial communities play a vital role in digestion and detoxification in mammalian herbivores and can therefore serve as biomarkers of health status. A large challenge faced by mammalian herbivores involves the dietary limitations created potentially toxic plant secondary metabolites (PSMs) which are energetically costly to detoxify, but often present in highly available food sources. Herbivores must therefore make difficult dietary choices to maximize the uptake of available nutrients, but also minimize the potentially harmful effects of PSM ingestion. The consequences of these dietary decisions are not limited to host-associated physiology. Experimental studies show that, gut microbial communities also respond to dietary variation experienced by the host. Experimental diet manipulation can be impractical in wild herbivore systems and may not capture natural in diet composition. Here we studied the relationship between the gut microbiome and the variation of nutritional status in a population of wild, free-ranging mammalian herbivores. We use a population of moose (*Alces alces*) ono Isle Royale National Park, Michigan studied during winter months over a 6-year period to assess the spatial and inter-annual variation in the gut microbiome and potential relationships with the nutritional host health and investment in detoxification of the host. The population serves as an ideal study system due to the natural inter-annual variation in

diet, nutritional condition, and investment in detoxification between moose subpopulations in the East and West regions. Prior analysis of fecal samples from this system revealed variation in prominent plant secondary metabolites as well as nutritional condition and investment in detoxification by moose among regions and years. This variation in moose nutritional condition and investment toward detoxification may be associated with the quantity and quality of plants available. In particular, balsam fir (*Abies balsamea*) is an important food source for *A.alces* in the winter months when available vegetation is limited, but has PSCs and varies spatially and temporally both in its availability and nutritional quality. Not only has relative abundance of *A. balsamea* been diminishing in the western portion of the island over time while the population in the east has remained stable, but this primary winter forage species also has a higher protein content in the western region than the eastern (Hoy et al., 2018). Given the high content of PSCs in balsam fir and previous studies which identified a trend of decreasing availability of balsam fir over time on the western region of the island, we predicted the moose in the western region to have a more diverse gut microbiome than their counterparts in the eastern region. Instead, our sequencing of 16S rRNA gene amplicons from moose fecal samples revealed moose gut microbial diversity remained lower in the western population than the eastern population over the 6-year moose feces sampling period. The data provides future opportunities for understanding the role of specific taxonomic groups in nutrient utilization and detoxification of PSMs as well as bringing insight into shifts in microbial diversity in response to diet variation. At a broader scale, the study findings that host-associated microbial communities are susceptible to variation in habitat is of value for those who study and monitor the health statuses of wildlife

herbivore populations as gut microbial insight provides another metric to characterize host health and identify when health is impacted by environmental stressors. With this added insight into animal well-being, monitoring gut microbial communities can be valuable in shifting the healthcare of wildlife herbivore populations towards more proactive and preventative strategies.

Introduction

Microbial communities are key contributors and regulators of various physiological processes that in turn shape the overall health of a host species (Moran et al., 2019) and as such can be evaluated as biomarkers of host health. This has been most thoroughly studied in humans, where disruption of the human adult gut microbiome has been associated with several disorders including allergy development, Celiac disease, Chron's disease, and both Types I and II diabetes. Many of the disorders associated with disruption of the gut microbiome, or dysbiosis, exhibit an overall reduction of microbial diversity or key functional groups and demonstrate an inflammatory response by the host (Heilbronn & Campbell, 2008; Lupp et al., 2007; Penders et al., 2007). Bacterial community composition has been identified as being directly involved in nutrient uptake from ingested food and dramatic shifts in community structure has been seen in human diseases such as obesity. In such studies, diet has been identified as a factor that can impact gut microbial community composition (Cani et al., 2007; Hildebrandt et al., 2009).

Significant findings of gut microbial structure and composition linked to nutritional variables in human subjects can give insight into understanding the health of wildlife animal species. As microbial communities are susceptible to the fluctuation of

abiotic and biotic factors that affect their host organisms, monitoring microbiota has allowed for a new approach to study the health and physiology of wildlife animals who face increasing environmental threats. More so, translating the use of microbes to predict health is particularly useful in vertebrate herbivores who face added complication in the physiological consequences of consuming plant secondary metabolites. These metabolites can be toxic to herbivores and can inhibit digestion, metabolism, nutrient assimilation, and retention (Au et al., 2013; Forbey et al., 2011; Kohl et al., 2015; Sorensen et al., 2005). For example, in mammalian herbivores, many host species lack endogenous enzymes for plant material degradation and depend upon symbiotic microbes for digestion of indigestible cellulose as well as metabolizing potentially toxic plant secondary metabolites (PSMs, (Kohl et al., 2016; Melody, 2017; Svartström et al., 2017; Tsuchida et al., 2017). For example, tolerance to toxins produced by the ragwort *Jacobea vulgaris* is thought to be due to the detoxification role of the rumen microbiota in sheep and goats (Rattray & Craig, 2007). Given that plant material is generally high in indigestible fiber, low in essential nutrients, often contains toxic defensive metabolites (Karasov & Rio, 2020), and the success of herbivore plant digestion is largely reliant upon enzymes produced by gut microbes (Stevens & Hume, 2004), disruption of these microbial communities could lead to health consequences for wild mammalian herbivores. the host.

While diet changes have been shown to impact community composition of the gut microbiome in wildlife species when studied in captivity (Fan et al., 2017; Youngblut et al., 2019), these studies have been limited by the inability to replicate naturally dynamic environmental conditions for species of interest. Studies in captivity are unable to

account for the difficult and often nutritionally complex decisions free-ranging animals make in response to shifts in abiotic and biotic factors such as predator threat, unpredictable climate periods, and food availability. For example, bacterial communities in the ceca of avian herbivores are observed to shift in response to their highly specialized seasonal diets, but show substantial differences in the gut microbiome profile of captive vs. wild grouse species (Drovetski et al., 2019; Wienemann et al., 2011). Gut microbial communities act as an extended phenotype of the host that could mediate dietary decisions and physiological tolerance to dietary constituents, particularly in vertebrate herbivores (Henry et al., 2021; Richards et al., n.d.). Plants synthesize a diversity of secondary, or specialized, metabolites (Lacchini & Goossens, 2020; Louveau & Osbourn, 2019; Moghe & Kruse, 2018; Weng et al., 2021), some of which are toxic to herbivores (Panda et al., 2021; Wittstock & Gershenzon, 2002; Zhou et al., 2015). Conversely, herbivores have adapted mechanisms to defend themselves against plant chemical warfare via detoxification mechanisms (Dearing & Cork, 1999; Kohl et al., 2016, 2018; Marsh, Wallis, McLean, et al., 2006) which may be, in part, co-mediated through the gut microbiome and host . Many free-ranging herbivores make dietary choices that balance the ingestion of necessary nutrients while minimizing exposure to potentially harmful PSMs (Frye et al., 2013). Thus, the microbial community within the wild herbivore digestive system could be instrumental to producing and maintaining detoxification processes. As the gut microbiomes of wild and captive animals can differ substantially (Ushida et al., 2016), there is a need for more studies that assess gut microbiome communities of wild, free-ranging animals and link these community profiles to demographic metrics used by practitioners (Tulchinsky & Varavikova, 2014)

to manage wildlife, and to establish successful releases from mammals bred in captivity and cultivate species' survival.

One opportunity to understand the link between the gut microbiome and host health is the temporal and spatial variation in plant-herbivore interactions. Seasonal, annual, and regional variation in the availability and quality of plants and how herbivores respond to that variation can reveal the role of gut microbial community composition across both time and space. Studies in sheep (*Ovis aries*) have demonstrated that varying nutritional levels can have significant effects on the ruminal microbial communities as higher nutritional dietary levels linearly increased the abundance of certain bacterial phyla of interest, but linearly decreased the community richness (Wang et al., 2017). In a study collected from moose (*Alces alces*) in Vermont, Alaska, and Norway, variation in the available forage among geographic locations played a large role in defining the core microbiome in the three isolated populations (Ishaq & Wright, 2014). Furthermore, a study on the Western capercaillie (*Tetrao urogallus*) revealed foraging limitations to coniferous needles during the winter resulting in reduced diversity of the cecal bacterial community compared to when birds have a more diverse diet (Wienemann et al., 2011). Not only do these studies highlight a few of the environmental variables impacting wildlife herbivore health, they also reveal a shift in the host gut microbiome community structure and composition in response to variations in these environmental variables. As such, characterization of herbivore gut microbiomes can allow for further interpretation of host health status and subsequently give insight into predicting host responses to perpetually fluctuating environmental factors.

To advance our characterization of the gut microbiomes of wild herbivores, we study the microbial communities of moose (*Alces alces*) in Isle Royale National Park, Michigan. This moose population naturally displays heterogeneous distribution of individuals across the island, resulting in the formation of two regional subpopulations of moose on the eastern and western sides of the island (Peterson, 1999). Moose predominately feed on tree shoots and shrubs, which they must consume in considerable amounts to uphold their body mass. However, in the winter months, food availability becomes highly restricted for the moose whose foraging options are generally limited to evergreen trees that are high in PSMs and vary in nutritional capacity. One such tree species is the balsam fir (*Abies balsamea*), a heavily chemically defended conifer. Balsam fir is the principal food source in winter for moose living on Isle Royale as it has greater concentration of protein, lower concentration of cellulose, and higher in vitro digestibility than the average deciduous species on which Isle Royale moose feed (McLaren & Peterson, 1994; Risenhoover, 1987). Fir typically represents 47% of winter diet, northern white cedar (Thuja occidentalis) represents 15%, and the remainder comprises a range of deciduous species (Parikh et al., 2017).

Previous studies of these moose have shown that there is spatial and temporal variation in both diet composition and quality (Melody, 2017). Diet composition was found to differ between the eastern and western regions of this study site, with moose in the east consuming substantially less cedar, and more balsam fir and deciduous forage. Host metabolism was compared by determining ratios of urea nitrogen to creatinine (UN:C) and glucuronic acid to creatinine (GA:C) in urine samples (Parikh et al., 2017). In wild herbivores, increased excretion of urea nitrogen in the urine during mid to late

winter is an indicator of increased catabolism of endogenous protein as a result of prolonged nutritional restriction (Delgiudice et al., n.d.,). While UN:C can be high in animals eating high protein foods, in the winter when food sources are limited, it is more likely that elevated UN:C ratios indicate poor nutritional condition (Parikh et al., 2017). Glucuronic acid excretion in the urine is positively correlated with increase intake of PSMs and is therefore an indicator of the organism's investment toward detoxification (Guglielmo et al., 1996; Marsh, Wallis, Andrew, et al., 2006). The relationship between nutritional stress (UN:C) and energy expended toward detoxification (GA:C) has been shown to vary spatially. In the eastern population, nutritional stress increased with greater investment toward detoxification, but in this correlation was not observed in the western population where forage protein content is higher (Hoy et al., 2018). Together, these results indicate that moose in the east consume a diet higher in PSMs that requires more energy expenditure toward detoxification and negatively impacts nutritional condition. Our objective was to investigate patterns in the gut microbiome relative to these regional patterns of diet, nutritional condition, and detoxification.

Host diet determines the availability of the nutritional components in food sources that in turn select for certain microbial taxa (Louis et al., 2007). More complex diets have been correlated with more diverse gut microbial communities (Greene et al., 2018) and in a previous study on this moose population, increased diet diversity was associated with less nutritional restriction (Parikh et al. 2017). We therefore hypothesized that moose in the east, who have poor nutrition and more narrowed diets, would have gut microbiomes characterized by lower bacterial diversity than in the west. Our rationale for this pattern is that diet diversity benefits the generalist herbivore because of interspecific variation in

nutritional content of foraging species (Nersesian et al., 2012) and diverse diets minimize the intake of any one type of PSM (Freeland & Janzen, 1974; Provenza et al., 2003). Secondly, during years where ratios of UN:C were the highest, we predicted decreased bacterial diversity. Third, during years where ratios of GA:C were the highest, we predicted decreased bacterial diversity. Our rationale for this pattern is that increased GA:C would indicate increased consumption of PSMs which requires more energy expenditure toward detoxification thereby negatively impacting nutritional condition and reduced bacterial diversity has been viewed to be a negative indicator of health (Clayton et al., 2018; Fujimura et al., 2010; McKenzie et al., 2017). Lastly, we predicted stronger correlations between bacterial diversity and UN:C rather than GA:C as a result of UN:C's indication of more long-term evidence of gut health linked to host nutritional health and GA:C's indication of consumptions of toxins on a more microscale, day-to-day level. (Kohl et al., 2018).

Methods

To test predictions, we sampled 326 moose fecal specimens (148 from the west and 173 from the east) for DNA extraction and PCR amplification for 16S rRNA gene sequencing and microbial DNA extraction.

Sample Collection

Both fecal and urine samples were collected in tandem over a 4-week period from January to February in Isle Royale in each of the six study years (2013, 2014, 2015, 2017, 2018, and 2019). Study period omits the year 2016 due to a shift in funding. Samples were collected in the snow along the tracks of a single moose to ensure it represented a single individual. Although care was taken to sample from across the area of the region and find distinct tracks, it was not possible to determine whether a pellet sample came from the

same individual moose each year. All collections were kept frozen after collection and during transport to the laboratory where they were stored at -20C. Fecal pellets were sampled from the core of the pellets under sterile conditions and kept frozen prior to DNA extraction and sequencing. All 326 samples were sent to and processed by the Center for Microbiome Innovation in San Diego, CA. Both fecal and urine samples were collected in tandem over a 4-week period in Isle Royale between late January and early February in each of the six study years (2013, 2014, 2015, 2017, 2018, and 2019). Fecal pellets were sampled from the core of the pellets under sterile conditions and subsequently frozen until DNA extraction and sequencing. For quality assurance, all 326 samples were sent to and processed by the Center for Microbiome Innovation in San Diego, CA. Methods for DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing were previously described in detail (Sinha et al., 2016; Vogtmann et al., 2017). Briefly, DNA extraction, PCR amplification of the V4 region of the 16S rRNA gene, and amplicon preparation were performed as described by (Caporaso et al., 2012) using the universal bacterial primer set 515F/806R (Walters et al., 2011) and can be found on the Earth Microbiome Project website (http://www.earthmicrobiome.org/emp-standard-protocols/dna-extraction-protocol/).

Sequencing and Microbial Analysis

PCR amplification of the V4 region of the 16S ribosomal RNA gene using the universal bacterial primer set 515F/806R (Walters et al., 2011) was followed by 250 × 2 paired-end sequencing on an Illumina HiSeq (Illumina, Inc., San Diego, CA, USA). Raw sequences were processed using QIIME2 (Estaki et al., 2020) Pipeline- Version 2.2020.6. Demultiplexed paired-end reads were trimmed to 150 bp, merged, and the resulting

sequences were denoised using the DADA2 pipeline plug-in (Callahan et al., 2016) within QIIME2 environment, to identify amplicon sequence variants (ASV). Sequence depths ranged between 53 and 24,683 per sample with a median value of 16,285. A total of 5,178,099 sequences clustered into 2,634 ASVs after quality control, denoising, and merging. ASVs were taxonomically classified in QIIME2 using the pre-formatted SILVA version 138 reference sequence and taxonomy files here that were processed using RESCRIPt (Robeson et al., 2020) via a trained classifier (Quast et al., 2013; Yilmaz et al., 2014) The resulting feature table and taxonomic assignments were analyzed in QIIME2 and using the R phyloseq package (McMurdie & Holmes, 2013).

The QIIME2 "diversity core-metrics-phylogenetic" command was used to calculate a series of diversity metrics, including several alpha and beta diversity metrics. Rarefaction analysis was also carried using the used the command "diversity alpha-rarefaction" to confirm sufficient sequencing depth. QIIME 2 core-metrics creates several alpha diversity metrics: observed ASVs (bacterial community richness), Shannon's index (bacterial community richness and evenness), Faith's phylogenetic diversity (PD) (bacterial community richness that incorporates phylogenetic relationships between taxa), and Pielou's species evenness (bacterial community even- ness) (Lozupone & Knight, 2005). Alpha diversity refers to metrics of diversity within a sample which includes the total number of species (richness) and how evenly distributed the members of a community are among the species present (evenness). We calculated alpha diversity using the Shannon index (a metric of both evenness and richness) with data rarefied to 5,900 sequences per sample. We used a two-way ANOVA to determine the significance of differences in alpha diversity when grouped by region as well as by year. Core-metrics

of beta diversity metrics included: Bray–Curtis distance (abundance without phylogeny), Jaccard distance (presence and absence of ASVs without phylogeny), unweighted UniFrac distance (presence and absence of ASVs with phylogeny), and weighted UniFrac distance (abundance of ASVs with phylogeny). Beta diversity refers to the biological diversity among environments or along a gradient and is a measure of the similarity or dissimilarity between bacterial communities (Lozupone Catherine A. et al., 2007). Beta diversity was calculated using unweighted UniFrac phylogenetic distances which consider species' presence and absence information and by comparing the length of phylogentic branching (Lozupone & Knight, 2005). To compare the dissimilarities of the bacterial community structures in the moose samples from eastern vs. western regions, a principal coordinate analysis (PCoA) (Lozupone & Knight, 2005) was performed based on unweighted Unifrac metric distances (Figure 2). Variation in community composition between years and region was quantified by measuring the distance to the centroid of east and west subgroups in each year (Anderson et al. 2006), using the betadisper function in the vegan package (Oksanen, 2015) (Figure 3). Statistical significance of dissimilarity in distances to centroids between regions and over time was assessed using an ANOVA. The phyloseq package in R was used for figure construction. Principal coordinates analysis (PCoA) was used to visualize sample dissimilarities (Caporaso et al., 2012; Vázquez-Baeza et al., 2013) based on the Bray–Curtis and unweighted UniFrac distance metrics.

Results

Sequencing

Sequence depths ranged between 53 and 24,683 per sample with a median value of 16,285. A total of 5,178,099 sequences clustered into 2,634 ASVs remained after quality control, denoising, and merging paired end reads using the DADA2 pipeline plug-in (Callahan et al., 2016) within QIIME2 environment.

Alpha diversity

Alpha diversity was measured to assess the gut microbial community composition within individual moose and identify the potential patterns of spatial (region) and temporal (yearly) variation. ANOVA Alpha diversity differed significantly between regions (p-value <0.0001, add F stats or other, Table 1) with moose in the east having higher Shannon Index values than moose in the west (Figure 1), indicating gut bacterial diversity was higher in eastern moose than western moose. Alpha diversity also significantly varied by year (p-value <0.01, add F stats or other) (Table 1) with the highest Shannon index values observed in the year 2015 and the lowest observed in 2019 (Figure 1). There was no region by year interaction (p-value > 0.05, add F stats or other).

Beta diversity

To explore compositional differences in the microbiomes based on region, we analyzed beta diversity to estimate the dissimilarity between the microbiomes found in the east and west fecal samples. Despite considerable overlap among groups in the PCoA (Figure 2), a PERMANOVA analysis the east and west region has detected significant differences (PERMANOVA; F-value 5.762, p=0.001). We also found a significant difference in beta diversity across years (PERMANOVA; F-value 3.712, p=0.001).

Additionally, there was a significant region by year interaction (PERMANOVA; F-value 1.289, p=0.005) indicating that annual variation in the condition of the moose or environment disparately affected the microbial community structure in the different regions. We did not find (dispersion) statistical significance of dissimilarity in distances to centroids between east and west regions (ANOVA; F-value 0.3582, p-value 0.545) indicating that regional differences in microbial communities was not the result of variation in dispersion. Although distances to centroid were generally consistent among years (Figure 3), We did find that unweighted UniFrac diversity index distances to centroid was lower in 2017 than 2019 in the western population (TukeyHSD, p-value 0.0045368, Table 4). There was no significantly difference in distances to centroid among years within moose in the east (TukeyHSD, p-value > 0.05, Tables 5).

UN:C Ratios Correlated to Alpha and Beta Diversity

To assess whether host nutrition may explain individual level and regional differences observed in microbial diversity, correlations between Urea Nitrogen: Creatinine ratios (a reported marker of nutritional stress for *Alces alces*) and alpha diversity was performed using Spearman rank correlation method ("Spearman Rank Correlation Coefficient," 2008). There was no significant correlation observed between Shannon Diversity Index values and UN:C ratios for all the data combined (Figure 4a). But when grouped by region, a negative correlation between UN:C values and Shannon Diversity index values was observed (Figure 4b), indicating that microbial diversity declines with greater nutritional stress within each region. Moose in the east had higher overall UN:C values and showed a significant negative correlation between UN:C values and Shannon Index values (R = -0.16, p-value of 0.04). Although a similar trend was

observed, UN:C values and Shannon Index values were not significantly correlated in moose in the west (R = -0.14, p-value = 0.1).

GA:C Ratios Correlated to Alpha and Beta Diversity

To assess whether investment in detoxification may explain individual level and regional differences observed in microbial diversity, correlations between Glucuronic Acid: Creatinine ratios (a reported marker of detoxification investment by *Alces* , REF*Alces alces*) and alpha diversity was performed using Spearman rank correlation method ("Spearman Rank Correlation Coefficient," 2008). There was no significant correlation observed between alpha or beta diversity index values and GA:C values for all data combined (Figure 5a) or when grouped by region (Figure 5b).

Discussion

Results revealed differences in gut microbial communities between eastern and western subpopulations of moose as well as interannual variation in microbial diversity. We predicted that moose in the east, who have poor nutrition and more narrowed diets, would have gut microbiomes characterized by lower bacterial diversity than in the west. In contrast with this prediction, all years moose in the eastern region had consistently higher alpha diversity of microbes than moose in the western region. This observed shift in alpha diversity indicates that differences in nutritional value of food on the two regions of the island correlate with significant shifts in the microbial diversity of the hosts fecal microbiomes.

Additionally, we predicted decreased bacterial diversity both during years where ratios of UN:C were the highest as well as during years where ratios of GA:C were the highest. Although regional comparisons showing eastern moose had higher microbial

diversity than western moose did not support prediction, individual moose in lower nutritional condition (i.e., higher UN:C) , particularly in the east, did have lower microbial diversity. Lastly, we predicted stronger correlations between bacterial diversity and UN:C rather than GA:C as a result of UN:C's indication of more long-term evidence of gut health linked to host nutritional health and GA:C's indication of consumptions of toxins on a more microscale, day-to-day level (Kohl et al., 2018). We found no evidence that investment in detoxification by moose (i.e., GA:C) was related to microbial diversity

The most likely explanation for interannual and regional differences in microbial diversity of moose is the known variation in diet composition and quality related to evenness. For example, the moose's winter diet is not only comprised of balsam fir, but additionally includes cedar (*Thuja occidentalis)* whose leaves have been found to contain volatile PSMs, such as limonene, alpha-pinene, and myrcene (Gao et al., 2005). Cedar encompasses 27% of the diet for moose in the west compared to only 0.02% to moose in the east (Parikh et al., 2017). In addition, moose in the east consume a higher proportion of deciduous species (42%) compared to western moose (29% deciduous). The deciduous portion of the diet could represent up to 15 unique plant species and indicates that moose in the east have a more diverse diet than in the west. While both balsam fir and cedar contain volatile toxins, it is possible that cedar contains either higher concentrations or more bioactive of PSMs which may select for less diverse microbial taxa in moose in the west. In addition, the overall higher cellulose content of deciduous and higher crude protein content of balsam fir compared to other plants consumed, both of which are in higher proportion in the diet of moose in the east, may support a more diverse microbial

community in eastern moose (Bel Lassen et al., 2021; Holscher, 2017; Wang et al., 2017).

Although we predicted that eastern moose that were in lower overall nutritional condition as indicated by higher UN:C values would have lower with microbial diversity than western moose, it was the eastern population that had the higher microbial diversity. This indicates that microbial diversity is related to more than just nutritional stress, at a regional scale. Several environmental conditions may contribute to distinct differences in both nutritional condition and microbial diversity between the two regions. Biotic factors that vary regionally and annually include density, predation, and diet composition. In the coming years, high herbivore densities are also expected to deplete and reduce the quality of forage available (DeAngelis et al., 2015). Herbivore intake rates can be reduced when the threat of predation leads to herbivores behaving more (Fortin et al., 2005; McArt et al., 2009). Furthermore, predation risk affects herbivore selectivity for forage types in turn affecting herbivore diet composition and thereby reducing diet quality. Predation risk can also affect how selective herbivores are for certain forage types (Camp et al., 2017) which can ultimately affect diet composition (Hoy et al., 2019) and reduce diet quality (Barnier et al., 2014). Abiotic factors varying regionally and annually include climatic factors such as snow depth and temperature. For example, nutritional restriction was found to be greater (as indicated by higher UN:C) for moose during winters with deeper snow and during winters that followed hotter summers (Hoy et al., 2018).

The relationship between environmental conditions and microbial diversity of hosts is further supported by interannual variation observed in alpha and beta diversity. For example, in the year 2015, both eastern and western populations had higher overall

alpha diversity values than in any other year. This could be attributed to relatively low snow depth, high summer precipitation and longer growing degree days in 2015 bacterial beta diversity, particularly when compared to 2017. Further exploration of these particular year-to-year findings could be conducted by examining other abiotic and biotic factors such as snow depth, predation, and precipitation (Montgomery et al., 2013). For example, the increased energetic cost of moving in deep snow may influence microbial diversity indirectly as such restricted movement influences food intake rates and availability of quality forage (Parker et al., 1984). Higher summer precipitation and longer growing season could not only result in moose experiencing more thermal stress in the form of increased metabolic and respiration rates (McCann et al., 2013), but allow for overgrowth of plant tissues grown at higher temperatures that tend to have increased PSM concentration and low crude protein content (Forbey et al., 2013). These changes in abiotic factors may influence microbial diversity indirectly as moose enter winter in a poorer nutritional state after experiencing thermal stress coupled with higher metabolic costs and reduced food intake experienced during hot summers. In 2019, moose on the west had a much wider variation in bacterial beta diversity and in particular when compared to 2017 which was much more uniform in unweighted unifrac distance measurements as indicated by the Tukey HSD.

Although our regional analysis did not support our prediction that poor nutritional condition of eastern moose would be associated with lower microbial diversity, we did support that prediction at the individual level in the eastern region. At the individual level, microbial diversity was negatively correlated with nutritional condition. While the relationship was only statistically significant for moose in the east, we observed a similar

trend for moose in the west. Nutritional condition explained 16% of the variation in alpha-diversity of gut microbes in moose. The weaker correlations in the western moose may indicate that microbial diversity is only influenced under composition more severe nutritional restriction experienced in individual moose in the east. In support, human studies have linked nutrition-related conditions such as Crohn's Disease and Type II Diabetes Mellitus with intestinal bacterial load, microbiome composition, and inflammation (Chakaroun et al., 2020). It is also possible that only specific taxonomic groups are influenced by nutritional condition which may not be captured by diversity indices. For example, urban house sparrows showed no significant differences in relative abundance of microbial taxa at the phylum level from rural house sparrows, but a more enriched analysis at the order level identified a higher abundance of microbes from the phylum *Proteobacteria* in urban house sparrows (Gadau et al., 2019).

Unlike nutritional condition, the investment in detoxification, as indicated by GA:C values, was not correlated with microbial diversity. GA:C is a measure of detoxification representing the day-to-day foraging decisions, specifically the daily amount of PSMs consumed, absorbed, and metabolized by the host. While shifts in microbial diversity do change relative to toxin intake, these changes likely occur over longer time periods, such as diet transitions associated with seasonal shifts. We propose that microbial communities may be more temporally stable relative to short term daily fluctuations in GA:C or lag daily dietary decisions. For example, previous research suggests that horse gut microbiota can adapt in response to new diets quickly within 4–6 days (Fernandes et al., 2014). It is also possible that the systemic PSM concentration that could be detoxified by the host is not the same PSM concentration experienced by gut

microbes. Shifts in microbial diversity in response to UN:C but not to GA:C strengthens the argument for more cumulative, long-term measures of host nutrition, and is more likely to be related to composition of microbial communities than daily measures of PSM exposure. Again, it is also possible that only specific taxonomic groups are influenced by PSM exposure in the host which may not be captured by diversity indices. For example, Integrated analysis showed microbes within *Proteobacteria* may be inhibited by the antibacterial property within flavonoid PSMs, whereas more members belonging to *Firmicutes* are favored selectively by the specific nutrients in flavonoids (Braune & Blaut, 2016).

Understanding how the shifts in gut microbial communities mediate the physiological responses of animals to abiotic and biotic stressors of mammals could have several benefits to conservation of wildlife. In human health, shifts in microbial diversity is an indicator of health status. In domestic and captive species, management of gut microbial communities through diet can result in more successful release from domestication or captivity. As such, there is great potential that monitoring and managing gut microbes could benefit the management of wild species. However, advances in microbial-mediated management of wildlife requires that we first identify how to best characterize microbial communities and identify how microbial diversity is impacted by environmental variables experiences by the host. We contribute to this goal by showing there was spatial variation in gut microbial communities of a large, free-range herbivore which indicates diet impacts microbial diversity. This is similar to what is seen in the Western capercaillie (*Tetrao urogallus*) when foraging in the winter (Wienemann et al., 2011) and in the moose (*Alces alces)* populations of Vermont, Alsaka and Norway where

variation in available forage among geographic locations played a large role in defining the core microbiome in the three isolated populations (Ishaq & Wright, 2014). Similar to sheep (*Orvis orvis)* who demonstrate bacterial diversity response to varying nutritional condition (Wang et al., 2017), we also found temporal variation in microbial communities which indicates a microbial diversity response to fluctuating abiotic and biotic factors experienced by the host. Monitoring the shifts in gut microbial diversity, coupled with future understanding of the functional role of specific microbial taxa that are gained or lost; composition can enhance our ability to predict regional and temporal predictions of demographic health outcomes for host species.

Figures and Tables

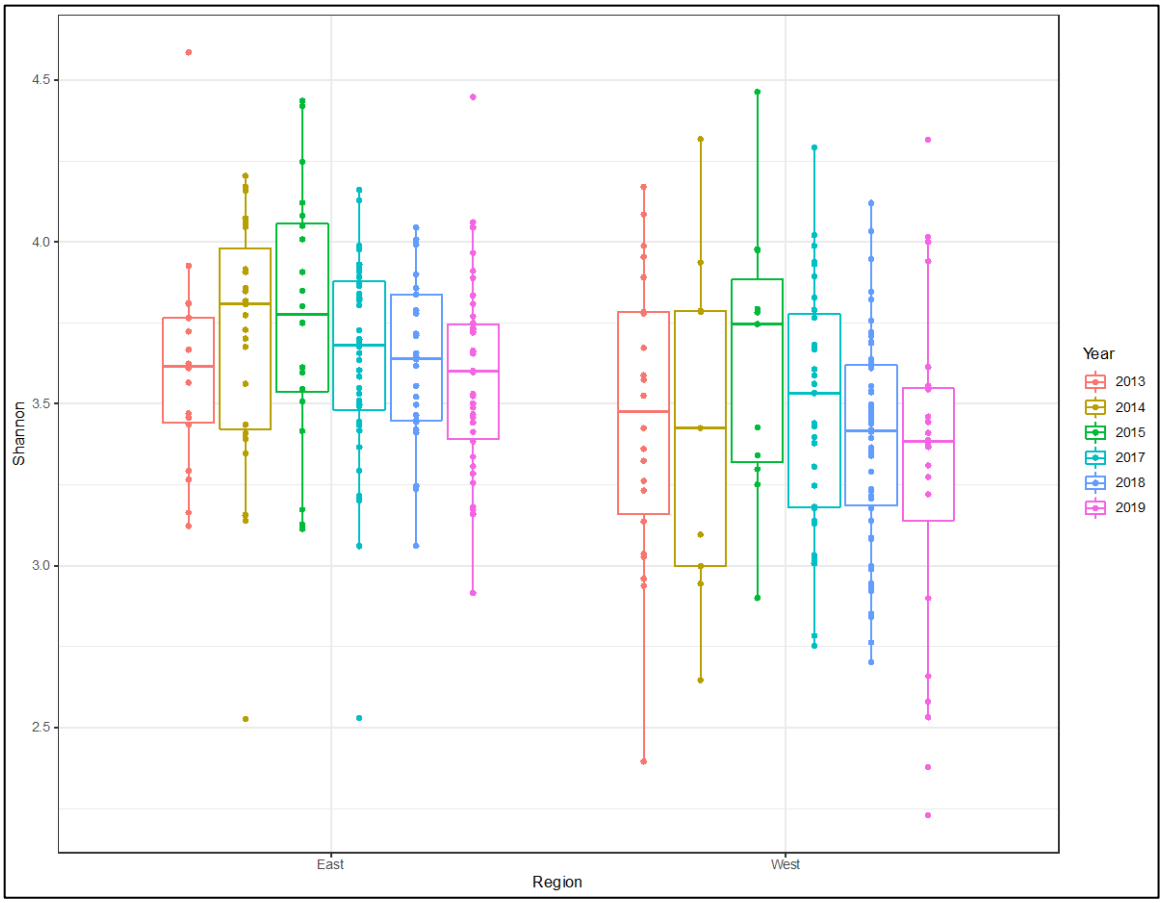


Figure 1.1. Box plots of α-diversity separated by region and year of collection; bacterial abundance and evenness assessed via Shannon indexes for geographic region by year. Shannon diversity metric showed significant differences in alpha diversity by region (Two-way ANOVA; F-value 44.17, $p<0.0001$) and by year (Two-way ANOVA; F-value 3.87, $p < 0.001$), but no interaction between region and year (Two-way ANOVA; F-value 1.423, $p > 0.05$). Moose on the east have a higher Shannon index indicating increased diversity when compared to moose in the west.

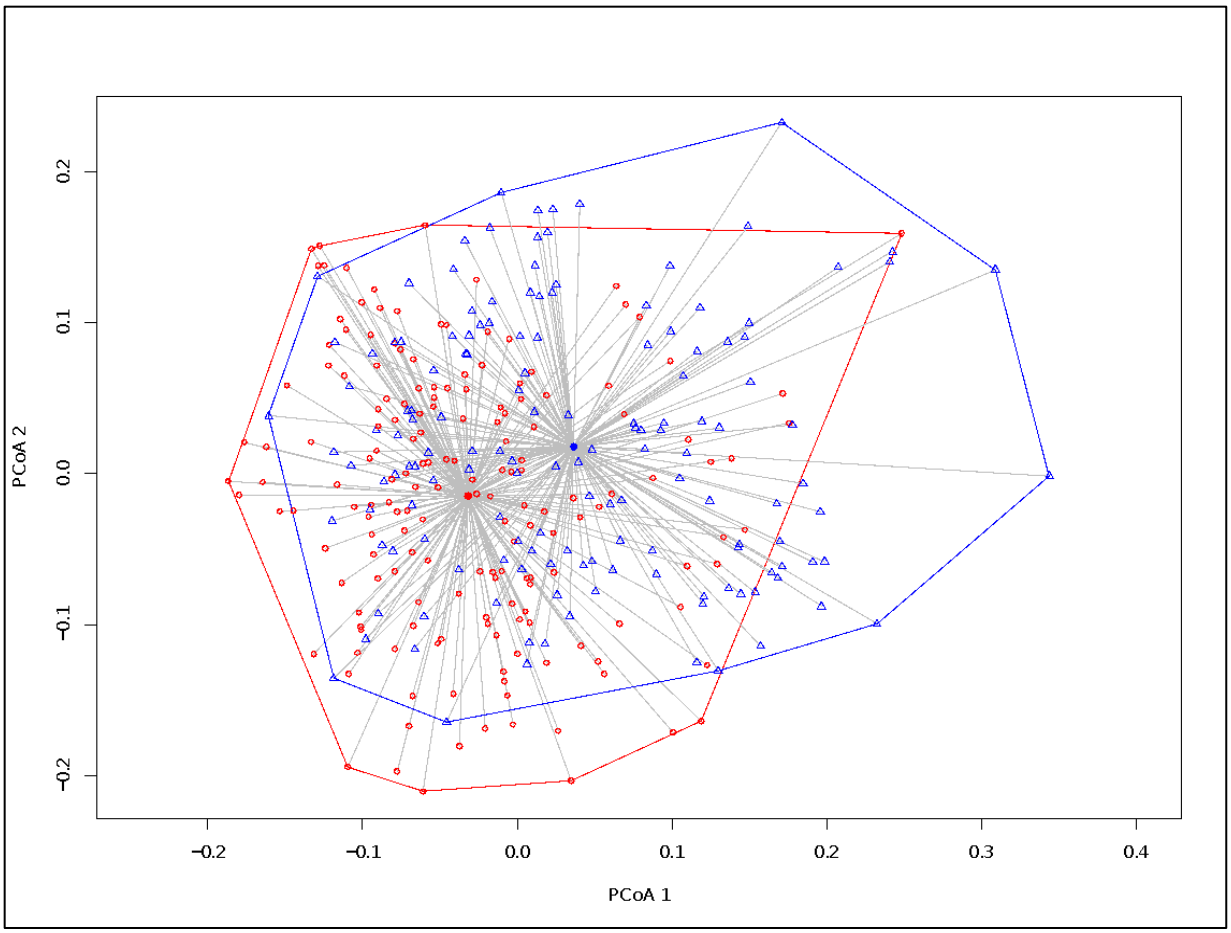


Figure 1.2. PCoA ordination of differences in β-diversity; unweighted UniFrac dissimilarities when grouped regionally by east (red circles) and west (blue triangles); lines connect samples to each group's median. Unweighted UniFrac diversity index showed significant differences in beta diversity when grouped by region (PERMANOVA; F-value 5.762, p=0.001) and by year (PERMANOVA; F-value 3.712, p=0.001). Additionally, there was significant interaction between region and year (PERMANOVA; F-value 1.289, p=0.005)

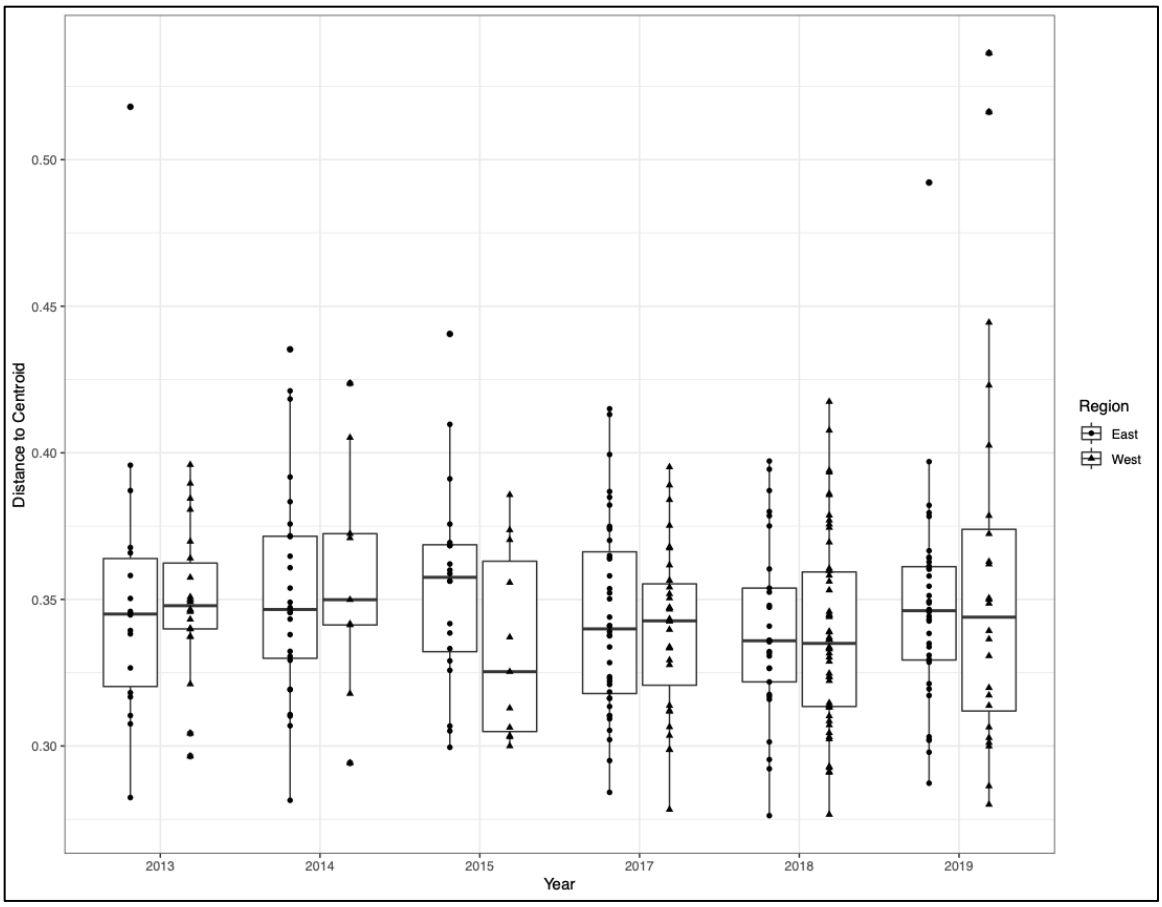


Figure 1.3. Differences in β-diversity dispersion; unweighted UniFrac dispersion (average distance to median) among years in west (circle) and east (triangles) regions. Unweighted UniFrac diversity index distances to centroid were not significant for variation in dispersion homogeneity by region (ANOVA; F-value 0.3582, p > 0.05). Unweighted UniFrac diversity index distances to centroid in the western moose group were significant when comparing years 2019 to 2017 (TukeyHSD, p-value < 0.05). Unweighted UniFrac diversity index distances to centroid in the eastern moose group were not significant for variation in dispersion homogeneity by year (TukeyHSD, p-value > 0.05). (Tables 4 and 5)

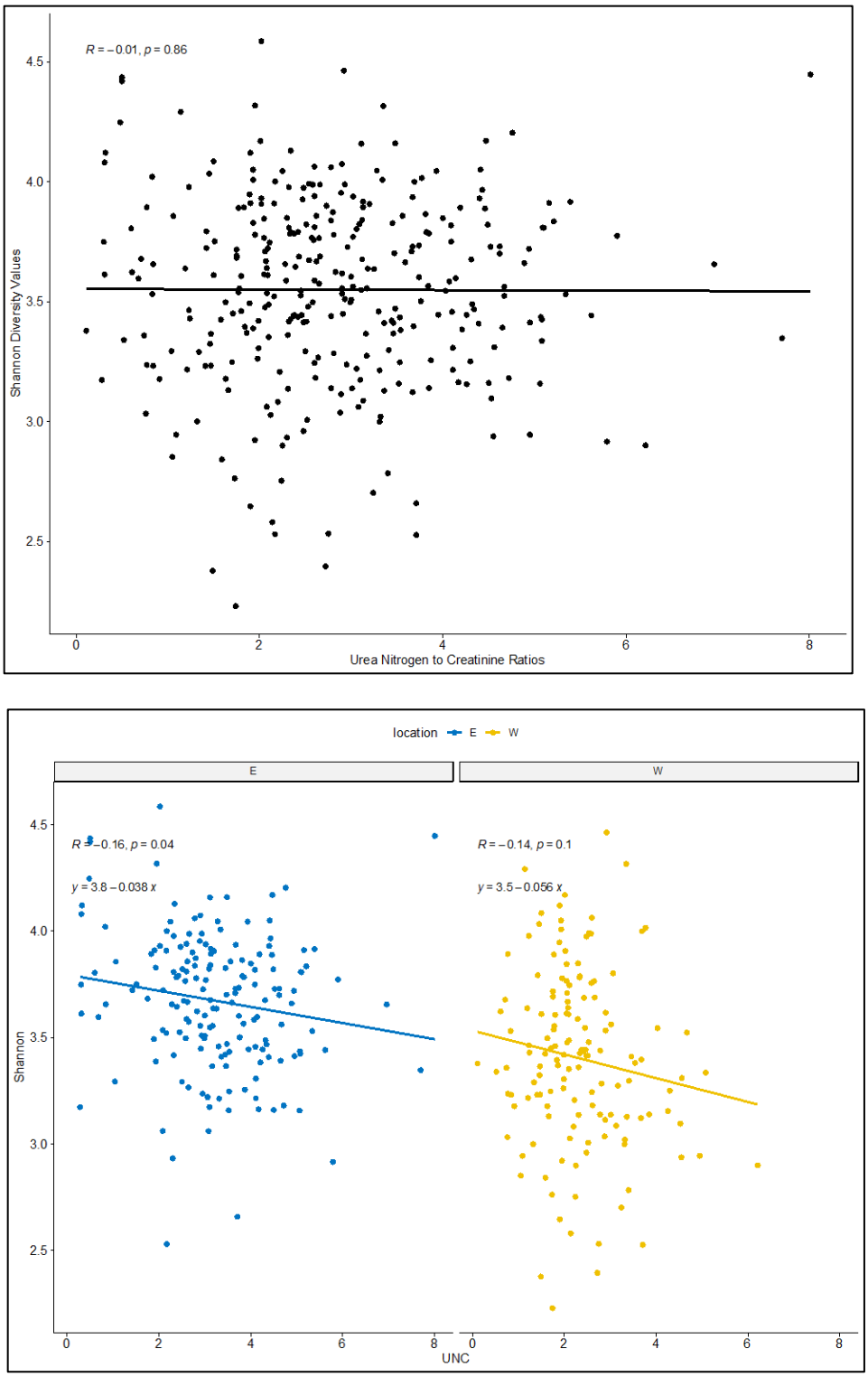
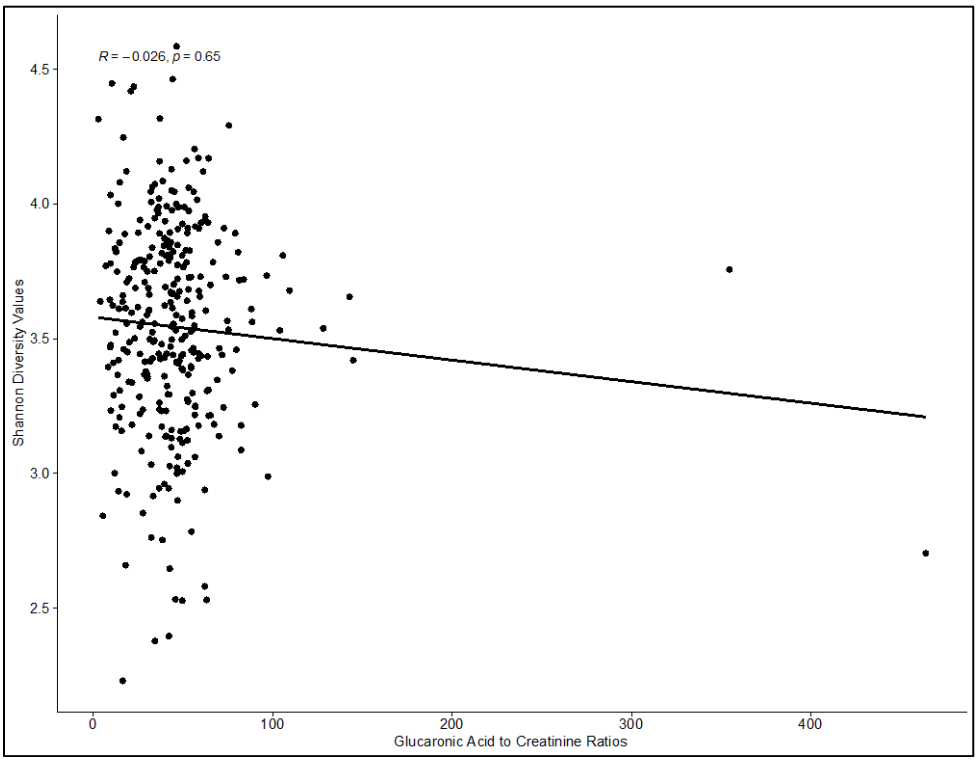


Figure 1.4. Spearman's Rank Correlation analysis between UN:C ratios and Microbial Diversity Index values from paired fecal and urine samples. (a) Shannon Diversity index values for all samples correlated to UN:C values. (b) Shannon Diversity Index values separated by east (blue) and west (yellow) correlated to UN:C values.



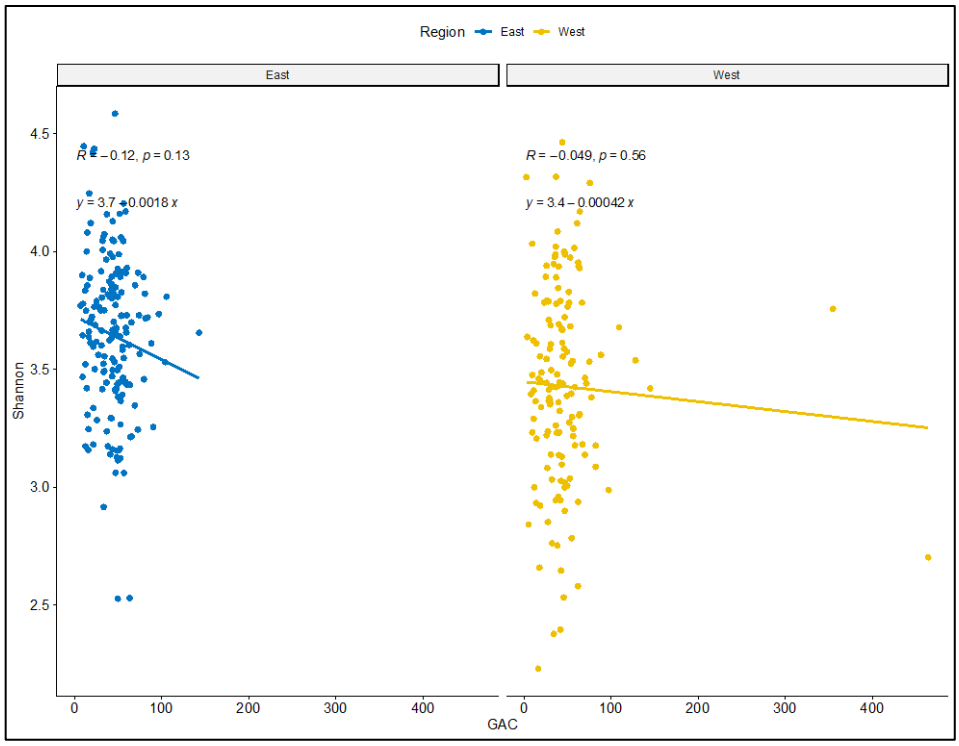


Figure 1.5. Spearman's Rank Correlation analysis between GA:C ratios and Microbial Diversity Index values from paired fecal and urine samples. (a) Shannon Diversity index values for all samples correlated to GA:C values. (b) Shannon Diversity Index values separated by east (blue) and west (yellow) correlated to GA:C values.

Table 1.1. Two-way ANOVA of Shannon Diversity Indexes by Region and Year

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Region	1	5.67	5.673***	44.174	1.37e-10
Year	5	2.49	0.497	3.874	0.00203
Region by Year	5	0.91	0.183	1.423	0.21576
Residuals	307	39.42	0.128		

Table 1.2. ANOVA of Unweighted UniFrac Distances by Region and Year

	Df	SumOfSqs	R2	F	Pr(>F)
Region	1	0.670	0.01706	5.7623	0.001 ***
Year	5	2.159	0.05495	3.7124	0.001 ***
Region by Year	5	0.750	0.01908	1.2890	0.005 **
Residual	307	35.704	0.90890		
Total	318	39.283	1.00000		

Table 1.3. Dispersion Homogeneity Unweighted UnifFrac distances by region

	Df	Sum Sq	Mean Sq	F	N.Perm	Pr(>F)
Region	1	0.00050	0.00049898	0.3582	999	0.545
Residuals	317	0.44155	0.00139290			

Table 1.4. TukeyHSD of Western Moose Unweighted Unifrac Dispersion by Year

Year Comparison	p adj
year2014-year2013	0.9810
year2015-year2013	0.9910
year2017-year2013	0.9102
year2018-year2013	0.9977
year2019-year2013	0.1577
year2015-year2014	0.9202
year2017-year2014	0.6066
year2018-year2014	0.9975
year2019-year2014	0.7727
year2017-year2015	0.9818
year2018-year2015	0.9539
year2019-year2015	0.0617
year2018-year2017	0.4564
year2019-year2017	0.0045
year2019-year2018	0.1240

Table 1.5. TukeyHSD of Eastern Moose Unweighted Unifrac Dispersion by Year

Year Comparison	p adj
year2014-year2013	0.9922
year2015-year2013	0.2586
year2017-year2013	0.2470
year2018-year2013	0.6627
year2019-year2013	0.3426
year2015-year2014	0.4960
year2017-year2014	0.5787
year2018-year2014	0.9274
year2019-year2014	0.7021
year2017-year2015	0.9835
year2018-year2015	0.9181
year2019-year2015	0.9650
year2018-year2017	0.9951
year2019-year2017	0.9999
year2019-year2018	0.9993

CHAPTER TWO: LAB-BASED UNDERGRADUATE RESEARCH EXPERIENCES: A VIRTUAL APPROACH TO "LURE" UNDERGRADUATES INTO WILDLIFE RESEARCH

Abstract

The classrooms' transition toward an online platform in the midst of a global pandemic identified a substantial gap in availability of incorporating research with remote interactive learning opportunities within the lab components of undergraduate science courses. In an effort to both enhance the student experience amidst the transition to online lab-based courses and connect undergraduates to active research on campus, three remote Lab-Based Undergraduate Research Experiences (LUREs) were developed. Modules centered around the analysis of mammalian gut microbial data allowed students to understand the relevance of toxin and nutrient absorbance in animals and understand how the environment and morphology can influence animal physiology. LUREs demonstrate how a diverse workforce of classroom scientists can be used to analyze data to monitor the health of wildlife, generate and test novel hypotheses, and share results with the broader scientific community. LUREs can provide students with the confidence to identify themselves as capable scientific researchers and thereby increase the recruitment and retention of a more diverse generation of wildlife researchers. Our LUREs demonstrated the capacity to generate a broad range of students with an arsenal of knowledge and research skills to think critically about science, wildlife populations, management agencies, and add meaningful contributions to active research studies.

Introduction

In the education of physical and natural sciences, the laboratory plays an important role by allowing students to dive into concepts learned in lecture and develop a better understanding through critical thinking and experimentation (Feig, 2010). Students are motivated to learn when provided the opportunity to employ concepts themselves and through the implementation of experiments. The lab component of the course not only allows for the development of technical skills, but also more broadly applicable skills such as communication and collaboration (Woods et al., 2000). Although there has been extensive research aimed at improving online courses in higher education, there has been less focus toward lab-based experiences. Of the abundant research advice for creating an effective learning environment in online lecture courses, a standout theme is active and visible engagement with students (Faulconer & Gruss, 2018).

Over the last decade, research experience has become an unspoken necessary requirement in gaining access to professional STEM careers. This is especially true in the field of wildlife biology, where professionals have voiced concern that students lack the skills necessary to address real-world scientific issues (Millspaugh & Millenbah, 2004). The Course-based undergraduate research experience (CURE) learning system has been shown to be highly efficacious toward enriching research-related skills, increasing understanding of the process of scientific discovery, and enhancing interest in STEM careers (Denofrio et al., 2007; Gentile, 2017). Implementation in several introductory science courses has revealed an advantage of CUREs when compared to traditional structured research experiences. Specifically,CUREs allow a wider range of students to gain research experience by moving authentic research into a teaching laboratory as part

of a required or elective course (Flaherty et al., 2017; Linn et al., 2015). Through CUREs, students are able to gain valuable skills which many professional STEM careers require from those practicing in the field (Figure 1).

While implementation of CUREs has several advantages, a major barrier identified has been the lack of time for faulty to develop in-class research experiences (Spell et al., 2014). Graduate teaching assistants (GTAs) can play a vital role in the development and implementation of CUREs, or in this case, shorter Lab-based undergraduate research experiences (LUREs) offered as modules within a course. GTAs offer sustainable mechanisms by which to deliver and revise LUREs sinceURE many institutions tend to appoint GTAs as laboratory instructors in STEM courses and GTAs constitute 50% of all instuctors at research universities (Baldwin & Wawrzynski, 2011). Research on -CUREs is primarily focused on the student impact while the impact on laboratory instructors and the potential benefit of CUREs and LUREs for GTAs has been widely overlooked (Brownell & Kloser, 2015; Gormally et al., 2009; Howard & Miskowski, 2005). GTAs are tasked with balancing the roles of a student and scientific researcher while meeting the time and effort requirementsassociated with a teaching assitantships. Not only does tasking GTAs to develop LUREs or CUREs that incoporate their research establish pedagogical training that graduate students will use throughout their programs, but it also helps to develop the professional skills necessary to communicate scientific research. The fusion of graduate research work into the teaching labs of required or elective science courses gives GTAs access to a larger pool of diverse student researchers who participate in data collection/analysis, can help to develop new hypotheses, and have the potential to become long-term collaborative researchers.

When a global pandemic limited on-campus, in-person operations as students and faculty were asked to remain home, it created a unique opportunity to transform the laboratory experience in upper division science courses while also progressing the work of active research studies. The purpose of this project was to create remote opportunities for authentic research by converting ongoing summer remote research into lab modules that coulde be delivered remotely while under national health restrictions in an effort to increase the accessibility of research and education. The goal was to provide opportunity for students to participate in reproducible scientific work that is of interest to stakeholders outside the classroom and helps to shape new research questions or directions. This project demonstrates how interactive virtual learning experiences in the form of LUREs can engage undergraduate students while strengthening educational outcomes through increased accessibility of research that increases workforce diversity, results in students with valuable workforce skills, and leads to professional development for GTAs.

Methods

To reach project goals, research-funded graduate students who would become GTAs the following semester were chosen to develop lab modules that incorporated their ongoing remote research work. As a result, three remote lab moules were developed and delered in two separate upper division biology courses in the Fall 2020 (Zoology 409 Animal Physiology and Nutrition) and Spring 2021 (Zoology 421 Mammalogy) academic semesters at Boise State University.

Morphology of Vertebrates LURE

Zoology 409: Animal Physiology and Nutrition is a 4-credit Finishing Foundations course typically taken by seniors and juniors that addresses the physiological

principles common to all forms of animal life with a focus on nutrition. In the Fall of 2020, a total of 24 students were enrolled in the course within a single lab section. In years past, the first lab module of the semester consisted of an in-person dissection of an avian specimen. Students carried out a protocol and took measurements of anatomic parts of interest to understand the link between morphology and physiology. Afterward, students used lecture material and the measurements taken to answer questions about the dissection. When the pandemic hit, in-person participation was limited and there were a subset of students who were enrolled in the course remotely. To adapt to circumstances, I performed and recorded dissection on campus and used the Panopto Video Editing Software (http://www.panopto.com) to create an interactive video with embedded quiz questions and accompanying slides. Students were tasked with watching and answering questions throughout the video and then using morphological measurements I took to answer post-lab discussion questions (Figure 2). Thus, students who were unable to perform in-person dissections were able to participate in a dissection experience while also meeting the learning objectives of the lab.

Bioinformatics LURE

In the same ZOOL 409 course, an additional lab module was adapted to be taught remotely. Pre-COVID, the lab module trained students how to quantify and compare toxin absorbance by mammals. This lab module required access to scientific equipment and materials not feasible for a remote student in the course to perform remotely. Instead, students gained knowledge, skills and abilities in basic bioinformatics in one module and then put those skills into practice in a second module where they analyzed metagenomic

data from the gut microbial community of moose (Chapter 1) using a bioinformatic pipeline based in the R coding language software.

For the bioinformatics lab module, students were provided with a command script, dataset, a Power Point presentation, and a tutorial video I created. Students were instructed to mirror and follow along with me in the video as I executed the commands on my own machine. The online tutorial allowed for flexibility in the pace and allowed students to repeat sections of the video as needed. Throughout the tutorial videos, I lectured about the subject matter as I performed the analysis. Then, upon completion of the tutorial with me, students were given a separate dataset and tasked with implementing the same analysis learned in the tutorial to the new dataset. Students provided a post-lab report which included the code they used for analysis and answered discussion questions about the datasets.

Microbial Community Analysis LURE

The lab module on analysis of mammalian gut microbiome data was adapted from my graduate research. Students were taught to analyze the fecal microbiome data from 326 moose (*Alces alces*) to compare regional and temporal variation in microbial communities. Students were given a lecture on the origins of the data set, the research questions being studied, the relevance of the study system and then taught to perform data analysis as well as pursue analysis questions I had yet to address. The diversity analysis protocol taught to students was adapted from the protocol used by Frankel-Bricker et al., 2020.

Zoology 421: Mammalogy is a 4-credit elective course typically taken by seniors and juniors focused on the ecology, life histories, reproduction, classification,

identification, distribution, and adaptations of mammals. In the Spring of 2021, a total of 26 students were enrolled in the course with a singular lab section. In a relatively similar fashion of delivery as in ZOOL 409, students performed a two-week lab module. In the first week, they used the Bioinformatics LURE to learn the R programing language and performed basic data analysis on a small data set where they calculated the summary statistics and created a histogram. Then in the second week, students used the Microbial Community LURE to analyze metagenomic data in R where they performed statistical testing for microbial diversity, created figures for both alpha and beta diversity results, and identified abundant bacterial families in the sample data.

These modules met objectives for both lab courses as the analysis of mammalian gut microbial data allowed students to understand the relevance of toxin and nutrient absorbance in animals and understand how the environment, morphology and extended phenotype of host-associated microbes can influence animal physiology.

Discussion

By watching and participating in an interactive chukar bird dissection video session, students were able to understand the relevancy of understanding morphology and physiology of game birds as part of a collaboration with hunters and state agencies to better monitor and manage natural resources. Students also gained scientific skills in virtual, as well as linking organ measurements that will prepare them for careers in STEM fields. Finally, they used digital images of morphology coupled with metadata on location where the bird was taken, body size, and diet composition to discover interactions between the environment and physiological function of animals. In this way,

students are able to meet lab objectives in the setting of remote learning and educational accessibility was expanded.

The virtual bioinformatics lab modules provided the unique opportunity for students to build computational skills via the R programming language, which is relevant both in professional workforce development and postgraduate education. Students were able to leverage knowledge of collaborative research projects on the gut microbiome of herbivores to discover patterns of microbial community composition and structure to understand the physiology of mammals. By demonstrating the ability to perform basic analysis of microbiome data, students were more engaged in their lab work as they developed increased self-efficacy as scientists. Self-efficacy is also more likely to retain undergraduates as long-term researchers thereby diversifying the scientific workforce (Lopatto, 2007; M. Mataka & Grunert Kowalske, 2015; Swan et al., 2018). The virtual component of the lab module leads to increased accessibility and reproducibility in undergraduate lab-based science courses, as any student with access to a computer with R installed (free software), can download the tutorial script and dataset, and follow along the video to learn and reproduce the same results (Table 1).

All three lab modules demonstrate the successful adaptation of current practices in lab-based science course for remote learning without sacrificing the educational experience for the student. Historically, opportunities for undergraduates to participate in research has involved competition for limited positions in labs which is often hindered by the social complexities of students who may not identify themselves capable of scientific research. Incorporating graduate student research into elective or required lab-based science courses gives access to a larger proportion of undergraduates who otherwise

might never have engaged in research on campus, and thereby creates a more inclusive and diverse pool of undergraduate research participation.

Figures

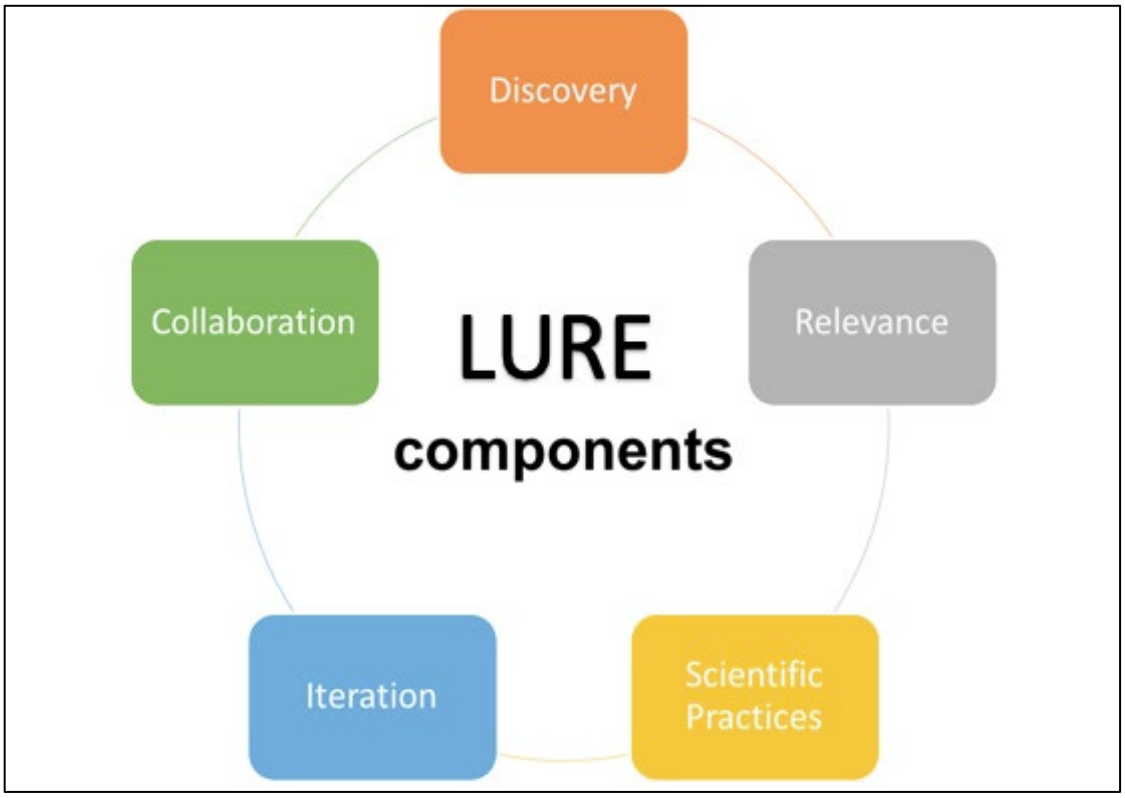


Figure 2.1. The components of a Lab-Based Undergraduate Research Experience (LURE) where students gain authentic research experiences that are Relevant to employers in the field, train them in real Scientific Practices, are Iterative because digital results are archived and can be revised and reanalyzed, represent Collaboration with graduate students or stakeholders and lead to Discovery of new information due to inclusion of a large cohort of undergraduates.

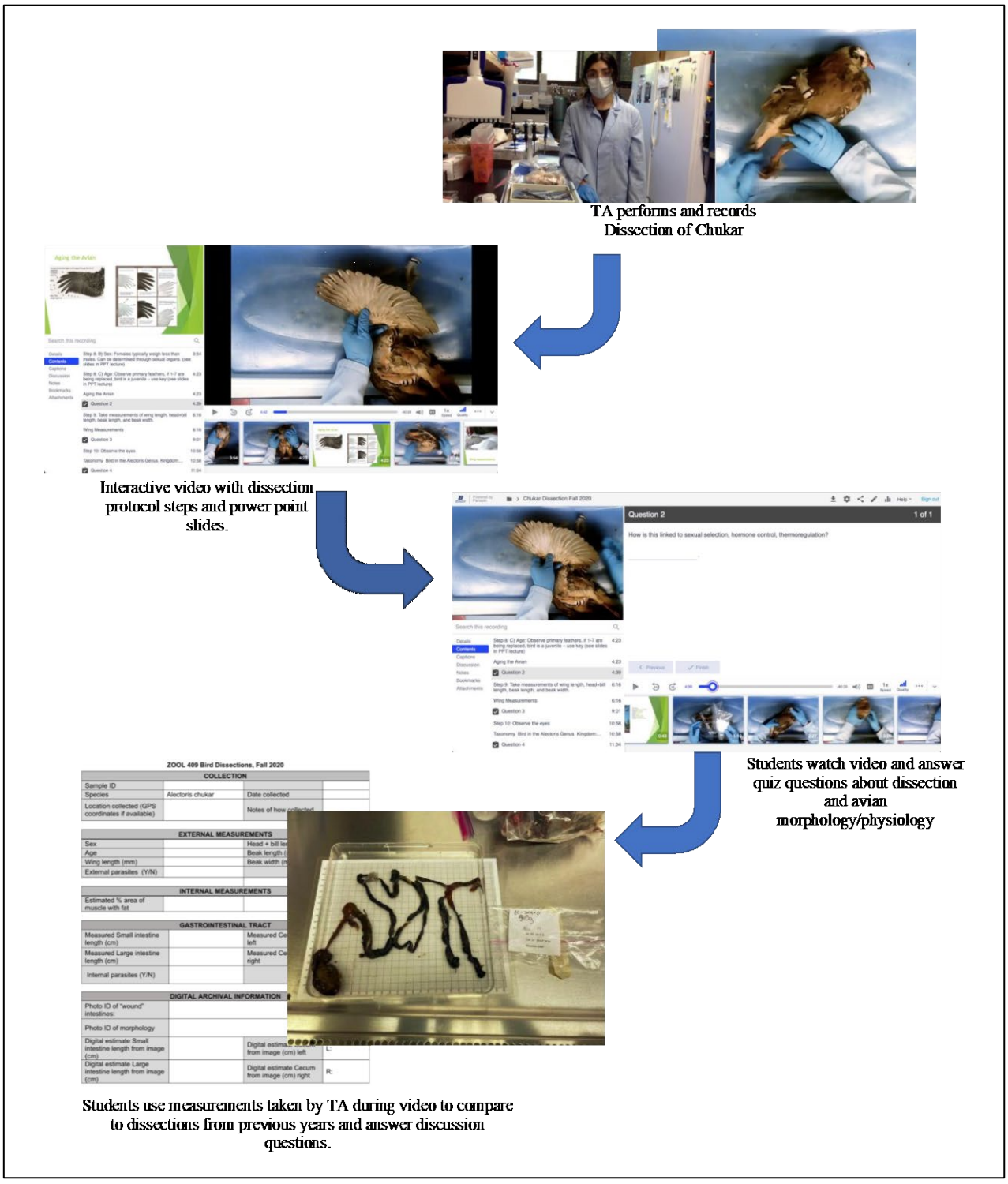


Figure 2.2. Example of a Lab-Based Undergraduate Research Experience (LURE) workflow from the perspective of the GTA.

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APPENDIX A

Alpha diversity analysis of moose (*alces alces*) fecal samples

Alpha diversity analysis of moose (*alces alces*) fecal samples

The QIIME2 "diversity core-metrics-phylogenetic" command was used to calculate a series of diversity metrics, including several alpha and beta diversity metrics. Rarefaction analysis was also carried using the used the command "diversity alpha-rarefaction" to confirm sufficient sequencing depth. QIIME 2 core-metrics creates several alpha diversity metrics: observed ASVs (bacterial community richness), Shannon's index (bacterial community richness and evenness), Faith's phylogenetic diversity (PD) (bacterial community richness that incorporates phylogenetic relationships between taxa), and Pielou's species evenness (bacterial community even- ness) (Lozupone & Knight, 2005). Alpha diversity refers to metrics of diversity within a sample which includes the total number of species (richness) and how evenly distributed the members of a community are among the species present (evenness). We calculated alpha diversity using the Shannon index (a metric of both evenness and richness) with data rarefied to 5,900 sequences per sample. We used a two-way ANOVA to determine the significance of differences in alpha diversity when grouped by region as well as by year.

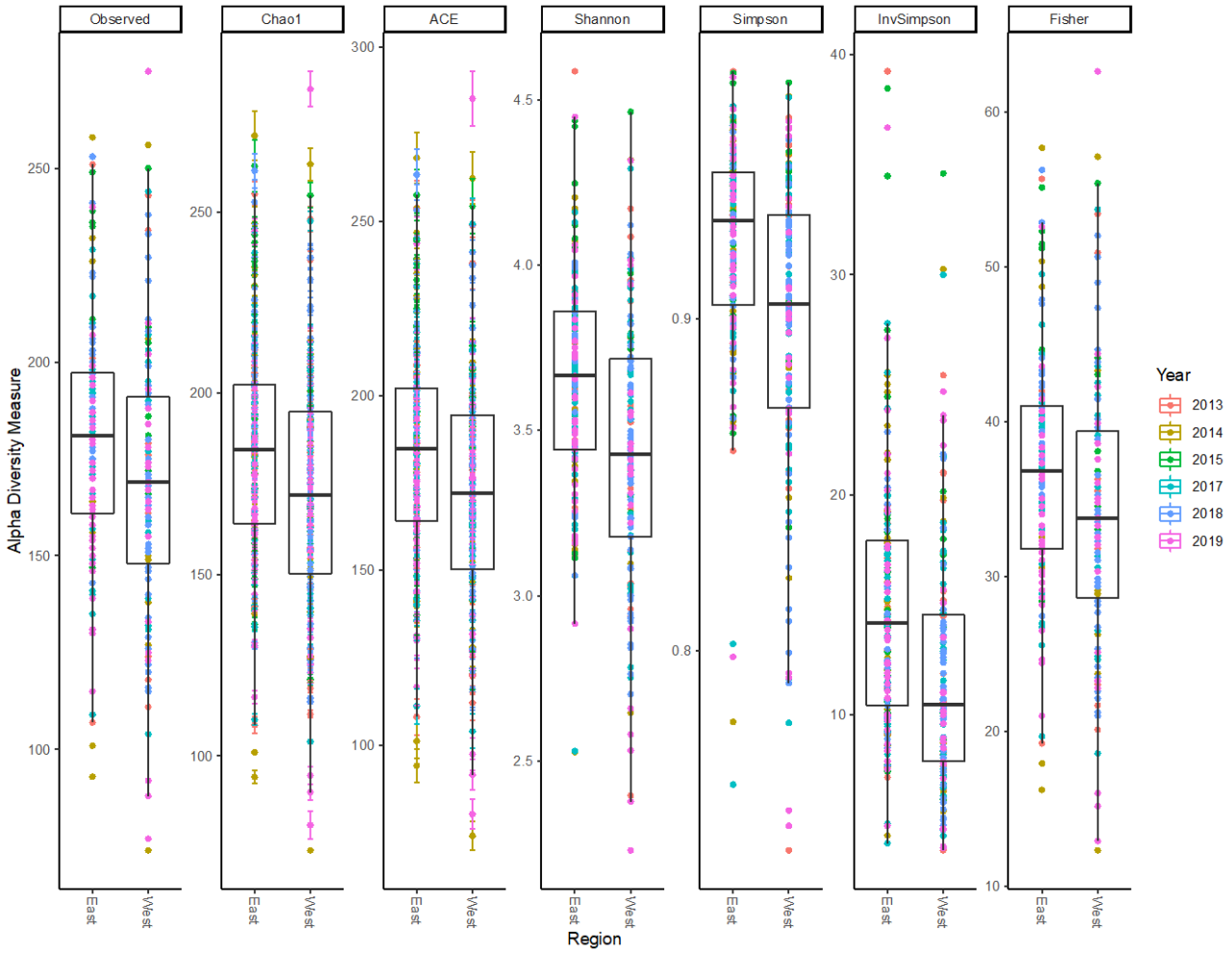

Figure A1.1. Box plots of α-diversity separated by region and year; bacterial abundance and species richness assessed via Observed ASVs, Chao1 Index, ACE Index, Shannon Index, Simpson Index, Inverse Simpson Index, and Fisher Index. Moose on the east have higher values of diversity in every index indicating increased diversity when compared to moose in the west.

APPENDIX B

Beta diversity analysis of moose (*alces alces*) fecal samples

Beta diversity analysis of moose (*alces alces*) fecal samples

Beta diversity refers to the biological diversity among environments or along a gradient and is a measure of the similarity or dissimilarity between bacterial communities (Lozupone Catherine A. et al., 2007). Beta diversity was calculated using unweighted UniFrac phylogenetic distances which consider species' presence and absence information and by comparing the length of phylogentic branching (Lozupone & Knight, 2005). To compare the dissimilarities of the bacterial community structures in the moose samples from eastern vs. western regions, a principal coordinate analysis (PCoA) (Lozupone & Knight, 2005) was performed based on unweighted Unifrac metric distances (Figure 2). Variation in community composition between years and region was quantified by measuring the distance to the centroid of east and west subgroups in each year (Elith et al., 2006), using the betadisper function in the vegan package (Oksanen et al. 2015) (Figure 3). Statistical significance of dissimilarity in distances to centroids between regions and over time was assessed using an ANOVA.

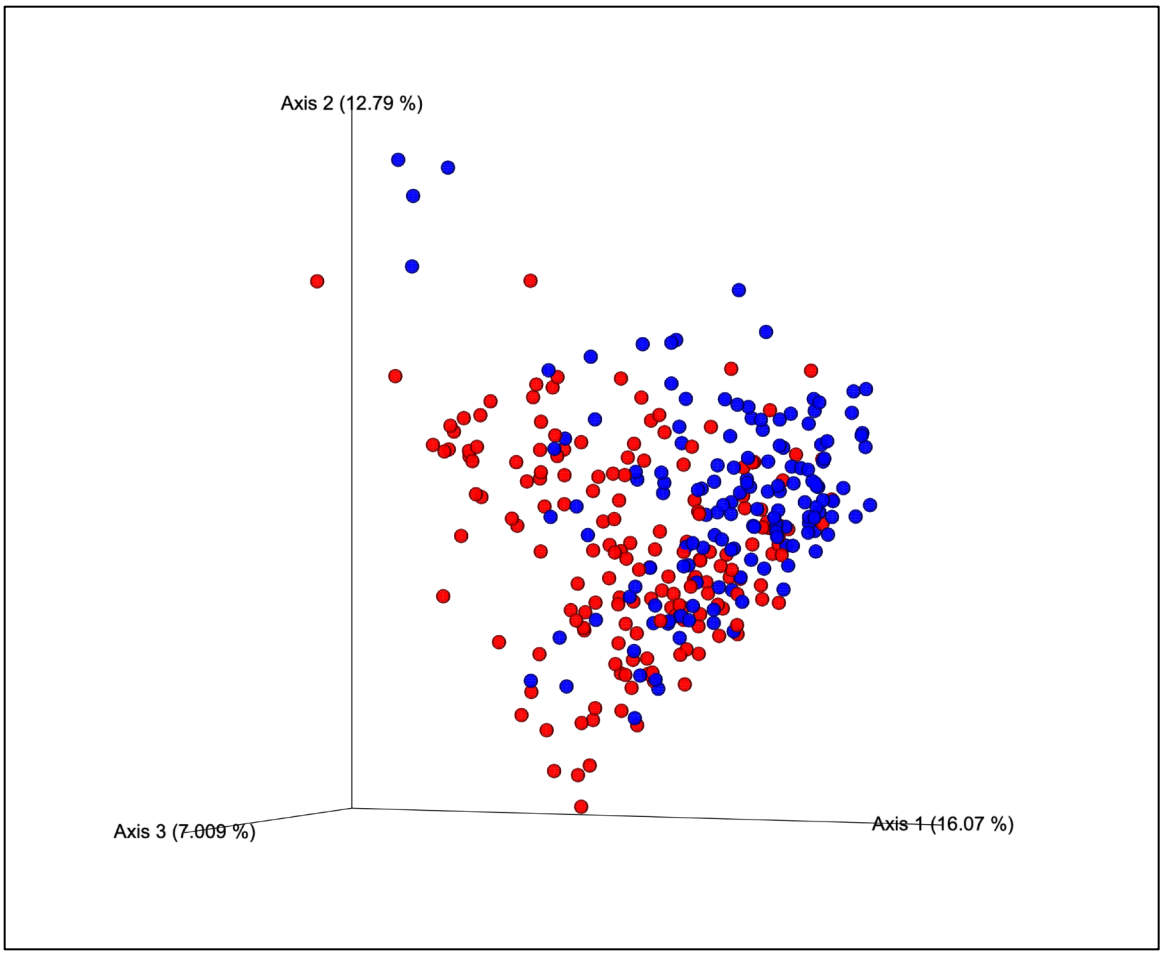

Figure B1.1 3D PCoA ordination of differences in β-diversity; Bray-Curtis dissimilarities when grouped regionally by east (red circles) and west (blue circles); Bray-Curtis diversity index showed significant differences in beta diversity when grouped by region (PERMANOVA; F-value 26.406, p=0.001). Additionally, there was significant interaction between region and year (PERMANOVA; F-value 2.130, p=0.001)

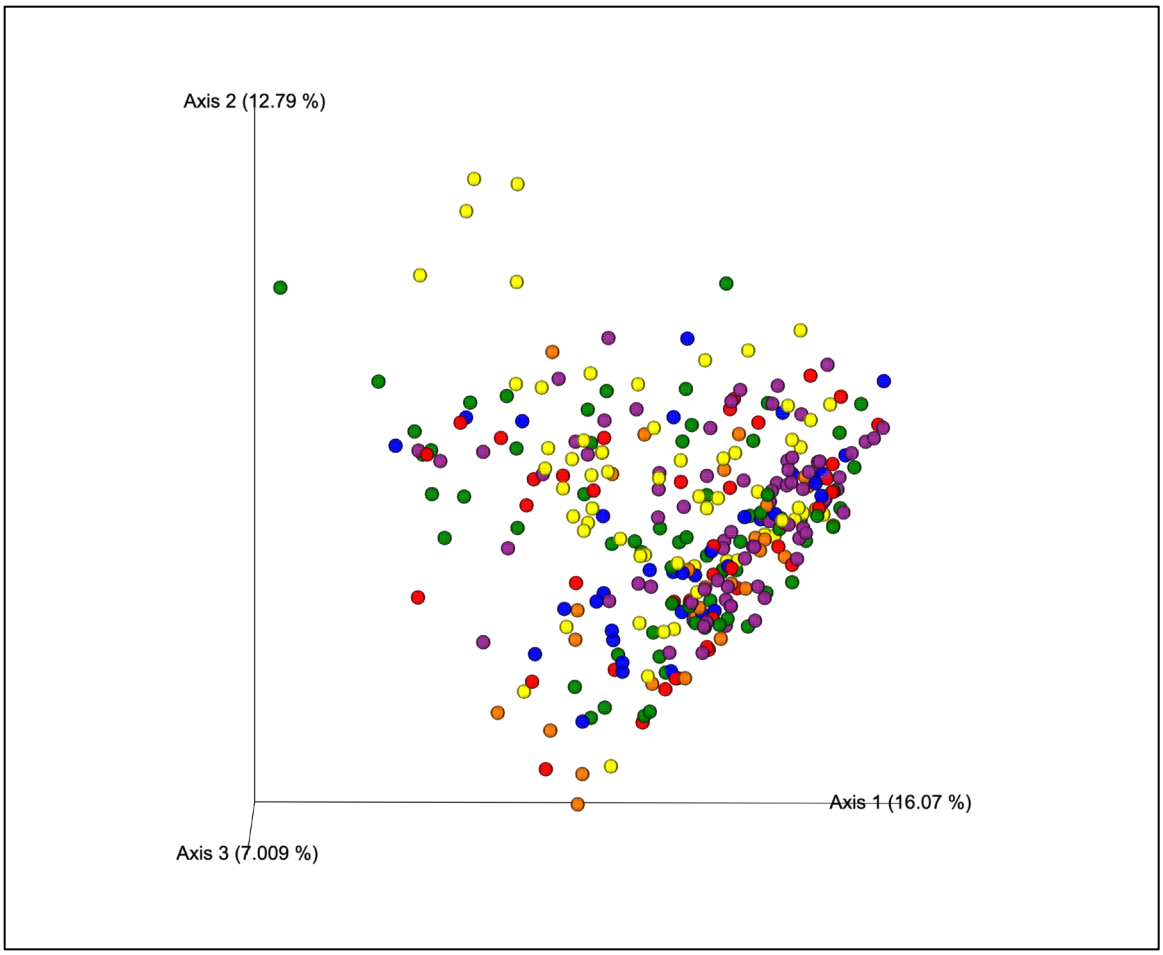

Figure B1.2. 3D PCoA ordination of differences in β-diversity; Bray-Curtis dissimilarities when grouped by year. Red (2013), Blue (2014), Orange (2015), Green (2017), Purple (2018), and Yellow (2019). Bray-Curtis diversity index showed significant differences in beta diversity when grouped by year (PERMANOVA; F-value 4.106, p=0.001). Additionally, there was significant interaction between region and year (PERMANOVA; F-value 2.130, p=0.001)

Table B1.1. ANOVA of Bray-Curtis Distances by Region and Year

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
location	1	2.28272	2.28272	26.405911	0.072427	0.001
year_text	5	1.77468	0.354936	4.105808	0.056308	0.001
location:year_text	5	0.920753	0.184151	2.130207	0.029214	0.001
Residuals	307	26.539323	0.086447	NaN	0.842051	NaN
Total	318	31.517477	NaN	NaN	1	NaN

Table B1.2. Dispersion Homogeneity Bray-Curtis distances by region

method name	PERMDISP
test statistic name	F-value
sample size	319
number of groups	2
test statistic	15.1686
p-value	0.001
number of permutations	999