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Close Quarters: Seasonal Cestode-microbe Interactions Within the Guts of Two North American Sorex Shrew Species

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CLOSE QUARTERS: SEASONAL CESTODE-MICROBE INTERACTIONS WITHIN THE GUTS OF TWO NORTH AMERICAN *SOREX* SHREW SPECIES

by

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(Under the Direction of Stephen Greiman)

ABSTRACT

The mammalian gut microbiome has shown to be essential to host fitness and health. It assists in both nutrient acquisition from diet, as well as, protection from pathogens and can include beneficial bacteria, as well as parasites such as helminths and protozoans. In this study, I analyzed the cestode and bacterial communities found in the intestines of two North American species of shrew, *Sorex monticola* and *Sorex cinereus*. Specimens were collected approximately every three weeks from May to October during 2016 and 2017 as well as during April and May of 2018 and September of 2009 from the Sangre de Cristo Mountains in Cowles, New Mexico. A total of 186 shrews were dissected and surveyed for cestode and bacterial taxa. I extracted DNA from the whole GI tract of each specimen and amplified 2 loci (28S rRNA for cestodes and 16S rRNA for bacteria) using one-step PCR amplification and sequencing on an Illumina MiSeq. Collection month, shrew species, sex, and weight class all significantly influenced the gut microbiome communities in *S. cinereus* and *S. monticola,* however some of the effect of collection month and shrew species on community composition is likely due to variances in within-group distributions. Cestode genera, *Mathevolepis* and *Monocercus,* both significantly altered the bacterial community composition of their hosts, but further analysis could not be conducted controlling for the presence of one or the other to parse out any confounding factors due to coinfection. This study provides novel descriptions of cestode taxa infecting *S. monticola* and provides a first survey of cestodes infecting shrews in New Mexico.

INDEX WORDS: Shrews, Gastrointestinal tract, Seasonality, Tapeworms, Cestodes, Intestinal microbiome, Helminth diversity

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CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

Microbiomes, communities of microorganisms that inhabit multicellular organisms, have been shown to directly influence host health and fitness (Turnbaugh et al. 2007, Nayfach et al. 2015). The microbiota of an organism includes communities of microorganisms colonizing the skin, gut, mouth, and genitals that are primarily composed of bacteria, but include Archaea, fungi, viruses, and protozoa (Glenndening et al., 2014). The presence of these symbionts in the gut serves a nutritional role that heightens the ability of the host to acquire energy and nutrients from its diet and is required for priming the development of immune response as well as the maintenance of the host immune homeostasis (Tremaroli et al., 2012, Brosschot 2018). Bacterial microbiota manage defense from colonization by undesired microbes by either generating toxins or outcompeting potential pathogens for resources such as available nutrients or space. Some taxa inadvertently provide host protection by supporting intestinal barrier function (Brusschot 2018). Although it is necessary for organisms to maintain a healthy, balanced gut flora, some studies have shown that not all populations within a microbiome may be favorable. Metabolic disorders, autoimmune diseases, and allergies have been shown to be correlated with alteration in microbial community composition (Backhed et al. 2012).

The evolutionary history of the mammalian microbiome displays patterns of co-phylogeny showing diversification parallel to the evolution and divergence of mammalian host species. In a study by Brooks et al (2016), four model taxa: deer mice, flies, mosquitoes, and wasps, were utilized to determine if microbial community assembly is random or displays community structures indicative of phylosymbiosis (congruency of the phylogeny of host group and microbiota). They

found that not only do hosts and microbiota undergo coevolution, transplanting microbial communities between taxa resulted in reduced ability to digest food in mice and lower survival rates in wasps (Brooks et al 2016).

1.2 SOURCES OF MICROBIOME DIVERSITY

A study by Nishida and Ochman (2018) investigated the different contributors to the fluctuation and diversification of mammalian gut microbiomes as well as the variabilities in rates and patterns of divergence in microbial communities. They collated the gut microbiota of 112 species representative of 14 of the 19 orders within Mammalia in order to assess the interactions of host and ecological elements that promote the diversification of the gut microbiome. They found that, largely, the same phyla dominate the gut microbiota of mammals with convergence of microbiota composition corresponding to category of diet observed across mammalian lineages. They also found that bacteria display a higher correlation with individual mammalian lineages than with diet type, further supporting other hypotheses of co-phylogeny. In spite of differences in physiology, the microbiota of many lineages of mammals have diverged at similar rates during the last 75 million years. The researchers found that rates of divergence of microbiota diversity are not influenced by the amount of dietary changes within a lineage, but instead the most drastic alterations are associated with the loss of bacterial taxa coinciding with major evolutionary events, such as, mammals transitioning from terrestrial to marine environments (Nishida and Ochman 2018).

There are competing hypotheses concerning the primary predictor of microbiota community structure in vertebrates and the degree of heritability versus environmental influence on gut microbe diversity (Moeller et al. 2018, Springer et al. 2017, Rothschild et al. 2018). A study by Moeller et al. (2018) found that the gut flora of mice is, largely, a heritable trait that changes very little across multiple generations. The researchers in this study gathered wild house mice, established 11 generations of inbred lines, and housed them in separate cages to prevent as much

horizontal transfer of bacteria as possible. After sequencing the microbiomes of the final generations of each inbred line, they found that most of the microbiome diversity was vertically transmitted. The flora of the last generations of mice most closely resembled that of their wild ancestors as opposed to other lines that shared the same lab environment and diet (Moeller et al. 2018). Other studies have suggested that one element of the heritability of microbiomes could simply be genetic susceptibility to colonization by specific microbial taxa (Springer et al. 2017). The element of the ecology of gut microbiota in mammals that is the most heritable is the environment provided by the host: the gut. Taxa of gut microbiomes share a phylosymbiotic relationship that has undergone co-evolution with their hosts.

1.3 SEASONALITY AND THE GUT MICROBIOME

Microbial community diversity within individuals is significantly dependent on environmental factors such as diet. Therefore, changes in environment and diet should result in changes in microbial community composition. Variations in microbiota between individuals of the same species and population have been attributed to age, sex, and seasonality (Springer et al. 2017). Several studies have shown that the gut microbiomes of organisms undergo seasonal changes influenced by changes in diet. A study by Bergmann et al. (2015) looked at whether or not seasonal patterns in diet elicit corresponding changes in gut microbial community composition in American bison. They collected fecal samples from the bison to track any changes in diet and gut flora from April to September, and they identified correlations between major bacteria phylum and primary dietary elements as well as changes in gut microbiota diversity that correspond to variations in the bison diet attributed to seasonality (Bergmann et al. 2015).

1.4 HELMINTH INTERACTIONS IN THE MAMMALIAN GUT

Another influencer of gut microbiome diversity is intestinal parasites, notably, the helminths (cestodes, trematodes, nematodes). These eukaryotic, multi-cellular organisms are known to utilize a diverse assemblage of hosts. Many species of helminths, including those that parasitize humans, are transmitted through the consumption of intermediate hosts such as fish, invertebrates,

other vertebrates, as well as, contact with soil. A number of factors could influence the relationship between helminths and the gut microbiota such as physiological changes of the host attributed to age, changes in diet, and the effects of helminths on the immune response of the host (Glendinning et al. 2014). A number of studies have shown seasonal variation in diversity of gut infecting helminths. One study by Salinas-Ramos et al. (2017) characterized the gastro-intestinal helminth load of three species of bats in order to test for variations attributed to environmental and bat prey fluctuations occurring seasonally. They observed no significant seasonal variation in the diversity of intestinal helminths. The researchers did, however, notice significant changes in relative abundance of four of the helminth taxa present in the bat guts. It is likely that the changes in abundance coincide with the availability of the insect intermediate host that varies seasonally (Salinas-Ramos et al. 2017).

Intestinal parasites occupy the same location within the host as the gut flora responsible for countless functions within multicellular organisms. It is likely that organisms that share such close quarters influence each other in some way, and it is possible that the interactions between helminths and the bacterial microbiome influence host health. Several studies have investigated the potential effects of one on the other. A study by Reynolds et al. (2015), aimed at using helminth excretory-secretory products to treat allergic diseases, revealed that gut helminths are capable of altering microbial community composition as one of the many methods used by helminths to modulate host immunity. They found that mice infected with Hpb (*Heligmosomoides polygrys bakeri*) experienced an increase in the abundance of bacteria belonging to Clostridiales compared to those that were uninfected. It is possible that persistent helminth infections affect the susceptibility of the host to overgrowth of bacteria within the gut, as helminth infection has been observed to negatively impact immunity to coinfection by other pathogens such as microbial parasites and bacteria (Reynolds et al. 2015).

1.5 STUDY SYSTEM

Substantial work relating the gut microbiome to helminth infections within humans has been done; however, a significant knowledge gap exists in regard to research conducted concerning helminths and microbiomes within wild systems of small mammals. Small mammals, such as shrews, and their parasites provide insights into the ecological and evolutionary processes that govern dynamics of terrestrial systems, and further investigations of these processes could have implications for conservation and medicine (Hope et al. 2016). In this study, I investigated the impacts of seasonal/temporal factors on the gut microbiome and helminth community of two sympatric shrew species: *Sorex monticola* and *Sorex cinereus.*

Sorex monticola is native to North America, and its range spans from the north of Mexico all the way to northern Alaska. This shrew is primarily insectivorous, but occasionally eats plant material such as seeds as well as lichens and fungi. The shrew inhabits boreal forests as well as mountainous habitats (Smith 1996). *Sorex cinereus* is also a native of North America and is more widely distributed than *S. monticola*. Its habitat ranges from Canada to the mountains of Appalachia, and its diet is similar to that of *S. monticola* (Whitaker Jr et al. 2004). Both *S. monticola* and *S. cinereus* share similar life history strategies. Shrews are short lived, and these shrews breed roughly the same time of year with most litters being born in the spring with the potential to birth multiple litters across their lifespan of approximately a year. These organisms have a fast metabolism and require calorie dense diets. They have to be eating almost constantly and are capable of dying of starvation in a couple of hours (Smith 1996, Whitaker Jr et al. 2004).

This study focused on native bacteria and parasitic cestode (tapeworm) communities within two *Sorex* species and the interactions between these communities in the shrew gut. Microbiome diversity is correlated with diet, and the microbiota of some organisms change seasonally due to dietary variation. In some cases, this dietary variation influences the ability of the host organism to respond to drastic season-driven environmental change (Amato et al. 2015, Krahicich et al. 2018). Sequencing of microbiota from shrews has enabled the novel description of microbial diversity within these small mammals, as well as provide an important baseline for future studies looking at

changes over space and time. Season-driven environmental changes are predicted to increase in the coming years as a direct result of anthropogenic environmental perturbations (Urban et al. 2016).

North American shrews host diverse communities of helminth parasites totaling more than 97 species including 9 trematodes, 34 cestodes, 50 nematodes, and 4 acanthocephalans (Kinsella & Tkach 2009). This work focused specifically on cestode diversity. Cestodes, tapeworms, are segmented flatworms belonging to the class Cestoda which contains over 6,000 species of parasitic worms (Ruppert et al. 2004). The lifecycles of tapeworms are complex and often differ slightly between species, but they largely follow the same life history strategy. First, the eggs of a tapeworm are ingested by an intermediate host (where a parasite grows but doesn't reach sexual maturity) such as an arthropod where the eggs develop into cysticercoids. The arthropod containing the cysticercoid is ingested by a definitive host (the host where the cestode reaches sexual maturity), after which the cysticercoid matures into an adult worm in the small intestine of the host. The adult worm produces gravid proglottids which release eggs that are passed in the stool of the definitive host.

The life cycles of helminth parasites that utilize mammalian hosts are closely tied to the conditions of their environments, and accelerating environmental change is a looming threat to species distribution that could lead to expansion or contraction of the ranges of hosts and parasites (Hope et al. 2016). It is possible that helminth diversity within shrews could directly influence microbe diversity. This study could provide insight into that relationship as well as allow for the generalization of the microbe/parasite relationship within other organisms.

Although shrews do not experience a seasonal change from arthropod to seed based diets, it is possible that they experience changes in the diversity of cestodes due to the change in seasonal abundance of their arthropod intermediate hosts. Seasonal variation in soil arthropods was observed in a forest-steppe ecotone in Northern Hebei, China (Zhu et al. 2010). Most of the variation observed as associated with changes in average air temperature and precipitation. Another study

that took place in 2013 was conducted in a desertified steppe habitat. They observed a significant decline in community indices of ground arthropods from spring through summer largely attributed to abiotic conditions (Liu et al. 2013).

1.6 HYPOTHESES

This work builds off of the following 2 questions related to the intestinal microbiome and helminth community composition of shrews. 1.) How do seasonal (monthly, yearly, etc.) factors effect the composition of the intestinal microbiome and cestode communities of shrews? and 2.) How do shrews differ in microbiome diversity, cestode species richness, and cestode prevalence when compared between shrew species, sex, and weight class? A total of eleven hypotheses were tested and divided into two main categories: 1.) Gut microbiome diversity including collection period, locality, host species, host sex, host weight group, cestode species richness, and 2.) cestode species richness and prevalence including collection period, locality, host species, host sex, and host weight group.

1.6.1 INTESTINAL CESTODE SPECIES RICHNESS AND PREVALENCE

1. *Collection Period:* The cestodes surveyed in this study utilize shrews as definitive hosts. Shrews become infected primarily through their arthropod based diet, as these insects act as intermediate hosts. Community composition of these arthropods shift throughout the year, related mostly to abiotic factors (precipitation, temperature, etc.). Therefore, it is expected that cestode species richness changes over time (weekly, monthly, annually). Specifically, helminth species richness increases as seasonality progresses.

2. *Locality*. Due to the close proximity of the sites to each other, there are no expected differences in cestode species richness between individuals trapped at the two localities.

3. *Host Species:* As helminths tend to exhibit high levels of host specificity, there is expected variation in cestode species richness between shrew species with some cestode taxa being more prevalent in one shrew species than the other (Springer et al. 2017; Hope et al. 2016; Smith and Belk 1996).

4. *Host Sex: Sorex* spp. are almost universally infected with intestinal helminths, regardless of sex, but a recent study by Hostert et al. 2018 showed sex specific preferences for specific prey items due to body size. This difference in prey items between individuals of different sexes creates the possibility for male and female shrews to host measurably different cestode communities. Therefore, it is expected that male and female shrews have different cestode species richness as well as varying prevalence of cestode genera.

5. *Host Weight Group:* Host weight group is most closely associated with specimen age, so there is a significant expected effect of weight group on helminth diversity due to an increased opportunity to accumulate helminth taxa over time. As weight class increases, cestode species richness increases (Sherrard-Smith et al. 2015).

1.6.2 GUT MICROBIOME DIVERSITY

1. *Collection Period:* Given that shrews do not transition from a more plant-based diet to a more protein rich (insect) diet, it's expected that there will be little change in primary diet composition. However, prey species composition (insect diversity) is likely to change dramatically over the different collection periods, due mostly to changes in temperature and precipitation. This change in dietary variety as well as changes in abiotic conditions will likely lead to differences in gut microbiome communities over time.

2. *Locality:* Individuals for this study were collected from two separate sites approximately one mile apart. Due to the close proximity of the sites to each other, there are no expected differences between individuals trapped at the two localities.

3. *Host Species:* Mammals have been shown to have a somewhat conserved microbiome among taxa (Maurice et al. 2015), so there are no expected significant differences in gut microbiome diversity between the two shrew species.

4. *Host Sex:* Markle et al. (2013) demonstrated an effect of sex on microbial community structure in mice associated with hormonal differences and sex-biased immunity, therefore it is expected that there are slight differences in microbial community structure between male and female shrews.

5. *Host Weight Group:* There is precedent for differences in microbial community diversity between organisms classified as obese and those with a healthy weight as well as differences between juvenile and adult gut microbiomes, therefore a correlation between weight class in shrews and bacterial diversity is expected (Hartstra et al. 2015; Ley et al. 2005).

6. *Cestode Species Richness:* Although previous studies have observed differences between individuals infected with intestinal helminths and those without in terms of microbiome diversity (reviewed above), there is no expected variation due to the observed universal infection rate of shrews with intestinal helminths.

CHAPTER 2

METHODS

2.1 SHREW SPECIMEN COLLECTION

Shrews were collected every three weeks from May to October 2017 via snap traps and pitfall traps from two sites in Cowles, New Mexico; Jacks Creek (Lat 35.831, Long: -105.659) and Windsor creek (Lat: 35.816, Long: -105.680). The selected sites were close enough together to ensure that specimens were from the same population but far enough apart to avoid oversampling from one location. The selection of the collection sites eliminated any significant confounding variables that might have been attributed to location and population differences. Trapping was largely done overnight with the collection of specimens occurring in the early morning. Traps that were set during the day were checked every three hours to ensure minimal degradation of tissue. Collected shrews were identified as either *S. monticola* or *S. cinereus* using morphological characteristics and COI mtDNA barcode sequencing. Any living specimens were euthanized using chloroform following IACUC protocol. Collected shrews were dissected in the field, and the entire GI tract of each specimen was removed and immediately frozen in liquid nitrogen.

2.2 DNA EXTRACTION OF GUT MICROBIOMES AND HELMINTH COMMUNITIES

DNA from whole Gi tracts of shrews was DNA extracted using a ZR Fecal DNA miniPrep kit (Zymo Research, Irbine, CA) to include DNA from microbiome and helminth communities. Although gut microbes are usually surveyed using fecal samples, the whole GI tract was processed instead of fecal samples to create a complete survey of gut microbes in the *Sorex* specimens. Whole shrew guts were removed from their storage at -80 degrees C and suspended on a petri dish under a dissecting microscope. The guts were elongated and cut in half in order to evenly distribute tissue between two vials during the DNA extraction steps. To ensure that as much of the gut contents are extracted as possible without overburdening the spin columns, each half of the shrew guts were

scraped into a tube with sterile utensils and filled with tissue lysis buffer and beads used to lyse cestode and bacterial cells. The two tubes were combined further downstream upon the elution of the DNA to make one complete extracted sample. All tools and containers were soaked in a 10% bleach solution between each sample to prevent cross-contamination.

The samples were processed mostly following the ZR Fecal DNA miniPrep kit protocol with a few alterations. Once the tissue was in the bead bashing tubes, the samples were processed with a tissue homogenizer (TissueLyser II, QIAGEN, Hilden, Germany) at maximum speed for a total of 30 minutes (two segments of 15 minutes). The samples were further processed according to the DNA extraction protocol with modifications made to the steps regarding the initial sample filtration with DNA binding buffer. I used 50uL less of the tissue/ binding buffer mixture (750uL instead of 800uL) to compensate for the high concentration of tissue in the spin columns. A second modification to the extraction protocol was made during the elution step in which I added 150uL of DNA Elution Buffer instead of 100uL.

2.3 MICROBIOME AND CESTODE COMMUNITY LIBRARY PREPARATION

Before PCR library preparation could take place, the quantity of DNA extracted from each sample was measured using a using the Qubit™ dsDNA Broad Range Assay Kit (ThermoFisher Scientific, Waltham, MA) on a Qubit™ fluorometer. Samples were then diluted and standardized to 50ng/μL for library preparation. For samples with a concentration of DNA less than 50ng/ μL, twice the volume of DNA was used. The 16s rRNA gene for bacteria and the 28s rRNA gene for cestodes were amplified in triplicate via PCR total reaction volumes of 25 μL with 10.1 μL deionized water, 2.5 μL of 10X PCR Buffer, 0.5 μL dNTP's (10 mM), 2.0 μL MgCl (25 mM), 0.5 μL BSA, 2.5 μL of forward primer (10 mM), 2.5 μL of reverse primer (10 mM), 0.2 μL of AmpliTaq Gold high fidelity polymerase (5 u/mL), and 4.0 μL of template DNA. The thermocycler protocol consisted of a cover temperature of 105°C and an initial denaturation temperature of 95°C for 10 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds,

and elongation at 72°C for 1 minute. Following amplification of the target genes, libraries were pooled and normalized. PCR amplification was confirmed via gel electrophoresis, and aliquots of the pooled libraries were cleaned using 1 µL ExoSAP-IT (ThermoFisher) per aliquot to remove impurities. Following the ExoSAP-IT protocol, the product was quantified via a Broad Range Quant-iT dsDNA assay kit (ThermoFisher Scientific, Waltham, MA) and normalized to a concentration of 50 ng/µL. The libraries were then pooled by genetic locus, 16s or 28s, into 1.5mL microcentrifuge tubes. A 25 µL aliquot from each normalized pool underwent gel purification in order to remove extra primers using a Qiagen MiniElute Gel Purification Kit (Qiagen, Valencia, California). A High Sensitivity Quant-it dsDNA assay kit (ThermoFisher) was used to quantify the cleaned samples which were then diluted to 4nM and pooled across all loci in equal quantities. Libraries were then sequenced using an Illumina MiSeq sequencer through the University of Georgia Genomics and Bioinformatics Core following the protocol outlined by Greiman et al. (2018).

2.4 BIOINFORMATIC ANALYSIS

Sequence libraries were processed using Mothur software package (v 1.37.3) as described in Kozich et al. (2013) for quality control, sequence filtering and alignment, clustering, and identification of operational taxonomic units (OTUs). For data from both cestode and bacterial samples, ambiguous nucleotide base calls were removed as well as forward and reverse sequences and paired-end reads were combined by extracting sequence reads and quality scores. The command “make.contigs” was used to extract the sequence reads and create contigs from the samples. The command “screen.seqs” was used to eliminate ambiguous base calls found outside of the anticipated sequence length (Kozich et al., 2013). For this study, bacterial 16S sequences that were over 320 base pairs and cestode 28S sequences that were over 300 base pairs in length were removed. Duplicate sequences were merged and grouped using the command “unique.seqs,” and a table quantifying the number of appearances of each unique sequence in each group was created

using the command “count.seqs” (Kozich et al., 2013). The command “pcr.seqs” was used to match sequences to reference alignments working in synergy with “align.seqs” and “screen.seqs” to make certain that the sequences are all roughly the same size and that the amplicons are all accurately positioned in accordance with each other (Kozich et al. 2013).

The database, SILVA (v132), was used as a reference alignment for processing of bacterial data. The command “filter.seqs” was used following sequence alignment to eliminate areas not included in the target region where sequence overlap occurred (Kozich et al. 2013). Following alignment, “unique.seqs” was used again to ensure there were no newly created duplicate groups following the previous steps. Sequences were split into new groups in accordance with similarity to one another and sorted from most to least abundant using the command “pre.cluster” allowing for specifications to be set regarding the maximum allowed base pair difference between sequences pre assortment. In this case, two base-pair differences were allowed for bacterial data, which is a standard for microbiome studies.

Sequences that were grouped independently but were made up of bits of different sequences, known as chimeric sequences, were identified using the command “chimera.vsearch” and removed using the command “remove.seqs”. Sequences from non-target organisms and those incorrectly amplified were identified and removed using “Classify.seqs” and “remove.linage” (Kozich et al. 2013). Prior to analysis, sequences needed to be separated into operational taxonomic units, OTU’s. This was accomplished using the command “cluster.split”. Sequences were grouped and then sorted according to relative distance between neighboring OTU’s. Following sorting, a taxonomy file was generated by first using the command “make.shared” to generate number of sequences and relative abundance at each OTU and then “classify.otu” to compile the taxon information at each OTU into a file (Kozich et al. 2013). Following analysis in Mothur, OTU read counts were filtered manually and samples with less than 0.1% of the average read count when compared with the negative control were removed.

2.4.1 CESTODE BIOINFORMATIC ANALYSIS USING GENEIOUS

Single gene reference sequences from morphologically identified cestode species (Table 2.1) were assembled and aligned into reference libraries using the software Geneious (www.geneious.com). Prior to analysis ins Geneious, some processing was performed in Mothur. Contigs were created from extracted sequence reads using the command "make.contigs". The command "screen.seqs" was then used to eliminate ambiguous base calls found outside of the anticipated sequence length (Kozich et al., 2013). In Geneious, a global BLASTN search against a database constructed from reference libraries of morphologically identified cestode species was conducted to assign haplotypes. The species reads identified in Geneious were grouped by genera and reads with less than 98% identity were placed in groups titled "Unknown Hymenolepididae or "Unknown Dilepididae."

2.5 STATISTICAL ANALYSIS

Data from mothur along with associated metadata were analyzed in the statistical programs R and Calypso (v 8.84). Detailed summary files from mothur analysis and metadata files were uploaded to Calypso, where the hypothesis testing for 16s bacterial data was conducted. Following the program suggested processing for community level data, data was normalized using total sum normalization (TSS) with a square root transformation (Hellinger transformation) to transform raw data values into relative abundances (Zakrzewski et al. 2016).

To test for any differences in microbiome diversity between collection periods, a Kruskal-Wallis test was used to compare values for Shannon diversity index between groups. Kruskal-Wallis was also used to compare diversity index values between weight classes (low ($\leq$ 4.0g), medium (4.1g – 5.9g), and high ($\geq$ 6.0g)), and cestode species richness. The Wilcoxon rank sum test was used to test for any differences between collection sites for microbiome diversity as well as to test for any differences in diversity between sex and species. These same tests were conducted using relative abundance of specific bacteria phyla to test for any phylum level changes in abundance and diversity between shrew species, collection period, weight class, and cestode species

richness. An FDR, false discovery rate, value was used for microbiome hypothesis testing in order to minimize false positives instead of a regular P value or Bonferroni correction.

Because the data collection method for cestode diversity relied on molecular identification instead of visual confirmation, only presence absence data was available. While the microbiome alpha diversity analysis used the Shannon diversity index, the cestode community analysis relied on species richness, the number of different cestode species/ genera infecting an individual shrew as a measure of diversity. Kruskal-Wallis tests were used to compare cestode species richness between collection period and weight class. Wilcoxon rank sum tests were used to compare cestode species richness between shrews of different species and sex. To test for any differences in prevalence of cestode genera between shrew species, a chi-squared test was conducted for each cestode genus.

For measurements of beta diversity, an ADONIS multivariate analysis (analogous to PERMANOVA) was used to test community level differences in gut microbiome diversity between shrews of different species, weight class, sex, collection period, cestode species richness, and ectoparasite infection status.

Positive Control Number	Extraction Number	Species
Helminth PC1	SG320	Unknown Cestode
	SG189	*Staphylocystoides sp.*
	SG297	*Skrjabinacanthus sp.*
	SG93	*Longistriata sp.*
	SG94	*Longistriata sp.*
	SG174	*Urocystis sp.*
	SG175	*Urocystis sp.*
	SG179	*Hymenolepis sp.*
Helminth PC2	SG95	*Longistriata sp.*
	SG96	*Longistriata sp.*
	SG177	*Urocystis sp.*
	SG187	*Neoglyphe sp.*
	SG188	*Staphylocystoides sp.*
	SG221	*Staphylocystoides sp. (longi?)*
	SG222	*Staphylocystoides sp. (oligospinosus?)*
	SG306	*Blarinolepis sp.*
	SG335	Cercariae and Sporocysts
Bacteria PC1	Bacterial mix	
Bacteria PC2	Bacterial mix	

TABLE 2.1: Library Preparation Positive Controls Composition

CHAPTER 3

INTESTINAL CESTODE SPECIES RICHNESS

3.1 INTRODUCTION

The class cestoda (Platyhelminthes), also known as tapeworms, are segmented intestinal flatworms that are introduced to the host gut through the consumption of an intermediate host. In the case of shrews, the intermediate host is most likely an arthropod. The life cycle of helminth parasites and their success or failure at completing their life cycle are bound to the conditions of their environment. Because cestodes can be fairly host specific (at least at a host generic or family level), precise interactions in space and time are necessary for infection of the parasite's target host species (definitive host). Several studies in insectivorous mammals have demonstrated that helminth community diversity undergoes seasonal changes. Blankespoor and Ulmer (1970) and Nickel and Hansen (1967) provided evidence that helminth prevalence and intensity in bats increases from spring to summer peaking in autumn, and Lord et al. (2012) suggested that seasonal variation in helminth abundance and diversity might be closely tied to changes in abundance and diversity of arthropod prey items that serve as intermediate hosts for helminths.

This chapter is focused on examining possible factors that may contribute to changes in the cestode communities within shrews over multiple months and years. Particularly we want to know if (1) cestode communities in shrews undergo seasonal change? And (2) what are the biggest influencers of cestode species richness or community composition in shrews? Above all, diet has been shown to be the biggest indicator of intestinal helminth diversity, prevalence, and intensity (Salinas-Ramos et al., 2017). Associated with these questions are several hypotheses (restated below) that are centered around variables with the potential to influence shrew diet through prey item preference due to host species/sex/size or prey item diversity and availability due to seasonal change.

3.1.1 HYPOTHESES

1. *Collection Period:* The cestodes surveyed in this study utilize shrews as definitive hosts. Shrews become infected primarily through their arthropod based diet, as these insects act as intermediate hosts. Community composition of these arthropods shift throughout the year, related mostly to abiotic factors (precipitation, temperature, etc.). Therefore, it is expected that cestode species richness will change over time (weekly, monthly, annually). Specifically, helminth species richness will increase as seasonality progresses.

2. *Locality*. Due to the close proximity of the two sites to each other, its expected that there will be no differences in cestode species richness between individuals trapped at the two localities.

3. *Host Species:* As helminths tend to exhibit high levels of host specificity, it's expected that there will be variation in cestode species richness between the two shrew species, with some cestode taxa being more prevalent in one shrew species than the other (Springer et al. 2017; Hope et al. 2016; Smith and Belk 1996).

4. *Host Sex: Sorex* spp. are almost universally infected with intestinal helminths, regardless of sex, but a recent study by Hostert et al. (2018) showed sex specific preferences for specific prey items due to body size. This difference in prey item selection between individuals of different sexes creates the possibility for male and female shrews to host measurably different cestode communities. Therefore, it is expected that male and female shrews will exhibit different cestode species richness, as well as varying prevalence of cestode genera.

5. *Host Weight Group:* Host weight group is most closely associated with specimen age, so there is a significant expected effect of weight group on helminth diversity due to an increased opportunity to accumulate helminth taxa over time. As weight class increases, cestode species richness will likely increase (Sherrard-Smith et al., 2015).

3.2 METHODS

The data collection method for cestode diversity relied on molecular identification instead of visual confirmation, and because cestodes are multi-celled organisms with multiple copies of the

28S rDNA genes, only presence absence data was obtained. The cestode community analysis relied on species richness as a measure of diversity. Kruskal-Wallis tests were used to compare cestode species richness between collection period and weight class. Wilcoxon rank sum tests were used to compare cestode species richness between shrews of different species and sex. To test for differences in prevalence of cestode genera between shrew species, a chi-squared test was conducted for each cestode genus.

3.3 RESULTS

3.3.1 CESTODE COMMUNITY STRUCTURE

Analysis in Geneious revealed that the communities of cestodes inhabiting *Sorex cinereus* and *Sorex monticola* at these two sampling sites consist largely of those belonging to the genera *Lineolepis*, *Mathevolepis*, *Monocercus*, *Staphylocystoides*, *Urocystis*, a new genus Hymenolepididae gen. nov., and *Ditestolepis*. We found several individuals from unknown genera within Hymenolepididae as well as Dilepididae. Those individuals were pooled with each other into groups titled “Hymenolepididae Unknown Genus” and “Dilepididae Unknown Genus”. In *S. cinereus,* the cestode communities were composed of approximately 18% *Lineolepis*, 16% *Mathevolepis*, 14% *Monocercus*, 13% *Staphylocystoides*, 11% Hymenolepididae unknown genus, 11% *Urocystis*, 4% Hymenolepididae gen. nov., 4% *Ditestolepis*, 2% *Skrjabinacanthus*, and a group of rare cestode taxa accounting for 7% of cases (Figure 3.1).

In *S. monticola,* the composition of the cestode communities remained largely the same, with some cestode taxa differing in relative abundance of infection: 23% *Lineolepis*, 17% *Monocercus*, 12% Hymenolepididae unknown genus, 11% *Mathevolepis*, 9% *Urocystis*, 8% *Staphylocystoides*, 8% *Dilepididae* unknown genus, 4% *Staphylocystis*, 4% *Ditestolepis*, 2% *Skrjabinacanthus*, and 2% belonging to other taxa (Figure 3.2).

3.3.2 CESTODE SPECIES RICHNESS AND SHREW DEMOGRAPHIC

Sorex cinereus and *S. monticola* differed in overall cestode species richness, with *S. cinereus* hosting a statistically significantly greater variety of tapeworms than *S.monticola* (Wilcoxon rank p=.0008) (Figure 3.3). When comparing species richness between the three shrew weight classes (low (≤ 4.0g), medium (4.1g – 5.9g), and high (≥ 6.0g)), there was an observable trend in cestode richness with richness increasing as weight increased. However, it was not statistically significant (Kruskal-Wallis p=.0627) (Figure 3.4). Male and female shrews displayed no significant differences in cestode species richness (Wilcoxon rank p=.0137) (Figure 3.5).

3.3.3 CESTODE SPECIES RICHNESS AND SEASONALITY

When comparing cestode species richness and collection year, shrews collected in 2009 did not differ significantly from those collected in 2016, 2017, or 2018. However, shrews collected in 2016 hosted a larger variety of cestode genera than those collected in 2017 and 2018 (Kruskal-Wallis p<.0001) (Figure 3.6) (Table 3.1).

Only 2016 and 2017 contained data that encompassed an entire sampling season (April – October), so comparisons between June and July, which contained the greatest difference in mean cestode richness, were made averaging those years. When pooling the data from each year, the greatest difference observable is between the months of June and July where mean cestode richness increased from 2.41 in June to 4.65 in July. (Kruskal-Wallis p=.0099). Mean richness values ranged from 2.25 in April 2018 to 4.65 in July, with values gradually decreasing from 4.00 in August through September and October. (Figure 3.7) (Table 3.2).

3.3.4 CESTODE PREVALENCE BETWEEN SHREW SPECIES

A contingency analysis of infection by shrew species revealed that of the 15 cestode genera observed, five showed differences in prevalence between *S. cinereus* and *S. monticola.* *Mathevolepis*, *Staphylocystoides*, *Urocystis*, and the Hymenolepididae gen. nov showed a higher prevalence in *S. cinereus* than in *S. monticola. Mathevolepis* infected 71.7% of *S. cinereus*, but only

34.9% of *S. monticola* ($X^2(1, N=182) = 20.89$, $p < .0001$). *Staphylocystoides* infected 58.5% of *S. cinereus* and only 25.6% of *S. monticola* ($X^2(1, N=182) = 17.395$, $p < .0001$). Hymenolepididae gen. nov infected 18.9% of *S. cinereus* and only 0.7% of *S. monticola* ($X^2(1, N=182) = 20.008$, $p < .0001$), and *Urocystis* infected 45.3% of *S. cinereus* and only 31% of *S. monticola* ($X^2(1, N=182) = 3.35$, $p = .049$). Cestodes from the Unknown Dilepididae genus were more prevalent in *S. monticola* infecting only 7.5% of *S. cinereus* and 24.8% of *S. monticola* ($X^2(1, N=182) = 8.14$, $p = .0043$) (Figure 3.8). Several cestode taxa showed similar prevalence within both shrew species. *Lineolepis* was present in 79.2% of *S. cinereus* and 75.2% of *S. monticola*, *Monocercus* was present in 60.4% of S. *cinereus* and 56.6% of *S. monticola,* and *Soricinia* was present in 3.8% of S. *cinereus* and 3.1% of S. *monticola* (Figure 3.8).

3.4 DISCUSSION

3.4.1 CESTODE COMMUNITY STRUCTURE

The cestode community structure observed here very closely matches that reported in (Kinsella and Tkach. 2009). *Cladotaenia*, *Ditestolepis*, *Lineolepis*, *Mathevolepis*, *Soricinia*, *Skrjabinacanthus*, *Staphylocystis*, and *Staphylocystoides* are reported by Kinsella and Tkach (2009) as being found *S. cinereus* in North America. However, the study does not include a list for *S. monticola.* It is possible that some cestode records from *S. monticola* are included, but they are likely misclassified due to the fact that *S. monticola* was formerly considered to be a subspecies of *Sorex vagrans* (Senger, 1955; Kinsella and Tkach, 2009). Also, at the time of Kinsella and Tkach (2009), none of the shrews surveyed for helminth fauna were collected in New Mexico. Of the cestode genera reported by Kinsella and Tkach (2009) for *S. cinereus,* all but *Cladotaenia* are found in of both *S. cinereus* and *S. monticola* in New Mexico. Also, *Monocercus* and *Urocystis* are found in substantial prevalence in both *S. cinereus* and *S. monticola* here and are unreported in *S. cinereus* in Kinsella and Tkach (2009).

3.4.2 CESTODE SPECIES RICHNESS AND SHREW DEMOGRAPHIC

There were no significant differences in cestode species richness between male and female shrews or shrew weight class, rejecting the hypotheses for both of these variables. Although it wasn't significant, there was a trend in weight class and cestode richness. Further analysis with larger sample size could reveal a relationship there. The observable differences in shrew weight class could also be attributed slight differences in weight between the two species of shrew, since there was a significant relationship there.

Although cestode taxa are somewhat host specific, both species of shrew surveyed housed many of the same cestode taxa. This is not surprising considering the evidence of shrews in the genus *Sorex* sharing much of their cestode fauna when not geographically distant (Haukisalmi, 2015; Kinsella and Tkach, 2009; Binkienė et al., 2011). However, *S.cinereus* and *S.monticola* differed in overall cestode species richness, with *S. cinereus* hosting a greater variety of tapeworms than *S.monticola.*

Little is known about how similar or dissimilar the diets of these two shrew species are. With diet being such a strong predictor of cestode community diversity and abundance, further investigation into the arthropod prey items utilized by these species could prove useful. Identification of cestode taxa to species could also shed light on the nuanced differences in the cestode communities in *S. cinereus* and *S. monticola*, as previous studies have shown that although many cestode genera are found across several shrew species in the same genus, there are cases where cestode species are found in some *Sorex* species and not others (Haukisalmi, 2015; Kinsella and Tkach, 2009; Binkienė et al., 2011). Also, description of the arthropod communities utilized as food sources by *Sorex* shrews, as well as, species level identification of their cestode taxa could address the currently lacking record of life cycles of cestode fauna parasitic to shrews in North America and more clearly define the microniches occupied by these two species of shrews (Kinsella and Tkach, 2009).

3.4.3 CESTODE SPECIES RICHNESS AND SEASONALITY

When comparing cestode species richness and collection year, shrews collected in 2009 did not differ significantly from those collected in 2016, 2017, or 2018. However, shrews collected in 2016 hosted a larger variety of cestode genera than those collected in 2017 and 2018. It is unclear whether such drastic changes are attributed to differences in abiotic conditions (i.e. temperature and precipitation) or changes in behavior, reproductive status etc. Likely, however, these differences are due to sampling bias. The specimens collected from 2009 were all from the month of September, and the specimens from 2018 only included individuals form April and May (due to forest fires preventing trapping).

Mean cestode richness values ranged from 2.25 in April 2018 to 4.65 in July (average 2016 and 2017), with values gradually decreasing from 4.00 in August through September and October (averaging data from 2009, 2016, and 2017). This trend closely matches changes in temperature and rainfall for the region (NOAA, 2020). Specifically, the dramatic increase in cestode species richness in July corresponds to a nearly doubling of average rainfall from June to July (Figure 3.9).

The observed increase in cestode species richness in the summer/fall months is similar to other studies concerning seasonal variation in diversity and prevalence in helminths as a broad group. However, some studies have demonstrated that specifically cestode richness and abundance decreases during rainy seasons and increase during dry periods (Viljoen et al., 2011; Coggins et al. 1982; Sissay et al., 2008). As cestode species richness in this study increased with average temperature and rainfall, further investigation is necessary. Although moisture availability and average temperature are factors demonstrated to influence arthropod diversity and helminth diversity, the variety in cestode species richness observed here is likely not all due to abiotic factors. Some studies have demonstrated that biotic factors such as reproductive status, behavior, and immunocompetence of hosts fluctuate with seasonality as well and can thus be attributed to at least some of the temporal variation in cestode community richness observed in *S. cinereus* and *S. monticola* (Šimková et al., 2005; Felis and Esch, 2004).

3.4.4 CESTODE PREVALENCE BETWEEN SHREW SPECIES

Mathevolepis, *Staphylocystoides*, *Urocystis*, and Hymenolepididae gen. nov. all showed a statistically higher prevalence in *S. cinereus* than *S. monticola.* The reasons for this are likely the same for the observed greater cestode species richness in *S. cinereus* discussed above. Decreased host fitness resulting from parasite infection is a selective pressure for resistance strategies. However, those same adaptive efforts of the host increase selection on parasites (Buckling and Rainey, 2002). The answer to these questions likely lies in the evolution of populations of S. *cinereus* and S. *monticola* in this region and requires consideration of how *S. cinereus* and *S. monticola* differ in exposure and susceptibility to the described cestode communities (Park et al., 2017).

3.5 CONCLUSIONS

Cestode communities observed in *Sorex cinereus* and *S. monticola* are similar to those observed in other *Sorex* shrews in North America. This study provides clearer discription of cestode taxa infecting *S. monticola* and provides a first survey of cestodes infecting shrews in New Mexico. Cestode taxa showed varying prevalence between the two shrew species, and *S. cinereus* hosted overall greater cestode richness than *S. monticola.* Further investigation into the arthropod prey items utilized by these species could provide useful insight into the microniches occupied by these shrew taxa, and identifying the cestode taxa harbored by *S. cinereus* and *S. monticola* to species could shed further light on the life cycles of cestodes infecting shrews which are better understood in the palearctic but under studied in North America.

The largest influencers of cestode species richness in shrews was collection period. Cestode species richness varied significantly over time with increased richness in the warmer rainy months of New Mexico. These findings differ from other reports describing cestode diversity and

abundance increasing in dry, cooler seasons, so further study on the consistency of seasonality of cestode communities across mammalian lineages is recommended.

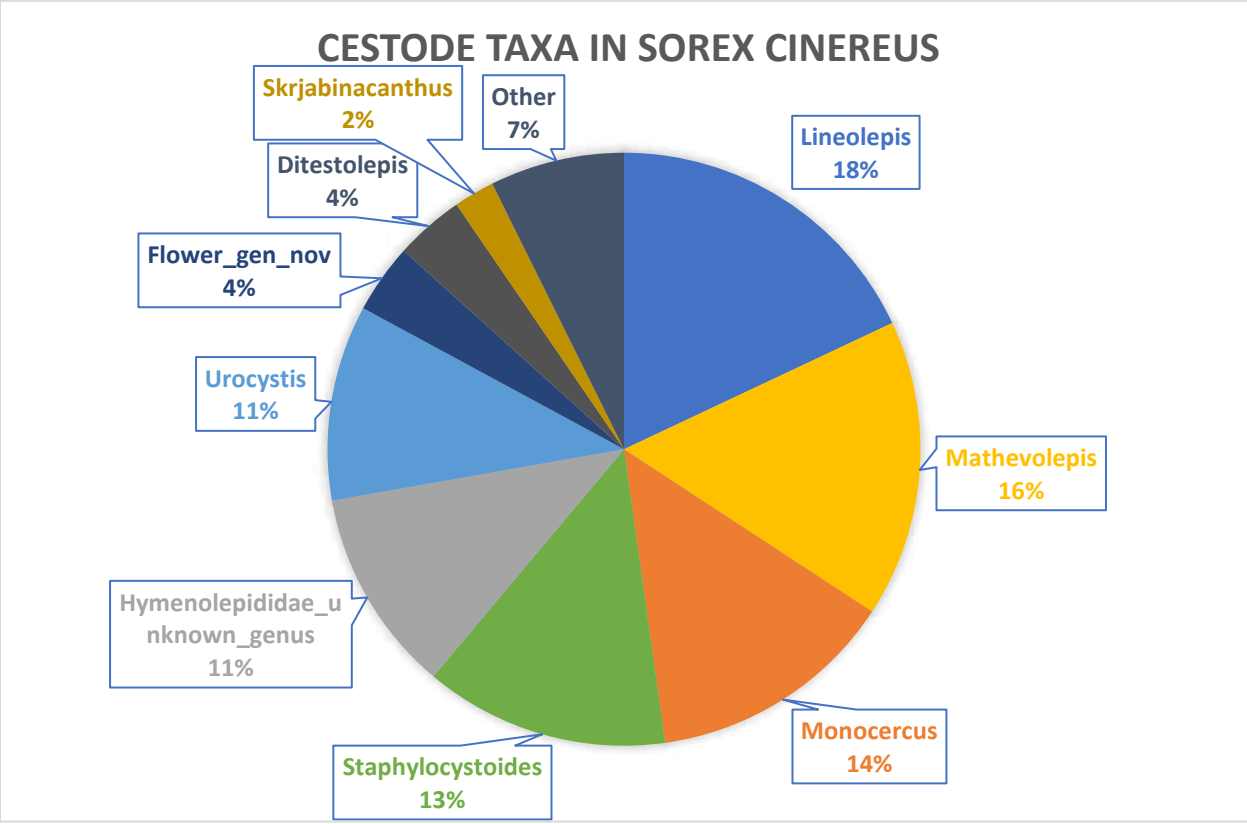

Figure 3.1: Pie chart depicting cestode taxa observed in *Sorex cinereus* grouped by percent of detected infections.

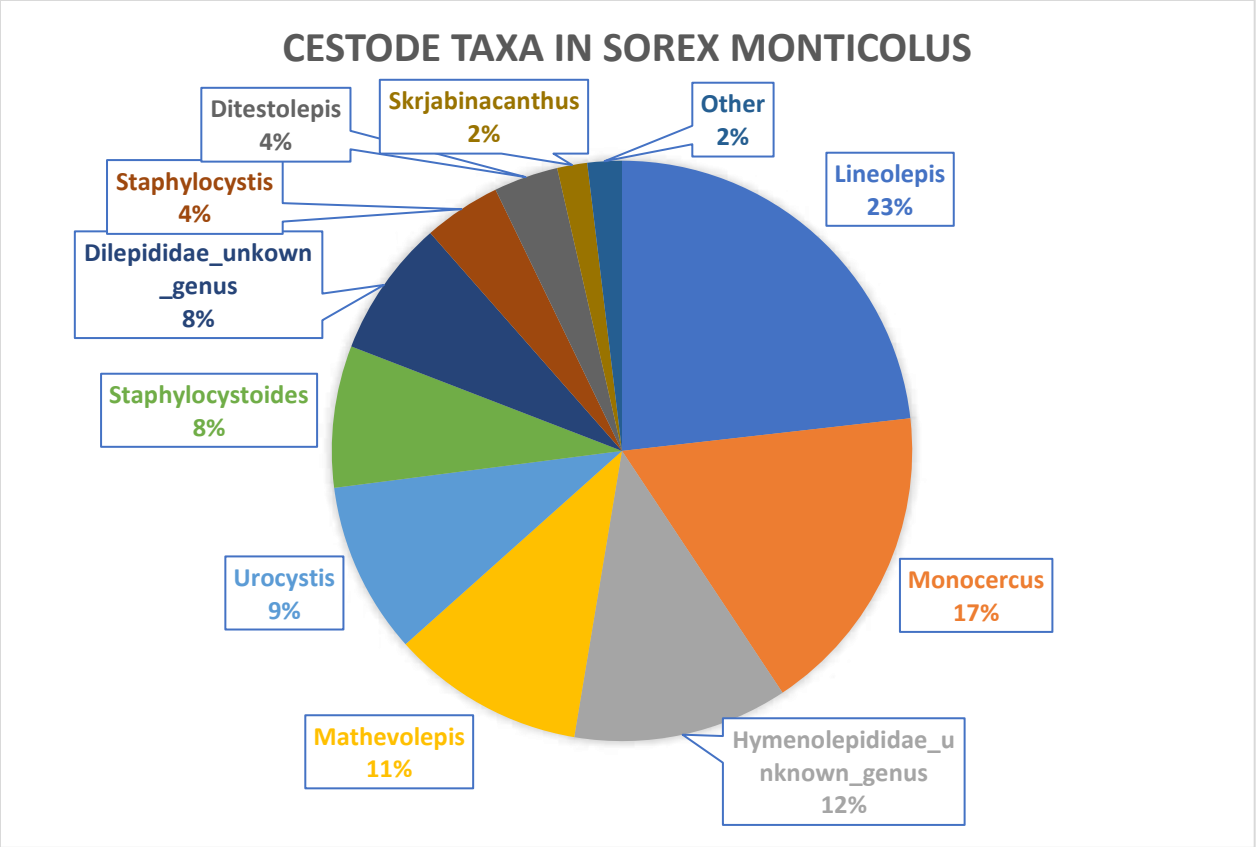

Figure 3.2: Pie chart depicting cestode taxa observed in *Sorex monticola* grouped by percent of detected infections.

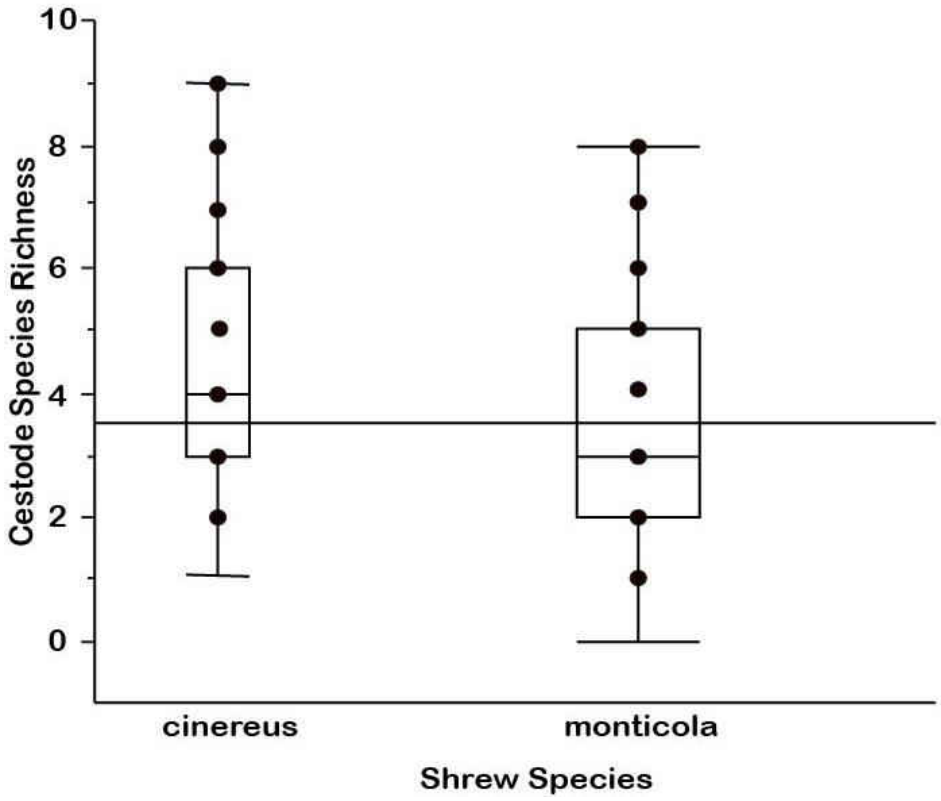

Figure 3.3: Wilcoxon rank sum test for cestode species richness in S. *cinereus* and S. *monticola.* Upper bars represent the 95th quartile, lower bars represent the 5th quartile, and middle bars represent the median for each species.

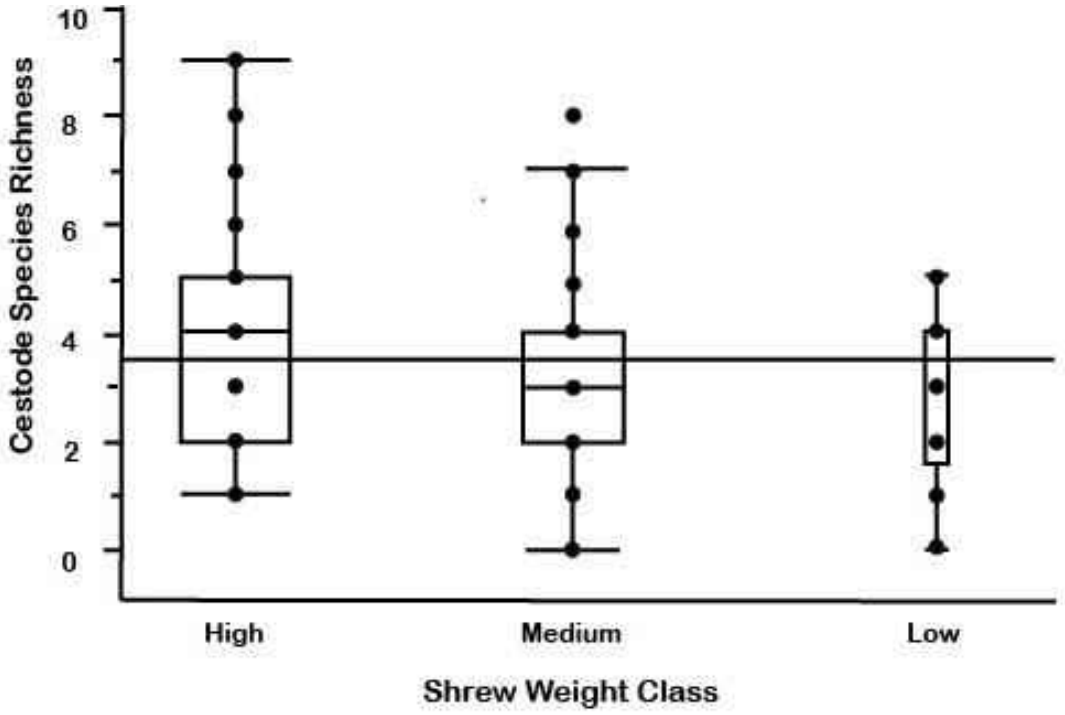

Figure 3.4: Wilcoxon rank sum test for cestode species richness and shrew weight class. Upper bars represent the 95th quartile, lower bars represent the 5th quartile, and middle bars represent the median for each weight class.

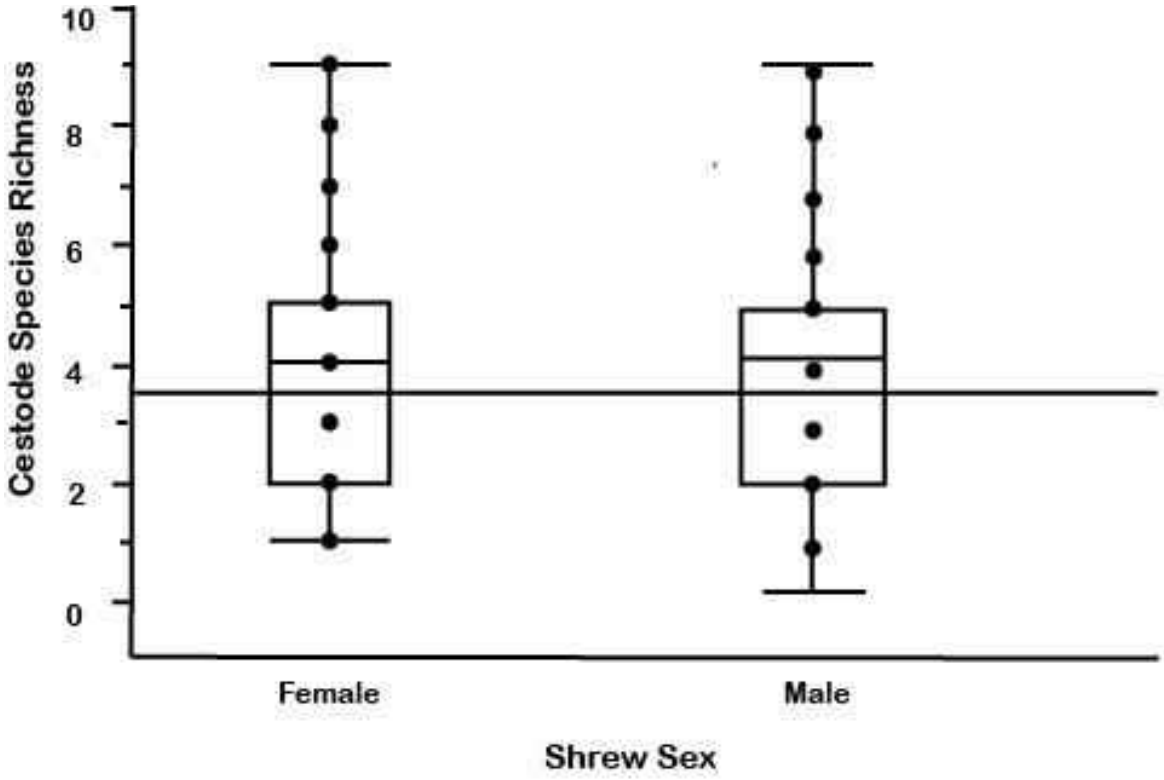

Figure 3.5: Wilcoxon rank sum test for cestode species richness and shrew sex. Upper bars represent the 95th quartile, lower bars represent the 5th quartile, and middle bars represent the median for each sex.

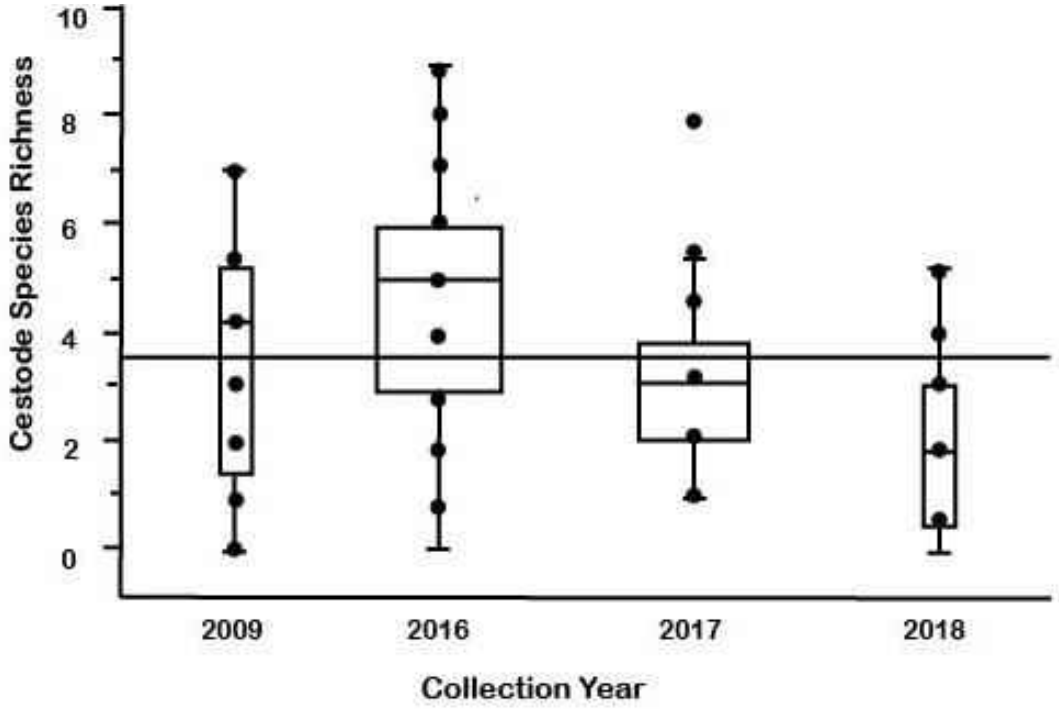

Figure 3.6: Kruskal-Wallis test for cestode species richness and collection year. Upper bars represent the 95th quartile, lower bars represent the 5th quartile, and middle bars represent the median for each year.

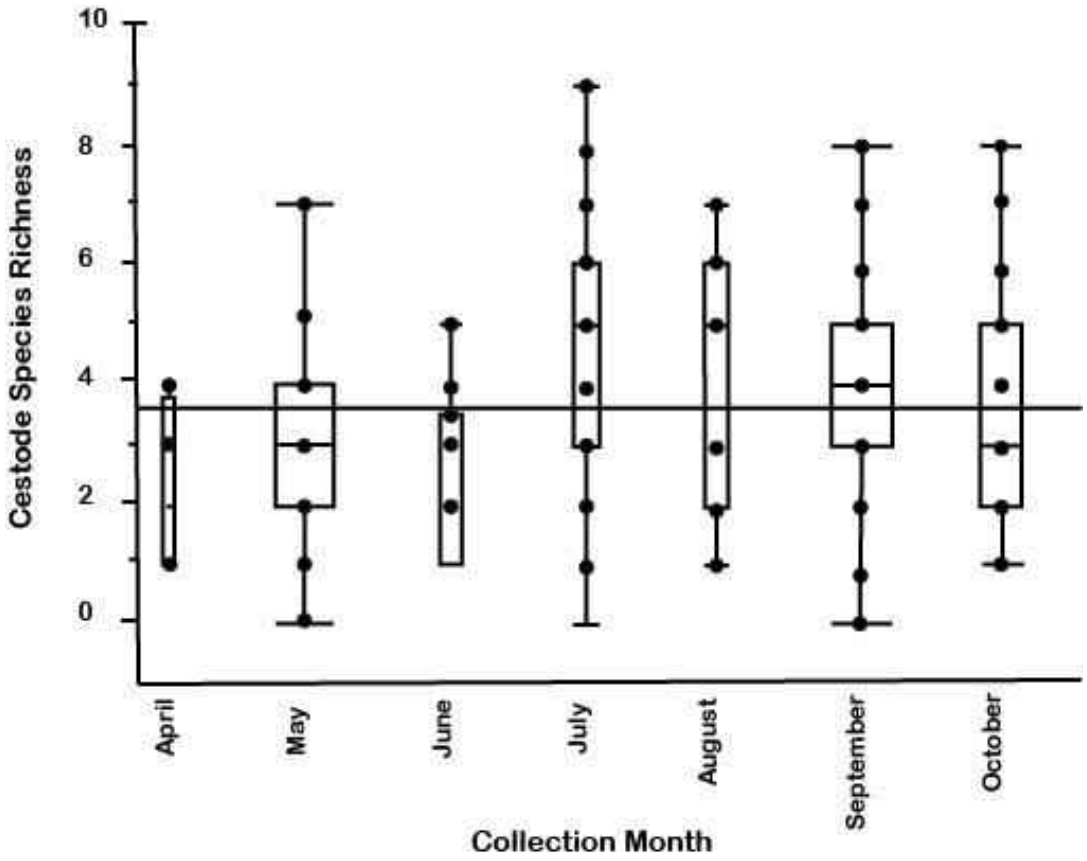

Figure 3.7: Kruskal-Wallis test for cestode species richness and month. Upper bars represent the 95th quartile, lower bars represent the 5th quartile, and middle bars represent the median for each month.

Mathevolepis Infection

ShrewSpecies	Count / Total % / Col % / Row %	0	1	Total
cinereus		15	38	53
		8.24	20.88	29.12
		15.15	45.78	
		28.30	71.70	
monticolus		84	45	129
		46.15	24.73	70.88
		84.85	54.22	
		65.12	34.88	
Total		99	83	182
		54.40	45.60	

Staphylocystoides Infection

ShrewSpecies	Count / Total % / Col % / Row %	0	1	Total
cinereus		22	31	53
		12.09	17.03	29.12
		18.64	48.44	
		41.51	58.49	
monticolus		96	33	129
		52.75	18.13	70.88
		81.36	51.56	
		74.42	25.58	
Total		118	64	182
		64.84	35.16	

Flower Genus Infection

ShrewSpecies	Count / Total % / Col % / Row %	0	1	Total
cinereus		43	10	53
		23.63	5.49	29.12
		25.15	90.91	
		81.13	18.87	
monticolus		128	1	129
		70.33	0.55	70.88
		74.85	9.09	
		99.22	0.78	
Total		171	11	182
		93.96	6.04	

Urocystis Infection

ShrewSpecies	Count / Total % / Col % / Row %	0	1	Total
cinereus		29	24	53
		15.93	13.19	29.12
		24.58	37.50	
		54.72	45.28	
monticolus		89	40	129
		48.90	21.98	70.88
		75.42	62.50	
		68.99	31.01	
Total		118	64	182
		64.84	35.16	

Unknown Dilepididae Infection

ShrewSpecies	Count / Total % / Col % / Row %	0	1	Total
cinereus		49	4	53
		26.92	2.20	29.12
		33.56	11.11	
		92.45	7.55	
monticolus		97	32	129
		53.30	17.58	70.88
		66.44	88.89	
		75.19	24.81	
Total		146	36	182
		80.22	19.78	

Figure 3.8: Contingency tables of infection with *Mathevolepis*, *Staphylocystoides*, Hymenolepididae gen. nov (Flower Genus), *Urocystis*, and Unknown Dilepididae between shrew species. Columns with "0" are uninfected and columns with "1" are infected.

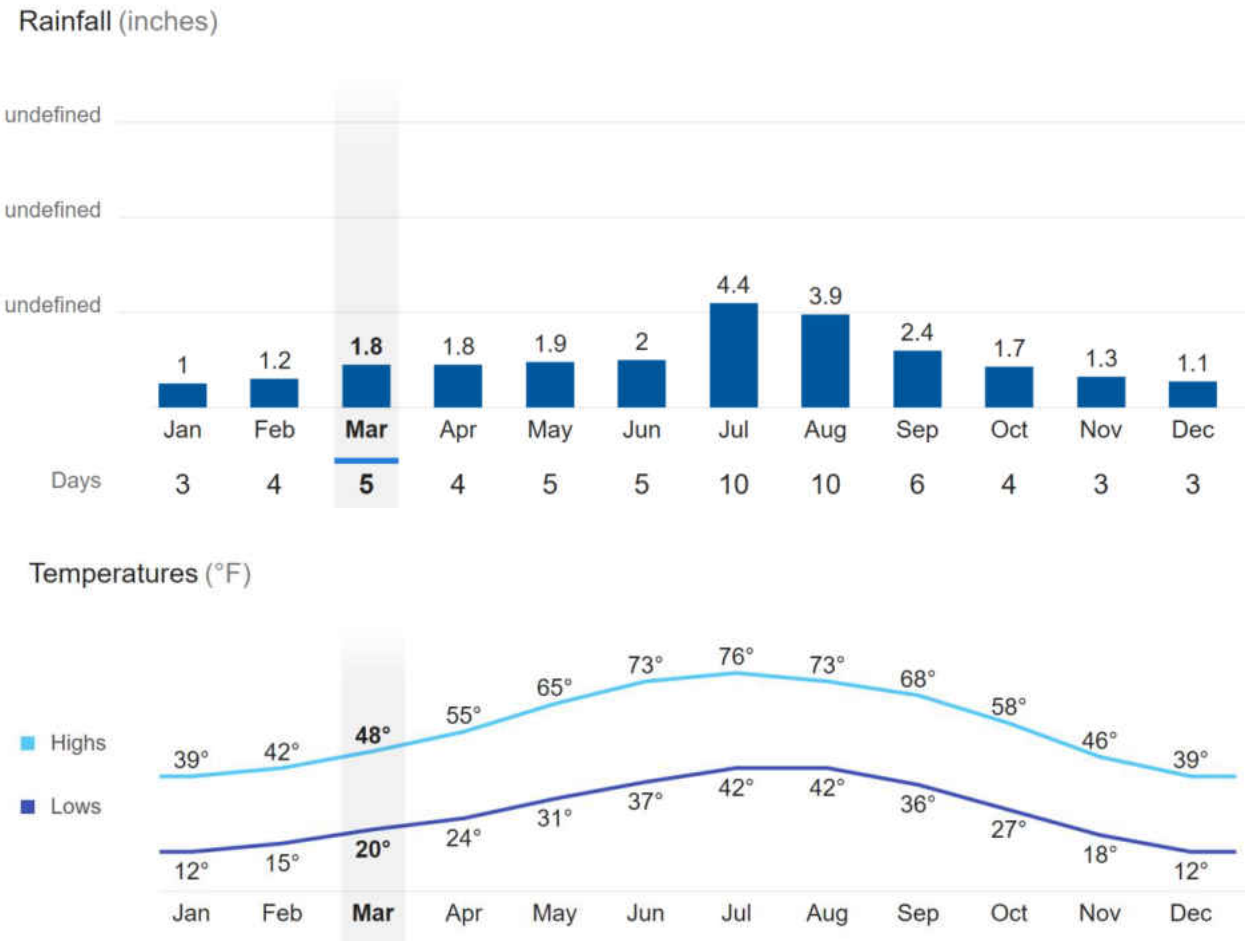

Figure 3.9: Average rainfall in inches and days per month in the top graph and temperature with high and low averages by month for Cowles, NM.

Level			Mean
2016	A		4.4500000
2009	A	B	3.4444444
2017		B	2.9589041
2018		B	2.1250000

Table 3.1: Connecting letters report for figure 3.6.

Level				Mean
July	A			4.6470588
September	A	B		4.0869565
August	A	B	C	4.0000000
October	A	B	C	3.7096774
May		B	C	2.9318182
June			C	2.4117647
April	A	B	C	2.2500000

Table 3.2: Connecting letters report for figure 3.7.

CHAPTER 4

GUT MICROBIOME DIVERSITY AND HELMINTH INTERACTIONS

4.1 INTRODUCTION

Microbiomes, communities of microorganisms that inhabit multicellular organisms, have been shown to directly influence host health and fitness and are crucial elements both in an organism's ability to acquire nutrients from its diet and the ability of an organism to fight off infection (Turnbaugh et al., 2007; Nayfach et al., 2015). The microbiota of an organism includes communities of microorganisms colonizing the skin, gut, mouth, and genitals that are primarily composed of bacteria, but include Archaea, fungi, viruses, and protozoa (Glenndening et al., 2014). The diversity of microbes within individuals are significantly reliant on heritable, behavioral, and environmental factors. Therefore, it is likely that changes in environment and diet result in changes in the diversity and abundance of taxa within the gut microbiome.

Another influencer of gut microbiome diversity is intestinal parasites, notably, those within the phylum Platyhelminthes. These eukaryotic, multi-cellular organisms are known to utilize a diverse assemblage of hosts. Many species of helminths, including those that parasitize humans, are transmitted through the consumption of intermediate hosts such as fish, invertebrates, other vertebrates, as well as, contact with soil. Because the life cycles of helminths are reliant on many external factors, such as, availability of intermediate hosts, the likelihood of ingestion by a viable definitive host, and changes in the environment, including; shifting biodiversity of arthropod communities, they could impact cestode diversity and the nuanced interactions occurring between parasitic helminths and the host gut flora. Additionally, a number of host specific factors could influence the relationship between helminths and the gut microbiota. These include physiological changes of the host attributed to age, changes in diet, changes in behavior, the effects of helminths

on the immune response of the host, and the influence of gut microbiota on the parasitic helminth (Glendinning et al. 2014).

This chapter is focused on examining possible factors that may contribute to changes in the bacterial communities within shrews over multiple months and years. Particularly we want to know if (1) bacterial communities in shrews undergo seasonal change, (2) what effect, if any, specific cestode taxa and cestode species richness have on microbiome diversity and composition, and (3) what factors contribute the most to shrew gut microbiome diversity and community composition. Associated with these questions are several hypotheses (restated below) that are centered around variables with the potential to influence gut microbiome diversity and community composition.

1. *Collection Period:* Given that shrews do not transition from a more plant-based diet to a more protein rich (insect) diet, it's expected that there will be little change in primary diet composition. However, prey species composition (insect diversity) is likely to change dramatically over the different collection periods, due mostly to changes in temperature and precipitation.
2. *Locality:* Individuals for this study were collected from two separate sites approximately one mile apart. Due to the close proximity of the sites to each other, there are no expected differences between individuals trapped at the two localities.
3. *Host Species:* Mammals have been shown to have a somewhat conserved microbiome among taxa (Maurice et al. 2015), so there are no expected significant differences in gut microbiome diversity between the two shrew species.
4. *Host Sex:* Markle et al. (2013) demonstrated an effect of sex on microbial community structure in mice associated with hormonal differences and sex-biased immunity, therefore it is expected that there are slight differences in microbial community structure between male and female shrews.
5. *Host Weight Group:* There is precedent for differences in microbial community diversity between organisms classified as obese and those with a healthy weight, therefore a weak correlation

between weight class in shrews and bacterial diversity is expected (Hartstra et al., 2015; Ley et al., 2005).

6. *Cestode Species Richness:* Specific cestode taxa may alter the landscape ecology of the gut. Although previous studies have observed differences between individuals infected with intestinal helminths and those without in terms of microbiome diversity (reviewed above), there is no expected variation in the specimens collected here due to the observed universal infection rate of shrews with intestinal helminths within our samples.

4.2 RESULTS

4.2.1 MICROBIOME DIVERSITY BETWEEN SHREW DEMOGRAPHICS

Analysis in Calypso revealed that the shrew gut microbe communities consisted largely of 16 bacterial phyla. The most abundant of these were Epsilonbacteraeota 19%, an unclassified phylum 18%, Proteobacteria 14%, Tenericutes 12%, Firmicutes 11%, and Actinobacteria 6%, and although samples varied in their relative abundance of these taxa amongst each other, the topmost abundant phyla remained the same (Figure 4.1).

Shrews collected from Jacks creek displayed no significant differences in relative bacteria abundance or diversity from those collected at Winsor creek. When comparing microbiome diversity and abundance between the two species, *S. cinereus* and *S. monticola* differed in abundance of two bacterial phyla with *S. cinereus* hosting a greater abundance of Epsolonbacteraeota than *S. monticola* and *S. monticola* hosting a greater abundance of Proteobacteria than *S. cinereus* (Wilcoxon rank: Epsilonbacteraeota P=.0016, FDR=.026; Proteobacteria P=.0045, FDR=.036) (Figure 4.1) (Figure 4.2).

Adonis multivariate analysis (analogous to perMANOVA) based on OTU Bray Curtis models revealed that there were distinct differences in bacterial community composition between shrew species (r^2 =0.0187, p=.0.005), shrew sex (r^2 =0.0233, p=.004), and weight class (r^2 =0.0673,

p<.0001) (Figure 4.8). However, when tested for homogeneity of variance, shrew species was significant (PERMDISP2: p=.0002), suggesting that differences in microbiome communities between shrew species could be due to variances in within-group distributions rather than variation in centroid location.

Male and female shrews did not differ in the amount of diversity present in their gut microbiomes or relative bacterial phyla abundance, but individuals from the three weight classes displayed varying abundances of bacterial phyla (Wilcoxon rank: Proteobacteria P=.0000022, FDR=.000035; Planktomycetes P=.00021, FDR=.0017; Cyanobacteria P=.00046, FDR=.0022; Acidobacteria P=.00055, FDR=.0022; Verrucomicrobia P=.0019, FDR=.0061; Dependentiae P=.00023, FDR=.0061; Actinobacteria P=.0027, FDR=.0062; Firmicutes P=.0031, FDR=.0062; Chloriflexi P=.oo39, FDR=.0069; Unclassified phylum P=.016, FDR=.026; Chlamdiae P=.02, FDR=.029; Gammatimonadetes P=.031, FDR=.041)(Figure 4.3 and 4.4).

Proteobacteria, Planctomycetes, Cyanobacteria, Acidobacteria, Verrucomicrobia, and Actinobacteria all displayed higher abundance in individuals in the “high” weight class. Dependentiae, Chlamydiae, and Gemmatimonadetes were present in higher abundance in individuals found in the “medium” weight class, and Firmicutes was found in increased abundance in individuals in the “low” weight class (Wilcoxon rank P<.05).

4.2.2 SHREW MICROBIOME DIVERSITY AND SEASONALITY

Among samples collected in 2009, 2016, 2017, and 2018, three bacteria phyla varied significantly between sampling years with the same general trend in relative abundance. Proteobacteria was more abundant between the three phyla followed by Actinobacteria and Cyanobacteria respectively (Wilcoxon rank: Actinobacteria P=.002, FDR=.019; Cyanobacteria P=.0038, FDR=.021; Proteobacteria P=.0039, FDR=.021). 2016 and 2017 saw significantly lower relative abundances in all three phyla when compared to 2009 and 2018 (Figure 4.5).

Shrew gut microbiome community composition varied over time (r^2 =0.155, p<.0.001) (Figure 4.8), however, a test for homogeneity of variance was significant for collection month (PERMDISP2: p=0.002). When compared by collection month, Tenericutes varied significantly with the greatest abundance occurring in the months of September and October in both 2016 and 2017 (Wilcoxon rank: p=.0077; FDR=.048). Proteobacteria varied significantly across collection periods but was not consistent in terms of repeated increases or decreases of abundance in specific months with the greatest abundance in June 2017 and September 2009 (Wilcoxon rank: p=.0092; FDR=.048). Actinobacteria also varied with collection period being dramatically more abundant in April 2018 than any other month but with no real repeated seasonal pattern (Wilcoxon rank: p=.014; FDR=.048) (Figure 4.7).

4.2.3 CESTODE-MICROBE INTERACTIONS

There was no difference in gut microbe phyla abundance or level of diversity among individuals found to be hosting ectoparasites (ticks, fleas, and lice) and those without, however, Bacterial community composition varied between shrews infected with ectoparasites of any sort and those without (r^2=.0185, p=.00533). Microbiome composition differed between individuals carrying ticks and those without ticks (r^2=.0389, p=.0283), and microbe communities in individuals carrying harboring fleas differed from those with no fleas (r^2=.0389, p=.0036) (Figure 4.9).

Analysis of bacterial communities of those infected with specific cestode taxa revealed that shrews infected with *Mathevolepis* hosted distinct microbiota from those not infected (r^2=.0202, p=.0043). Shrews infected with cestodes in the genus Mathevolepis showed a less diverse gut flora than those without the parasite (Kruskal-Wallis: P=.0029) (Figure 4.8). Specifically, Actinobacteria, Chloroflexi, Dependenteae, Verrucomicrobia, Planctomycetes, and Cyanobacteria showed decreased abundance in shrews infected with Mathevolepis (Kruskal-Wallis: Actinobacteria P=.001, FDR=.012; Chloroflexi P=.0015, FDR=.012; Dependenteae P=.0073, FDR=.032; Verrucomicrobia P=.0097, FDR=.032; Planctomycetes P=.01, FDR=.035). Surprisingly, the

bacterial communities of shrews infected with *Monocercus* were distinct from those not infected with *Monocercus* (r^2=.0144, p=.0043), even though there were no differences the abundance of individual bacterial phyla (Figure 4.10).

4.3 DISCUSSION

4.3.1 SHREW MICROBIOME LITERATURE COMPARISON

The shrew microbiome composition observed in this study is consistent with published gut microbiomes of shrews as well as other mammals with the five most abundant phyla observed here, Epsilonbacteraeota, Firmicutes, Tenericutes, Proteobacteria, and Bacteriodetes, existing as key taxa across mammalian lineages (Nishida and Ochman, 2018; Bergmann et al., 2015; Maurice et al., 2015; Zhao et al. 2015; Davenport et al., 2014). The presence of Cyanobacteria in the microbiome is not uncommon in mammals is usually a result of drinking water or eating invertebrates from water containing blooms of Cyanobacteria. Heavy infection with Cyanobacteria can lead to GI distress and disease (Kubickova et al., 2019).

Knowles et al. (2019) published the microbiome for *Sorex araneus* and described similar microbiome composition to those seen in *S. cinereus* and *S, monticola,* with one difference being the larger proportion of Proteobacteria (~50%) in their study resulting from not separating Epsilonproteobacteraeota from Proteobacteria following Epsilonproteobacteraeota being classified as its own phylum by Waite et al. (2017). For this analysis, we separated Epsilonbacteraeota from Proteobacteria accounting for our smaller proportion (~14%) of Proteobacteria (Knowles et al., 2019; Waite et al., 2017).

4.3.2 WEIGHT CLASS

Larger shrews harbored a greater abundance of bacterial phyla such as Proteobacteria, Planctomycetes, Cyanobacteria, Acidobacteria, Verrucomicrobia, and Actinobacteria. The life cycle of *Sorex* shrews follows a pattern of birthing in the spring and summer and the death of the parent

generation by the following fall and winter. Because the odds of collecting both old and young shrews during the periods we sampled are fairly high, the observed differences in weight could be due mostly to the age of the shrews collected. Some of the variation in gut microbiome diversity and abundance between the weight groups could also be a result of the maturation of the host immune system or the possibility that larger shrews are utilizing different/ larger prey items than those in the lower weight groups resulting in subtle differences in microbiome diversity through introduced flora.

Individuals in the medium weight class displayed higher abundances of Dependentiae, Chlamydiae, and Gemmatimonadetes. Dependentiae is a parasitic microbe. Bacteria in this phylum feature adaptations such as degenerated cell envelopes and ATP/ADP translocase for utilizing host ATP pools. The relationship between Dependentiae and shrews is likely parasitic, but further studies are required to determine why a microbe "dependent" on host organisms would increase in abundance specifically in shrews of the "medium" weight class (Yeoh, 2016). The literature discussing the role of Gemmatimonadetes in the mammalian gut microbiome is sparse, but bacteria belonging to Gemmatimonadetes make up approximately 2% of soil bacteria and consistently make up a small percentage of mammalian gut bacteria (Davenport, 2014; DeBruyn, 2011). Curiously, Firmicutes was present in higher abundances in shrews in the "low" weight class. Increased abundance of Firmicutes was observed in undernourished children in a study looking at the effects of obesity and undernourishment, so we could be seeing a manifestation of the same relationship in shrews (Méndez-Salazar, 2018). It is possible that the differences observed in the diversity and abundance of bacteria in the different weight classes could be alluding to natural progressions of shrew gut diversity as the shrews age.

4.3.3 SEX

Previous research has demonstrated an effect of sex on microbial community structure in mice associated with hormonal differences and sex-biased immunity (Markle 2013). However,

shrew microbe diversity and abundance did not significantly differ between male and female specimens. There was no significant relationship between shrew weight class and sex, rejecting the idea that male and female shrews were utilizing different prey items as a result of sexual dimorphism. Part of this homogeneity of male and female microbiota could be attributed to misidentification of male and female specimens since *Sorex* shrews are notoriously difficult to sex in the field (Carraway 2009). Further analysis with a larger sample size and concrete identification of sex through methods such as the usage of the SRY gene could reveal any presently hidden relationships.

4.3.4 SEASONALITY

Although individual phyla (Tenericutes, Proteobacteria, and Actinobacteria) varied significantly across sampling period, there were no significant trends in Shannon diversity (levels of diversity) between collection month. Collection period was significant in analysis of microbiome community composition, but some of that diversity may have been due to inherent variances in within-group distributions. Because there are no differences in quantified diversity, but differences in community composition, it is possible that seasonal shifts in community composition are occurring without significantly changing how diverse the community is.

4.3.5 CESTODE MICROBE INTERACTIONS IN THE GUT

The ecology of the vertebrate gut has undergone dynamic changes over hundreds of millions of years serving as a theater of interactions and struggles between prokaryotes and parasitic eukaryotes (Jackson et al., 2009). GI helminths and gut microbes have, independently of each other, earned credit for the immunomodulatory influences and shaping of the immune homeostasis of their host organisms (Leung et al. 2018). Our study showed that shrews infected with cestodes in the genus *Mathevolepis* demonstrated a decreased abundance of Actinobacteria, Chloroflexi, Dependenteae, Verrucomicrobia, Planctomycetes, and Cyanobacteria. Pinpointing the

source of this repression of bacteria taxa is difficult. Although recent years have seen an influx of studies demonstrating the effects of parasite infections on gut microbiota due to more readily available next-generation sequencing technology, the variation in results across studies due to differences in experimental design such as sampling techniques and the environment in which animals are kept prevent a clear and concise picture of microbe-parasite interactions (Peachey et al., 2017).

One explanation is that colonization of the GI tract by a parasite alters the ecology of the host gut by affecting mucus production and composition and epithelial cell turnover, thus serving as an ecosystem engineer of the host gut making changes in the landscape of the GI tract with far reaching implications since many microbial taxa are fed and housed by the outer mucus layer of the gut (Peachey et al., 2017). A study by Theodoropoulos et al. (2001) demonstrated that some helminth taxa can produce molecules similar to mucus themselves as a part of host cell attachment and evasion of the host immunity. Bacteroidetes, Firmicutes, Actinobacteria, and Verrucomicrobia, all shown to be negatively impacted by *Mathevolepis* infection, use carbohydrates from mucus to acquire carbon and could be experiencing environmental stress driven by *Mathevolepis* (Tailford et al., 2015). It is unclear why individuals with *Monocercus* infections displayed altered gut microbe community composition. There were no significant relationships between *Monocercus* infection and Shannon diversity or changes in abundance of any bacterial phyla. It is possible that there are more nuanced differences at lower taxonomic levels not revealed by this study.

Another variable capable of altering gut microbe composition is innate immune response. One study of *H. diminuta* in rats showed upregulation of TLR2 and TLR4 in the jejunum and colon (Kosik-Bogacka et al., 2012). There is evidence that upregulation of TLRs leads to the production of pro-inflammatory cytokines that influence bacterial infection. This innate immune response from the host induced by parasite infections could be a method by which helminths mediate mucosal

inflammation against the microbes in the gut as a mechanism to control immune homeostasis (Ince et al., 2006).

As anticipated, cestode species richness did not negatively impact gut microbiome diversity. It is likely that these species of shrew coevolved with their cestode communities. Also, because of the universal infection rate of these shrews sampled here, detecting any real effect for species richness and microbiome diversity is difficult because there are too few specimens with which to compare infected v. uninfected/low gut microbe diversity.

4.4 CONCLUSIONS

Although three bacterial phyla (Tenericutes, Proteobacteria, and Actinobacteria) varied significantly in abundance across sampling period, and the bacterial community composition in *Sorex cinereus* and *S. monticola* varied significantly by collection month, there was no significant difference in Shannon diversity index between shrews collected during the different sampling periods suggesting changes in community composition but not necessarily changes in the amount of diversity within the bacterial communities. Collection month, shrew species, sex, and weight class all significantly influenced the gut microbiome communities in *S. cinereus* and *S. monticola,* however some of the effect of collection month and shrew species on community composition is likely due to variances in within-group distributions. *Mathevolepis* and *Monocercus* were both associated with altered bacterial community composition, but further analysis could be conducted controlling for the presence of one or the other to parse out any confounding due to coinfection.

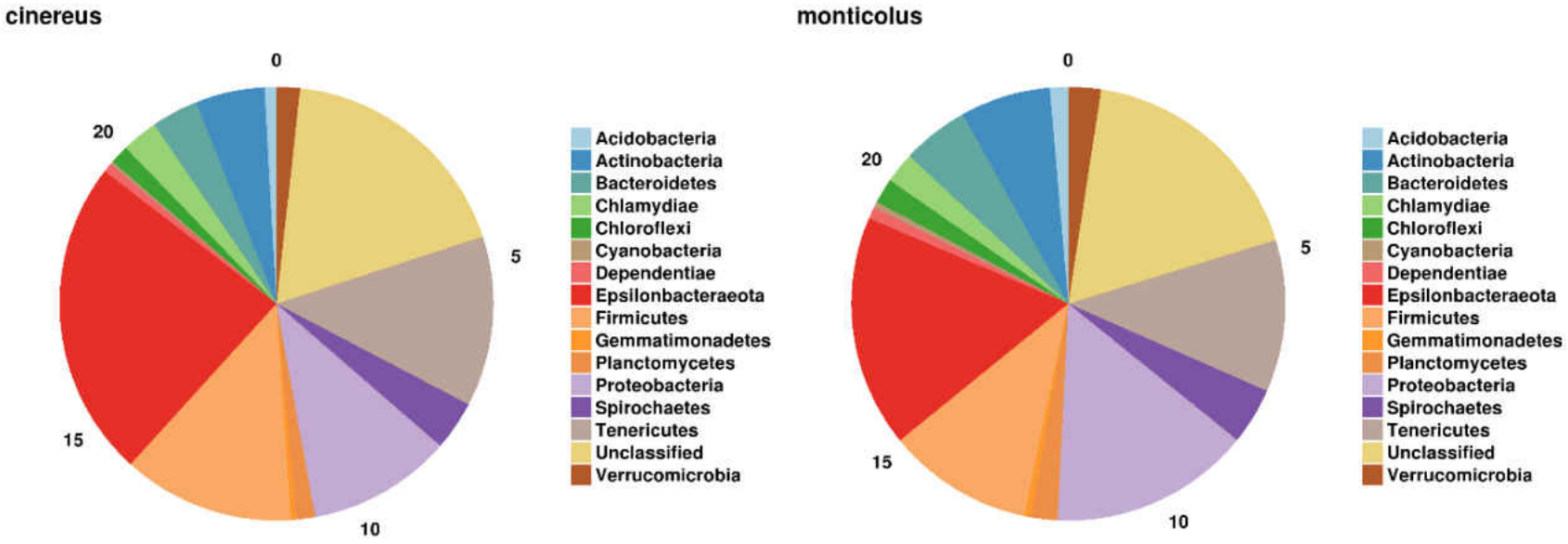

Figure 4.1: Pie chart depicting relative abundance of bacterial phyla in *S. monticola* and *S. cinereus.*

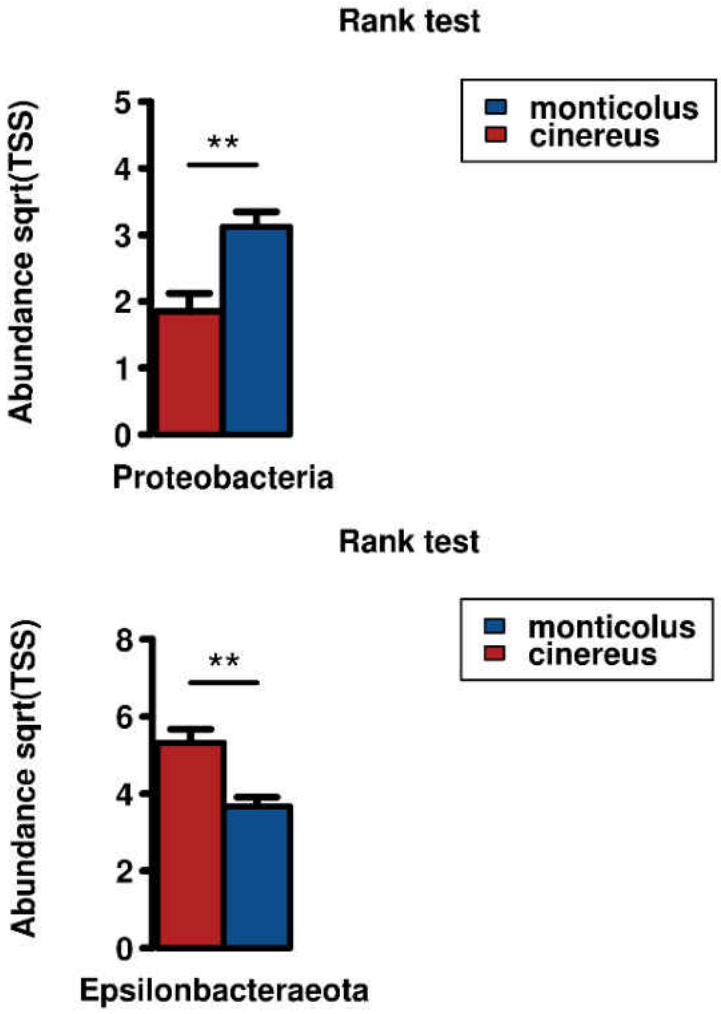

Figure 4.2: Relative abundance of Proteobacteria and Epsilonbacteraeota in *S. monticola* and *S. cinereus*.

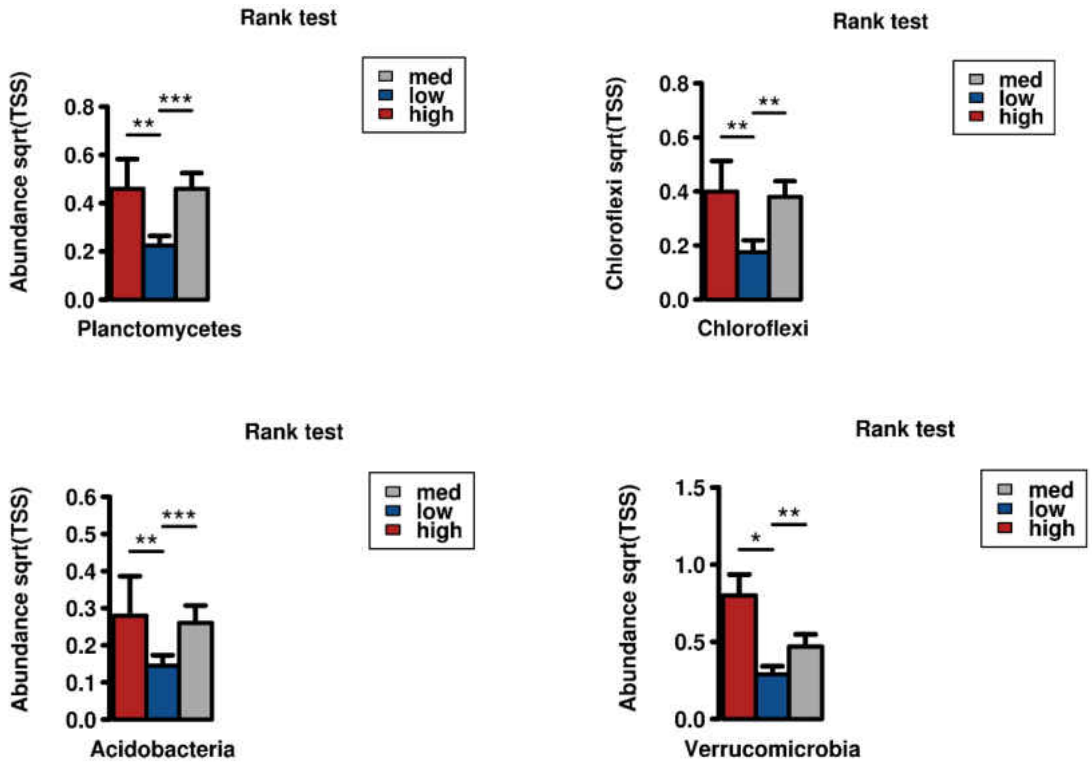

Figure 4.3: Relative abundance of Planctomycetes, Chloroflexi, Acidobacteria, and Verrucomicrobia in relation to shrew weight class.

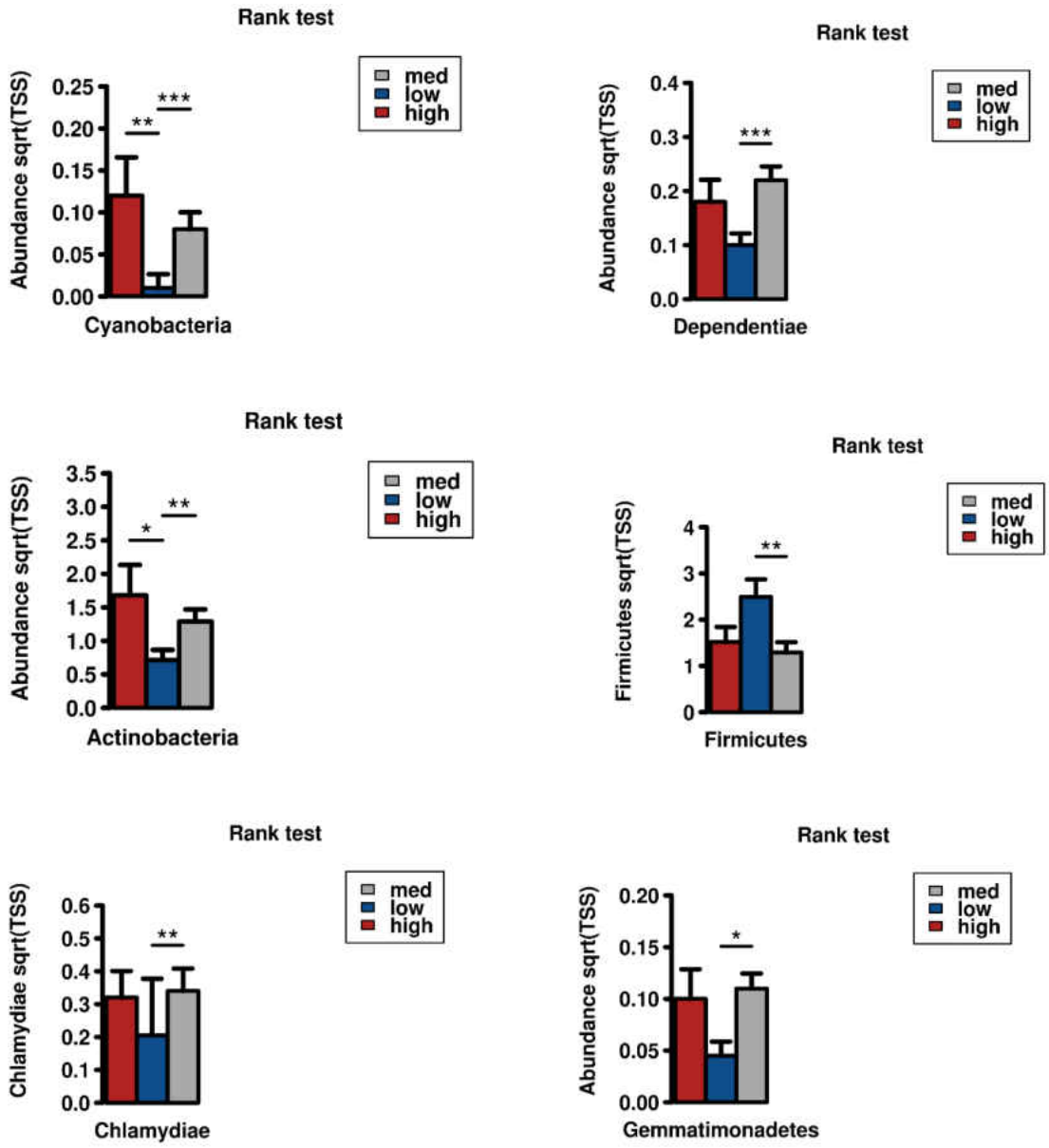

Figure 4.4: Relative abundance of Cyanobacteria, Dependentiae, Actinobacteria, Firmicutes, Chlamydiae, and Gammatimonadetes in relation to shrew weight class.

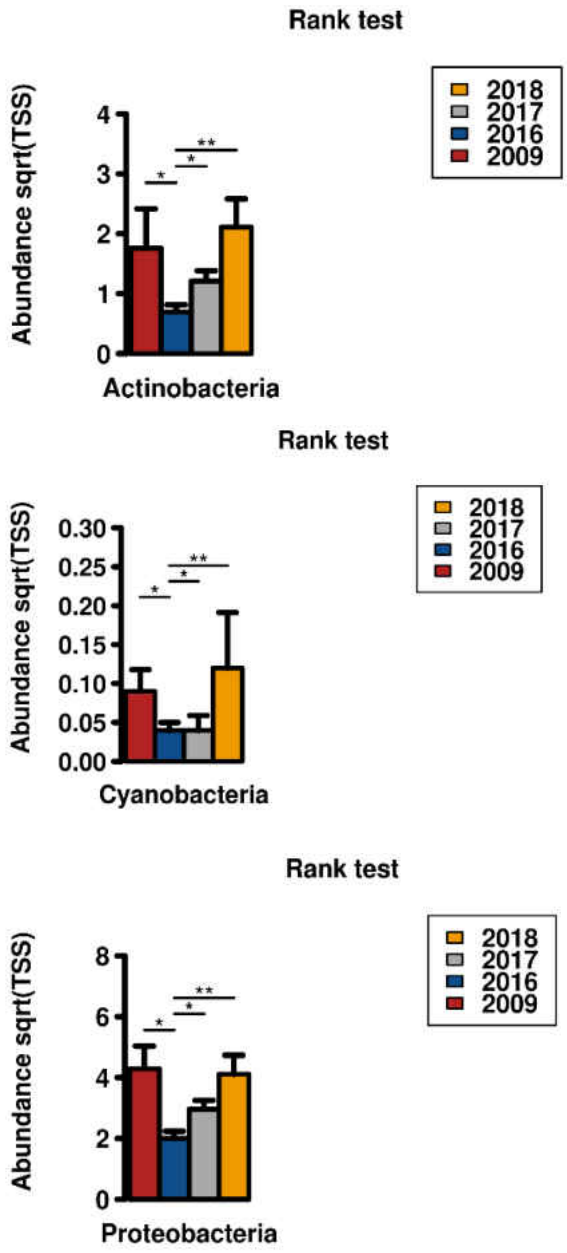

Figure 4.5: Relative abundance of phyla in relation to year for Actinobacteria, Cyanobacteria, and Proteobacteria.

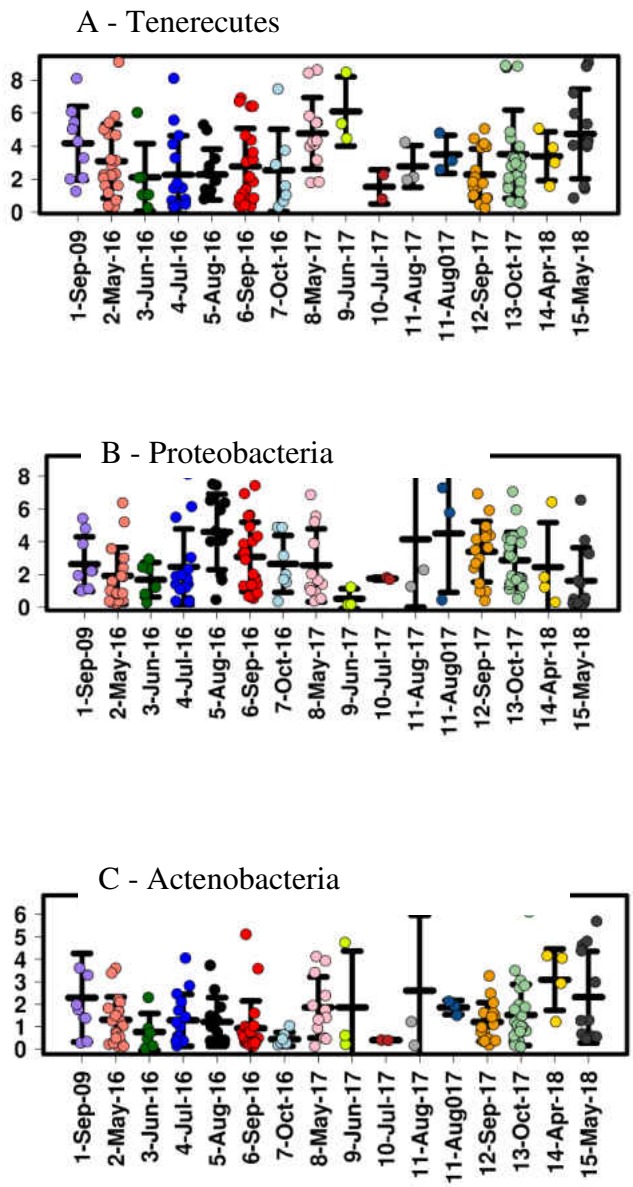

Figure 4.6: Strip plots depicting variation in abundance of three bacterial phyla across collection period, (A) Tenericutes, (B) Proteobacteria, and (C) Actinobacteria across 15 collection periods. Each dot corresponds to an individual shrew. Upper bars represent the 95th quartile, lower bars represent the 5th quartile, and middle bars represent the median for each respective collection period.

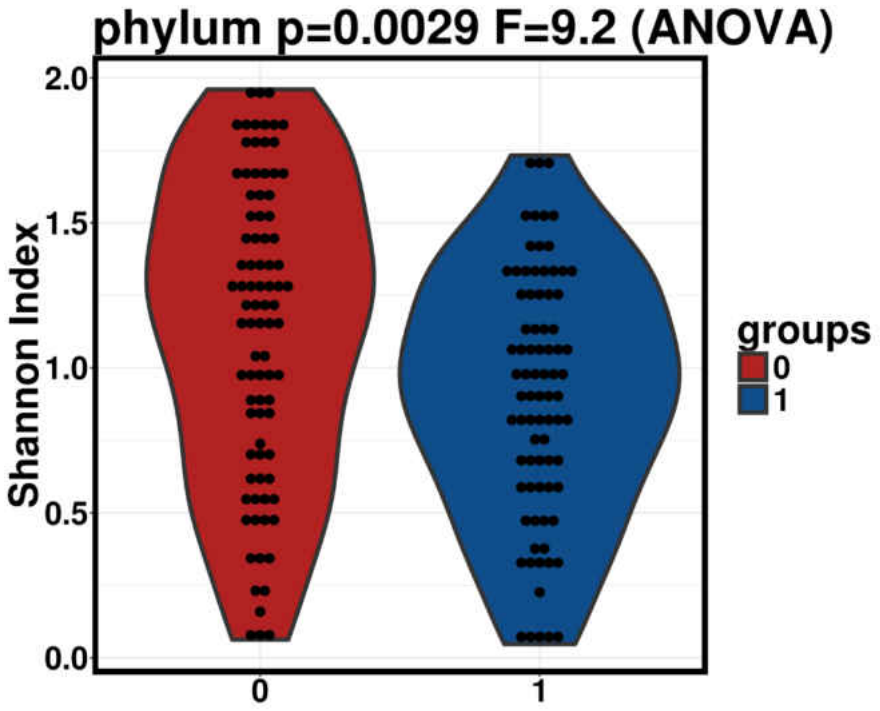

Figure 4.7: Violin plot depicting Shannon diversity index between shrews infected with *Mathevolepis* and those without with 0 representing no infection and 1 representing those infected with *Mathevolepis.*

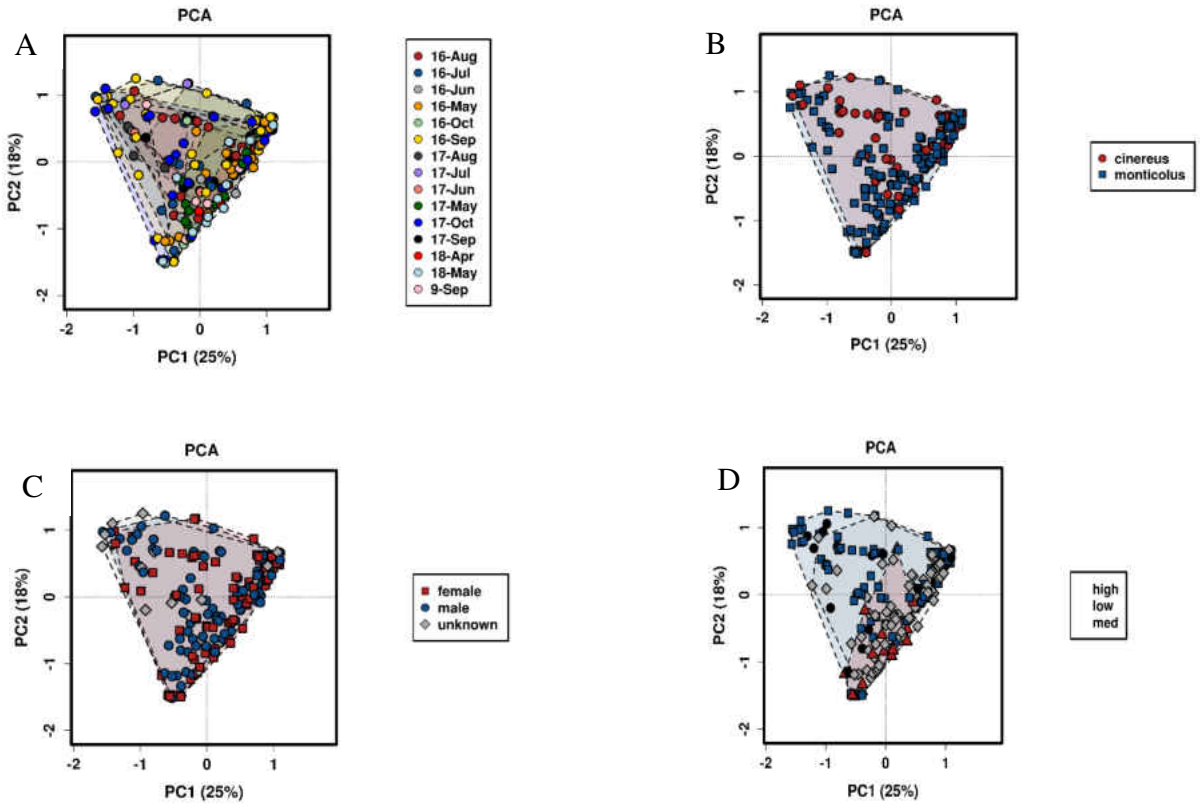

Figure 4.8: Principal coordinate analysis plots of (A) collection month, (B) shrew species, (C) shrew sex, and (D) shrew weight class depicting bacterial beta diversity gut microbiomes of shrews from varying collection periods, species, sex, and weight class.

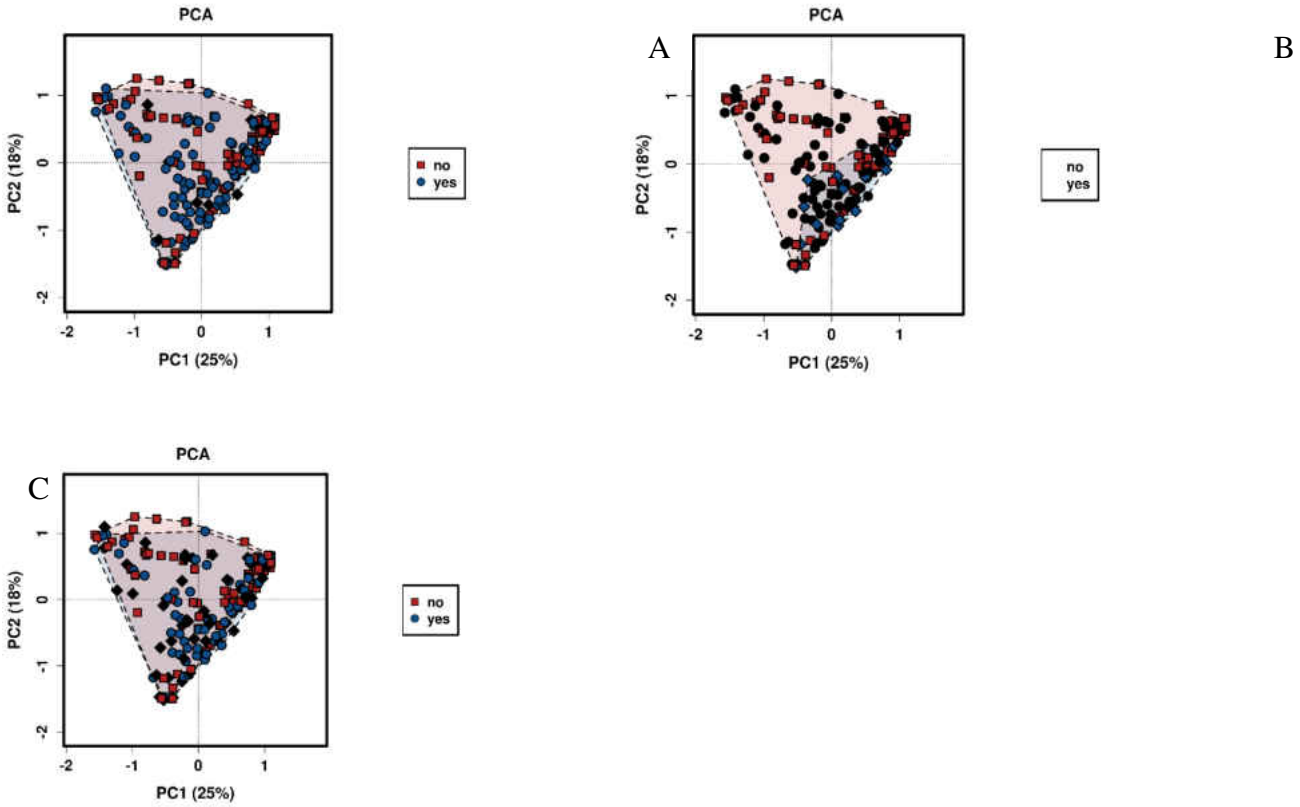

Figure 4.9: Principal coordinate analysis plots of (A) infection with or without ectoparasites, (B) infection with fleas, and (C) infection with ticks, depicting bacterial beta diversity of gut microbiomes of shrews. Color and shape indicate infection with red squares being no infection and blue squares being positive infection.

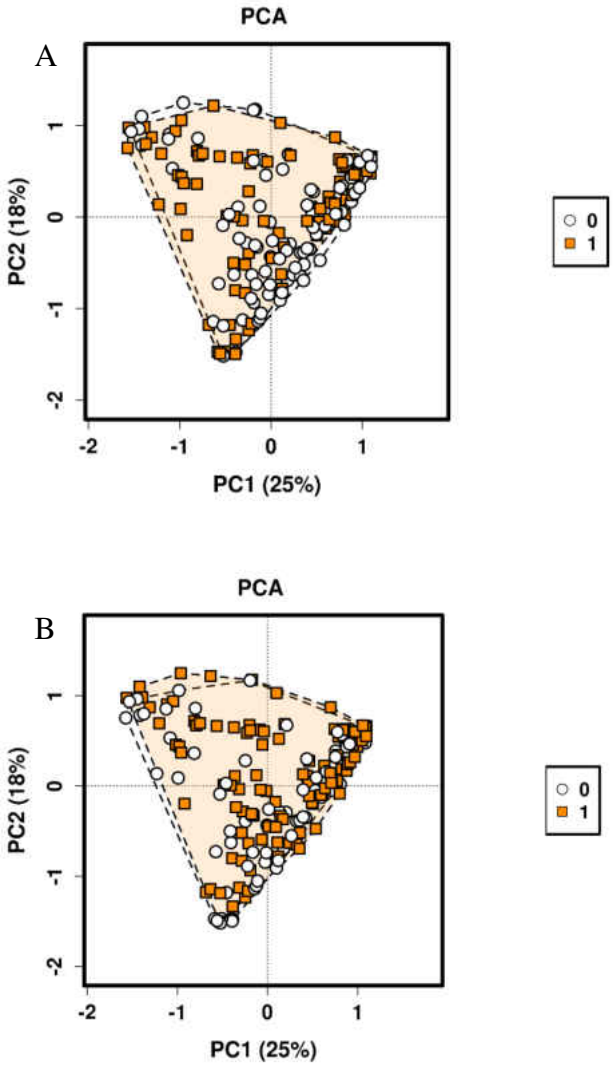

Figure 4.10: Principal coordinate analysis plots of (A) infection with *Mathevolepis*, and (B) infection with *Monocercus*, depicting bacterial beta diversity of gut microbiomes of shrews. Color and shape indicate infection with 0 being negative and 1 being positive.

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