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Bacterial and parasitic microbiome of mantled howler monkeys: interactions and implications of human disturbance

Macfarland, Colin Evan

UNIVERSITY OF CALGARY

Bacterial and parasitic microbiome of mantled howler monkeys: interactions and implications of human disturbance

by

Colin Evan Macfarland

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Abstract

The gastrointestinal (GI) microbiome plays a significant role in contributing to the digestive health of the host. The community of bacteria, parasites and other microorganisms that make up the GI microbiome are known to change in response to external and internal factors including diet, stress, infection, and other environmental conditions. GI parasites are typically harmful organisms that affect hosts through triggering inflammatory response, reducing nutrient availability, and change mutualistic bacterial communities in the gut. However, they have been found to benefit the host in some circumstances. For example, helminthic parasites may have a role protecting the host from chronic inflammation caused by pathogenic bacteria or inflammatory bowel disease. In this thesis, I study the gut bacteria and parasites of populations of mantled howler monkeys (*Alouatta palliata*) in northwestern Costa Rica. I examine the extent to which the bacterial microbiome of howler monkeys is different in the presence of infection by helminths, and if howlers living in areas of anthropogenic habitat fragmentation have observable differences in the bacterial microbiome or helminth parasites. I used coprological examination of fecal flotations to assess parasitism, and 16S high-throughput sequencing to assess bacterial abundances. I found evidence of several helminth taxa infecting these howlers, including from the genera *Controrchis*, *Strongyloides*, *Enterobius*, and an unidentified Trematode. Howler monkeys infected with helminth parasites did not show a significantly different bacterial diversity; however, helminth-positive howlers have significant differences in relative abundances of Clostridiales and Bacteroidales bacteria. Furthermore, I found that howlers living in areas with anthropogenic habitat fragmentation had significantly lower diversity of gastrointestinal bacteria than howlers living in continuous forest. Lastly, I found that the howler populations within Sector Santa Rosa had a higher density of parasite infection, compared to those living in

anthropogenically fragmented habitats. My research on the gastrointestinal and parasite microbiome of howlers provides new insights into the health and ecology of wild primates; further research that compares phenomena occurring in human populations and non-human primates is likely to be fruitful. My work provides new data on the impacts of habitat fragmentation that can inform primate conservation efforts, health monitoring efforts, and management decisions.

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Preface

This thesis is original, unpublished, independent work by the author, C. E. Macfarland

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Chapter 1: Introduction

The Role of the Gut Microbiome in Health and Disease

The microbiome of the gastrointestinal tract contains a massive community of bacteria that contains a collective genome thousands of times larger than our own[1]. It has evolved a mutualistic relationship with, and provides important functions in, healthy humans. These functions include maturation and shaping of the immune system[2]; aiding digestion of macronutrients that our digestive tract cannot natively break down[3]; utilizing an independent network of metabolic pathways to yield important micronutrients[4]; protecting from opportunistic pathogens through protective colonization[5]; and even modulation of the central nervous system through the gut-brain axis[6]. These studies indicate that the bacterial microbiome interacts with the CNS through immune activation, signalling via the vagus nerve, and metabolites interacting with cytokines and other immune cells[7]. The bacterial communities that exist in our GI tract are dynamic, changing over time in response to various stimuli. One major stimulus that can modulate the bacterial microbiome is diet[3,8,9]. Dietary changes can impact communities of bacteria in the gut within days[10], mainly through altering the accessibility of non-digestible carbohydrates, which common taxa such as Bacteroides and Firmicutes, bacterial phyla that include many species, and the genus *Bifidobacterium,* utilize in their own metabolism. In this way a change in host diet can promote or impede bacterial growth[10]. Other external factors have been shown to alter the bacterial microbiome, including stress from heat[11] or cold[12], pathogens[13,14] and antibiotic treatments of those pathogens[15], as well as stress from physical activity[16] or disease elsewhere in the host[17].

Though dynamic and diverse, common human GI microbiomes are comprised of approximately 90% of the bacterial phyla Bacteroides and Firmicutes[10]. Though these two taxa are highly prevalent, most bacterial microbiomes have over 100 different species of bacteria[1], and a higher diversity of bacteria can be an indicator of health[18] as it has been hypothesized that more diverse microbiomes are more resilient to detrimental alterations, and have a greater chance of recovery to homeostasis[9,10]. Within the GI tract, certain genera such as *Lactobacillus* and *Bifidobacterium* are associated with a healthy lifestyle or diet[3] and are commonly administered as probiotics to increase GI health[19]. Due to the benefits that are provided through a healthy community of bacteria living in our gut, dysbiosis, or the destabilization of the core community of bacteria, can be detrimental to health, beyond just poor digestion. As a consequence of the interconnected nature of the bacteria in our GI tract and our immune system, an unbalanced microbiome can induce inflammation[2]. In times of immune related stress on the GI tract, external pathogens can establish infection, or mutualistic bacteria present in the GI tract can become more prominent, become inflammatory, and lead to secondary infections in the body[14,20]. In humans, obesity, inflammatory bowel disease (Crohn's and Colitis)[21], autoimmune disorders such as diabetes, and cardiovascular disease, have all been correlated to poor gut health and a destabilization of the bacterial microbiome[22]. Many of these conditions can be attributed to immune response to modulate or alter the bacterial microbiome in the GI tract, and due to the bi-directional nature of the interaction of the GI microbiome and the host, it is clear that the delicate balance of the bacterial microbiome is important to uphold for the health of the host.

Helminth Interactions with the Microbiome

When examining the homeostatic balance of the bacterial microbiome in the gastrointestinal tract, non-bacterial pathogenic organisms such as parasites, and the role they play, become important considerations. Parasitic infections in the GI tract of humans is extremely common[23], especially helminth parasites. Helminths are a group of wormy parasites that are split into two groups, Platyhelminthes (flatworms (cestodes), incl. tapeworms, as well as flukes (trematodes)), and Nematoda (roundworms). These parasites can infect a variety of internal locations in the body including the lungs, liver, central nervous system, and skin, but commonly inhabit the gastrointestinal tract, and are largely transmitted through ingestion of eggs or larvae[24]. Common helminthic diseases such as ascariasis; an infection of the small intestine with *Ascaris lumbricoides*, hookworm infection; infection of the intestines caused by *Ancylostoma duodenale* or *Necator americanus*, and trichuriasis; an infection of the GI tract by *Trichuris trichiura*, impose an intense burden on humans; nearly one quarter of the world's population is estimated to be infected with a soil-transmitted helminth[25,26]. There are other varieties of common parasite infections that are known as neglected tropical diseases, including bloodborne parasites that contribute to diseases such as malaria, leishmaniasis, or Chagas disease, and together with soil-transmitted helminths, contribute to over 2.7 billion infections worldwide and over half a million deaths per year[27].

Helminthic parasites can also be located in the gastrointestinal tract, and can disrupt the homeostatic balance of bacteria within the microbiome[28,29]. These parasites can disrupt and change the bacterial communities in the mammalian gut[29–31] altering host nutritional uptake[14], triggering inflammatory response themselves or instigate commensal bacteria to induce inflammation[13], and actively compete for space in the gut[29]; causing a cascade of proinflammatory reactions from the host that lead to poor health outcomes[28], including to diarrheal diseases, malnutrition, and cognitive impairment[23].

As detrimental as many helminths are, humans and other mammals have co-evolved over thousands of years with these parasites[32], and recent outlook on certain helminth interactions with their hosts have suggested mutual benefit[33] to infection. Many developing countries' populations have a higher incidence of infection with helminth parasites[25] compared to developed countries, a subset of which are slow to show typical symptoms associated with infection, or are asymptomatic. When these parasites are removed, gut health can be negatively affected, and drug administration does not prevent rapid re-infection[34], suggesting an often overlooked protective benefit to having helminths in the GI tract[35], and the need for better strategies for removing harmful infections in developing countries. Harboring certain helminth parasites has been hypothesized to block colonization of pathogenic bacteria, protecting the hosts from diarrheal diseases and inflammation[35,36]. Even in countries with the infrastructure to combat infectious diseases at a large scale, it has been postulated that the widespread use of anti-helminthic antibiotics and vaccines, as well as an increase in sanitary practices has led to an unanticipated increased rate of inflammatory disorders and sensitivities in the gastrointestinal tract[34]. Extensive research has demonstrated that helminth colonization can have protective benefits against these disorders[37], and increase in the bacterial diversity in the GI tract, as well as have a positive impact on autoimmune disorders (including allergies)[38]. Experimentation with helminth therapies as treatments of IBDs (Crohn's and Colitis)[39,40], have led to successes in the lab, but no widespread medical usage as of yet.

Significance of using Non-Human Primates to Study the GI Microbiome

Gastrointestinal microbiome research has largely been carried out in the lab with controlled murine models[18,41], focusing on how mechanisms of the gut affect health in humans. These model systems have allowed researchers to understand how large a role the bacterial microbiome plays in human health, far beyond the gut[7]. Understanding of the role commensal bacteria play in inflammation, and creating a dysbiotic gut environment[42], how nutrients are broken down and adsorbed through the gut via bacteria[4], and how this can be disrupted with infection with pathogenic bacteria[14], has benefitted from murine model systems. Furthermore, the impact of parasites on the bacterial microbiome has additionally been studied in these systems, including how protozoan parasites such as *Giardia*, can disrupt bacterial communities[13,30], helminths having beneficial relationships in mammalian hosts, resulting in a protective effect against inflammation[29,35], and reducing symptoms of inflammatory bowel disease[33]. Although the importance of laboratory model systems on advancing our understanding of the mechanisms of gastrointestinal health cannot be understated, they are limited in their ability to replicate the complexity of wild settings. To alleviate some of these difficulties, the bacterial gut microbiome of wild mice has been comparatively studied to laboratory mice, and wild mice that are closely related to laboratory mice strains have been found to have a significantly different bacterial community[43], the wild bacterial microbiome was more resilient to disruption through environmental stress[44], and there was a large variation in bacterial diversity between wild populations[45]. This approach helps connect our understanding of what is seen in the lab, and what can be applied in medicine, as you can study observations seen in wild mice in controlled laboratory strains and suss out mechanisms of GI health.

Many phenomena in murine studies are also observed in non-human primates, and the gut microbiome is not an exception. Humans and non-human primates share many physiological similarities and can be infected by the same pathogens, including bacteria and parasites[46]. Studying non-human primates can also grant greater understanding of human health[47,48], and lessons learned focused on human health may be applicable to non-human primates. One study that found helminth infections were associated with an increase in bacterial diversity[36] was inspired by research on rhesus macaques (*Macaca mulatta*)[49]. Other than the benefit to understand our own health through studying non-human primates, we can also better understand our evolutionary history, and how parasites may have co-evolved among primate populations[50].

Other primate studies have demonstrated the vital roles the microbiome plays in primate health, including dietary impacts on bacterial communities[51], nutritional demands met with gut bacteria[52], and how primate phylogeny plays a large role in determining diversity of bacterial gastrointestinal communities[53]. Many of the outcomes of those studies show similarities between the microbiome of non-human primates and humans. Along with studies of primate bacterial microbiomes, primatologists have had an interest in parasites that commonly infect wild populations of monkeys and apes, conducting population wide surveys[54–56], tracking health implications of parasite infections[57], and more recently, started surveying the microbiome, similar to how studies on humans can look to the gut microbiome for indicators of health[58–60]. Early studies looking into the microbiome were sampled exclusively from captive primates[53,61]. Researchers are now more aware of the impact that external stressors, such as captivity and deforestation, have on the bacterial microbiome and parasite infection, and their effect on the gastrointestinal health of these primates. Although parasite colonization within non-human primates and the bacterial microbiome has been extensively studied, and bacterial communities within primates show many the same relationships and dynamics as in humans, few studies have integrated examination of parasites and bacteria together, analyzing their relationship and impact on the primate host.

Howlers as a Study Organism

I studied the GI microbiome and parasite community of a Central American platyrrhine primate, the mantled howler monkey (*Alouatta palliata*). Howler monkeys, in general, live in small social groups of around 6-15 members, with some howlers living in larger groups up to 20 individuals[62]. In contrast to many other platyrrhine primates, howler males and females both disperse, and are the most folivorous Central and South American monkey, with their diet made up of leaves, flowers, and sometimes seeds and fruits[62,63]. Howlers are known to rest for more than 80% of the day, with most of the rest of that time spent eating leaves[63]. Howler monkeys are an accessible study species, as they are relatively slow moving, easily habituated to humans, and easy to track through their habitat because they rest for long periods of time. Howlers are also found to be commonly infected with parasites[56,57,64,65], and many studies have been done characterizing their gastrointestinal bacterial microbiome[51,52,66]. Through non-invasive examination of fecal samples from different social groups of howler monkeys, comparisons between their GI microbiomes can be made without the need for captivity or direct human contact. Although sampling is more difficult in a natural system without direct contact, using a non-captive, indirect approach is vital for understanding the microbiome without introducing unnecessary variables of stress, artificial diet, or excessive human interaction, as captivity has been shown to alter the bacterial microbiomes of primates[61,67]. Due to the nature of the howler monkey diet being primarily folivorous, the bacterial communities and their interaction with parasites in their gut play an essential role in maintaining their dietary ecology and health.

Folivores have the difficult job of breaking down leaves, stems, and cellulose loaded plant material to obtain proportionally less nutritional value compared to omnivorous diets that include insects, and small vertebrates. Howlers, as well as other folivorous primates such as colobus monkeys, have specific adaptations to their GI tract, including an enlarged hindgut[52,63]. This extends the transit time of food through their digestive tract, allowing extended fermentation. The breakdown of tough foods is largely handled by the bacteria in their hindgut[51,52], these bacteria being a necessity for accessing all the potential nutrients of their nutrient poor diet. Additionally their diets are seasonal, opportunistically feeding on fruits when available[51,68] , thus changes of the bacterial communities in the howler gut is common, and a diverse bacterial community is required for the meeting nutritional demands when transitioning to different food types.

As with other mammals, modulation of the gut microbiome through parasite interaction could play a vital role in homeostasis. Howlers have been known to be infected with many parasites, including *Giardia*[46,56,57], *Controrchis*[56,57,64], *Trypanoxyuris*[56,57], *Entamoeba*[57], *Ascaridae*[57], and *Strongyloides*[65]. *Giardia* and helminth parasites, have been implicated in inflammatory disruptions and modulation of the bacterial microbiome[28,30], and could have an important role in the maintenance or disruption of howler microbiomes, affecting the nutritional uptake, and overall health of these primates. In addition, habitat fragmentation as it relates to the microbiome of primates, including howlers has been previously studied, finding fragmentation, and degradation of habitat having a negative impact on the diversity of bacteria, an increase in parasite infection, and lowered health[59,66,69]. These studies present a good starting point for further research in this area. My present study builds on this past research on howler helminth parasites and gut microbiota by studying wild mantled howler monkeys in the Guanacaste Province of Costa Rica.

Figure 1: A wild mantled howler (*Alouatta palliata*) in a cecropia tree (*Cecropia peltata*) from our sample study population. Image credit: Amanda Melin.

Study Location

Samples from this study were collected in the northwestern Costa Rican Guanacaste province. Samples were collected from three locations: 1) Sector Santa Rosa (SSR), Area de Conservación Guanacaste (ACG), a nationally protected tropical dry forest; 2) near the tourist town of Tamarindo (a fragmented habitat approximately 60 km South of SSR); and 3) from Eladio's Ranch, a small ranch interspersed with fragmented forest (Figure 2). The age-sex composition of these groups was not well known, but each contained multiple adult males and females, along with juveniles (Suppl. Table 1). These three sites differ markedly in their degree of anthropogenic disturbance and forest intactness. SSR represents an intact forest with minimal human or agricultural disturbance; Tamarindo represents a highly disturbed, semi-urban fragmentary habitat with considerable potential for human disturbance, and Eladio's Ranch represents a fragmentary forest with a large amount of agricultural disturbance, and low human interaction. The ACG is a UNESCO world heritage site, with over 104,000 ha of protected land. This biodiversity hotspot is a remediated ecosystem, largely recovered from extensive use for ranching, where a patchwork of forests and savannah ecosystems gave way to tropical dry forests[70], one of the most endangered tropical habitats[71]. The protection of these forests allowed primate species, including howlers[72], to recover from low populations. Sector Santa Rosa is one of 18 biological and geographically distinct sectors of the ACG, beginning as a national park in 1971[70], and initiating the conservation effort that eventually led to the formation of the ACG in 1986. Tropical dry forests are harsh environments, with extreme variation in rainfall, where a six-month wet season provides almost all of the rain for the entire year, followed by a six-month dry season, where food and water availability can be sparse[73,74]

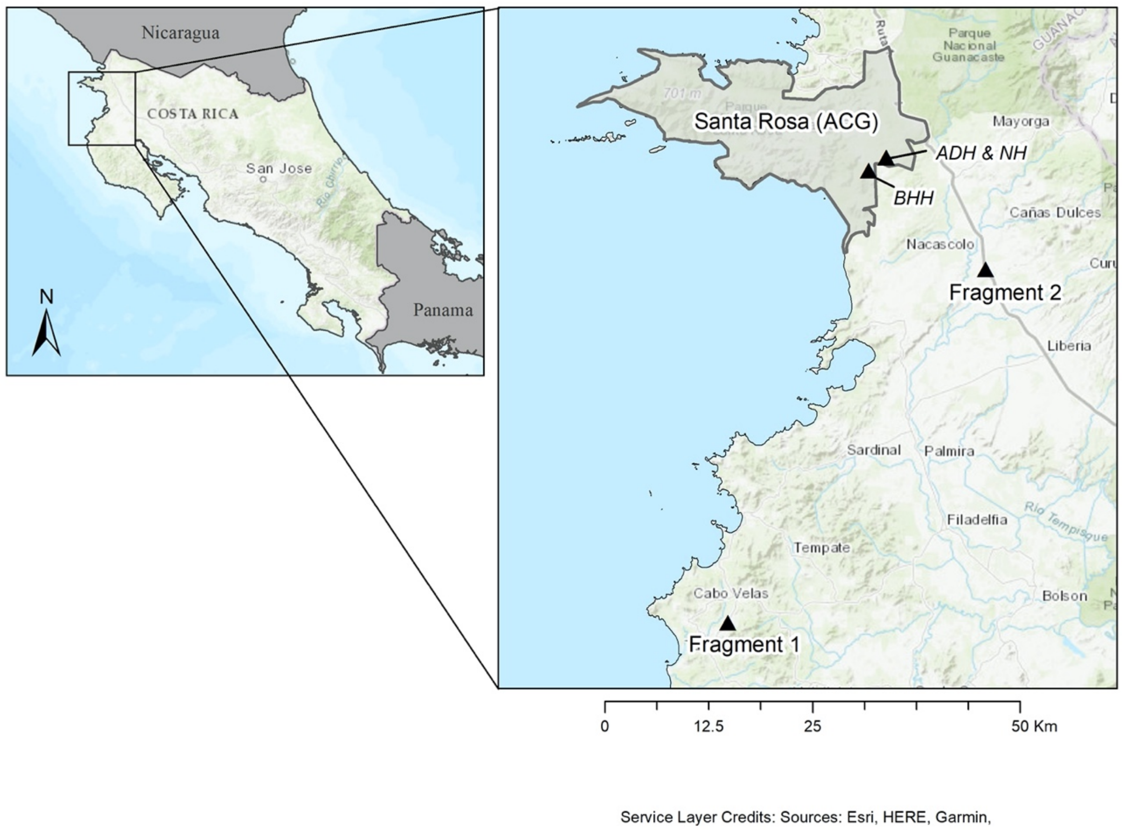

Figure 2: Inset map of sampling locations within northwestern province of Guanacaste, Costa Rica. Sector Santa Rosa, part of the ACG, has 3 social groups of howlers that live in close proximity. Fragment 1 corresponds to howlers living near Tamarindo, and Fragment 2 correspond to howlers living near Eladio's Ranch. Map courtesy of Tracy Wyman, this map was created in ArcGIS v 1.7.1.

Thesis Focus, and Data Chapters

This study aims to bridge a gap in knowledge between the bacterial microbiome of howlers, and the helminth parasites that infect them. I will do this by analyzing 1) the relationship helminth presence has on the diversity and abundance of bacterial taxa, and 2) interactions between the bacterial microbiome and helminth parasites in these primates. Additionally, examining the impact that anthropogenic fragmentation of habitat has on the bacteria and helminth parasites by comparing samples collected in different environments. In chapter 3, I examine the relationship between helminth infections and the bacterial makeup of the gut microbiome of howler monkeys, by looking at those that are presently infected, and comparing to howlers with no detectable helminth infection. I hypothesize that helminth infections correlate to a difference in bacterial diversity and abundance, including bacterial groups that may play a protective role in the gastrointestinal tract. In these two groups, I will test for significant differences in the bacterial communities, both in diversity and abundance. Further, I will examine the prevalence of helminth infection in these sample populations of howlers in general, giving insight into the potential effects parasites have on the bacterial makeup of the microbiome, and the possibility of a beneficial relationship that helminths may have in howlers. In chapter 4, I examine bacterial diversity of howler fecal samples collected in three contexts, two anthropogenically disturbed habitats, and one intact, protected forest habitat. Additionally, I will analyze helminth parasite prevalence in these habitats, examining the relationship between intact and fragmented forest habitat effects on the bacterial and parasite microbiome of howlers. I hypothesize that anthropogenic disturbance has negative health consequences in terms of bacterial diversity and parasite density of infection. These will provide new data on the effect that parasites have in the primate GI microbiome, and inform future comparative research programs and conservation efforts for monitoring the gastrointestinal health of wild primates. Furthermore, I seek to promote the practice of studying wild primates to deepen our understanding of our relationship with parasites, and how they may impact our bacterial microbiome in the gastrointestinal tract.

Chapter 2: Methods

Study Species and Collection Area

Howler monkeys (genus *Alouatta*) are medium-sized, arboreal, folivorous-frugivorous primates found in Mexico and throughout the tropical forests of Central and South America. Howlers live in multi-male, multi-female groups in which both sexes disperse from their natal group. They are known to naturally vary in terms of the presence of gastrointestinal helminth parasites[56,65] and have been an important primate model species for understanding the gut bacterial microbiome[51,52]. Samples were collected from five social groups of wild mantled howler monkeys (*Alouatta palliata*) in the northwestern province of Guanacaste in Costa Rica. These mantled howlers have moderate sexual dimorphism, live in medium size groups of 15-30 mixed sex individuals[72], with adult females (4 Kg) smaller than adult males (6.5 Kg)[62,72,75]. These five social groups were found in two different types of habitat; one group was living on the border of an active cattle ranch (Eladio's Ranch) and eco-tourist "airbnb". This site was located off the interamerican highway, route 1, approximately 40km south of the entrance to Sector Santa Rosa. The habitat type was cattle pasture, and the howler group lived in the trees, primarily mango trees that were planted around the border to provide shade. The second fragmented site was a suburban area near the town of Tamarindo, on the Nicoya peninsula of the Guanacaste Province. This group traveled among a mosaic of natural and cultivated fruit trees located near a condominium complex and small residential houses. The last three groups reside in a continuous forest within Sector Santa Rosa, one of 18 sectors that make up the Área de Conservación Guanacaste (ACG) in northwestern Costa Rica. This habitat is a tropical dry forest, a UNESCO world heritage site, a biodiversity hotspot, and an example of a successful ecological recovery of one of the worlds most endangered habitats[70,71]. The ACG, through skillful planning, conservation, and regrowth, transitioned from a severely anthropogenically disturbed habitat, with fragmented patches of forest and savannah type environments for cattle, to a variety of heavily continuously forested areas, rich in biodiversity[70,74]. Sector Santa Rosa (SSR) is a national park founded in 1971 to help protect and regenerate the tropical dry forests of Costa Rica, is within the ACG. Guanacaste province is known to have large seasonal variation[73], oscillating between extreme dry and wet seasons, where almost the entire year's precipitation falls within a rough 6 month window[76,77].

My study followed the guidelines of the Canadian Council of Animal Care, and was approved by the Animal Care Committee (AC15-0161) of the University of Calgary, and by the Sistema Nacional de Área de Conservación (R-ACG-PI-004-2017) in Costa Rica.

Sample Collection

One fecal sample per individual was collected from 25 individual howlers in 5 social groups immediately following defecation (Suppl. Table 1). Samples were collected by Amanda Melin and Shasta Webb during the rainy season in July of 2017 within 2 weeks of each other to control for variation due to seasonality. Each fecal sample upon collection was divided into two treatments; one portion was stored in 8 mL of pre-aliquoted 96% ethanol for coprological study to identify helminths and stored at room temperature. The second portion was placed in a 2ml sterile cryotube on ice. Within 4 hours the cryotubes were placed in a liquid nitrogen dry-shipper and reserved for genomic microbiome analysis. All samples were subsequently transported to the primate genomics laboratory at University of Calgary. Upon arrival ethanol samples were kept at room temperature away from direct light, and cryotubes were transferred to a -80 C freezer for storage. Four ethanol samples were exhausted during coprological preparation before an aliquot could be taken for use in sequencing, therefore sample sizes reported for coprological portions of this study are n = 21, whereas sample sizes for bacterial microbiome portions that utilized the frozen samples are n = 25.

Parasite Flotation and Identification

I identified parasites in the Kutz Research Laboratory through sucrose flotation and coprological microscopy following the Canadian Wildlife Health Cooperative's standard operating procedures. In brief, the samples were homogenized by pulverizing and mixing in a disposable cup with a tongue depressor, during which, we added approximately 12 ml of reverse osmosis water. This mixture was then filtered through a double layer of cheesecloth to separate out large solids, and the cup was rinsed to wash any extra fecal slurry though the filter. The filtrate was then poured into a test tube, and centrifuged for 10 minutes at 500 G and the supernatant was poured off leaving the precipitate of fecal slurry. Next, a small volume of Sheather's sugar solution[78] was added and the tube was vortexed to mix the fecal slurry with the sugar solution, then the tubes were filled with Sheather's until a meniscus was visible above the test tube. A coverslip was set on top of each tube, and they were spun for another 10 minutes at 500 G to allow any parasitic eggs to attach to the coverslip. The coverslips were then taken off and placed on a slide for microscopic analysis.

I inspected the slides under 400x magnification counted all eggs and cysts, and photos of suspected parasites were taken for identification. The density of parasites was calculated as eggs per millilitre, using the original total volume of feces collected, which was calculated based on the amount of displacement the sample had in 8 mL of ethanol in the collection tubes.

DNA Extraction, Library Preparation, and Sequencing for Microbial Identification

Extraction

DNA was extracted in duplicate alongside 7 blanks (made using molecular biology grade water in place of fecal sample) and the 25 cryo-frozen and ethanol stored fecal samples using a phenol-chloroform bead beating extraction protocol[79]. These blanks were treated identically to fecal samples, and were carried through every step in the procedure. Briefly, I added 500 µl phenol-chloroform:IAA to approximately 200 ul of fecal sample and an equal proportion of zirconium beads. The standard bead-beating method of extraction was modified by adding 20 µl of Proteinase K to each sample and left to digest for 2 hours at 56 degrees C. This Proteinase K step was performed to allow the more robust outer casing of potential parasitic eggs and cysts to be digested, releasing a greater amount of DNA, so that future work may also examine 18s sequences for taxonomical parasite identification. After two rounds of successive bead beating and chilling on ice, the supernatant was then mixed with another aliquot of phenol chloroform and centrifuged to leave a supernatant of solubilized DNA. This was left to precipitate overnight at -20 C with isopropanol and sodium acetate. The following day, chilled samples were centrifuged to pellet DNA and washed with ethanol. Then, washed DNA was solubilized in TE buffer, and purified using a spin column purification as per the Invitrogen Quick PCR Purification Kit purification procedure. Extracted and purified DNA was then frozen at -20 C until ready to be quantified using a standard Qubit 3 Fluorometer procedure, and normalized based on Qubit readings to 1ng/µl prior to library preparation.

Library Preparation and 16s Sequencing

I prepared 16s libraries for sequencing using a two-step amplification protocol.

For PCR1, the following primers with redundant bases (Y = T or C, M = A or C, H = A or C or T, V = G or C or A, W = A or T) were used from the University of Texas genomics core[80]:

Hyb515F_rRNA: 5'-
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGYCAGCMGCCGCGGTA** -3'

Hyb806R_rRNA: 5'-
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGACTACHVGGGTWTCTAAT**-3'

These primers amplify a 292 base pair portion of the V4 region of the 16S gene in *E. coli*. Bolded regions of the primer are complimentary to the bacterial 16S rRNA gene, un-bolded regions are Illumina specific primers for sequencing priming, and have complementary sites to the index/barcode/flow cell binding nucleotides in PCR2. This PCR reaction was run in triplicate to minimize jackpot effects and other biases that can occur during PCR that could affect the estimated abundance of different taxa during analysis. PCR 1 had a unique reagent mix that was optimized in house (Supp. Table 2), and utilized settings that were also optimized (Supp. Table 2).

Amplified DNA from PCR1 was purified following a bead-based purification protocol based on Agencourt's AMPure XP magnetic Bead Based Purification, but I utilized Sera-Mag Magnetic Speed Beads to decrease costs. In brief, I combined 20 µl of each sample in triplicate into a single well, with 40 µl of molecular biology grade water to bring the volume to 100 µl. 80 µl of room temperature beads were added, mixed with the samples, and incubated for 5 minutes. Supernatant was discarded by placing the plate on a magnetic stand and pipetting out the liquid, leaving the beads undisturbed. Fresh 80% ethanol was then used to wash the wells twice (while still on the magnet), and then the beads were left to air dry for 3-5 minutes. Beads were resuspended and DNA was solubilized in molecular biology grade water off the magnetic stand, and then placed back on the stand and 32.5 µl of each sample was collected for use in PCR2, with portions of some samples used for quality checks via Qubit and gel visualization. Illumina i7 and i5 barcoded primers were attached during PCR2 using a different set of reagents than PCR 1 (Supp. Table 4), and used reaction conditions that were optimized for the change in reagents (Supp. Table 5).

After the second PCR, I performed a second bead-based purification as per the protocol described for the first. Quantification of all samples after purification 2 was done via standard Qubit quantification protocols, and 50 ng of DNA for each sample was used in creating a pooled library. In the case of there being no Qubit detectable DNA in blanks and low quantity samples, 20 µl of each of these samples were added to the pooled library.

The pooled samples were analyzed using a KAPA qPCR Library Quantification Kit to determine the DNA concentration of the prepared library. The working concentration calculated by the qPCR was 18.4 nM and the library was diluted down to 4 nM for sequencing. After library preparation, I sequenced was performed on an Illumina MiSeq Sequencer following the standard protocol and using a MiSeq Reagent Kit v2 (500-cycles) kit.

Statistic and Bioinformatic Analysis

I performed post-sequencing quality checks and demultiplexing of raw reads using Illumina BaseSpace, and FASTQ files were downloaded for further processing. I conducted all statistical and bioinformatic analysis in R version 4.0.3. The FASTQ sequences were processed on University of Calgary's Synergy Server, primer removal and pre-filtering was done using cutadapt[81] (v2.7). Reads shorter than 150 nucleotides were removed before being run through cutadapt, and further filtering and processing was done using DADA2[82] (v1.14). Filtering parameters for DADA2 were as follows: forward and reverse reads were truncated at 220 and 190 nucleotides respectively, max number of "N's" was set to 0, maximum errors for forward and reverse reads were set and 2 and 3 respectively, with the rest of the parameters set to their default values. Standard dereplication, merging, chimera removal, and assignment of amplicon sequence variants (ASVs) using the "pseudo" pooling method was performed. ASVs are utilized by the DADA2 protocol rather than operational taxonomic units (OTUs). ASVs are a higher resolution assignment, where reads are grouped based on variants in amplicon sequences, and differences as small as one nucleotide can be identified, this is different than OTU classification, as that method bins reads based on a fixed threshold of dissimilarity[83]. I used the Silva database[84] v132 as a reference library for bacterial taxonomy assignment, and performed the optional species assignment step using the Silva Species assignment database v132.

After processing, I used decontam (v1.4.0)[85] to remove contaminants found in blank samples, and Phyloseq[86] (v1.30) to arrange and organize 16S data generated by DADA2, as well as to calculate and visualize alpha and beta diversity of bacterial species. Statistical analysis of alpha diversity was calculated using a wilcox test, a non-parametric version of a t-test, and beta diversity was calculated with a PERMANOVA test using the pairwise adonis package[87] (v0.4) Differential abundance testing was carried out with DESeq2[88] (v1.26) to test for different bacterial ASVs abundance in samples with, versus without detectable helminth eggs present. The prevalence of different types of parasites and density of parasite infection (in eggs/mL) for different locations of howlers were visualized using ggplot2[89] (v3.2.1). The full code used for sequence processing and statistical analysis and visualization is available as a repository on GitHub. (https://github.com/cemacfar/ThesisAnalysis2021)

<u>**Chapter 3: Parasite Interactions with the Gastrointestinal Bacterial Microbiome**</u>

Abstract

The gastrointestinal (GI) microbiome is a key regulator of health and disease and composed of diverse bacteria, viruses, fungi, and parasites. While parasitism often leads to negative health outcomes, recent studies on humans and mice have also shown that some helminth infections can be beneficial by reducing inflammation. Specifically, a change in the relative abundance of Bacteroidales and Clostridiales significantly shifts the microbiome, disrupting the symbiotic bacteria, triggering an immune response, and leading to chronic inflammation. I ask whether natural infection by helminth parasites influences the bacterial microbiome in a natural environment. I study the intestinal bacteria and helminthic parasites of wild Costa Rican howler monkeys (*Alouatta palliata*). I find that the helminth *Controrchis* sp. was the most prevalent parasite in my study of 21 howlers, and while the overall alpha and beta diversity did not statistically significantly change in association with detectable presence of helminth infection, the differential abundance of two bacterial phyla, Bacteroidales and Clostridiales, changed significantly. These data support the hypothesis that the gut microbiome is influenced by parasite infection in ways that are consistent with relative abundances of bacteria involved in inflammatory responses, or the bacterial microbiome community is permissive of helminth infection. This provides tentative new support of the potential protective roles of helminth parasites in reducing inflammation.

Introduction

The gastrointestinal (GI) microbiome of mammals is comprised of prokaryotic and eukaryotic organisms that form a complex community and play essential roles in health and digestion[90]. Conversely, microbiomes that have had disruptions in the homeostatic balance of their bacterial communities have been implicated in a broad range of disorders. Microbiota bacteria dynamically shift in response to changes in diet[51], environmental stressors[66], pathogenic infection[29,10] and interactions between bacteria and parasites[13]. These inter-domain interactions in the gut have garnered much recent interest in mammalian research[28,29]. Helminths are parasitic worms that can infect almost every organ and system in the host[91], and are common in the GI tract. Studies of germ-free mice have shown that bacteria play a role in modulating the GI tract to in response to infection by a variety of helminth parasites (e.g. *Heligmosomoides polygyrus bakeri, Nippostrongylus brasiliensis* and *Trichinella spiralis*) in ways that may facilitate – or alternatively – bolster defense against helminth infection[28]. Furthermore, while parasitic infection often negatively impacts hosts, some studies find evidence that GI parasites, including helminths, may have a positive role in promoting a healthy gut microbiome. These studies have shown helminth infection to increase bacterial diversity in the gut[36,29], and modulate existing communities of bacteria[28]. Importantly, recent evidence suggests that helminth parasites may decrease severity of inflammatory diseases through moderating bacterial communities in the gut, leading to healthier GI outcomes[38].

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis[9], are a large health concern, decreasing quality of life for approximately 3.9 million females and 3 million males globally, with the majority of cases in developed countries, and placing large burdens on public health systems[92]. A growing body of research indicates that parasites, particularly helminths, play a role in shaping the GI microbiome community structure [28] and this includes assisting in modulating IBD. Helminthic therapies – the intentional introduction of helminths into the GI tract – are being investigated for inflammatory bowel disease, as they appear to promote homeostatic microbiota and reduce pro-inflammatory bacterial species[37,40,49]; however support for this is conflicting[93] and the interaction may be specific to the bacteria-parasite species involved in the interaction, and widespread clinical usage has not been implemented. Ramanan et. al.[35] looked in depth at the protective benefits that helminths can have for humans in developing countries, where natural helminth infections of the GI tract are very common[25]. The authors demonstrated that when helminths were removed via anti-helminthic drugs, a significant increase in a taxa of a proinflammatory gram-negative Bacteroidales bacteria was observed. In the same study, mice with initially inflamed GI tracts colonized with helminthic parasites showed reduced inflammation, as well as a decrease in proportion of inflammatory Bacteroidales, and an increase in a different order of gram-positive bacteria, Clostridiales, a group of bacteria that have been shown to have an inverse relationship in respect to general abundance with Bacteroidales. Overall, these two groups of bacteria have shown significant modulation in abundance linked to the presence of helminths[35,94,95], as well as being associated with inflammation in the gut in mice and humans[21,41,96].

These experimental studies are insightful, however, there is a dearth of studies that have attempted to study parasite-bacteria-host interactions in natural systems, as appropriate data sets of samples consistently collected and appropriately stored that show natural variation in presence of parasites are difficult to obtain[61]. In particular, studies of primates are needed as conservation and monitoring of their health and habitat are a concern[97], and can be valuable because of their physiological and genetic similarities to humans, and their widespread use as model systems for studying immunology and infection[48,98]. This study assesses the helminthic parasites and bacterial microbiome of wild howler monkeys. I predict that there will be a high proportion of howlers that are positive for helminth infection, and that alpha and beta diversity of bacterial communities in howlers that are infected by helminths will significantly differ; specifically those with helminths will show a higher bacterial diversity measure than those without, as this relationship has previously been implicated in humans, as well as non-human primate species[36,49]. I further predict that helminth infections correlate with shifts in relative abundance of bacterial taxa that may be protective. Specifically, I predict that there will be a significant change in the abundance of bacteria in the orders Bacteroidales and Clostridales linked to helminth infection.

Methods

Sample Collection

Howler monkey fecal samples were collected during the rainy season in July 2017 from three study locations in the Guanacaste province of Costa Rica; Sector Santa Rosa (SSR), a protected tropical dry forest, and two forest fragments, one located outside the town of Tamarindo, and one near an active Ranch and 'airbnb'. One fecal sample was collected from 25 individual howler monkeys, split, and stored in either 96% ethanol, or set on ice to be frozen in liquid nitrogen.

Parasite Flotation and Identification

Homogenized samples were floated in Sheather's sucrose solution, fixed to slides, and inspected under 400x magnification. Egg counts were recorded, and photos were taken for morphological identification with the aid of the Kutz Research Group.

DNA Extraction, Library Preparation, and Sequencing for Microbial Identification

DNA was extracted from fecal samples using a bead-beater based extraction method with an additional incubation with proteinase K. Blanks were extracted alongside DNA to limit contamination. DNA was purified using an Invitrogen quick PCR purification kit, and quantified before amplification with a Qubit 3 Fluorometer, and normalized to 1ng/µl with molecular biology grade water.

The V4 region of the 16S genome was amplified using a two-step amplification protocol, and utilizing primers from the University of Texas genomics core and Illumina i7 and i5 barcoded primers, with a bead based purification preformed after each amplification. Amplified DNA was then quantified using a Qubit 3 Fluorometer, and pooled to be analyzed with a KAPA qPCR Library Quantification Kit. The pooled library was diluted to 4nM for sequencing on an Illumina MiSeq Sequencer, using the MiSeq Reagent Kit v2 (500 cycles) kit.

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FASTQ files were taken from Illumina BaseSpace for processing, which included pre-filtering and primer removal through cutadapt (v2.7), and further processing with DADA2 (v1.14). ASVs were assigned by DADA2 using the Silva v132 database as a reference library. Packages for analysis used in R (v 4.0.3) included decontam (v1.4.0) for removing blanks, Phyloseq (v1.30) for organizing data and assessing alpha diversity, pairwise adonis (v0.4) package for beta diversity testing, DESeq2 (v1.26) for differential abundance analysis, and ggplot2 (v3.2.1) for al visualization.

See *Chapter 2: Methods*, for a detailed explanation of procedures used in this study.

<u>*Results*</u>

Parasite Prevalence

I found a detectable helminth infection in 15 out of the 21 howler fecal samples examined. Through coprological examination I found that this sample population of howlers were infected with four identifiable helminth parasites: *Contorchis biliophilus*, *Strongyloides sp*., an unidentified Trematode, and a Pinworm (*Enterobius sp.*) (Figure 3). The trematode, *Controrchis biliophilus*, was the most prevalent, being observed in 9 out of 21 samples, and a separate unidentified Trematode was second, which we found in 6 samples (Figure 3).

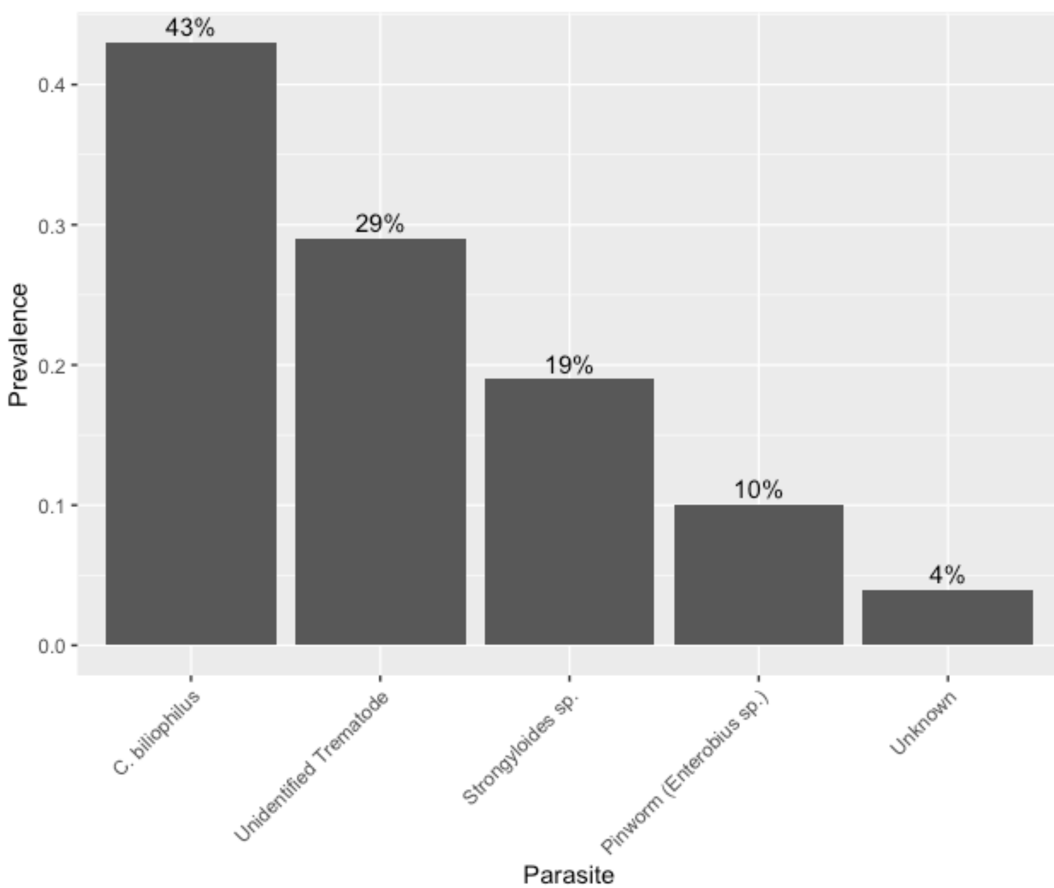

Figure 3: Prevalence (the percent of samples that contained at least one of the identified parasites) of helminth parasites found within the 21 fecal samples collected from wild howler monkeys. The number above each bar represents the prevalence of that parasite.

Alpha and Beta Diversity

After filtering all bacterial ASVs to only include ASVs belonging to samples with a read count of over 500, a total of 184 ASVs were resolved to at least the taxonomical level of Family. I found there to be no significant difference in alpha diversity of the bacterial communities within those individuals that were found to be infected with helminths ($n = 15$) to those negative for infection ($n = 6$), in both richness (Chao1 estimate of richness $p = 0.85$) and diversity (Shannon estimate of diversity $p = 0.62$) measures (Figure 4). To test for differences in beta diversity in the composition of the bacterial microbiome between parasite positive and negative groups, I used a Principal Coordinates Analysis (PCoA) to test for dissimilarities between howler samples using Bray-Curtis dissimilarity method (Figure 5). Helminth-infected howlers showed no clear clustering pattern, and no significant difference was seen between individuals with and without infection when a nonparametric PERMANOVA test was applied to these two groups ($p = 0.111$)

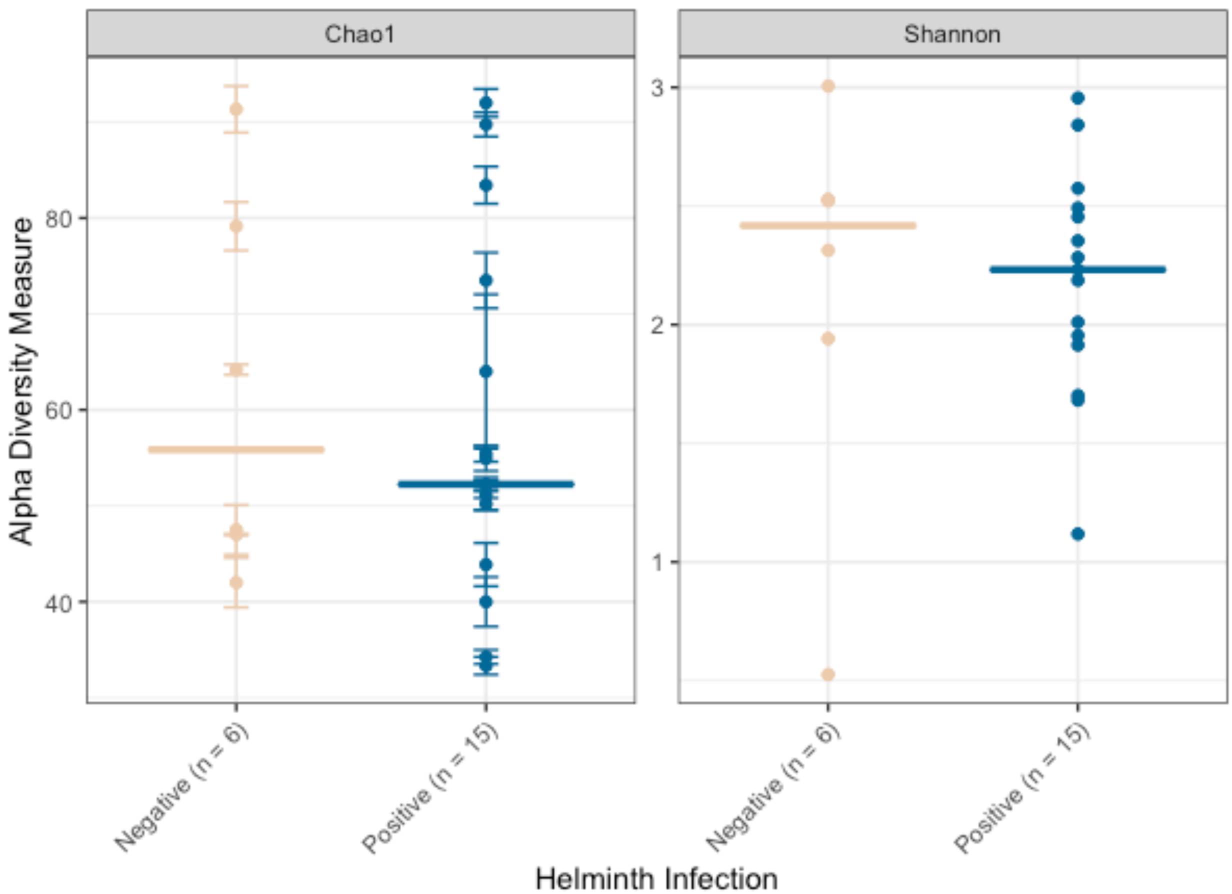

Figure 4: Alpha diversity (Chao1 richness and Shannon diversity) measures for howler monkeys positive or negative for helminth infections. Chao1 test calculates an estimated richness of the assigned ASVs of each howler, as well as variance, seen as bars placed around each point; Shannon estimates diversity (both richness and evenness of assigned ASVs). The large horizontal line represents the median and dots represent individual howlers. No significance in alpha diversity measures were seen in richness ($p_{Chao1} = 0.85$) or diversity ($p_{Shannon} = 0.62$). 15 howler monkeys were positive for helminths, and 6 were negative.

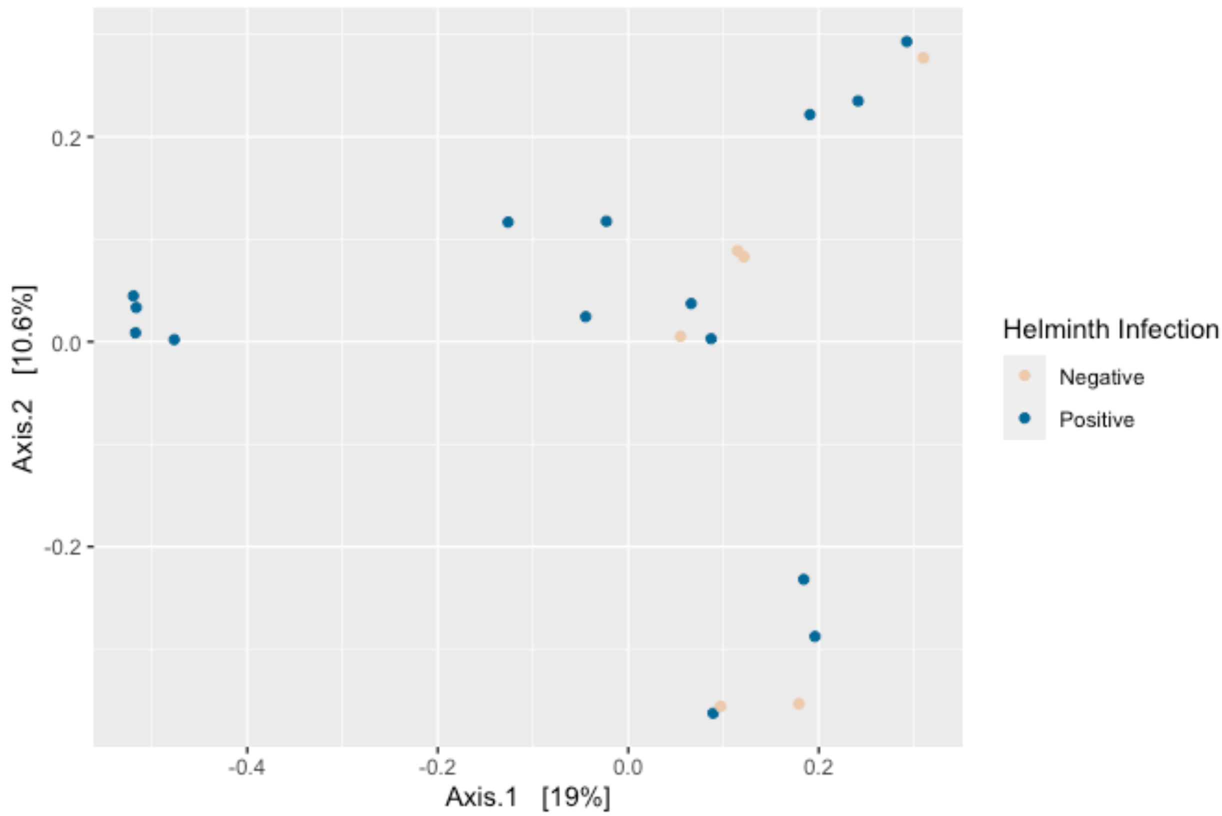

Figure 5: Principal Coordinates Analysis (PCoA) plot performed with Bray-Curtis dissimilarity. The points represent the clustering of howler monkeys bacterial microbiomes that are positive for helminth infection, or negative. The more similar the microbial composition, the closer the points will cluster. No significant difference between clusters could be found ($p_{adonis} = 0.111$)

Differential Abundance

I examined differential abundance between infected (n = 15) and non-infected (n = 6) monkeys. Of the 356 ASVs identified, only seven ASVs were significantly differentially abundant (Table 1) between two conditions, helminth positive or helminth negative (Figure 6A). All seven were members of two bacterial orders: Bacteroidales and Clostridiales (Figure 6B).

Table 1: Summary of ASVs, log fold change, adjusted p-value, and their assigned taxonomic Order, that were significantly different between howler monkeys with no current helminth infection and those with a detected helminth infection.

ASV	Log Fold Change	Adjusted P-Value	Order
ASV 141	-26.62864	6.84E-14	Bacteroidales
ASV 342	24.06924	4.23E-11	Bacteroidales
ASV 349	19.86453	9.09E-08	Bacteroidales
ASV 366	23.16081	1.61E-10	Clostridiales
ASV 381	23.73451	5.73E-11	Bacteroidales
ASV 567	-23.54174	4.23E-11	Bacteroidales
ASV 664	-22.44202	2.51E-10	Clostridiales

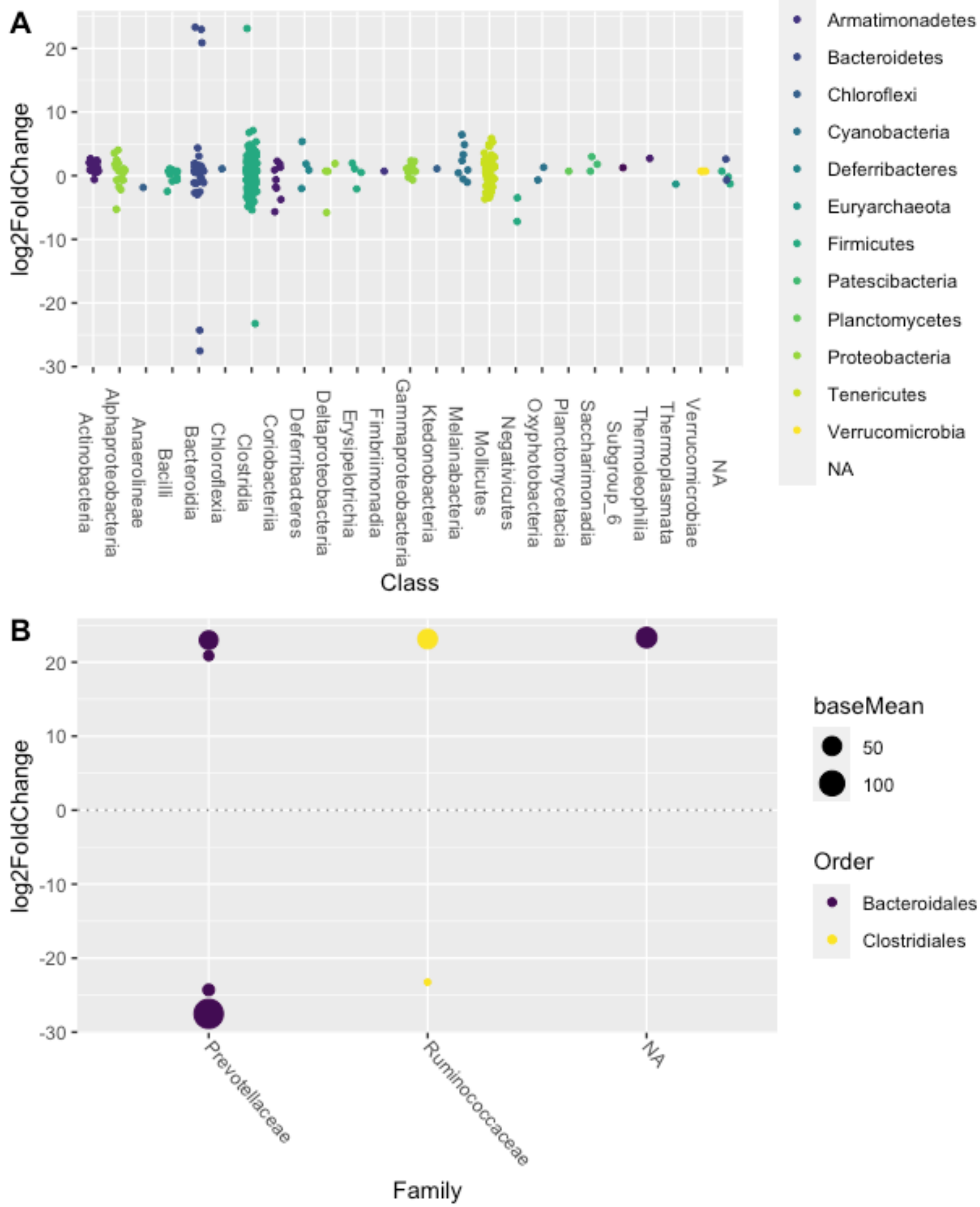

Figure 6: Differential abundance analysis preformed by DESeq2, testing the howler gut microbiome for differences in bacterial abundance between howlers that were positive for helminth infection, and those negative for helminth infection. A) The points represent the log fold change of all 356 bacterial ASVs. The colour of the points separates them into phylum, and

they are further separated by class. Only 7 found to be significant between those with helminths present and those with no detectable helminths (Table 1)(alpha < 0.01) B). Clostridiales and Bacteroidales were the only orders of bacteria found to have significant differences.

Discussion

My main results are two-fold. 1) I find no differences in diversity or richness of the bacterial community between howlers with active helminth infections and those without; 2) I find 7 ASVs are significantly difference in abundance between howlers with active helminth infections and those without. My results suggest that helminthic parasites interact with specific bacterial taxa in the gastrointestinal tract in ways that might be similar to those seen in human and mice studies[99,33]

Parasite Prevalence

Helminthic parasites are well known to inhibit inflammatory responses, which may have evolved as a survival mechanism to attenuate host response to helminthic infection[33]. The high prevalence of helminths I document in this study of a small sample sampling of primates, is supported in many other studies[54,56,65]. *Controrchis* has been reported as a common parasite to howlers[56,64], and the high prevalence hypothesized to be associated with food sources harboring these parasites. A study of howler monkeys in Belize found 89% of individuals infected with *Controrchis*[64], and another from Mexico found 76.7% of *A. palliata mexicana* to be infected with this helminth[57]. My study finds a large proportion of howlers (46%) to be infected with *Controrchis*, lower than the previous mentioned studies, but higher than what has been found in a study on *A. pigra*, located in Mexico, which found only 22.9% of these howlers to be infected[57]. The widespread nature of this parasite should be examined in more detail, as it could be an indirect consequence of these howlers consuming ants that *Controchis* parasitize, that reside on leaves which form a large portion of their diet. This parasite may have a large impact on the health and homeostatic balance on the howler monkey GI microbiome, as it is highly prevalent in multiple howler species in Central America, and warrants further investigation.

Alpha and Beta Diversity

The diversity of bacteria was not found to significantly differ between howlers with and within detected helminth infections. This differs from other studies[36,49] where both alpha and beta diversity measures have been shown to be significantly affected by parasite presence. As with many wild primate studied, low sample sizes likely impeded my ability to detect small differences. Another possible explanation could be that while I did not positively identify helminths in our coprological analysis, a low intensity of helminth infection in individual monkeys, or in the population we studied, could lead to false negatives, a shortcoming that could be studied with the application of a genetic screening method. Additionally, due to my study only sampling over one time point, howlers that were negative for helminth infection could have recently cleared a helminth infection, or currently been infected with a parasite that was not screened for. Further studies on these howler groups should consider a sampling strategy that takes place over the course of a season, multiple seasons, and includes the collection of multiple samples per individual. These findings introduce questions for further investigation to determine if these same parasite and bacterial relationships coincide with an increase or decrease in gut inflammation, potentially by testing for biomarkers of inflammation such as lactoferrin or calprotectin[100,101], with the potential to become a tool in monitoring intestinal health of these howlers.

Differential Abundance

I found seven bacterial ASVs with significantly different abundance between helminth infected howlers and howlers without detected helminth infection. Despite my relatively small sample sizes, I also show evidence of a relationship between the presence of helminth parasites and the relative abundances of bacterial taxa in the orders Clostridiales and Bacteroidales. My results are consistent with the findings of others who have studied this interaction in murine models, and support the interpretation that parasitic infections can affect the homeostatic balance of bacteria[35], with the abundance of Bacteroidales and Clostridiales significantly shifting between those with and without helminth parasites. However, the large log-fold differences (e.g. > 20 fold) reported are surprising. This could be an artifact of having a low sample size as differences between individual samples on relatively rare ASVs can have a large overall impact. Supplementary Table 7 shows that reads across the samples positive for helminths were relatively similar, with an average of 4556 reads, while the samples negative for helminth infection were more varied, with the lowest read count being 1696, the highest 15012, and an average read count of 8151. A limitation of my study is that direct comparisons of the functional relationship of these bacteria in the gut of howlers is difficult, as I only examined 16S data to assign taxonomy.

Overall, I provide new data on the bacterial and parasitic gut microbiome of howler monkeys, and their inter-relationships. Although my study is limited in scope, I join others in suggesting that studies of wild primates in their natural environment can be better understood from drawing on our breadth of knowledge from human microbiome studies[47,48,98], as well as methods and tools used to monitor the GI tract in humans can be utilized in non-human primates such as howlers. A more comprehensive assessment of immune responses and GI response in the context of parasitic infection in these primates is warranted, including non-invasive assessments of inflammation through fecal biomarkers enabling us to determine if helminthic infection creates or subdues an inflammatory response in these howlers[100,101]. Examining the severity of inflammation in wild populations of howlers in the context of helminth infection would allow further assessment of potential protective benefits of helminths, and deepen our understanding of the complex relationship parasites and bacteria play in maintaining howler, and primate health. In the future, genomic sequencing, for example using 18S or ITS barcoding, of samples with parasites – or the parasites themselves - would also allow refinement of information generated by the coprological data, as well as enabling larger study sizes, and resolve parasite taxonomy that was not possible during this study using only morphological identification. Such studies will help improve our understanding of the ecological relationship between helminths parasites, bacterial communities, and their non-human primate hosts.

<u>**Chapter 4: Effects of Anthropogenic Forest Fragmentation on the Gastrointestinal Bacterial Microbiome**</u>

<u>*Abstract*</u>

The gastrointestinal bacterial microbiome is a dynamic community of bacterial that serves vital functions in maintaining health and uptake of nutrition to its host. Anthropogenic disturbance negatively affects the health of primates in various ways. For example, human development and activity is a major factor in the fragmentation and degradation of primate habitat worldwide. Specifically, fragmentation and human disturbance has shown to increase the risk of parasite infection and pathogenic bacterial infection. I examine whether habitat fragmentation plays a role in lowering the diversity of the bacterial gastrointestinal microbiome, and whether anthropogenic isolation has an effect on the prevalence of helminth infection. Through coprological analysis and 16S high throughput sequencing of fecal samples from wild Costa Rican mantled howler monkeys (*Alouatta palliata*), I find that the alpha diversity in howlers gut bacteria microbiome is significantly lower in the population in a fragmented habitat near an active ranch. I find the beta diversity does not significantly differ between populations in different habitats. Further, I found that howlers in an intact forest biome have a higher prevalence of helminth parasite infections than those in the fragmented habitats near anthropogenic sources. These data support the hypothesis that the bacterial microbiome is influenced by habitat fragmentation through anthropogenic sources, consistent with other studies. Surprisingly, intensity of helminth infection is highest in populations of howler monkeys living in intact forest biomes, which does not support my hypothesis that fragmented habitats are associated with increased parasite infection. This results might reflect the more complicated

parasite community dynamics of a biodiverse ecosystem. This study has implications for health and conservation of wild primate populations.

Introduction

Anthropogenic disturbance of habitats can have significant health implications for resident animals[102]. Agriculture and urbanization reduce natural landscapes for wild animals, and increased potential to leech harmful chemicals in the form of pesticides, herbicides or fertilizers[103]. In addition, the presence of humans in the habitat of animals can reduce animals' ability to forage successfully via increasing demand for vigilance[104]. The expansion of human activity has led to multiple mammalian species changing activity patterns to avoid exposure to humans during daylight hours[105]. Disturbance of wild habitats by human activity is also linked to an increase in the rate of extinctions[103] and speciation[106], demonstrating the large impact humans have on biodiversity. This impact on wild populations is seen in the declining numbers of primates in the wild, and an increase in the number of primate species that are threatened or endangered[97]. Human disturbances near or within habitats of wild primates may shrink the available area to primates, which can lower the carrying capacity of the ecosystem, and decrease habitat quality (e.g. resource availability, habitat size, biodiversity) through forest fragmentation[66]. As a result researchers have proposed careful study of the links between anthropogenic disturbance and effects on populations of mammals and primates[107]. Many of the mentioned studies focus on primate population density, ranging, and diet, but it is also important to look at biological samples, which provide more direct insights into health of wild populations.

Non-invasive biological sampling of feces grants the ability to study the gastrointestinal microbiome of wild primates; an ever growing area in primatology for understanding overall health of these animals[60]. The GI microbiome has been extensively studied in humans in a health context[3,5,18], and these studies provided a great stepping stone for primatologists, as many of the same principles of human GI health may apply to non-human primates as well[47,50]. The bacterial microbiome shifts dynamically in response to age, season, changes in diet, and environmental stress in primates much the same way as humans[9,51,58]. In humans, a decrease in the diversity of the gut microbiome is generally associated with decreased microbiome functionality, affecting nutritional uptake, and altering community composition[22]. Additionally, lowered diversity of bacteria in the GI tract has been associated with chronic inflammation in the gut including IBD, diabetes, and cardiovascular disease[22,108], which shows the importance of bacterial diversity and homeostasis in maintaining proper gut health. In primates, the gut microbiome composition has been known to be affected by captivity[61]. Furthermore, habitat disturbance may also influence the gastrointestinal microbiome, as the microbiome of free-living primates is reported to be more diverse in habitats with less anthropogenic disturbance[59]. One of the many ways habitat quality has the potential to indirectly impact the microbiome is through food availability, and diet is one of the driving factors in bacterial makeup of the GI tract[18,51]. Habitat fragmentation can influence the quality and quantity of foliage, fruit, invertebrates, water, and other sources of nutrients[52,63], and primates in more disturbed and fragmented areas may have lower microbiome diversity. Lowered habitat quality from human disturbance can also affect the microbiome through introducing pathogenic bacteria[102], and zoonotic parasites[55] to wild populations of primates.

Parasites, and the impact that parasites have on the GI tract is another significant area of study for wild primates[54,64,65,109], including how primate parasites are transmitted, the rates of parasite infections in wild populations, interactions between parasites and bacteria, and how anthropogenic disturbance can increase risk of parasite infection. Parasitic surveys of primate populations have shown that many populations live with persistent parasite infections, and while parasites are not uncommon in wild populations of mammals[54,65,110], they often do not cause any observable, external, detrimental signs of infection[54,64,65]. However, GI parasites have been shown to have a broad impact on modulating the makeup of the bacterial microbiome[13,28,94], changing prominent species, triggering antagonistic responses, or impacting gut function. In these cases, gastrointestinal parasites can be detrimental, triggering inflammation, and destabilizing the homeostatic balance of the bacterial communities[30,31] Studying the parasitic microbiome is an important component towards understanding a primates gastrointestinal microbial diversity, and may also provide an ecological snapshot of overall health.

As detrimental as parasites may be to the health of the host, some helminthic parasite infections have been found to be beneficial, inhibiting inflammatory bacteria from colonizing the gut[34], or worsening effects of viral infections[37]. Furthermore, individuals that have serious inflammatory diseases such as Crohn's or colitis have benefited from helminthic parasite infection. In a subset of these patients, helminth infection was associated with a decrease in inflammatory bacteria[33,35,36], and a potential increase in colonization of non-inflammatory bacteria. In humans in developing countries, these types of helminth infections can be widespread, but not always detrimental[25,94,111], and hypothesized to be protective against colonization of dangerous bacteria, and this is possibly the same mechanism occurring in primate populations with low, but persistent parasite infection as well.

Within primate populations, susceptibility to disease, and infection by parasites, increases in fragmented, or disturbed environments both from natural causes such as a hurricanes[69], or from humans[55,64,102]. In these fragmented habitats, additional energy expenditure is needed for the acquisition of resources, placing an increased burden on primate groups, negatively affecting their overall fitness[63]. Additionally, in some cases of human disturbance, foreign pathogens can be introduced, further reducing primate fitness[102]. Together, habitat quality and health directly impacts primate fitness. However, data from wild primates is still sparse as it pertains to the relationship between habitat and health, parasites, and bacteria. A plethora of studies focus on modulation of the primate bacterial microbiome, through fragmentation[59,66], temporal changes in diet[51,53], human interaction and captivity[61], and characterization of the bacterial communities present[112,113]; or surveys of parasites among primate populations[46,54–56,64,114], suggesting negative health impacts. However these studies fail to incorporate bacterial microbiome with parasite infection data, and given how common parasites are among wild primates, their modulatory impact on the bacteria should not be ignored. This study seeks to clarify the ways in which anthropogenic fragmentation of habitats affect one important aspect of primate health: density of parasite infection, and the relationship fragmentation has on diversity of the bacterial microbiome.

Howler monkeys as a study system are well suited to this research for three reasons; firstly, they are easy to track and follow because they are slow moving, spend the majority of their day resting in one area, and are easy to habituate; second, howlers are tolerant to many types of habitats, and can be found in a variety of primary forest and anthropogenically modified habitats[62]; lastly, they are relatively well-studied in terms of the parasites that commonly infect them, and the microbes that contribute to their gastrointestinal and overall health[51,52], enabling comparisons to be made to previous work.

In this study I specifically examine wild howler monkeys in northwestern Costa Rica living in two distinct conditions: 1) a primary forest; Sector Santa Rosa, Área de Conservación

Guanacaste (a protected conservation area), and 2) two anthropogenically fragmented forests, one near a working ranch, and the other near the tourist town of Tamarindo, Guanacaste. Though not specifically measured in this study in terms of habitat size, food availability, or biodiversity, Sector Santa Rosa is the higher quality habitat, as it has low forest fragmentation, and far fewer anthropogenic disturbances compared to the fragmented habitats of Eladio's and Tamarindo. I test the alternate hypothesis that anthropogenic disturbance has negative health consequences in terms of bacterial diversity and parasite density, impacting the dynamics of bacteria and parasites in the gastrointestinal tract, against the null hypotheses of no systematic variation correlating with habitat. I predict that there will be higher alpha diversity and distinct beta diversity of gut bacteria in the Sector Santa Rosa, the undisturbed forest that is located within a protected conservation area, and lower alpha diversity, and distinct beta diversity in the anthropogenically fragmented habitats, near the ranch, and near the town of Tamarindo. High alpha diversity will correlate to an increased richness (number of species), and evenness (if species are equally distributed or if few dominate the community) of bacterial species, and distinct beta diversity will demonstrate unique bacterial community composition between locations via clustering of similar community structures. Additionally, I expect there to be a lower density of parasites in primates living in the high-quality, undisturbed forest habitat, and higher in the two anthropogenic habitats, as fragmentation has been shown to increase risk of parasite infection[57,69,115].

<u>*Methods*</u>

Sample Collection

Howler monkey fecal samples were collected during the rainy season in July 2017 from three study locations in the Guanacaste province of Costa Rica; Sector Santa Rosa (SSR), a protected tropical dry forest, and two forest fragments, one located outside the town of Tamarindo, and one near an active Ranch and 'airbnb'. One fecal sample was collected from 25 individual howler monkeys, split, and stored in either 96% ethanol, or set on ice to be frozen in liquid nitrogen.

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See *Chapter 2: Methods*, for a detailed explanation of procedures used in this study.

<u>Results</u>

Bacterial Diversity

I first tested the impact of storage media, ethanol versus frozen in liquid nitrogen, to determine the impact on the bacterial diversity. Supplementary Figure 1 and Figure 2 show the result of this analysis. I found a significant effect on richness and beta diversity of bacterial communities. The alpha diversity of bacterial communities in 25 frozen fecal samples and 21 ethanol stored fecal samples of howler monkeys living in 2 distinct habitats, fragmented (Eladio's Ranch and Tamarindo), and unfragmented, continuous forest (Sector Santa Rosa), was tested using a Wilcox test. . Samples stored in ethanol had a lower diversity on average than samples stored in liquid nitrogen (when comparing Shannon alpha diversity) and similar diversities when compared with Simpson measures. I used frozen samples, the gold standard for microbiome research, to compare habitat conditions. For frozen analysis, Santa Rosa had a sample size of 17, and Eladio's and Tamarindo both had samples sizes of 4. The sample size for ethanol analysis was 13 for Santa Rosa, and 4 for both Eladio's and Tamarindo. Below statistical tests are reported for samples frozen in liquid nitrogen. I chose this because this is the gold standard for microbiome research and yielded the greater sample size.

Richness (Chao1) was not found to significantly differ between the sites, while measures for alpha diversity (factoring in species evenness) found that diversity was significantly lower in the bacterial communities of the howlers living in the fragmented habitat of Eladio's Ranch compared to Santa Rosa (alpha = 0.05, Santa Rosa (n=17) and Eladio's (n=4) $p_{Shannon} = 0.02373$, $p_{Simpson} = 0.03141$). This decrease in diversity was not seen between Santa Rosa and Tamarindo howlers (alpha = 0.05, Santa Rosa (n=17) vs. Tamarindo (n=4) p = 0.6353) or Tamarindo and Eladio's (alpha = 0.05, Tamarindo (n=4) vs. Eladio's (n=4) p = 0.3429) (Figure 7A). This decrease in diversity was not found when fragmented habitats, Eladio's and Tamarindo, were combined, and directly compared to Sector Santa Rosa ($p_{Shannon} = 0.07523$) (Figure 8A).

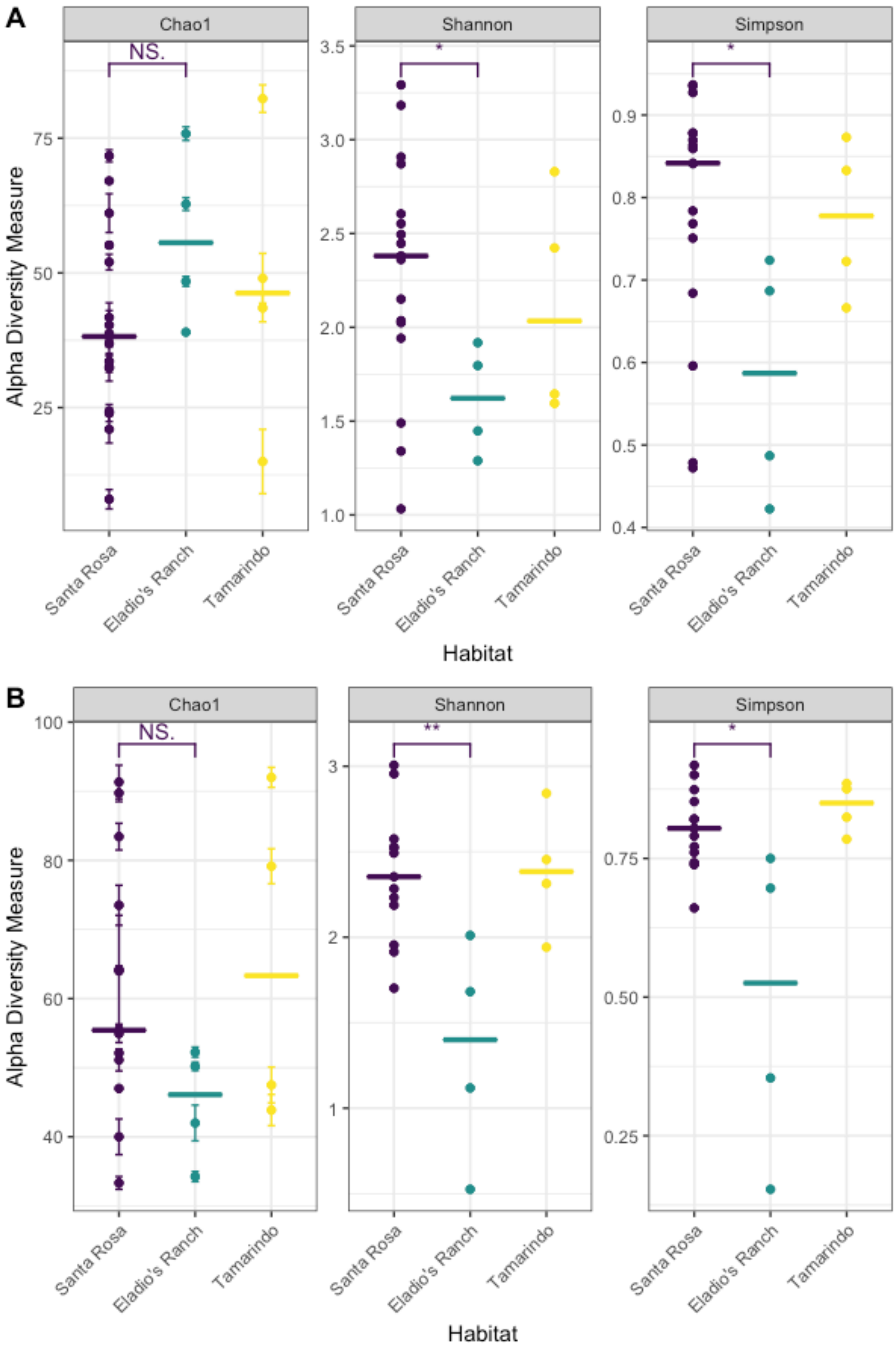
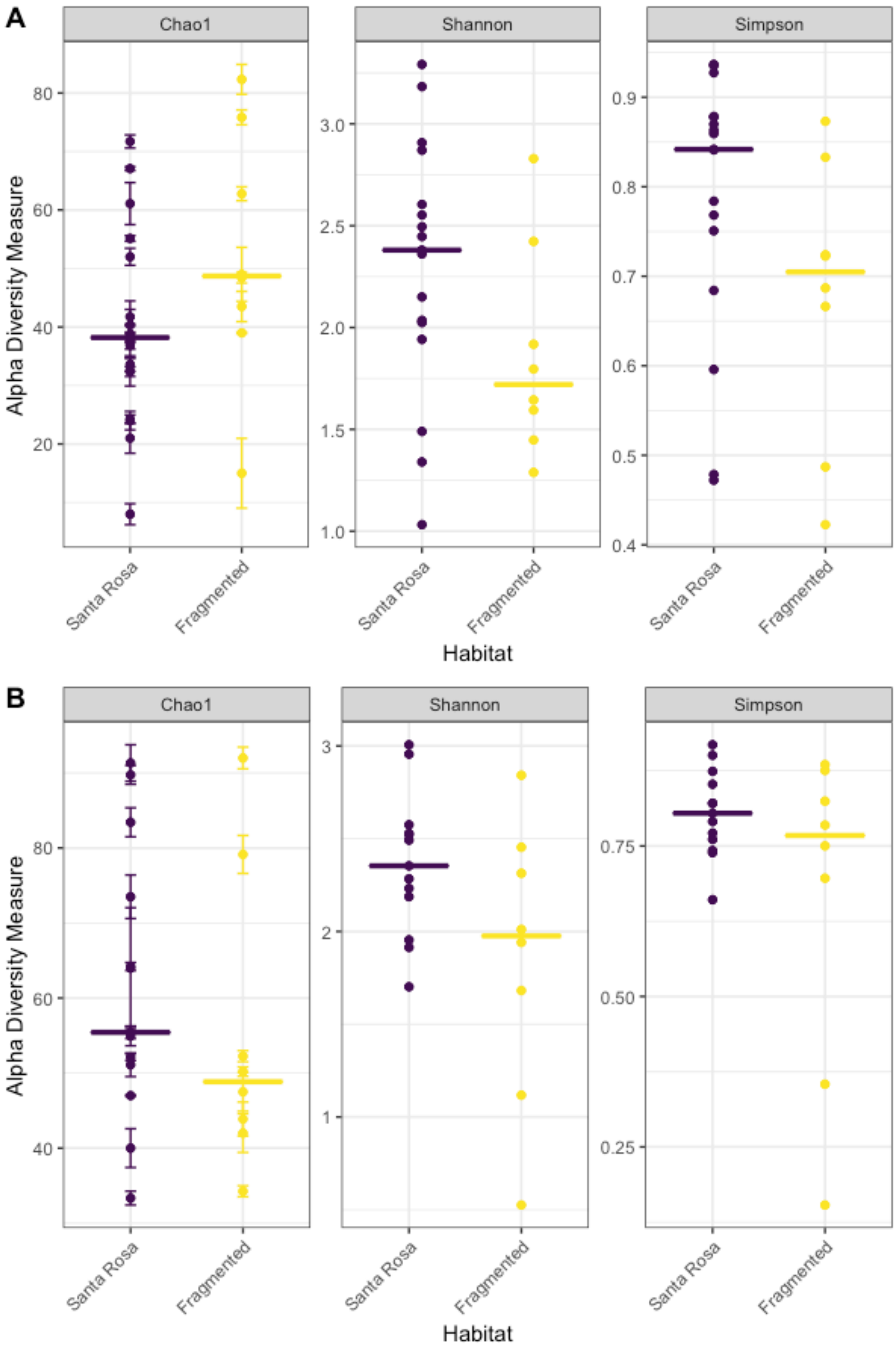

A
Chao1
Shannon
Simpson
NS.
*
*
Alpha Diversity Measure
Santa Rosa
Eladio's Ranch
Tamarindo
Habitat
B
Chao1
Shannon
Simpson
NS.
**
*
Alpha Diversity Measure
Santa Rosa
Eladio's Ranch
Tamarindo
Habitat

Figure 7: Alpha diversity measures of fecal samples for howler monkeys based on habitat location. Chao1 estimates richness, and Shannon and Simpson both estimate diversity (both richness and evenness), with Simpson allowing rare species to not influence the alpha value as much as Shannon. The horizontal line represents the median, and dots visible represent individual howler samples. A) Alpha diversity analysis of frozen fecal samples (n = 25). With an alpha of 0.05, no significance (N.S.) in alpha diversity measures were seen in richness (Chao1). In diversity measures, Eladio's Ranch was found to be significantly lower than howlers located in Santa Rosa, but not Tamarindo (Santa Rosa and Eladio's $p_{Shannon} = 0.02373$, Tamarindo and Eladio's $p_{Shannon} = 0.3429$). B) Alpha diversity analysis of ethanol fecal samples. Chao1 richness test again found no significance (N.S.) between Santa Rosa and Eladio's Ranch or Tamarindo habitats, and Santa Rosa populations, were found to have a significantly higher alpha diversity than Eladio's Ranch, but not Tamarindo (Santa Rosa vs. Eladio's Ranch $p_{Shannon} = 0.005882$, Santa Rosa vs. Tamarindo $p_{Shannon} = 1$, Tamarindo vs. Eladio's Ranch $p_{Shannon} = 0.05714$).



A
Chao1
Shannon
Simpson
Alpha Diversity Measure
80
60
40
20
3.0
2.5
2.0
1.5
1.0
0.9
0.8
0.7
0.6
0.5
0.4
Santa Rosa
Fragmented
Habitat
B
Chao1
Shannon
Simpson
Alpha Diversity Measure
80
60
40
3
2
1
0.75
0.50
0.25
Santa Rosa
Fragmented
Habitat

Figure 8: Alpha diversity measures of fecal samples for howler monkeys with fragmented habitats of Eladio's and Tamarindo combined. Chao1 estimates richness, and Shannon and Simpson both estimate diversity (both richness and evenness), with Simpson allowing rare species to not influence the alpha value as much as Shannon. The horizontal line represents the median, and dots visible represent individual howler samples. A) Alpha diversity analysis of frozen fecal samples (n = 25). With an alpha of 0.05, no significance in alpha diversity measures were seen in richness or in diversity ($p_{Shannon} = 0.7523$, $p_{Simpson} = 0.6569$) measures in howlers when comparing Santa Rosa to all fragmented habitat samples. B) Alpha diversity analysis of ethanol-preserved fecal samples (n = 21). Chao1 richness test again found no significant differences between Santa Rosa and all fragmented samples ($p_{Shannon} = 0.1042$, $p_{Simpson} = 0.4137$).

Differences in beta diversity of bacterial from howler fecal samples between fragmented and unfragmented habitats was tested using the 25 frozen samples. First, these samples were tested for homogenous dispersal between groups through Betadisper in Vegan, followed by the non-parametric PERMANOVA test. No howler group significantly differed with respect to beta diversity of their bacterial microbiome (SSR and Eladio's p = 0.48, SSR and Tamarindo p = 0.078, Eladio's and Tamarindo p = 0.108) (Figure 9). Clustering of these howler bacterial groups (Figure 9) shows a high degree of overlap among howlers in Santa Rosa, Eladio's Ranch and Tamarindo.

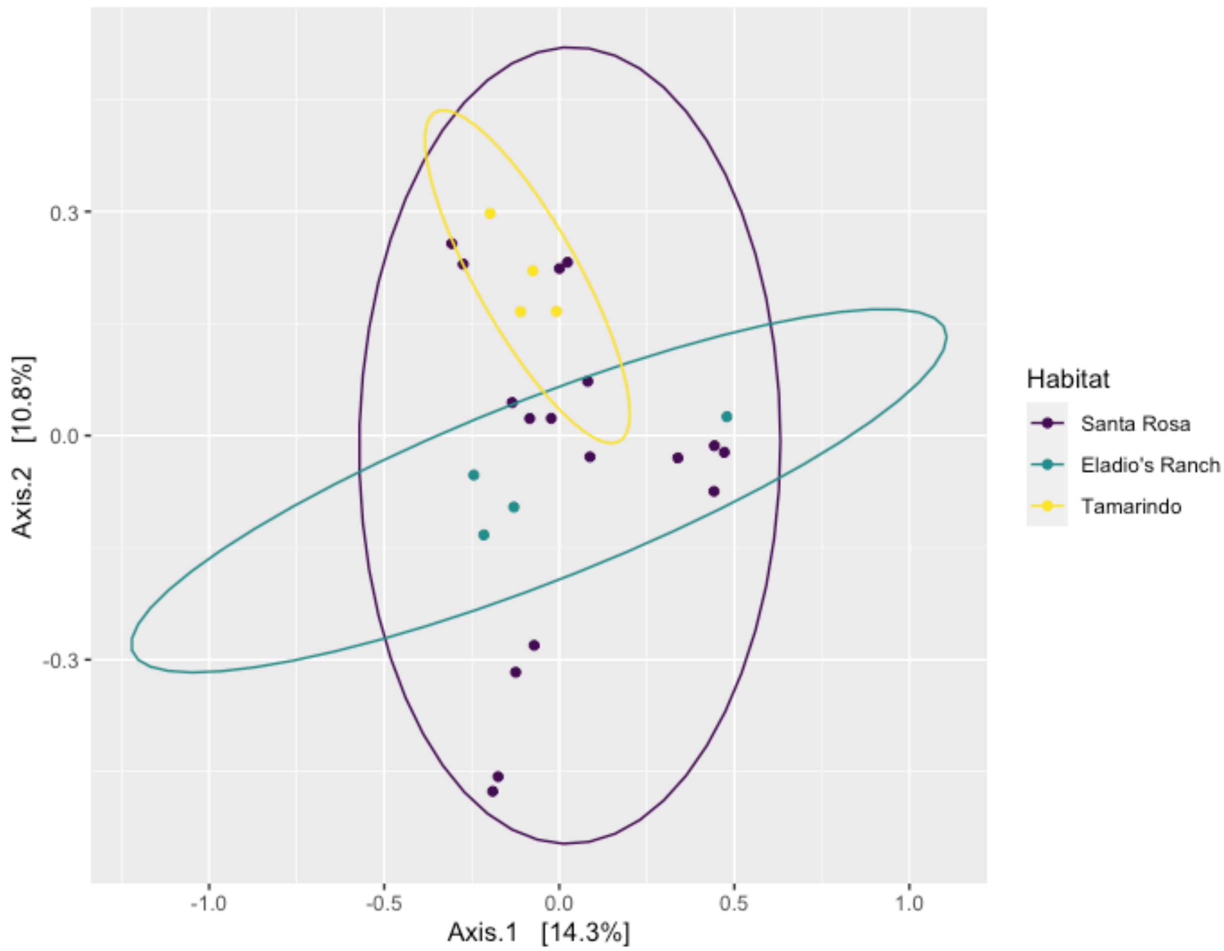


Figure 9: PCoA plot of the beta diversity of bacterial communities in howler gut microbiome performed with Bray-Curtis dissimilarity. The points represent the clustering of howler monkeys bacterial microbiomes based on their habitat location. The more similar the microbial composition, the closer the points will cluster. The ellipses is a statistical approximation of clustering. All three locations were determined to not be significantly different with PERMANOVA test (SSR and Eladio's p = 0.48, SSR and Tamarindo p = 0.078, Eladio's and Tamarindo p = 0.108).

Parasite Prevalence and Density

In the 21 samples coprologically inspected, positive parasite infection was reported in 71% (15 out of 21) of the sample population. The most prevalent (a count of at least one in a howler sample) parasite found was *Controrchis* spp. at 43%, with the second most prevalent parasite being a Trematode (29%). *Strongyloides* spp. and *Enterobius* spp. (Pinworm) were also detected with a prevalence of 19% and 10% (Table 2). Prevalence of parasites was also found to be higher among howlers that were located in Santa Rosa, compared to howlers in fragmented habitats of Tamarindo and Eladio's Ranch (Supp. Table 6).

Table 2: Sample prevalence for each of the four morphologically identified parasites, and an unidentified parasite egg. Prevalence was calculated by counting the presence of at least one parasite in each of the 21 samples

Parasite	**Prevalence**
Controrchis biliophilus	43%
Unidentified Trematode	29%
Strongyloides sp.	19%
Pinworm (*Enterobius sp.)*	10%
Unknown	4%

Additionally, the density of parasite eggs, the average number of eggs found per millilitre of fecal sample, were counted through visual inspection of fecal flotations. Howlers located in Sector Santa Rosa had significantly higher density of infection, more than 3x more parasites when compared to howlers in fragmented habitats of Tamarindo and Eladio's Ranch (Figure 10). Additionally, when examined separately, both Tamarindo and Eladio's Ranch have lower parasite densities than Santa Rosa (Supp. Figure 3). Together, howlers in Sector Santa Rosa have both a higher prevalence of parasite infection, and those infections are denser.

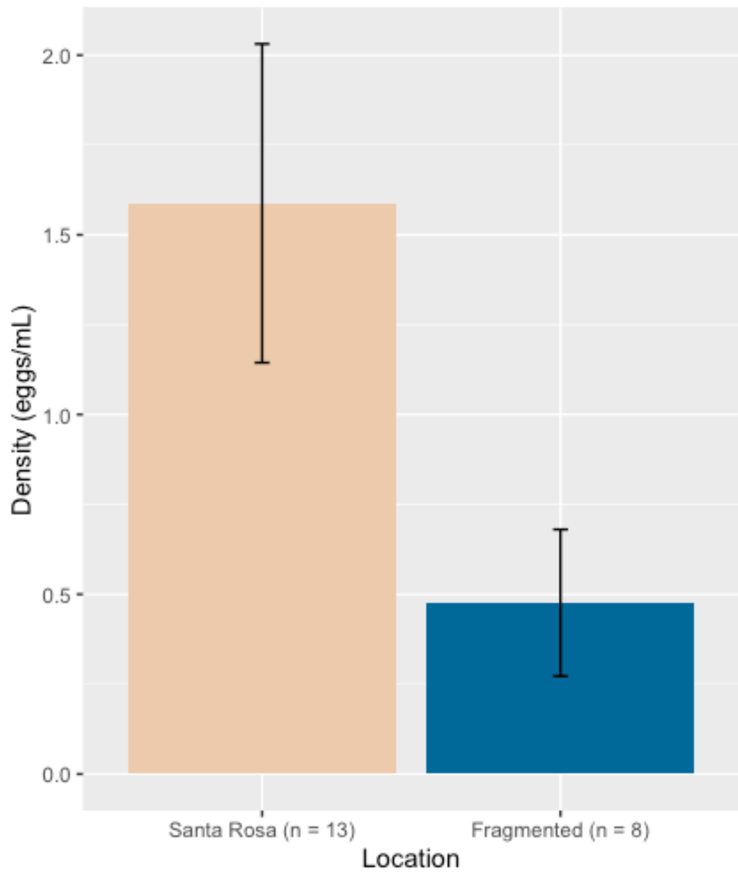


Figure 10: Density of parasites eggs counted (all parasite eggs per millilitre of fecal sample) found in howlers residing in the two study habitats, the protected tropical dry forest of Santa Rosa, and the fragmented habitat, combining the groups from Eladio's and Tamarindo. Error bar represents the standard error from the mean (SEM) for 21 samples.

Discussion

In this study I examined the extent to which variation in habitat affects the helminth and bacterial microbiome within the gastrointestinal tract of wild howler monkeys. This was evaluated through alpha and beta diversity measures, and examination of helminth eggs in fecal samples of howlers across fragmented and continuous forest habitats. I found that howlers living in close proximity to the site of a ranch, a fragmented habitat, had significantly lower bacterial alpha diversity compared to continuous protected forest or a town. Furthermore, I found that parasite infection was denser, and more prevalent amongst howlers living in Sector Santa Rosa, the protected, continuous forest habitat, isolated from anthropogenic disturbances.

Bacterial Diversity

I predicted that howlers living further from anthropogenic disruptions would have significantly increased alpha diversity, and distinct beta diversity among their bacterial communities. While I found alpha diversity of howlers living in Santa Rosa and Tamarindo locations to not differ significantly, those among the fragmented forest of Eladio's Ranch had a significantly lower alpha diversity, suggesting that habitat fragmentation and isolation from humans may not be the only variables of interest when studying habitat impact on the bacterial microbiome. Alpha diversity being significantly lower in the social group at Eladio's Ranch could be a reflection of a fragmented habitat, with smaller pockets of trees, and a lower quality and abundance of nutritional resources. There has been relevant research that reinforces this idea, that diet and quality of habitat significantly impacts the bacterial microbiome[57,59,66], and among capuchins living in the same area, diet significantly changes temporally, and also impacts the gut microbiome[73]. My evidence here suggests the same, reinforcing the large impact of habitat fragmentation and its indirect effects on habitat have on the bacterial community within wild primates. Though, overall beta diversity between all three locations did not significantly differ, and taken together, this only partially supports the alternate hypothesis that bacterial diversity significantly shifts dependent upon the habitat location of howler monkeys. The results of the beta diversity test may be impacted by the small sample sizes of the fragmented habitats (only four howlers each), as other studies do support significant impacts of fragmentation and habitat location.

From previous work done on primate populations, anthropogenic modulation on the microbiome can be significant, and the fragmented habitat that howlers occupy in Tamarindo is a much more populous location, and for lack of quality plants, flowers, and fruits, could be compensated through interactions with humans, potentially feeding, foraging in trash, or human horticulture providing unique food sources. This study did not examine variables such as behaviour or foraging strategies within the howler sample populations, and further study of these habitats should include observations of fruit and vegetation availability, as well as howler foraging activity, to allow us the ability to resolve the variability in these different locations, and how they can affect the howler microbiome. While a good starting point for examining the diversity of the bacterial microbiome in these three small populations, a more intensive study is warranted to fully understand why Tamarindo howlers and Santa Rosa howlers exhibit similar alpha diversities, and if beta diversity is truly not significantly different, or if this is a consequence of sample size, seasonality, or other variables.

Parasite Prevalence and Density

My prediction for parasite infection to be more dense among the fragmented, anthropogenically disturbed habitat, was not supported, even though literature has found that disturbed habitat has been heavily associated with poor health in wild primates[55,102], and further associated with increased risk of parasite infection[69]. Surprisingly, my results for parasite density and prevalence in fragmented versus continuous forest, found significant evidence that howler populations more isolated from human interaction, had a higher density of parasites than those near to human activity or in fragmented forests and a higher prevalence of parasites in general. Although habitat degradation has been linked to parasite infection, and poorer health in primates, helminths have been found to have a beneficial impact in macaques[49], and parasite infections in wild populations of primates is extremely common. Possible explanations explaining the higher incidence of infection in isolated forests could be linked to food sources, water availability, and interspecies transmission. For example, one such plant, Cecropia, is a species of neotropical tree that howlers feed on, and it displays myrmecophytism, a type of defense that utilizes colonies of ants for natural warding of herbivores. These ants commonly carry parasites that could incidentally infect howlers eating those plants[64]. Supporting this idea, a parasite that I found to be in high prevalence in this study of howlers was *Controrchis* spp., which was studied for its association with *Azteca sp*. ants as a transmission source for *Controrchis sp*. [64] While *Controrchis* has been extensively studied in relation to howlers, this parasite is not at common in human populations, and in this study, was not associated with howler habitats that were in closer relation to anthropomorphic sources. Additionally, capuchin monkeys and spider monkeys, other Central American primates that shares habitat with howlers within SSR, have been studied for their associations with high rates of parasite infection[54,65]. Many of the parasites found to infect these two species, were also found in this study affecting howlers, and transmission between species of primates is a possibility. Finally, during the dry season in SSR, some of the only sources of water are ground based pools, which capuchins and spiders, as well as other animal species visit, increasing the changes of parasite transmission within the community. Although routes of transmission were not directly observed or measured in this study, transmission between species, including from food sources and water, could be important sources of parasites for howler monkeys. Among howlers in fragmented habitats, lower rates of parasite infection were found. However, if helminths are beneficial to the gastrointestinal microbiome of howlers, this could be detrimental, and these populations of howlers could have increased inflammation, and worse overall health. Though many of the parasites that were detected in this study, pinworms, trematodes, and strongyles, are all parasites that infect humans, and thus cannot be ruled out as a source of transmission. Zoonotic spread of parasites has been found in primates. For example *Giardia* and *Cryptosporidium* are of well-studied parasites known for zoonotic transmission[46,55,116–118], these protozoans being common in humans, primates, and mammalian populations. If the source of these helminths that were found in howler populations were found among the human populations of Costa Rica, it would could impact our view of conservation of these howler populations, demonstrating further negative impacts anthropogenic influence has on howler populations. Interactions with these primates would have to be taken more cautiously, and education of the dangerous of zoonotic transmission could be implemented to limit the spread of parasites in humans and primates.

Although answering questions with regards to how fragmentation affects parasite prevalence and bacterial diversity, this study raises many questions that remain unanswered. Larger sample populations could strengthen the arguments made throughout this study, as we were limited to a population of 25 howlers. If a wider sampling approach was adopted, it would also be pertinent to consider behavioural observations such as foraging, food intake, activity, and interactions with other species. This would allow a deeper understanding about what most significantly impacts the bacterial microbiome and helminth prevalence and density. Further study characterizing the bacterial communities within howler populations is needed to understand the functional differences between gut microbiomes, if howlers in fragmented habitats have increased markers for inflammation, and if this inflammation can be linked to a lower prevalence of helminths, or other causal effects of living in a low-quality habitat. Lastly, investigation is warranted to understand if human populations living in close relation to howler populated forests share these parasitic infections more often than those that are anthropogenically isolated, with a focus on determining parasite transmission sources.

In conclusion, primatological studies that integrate microbial and parasite data are needed to strengthen our ecological understanding of the impacts that human disturbances have on wild populations of at risk and endangered mammals. A diverse and healthy bacterial microbiome is critical for overall health of mammals, including primates such as howlers, and modulation through anthropogenic disturbance, such as forest fragmentation, has negative consequences on mammalian health. In addition, parasites are extremely prevalent in primates, exemplified through study, and many others, and further modulate the gastrointestinal bacterial microbiome. Studies in humans and non-human primates show a similar beneficial relationship between helminth infection and gut health, indirectly supported here by increased helminth prevalence within the protected conservation area of Sector Santa Rosa, and parasites need to be taken into consideration when looking at GI microbiome health of wild primates. Finally, conscious conservation of wild habitat needs to be considered with regards to anthropogenic activity and development, to preserve biodiversity and maintain healthy ecosystems, including resource availability for primates, parasites that could be beneficial, and to maintain bacterial diversity in these populations.

Chapter 5 - Discussion

Research Goals

My goal in conducting this thesis research was to build upon primate gastrointestinal microbiome research. I sought for evidence that non-human primate microbiomes can be affected by similar mechanisms to human microbiomes, and to push future research into examining the relationship between helminth parasites and non-human primates. Additionally, I wanted to examine how human populations affect the gastrointestinal health of primates, through fragmentation of habitat, or direct interaction. Ultimately, I sought to provide a framework for future studies to examine non-human primates, or even this howler population, in greater depth, in the context of comparative analysis to human studies and human microbiome mechanisms. This area of research, comparing the human gastrointestinal microbiome to non-human primate microbiomes, can aid in developing our understanding of our bacterial microbiome's relationship with helminth parasites, and how helminths may have a bigger role in moderating the human gastrointestinal inflammatory response throughout our history.

In my thesis research, I found evidence that the howler gastrointestinal microbiome changes in response to helminth parasites in a way that is strikingly similar to what is found in humans; in particular that the presence of helminths significantly correlated with changes in two orders of bacteria, Bacteroidales and Clostridiales. Furthermore, I found fragmentation to have a significant impact on howler gastrointestinal microbiomes; decreasing diversity of bacterial communities, and increasing density of parasite infection in continuous forest habitats, implicating human activity in regards to fragmentation and disruption of primate habitat a significant factor in gastrointestinal diversity of howlers.

Parasite Interactions with the Bacterial Microbiome

In chapter three, I studied how helminth infection shapes the howler gastrointestinal tract. I hypothesized that helminth infections correlate with differences in relative abundance of bacterial orders potentially linked to provide a protective benefit. I predicted that helminth infected howlers would have a significant difference in abundance of two bacterial orders a decreased abundance of Bacteroidales and increased abundance of Clostridiales. I further predicted that a significant portion of howlers would be positive for helminth infection, and those that were positive for parasites would have increased bacterial diversity. I found my first prediction was supported, as I was able to demonstrate a significant difference in abundance of Bacteroidales and Clostridiales when comparing helminth infected and uninfected howlers, though coarse resolution of taxonomy lead to ASVs within the same order both increased and decreased abundance. There could be functional differences within these ASVs, and still a significant result that only two orders were significantly affected by helminth presence. These results have implications for future studies into howler gastrointestinal diversity as they are consistent with results found in humans. A study by Ramanan et. al. 2016[35] strongly supported the conclusion that helminth infections were protective in humans in locales with less infrastructure dedicated to anti-helminthic treatments and sanitization, demonstrating a marked increase in inflammation when helminths were removed from humans living in these regions, as well as helminth infection being a significant factor in affecting the abundance of Bacteroidales and Clostridiales. In this study, the relationship is appears similar in a non-human primate as well, and protective helminths infections could be important to the diversity of howler gastrointestinal bacterial communities. Primates, including humans, have a long evolutionary history with parasites, and these results indicate the potential for protective effects of parasites against inflammation, a consideration to conservation efforts and future studies focused on howlers or other primates, that helminths may have a bigger role to play in overall primate fitness and gastrointestinal health.

My second prediction, that parasites are very common among wild primates, was supported. I found significantly more howlers in my sample population (71%) that were, at the time of sampling, positive for helminth infection than free of infection. Other primatological parasite surveys found similar prevalence; capuchins[54] within Santa Rosa, and howler monkeys[56,64,65] in Mexico and Belize, all found that a high proportion of their sample population, near 80% and above, were infected with helminth parasites. High prevalence of parasites reported in multiple primate species could support an important ecological relationship, but further study examining the direct health effects (inflammation, protective colonization) of helminths will need to be conducted.

My last prediction, that howlers will show a significant increase in overall bacterial diversity in helminth infected subjects, was not supported, as I did not find significant alpha diversity change related to helminth infection. Beta diversity measures were also shown to not be significant in association with helminths. A possible reason for not finding significance in diversity is the small sample size I was working with, only 21 howlers were successfully coprologically examined for parasites, and of those, only 6 were found to be uninfected at the time of sampling. With a larger study population, or a study that sampled howlers at multiple time intervals, actively helminth infected howlers may show increased diversity, as this is relationship has previously been examined in both humans[36], and nonhuman primates[49].

Effect of Anthropogenic Fragmentation on the Bacterial Microbiome

In chapter four, I examined the effects that living in different forest habitats; anthropogenic fragmented forest, and anthropogenically isolated, has on the howler gastrointestinal microbiome. I hypothesized that living among fragmented forest near anthropogenic sources would have a negative impact on howler health, predicting a lower diversity of bacteria, distinct bacterial communities, and higher density of parasite infection in the anthropogenically disturbed habitat. I demonstrated a higher alpha diversity of bacteria in the gut microbiome of howlers living in the isolated Sector Santa Rosa habitat when compared to one of the fragmented habitats, Eladio's Ranch. I found no differences in beta diversity of the bacterial communities among howlers living in different habitats. This was somewhat surprising given that bacterial communities have been previously reported to be significantly changed, including a decrease in diversity, in habitats that are more anthropogenically disturbed, potentially exposing primates to pathogenic bacteria[102], captive primates have a significantly changed bacterial diversity[61], and fragmentation due to human activity have been shown to alter the gut microbiota[36,47,113].

My second prediction was not supported, as I found parasite prevalence and density was higher in Sector Santa Rosa, the anthropogenically isolated habitat. While this result was surprising, given that fragmentation increases parasite infection risk[57,69], and human disturbance can introduce zoonotic spread of protozoan parasites[46,55], there are some potential explanations. Helminths have been positively correlated with increased bacteria diversity in humans and primates[93], and primates closer to human populations[57], fragmented environments[59], or in captivity[62] have decreased bacterial diversity. In this study I found that the isolated Sector Santa

Rosa sample population of howlers had an increased diversity of bacteria, which could potentially be liked to increased prevalence of helminth infection, though this was not directly tested.

These results raise interesting questions, as there are more variables at play that could be tested, for example, continuous forests have higher quality food resources, and diet has been shown to significantly impact the bacterial communities found within the gastrointestinal microbiome[51]. Furthermore, the significantly higher prevalence of howler parasite infections in an isolated habitat could be supported by intake of food resources that are intermediate hosts for parasites such as *Controrchis*[50]. This hypothesis is not new, and studies of howlers eating cecropia plants and have shown increased *Controrchis* infections, and a direct study of this interaction on this population of howlers in Sector Santa Rosa could be an area of interest in the future.

Future Areas of Study

Though many links between parasites and the bacterial microbiome were found in these studies, there is many areas where further investigation would allow a better understanding of how helminths effect the overall health of howler monkeys. One such area is inflammation; though I found helminths to significantly alter the abundance of Bacteroidales and Clostridiales, demonstrating a similarity between human microbiome mechanisms and non-human primates, I did not test for changes in gastrointestinal inflammation. In order to support the prediction that helminths have a protective role in in the primate GI tract, decreasing inflammation, a direct relationship of lowered inflammation and helminth presence should be examined. This question could be studied by measuring levels of biomarkers of gastrointestinal inflammation, such as calprotectin[120] or lactoferrin[121]. These biomarkers have been used in murine models and human studies, enabling a quantifiable method to measure the effect helminths have on GI inflammation. This relationship between protective helminths and the gastrointestinal tract has been found in human studies trying to exploit this phenomena in the hopes of using helminths as therapeutic tools in relieving chronic intestinal inflammation[33,39,40].

Furthermore, beyond solidifying this positive host-parasite interaction across multiple mammalian species, there is an opportunity to establish a link between helminth infections and healthy primate populations. A study examining the overall health of populations, and the prevalence of parasites could be informative for primate conservation efforts, as work limiting the spread of parasite infection (specifically helminths) may not be necessary if the parasite plays a role in maintaining healthy gastrointestinal tract. Further microbiome surveys of larger populations of howlers or other Costa Rican primates need to be done. A larger survey will deepen understanding of the relationship that helminth parasites have with primate populations and will increase our evolutionary understanding of the relationship parasites have with humans. In general, to enable more precise research questions, and strengthen our understanding of howler health in Costa Rica, an extended survey and monitoring of Costa Rican howlers should be done. A up to date and general study to assess age, sex, population, diet and food availability, inter and intrapopulation interactions, as well as any significant interspecies interactions, would allow refinement of the questions asked in this thesis, and future research on these howlers.

While I did not investigate this in this study, a differential abundance analysis between howlers in Santa Rosa and fragmented forests such as Tamarindo could be done to examine if they have significantly different species present, such as pathogenic enterobacteria inducing inflammation, as this has been shown to occur in other primate populations near humans[102].

Additionally, a survey of the plant and fruit species that are available and eaten by howlers in these different habitats could be studied, as diet plays such a large role in the dynamic changes of the microbiome, and changes in bacterial diversity could be strongly associated with diet. Certainly, many confounding variables could be at play, and difficult to control for in a wild system. As mentioned, food availability, as well as human effects on foraging behaviour in fragmented and unfragmented forests, could contribute to bacterial diversity, gut health, and parasite prevalence. Further study to isolate and study the impacts that these other factors have need to be done in order to confidently correlate fragmentation, diet, helminths and increased bacterial diversity.

Conclusion

Together, these two studies of the gastrointestinal microbiomes of howlers grants much needed insight into how parasites and anthropogenic habitat fragmentation play a significant role in the health and ecology of howler monkeys. We already knew from a large variety of studies that the bacterial microbiome of howlers is dynamic, changing with diet[51], activity[49], as well as age/sex[32], but this study additionally evaluated the impacts of parasites and habitat fragmentation. Fragmentation of primate habitat has demonstrated decreased diversity of gastrointestinal bacteria in Red Colobus monkeys, an African primate that is largely folivorous, similar to howlers[59]. Furthermore, a decreased diversity of bacteria has been hypothesized to reduce the ability of the gut microbes to break down folivorous diets, decreasing availability of nutrients to these monkeys[59]. Increased parasite infection has also been linked to fragmentation. In these same African Red Colobus monkeys, increased parasite infection was seen in populations in fragmented habitats[122], howler monkeys in Mexico were found to be more likely infected with parasites in fragmented habitats[57], and in a hurricane effected forest, a similar result of elevated levels of parasitism[69]. Though in these studies, these changes in host-parasite dynamics were hypothesized to be due to indirect effects of forest fragmentation; decreased population size, or in the howler studies, *Controrchis biliophilus*, a nematode known to be transmitted via Cecropia plants, was found in high abundance, and in the hurricane damaged forest, Cecropia was found to be increased in abundance.

Additionally, human fragmentation with wild populations of mammals of other species show similar results. In an African mouse, higher parasite species richness was found in fragmented habitat, but parasite abundance was not significantly different, as these rodents were hypothesized to have a potentially permanent association with parasites[115]. The Eurasian red squirrel and common fruit bat show a similar relationship, increased parasite abundance in fragmented forest habitats, but this was again hypothesized to be due to indirect factors of nutritional stress related to lower resource availability[123], or less species diversity within the fragments[124]. Degradation in habitat quality has been shown to negatively impact the diversity of bacteria in multiple species, a relationship I further support within howler populations in this thesis, and fragmentation has indirect effects on the species living in those habitats, which may lead to increased risk of parasitism. These studies further implicate the importance of examining diet and other behavioural changes when studying parasitism and the gastrointestinal microbiome, as fragmentation has many cascading effects on primate habitat.

My research indicates that parasites affect the howler microbiome in similar ways as they do in humans. These studies support that valuable insight can be taken from non-human primates as a study system for human health research, shedding light on the evolutionary relationship parasites have with human hosts, through examination of closely related non-human primate populations. Further revealing that helminth infection could be used as a therapeutic tool in monitoring primate health, showing a high degree of similarity to helminth effects of the human gut microbiome[33]. Primates can help our understanding of the evolutionary history of human physiology, as they have lived mutualistically with parasites, and only in recent industrialized human history have we made parasites uncommon[74]. It is quite suggestive that countries with lower rates of parasite infection also have higher rates of inflammatory diseases and autoimmune disorders[92], and it is possible that parasites play a more complex role in human health than currently understood, and clues to the full gastrointestinal impact they have could be uncovered through study of primate populations. To conclude, research in the health of wild populations of primates should take note of the wealth of research already conducted for in humans for understanding biological mechanisms and health indicators, and studies working for the benefit of human health should take note of primatological studies performed to more fully understand the ecological evolutionary role that parasites have on the gastrointestinal tract in humans.

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Appendix:

Supplementary Tables

Supp. Table 1: Total metadata for howlers that were sampled from Costa Rica in each of the sampling locations; Sector Santa Rosa, Eladio's Ranch, and near the town of Tamarindo. Sample code was used internally to organize samples. "NA" was used for helminth infections for samples that were stored in Liquid Nitrogen as they were not examined for helminths.

Sample Code	Age	Sex	Field Site	Storage Method	Helminth Infection
10f	Adult	Female	Santa Rosa	Liquid Nitrogen	NA
11f	Adult	Male	Santa Rosa	Liquid Nitrogen	NA
12f	Adult	Male	Santa Rosa	Liquid Nitrogen	NA
13f	Adult	Female	Santa Rosa	Liquid Nitrogen	NA
14f	Unknown	Unknown	Santa Rosa	Liquid Nitrogen	NA
15f	Unknown	Unknown	Santa Rosa	Liquid Nitrogen	NA
16f	Adult	Female	Santa Rosa	Liquid Nitrogen	NA
17f	Adult	Female	Santa Rosa	Liquid Nitrogen	NA
18f	Adult	Female	Santa Rosa	Liquid Nitrogen	NA
19f	Unknown	Unknown	Santa Rosa	Liquid Nitrogen	NA
20f	Adult	Male	Santa Rosa	Liquid Nitrogen	NA
21f	Adult	Male	Santa Rosa	Liquid Nitrogen	NA
26f	Adult	Female	Eladio's Ranch	Liquid Nitrogen	NA
27f	Adult	Male	Eladio's Ranch	Liquid Nitrogen	NA
28f	Unknown	Unknown	Eladio's Ranch	Liquid Nitrogen	NA
29f	Unknown	Unknown	Eladio's Ranch	Liquid Nitrogen	NA
32f	Unknown	Unknown	Tamarindo	Liquid Nitrogen	NA
33f	Unknown	Unknown	Tamarindo	Liquid Nitrogen	NA
34f	Unknown	Unknown	Tamarindo	Liquid Nitrogen	NA
35f	Juvenile	Unknown	Tamarindo	Liquid Nitrogen	NA
36f	Unknown	Unknown	Santa Rosa	Liquid Nitrogen	NA
37f	Unknown	Unknown	Santa Rosa	Liquid Nitrogen	NA
38f	Unknown	Unknown	Santa Rosa	Liquid Nitrogen	NA

39f	Adult	Male	Santa Rosa	Liquid Nitrogen	NA
40f	Unknown	Unknown	Santa Rosa	Liquid Nitrogen	NA
14e	Unknown	Unknown	Santa Rosa	Ethanol	Helminth
15e	Unknown	Unknown	Santa Rosa	Ethanol	Helminth
16e	Adult	Female	Santa Rosa	Ethanol	Helminth
17e	Adult	Female	Santa Rosa	Ethanol	Helminth
18e	Adult	Female	Santa Rosa	Ethanol	Helminth
19e	Unknown	Unknown	Santa Rosa	Ethanol	Helminth
20e	Adult	Male	Santa Rosa	Ethanol	Helminth
21e	Adult	Male	Santa Rosa	Ethanol	None
26e	Adult	Female	Eladio's Ranch	Ethanol	Helminth
27e	Adult	Male	Eladio's Ranch	Ethanol	Helminth
28e	Unknown	Unknown	Eladio's Ranch	Ethanol	None
29e	Unknown	Unknown	Eladio's Ranch	Ethanol	Helminth
32e	Unknown	Unknown	Tamarindo	Ethanol	Helminth
33e	Unknown	Unknown	Tamarindo	Ethanol	Helminth
34e	Unknown	Unknown	Tamarindo	Ethanol	None
35e	Juvenile	Unknown	Tamarindo	Ethanol	None
36e	Unknown	Unknown	Santa Rosa	Ethanol	None
37e	Unknown	Unknown	Santa Rosa	Ethanol	None
38e	Unknown	Unknown	Santa Rosa	Ethanol	Helminth
39e	Adult	Male	Santa Rosa	Ethanol	Helminth
40e	Unknown	Unknown	Santa Rosa	Ethanol	Helminth

Supp. Table 2: PCR 1 reagent quantities per sample for library preparation.

Item	**Quantity per sample (µl)**	**Final concentration**
2X NEBNext HiFi MasterMix	10	1X
NEB BSA (20 mg/ml)	0.1	0.1 mg/ml

Forward primer+Adapter (5 μM)	1	0.25μM
Reverse primer+Adapter (5 μM)	1	0.25μM
ddH_2O	3.9	N/A
TOTAL	**16**	
DNA lysate	4	1 ng/μl

Supp. Table 3: PCR 1 reaction conditions during library preparation.

		Temperature	**Time**
INITIAL DENATURATION		98°C	30 sec
DENATURATION	20 cycles	98°C	30 sec
ANNEALING		62°C	30 sec
EXTENSION		72°C	30 sec
FINAL EXTENSION		72°C	5 min
Hold		4°C	∞

Supp. Table 4: PCR 2 reagent quantities during the second stage of library preparation.

Item	**Quantity per sample (μl)**	**Final concentration**
2X NEBNext HiFi MasterMix	15	1X
Forward primer+Adapter (10 μM)	2.5	0.33μM
Reverse primer+Adapter (10 μM)	2.5	0.33μM
TOTAL	**20**	
DNA lysate	10	1 ng/μl

Supp. Table 5: PCR 2 reaction conditions for the second stage of library preparation.

		Temperature	Time
INITIAL DENATURATION		98°C	45 sec
DENATURATION	20 cycles	98°C	20 sec
ANNEALING		63°C	20 sec
EXTENSION		72°C	20 sec
FINAL EXTENSION		72°C	2 min
Hold		4°C	∞

Supp. Table 6: Parasite prevalence among howlers in Santa Rosa, Eladio's, Tamarindo, combined fragmented sites, and among all samples. Counts are of finding at least one parasite in each fecal sample, represented as present or not detected among each location.

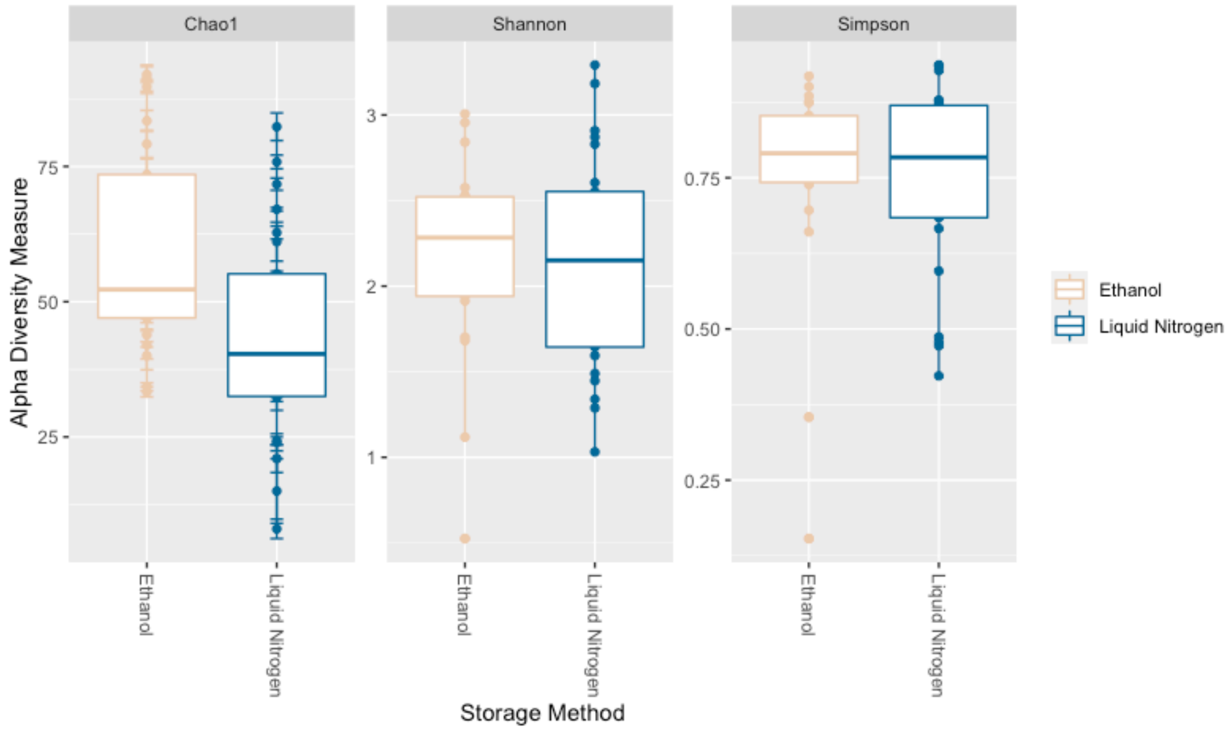
Habitat	Helminth Present	Helminth Not Detected	Prevalence
Santa Rosa	10	3	77%
Eladio's	3	1	75%
Tamarindo	2	2	50%
Fragmented Combined	5	3	63%
All Combined	15	6	71%

Supp. Table 7: Read counts per sample of the fecal samples used in differential abundance testing, sorted by helminth infection status. The average read count for positive samples was 4556 reads, and 8151 reads for negative samples.

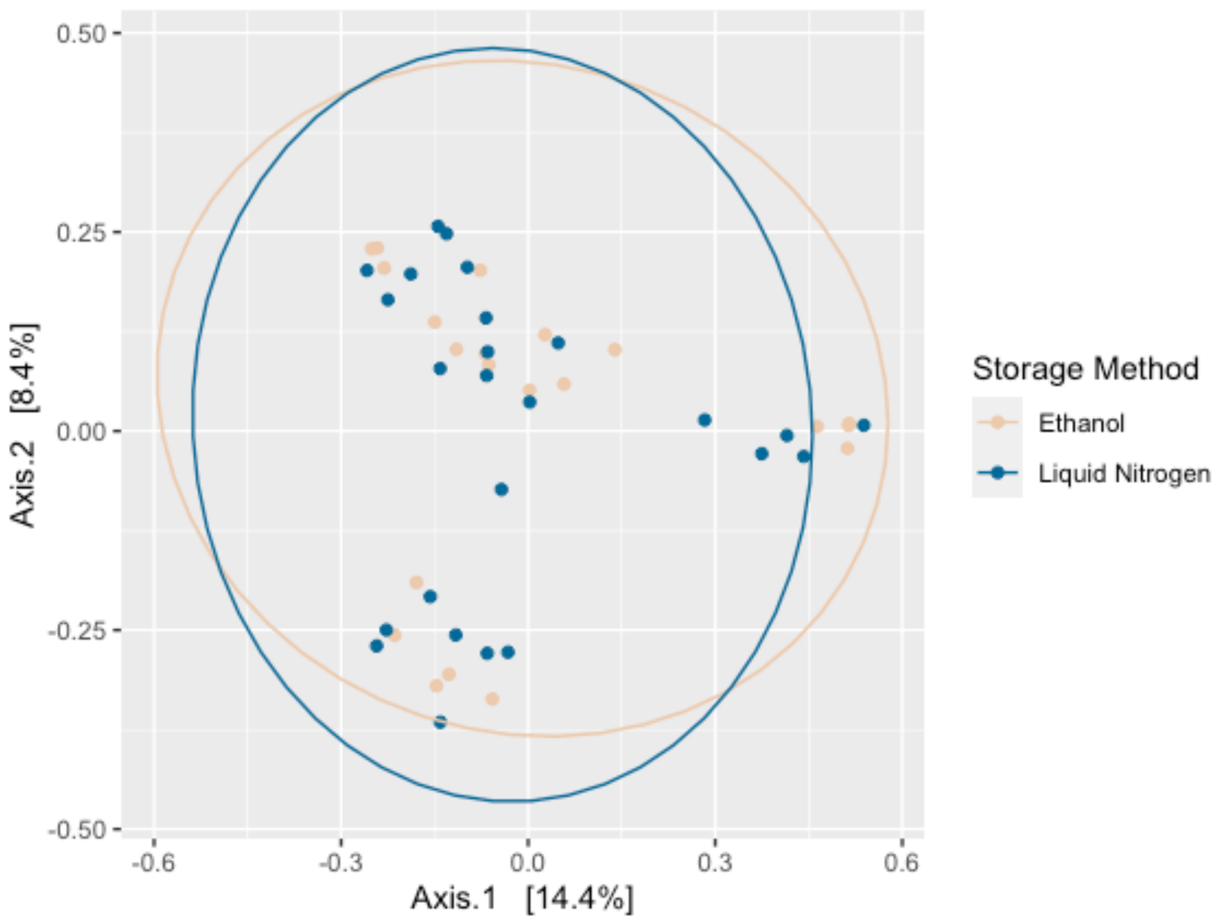
Sample	Read Count	Helminth Infection
14e	3883	Positive
15e	4394	Positive
16e	3339	Positive

17e	4064	Positive
18e	7011	Positive
19e	2303	Positive
20e	3479	Positive
26e	5755	Positive
27e	5325	Positive
29e	6242	Positive
32e	6853	Positive
33e	2363	Positive
38e	2410	Positive
39e	6506	Positive
40e	4415	Positive
21e	5493	Negative
28e	9215	Negative
34e	15012	Negative
35e	14842	Negative
36e	1696	Negative
37e	2649	Negative

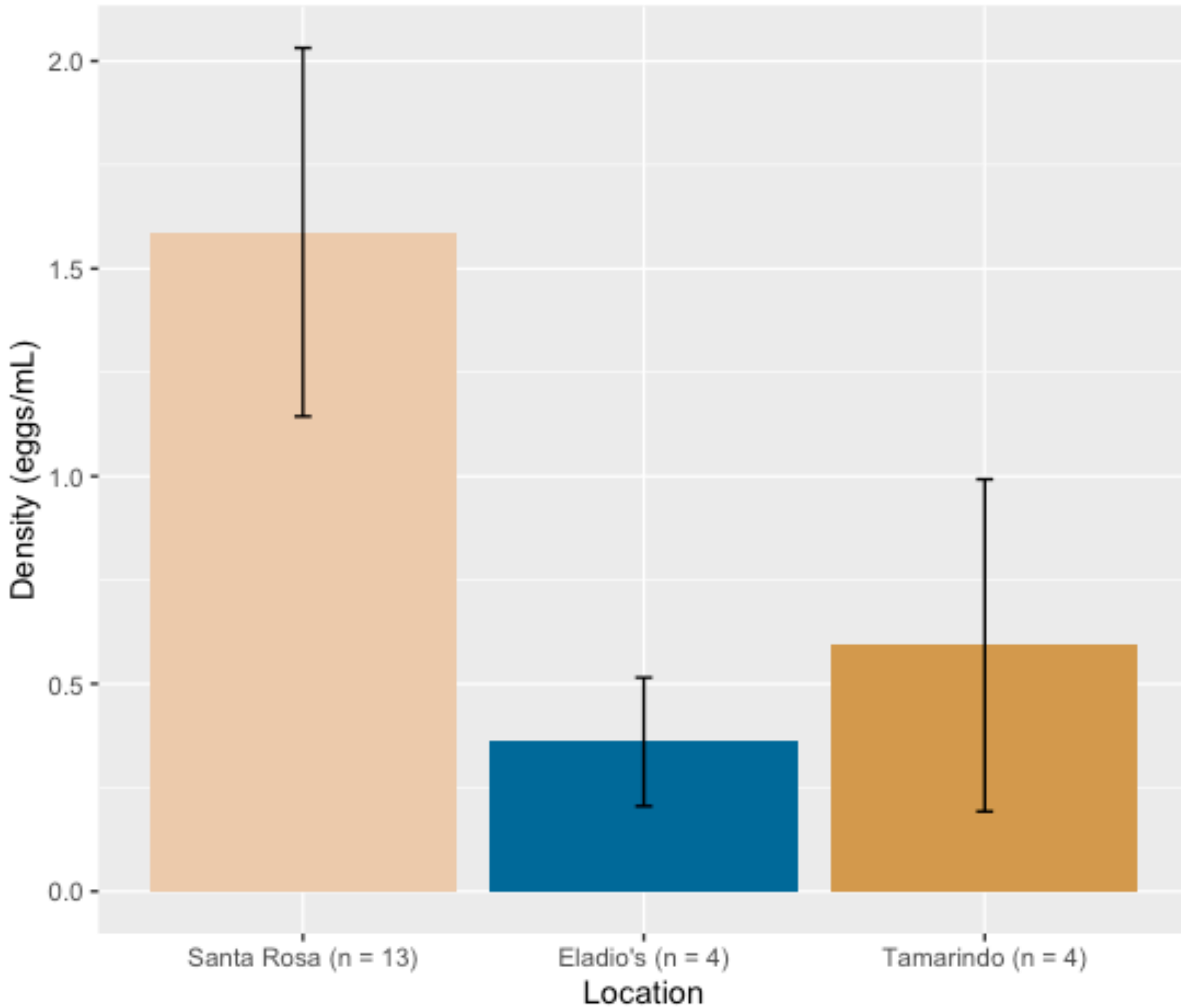
Supplementary Figures



Supp. Figure 1: Alpha diversity measures of fecal samples for howler monkeys based on storage method Chao1 estimates species richness, and Shannon and Simpson both estimate diversity (both richness and evenness), with Simpson allowing rare species to not influence the alpha value as much as Shannon. With an alpha of 0.05, a significant decrease in richness was seen in ($p_{Chao1} = 0.0078$) but was found to not be significantly different in diversity measures ($p_{Shannon} = 0.83$, $p_{Simpson} = 0.95$).



Supp. Figure 2: PCoA of log10 transformed data with a Bray-Curtis dissimilarity method used to visualize beta diversity measures for storage methods. Points represent individual fecal samples for howler monkeys. Though there is a high degree of overlap, statistical testing through a pairwise adonis shows a repeatable p value of ~0.05, suggesting significant difference in the bacterial beta diversity.



Supp. Figure 3: Density of parasites eggs counted (all parasite eggs per millilitre of fecal sample) found in howlers residing in the three study locations, the protected tropical dry forest of Santa Rosa, the town of Tamarindo, and Eladio's Ranch. Error bar represents the standard error from the mean (SEM) for samples in each group; Santa Rosa n = 13, Eladio's n = 4, and Tamarindo n = 4.