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Assessing the Role of the Microbiome, Parasite Infections, and Movement in Avian Health

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ASSESSING THE ROLE OF THE MICROBIOME, PARASITE INFECTIONS, AND MOVEMENT IN AVIAN HEALTH

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A DISSERTATION

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Dissertation Advisor: Dr. Pauline Kamath

An Abstract of the Dissertation Presented
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Avian health encompasses the physical, physiological, and behavioral well-being of birds. Assessing avian health is not only important for the conservation and management of wild birds and the recreational economy, but also for the management of infectious diseases that threaten public health and agriculture. Birds, comprising approximately 10,000 species and an estimated 50 billion individuals worldwide, are known to be involved in the spread of pathogens, some of which are zoonotic (from animals to humans), such as avian influenza and West Nile viruses. Individual measures of avian health may include physical measurements (e.g., body mass, wing length), pathogen infection status, the host-associated microbial community (the microbiome), and behavior (e.g., movement, migratory status). In particular, the microbiome is known to play diverse functional roles in individuals, including in immune function, growth, and physiology, however little is known about the relationships between the microbiome, pathogen infection, and fitness in wild birds. Here, we sought to evaluate indicators of avian health and the factors that drive them by (1) defining the "core" microbiome of mallard ducks (*Anas platyrhynchos*), (2) demonstrating the utility of microbiome data for pathogen detection in barn swallows (*Hirundo rustica*), (3) identifying predictors of parasite infection intensity and

relationships with the microbiome in Maine waterfowl, and (4) examining whether trait variation (plumage coloration) predicts potential indicators of avian health (pathogen infection, microbiome, movement), and whether these health indicators affect reproductive success in barn owls (*Tyto alba*). We collected cloacal swabs from multiple wild bird species to characterize the cloacal bacterial microbiome through 16S rRNA sequencing. We also collected biological samples for the detection and/or quantification of pathogen infections: a cloacal swab in nutrient broth for *Salmonella* (barn swallows) and whole blood for avian haemosporidian parasite (all others). We recorded host ecological data (all species), as well as movement and/or reproductive data (barn owls only) and conducted statistical analyses to identify potential drivers of pathogen infection, microbiome diversity and composition, movement and/or fitness. We found that although six taxa were identified as part of the core cloacal microbiome of mallard ducks, they were not universally prominent across three represented flyways (Obj. 1). Rather, sampling location was found to significantly influence the bacterial microbiome alpha diversity (Chao1; χ^2 = 71.218, p = 3.43e-16) of mallards. We also detected *Salmonella* in 23.1% (25) barn swallow samples and found a significant relationship between the presence of *Salmonella* and microbiome alpha diversity in swallows (Obj. 2). Location was the primary driver for avian haemosporidian parasite infection intensity in Maine waterfowl, followed by age (Obj. 3). While we found no consistent relationship between parasite infection and the avian microbiome across duck species, we did observe a significant relationship between parasite infection intensity and microbiome composition (beta diversity) using the weighted UniFrac measure (F = 3.02, p = 0.013). Finally, we found no relationship between plumage coloration and indicators of avian health in barn owls (Obj. 4). However, female owl movement, as reflected by home range area, was inversely related to measures of reproductive success (clutch size and fledge success.

Furthermore, microbiome alpha diversity was significantly correlated with Julian laying date, such that individuals with higher microbiome diversity laid their eggs earlier, thereby potentially enhancing their reproductive potential. Collectively, this thesis evaluates multiple indicators of avian health, including the microbiome diversity, parasite infections, and movement ecology, and provides valuable insight into the ecological drivers and dynamics of host-microbe interactions.

DEDICATION

For my family, for teaching me to never give up and always encouraging me. For my friends, for their overwhelming support, unforgettable memories, and keeping me sane throughout this entire process. To *Indomitus Imperium*, for the adventures that shaped my reality and the dragons that showed me courage in the face of fire.

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CHAPTER 1

THE ROLE OF ENVIRONMENT VERSUS HOST FACTORS IN SHAPING THE MALLARD CORE MICROBIOME

The gastrointestinal tract of birds is home to a wide variety of microorganisms, also known as the microbiome, that are involved in multiple functions including development, digestion, and immune function. Given the diverse functions played by the microbiome, identifying taxa that make up core microbiome (shared microbial taxa within a specific host group) may improve our understanding of the role of prominent microorganisms in host evolution and health. Migratory birds, like mallard ducks (*Anas platyrhynchos*), provide a unique avenue to explore the core microbiome as well as factors that drive microbiome diversity, such as age, location, and phylogeny. In this study we aimed to 1) describe the core cloacal bacterial microbiome of mallard ducks across three sampling regions (Israel, California and Maine, USA) and 2) examine relationships between the microbiome and both host (age and sex) and environmental (location) factors. We obtained 16S rRNA sequencing data from 382 mallards, 114 of which were from the previously published literature, to examine the core cloacal microbiome of mallard ducks. We identified six potential taxa, representing the core cloacal microbiome of mallard ducks, which were found in two of our three populations (Maine and Israel). We also found location to significantly influence bacterial microbiome alpha diversity (Chao1; $\chi^2 = 71.218$, $p = 3.43e\text{-}16$) and beta diversity, with 50 differentially abundant taxa among the three locations. Our results reveal key bacterial taxa that may make up the mallard core microbiome, and therefore may be involved in important host functions. These data also highlight the significance of the environment in shaping variation in the microbiome across host populations, complicating interpretations of core microbiome studies in migratory birds.

Introduction

The gastrointestinal tract of birds hosts a diverse microbiome (herein defined as the community of microorganisms inhabiting a specific environment) that serves various functions, including development, digestion, and immune function (Kohl 2012). Given the importance of the microbiome in individual health and biological functions, an increasing number of studies have emerged aiming to identify factors that influence microbiome diversity and composition (Dewar et al. 2017; Gallardo et al. 2017; Ambrosini et al. 2019; Corl et al. 2020; Turjeman et al. 2020; Choi et al. 2021; Pekarsky et al. 2021; Thie et al. 2022). A common area of focus has been to identify the "core microbiome" of specific species or populations, with the core broadly defined as the taxa shared by most or all individuals within a group of interest (Turnbaugh et al. 2007; Hamady and Knight 2009; Shade and Handelsman 2012). However, there are different definitions of what makes up this "core" (see Risely 2020; Neu et al. 2021) and how to measure or quantify it (Neu et al. 2021; Custer et al. 2023). These previous definitions of the core microbiome have varied with respect to its spatial or temporal stability, as well as its role in host evolution or function. Here, we define the core microbiome as the universal common core as described by Risely (2020) – taxa present in a significant proportion of a specific host population or species – which is suggested to reflect the portion of the microbiome with particular relevance to host biological function.

Studies into the common core microbiome have focused primarily on humans and mammals (~35%) (Neu et al. 2021), followed by invertebrates, plants, fish, reptiles, and birds, with avian studies comprising less than 5%. Waite and Taylor (2014) conducted a meta-analysis of 18 species of wild birds and chickens, and concluded that the core avian gut microbiome includes taxa mostly from Firmicutes with smaller representation from phyla such as

Actinobacteria, Bacteriodetes, and Proteobacteria; further subsequent reviews have confirmed these results (Grond et al. 2018; Sun et al. 2022). However, the avian gut microbiome has also been found to vary considerably across species (Godoy-Vitorino et al. 2012; Hird et al. 2015) and can be influenced by multiple host and environmental factors, such as diet (Pekarsky et al. 2021; Lu et al. 2022), age (Waite and Taylor 2015; Dewar et al. 2017; Zhou et al. 2020), sex (Corl et al. 2020; Liu et al. 2020; Góngora et al. 2021), movement (Corl et al. 2020; Turjeman et al. 2020; Thie et al. 2022), location (Hird et al. 2014), and phylogeny (Waite and Taylor 2014; Hird et al. 2015; Song et al. 2020; Lu et al. 2022). Together, these studies highlight the variable mechanisms driving microbiome composition and diversity across avian host populations. Further, core microbiome studies primarily have focused on a single population or populations within a limited geographic area. Given that microbiome diversity is known to correlate with both host evolutionary history (Brooks et al. 2016; Koskella, Hall, and Metcalf 2017; Sharpton 2018) and environment, it is important to explore microbiomes of a single species across large spatial scales to make species or population level inferences into key taxa comprising the core microbiome and their functional roles. A better understanding of the core microbial taxa associated with a host species of interest can also provide insight into the co-evolution of hosts and their microbes.

Migratory birds are of significant interest in core microbiome studies due to their remarkable physiology and extensive movement. During migration, these birds undergo substantial physiological changes, which likely influence their microbiome composition (Skeen et al. 2021; Zhang, Yang, and Zhu 2021). Additionally, migration exposes them to various environments with a diverse array of microorganisms, which has been reflected by distinct microbiome compositions between migrant and resident populations of the same species

(Turjeman et al. 2020; Obrochta et al. 2022). For instance, in barn swallows (*Hirundo rustica*) an increased abundance of *Mycoplasma* spp. and *Corynebacterium* spp. was reported in migrant swallows compared to their resident counterparts (Turjeman et al. 2020). Similarly, in urban Canada geese (*Branta canadensis*), migrant geese were found to have higher abundances of Firmicutes, as well as *Terrisporobacter*, *Turicibacter*, and *Cellulosilyticum* species (Obrochta et al. 2022). Thus, investigations into the microbiome composition in migratory birds can shed light on the factors driving core microbiome composition, making these birds compelling candidates for such research.

Atlantic Flyway waterfowl, including mallards, play a significant role in bolstering the eco-tourism and hunting economy of the eastern United States. Extensive monitoring data across the entire flyway indicates a 50% decrease in mallard populations over the last two decades, prompting reductions in hunting bag limits for mallards across the entire flyway (Heusmann 2017; SUNY College of Environmental Science and Forestry n.d.). In Maine specifically, Breeding Bird Survey counts within the state show evidence of a population decline in mallards in recent years ("BBS - USGS Patuxent Wildlife Research Center" n.d.). In contrast, in California, which is part of the Pacific Flyway, mallard abundance has increased by 13% and is the most abundant breeding duck species ("CDFW News | Breeding Ducks Increase by 30 Percent in Annual CDFW Waterfowl Breeding Population Survey" n.d.). Given the importance of these birds for eco-tourism and hunting, insight into characteristics and drivers of the host species core microbiome may prove to be valuable for monitoring the health of these populations. Their widespread distribution also allows for addressing compelling questions regarding the influence of environment versus phylogeny on the core microbiome at the species and population level.

In this study, we targeted three spatially disparate populations of mallards to assess the existence of a phylogenetically conserved core microbiome for the species, as well as to examine factors shaping variability in the host microbiome across populations. Specifically, our two main objectives were to (1) define the core cloacal bacterial microbiome, using 16S rRNA sequencing data, of mallard ducks sampled across three migratory flyways, the Pacific Americas Flyway (California), Atlantic Americas Flyway (Maine), and Black Sea/Mediterranean Flyway (Israel), and 2) explore relationships between microbiome community diversity and composition and host factors (age, sex) across the three study regions. These results are expected to identify core taxa crucial for the normal functioning of the mallard gut microbiome and may reveal potential bioindicators that could be valuable for monitoring mallard population health.

Methods

Data Collection

We combined previously collected and newly generated 16S rRNA sequencing data to characterize the microbiome of mallard ducks from two populations in the United States (California, Figure 1.1B; Maine, Figure 1.1C-D) and a population in Israel (Figure 1.1E), representing the Pacific Americas, Atlantic Americas, and Black Sea/Mediterranean flyways, respectively. Microbiome sequence data from California mallards (n = 114) were obtained from a previous study and sample collection and sequencing methods are described in Ganz et al. (2017).

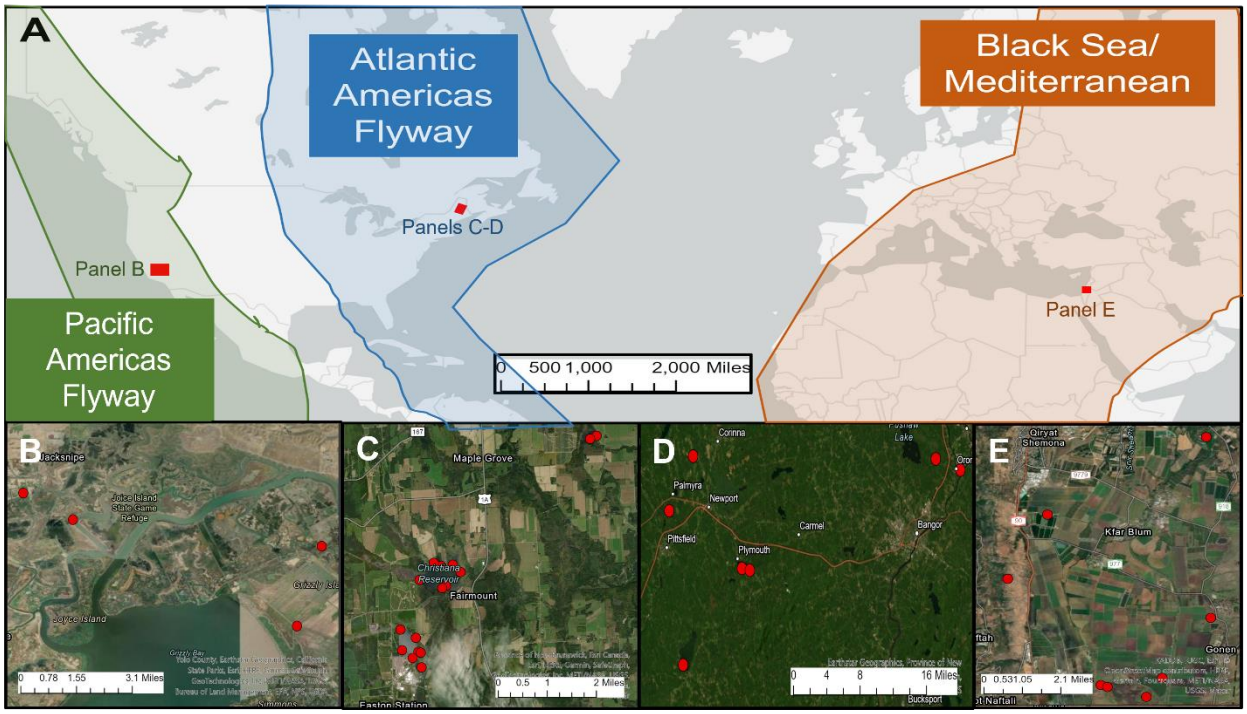

Figure 1.1. Sampling locations of mallard ducks (*Anas platyrhynchos*). Map shows A) three represented flyways, the Pacific Americas (green), Atlantic Americas (blue), and Black/Mediterranean (orange), with sampling regions shown by red boxes and corresponding panels for B) California, C-D) Maine, and E) Israel mallard populations. Red dots in panels B-E represent specific duck sampling sites.

We collected cloacal swabs to characterize the microbiome of mallards from two additional locations: Maine, USA, and Israel. We captured and sampled mallards in two areas of Maine during pre- to early- migration (August and September) over a three-year period (2018 – 2020). In 2018 and 2019, we sampled birds at sites around Lake Christina (Fort Fairfield), Lake Josephine (Eason) and Dorsey Pond (Fort Fairfield), located in northern Maine (Figure 1.1C), and in 2020, we sampled sites throughout southern Maine, ranging from Unity north to Orono (Figure 1.1D). We captured 168 mallard ducks using modified clover leaf traps (North and Hicks

2017) at sites along the edges of each body of water. During capture, each mallard cloaca was swabbed for obtaining sequence data on the host-associated microbiome. Briefly, a sterile swab was inserted into the cloaca of the bird and then swirled three times before storage in 95% EtOH and transfer to a -80℃ freezer until DNA extraction. From each bird, we also collected data on the individual's age (hatch-year or after hatch-year), sex, weight, bill length, and wing length.

In Israel, samples were collected from 114 wild mallards in two locations, the Hula Valley (Figure 1.1E) and Bet She'an Valley (Figure 1.1A). Both locations consist of a large variety of agricultural fields and a network of narrow canals, natural springs, fishponds, water reservoirs, and nature reserves. Such complex wetland systems are a favorable habitat for mallards (Pearse et al. 2012) and serve as an important stopover and/or wintering habitat for several thousands of migrating mallards each year (Shihirai et al. 1996). In the Hula Valley, all mallards were captured and live sampled in shallow waterbodies or agricultural fields during the winter months (November - February) of 2017-2018 (n = 6), 2018-2019 (n = 21), and 2019-20 (n = 47) except for two mallards found dead in the Hula Valley (n = 1 for 2018 and n = 1 for 2019). The live sampled mallards were caught using either a whoosh net (n = 4), baited swim-in traps (n = 18), or cannon net (n = 52) (Whitworth et al. 2007; Dmytryk 2012). Captured individuals were extracted from the traps and kept in cloth bags (1-3 individuals/bag) until processing. We additionally sampled carcasses of recently (< 2 hours) hunted mallards in the Bet She'an Valley (n = 19 for 2018-2019 and n = 19 for 2019-2020). Live caught mallards were banded, sexed, and aged based on plumage characteristics (Carney 1992). A cloacal swab for microbiome analysis (stored into 95% EtOH in 1.5 mL Eppendorf tubes) was collected for each bird and immediately transferred into -20℃ in temporary storage in the field, and then transferred to -80℃ within 5 days for long-term storage until processing in the laboratory.

Microbiome Extraction and Sequencing

DNA was extracted from cloacal swabs collected from Maine and Israel mallards for 16S rRNA gene sequencing of the microbiota, using DNeasy PowerLyzer PowerSoil kits (Qiagen Inc., Germantown, MD) and following the manufacturer's protocol with modifications as detailed in Corl et al. (2020). Extractions for samples collected in Maine were completed separately from extractions for samples collected in Israel. For each population, samples were extracted in batches randomized across local sampling sites and collection years. An empty tube was included with each extraction batch as a negative control to account for potential contamination introduced by the kit or laboratory environment. After extraction, samples were shipped to Argonne National Laboratory (Lemont, IL) for PCR-amplification (in triplicate) using the primer pair 515F/806R (Caporaso et al. 2012) of the 151 bp V4 region of the bacterial 16S rRNA gene; PCR products were then sequenced on an Illumina MiSeq in both directions to obtain pair-end reads.

Microbiome Quality Control and Data Filtering

We used demultiplexed 16S rRNA sequences and R v.4.3.0 (R Core Team 2023) for analysis of all microbiome data, following the workflow detailed by Callahan et al. (2016) and described in Choi et al. (2021) for quality control and filtering. We used DADA2 (Callahan et al. 2016) to identify amplicon sequence variants (ASVs) and DECIPHER (Wright 2015) to align sequences. We assigned taxonomy with SILVA 138.1 taxonomy database (Quast et al. 2013) and included species level assignments. We used the negative controls as reference for removal of contaminant sequences with the decontam package (Davis et al. 2018); for this analysis, a prevalence threshold of 0.5 was used to remove sequences more common in the negative controls than in our samples. We also removed any ASVs not belonging to the kingdom

Bacteria. We treated each dataset separately (California, Maine, Israel) up to this point, before combining them into a single phyloseq object to build a maximum likelihood phylogenetic tree with the package phangorn (Schliep 2011) and merged all files (sequence variant table, taxonomy table, and phylogenetic tree) with the metadata using the phyloseq package (McMurdie and Holmes 2013) for statistical analyses.

We assessed the cloacal bacterial community diversity for each sample as well as between samples after rarefaction to 4000 reads based on the values of mean alpha diversity where the rarefaction curves (rngseed = 711) plateaued. At these sampling depths, even the most diverse samples plateaued on rarefaction curves and thus samples that fell below these depths were removed leaving 114 mallards from California, 168 from Maine, and 100 from Israel for a total of 382 mallards that were included in downstream microbiome analyses.

Identifying the Core Microbiome

Neu et al. (2021) describes three methods to identify the common core microbiome: occurrence only, relative abundance only, and abundance-occurrence combined. Occurrence is the number of samples that a specific taxon is present in or the prevalence. Abundance only methods focus on the proportion of reads that a specific taxon occupies within a sample. Abundance-occurrence factors in both abundance and occurrence to identify core taxa. For all three methods, results will vary based on the cutoffs set for core membership. Based on reviews (Risely 2020; Neu et al. 2021; Custer et al. 2023), there seems to be no consensus on what thresholds to set for abundance and occurrence, however, Custer et al. (2023) found occurrence based methods to be the most accurate for identifying a core microbiome, thus we used occurrence to define our common core microbiome.

We used the microbiome (Lahti and Sudarshan 2017) package to determine core cloacal microbiome taxa for mallard ducks across all three sampling locations. First, using the prevalence (occurrence) only method, we set a minimum prevalence threshold of 50% for which any taxa below this threshold at the lowest abundance (in this case relative abundance >0% for the prevalence only method) would be excluded from core taxa as per Custer et al. (2023). This was done to identify only taxa that are found in a majority (>50%) of samples as core taxa at the species level. Once taxa below the 50% prevalence threshold were filtered out, only core taxa remained, all of which had a prevalence >50%. We then explored how the prevalence of the identified core taxa changed across a range of abundance parameters – ASV minimum relative abundance from >0.0% (to represent prevalence only method) to 0.5% of the total reads – by creating a heatmap. The range of prevalence or occurrence displayed on the heatmap demonstrates how the prevalence of the core taxa changes with relative abundance. We set our maximum prevalence cutoff to 70% as previous research (Risely et al. 2021) determined that thresholds above 70% became inconsistent and unpredictable for diversity measures. We then used the microbiome and eulerr (Larsson 2022) packages to construct a Venn diagram displaying the core microbiome for each location as well as taxa shared between locations, reflecting the distribution of core taxa at the population level. We again used a prevalence (occurrence) only method, with a threshold of 50% of samples within that location, to determine core taxa per location. We then built separate phylogenetic trees with the package phangorn (Schliep 2011) of the core microbiome for each location, annotating core taxa shared between locations.

Exploring the Effects of Location and Host Factors on the Cloacal Bacterial Community in Mallards

We filtered the dataset to include only individuals with data for sex (n = 372) to examine whether microbiome alpha diversity, using the Chao1 measure (Chao 1984), differed between male and female mallard ducks. We applied the Chao1 measure as it includes rare and missing species (Chao and Shen 2003), which are informative for diversity comparisons. We used a Wilcoxon rank sum test (Mann-Whitney U test; Mann and Whitney 1947) to test for significant differences in Chao1 alpha diversity between male and female mallard ducks. For age, we used a linear model with an interaction between age and location as California samples only included juvenile mallards. We ran a Kruskal-Wallis test (Kruskal and Wallis 1952) to test for differences in Chao1 alpha diversity across locations and then ran pairwise comparisons using the Wilcoxon rank sum test (Mann and Whitney 1947).

We examined the variation in cloacal bacterial community composition (beta diversity) among locations for mallards using principal coordinate analysis. Using adonis from the vegan package (Oksanen et al. 2020), we calculated permutational multivariate analysis of variance (PERMANOVA) for both the weighted and unweighted UniFrac metrics. UniFrac compares communities by utilizing phylogenetic information to estimate sample distances (Lozupone and Knight 2005). The unweighted UniFrac relies solely on presence/absence data, while the weighted UniFrac takes into account the relative abundance of ASVs to calculate distances(C. A. Lozupone et al. 2007).

Lastly, we used the ancombc2 function in the ANCOM-BC package (Lin and Peddada 2020; Lin et al. 2022) to run a pattern analysis following Lin and Peddada (2023) using the code provided on their GitHub repository (“ Multi-Group Analysis of Compositions of Microbiomes

with Covariate Adjustments and Repeated Measures" n.d.) with respect to location. We identified differentially abundant taxa at the Family level, using the location Israel as the reference, given that mallards from this location had the most diverse microbiomes. We built a plot displaying differentially abundant taxa with respect to Israel, using the Holm-Bonferroni method (Holm 1979) to correct the significance threshold for multiple comparisons.

Results

Core Mallard Cloacal Microbiome

At the species level, our analysis identified six core taxa present in the mallard cloacal microbiome (Figure 1.2). These six taxa included a *Helicobacter* species, a *Fusobacterium* species, an unidentified bacterium from the family Leptotrichiaceae, *Megamonas funiformis*, an unidentified bacterium from the family Lachnospiraceae, and *Clostridium sensu stricto 1*. With prevalence only (set at 50%), the most prevalent taxa (65.9%) was the unknown *Helicobacter* species, whereas the least prevalent (53.7%) was *Clostridium sensu stricto 1*. The most abundant taxon was the unknown *Fusobacterium* species (6.31%), while the unknown Lachnospiraceae species was the least abundant (0.007%).

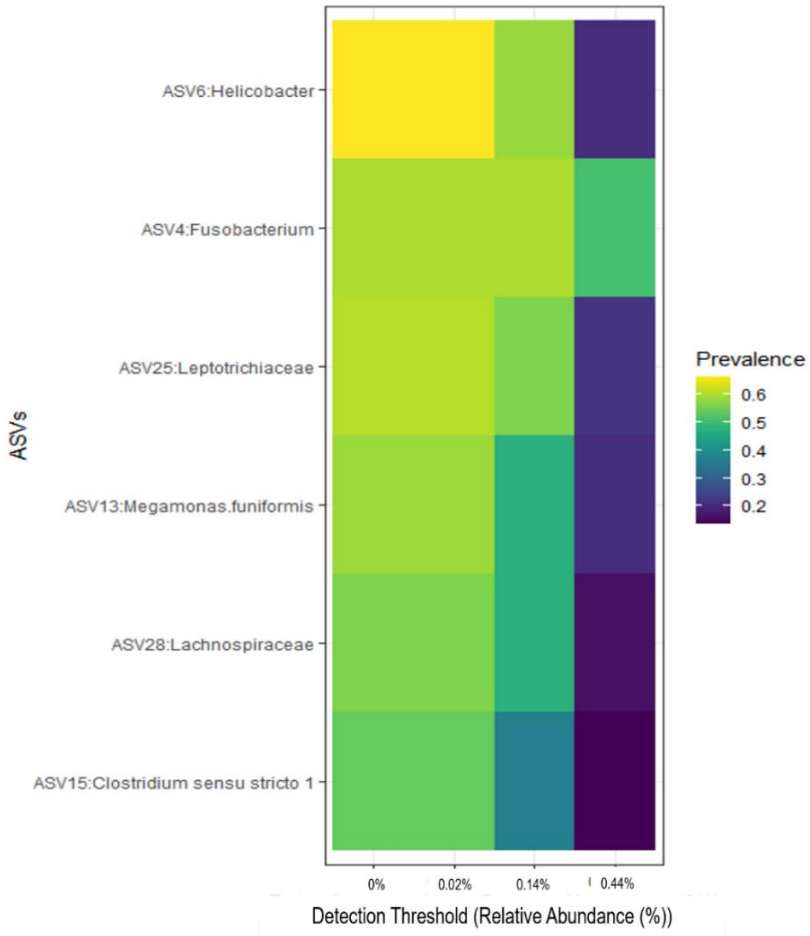

Figure 1.2. Heatmap displaying core cloacal microbiome taxa for mallards. Threshold taxonomic detection parameters were set to range from 20% to 70% for ASV prevalence and from >0.007% to 0.44% for ASV relative abundance. Blocks from left to right reflect increasing relative abundance and colors correspond to prevalence, from low (indigo) to high (yellow). Zero taxa matched our maximum prevalence of 70% thus taxa at 70% are not displayed here.

At the location level and assuming a minimum prevalence of 50%, we identified ten core taxa in California mallards, none of which were shared with any other location; for Israel and Maine mallards, 32 and 31 core taxa were present, respectively, with 15 taxa shared between the two locations (Figure 1.3). Shared taxa between Maine and Israel were as follows:

Prevotellaceae Ga6A1 group, a unknown bacterium from the family Porphyromonadaceae, a *Peptoniphilus* species, a *Parvimonas* species, an unknown bacterium from the family Peptosteptococcaceae, a *Helicobacter* species, *Fusobacterium mortiferum*, a *Fusobacterium* species, *Megamonas funiformis*, an unknown bacterium from the family Leptotrichiaceae, *Clostridium sensu stricto 1*, an *Intestinimonas* species, *Subdoligrahulum variabile*, and two unknown bacteria from the family Lachnospiraceae (Figure 1.4). All six bacteria identified as part of the core mallard microbiome at the species level were also identified in the core taxa of both the Maine and Israel mallard populations, but not found in the California population. The remaining nine, although shared between Maine and California, fell below our initial minimum prevalence threshold of 50% at the species level and thus were not found on our heatmap (while above 50% prevalence for each of the two locations, these nine taxa were not above 50% prevalence at the species level and thus excluded from the heatmap). Taxa found in the core microbiome of California mallards is displayed in Supplemental Figure A.1.

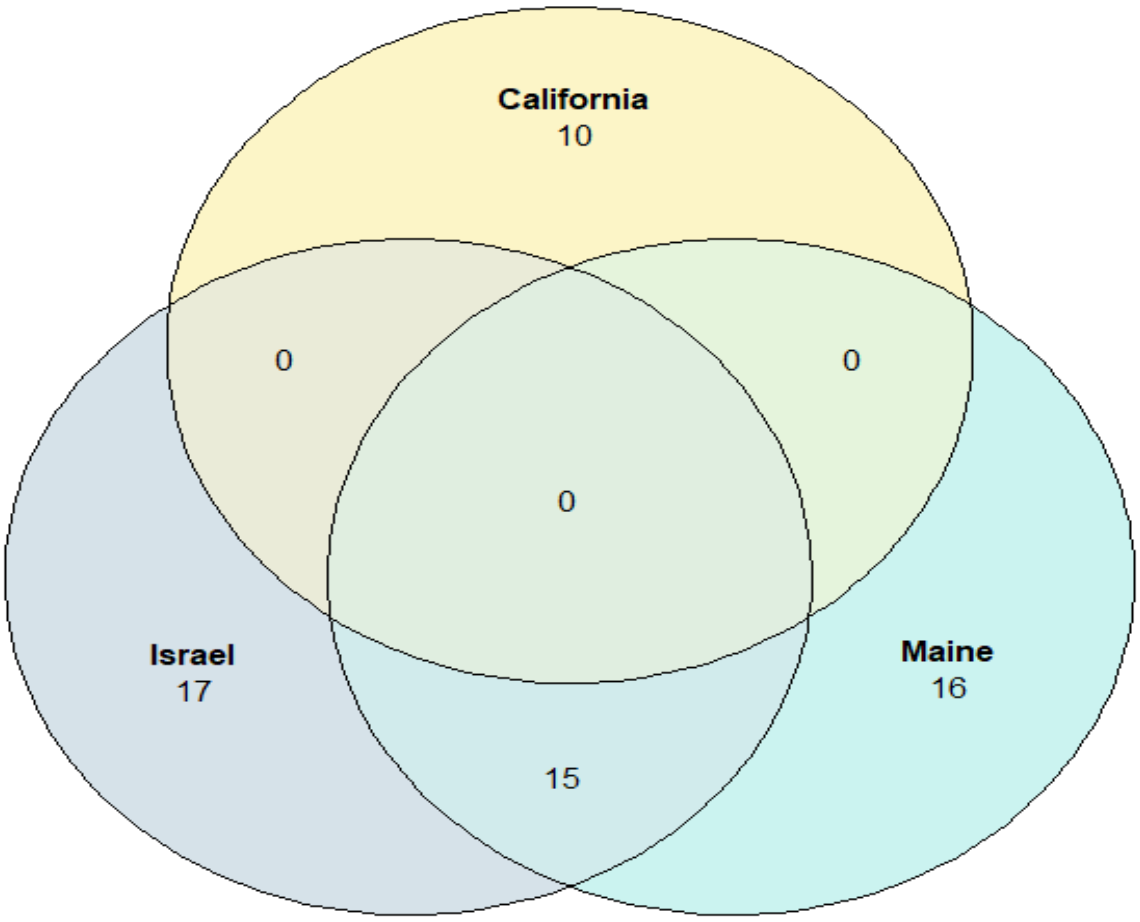

Figure 1.3. Venn diagrams of core cloacal microbiome taxa based on 50% prevalence (occurrence) across samples for each sampling location. At 50% prevalence, California mallards (yellow) had 10 core taxa with none shared; whereas Israel (gray) mallards had 32 core taxa and Maine (blue) mallards had 31 core taxa, with 15 taxa shared between the two locations.

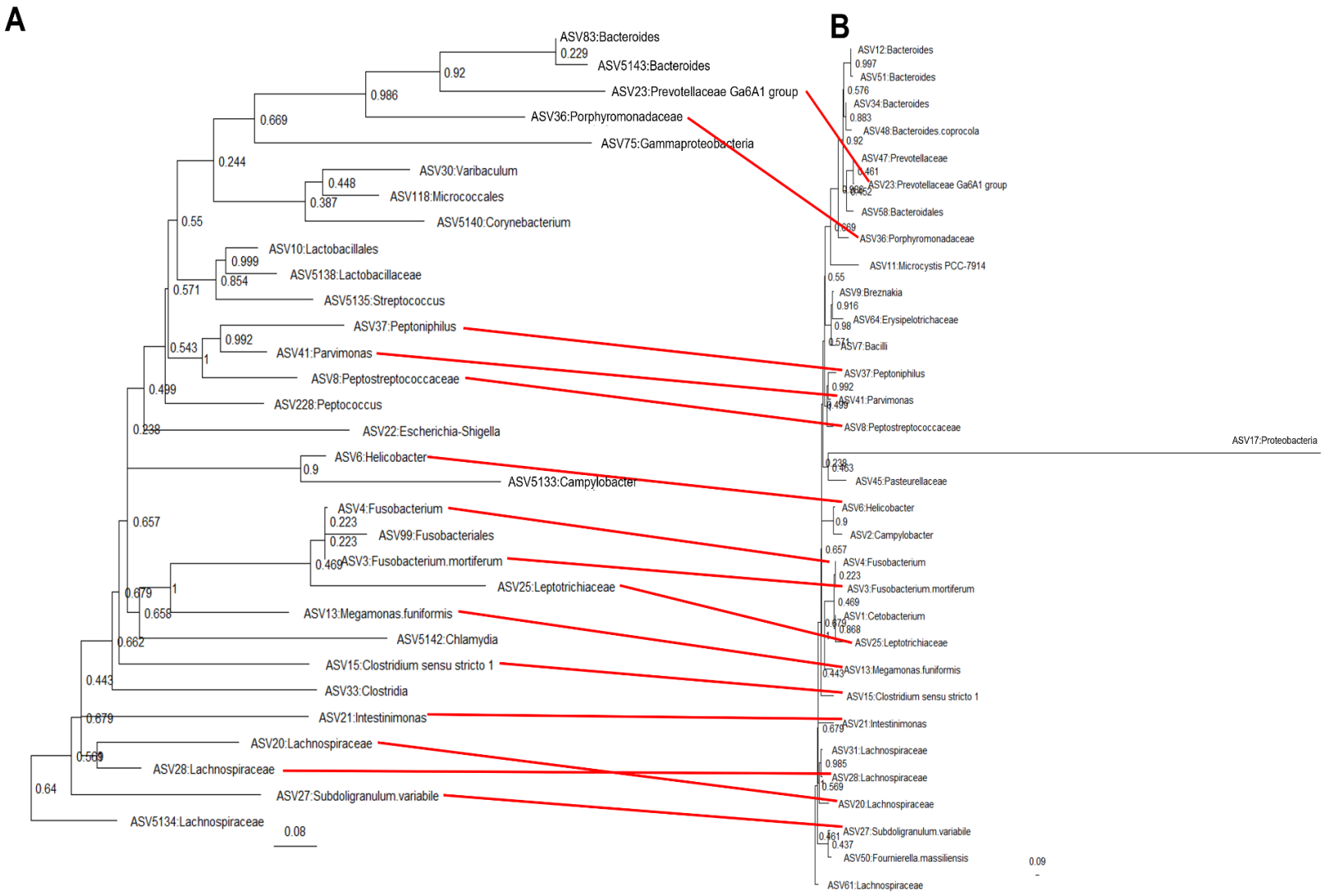

Figure 1.4. Phylogenetic trees of core cloacal microbiome taxa for mallards sampled in A) Maine, and B) Israel. Red lines indicate shared taxa between locations. Maine and Israel shared 15 core taxa.

Location Drives Cloacal Bacterial Community Alpha and Beta Diversity

Chao1 alpha diversity did not differ between male and female mallards (W = 17,895, $p = 0.47$; Figure A.2). Male mallards had a mean Chao1 estimate of 88.33, whereas females had a mean estimate of 96.52. A linear model revealed a significant interaction between age and location for Chao1 alpha diversity ($R^2 = 0.19$, $F = 22.16$, $p = 2.28^{-16}$) with adult mallard ducks having a higher mean Chao1 (116.42) than juveniles (75.43, W = 823, $p = 6.34^{-12}$; Figure 1.5); however, Chao1 alpha diversity did not differ (W = 823, $p = 0.71$; Figure 6) between adult (131.69) and juvenile mallard ducks from Israel (124.35), but was significantly higher in adults (102.39) than in juveniles (82.49) in Maine (W = 3929, $p = 0.0068$; Figure 1.6). Chao1 alpha diversity was significantly different among locations ($\chi^2 = 71.218$, $p = 3.43e\text{-}16$; Figure 1.7). Pairwise location comparisons revealed that microbial alpha diversity was also significantly different in Israel versus Maine (W = 11188.5, $p = 5.54e\text{-}6$), Israel versus California (W = 9519, $p = 2.93e\text{-}18$), and Maine versus California (W = 12764.5, $p = 2.10e\text{-}6$).

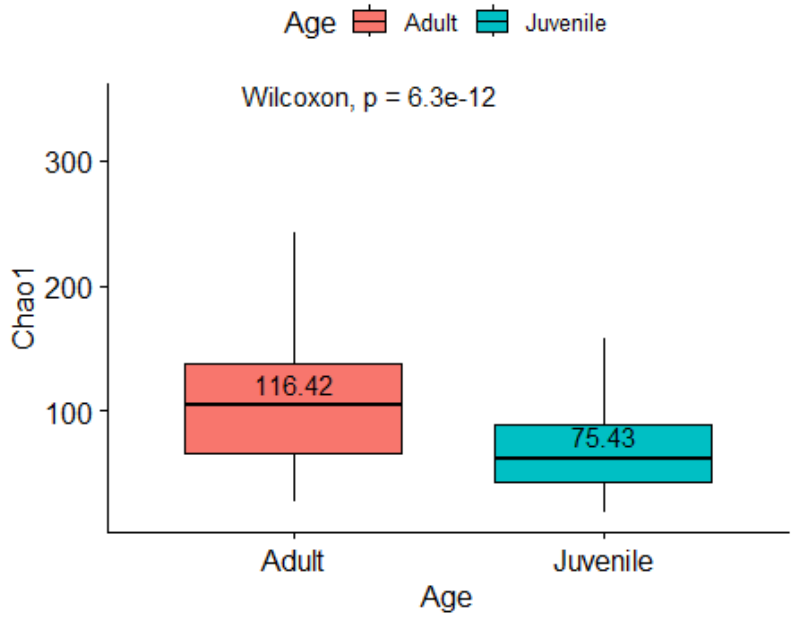

Figure 1.5. Boxplot comparison of Chao1 alpha diversity in adult (red) and juvenile (blue) mallard ducks. Adult mallard ducks had higher Chao1 alpha diversity (116.42) than juvenile mallard ducks (75.43, W = 823, p = 0.71).

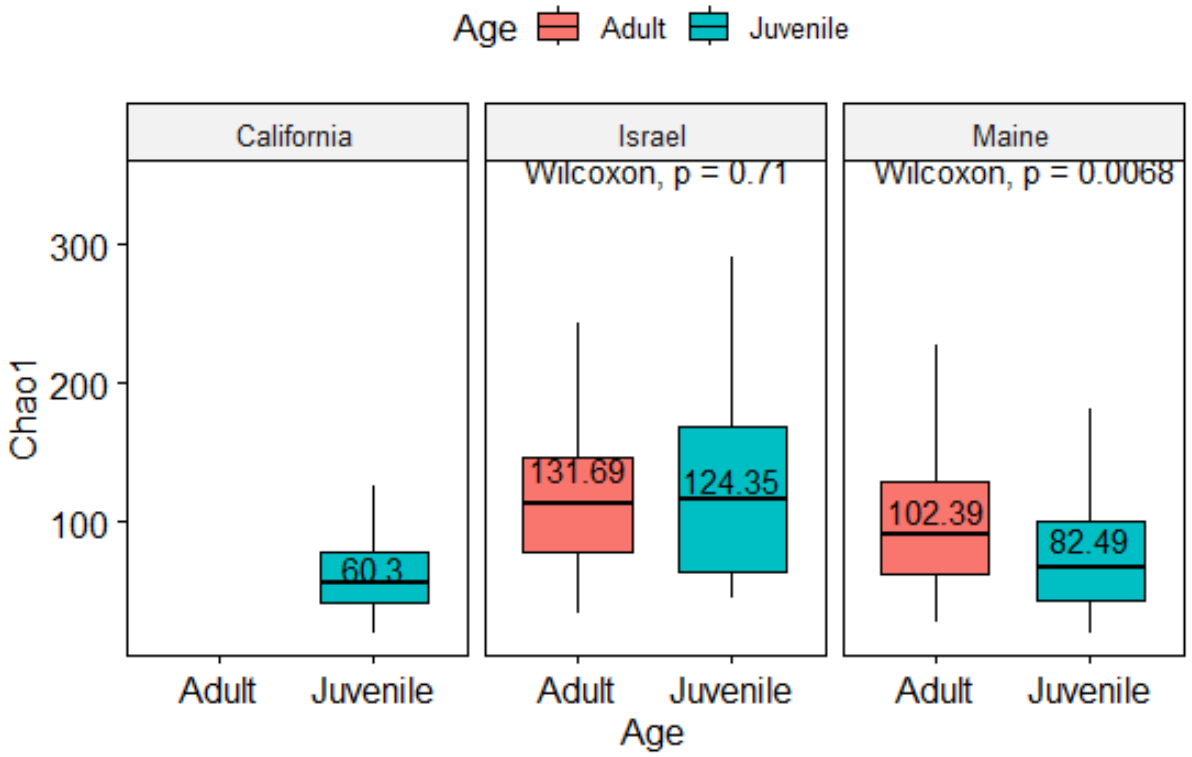

Figure 1.6. Boxplot comparison of Chao1 alpha diversity in adult (red) and juvenile (blue) mallard ducks by location. Juvenile mallard ducks from California (left panel) had a mean Chao1 of 60.3. Adult mallard ducks from Israel (middle panel) did not differ in alpha diversity from juvenile mallard ducks (W = 823, p = 0.71). For Maine (right panel), adult mallard ducks (102.39) had higher Chao1 alpha diversity than juvenile mallard ducks (82.49, W = 3,929, p = 0.0068).

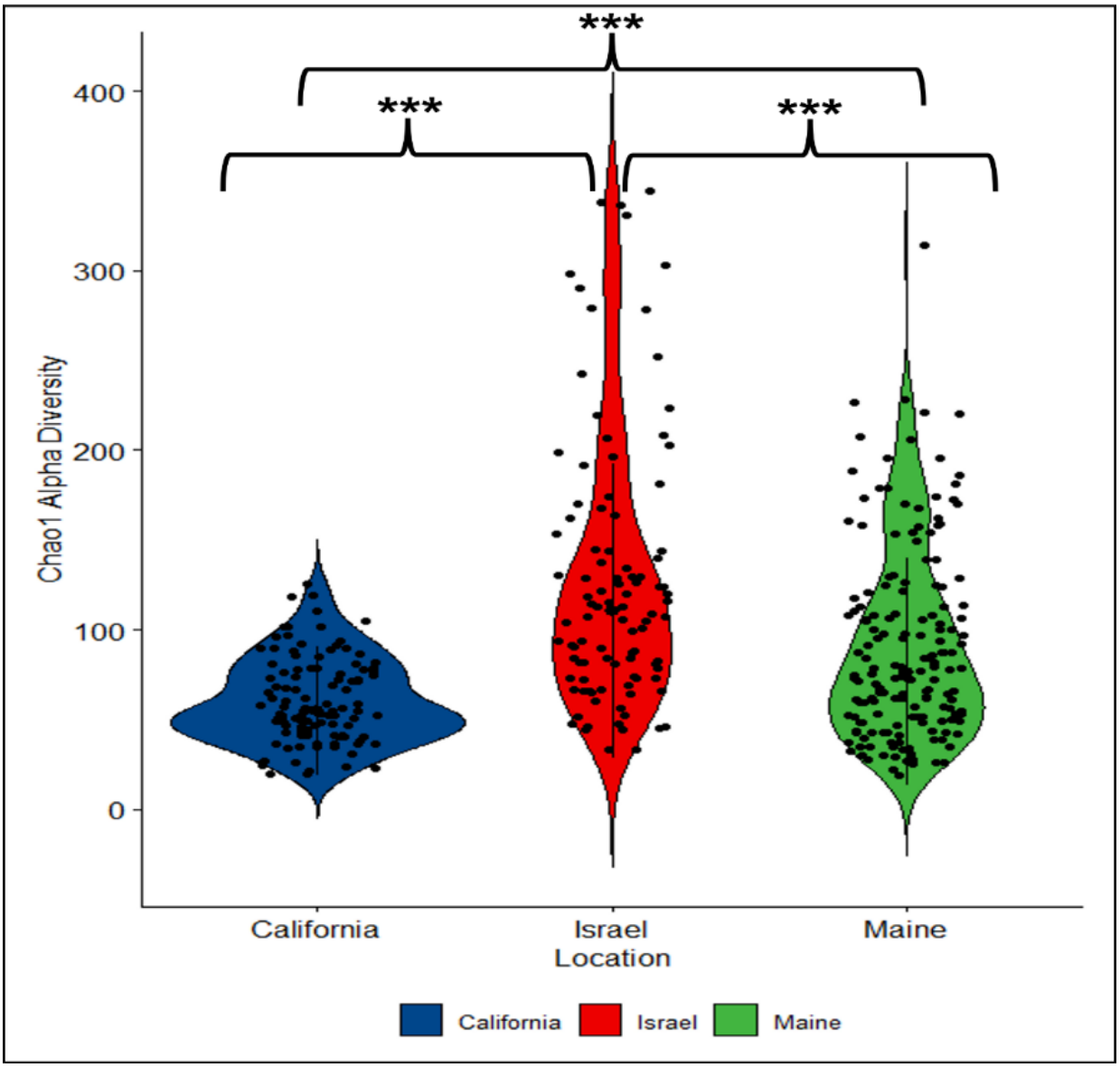

Figure 1.7. Violin plot comparison of Chao1 alpha diversity in mallard ducks by sampling location region. Alpha diversity varied significantly by location (χ^2 = 71.218, p = 3.43e-16). Pairwise comparisons were also significant for Israel (red) versus Maine (green; W = 11188.5, p = 5.54e-6), Israel versus California (blue; W = 9519, p = 2.93e-18), and Maine versus California (W = 12764.5, p = 2.10e-6).

Bacterial community composition, accounting for relative taxonomic abundance (beta-diversity), was also found to differ significantly among locations, based on both the unweighted (F = 203.4, p = 0.001; Figure 1.8A) and weighted UniFrac measure (F = 919.93, p = 0.001; Figure 1.8B). Our ancombc2 analysis revealed 65 differentially abundant taxa, 50 of which were differed significantly among locations (Figure 1.9).

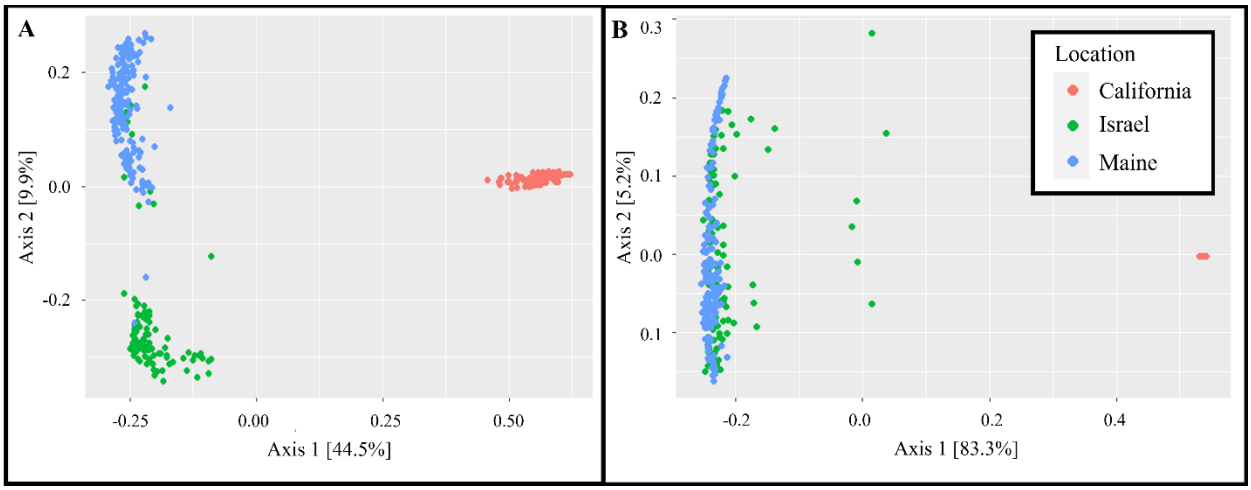

Figure 1.8. PCoA of beta diversity colored by sampling location for mallard ducks based on the A) Unweighted and B) Weighted UniFrac measures. Bacterial community composition varied significantly with location for both the unweighted (F = 203.4, p = 0.001) and weighted UniFrac measures (F = 919.93, p = 0.001).

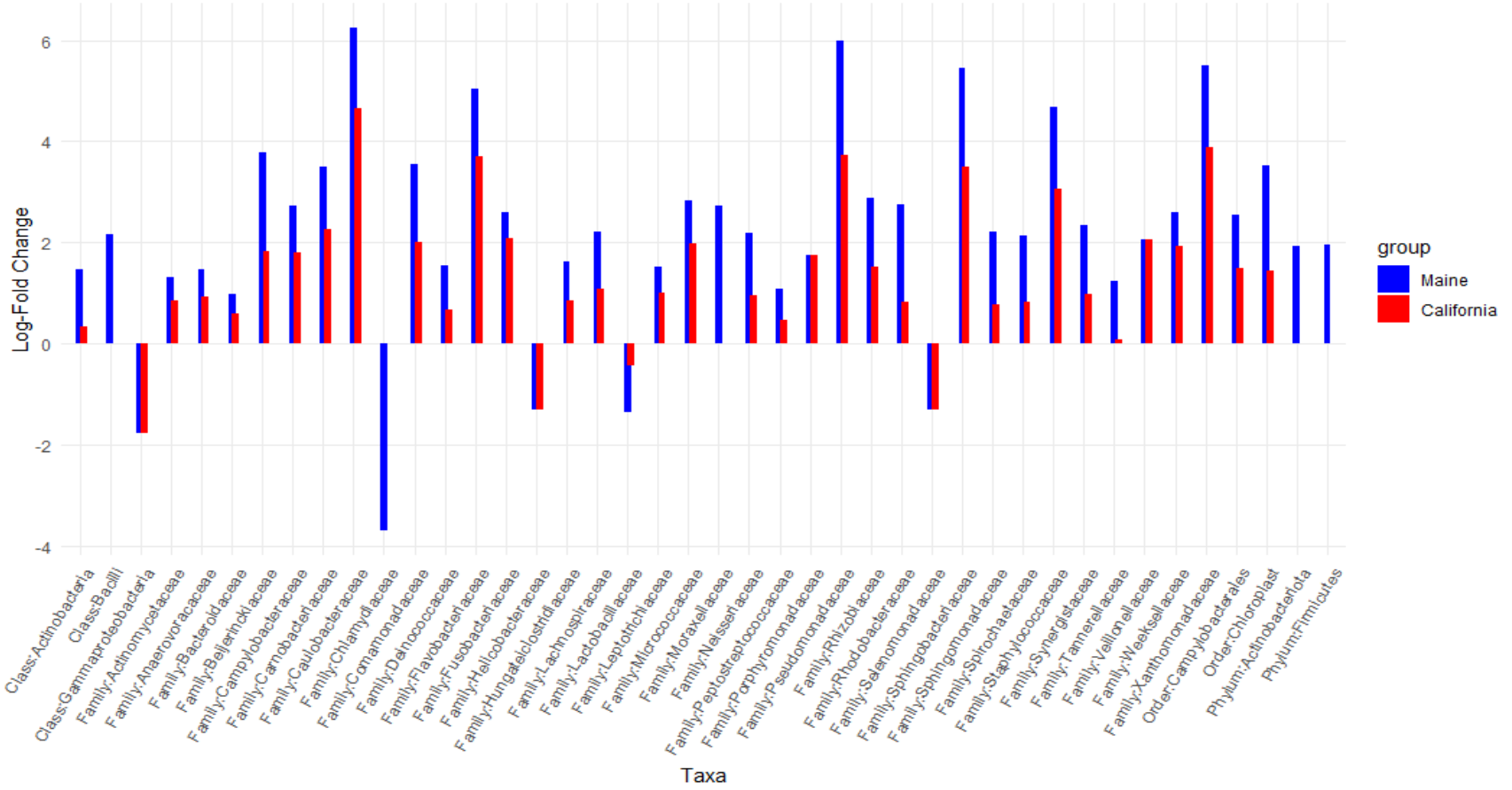

Figure 1.9. Barplot of ANCOM-BC2 differential abundance analysis with respect to location. With Israel as the reference category for abundance, 42 taxa were identified as differentially abundant with the Holm-Bonferroni correction of 34 taxa as significant. Maine (blue) had 42 differentially abundant taxa from Israel while California (red) had 38 differentially abundant taxa. Bars above the x-axis represent increased abundance, bars below represent decreased abundance with the log fold-change relative to the Israel reference group.

Discussion

We identified the core cloacal microbiome of mallard duck samples collected from three different populations across the globe, and examined both environmental and host factors, including location, sex and age, shaping the mallard microbiome taxonomic composition and diversity. Core microbiome research provides insight into the stable microbiome of individuals and hosts, highlighting potential taxa of functional importance. Studying the "typical" microbiome of hosts could also be useful as potential bioindicators of health as well to identify factors that may affect a "healthy" diverse microbiome. Here, using an "occurrence only" approach, we identified six possible taxa comprising the core microbiome of mallards at the species level when our prevalence threshold is set to 50%. We also found that Maine and Israel populations shared core taxa, however mallards from California shared no core taxa with the other mallard populations regardless of threshold parameters applied. Microbiome alpha diversity (Chao1) did not differ by sex but did differ by age and among locations. Microbiome composition (beta diversity) differed by location, with 50 taxa identified as being differentially abundant among populations. Our results showing location as a strong predictor of variation in the mallard microbiome suggests that the environment likely plays a central role in influencing host-associated microbial communities. Further, while we found weak support for a stable core microbiome in Israel and Maine mallards, no taxa were shared across all host populations, suggesting that a phylogenetically conserved core microbiome may not exist at the host species level.

Using a prevalence threshold of 50%, we identified six potential taxa as candidates for the species level core cloacal microbiome of mallard ducks: an unknown *Helicobacter* species, an unknown *Fusobacterium* species, an unidentified bacterium from the family Leptotrichiaceae,

Megamonas funiformis, an unidentified bacterium from the family Lachnospiraceae, and *Clostridium sensu stricto 1*. Although *Fusobacteria* are generally considered to be pathogenic to vertebrates (Roggenbuck et al. 2014), it is common in the microbiota of carnivorous and omnivorous birds (Waite and Taylor 2015) such as mallards. Members of the *Helicobacter* bacterial genus are also known to be pathogenic and associated with gasteroenteritis (e.g., *H. pylori*) in mammals and poultry, but other species in the genus are prevalent in the microbiome of birds (Whary and Fox 2004; Fox et al. 2006; García-Amado et al. 2013). *Megamonas funiformis* has been identified as a core microbiota for laying hens (Roth et al. 2022), Leptotrichiaceae have previously been found to be prevalent in the microbiota of other birds (Cho and Lee 2020; Dietz et al. 2019), and Lachnospiraceae have been linked to productivity in poultry (Lundberg, Scharch, and Sandvang 2021) likely due to their anti-inflammatory potential (Biddle et al. 2013).

Although the six core microbiome taxa were identified in over 50% of our samples, the lack of shared core taxa among all three locations suggests that core taxa are likely population, location, or environment-specific for mallard ducks, rather than species-specific. Our results highlighting differences in Chao1 alpha diversity, community structure (beta diversity), and differential abundance across the three locations provide further evidence for the environment being a strong driver of microbiome diversity and composition. Our three mallard study populations could have differences in diet, migratory behavior, and habitat types, factors known to influence microbiome diversity and composition. These results align with previous work done in brown-headed cowbirds (*Molothrus ater*) where no support was found for cowbird genetics, but rather location was a significant explanatory variable predicting microbiome diversity and composition (Hird et al. 2014). Even in closely related *Plethodon* salamander species,

individuals living in the same environment shared similar microbiomes compared to individuals of the same species in different environments (Muletz Wolz et al. 2018). However, another study in brown lemurs (*Eulemur* spp.) and sifakas (*Propithecus* spp.) found conflicting results, with brown lemurs sharing similar microbiomes despite location, whereas sifaka microbiomes varied between habitats (Greene et al. 2019). These conflicting results highlight the complexity in identifying drivers of microbiome diversity and emphasize the need for system and population specific studies.

Our results also support the idea that defining the core microbiome is method and parameter specific. Reviews of core microbiome studies have found that prevalence (occurrence) thresholds used to define core taxa ranged from 30% to 100%, with 100% being the most common (Neu et al. 2021; Custer et al. 2023) . However, a 100% threshold risks losing taxa that could serve important functional or ecological roles and, thus, is not recommended (Neu et al. 2021). Abundance only methods included taxa as core if they were the most abundant in samples (e.g. top 10 most abundant taxa) (Custer et al. 2023) or were more abundant compared to the surrounding environment (Neu et al. 2021). However, like the occurrence only method, rare taxa are missed with this method, results vary based on thresholds set, and are sample size dependent. The combined abundance-occurrence method accounts for sample size, however the core taxa may represent only a snapshot of the normal function of the microbiome and lacks the temporal scale that could be important for identifying a core microbiome taxa (Risely 2020). Thus, establishing standardized guidelines for identifying core microbial taxa (Neu et al. 2021; Custer et al. 2023) is crucial for ensuring study comparability and advancing our understanding of the core microbiome.

Sample preparation and sequencing can alter microbiome diversity and composition results (Nearing et al. 2021). One explanation for our finding of shared taxa between two of our locations (Maine, Israel), but no shared taxa with California, could be due to differences introduced during sample preparation and sequencing. The Maine and Israel samples used in this study were extracted and sequenced at the same facilities, whereas the California samples were derived from a previously published study (Ganz et al. 2017), and therefore extracted and sequenced at a separate facility. Thus, the lack of shared taxa among our three mallard study populations could be due to differences in the extraction kits, PCR reagents, and sequencing facilities and their associated bacteria. Likewise, the shared taxa between Maine and Israel could be taxa associated with shared reagents and facilities; however, given that the six core taxa identified are also common to the avian microbiome, we expect this potential explanation to be unlikely.

The avian microbiome is known to be influenced by host factors such as diet (Kohl 2012; Grond et al. 2018; Pekarsky et al. 2021), age (Dewar et al. 2017), and sex (Liu et al. 2020). We found that Chao1 alpha diversity did not vary by sex, but did vary by age, with adults having higher diversity than juvenile mallard ducks. Adults, having been exposed to more environments are likely to encounter a diverse range of bacterial taxa when compared to juveniles with less exposure to different environments. Our results are consistent with other studies in birds that have found associations between age and bacterial diversity (Dewar et al. 2017; Zhou et al. 2020; Márcia Barbosa et al. 2013), however the direction of this relationship has varied. For example, Zhou et al. (2020) found an opposing pattern, with juvenile Eurasian kestrels (*Falco tinnunculus*) having more diverse microbiome communities than adults. However, Zhou et al. (2020)

characterized the fecal microbiome, whereas our study used the cloacal microbiome which are known to be different (Videvall et al. 2018).

We also found that the relationship between age and microbiome diversity was not consistent across sampling locations. This difference by age was primarily driven by birds from Maine. The differences in age found in Maine might be due to unique environmental conditions affecting microbiome diversity. A previous study in cowbirds (*M. ater*) found both physical location and age to be strong drivers of microbiome diversity, with environment possibly being the strongest (Hird et al. 2014). Another study in zebra (*Taeniopygia guttata*) and Bengalese (*Lonchura striata domestica*) finches reported juvenile finches as having higher alpha diversity than adult finches, but finches living in the same environment tended to share more similar microbiomes (Maraci et al. 2022). Lastly, major microbiome differences between adult and juvenile chinstrap penguins (*Pygoscelis antarctica*) have been associated with food metabolism (Tian et al. 2021). As our Maine study area included more sites (23) than either of the other two locations (Israel – 8 sites, California – 4 sites) and the distribution of adults and juveniles was heavily skewed (> 66.7%) for most sites (17), we speculate that diet and environment could drive the microbiome differences observed between juvenile and adult mallards. The greater number of sites could represent a broader range of habitats and environments that then influence age-driven differences in alpha diversity.

Conclusions and Future Directions

We identified six potential taxa for the core cloacal microbiome of mallard ducks across two sampling locations (Israel and Maine). In addition, location was a strong driver of alpha and beta diversity, with multiple bacterial taxa differing in abundance across mallard populations, which is consistent with other studies (Hird et al. 2014; Muletz Wolz et al. 2018). We also found age to

influence alpha diversity but not sex which differs from some avian studies (Liu et al. 2020; Góngora et al. 2021), but is consistent with others (Kreisinger et al. 2017). However, the differences we observed related to age primarily stem from a single location (Maine), underscoring the significant influence of location on microbiome composition. Our results highlight the key role of the environment in shaping the avian microbiome. They also emphasize the need for future microbiome studies to follow previously published guidelines for better synthesis in the identification of core host-associated microbial taxa, data which will improve our understanding of the importance and functional role of the microbiome in host fitness and health.

CHAPTER 2

HIGH-THROUGHPUT SEQUENCING FOR EXAMINING *SALMONELLA* PREVALENCE AND PATHOGEN – MICROBIOTA RELATIONSHIPS IN BARN SWALLOWS

Studies in both humans and model organisms suggest that the microbiome may play a significant role in host health, including digestion and immune function. Microbiota can offer protection from exogenous pathogens through colonization resistance, but microbial dysbiosis in the gastrointestinal tract can decrease resistance and is associated with pathogenesis. Little is known about the effects of potential pathogens, such as *Salmonella*, on the microbiome in wildlife, which are known to play an important role in disease transmission to humans. Culturing techniques have traditionally been used to detect pathogens, but recent studies have utilized high throughput sequencing of the 16S rRNA gene to characterize host-associated microbial communities (i.e., the microbiome) and to detect specific bacteria. Building upon this work, we evaluated the utility of high throughput 16S rRNA gene sequencing for potential bacterial pathogen detection in barn swallows (*Hirundo rustica*) and used these data to explore relationships between potential pathogens and microbiota. To accomplish this, we first compared the detection of *Salmonella* spp. in swallows using 16S rRNA data with standard culture techniques. Second, we examined the prevalence of *Salmonella* using 16S rRNA data and examined the relationship between *Salmonella*-presence or -absence and individual host factors. Lastly, we evaluated host-

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associated bacterial diversity and community composition in *Salmonella*-present vs. -absent birds. Out of 108 samples, we detected *Salmonella* in six (5.6%) samples based on culture, 25 (23.1%) samples with unrarefied 16S rRNA gene sequencing data, and three (2.8%) samples with both techniques. We found that sex, migratory status, and weight were correlated with *Salmonella* presence in swallows. In addition, bacterial community composition and diversity differed between birds based on *Salmonella* status. This study highlights the value of 16S rRNA gene sequencing data for monitoring pathogens in wild birds and investigating the ecology of host microbe-pathogen relationships, data which are important for prediction and mitigation of disease spillover into domestic animals and humans.

Introduction

The gut microbiome, defined as the community of living microorganisms (e.g., bacteria) and non-living genetic elements (e.g., relic DNA) inhabiting the gastrointestinal tract (Berg et al. 2020), plays an important role in an individual's development, digestion, and immune function (van der Waaij 1989; Kohl 2012). Intestinal microbial communities provide different functions for the host and are influenced by host diet, physiology, environment, and taxonomy (Kohl 2012; Hird et al. 2015). Further, the microbiome is a key within- host trait that is associated with host-pathogen interactions. Infection with pathogens is linked to changes in the microbiome, as seen in mallards infected with low-pathogenic avian influenza virus (Ganz et al. 2017), as well as Marek's disease virus (Perumbakkam et al. 2014) and *Salmonella* infection in chickens (Videnska et al. 2013). It is unclear whether microbial dysbiosis is a result of or a precursor to pathogen infection: commensal microbiota can promote colonization resistance, but changes in the normal microbial community can decrease colonization resistance allowing for pathogen infection (Sorbara and Pamer 2019). Alternatively, pathogen infection can disrupt the microbial

balance in the gut, or a state of dysbiosis (Lupp et al. 2007). In either case, the relationship between pathogen infection and microbiome composition highlights the need to improve our understanding of the how and when the microbiome is influenced by pathogen infections.

The introduction of massive-parallel genetic sequencing methods has dramatically advanced the field of microbial ecology, allowing for a deeper examination of the microbial communities in both humans and other animals. Traditional culture techniques are time-intensive and tend to favor microbial species that thrive in laboratory settings (Davies et al. 2000), thereby excluding the vast majority of microbial diversity present within a community (Rhoads et al. 2012). Molecular techniques, such as low- and high-throughput 16S rRNA gene metabarcoding, have helped address the underestimation of community microbial diversity using culture methods, because they increase sensitivity through sequencing a small DNA region from all bacteria, and are more time efficient (Felske et al. 1998; Schwieger and Tebbe, 1998). The advent of next- generation sequencing has greatly accelerated the number of studies characterizing the microbiome of specific organisms and environments, with some projects achieving this at a global scale (e.g., The Earth Microbiome Project, Thompson et al. 2017). This has led to many new insights into host-microbe interactions at the molecular, individual, and community levels (Rosario and Breitbart 2011), as well as the role of the microbiome in fighting disease and stimulating the host immune response (Kohl 2012).

Recent studies have expanded the use of 16S rRNA gene amplicon data beyond the characterization of host-associated microbiomes to the detection of bacterial pathogens with greater taxonomic specificity (Srinivasan et al. 2014; Banskar et al. 2016). Many studies comparing traditional culture techniques to sequencing methods have found that 16S rRNA gene sequencing is more sensitive and can capture a greater proportion of the microbial diversity than

culture techniques (Westergren et al. 2009; Rhoads et al. 2012; Park et al. 2014; Gupta et al. 2019), although comparative results were more equivocal (Wilson et al. 2018) with differing results based on the study. In part, 16S rRNA gene amplicon sequencing might increase sensitivity because DNA can be detected from living and dead cells, as well as from residual DNA present in the environment, whereas culturing is restricted to living cells. Most studies comparing culture to 16S sequencing have been limited to humans and have focused on commensal bacteria (Rhoads et al. 2012; Gupta et al. 2019), rather than bacterial pathogens (Westergren et al. 2009). Those studies outside of humans have focused on livestock (Park et al. 2014; Wilson et al. 2018) and not wildlife, despite wild populations being important reservoirs of zoonotic disease that can spill over into humans or livestock (e.g., COVID-19 likely originated from a bat host; Lu et al. 2020; Zhou et al. 2020).

Salmonella is a genus of bacteria that contains several pathogenic strains capable of being transmitted among wildlife, domestic animals, and humans as well as through the environment where it naturally resides in the soil (Wiedemann et al. 2014; Aung et al. 2020). *Salmonella* may be naturally occurring in the gastrointestinal tract and harmless in small quantities, but can become pathogenic when significantly increased in abundance, as is the case with some strains of Escherichia coli (Tizard 2004). When *Salmonella* becomes pathogenic, it causes the disease known as salmonellosis and can result in symptoms such as diarrhea, fever, and lethargy, although some infected individuals may be asymptomatic. Infections can occur through fecal-oral contact via contaminated food or water, direct animal contact, and occasionally from person to person (Tizard 2004). Wild avian hosts often serve as reservoirs for *Salmonella* (Gargiulo et al. 2018), and sometimes are the source of outbreaks in human and livestock populations (e.g., Foti et al. 2009). Furthermore, *Salmonella* outbreaks have been known to rapidly emerge in

songbird (Passeriformes) populations largely due to the use of bird feeders, where high-density aggregations of birds increase the likelihood of transmission to other wild and domestic species (Tizard 2004; Giovannini et al. 2012). A recent *Salmonella* outbreak resulted in die-offs of songbirds across multiple states within the United States (Machemer 2021; Mansfield and Lehman 2021). Therefore, pathogen monitoring in birds is critical for reducing the likelihood of spillover events to susceptible wildlife, domestic animals, and humans. Monitoring can entail not just estimating the prevalence of a bacterial pathogen, but also examining its relationship with the host's microbial community.

A healthy microbiome may affect host health and immune response by preventing successful colonization of invading bacteria (colonization resistance), acting as a first line of defense against pathogens, and by modulating immune signaling (Sorbara and Pamer 2019; Kogut et al. 2020; Rogers et al. 2020). Infection by pathogens, such as *Salmonella*, triggers an immune response leading to inflammation within the gut that alters the microbiome composition to favorable conditions (e.g., changes in pH) for pathogen colonization and reproduction, reducing competitors and decreasing the overall community diversity (i.e., α-diversity; Lupp et al. 2007). This dysbiosis allows invading pathogens to persist and may lead to differences in the microbiome between infected and uninfected individuals, as seen in waterfowl infected with avian influenza viruses (Ganz et al. 2017; Hird et al. 2018). While studies on the relationship between *Salmonella* infections and the microbiome are extensive in vertebrates (Bratburd et al. 2018, reviewed by Rogers et al. 2020), little is known on the effects of the presence of *Salmonella* itself on the microbiome of wild species regardless of pathogen status (i.e., carriers of *Salmonella* that may or may not be diseased). With this in mind, we sought to explore the microbiome of barn swallows (*Hirundo rustica*) with or without the presence of *Salmonella*. We

hypothesized that the microbiome of *Salmonella*-present (pathogenic or non- pathogenic) barn swallows will have decreased alpha diversity compared to the microbiome of *Salmonella*-absent birds.

The barn swallow is a widespread ubiquitous passerine species well studied in terms of its life history (Balbontin et al. 2012; Møller 2014), behavior (Saino et al. 2002; Lifjeld et al. 2011), physiology (de Ayala et al. 2006; Schmidt-Wellenburg et al. 2007; Safran et al. 2008), migration ecology (Altwegg et al. 2011; Liechti et al. 2014; Pancerasa et al. 2018), and host-associated microbiomes (Kreisinger et al. 2015, 2017; Ambrosini et al. 2019; Turjeman et al. 2020), which make the species an ideal exemplar for this study. The breadth of knowledge available on barn swallows allows for the integration of information across fields and more in-depth conclusions from our findings. In particular, the migratory distance of barn swallows could serve as an indicator of the potential for transmission of *Salmonella* spp. across broad geographic scales, as seen in bar-headed geese (*Anser indicus*) infected with highly pathogenic avian influenza virus H5N1 (Prosser et al. 2011) and passerine birds with the parasite *Babesia venatorum* (Hasle et al. 2011). Although *Salmonella* spillover into humans and domestic animals has not been linked with barn swallows, one study found barn swallows carried strains of the bacterial pathogen, *Clostridium difficile*, that were also found in humans and farm animals, implicating swallows as a potential source of spillover (Bandelj et al. 2014). Further, barn swallows migrate along the Palearctic- African flyway, the world's largest bird migration network. Israel, our sampling area, serves as a migratory bottleneck along the flyway linking Eurasia and Africa, where birds are able to avoid crossing large ecological barriers along their migration route (Collins-Kreiner et al. 2013). More specifically, an estimated 500 million birds travel through the Hula Valley region of Israel each season (Gophen 2015) where dense

congregations of birds create opportunities for pathogen transmission and spillover to agricultural operations in the area and beyond.

In this study, we aimed to: (1) explore the prevalence of *Salmonella* from fecal microbiome data within barn swallows, (2) assess the utility of microbiome data for accurately detecting potentially pathogenic bacteria (*Salmonella* spp.), by comparing results from traditional culture techniques to those from 16S rRNA gene sequencing, (3) evaluate different host ecological factors predicting *Salmonella* presence or absence, and (4) analyze the diversity of total (living and relic) microbial communities with and without the presence of *Salmonella* in barn swallows. Our results highlight the value of 16S rRNA gene sequencing not just for monitoring potential bacterial pathogens, but also for better understanding the ecology and role of microbial communities in pathogen infections.

Methods

Study Area and Sample Collection

We captured 159 adult birds with mist-nets from four sites (Beit She'an, Haifa, Hula, Shefayim, Figure 2.1) in the northern half of Israel from November of 2016 to the end of November 2017. All fieldwork was conducted using permit number 2017/41764 issued by the Israel National Protection Authority and approved by Hebrew University ethics committee according to institutional and national guidelines. We placed captured birds in clean plastic cups inside individual bags to collect fecal samples for microbiome characterization. All fecal samples were collected using a sterile swab and stored in 95% EtOH and immediately frozen in a −20°C portable freezer for up to 7 days in the field before transfer to a −80°C freezer for long-term storage until extraction. For all individuals, an additional swab (fecal) was taken and stored in a glycerol-LB mixture for culture detection of *Salmonella* spp. Where possible, we collected

associated data on age, sex, season of capture, and site for all the birds. Sexing was completed according to standard protocols described in Turjeman et al. (2020). We estimated, as described below, *Salmonella* prevalence for each site using a filtered dataset (n = 108) and visualized the prevalence distribution over our study area by generating a map using QGIS version 2.16.3 with the Apple iPhoto basemap (QGIS Development Team, 2009). Sample sizes, summary statistics on the number of *Salmonella* reads, and GPS location details for each site are available in Table B.1.

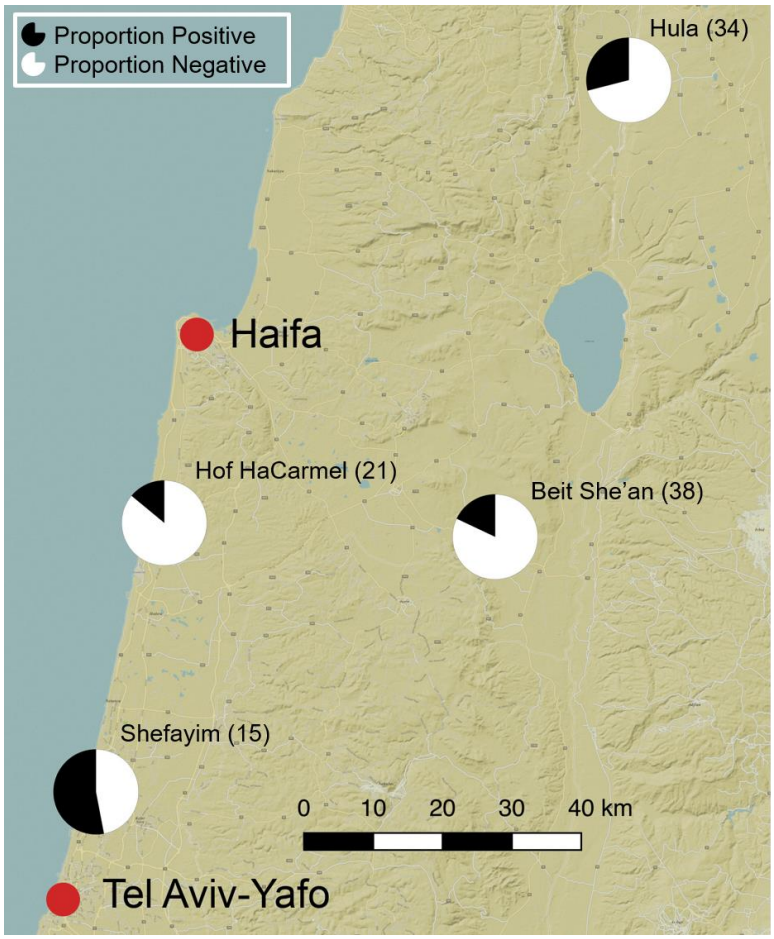


Figure 2.1. Sample collections sites by locality (4 total) showing proportion of birds positive (black) and negative (white) for *Salmonella*, based on 16S rRNA sequencing; samples sizes are shown in parentheses.

Culture Techniques for *Salmonella* Detection

Fecal swabs were stored in glycerol-LB and cultured to test for *Salmonella* spp. through one of two culturing workflows. The first workflow (workflow 1), done at the Kimron Veterinary Institute, Israel, used a culture-based approach and was performed on a subset of samples (n = 74) prior to 16S rRNA gene sequencing and did not detect *Salmonella* in any of the samples. However, sequencing revealed the presence of *Salmonella* in some samples and therefore we utilized a second culturing workflow (workflow 2) on the remaining samples (n = 34) to determine if *Salmonella* could be detected using a different culturing protocol. For the workflow 1, swabs were incubated in 10 mL buffered peptone water, followed by 1 mL tetrathionate-brilliant green broth, then XLT4 agar and Enteroplus slant agar. Each step included a 24 h incubation at 37°C. Cultures were considered *Salmonella*-present if they exhibited a characteristic tri-color appearance of *Salmonella* (red, black, yellow; ISO 6579- 1 2017). Workflow 2 was completed at the Hebrew University of Jerusalem, Israel. Swabs were incubated in 10 mL buffered peptone water, followed by 1 mL Rappaport-Vassiliadis broth, then brilliant green agar, and triple sugar iron agar slants. Each step included an 18–24 h incubation step at 37°C. Colonies suspected of being *Salmonella* were verified through PCR. DNA was extracted using DNeasy Blood and Tissue kits (Qiagen Inc., Germantown, MD) following the gram-negative bacteria protocol. PCR was conducted following Halatsi et al. (2006) using primer pair SdiA1 (AATATCGCTTCGTACCAC) and SdiA2 (GTAGGTAAACGAGGAGCAG). Reaction volume was20 µL: 3 µL DNA template, 1 µL of each primer, 10 µL of OneTaq Master Mix, and 5 µL of molecular grade water. Cycling conditions were as follows: 5 min of denaturation at 94°C, then 30 cycles of 94°C for 30 s, 52°C for 40 s, and 72°C for 30 s

ending with a final extension at 72°C for 7 min. PCR products were visualized by gel electrophoresis for the determination of the presence/absence of *Salmonella*.

Microbiome Extraction and Sequencing

A detailed protocol for 16S rRNA gene sequencing and processing of resulting reads is available in Corl et al. (2020) and Turjeman et al. (2020). Briefly, frozen swabs stored in 95% EtOH were extracted using DNeasy PowerLyzer PowerSoil kits (Qiagen Inc., Germantown, MD). Each DNA extraction contained a set of individuals randomized across sites and times of collection to control for any batch effects during extraction. Along with the barn swallow samples, three negative control samples were processed through the DNA extraction workflow in the same manner as for the fecal samples. These negative controls were included to offset the ubiquitous nature of bacteria and to account for possible contamination from laboratory plastics and consumables used throughout the collection and extraction processes. DNA extractions were shipped to Argonne National Laboratory (Lemont, IL) for PCR-amplification and DNA sequencing. Primers 515F and 806R (Caporaso et al. 2012), targeting the variable V4 region of the bacterial 16S rRNA gene were used for Illumina sequencing. Paired-end reads of 151 base pairs (bp) sequences were generated on two runs of an Illumina MiSeq.

Microbiome Quality Control, and Data Filtering

Sequences were demultiplexed using QIIME2 (Bolyen et al. 2019). Analyses of the data were conducted in R 3.6.3 (R Core Team 2020) following the R workflow for processing 16S data published by Callahan et al. (2016). The first 10 bases were removed from all reads and DADA2 (Callahan et al. 2016) was used to identify amplicon sequence variants (ASVs), which were unique sequences that were statistically unlikely to be due to sequencing error. The SILVA 132 taxonomy database was used to assign taxonomy, DECIPHER (Wright 2015) was

used to align sequences and the package phangorn (Schliep 2010) was used to build a maximum likelihood phylogenetic tree. All parts of the data (sequence variant table, taxonomy table, phylogentic tree) were combined along with the metadata using the phyloseq package (McMurdie and Holmes 2013) for statistical analyses. Contaminants were removed from the data set using the decontam package (Davis et al. 2018). The prevalence filter was used with a threshold set at 0.5 to remove all sequences that were more prevalent in the three negative controls than in samples from birds. The negative control samples did not have any *Salmonella* ASVs within them. In total, 518 ASVs were removed as contaminants. In addition, any ASVs not of the kingdom Bacteria were also removed, as well as any sequences matching mitochondria or chloroplasts. Lastly, we removed samples with poor quality PCR (n = 45) and samples without culture results (n = 2). The remaining 108 samples were analyzed to compare the sensitivity of culturing to 16S rRNA gene sequencing in detecting *Salmonella*. Throughout this manuscript, we use *Salmonella* presence/absence to indicate *Salmonella* spp. ASVs present/absent in the microbiome and note that it is unknown whether the *Salmonella* strains detected are pathogenic. We examined the bacterial community diversity within and across samples after rarefying the data to a sampling depth of 12,000 reads. A threshold of 12,000 reads was chosen after examining rarefaction curves (Figure B.1, rngseed = 711) that showed even the most diverse samples leveled off at this threshold and we would only lose seven samples, five of which had fewer than 5,000 reads. Therefore, our analyses of microbial diversity were conducted on 101 samples, after removing these seven samples from the dataset. To test for sampling effects during rarefaction, we ran the rarefaction analyses 100 times using different random seeds (1:100) and then determined the consistency across runs in detecting *Salmonella*.

Salmonella Prevalence and Comparison of Detection Methods

We estimated the prevalence of *Salmonella* spp. by detection method for all swallows with paired culture and 16S rRNA gene data (n = 108). Given that we used two different culture workflows, we ran separate sets of analyses for each workflow ($n_{\text{workflow 1}}$ = 74, $n_{\text{workflow 2}}$ = 34). A McNemar's exact test, assuming non-independence of samples that is appropriate for smaller sample sizes (Fagerland et al. 2013), was used to compare detection probabilities of culture and 16S rRNA gene sequencing for both workflow one and two. We used a generalized linear model (GLM) with a quasipoisson distribution from the car package (Fox and Weisberg 2011) followed by a Type II ANOVA with the smaller dataset of workflow 2 and the combined dataset to test two hypotheses. First, we tested whether culture success or failure could predict the absolute abundance of *Salmonella* as measured by the number of 16S rRNA sequencing reads, including the total number of reads per sample as a covariate to account for sequencing depth. We used absolute abundance for this model to maximize detection power while acknowledging that samples did not have equal sequencing effort (unrarefied). Second, to address how sequencing depth affects our detection probability, we tested whether *Salmonella*- present vs. -absent status in the host, as measured by the 16S data, was correlated with the total number of reads sequenced for the sample. We do not report the results for rarefied data as the results did not differ for either the culture success and absolute abundance or *Salmonella* presence and total reads tests. To determine the sensitivity of *Salmonella* detection to 16S sequencing depth, we used all samples with at least a single read for *Salmonella* and greater than 45,000 reads (n = 18). We chose 45,000 as a cutoff because at higher depths many samples were lost during rarefaction as they did not meet the required number of reads. We rarefied the data for these 18 *Salmonella*- positive samples

between depths of 5,000–45,000, in increments of 5,000 reads, using the default seed (711). For each depth, we calculated the proportion of samples with at least a single read of *Salmonella*. *Salmonella* detection, prevalence estimation, and statistical analyses were conducted in R 3.6.3 (R Core Team 2020). Figures displaying prevalence between detection methods were generated using the ggplot2 (Wickham, 2016) and phyloseq (McMurdie and Holmes 2013) packages in R.

Relationships between Host Ecology and *Salmonella* Status

We explored whether *Salmonella* presence in the gut microbiome was correlated with host characteristics to highlight how 16S data can enhance our understanding of host ecology in relation to the presence of potential pathogens. We tested whether the presence or absence of *Salmonella* was dependent on three host factors: migratory status, weight, and sex. A generalized linear model with a binomial distribution was run for each factor, with the total number of reads for every sample included as a covariate to control for sequencing depth, which likely influences the detection of *Salmonella* in the hosts. For this analysis and all subsequent analyses, we initially assigned *Salmonella*-present samples as samples that had one or more *Salmonella* reads. We also repeated each analysis with a higher threshold of two or more *Salmonella* reads to assign a *Salmonella*-present sample. We did this to account for possible false positives due to PCR or sequencing error. When using a two read threshold, we removed seven samples that had only a single *Salmonella* read from the analyses. We were not able to explore thresholds greater than two reads due to the need to maintain a sufficient sample size of *Salmonella*-present samples. Data was available for 92 samples to test for the effects of weight and sex at read threshold one, while 88 samples were used for read threshold two. Data for migratory status was available for 37 (one read) and 33 (two reads) samples that were classified as migrant or resident using both feather molt pattern evaluated during field capture and stable

isotope analyses (see Turjeman et al. 2020). For each model, we used an ANOVA to identify significant relationships.

Relationships Between the Microbiome and *Salmonella* Status

We estimated the diversity and bacterial species composition in all samples post rarefaction (rngseed = default of 711, $n = 101$) to examine host microbiome relationships with the presence of *Salmonella*. We used the Chao1 estimator of the number of species (Chao, 1984) to measure alpha diversity and used a Mann-Whitney-Wilcoxon test (MWW) to test for differences in alpha diversity of the microbiota between birds with and without *Salmonella* sequence reads. We chose to use Chao1 as an estimator for alpha diversity, because it accounts for rare and missing species (Chao and Shen 2003). To test for confounding factors that may also influence alpha diversity, we used a linear model and ANOVA, including sex, site, and season as additional factors. Migration status was not included because our previous work showed no differences in alpha diversity between migrants and residents in our study population (Turjeman et al. 2020).

Differences in bacterial communities between *Salmonella*- present and -absent birds were visualized using bar plots and principal coordinates analysis (PCoA) with the phyloseq package. PCoA chooses axes that explain most of the variation in the entire dataset without reference to the particular factors that may distinguish two groups of samples. Permutational multivariate analysis of variance (PERMANOVA) tests were calculated with adonis from the vegan package (Oksanen et al. 2019) with 9,999 permutations. This was done on both weighted and unweighted UniFrac metrics. UniFrac is a distance metric comparing bacterial communities that uses phylogenetic information to measure distance between samples (Lozupone and Knight 2005). The unweighted UniFrac uses only presence/absence data, whereas weighted UniFrac also

incorporates relative abundance of ASVs to measure distance between samples (Lozupone et al. 2007). To account for potentially significant differences based on location or dispersion, we tested for homogeneity of group dispersions using the betadisper function in the vegan package. When dispersions are significantly different, then significant differences in communities as found by PERMANOVA can be the result of differences in either dispersion or both dispersion and location.

Results

16S rRNA Gene Sequencing Detected Higher *Salmonella* Prevalence Than Culture-Based Methods

Culture results revealed a relatively low prevalence of *Salmonella,* with zero out of 74 birds positive for *Salmonella* presence in workflow 1 (0%, Figure 2.2A) and six out of 34 birds positive in workflow 2 (17.6%; Figure 2.2B). In comparison, results based on unrarefied 16S rRNA gene data identified *Salmonella* in ten swallows (13.5%, Figure 2.2A) for workflow 1 and 15 swallows (44.1%, Figure 2.2B) in workflow 2. The McNemar's exact test revealed a significant difference in *Salmonella* detection probability between culturing vs. 16S rRNA gene sequencing for both culture workflow 1 (Figure 2.2A, $p = 0.002$) and workflow 2 (Figure 2.2B, $p = 0.035$).

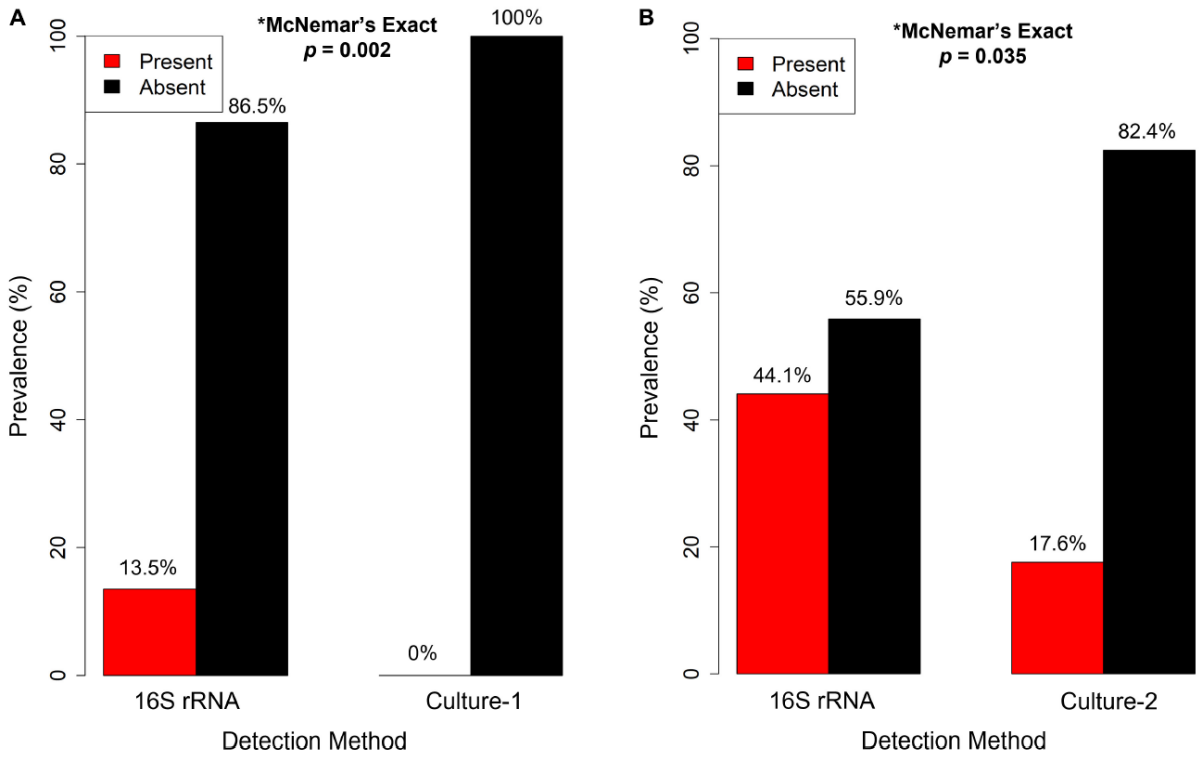


Figure 2.2. *Salmonella* spp. prevalence in barn swallows (*Hirundo rustica*) from Israel, estimated using unrarefied 16S rRNA gene sequencing (16S rRNA) and two culture methods: (A) culture workflow 1 (Culture-1; n = 74) and (B) culture workflow 2 (Culture-2; n = 34). The proportion of samples positive for the presence of Salmonella (red bars) detected by 16S rRNA gene sequencing was significantly higher than those obtained by culturing. This analysis was conducted on paired samples from the same individuals that had both culture and 16S rRNA gene data collected.

A GLM showed no significant relationship between *Salmonella* culture results and the absolute abundance of *Salmonella* as measured by unrarefied reads of 16S rRNA data for workflow 2 ($p = 0.58$) and both workflows combined ($p = 0.96$). We did not use workflow 1 for this analysis as there were no culture positive samples for comparison. Samples that were identified as having at least one *Salmonella* sequence read in the 16S rRNA data had

significantly higher sequencing depths than samples where no *Salmonella* sequences were detected (Figure B.2, $p = 0.002$). The mean absolute abundance of *Salmonella*, as measured by unrarefied 16S rRNA in culture-positive samples for workflow 2 (mean = 3.67, std dev = 7.12) and both workflows combined (same as workflow 2 as culture-positive samples did not change), did not significantly differ from mean abundance in culture-negative samples for workflow 2 (mean = 10.71, std dev = 38.87) (ANOVA, $F = 0.19$, $p = 0.66$) and the combined workflows (mean = 3.44, std dev = 20.83) (ANOVA, $F = 0.00069$, $p = 0.98$), respectively. *Salmonella* abundance was low, ranging from 1–199 reads (<0.007%, mean = 14.92) for samples positive for its presence based on 16S rRNA sequencing (Figure B.3A). Seven out of the 25 samples with *Salmonella* present in the microbiome had only a single *Salmonella* read.

We explored how *Salmonella* was affected by rarefaction, given that *Salmonella* presence status was related to sequencing depth. After rarefying to 12,000 reads, we detected *Salmonella* in an average of 12.95 samples (down from 25 samples pre-rarefying), with variability across runs (9–17 samples positive per run) due to random sampling (Figure B.3). The median value was 13 positive samples with a 95% confidence interval range of 12.6–13.3 samples after 100 iterations, so rarefaction reduced the number of *Salmonella*-present samples by about half. Samples with higher numbers of *Salmonella* reads before rarefying consistently had higher numbers of reads after rarefying. Post- rarefaction, one to three cultured samples were confirmed for *Salmonella* presence with 16S rRNA data, whereas three of the six culture positive samples were confirmed prior to rarefaction. In terms of sequencing depth and *Salmonella* detection, a high depth (>32,000) was required to obtain 80% detection within this system (Figure B.4) suggesting that a very high sequencing depth is required to consistently detect *Salmonella* in our data.

Salmonella Presence Differed Between Birds by Migratory Status and Weight

Using a *Salmonella* detection threshold of one read, we found a marginally significant difference in *Salmonella* presence ($p = 0.056$) between male and female birds. For host weight, *Salmonella*-present birds were slightly heavier (mean = 19.1 grams) in weight than those without *Salmonella* (mean = 18.1 grams) ($p = 0.012$). For migratory status, migrant swallows were significantly more likely to have *Salmonella* in their microbiome than resident swallows ($p = 0.041$), with a *Salmonella* prevalence of 52% in migrants and 16.7% in resident swallows.

When applying a *Salmonella* detection threshold of two reads, there was similarly a significant difference in *Salmonella* presence ($p = 0.015$) between male and female birds. *Salmonella* was present in seventeen of 37 (31.5%) male birds, in comparison to four of 39 (9.3%) females. For both weight and migratory status, there was no significant difference between *Salmonella*-present and -absent birds ($p = 0.081$ and 0.099, respectively).

Alpha Diversity Differed among Birds Based on *Salmonella* Presence and Absence

When examining relationships between *Salmonella* and microbial communities, our set of positive samples were all samples with *Salmonella* sequence reads identified by 16S rRNA sequencing (pre-rarefaction; $n = 25$), plus the samples that were only determined to be positive by culture ($n = 3$). One sample with *Salmonella* identified pre-rarefaction did not meet the required threshold inclusion and was removed during rarefaction; this left 27 *Salmonella*-present samples for the remainder of the analyses, when applying a detection threshold of one read. For a detection threshold of two reads, six additional samples were removed, leaving 21 *Salmonella*-present samples in the remaining analyses. At *Salmonella* read threshold one, individuals with *Salmonella* ($n = 27$) had higher levels of alpha diversity than individuals without *Salmonella* (Figure 2.3, MWW, $p = 0.001$). Both *Salmonella*

presence/absence (ANOVA, p = 0.047, F = 4.044, df = 1) and site (ANOVA, p = 0.048, F = 2.739, df = 3) were significant predictors of microbial community alpha diversity (Model R^2 = 0.18). Sex (ANOVA, p = 0.509, F = 0.44, df = 1), age (ANOVA, p = 0.55, F = 0.553, df = 1) and season (ANOVA, p = 0.92, F = 0.084, df = 2) were not significantly correlated with alpha diversity. *Salmonella* read threshold two yielded similar results: individuals with *Salmonella* had higher levels of alpha diversity than individuals without *Salmonella* (Figure B.5, MWW, p = 0.003), and both *Salmonella* presence/absence (ANOVA, p = 0.028, F = 4.970, df = 1) and site (ANOVA, p = 0.042, F = 2.852, df = 3) were significant predictors of microbial community alpha diversity (Model R^2 = 0.112). In addition, sex (ANOVA, p = 0.225, F = 1.49, df = 1), age (ANOVA, p = 0.60, F = 0.280, df = 1), and season (ANOVA, p = 0.93, F = 0.074, df = 2) were not significantly correlated with alpha diversity.

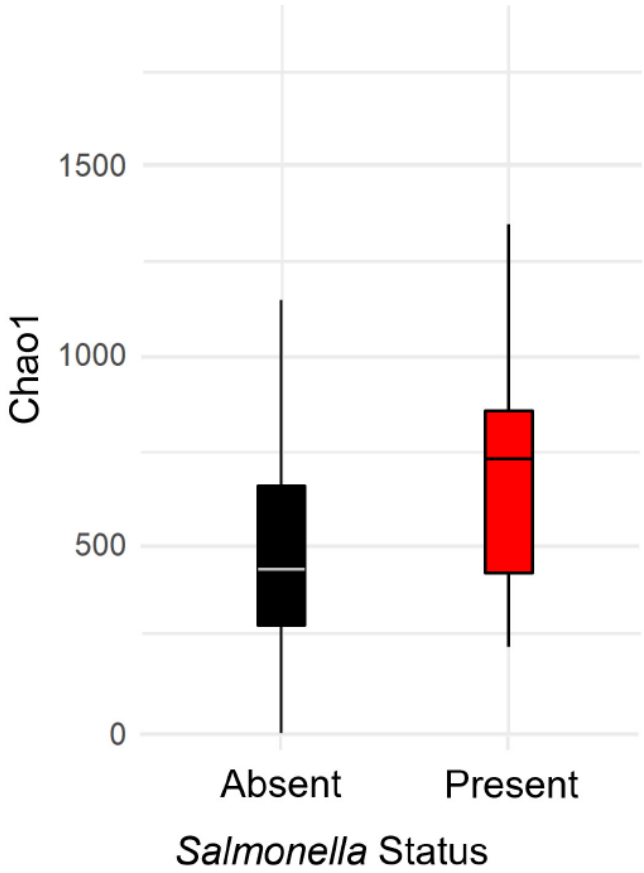


Figure 2.3. Bacterial alpha diversity, as estimated by the Chao1 statistic, by *Salmonella* spp. status (absent, present) in barn swallow (*Hirundo rustica*) fecal samples collected in Israel. Salmonella-absent samples had significantly lower diversity than -present samples (Mann-Whitney-Wilcoxon test, p = 0.008).

Across all samples, 16S rRNA data revealed that bacterial communities were comprised mainly of the phyla Proteobacteria, Tenericutes, Firmicutes, and Actinobacteria (Figure B.6). The microbial communities of *Salmonella*-present and -absent birds were significantly different in PERMANOVA tests using both unweighted (Figure 2.4A, p = 0.001, F = 2.637, df = 1, R^2 = 0.260) and weighted (Figure 2.4B, p = 0.013, F = 2.821, df = 1, R^2 = 0.028) UniFrac distances at read threshold one. Homogeneity of dispersion between the two

groups was rejected for both unweighted and weighted UniFrac distances (unweighted: F = 24.786, df = 1, p = 0.001; weighted: F = 12.506, df = 1, p = 0.001). Thus, significant differences in the bacterial communities of *Salmonella*-present and -absent birds are driven at least in part by different dispersion of the two groups. However, the principal coordinates analysis plot for the unweighted UniFrac (Figure 2.4A) suggests that there may also be differences in the location of points within the plot for the two groups, because many birds with *Salmonella* reads have bacterial communities that occupy a space that is distinct from birds without *Salmonella* reads. Read threshold two yielded similar results and thus are not reported here (Figure B.7).

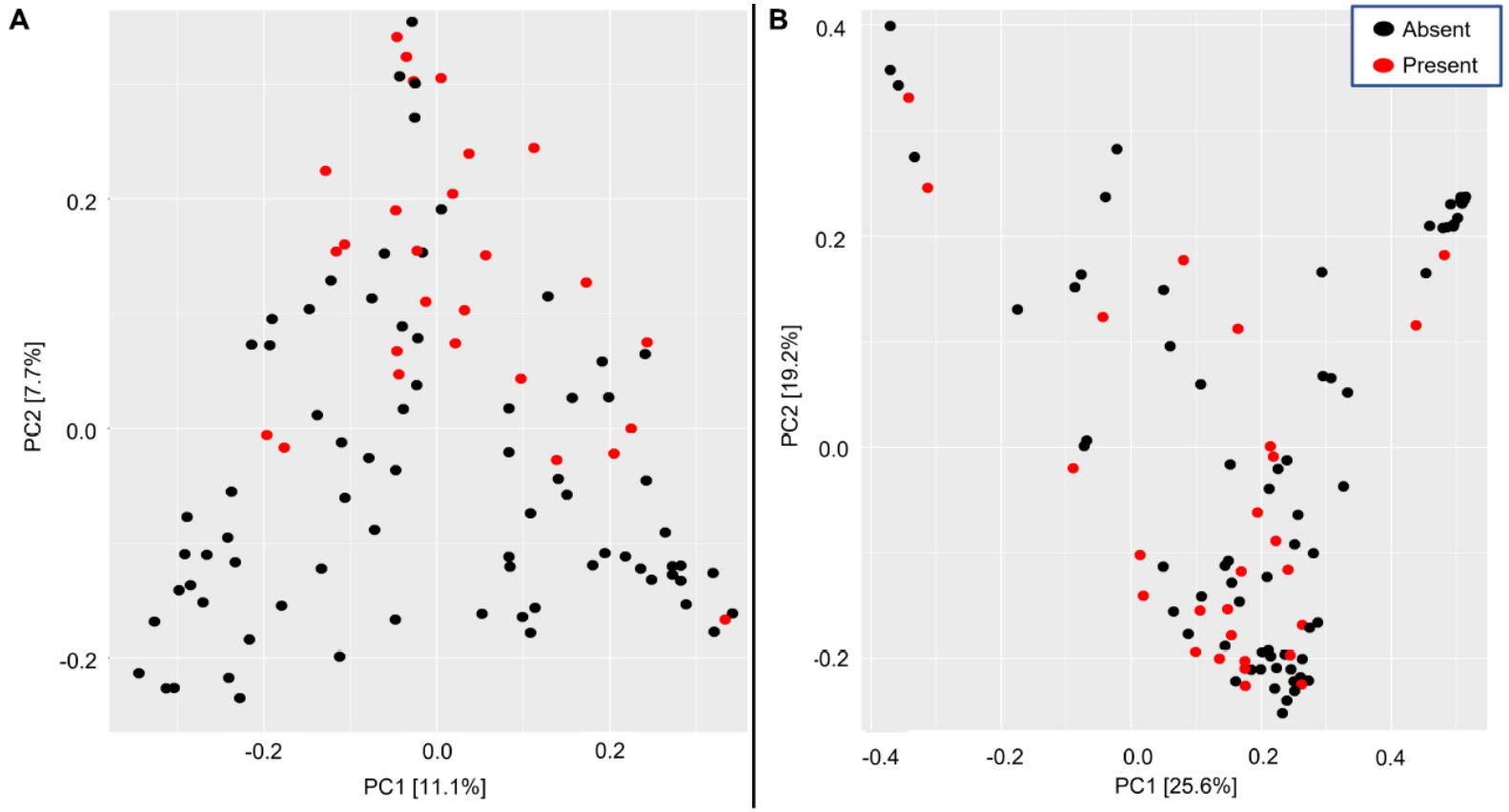


Figure 2.4. Principal coordinate analysis plots showing the bacterial communities in barn swallow (Hirundo rustica) fecal samples with Salmonella (present: red dots) and without (absent: black dots) by (A) unweighted UniFrac and (B) weighted UniFrac metrics. The amount of variation explained by each axis is in brackets. Bacterial beta diversity significantly differed for both metrics (p = 0.001 for both) between Salmonella-present and -absent birds.

Discussion

Salmonella is a bacterial genus that includes several pathogenic serotypes of concern to both public health and agriculture. As wild bird species can act as reservoirs or carriers for these pathogenic bacteria, continued *Salmonella* surveillance is critical for preventing spillovers into humans and domestic animals (Giovannini et al. 2012). In barn swallows, *Salmonella* was not previously detected when using a culture diagnostic approach for screening (Haemig et al. 2008). However, our data suggest that *Salmonella* can be missed by culturing (e.g., culture workflow 1), and therefore microbiome data could enhance efforts to monitor *Salmonella* and other bacterial pathogens in wild birds for the purposes of both human and avian health. Further, our results demonstrate an approach that can be used not only for monitoring potential pathogens, but also for addressing key questions in disease ecology, such as the relationship between individual host factors (e.g., age, sex, and microbial community diversity) and pathogen infection.

We found that detection of *Salmonella* was significantly better with 16S rRNA gene sequencing data than using culture techniques (Figure 2). Our finding that 16S rRNA sequence data detected *Salmonella* in more birds than either respective culture workflow suggests that sequencing approaches could be an important tool for the detection and surveillance of bacterial pathogens like *Salmonella* in wildlife populations. Higher sensitivity of microbe detection using 16S rRNA gene sequencing data could be due to the detection of both residual or dead cells. For the purposes of this study, dead cell DNA is still of interest in terms of the potential for carrier status, because these cells could represent bacteria that are alive in the gastrointestinal tract, but unable to survive the semi-aerobic conditions of the cloaca (Grond et al. 2018), where fecal material passes prior to sample collection. However, if detection of only live potential

pathogens is desired, a pre-enrichment step using ethidium monoazide (Nogva et al. 2003) or propidium monoazide (Nocker et al. 2006) can be included to remove non- viable DNA.

The detection of *Salmonella* or other bacteria in 16S rRNA sequencing data will depend on the sequencing depth and the abundance of the bacteria in the sample. We found that *Salmonella* presence in barn swallows was related to sequencing depth (Figure B.2), that rarefaction to 12,000 reads resulted in *Salmonella* detection in only half the total samples observed when using the full data set, and that a high sequencing depth was required for reliable detection of *Salmonella* spp. (Figures B4, B5). This high sequencing depth requirement may explain the discrepancy in *Salmonella* detection for the three false negative samples that were culture positive, but 16S negative. Further, these data highlight two vital factors to keep in mind when using 16S rRNA gene data for disease detection. First, sequencing depth will determine the power to detect taxa, especially those in low abundance, as we observed here when increasing the *Salmonella* detection threshold. The minimum sequencing depth would change for other host species, which may not have a similarly low level of *Salmonella* abundance, especially if they are in a diseased state. Our results are consistent with these swallows potentially being infected or carriers of *Salmonella*, rather than experiencing disease, due to extremely low levels of abundance. Thus, we recommend high sequencing depths unless it is known *a priori* that the pathogen is abundant. A cost-effective approach would be to first sequence a subset of samples at high sequencing depth to determine the optimal level of sequencing for the remaining samples. Second, we recommend that all reads be used for detection of rare pathogens, because this approach maximizes the power for detection within a dataset. If this approach is taken, it could be useful to withhold some DNA before 16S library preparation that could be used for further PCR or sequencing of the target pathogen to

confirm positive samples that have very few reads from the bacteria of interest. A previous study showed that with a proper enrichment step, PCR detected *Salmonella* from as little as one colony forming unit (CFU) in food (Ferretti et al. 2001), suggesting that even in low abundance, *Salmonella* can be detected. PCR and Sanger sequencing of 16S positive samples could also be useful for determining whether the bacterial strain is pathogenic, which often requires longer reads than are typical with 16S rRNA amplicon sequencing.

Our data showed that the presence of *Salmonella* was correlated with multiple differences in host ecology and microbiota. When using a detection threshold of one read, birds with *Salmonella* weighed more than birds without *Salmonella*, and *Salmonella* was present in more migrants than resident barn swallows. These two factors were related to one another, because migrant birds were heavier than resident barn swallows (mean 20.2 vs. 17.5 grams). Barn swallows have been known to accumulate fat in preparation for migration (Pilastro and Spina 1999), which suggests that migrants might have differing foraging behaviors that could lead to a higher prevalence of *Salmonella.* Another potential reason why migrants have a higher prevalence of *Salmonella* is that they travel through multiple environments, which could increase their encounters with *Salmonella* species. This sort of pattern has been observed in barn owls (*Tyto alba*), where owls that traveled greater distances from their nests had more diverse microbiota (Corl et al. 2020). However, migration does not necessarily lead to changes in the microbiota as microbial diversity did not vary between fecal samples collected from common cranes (*Grus grus*) before and after migration (Pekarsky et al. 2021). Dietary, physiological, or subspecies differences might also explain differences in *Salmonella* prevalence between migrants and resident barn swallows. A caveat to the models correlating *Salmonella* presence with host ecological variables is they were sensitive to the read threshold and/or sample size. With

read threshold two, the relationships with both migratory status and weight were not significant (migratory status, $p = 0.099$, weight $p = 0.081$), whereas a read threshold of one revealed a significant relationship ($p < 0.05$) between these factors and *Salmonella* status. In contrast, the relationship with sex changed from marginally significant ($p = 0.056$) to significant ($p = 0.012$) when increasing the *Salmonella* detection threshold to two reads. Thus, similar trends were found for all three traits, but the statistical significance of the correlations is tentative. However, these results do highlight a set of traits to target for future study to elucidate the relationship between host ecology, microbiota, and *Salmonella* status. In addition, these results underscore the need for high sequencing depths and sufficient numbers of positive samples when investigating relationships between *Salmonella* status and host ecology.

We found that birds with *Salmonella* had more diverse bacterial communities, suggesting that *Salmonella* may alter community level interactions among bacterial taxa in the gut microbiome or that *Salmonella*-presence is correlated with other factors that alter bacterial communities. Increased diversity in *Salmonella*-present birds may be related to the vital role the gut microbiome plays in immune response and health. Hosts and their microbiota can work together to promote colonization resistance (Sorbara and Pamer 2019; Rogers et al. 2020), while infection by *Salmonella* spp. can cause dysbiosis in favor of *Salmonella* growth (Lupp et al. 2007) and a decrease in diversity that is not seen here. For example, microbial diversity was reduced in American white ibis (*Eudocimus albus*) shedding *Salmonella enterica*, relative to the diversity observed in healthy ibis (Murray et al. 2020). Similarly, microbial diversity was reduced in mallard ducks infected with avian influenza viruses (Ganz et al. 2017). However, pathogen relationships with microbial diversity are species-specific, as seen in waterfowl where influenza A virus infection was

negatively correlated with alpha diversity in only two of five species (Hird et al. 2018). However, our results contrast with these previous studies and the apparent increased microbial diversity in individuals with *Salmonella* may be due to avirulent or low abundance strains failing to trigger colitis, and thus being outcompeted by other bacterial species (Stecher et al. 2007). Another explanation is that hosts with *Salmonella* could have more varied diets, resulting in an increased chance of being colonized by diverse bacteria, including *Salmonella*. In addition, hosts may differ in their movement patterns (e.g., migrant vs. resident), which could affect their exposure and colonization by a particular bacterium: the use of more diverse habitats (long-distance migrants) may lead to more exposures and colonization by diverse bacteria as supported by our finding that migrants were more likely to have *Salmonella*.

We found that bacterial communities differed in individuals with and without *Salmonella*, but we were unable to disentangle whether the significance of these tests is driven in part by community-level differences or by significant differences in dispersion between *Salmonella*-present and -absent groups. With that caveat in mind, the PCoA of unweighted UniFrac suggests that there are both differences in location (i.e., position of the points) and dispersion (i.e., the spread of the points) of the two bacterial communities. Therefore, dispersion might not be the only factor separating bacterial communities with and without *Salmonella*, and *Salmonella*-present hosts may vary in their bacterial communities.

Our work demonstrates the potential for 16S rRNA gene sequencing data collected for microbiome studies to also be used for monitoring *Salmonella* and other pathogens in wild bird populations. The observed variation in microbial communities and host traits by *Salmonella* status also suggests that studies on pathogen transmission and host microbial ecology can mutually inform one another. Pathogens should be considered as one of the many plausible causal

explanations for the differences in the host microbiota. The few studies focused on pathogen-microbiome interactions in wildlife highlight a need for further research to elucidate the relationships between microbiota, pathogen infections, and disease. We propose that future microbiome studies of wild animals have great potential to be used to better understand disease epidemiology and ecology in wild populations, as well as aid in the identification of potential reservoir species for pathogenic bacteria. Microbiome studies could thus be a rich, but untapped source of data for better understanding the distribution and ecological dynamics of pathogens in the environment.

CHAPTER 3

ECOLOGICAL PREDICTORS OF HAEMOSPORIDIAN PARASITE INFECTIONS IN WATERFOWL: A ROLE FOR THE MICROBIOME AS AN INDICATOR OF HEALTH?

Avian haemosporidians are widespread parasites known to infect the blood cells of birds. Although infections are often chronic, the drivers and effects of these parasite infections on the host immunity and associated microorganisms are unknown in many host species. The gut microbial community (i.e., microbiome) is believed to play an important role in a host's immune function and previous studies found altered microbiomes in waterfowl with viral infections; however, little is known about the relationship between eukaryotic parasites and the microbiome. Our objectives were to 1) to determine the prevalence and intensity of haemosporidian infections (*Haemoproteus*, *Leucocytozoon,* and *Plasmodium* spp.), 2) identify host ecological predictors (age, sex, weight, wing length, location) of haemosporidian infection, and 3) examine relationships between the cloacal bacterial microbiome, characterized by 16S rRNA sequencing, and haemosporidian parasite infections in 333 wild ducks in Maine, representing four waterfowl species (*Aix sponsa , Anas carolinensis, A. platyrhynchos,* and *A. rubripes*). Overall, 96.7% of birds (322) tested positive for haemosporidian parasites with an average qPCR cycle threshold (C_T) value (index of parasite intensity) of 24.0. In general, adult ducks had higher haemosporidian infection intensity levels (i.e., lower C_T) than juveniles; however, potential predictors of infection (e.g., location, age) varied across species. The microbiome Chao1 alpha diversity did not differ between infected and uninfected birds, nor by infection intensity. Beta diversity, reflecting microbial community composition, varied with infection intensity for the weighted UniFrac measure ($F = 3.02$, $p = 0.013$) but not the unweighted ($F = 1.14$, $p = 0.26$) in

mallards suggesting that abundance of specific are driving this relationship. Our results reveal variation among host species in the risk factors predicting haemosporidian parasite infections in wild birds, and weak evidence to suggest microbiome communities could be influenced indirectly by these infections or vice versa. Despite little evidence for the microbiome as an indicator of haemosporidian parasite infections, further research is needed to understand its role in immune response and disease dynamics in wild birds.

Introduction

Haemosporidian parasites play a significant role in avian health and can have profound effects on bird populations. Infections of these parasites often persist chronically, especially in birds, with individuals exhibiting minimal or no symptoms while serving as asymptomatic carriers (Sato 2021; Thurber et al. 2014). However, infection in some individuals can also diminish fitness by impacting reproductive success (Knowles et al. 2010; Korpimaki, Hakkarainen, and Bennett 1993), survival (la Puente et al. 2010), and body condition (Dyrcz et al. 2005). For example, a study in Tengmalm's owls (*Aegolius funereus*) found that when food abundance was low, females infected with haemosporidian parasites had reduced clutch sizes (Korpimaki et al. 1993). Further, blue tits (*Cyanistes caeruleus*) treated for chronic *Plasmodium* spp. infections had increased hatching, fledging success and provisioning rates in comparison to untreated birds (Knowles et al. 2010). These data suggest that haemosporidian parasites can play a role in the overall health of birds and should be monitored in wild bird populations.

Haemosporidian parasites belong to the order Haemosporida and encompass species from the genera, *Haemoproteus*, *Leucocytozoon*, and *Plasmodium* (Garnham 1966; Ishtiaq et al. 2017). Among these genera, *Leucocytozoon* parasites are relatively large and primarily infect white and red blood cells, while *Haemoproteus* and *Plasmodium* parasites infect red blood cells,

with *Plasmodium* spp. exhibiting the greatest diversity. Notably, *Plasmodium* spp. have been implicated in significant population declines of birds such as Hawaiian honeycreepers (Atkinson and LaPointe 2009), underscoring their importance to wildlife conservation and management.

Studies have demonstrated that haemosporidian parasites infect many species, including waterfowl, which are important game species in the United States. Greiner et al. (1975) estimated a parasite prevalence of 31.9% in 13,114 birds from the family Anatidae that nest in northeastern North America with at least one individual from each focal species testing positive for haemosporidians. Reeves et al. (2015) found haemosporidian parasites in 77.9% and 70.3% of waterfowl tested in Alaska and California respectively. In contrast, Annetti et al. (2017) detected haemosporidians in only 1.6% of wood ducks sampled in Illinois. It is also notable that only Annetti et al. (2017) examined patterns of infection based on haemosporidian infection intensity in North American waterfowl. These results highlight the value of improving our understanding of the distribution and intensities of these parasites across waterfowl species in North America for game species management.

Haemosporidian parasite infection distributions and intensities can vary based on host age (Annetti et al. 2017; Meixell et al. 2016), body mass and condition (Meixell et al. 2016; Fleskes et al. 2017), but these relationships may differ by host and parasite species. For example, Fleskes et al. (2017) found that wing length, body mass, and body condition index decreased with *Leucocytozoon* infection but not with either *Plasmodium* or *Haemoproteus* infection in five species of dabbling ducks. In contrast, Meixell et al. (2016) reported a negative relationship between body condition and *Haemoproteus* infection in juvenile ducks only, and the relationship between *Leucocytozoon* infection and body condition differed among duck host species. These contrasting results highlight the need for system specific studies of parasite dynamics in

waterfowl and investigation into other potential drivers of infection heterogeneity that have not been explored, such as host-associated microbes.

The gut microbiome (community of living microorganisms within the gastrointestinal tract) plays an important role in an organism's development, digestion, and immune function (Kohl 2012), and therefore could be a promising indicator of animal health. Although the roles and functions of intestinal microbe species are well characterized in mammals (>90% of studies in existence), less is known about microbial communities (i.e., the microbiome) associated with birds, particularly wild birds (Colston and Jackson 2016; Grond et al. 2018). Of the emerging research focused on the avian microbiome, a majority of studies have focused on characterizing the microbiome composition and diversity in single or multiple host species, with limited focus on relationships with pathogen infection or health (Ganz et al. 2017; Hird et al. 2018; Choi et al. 2021; Sun et al. 2022). Given that wild birds, particularly waterfowl, are known to contribute to the global spread of zoonotic pathogens (Gaidet et al. 2010; The Global Consortium for H5N8 and Related Influenza Viruses 2016), a better understanding of the relationship between the microbiome and pathogen infection could be informative for examining drivers of disease transmission in host populations.

Previous studies have shown that host and ecological factors, such as species (Kohl 2012; Colston and Jackson 2016; Grond et al. 2018), diet (Kohl 2012; Grond et al. 2018; Pekarsky et al. 2021), behavior (Sun et al. 2022; Turjeman et al. 2020; Thie et al. 2022; Lucas and Heeb 2005), pathogen infection (Ganz et al. 2017; Hird et al. 2018; Choi et al. 2021) and the environment (Grond et al. 2018; Kohl 2012; Hird et al. 2015), may play a significant role in shaping microbiome diversity and community structure. For example, a study in cranes found season and food supplementation to influence the abundance of genera mostly from Firmicutes

(Pekarsky et al. 2021). In terms of behavior and environment, nest sharing had an effect on the microbiome structure of great tits (*Parus major*) and blue tits (*P. caeruleus*), even more so than the identity of the host species (Lucas and Heeb 2005). Further, studies in barn swallows (Turjeman et al. 2020) and buzzards (Thie et al. 2022) found evidence for migratory behavior (migratory status and arrival time, respectively) influencing microbiome community composition. Studies have also found that host microbiome communities differed between pathogen-infected and uninfected individuals (Ganz et al. 2017; Choi et al. 2021; Bavananthasivam et al. 2021), suggesting a relationship between the microbiome and disease. For example, the microbiomes of mallard ducks (*Anas platyrhynchos*) infected with avian influenza virus (AIV) were characterized by lower species diversity, richness, and evenness than those of uninfected ducks (Ganz et al. 2017) while another study in barn swallows found decreased microbial diversity in birds without *Salmonella* spp. present in their microbiomes (Choi et al. 2021).

While some studies have focused on the relationship between the avian gut microbiome and gut-associated pathogens (Bavananthasivam et al. 2021; Ganz et al. 2017; Hird et al. 2018; Ma et al. 2017; Murray et al. 2020), it remains unclear whether a relationship exists between the microbiome and those pathogens that invade animal tissues outside of the gastrointestinal tract, such as haemosporidian (blood) parasites. Previous work found no relationship between *Plasmodium* infection and microbial diversity (Aželytė et al. 2023; Rohrer et al. 2023); however, these studies found compositional and abundance differences in the fecal microbiome with *Plasmodium homocircumflexum* infection in canaries (*Serinus canaria domestica*) (Aželytė et al. 2023) and *Plasmodium* infection in Eurasian tree sparrows (Rohrer et al. 2023), respectively. If a relationship exists between the microbiome and pathogen infections, this could suggest an

indirect interaction through the host's physiological and immune responses rather than a direct interaction between microbes at the site of infection. For example, Palinauskas et al. (2022) theorized that the gut microbiota in birds expresses genes similar to the surface proteins of *Plasmodium* sporozoites and could thus induce the immune system to produce antibodies that aid in resistance to *Plasmodium* infection. While the role the avian microbiome plays in *Plasmodium* infection is unknown, there is strong support for a link between the mammalian gut microbiome and *Plasmodium* infections in mouse models where specific taxa were differentially abundant in mice susceptible and resistant to *Plasmodium* infection (Morffy Smith et al. 2019; Stough et al. 2016; Villarino et al. 2016).

Waterfowl of the Atlantic Flyway make an important contribution to the eco-tourism and hunting economy along the eastern United States. Long-term data from the Atlantic Flyway estimates that mallard populations have declined 50% in the last twenty years, leading to reductions in hunting bag limits for the species across the entire flyway (Heusmann 2017; SUNY College of Environmental Science and Forestry n.d.). Black ducks were once the most abundant dabbling duck along the Atlantic Flyway, however the population has experienced declines of over 50% since the 1980s (Ringelman et al. 2015; Sebastian 2023) and their abundance remains well below historic levels despite a 1.06% increase in their population from 2012 to 2021 (U.S. Fish and Wildlife Service 2022). In Maine specifically, Breeding Bird Survey counts within the state show evidence of a population decline in both mallards and black ducks, as well as consistently low numbers for wood ducks and green-winged teals in recent years ("BBS - USGS Patuxent Wildlife Research Center" n.d.). Given the importance of these birds for eco-tourism and hunting, parasite surveillance and the identification of potential health metrics, including the

host microbiome, for monitoring these populations is essential for informing game bird management.

In this study, we had three main objectives: 1) determine the prevalence and intensity of haemosporidians in four waterfowl species (wood ducks, green-winged teals, mallards, black ducks) found in Maine using molecular diagnostic techniques, 2) identify host ecological predictors (age, sex, weight, wing length, location) of haemosporidian infections, and 3) explore relationships between these parasite infections and the cloacal bacterial microbiome community, through 16S rRNA sequencing, in wild ducks. These results are expected to identify patterns and drivers of haemosporidian parasite infections in game birds important to the Atlantic Flyway; they may also inform management decisions by identifying important bioindicators for evaluating waterfowl population health.

Methods

Ethics Statement

All fieldwork was conducted in conjunction with the Maine Department of Inland Fisheries and Wildlife (MDIFW), under the MDIFW Wildlife Scientific Collection Permit No. 2018-552 and U.S. Fish and Wildlife Service's Migratory Bird Scientific Collection Permit No. MB99205C-0. An animal ethical handling and use protocol for this work was approved by the University of Maine's Institutional Animal Care and Use Committee (A2018-07-02).

Study Area

We trapped and sampled waterfowl in two areas of Maine during pre- to early- migration (August and September) over a three-year period (2018 – 2020). In 2018 and 2019, we sampled birds at sites around Lake Christina (Fort Fairfield), Lake Josephine (Eason) and Dorsey Pond (Fort Fairfield), located in northern Maine, and in 2020, we sampled sites throughout southern

Maine, ranging from Unity north to Orono (Figure 3.1). Our northern sampling occurred along water bodies primarily surrounded by agricultural fields (potato) and lumber mills. Capture sites in our southern sampling region were primarily at wildlife management areas or recreational areas with less disturbance in surrounding areas.

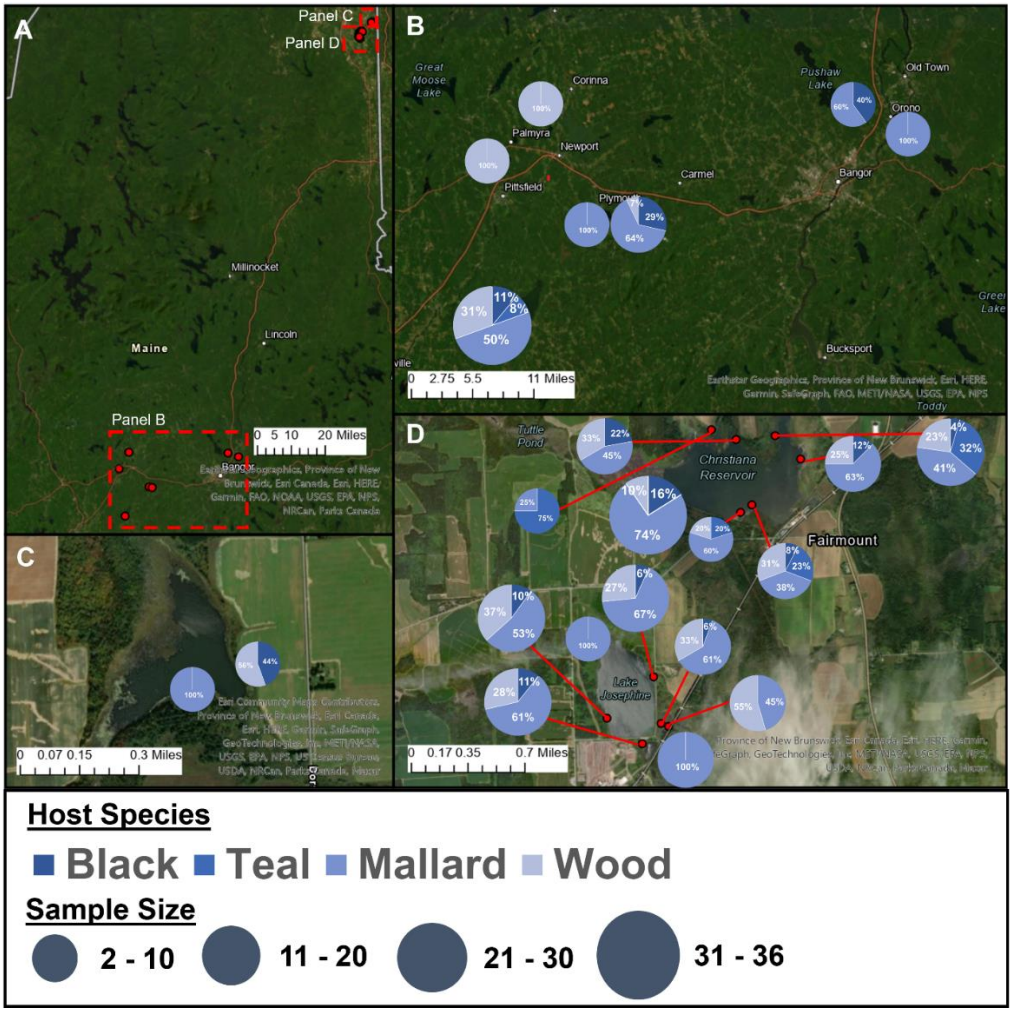


Figure 3.1. Map of Maine (A) with two focal sampling areas (dotted lines) and zoomed in panels for (B) southern ME (2020) and (C-D) northern ME (2018-2019) sites. Species distributions at each trap site are shown by pie charts with chart size relative to the total sample size at a particular site. Red lines connect pie charts to specific capture site locations (red dots). The different shades of blue in pie charts represent specific species from lightest blue to darkest blue: wood duck, mallard duck, green-winged teal, and black duck.

Sample Collection

We focused our sampling efforts on four species of waterfowl common to Maine, including wood ducks, green-winged teal, mallard ducks, and American black ducks. In total, we captured 333 ducks (90 wood ducks, 20 teal, 187 mallards, and 36 black ducks) using modified clover leaf traps (North and Hicks 2017) at multiple sites along the edges of each body of water. During capture, birds were banded with a standard waterfowl band, after which a blood sample was collected from the non-banded leg of each captured bird using a sterile needle, and a capillary tube was used to transfer the sample into a vial of Queen's Lysis Buffer or onto Whatman's FTA cards (GE Healthcare Life Sciences Marlborough, MA) to preserve the sample for downstream molecular applications. Where possible, a second blood tube was collected for haemosporidian diagnostics by microscopy (described below). In addition, cloacal swab samples were taken for obtaining data on host-associated microbiome. Briefly, a sterile swab was inserted into the cloaca of the bird and then swirled three times before storage in 95% EtOH and transfer to a -80°C freezer until DNA extraction. We chose the cloaca as a non-lethal sample that is expected to provide an index of the diversity present within the gastrointestinal tract. From each bird, we also collected data on the individual's age (hatch-year or after hatch-year), sex, weight, bill length, and wing length.

Haemosporidian Diagnostics

Avian haemosporidian diagnostics commonly entails microscopy and molecular techniques such as PCR (Ishtiaq et al. 2017). Following standard protocols, we made thin blood smears with a feathered edge within 24 hours of blood collection. Slides were fixed immediately using a Diff-Quik Kit (Electron Microscopy Sciences Hatfield, PA), and then stored at room temperature until they were scanned for haemosporidian diagnostics. A subset of samples (n = 233) with good

quality blood smears were screened for parasite presence/absence and quantification of an individual's parasite load (or parasite intensity), which was estimated as the number of parasites counted per 10,000 red blood cells following Godfrey et al. (1987). For the purposes of this study, we quantified the total haemosporidian parasite load, combining all genera together. We screened slides under an Olympus light microscope (Olympus, Tokyo, Japan) at 400X and then 1,000X with around 50 fields examined per slide for an estimated total of approximately 10,000 red blood cells screened for each sample.

In addition to microscopy, we used a quantitative PCR (qPCR) molecular diagnostic approach to enable detection of low-intensity haemosporidian infections that may be missed through microscopy (Fallon et al. 2003). qPCR has been more recently used for haemosporidian diagnostics not only because it is more sensitive, but also because it is less time-consuming (Ishtiaq et al. 2017; Bell et al. 2015), with multiplexing allowing for simultaneous detection of all three parasite genera in a single reaction (Ishtiaq et al. 2017). Furthermore, Ishtiaq et al. (2017) demonstrated a relationship between qPCR cycle threshold (C_T) values and the infection intensity estimated by microscopy, validating the use of the qPCR C_T values as a quantitative index of parasite load. Because neither microscopy nor PCR provides a complete picture, the two methods are often used in conjunction for improved detection and identification (Valkiunas et al. 2008).

For qPCR diagnostics, we extracted DNA from all samples (n = 333) using DNeasy Blood and Tissue kits (Qiagen Inc., Germantown, MD) following the blood or filter paper protocols for samples stored in Queen's Lysis Buffer or on Whatman FTA cards, respectively. SYBR Green qPCR was conducted following Ishtiaq et al. (2017) with primer pairs 343F/496R (Fallon et al. 2003) targeting a 153 bp (base pair) fragment of mitochondrial rRNA of all three

haemosporidian genera. Reaction volume was 10 µl and consisted of: 2 µl (5-25 ng/µl) of DNA template, 0.2 µM each of forward and reverse primer (0.5 µl each), 2 µl of nuclease free water, and 5 µl of 2X Luna Universal qPCR Master Mix (New England BioLabs, Ipswich, MA). qPCR cycling conditions were 50°C for 2 min and 95°C for 2 min, followed by 43 cycles of 95°C for 15 sec, 57°C for 45 sec, and 72°C for 30 sec, and a melt curve analysis. Each qPCR reaction plate included a negative control (water), a positive control for *Plasmodium relictum* from a bird confirmed to have an active infection, and two synthetic positive controls for *Haemoproteus columbae* and *Leucocytozoon majoris*, respectively (IDT, Coralville, IA); synthetic positive controls were designed using sequence data from the target mitochondrial rRNA region, amplified by the 343F/496R primer pair. All samples were run in duplicate and a bird was considered to be infected if both runs yielded a C_T of 38 or less (Ishtiaq et al. 2017) and were no more than 2 C_T values apart. Samples with inconsistent results were run again in duplicate and those still with inconsistent results were removed from downstream analyses.

Estimates of Haemosporidian Parasite Prevalence and Intensity

The qPCR assay used did not differentiate between the three genera, and thus for infected birds (with C_T value $\leq$38 cycles), the C_T value was used as an index of overall haemosporidian parasite infection intensity, rather than at the genus or species level. Lower C_T values are reflective of higher levels of infection and vice versa. Percent parasite prevalence was calculated as the number of infected individuals divided by the total number of samples and multiplied by 100. We performed a McNemar's test (Fagerland et al. 2013) to evaluate whether there was a significant difference in haemosporidian spp. prevalence estimates by diagnostic method, using paired microscopy and qPCR diagnostic data (n = 233).

Relationship between Host Ecology and Haemosporidian Parasite Infection

We examined whether haemosporidian parasite infection intensity was correlated with host characteristics to enhance our understanding of host-parasite ecology. We used parasite infection intensity, estimated by qPCR C_T values, as the model response variable given the high infection prevalence in our data. Given that all microscopy positive samples were also positive by qPCR, qPCR data was used in all subsequent analyses. We used a generalized linear model (GLM) to evaluate whether haemosporidian infection intensity (C_T) was dependent on five host variables: age, body mass, wing length, location, and sex. For location, we categorized the samples into the two general sampling areas: northern and southern Maine (Figure 3.1). Due to a high correlation between wing length and body mass ($R^2 = 0.61$, $p = 2.2^{-16}$), we excluded body mass from the models as wing length had fewer missing data. We tested for significance with a Wilcoxon rank sum tests (Mann-Whitney U test) (Mann and Whitney 1947) in R v4.3.0 (R Core Team 2023). We ran separate GLMs for each species, as well as a combined generalized linear mixed model (GLMM) that included host species as a random factor, and applied Akaike information criterion (AIC) (Akaike 1976) for model selection to identify the best models. A null model was included for each species level and combined species analysis. Sample sizes and distribution varied for age, sex, and wing length as not all individuals sampled had all associated host data. Thus, only individuals with data available for all host factors were included in our models, with the following sample sizes: 78 wood ducks, 17 teals, 171 mallards, and 33 black ducks.

Microbiome Extraction and Sequencing

We extracted DNA from cloacal swabs for 16S rRNA gene sequencing of the host-associated bacterial community. Whole genomic DNA was extracted using DNeasy PowerLyzer PowerSoil kits (Qiagen Inc., Germantown, MD) following the manufacturer's protocol with modifications

as detailed in Corl et al. (2020). To account for batch effects, we completed extractions in batches, separating species but randomizing individuals within species across sampling sites and collection years. For each extraction batch, we included an empty extraction tube as a negative control for potential contamination introduced by the kit or laboratory environment. Upon extraction, samples were sent to Argonne National Laboratory (Lemont, IL) for PCR-amplification (in triplicate) using the primer pair 515F/806R (Caporaso et al. 2012) of the 151 bp V4 region of the bacterial 16S rRNA gene; PCR products were then sequenced on an Illumina MiSeq in both directions for pair-end reads.

Microbiome Quality Control and Data Filtering

We used demultiplexed 16S rRNA sequences and R v.4.3.0 (R Core Team 2023) for analysis of microbiome data, modeling the workflow detailed by Callahan et al. (2016) and described in Choi et al. (2021) for quality control and filtering. We used DADA2 (Callahan et al. 2016) to identify amplicon sequence variants (ASVs) and DECIPHER (Wright 2015) to align sequences. We assigned taxonomy with SILVA 138.1 taxonomy database (Quast et al. 2013) and included species level assignments. We built a maximum likelihood phylogenetic tree with the package phangorn (Schliep 2011) and merged all files (sequence variant table, taxonomy table, and phylogenetic tree) with the metadata using the phyloseq package (McMurdie and Holmes 2013) for statistical analyses. We separated datasets by host species and then used the negative controls as reference for removal of contaminant sequences with the decontam package (Davis et al. 2018); for this analysis, a prevalence threshold of 0.5 was used to remove sequences more common in the negative controls than in our samples. We also removed any ASVs not belonging to the kingdom Bacteria. The remaining sequence data were used in all subsequent analyses.

We assessed the cloacal bacterial community diversity for each sample as well as between samples after rarefaction to 4000 reads for teals and mallards, 5000 reads for black and wood ducks, and 2000 reads for the combined species. These read thresholds used were based on the values of mean alpha diversity where the rarefaction curves (rngseed = 711) plateaued on plots for individual species (Figure C.1A-D) and all species combined (Figure C.1E). At these sampling depths, even the most diverse samples plateaued on rarefaction curves and thus samples that fell below these depths were removed leaving 19 teals, 33 black ducks, 85 wood ducks, 186 mallards for species level downstream bacterial diversity analyses, and 303 ducks for all species analyses.

Relationships Between the Cloacal Bacterial Community and Haemosporidian Infection Intensity

We measured the alpha diversity of the cloacal microbiome in all rarefied samples (rngseed = 711) using the Chao1 estimator (Chao 1984), and running separate analyses for each host species as well as a combined species analysis. Chao1 factors in rare and missing species (Chao and Shen 2003), which is useful for diversity comparisons. We investigated the relationship between Chao1 alpha diversity and haemosporidian infection intensity as reflected by qPCR C_T values. We ran linear regressions to examine relationships between Chao1 and haemosporidian infection intensity for the individual species and combined species datasets.

We applied a principal coordinate analysis (PCoA) in phyloseq to examine variation in beta diversity, reflecting the cloacal bacterial community composition, of birds in relation to haemosporidian infection intensity. We used adonis from the vegan package (Oksanen et al. 2020) to calculate permutational multivariate analysis of variance (PERMANOVA) for weighted and unweighted UniFrac metrics. UniFrac compares communities using phylogenetic

information to estimate distance between samples (Lozupone and Knight 2005); the unweighted version using only presence/absence data, whereas the weighted version incorporates relative abundance of ASVs to estimate distance (Lozupone et al. 2007).

Results

Haemosporidian Detection, Prevalence, and Intensity Varied by Host Species

Across all species, the probability of detecting haemosporidian infections in waterfowl was higher using qPCR than with microscopy, ($p = 2.02^{-16}$; Figure 3.2). At the species level, qPCR consistently detected more haemosporidian infections than microscopy ($p = 0.016$ teals, $p = 6.1^{-5}$ all other species). Overall ($n = 333$), qPCR detected haemosporidian parasite infections in 96.7% ($n = 322$) of birds, with only 11 birds testing negative (two teals, three mallards, and six wood ducks). Using a subset of samples evaluated by both methods ($n = 233$), haemosporidian infection prevalence was 48.1% based on microscopy, whereas in the same subset 100% of birds were determined to be infected based on qPCR. In addition, prevalence estimates based on microscopy varied more greatly (range: 37.9% - 71.4%) in comparison to qPCR-based estimates, which were more consistently high (range: 90-100%) across species (Figure 3.2).

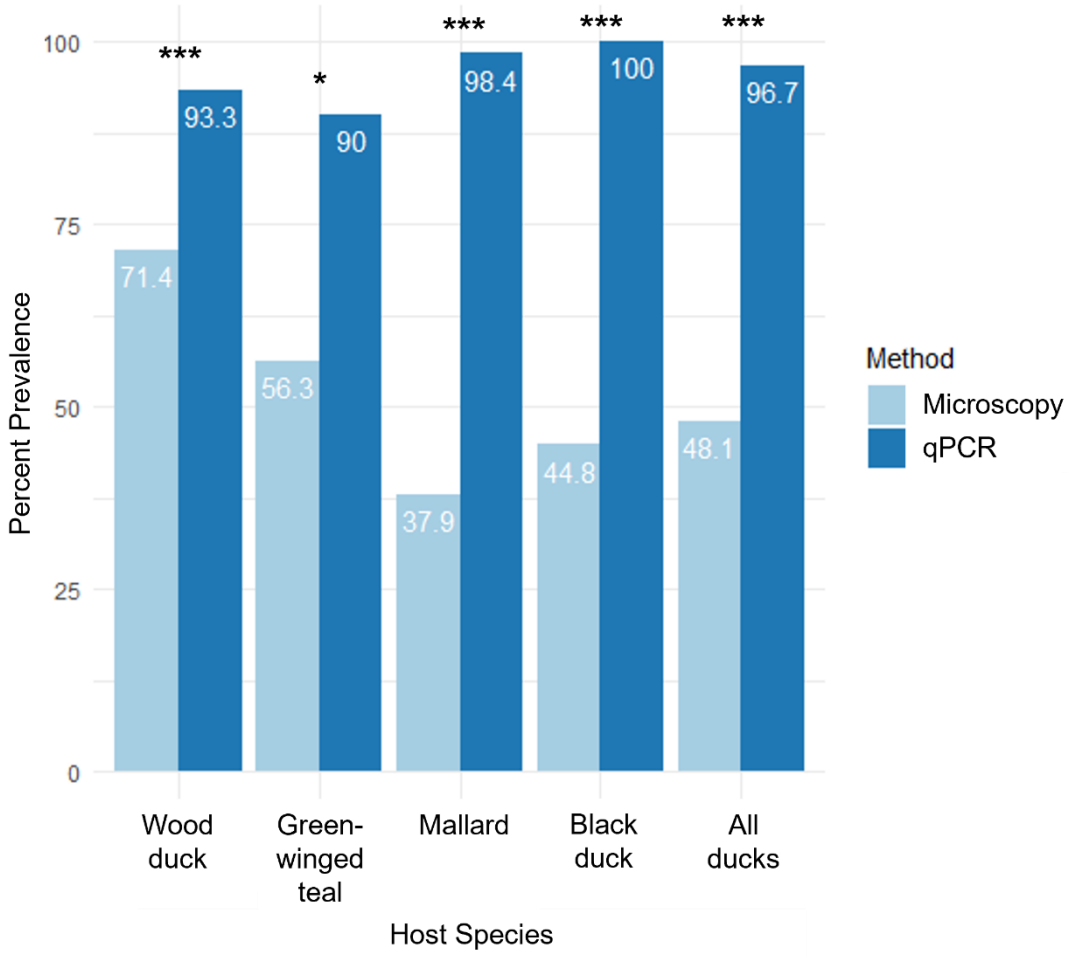


Figure 3.2. Avian haemosporidian parasite prevalence by waterfowl species and diagnostic approach. The percent prevalence is shown for each species (wood duck, green-winged teal, mallard duck, black duck) and overall waterfowl sampled (all ducks). Prevalence was estimated by two diagnostic approaches (microscopy: light blue, qPCR: dark blue), with estimates reported in white font near the top of each bar. Using paired samples from individuals with both qPCR and microscopy data available (n = 233), we found that parasite prevalence estimated by qPCR was significantly higher than that estimated by microscopy in the overall dataset ($p = 2.02^{-16}$) and by species (p = 0.016 teals, $p = 6.1^{-5}$ all other species). * $p < 0.05$, *** $p < 0.001$.

Haemosporidian parasite infection intensity differed by host species ($\chi^2 = 19.7$, $p = 0.00019$; Figure 3.3). Of the four species, wood ducks had the highest mean infection intensity (Mean $C_T = 23.02$), which was significantly greater than parasite intensity in mallards ($W = 9891.5$, $p = 0.00024$) and black ducks ($W = 2115.5$, $p = 0.00055$). Green-winged teals, however, also exhibited a high mean infection intensity (Mean $C_T = 23.12$) that did not did not differ significantly from wood ducks ($W = 749.5$, $p = 0.96$; Figure 3.3). Black ducks had the lowest estimates of parasite infection intensity (Mean $C_T = 25.42$) but did not differ from mallards ($W = 3757.5$, $p = 0.20$), which also had lower infection intensity (Mean $C_T = 24.29$). Interestingly, while black ducks had the lowest parasite intensity (Mean $C_T = 25.42$; Figure 3.3), the species also had the highest parasite prevalence (100%; Figure 3.2). Conversely, the two species with the highest parasite intensity (teal, wood duck) had the lowest parasite prevalence (~90-93%; Figure 3.2).

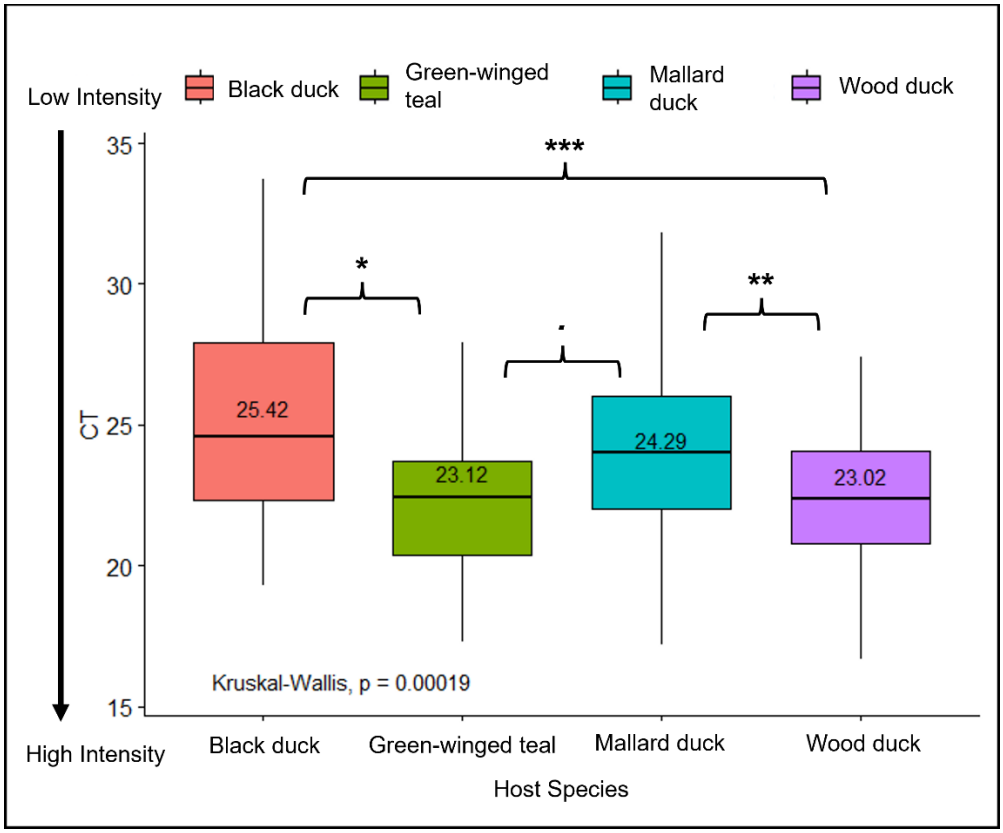


Figure 3.3. Boxplot comparisons of haemosporidian parasite infection intensity (C_T value) by waterfowl host species. Infection intensity varied by host species (χ^2 = 19.7, p = 0.00019). Wood ducks (purple) and green-winged teal (green) had the highest average infection intensity as portrayed by the lowest average C_T values (~23), while black ducks (red) and mallards (blue) had the lowest average infection intensity as portrayed by the highest average C_T values (~24-25). Asterisks mark significance with * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and . = $p < 0.1$.

Predictors of Haemosporidian Infection Intensity Varied by Host Species

For the combined species analyses (n = 299), body mass and wing length were correlated (cor = 0.61, $p = 2.2^{-16}$); therefore, body mass was excluded from the models as wing length had fewer missing data than body mass. The model including age and location, and with species as a random factor, was the best fitting model (AIC = 1448.4; Table 3.1); both age (W = 6937.5, p =

0.0011; Figure 3.4A) and location (W = 3460, $p = 3.84^{-8}$; Figure 3.5E) were significant predictors of parasite infection intensity in all ducks.

Table 3.1. AIC selection of generalized linear mixed models to identify variables associated with haemosporidian parasite infection intensity in all species combined.

Model	**Predictor Variables**	**AIC**	**ΔAIC**
Model 13	Age + Location + Species*	1448.4	0.0
Model 11	Age + Wing Length + Location + Species*	1448.5	0.1
Model 15	Wing Length + Location + Species*	1448.8	0.4
Model 17	Location + Species*	1449.8	1.4
Model 5	Age + Sex + Location + Species*	1452.2	3.9
Full Model	Age + Sex + Wing Length + Location + Species*	1452.5	4.1
Model 7	Sex + Wing Length + Location + Species	1452.8	4.4
Model 9	Sex + Location + Species*	1453.5	5.1
Model 3	Age + Sex + Wing Length + Location	1457.3	8.9
Model 14	Age + Species*	1492.3	43.9
Model 12	Age + Wing Length + Species*	1494.1	45.8
Model 6	Age + Sex + Species	1495.8	47.4
Model 4	Age + Sex + Wing Length + Species*	1497.4	49.1
Model 2	Species*	1498.3	49.9
Null Model	null model	1499.6	51.2
Model 16	Wing Length + Species*	1500.3	51.9
Model 10	Sex + Species*	1501.4	53.0
Model 8	Sex + Wing Length + Species*	1503.4	55.0

*Species was included as a random factor in the glmm

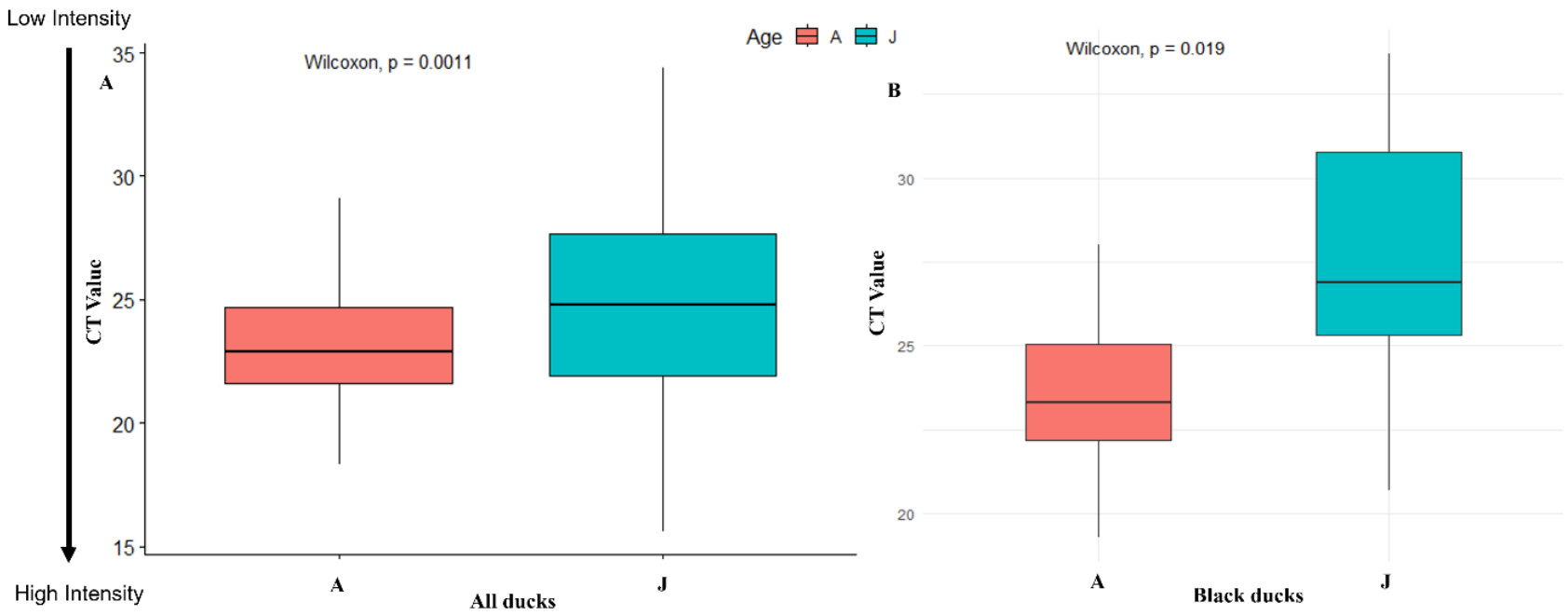


Figure 3.4. Boxplot comparison of haemosporidian parasite infection intensity (C_T value) by age for A) all ducks and B) black ducks. Adult ducks (A – red) had a higher infection intensity (lower mean C_T value) than juvenile ducks (J – blue) (W = 6937.5, p = 0.0011). A Wilcoxon rank sum test showed that adult black ducks (A – red) have higher parasite infection intensity than juvenile (J – blue) black ducks (W = 68, p = 0.019).

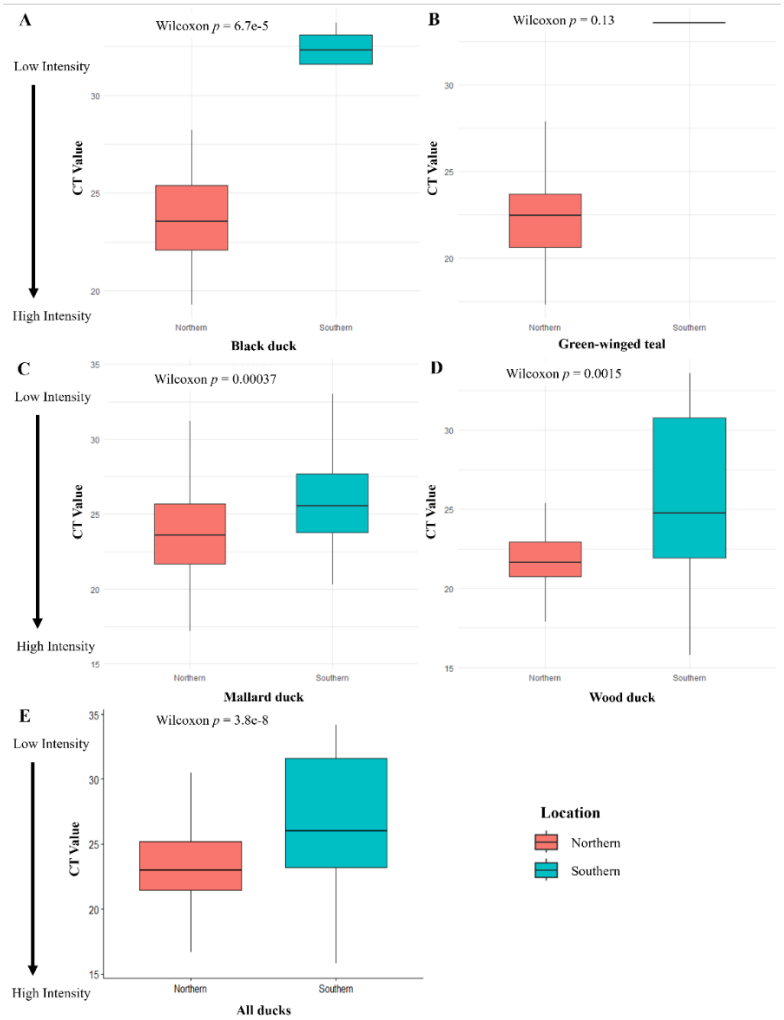


Figure 3.5. Boxplot comparison of haemosporidian parasite infection intensity (C_T value) in the northern and southern sampling regions in Maine for A) black ducks, B) green-winged teal, C) mallard ducks, D) wood ducks, and E) all ducks. All ducks (E) from the northern region (red) had higher parasite infection intensity than ducks from the southern region (blue) (W = 3460, $p = 3.8^{-8}$). Black ducks (A) sampled in the northern region (red) of Maine had higher parasite infection intensity than those from the southern region (blue) (W = 0, $p = 6.7^{-05}$). Mallards (C) from the northern region (red) had higher parasite infection intensity than mallards from the southern sampling region (blue) (W = 1408.5, $p = 3.7^{-4}$). Wood ducks (D) from the northern sampling region (red) had higher infection intensity than those sampled in the southern region (blue) (W = 330, $p = 0.0015$).

In the black duck models, no variables were correlated and thus all five variables were included as predictors in these models. The best fitting black duck model included age, sex, weight, and location as predictor variables (AIC = 87.3, Table C.1). However, two other models had ΔAICs that were less than 2 (Table C.1). Although the best fit model included four of the five predictor variables, only age (W = 68, p = 0.019; Figure 3.4B) and location (W = 0, $p = 6.7^{-05}$; Figure 3.5A) were significant, whereas weight (R^2 = 0.04, p = 0.29; Figure C.2) and sex (W = 93, p = 0.29; Figure C.3) were not. Adult black ducks had higher parasite infection intensity than juveniles, and black ducks sampled in the northern sampling region had higher parasite infection intensity than those sampled in the southern sampling region.

For green-winged teal models, there were no correlations between any of the predictor variables and thus all five variables were included in model comparisons. The best fitting model for teals included weight, wing length, and location (AIC = 75.0; Table C.2), however none of the variables were statistically significant (wing length: R^2 = 0.002, p = 0.85, Fig. C4; weight: R^2 = 0.03, p = 0.57, Fig. C5; location: W = 35, p = 0.96, Figure 3.5B). In addition, four models were < 2 ΔAIC values from the top model (Table C.2).

For mallard duck models, body mass and wing length were correlated (cor = 0.24, p = 0.012) and therefore body mass was excluded from the models. The best fitting model included wing length and location (AIC = 884.7; Table C.3). Infection intensity did not differ significantly by wing length (R^2 = 0.002, p = 0.6; Supplemental Fig. C6), however, mallards in the northern region had higher parasite infection intensity than mallards in the southern region (W = 1408, $p = 3.7^{-4}$, Fig. 3.5C). Four models had < 2 ΔAICs of the top model: location (ΔAIC = 0.1), age + location (ΔAIC = 0.5), age + wing length + location (ΔAIC = 1.2) and sex + wing length + location (ΔAIC= 1.9).

In the wood duck models, body mass and wing length were correlated (cor = 0.28, p = 0.021); thus, body mass was excluded from the models. For wood ducks, the best fitting model included location as the only predictor variable (AIC = 402.4; Table C.4). However, seven models were within < 2 ΔAICs of the top model (ΔAIC 0.2 to 1.5; Table C.4). Haemosporidian infection intensity was higher in wood ducks sampled in the northern region than in the southern region (W = 330, p = 0.0015, Fig. 3.5D).

Cloacal Bacterial Community Alpha Diversity did not Vary with Haemosporidian Infection

There was no significant relationship between Chao1 diversity and haemosporidian infection intensity (qPCR C_T values) in both the combined species model (R^2 = 0.005, p = 0.24; Figure C.7) as well as for the individual host species models (black ducks: R^2 = 0.04, p = 0.25, green-winged teals: R^2 = 0.0002, p = 0.97, mallards: R^2 = 0.0081, p = 0.25, wood ducks: R^2 = 0.0006, p = 0.83; Figure C.8).

Beta Diversity Varied with Haemosporidian Infection for the Weighted UniFrac in Mallards Only

For mallards, a significant relationship between infection intensity and beta diversity was observed based on the weighted UniFrac measure (F = 3.02, p = 0.013; Fig. 3.6B), but not the unweighted UniFrac measure (F = 1.14, p = 0.26; Fig. 3.6A) suggesting that relative abundance of specific taxa may drive beta diversity in mallards. There were no significant relationships between haemosporidian infection intensity and bacterial community composition (beta-diversity), based on both the unweighted and weighted UniFrac measures, for all other duck species: black ducks (unweighted: F = 0.997, p = 0.42, weighted: F = 0.282 p = 0.96; Figure C.9), green-winged teal (unweighted: F = 0.651, p = 0.47, weighted: F = 0.651 p = 0.46; Figure C.10), and wood ducks (unweighted: F = 1.56, p = 0.07, weighted: F = 1.56, p = 0.07; Fig. C11).

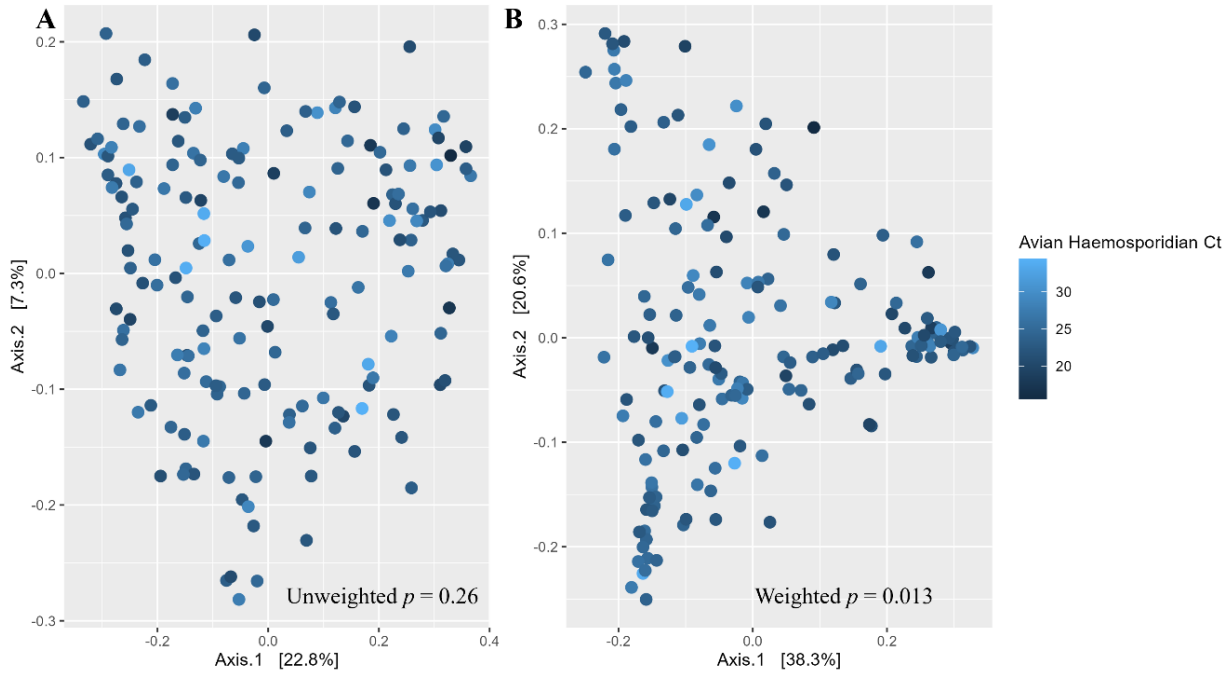


Figure 3.6. PCoA of beta diversity colored by haemosporidian parasite infection intensity (C_T value) for mallard ducks based on the A) Unweighted and B) Weighted UniFrac measures. Bacterial community composition varied significantly with host infection intensity when using the weighted UniFrac measure (F = 3.02, p = 0.013), whereas no relationship was found when using the unweighted UniFrac measure (F = 1.14, p = 0.26).

Discussion

In this study, we examined avian haemosporidian parasite prevalence and infection intensities in four waterfowl species common in Maine and evaluated ecological and host predictors of infection, including microbiome diversity and composition. We compared two commonly used parasite diagnostic approaches, microscopy and qPCR, and found that qPCR detected more haemosporidian infections than microscopy and consistently estimated high levels of prevalence in waterfowl, with infection intensity varying among host species. Location was the most significant risk factor predicting parasite infection across all models followed by age, but the relationship between age and infection varied by species. While we did not observe a

relationship between microbiome diversity and parasite infection, the bacterial community composition (weighted UniFrac measure of beta diversity) varied significantly with parasite infection intensity in mallards, suggesting the microbiome may be a weak, but unreliable predictor of haemosporidian parasite infections in waterfowl. Taken together, these results indicate that haemosporidian parasite-host interactions are complex and likely species specific, but location and age are important predictors of infection in waterfowl species. Given the evidence of declines in some waterfowl species (e.g., mallard ducks), there is a pressing need for parasite surveillance and the identification of infection risk factors for managing the health of these important game species.

We found that qPCR was significantly better at detecting haemosporidian parasites than microscopy and could also provide a valuable metric for quantifying parasite infection intensities. Our findings align with those of a previous study conducted by Ishtiaq et al. (2017), which showed qPCR had a higher sensitivity over microscopy. Parasite detection by microscopy is very time intensive and accurate quantification may depend on personnel experience; thus, the ability to make high quality slides and identify haemosporidian parasites via microscopy can affect parasite detection. Further, because haemosporidian infection are often chronic with parasites infecting few blood cells (low intensity), these low intensity infections may fall below the detection limit via microscopy (Ishtiaq et al. 2017), but are likely to still be detected by qPCR (Logan and Kirstin 2009). Using microscopy in conjunction with qPCR, however, can provide a more complete picture of parasite infection prevalence and intensity than either technique on its own. Taken together, qPCR is a valuable tool for avian haemosporidian detection that can supplement traditional microscopy and could be particularly useful in scenarios where personnel time is limited (e.g., parasite surveillance and ecological studies).

We found a high prevalence of haemosporidian blood parasites in all waterfowl host species in Maine. To the extent of our knowledge, this study is the first to estimate haemosporidian prevalence in waterfowl populations in the state. The prevalence of haemosporidian parasites in this system is high relative to studies conducted in other systems (Annetti et al. 2017; Reinoso-Pérez et al. 2016), however, this could be typical for our study system given no recent previous studies exist regarding haemosporidian prevalence in Maine waterfowl. The observed high prevalence could be attributed to season, as previous studies have shown haemosporidian prevalence is higher during the autumn (Schumm et al. 2021) and breeding season (Hellgren et al. 2013; Pulgarín et al. 2019), which coincides with the timing of sampling in our study. Sampling at the end of the breeding season and into early autumn increases the likelihood of sampling juvenile birds that have not been previously infected, and thus lack parasite immunity, and previous research has shown juvenile birds exhibit an increased risk of infection (Grilo et al. 2016). However, our data do not support this explanation for high prevalence, as we found that adult birds generally had higher parasite infection intensities than juveniles, and our sample sizes by age were relatively well balanced (n = 124 juveniles, n = 163 adults). Also, while observations of seasonal variation in parasite infections can vary by host genus and species (Schumm et al. 2021), other studies have found no differences among seasons (Ishtiaq, Bowden, and Jhala 2017). Another possible explanation for high prevalence may be due to coinfection; our qPCR assay did not differentiate between the three genera and thus prevalence was a combined measure of all haemosporidians genera present, which is in contrast to previous studies that have measured prevalence by haemosporidian genus (Greiner et al. 1975; Reinoso-Pérez et al. 2016; Annetti et al. 2017).

We found that parasite infection intensity varied by host species, with wood ducks and green-winged teal having the highest levels of infection and black ducks and mallards the lowest. Both wood ducks and teal species are solely migrants in Maine ("Maine Dept of Inland Fisheries and Wildlife" n.d.) and thus the increased intensity of infection could be explained by migratory behavior, which could result in increased parasite exposure and susceptibility. The energy demands of migration (Owen and Moore 2008) may result in a tradeoff in immune response, with this cost of migration particularly affecting a host's immune response to chronic parasite infections. Further, migrants stop at many stopover sites along their routes, visiting diverse environments and interacting with more avian species, thereby increasing their exposure to other hosts, vectors, and parasite species (Teitelbaum et al. 2018; de Angeli Dutra et al. 2021). In addition, migrants travel south to winter in habitats where vectors may still thrive, further increasing exposure whereas resident species are given a temporary reprieve as vector populations become dormant in the winter. Migrants may also carry parasites with them throughout their journey and increase the probability of parasite transmission to other individuals (McKay and Hoye 2016), as seen with avian influenza virus in birds (Weber and Stilianakis 2007). However, in some cases birds may increase their immune function at stopover sites and upon reaching their breeding grounds (Eikenaar et al. 2020), and migratory birds could have a lower prevalence of pathogen infection due to migratory escape (where migrants avoid unfavorable conditions, such as disease risk, in their habitats and travel to habitats with more favorable conditions) and migratory culling (where diseased migrants are unable to survive the journey and are thus culled from populations) (Altizer et al. 2011; Hall et al. 2014).

All our best models found sampling location to be a significant predictor in haemosporidian infection intensity, except for in teals. However, our teal dataset only had a

single individual for the southern region, which prohibits making a conclusion about the role of location on infection prevalence in this species. Reinoso-Peréz et al. (2016) found highly degraded shrublands (intense grazing, no tree layer) to have higher levels of infection intensity than moderately degraded shrublands. Our northern sampling region consisted of agriculture and timber mills could be considered as highly degraded, while the southern region of protected areas and large public ponds could be considered moderately degraded, aligning with these previous results. Further, Fecchio et al. (2021) found regional-scale evidence for a relationship between parasite transmission and landscape, climate, and host ecological traits and highlighted the importance of examining these interactions on a system by system basis due to variation among regions. Lastly, these parasites are transmitted by insect vectors (Valkiūnas 2005) and thus differences in the distribution and abundance of these vectors could explain the differences in parasite intensity seen based on sampling location region. However, our models did not include sampling year, an important consideration given that our two locations were not sampled in the same year, making it impossible to disentangle the effects of year versus environment. Therefore, while location appears to be the primary predictor of infection, we cannot exclude the possibility of annual variations in climate variables rather than, or in addition to, location as the primary driver of haemosporidian infections in waterfowl.

We found age was a significant predictor for the combined species model and for black ducks, with juveniles having lower parasite infection intensity than adults. This result contradicted previous studies that found juveniles to show increased risk of *Plasmodium* infection in penguins (Grilo et al. 2016). It is possible that many of the juvenile birds were likely newly infected individuals in the early stages of infection when parasite numbers are low and undetectable. High infection levels in adults also support previous findings that haemosporidian

infections may be chronic (la Puente et al. 2010; Grilo et al. 2016), such that individuals become infected over their lifetime by repeated parasite exposure, but are not able to easily recover from infection. In our study, 100% of black ducks were infected with haemosporidian parasites but the species also had lowest intensity of infection, suggesting the species may be able to maintain low levels of chronic infections. Again, it is plausible that adult ducks could have higher infection intensity compared to juveniles due to coinfection as we did not differentiate between the three genera. Previous research has shown that coinfecting haemosporidian parasites can have a positive effect on one another in adult sparrows, but not juveniles (Garcia-Longoria et al. 2022), suggesting that one haemosporidian species could facilitate infection by another species, driving higher infection prevalence in adult birds.

We found little evidence to support a relationship between the cloacal microbiome and haemosporidian parasite infection. In particular, Chao1 alpha diversity of the bacterial community was not related to haemosporidian infection status or intensity. Although no relationship was found with microbiome diversity, this does not mean an interaction does not exist between specific host-associated microorganisms and haemosporidian parasites. For example, Palinauskas et al. (2022) showed that specific microbial taxa in the gut express genes similar to the surface proteins of *Plasmodium* sporozoites and thus induce the host immune system to produce antibodies that aid in resistance to *Plasmodium* infection. Thus, individuals expressing these microbial genes would be expected to have a higher abundance of these specific taxa, and a lower probability of parasite infection. However, under this theory, we would not expect to see a relationship with microbial diversity, but rather microbial community composition or beta-diversity. In our study, the weighted UniFrac beta-diversity measure in only mallards was found to vary significantly with infection intensity. Since the weighted UniFrac

index accounts for relative abundance, there could be specific taxa that have substantial differences in abundance across the infection intensity spectrum that is driving this significant relationship in mallards as seen in tree sparrows (Rohrer et al. 2023) and canaries (Aželytė et al. 2023). Further experimental genomic and transcriptomic research is needed to shed light on this theory, and to identify specific bacterial taxa associated with infection intensity in mallards.

Conclusions and Future Directions

We found high haemosporidian infection prevalence among waterfowl species in Maine. Infection intensity and host ecological predictors varied by host species, with sampling location and age being key drivers in our system. These results add to the conflicting research that have previously found infection to vary by host age (Annetti et al. 2017; Meixell et al. 2016), body mass and condition (Meixell et al. 2016; Fleskes et al. 2017), highlighting the complexity of the relationship between haemosporidian infection and ecological factors. The observed high prevalence in waterfowl, and particularly in adults, suggests that haemosporidian infections may be chronic (Korpimaki, Hakkarainen, and Bennett 1993; Knowles, Palinauskas, and Sheldon 2010; la Puente et al. 2010). Further, we considered all three haemosporidian parasite genera together as a response variable, which could obscure insights into host-parasite relationships that are often species-specific. Because infection by one parasite species may facilitate infection by another (Garcia-Longoria et al. 2022), the taxonomic identity of haemosporidian parasite genera and species and their interactions should be accounted for in future ecological and epidemiological studies. In addition, differential abundance analysis to identify significant bacterial taxa or gene expression associated with infections across the intensity scale could provide further insight into the relationship between the microbiome and haemosporidian

parasites. These results will provide valuable information to guide the management of these important game species.

CHAPTER 4

MOVEMENT AFFECTS PARASITE INFECTION, PLUMAGE COLORATION, AND REPRODUCTIVE SUCCESS IN BARN OWLS

The Hamilton-Zuk hypothesis posits that phenotypic variation serves as an 'honest signal' of condition, thereby influencing females to select mates with lower parasite loads which in turn increases offspring fitness. Accordingly, individuals resistant to parasites, such as avian haemosporidians, which infect the blood cells of birds, should display showier ornamental traits as a reflection of generally better health and increased vigor. Movement behavior can potentially act as an indicator of health and previous work has linked distance traveled with reproductive success; thus, following Hamilton-Zuk theory, birds with increased movement may have increased parasite resistance, reflected by more showy phenotypic traits. Further the diversity of the microbiome, or community of microorganisms associated with a host, may also be an indicator of health. Our objectives were to (1) determine the prevalence and intensity of haemosporidian parasite infection in a population of barn owls, (2) assess relationships between parasite infection and other potential health indicators including movement and the microbiome, (3) examine whether an individual's plumage melanization extent vary with either parasite infection intensity, microbiome diversity, or movement, while also accounting for sex and mass as predictor variables, and (4) evaluate whether potential indicators of health (parasite infection intensity, microbiome diversity, movement) predict reproductive success (clutch size, fledge success, Julian laying date). Blood samples (avian haemosporidian screening) and cloacal swabs (bacterial microbiome) were collected from 61 adult barn owls during the breeding season in the Hula Valley, Israel, along with associated data on sex, age, movement, reproductive traits, and plumage traits. We found a high

prevalence of haemosporidian parasites, 96.7% (59/ 61) positive with an average C_T of 23.6. Our models revealed home range was negatively correlated with haemosporidian infection intensity (C_T value; $\beta = 0.44$, $R^2 = 0.30$, $p = 1.2e^{-3}$) and positively correlated with plumage melanization extent and mass. We also found home range area was inversely correlated with clutch size ($\beta = -0.12$, $R^2 = 0.27$, $p = 0.02$). Our results do not align with previous work in barn owls supporting plumage ornamentation traits as a signal of parasite resistance but do link plumage coloration with movement. Our study suggests movement behavior is related to the health and fitness of barn owls. Further experimental research is needed to disentangle the relationships between the host phenotypic variation, health indices, and fitness, in order to improve our understanding of parasite resistance and mate selection in birds.

Introduction

The Hamilton and Zuk hypothesis suggests that females in a population choose mates to increase reproductive success, by examining the male's physical and/or behavioral characteristics, which serve as an 'honest signal' of their health and ability to resist parasites (Hamilton and Zuk 1982; Balenger and Zuk 2014). In male birds, mating displays, song, and plumage features, such as bright coloration or spot patterns, size, and abundance have been shown to be associated with mating success (Møller 1990). It has been thought that females will therefore use these qualities to select mates with lower parasite loads to increase the fitness of their offspring through increased parasite resistance (Balenger and Zuk 2014). Both ornament (physical marking) size and quantity of an individual have been thought to be related to parasite resistance signaling in male birds (Borgia and Collis 1990; Saino et al. 2002; Lumpkin, Murphy, and Tarvin 2014). Previous studies have investigated this hypothesis with respect to ectoparasite (Borgia and Collis 1990; Alexandre Roulin et al. 2001) and blood

parasite infections (Lumpkin, Murphy, and Tarvin 2014; Garant et al. 2018), as well as general health indicators, such as corticosterone levels (Saino et al. 2002) and antibody production (Saino et al. 2002), but results vary depending on the phenotypic trait of interest (e.g. number of spots vs. bill color), parasite or outcome of interest, and study system, highlighting variation in this relationship.

Previous studies have provided substantial evidence for male ornamentation as signals of parasite resistance and reproductive success (Borgia and Collis 1990; Saino et al. 2002; Lumpkin, Murphy, and Tarvin 2014; Borgia and Collis 1989; Hill and Farmer 2005; MerilÄ et al. 1999). For example, a study in house finches (*Carpodacus mexicanus*) found that males with redder feather coloration cleared *Mycoplasma gallicepticum* infection faster (Hill and Farmer 2005). Similarly, in greenfinches (*Carduelis chloris*), a strong negative correlation was observed between plumage brightness and haemosporidian parasite infection intensity (MerilÄ et al. 1999). Further, studies have gone beyond parasite infection intensity and focused on immunity itself to represent parasite resistance. In barn swallows (*Hirundo rustica*), male swallows with longer tails, a trait known to undergo sexual selection, had a higher immune response than males with shorter tails (Saino et al. 2002). In blue-black grassquits (*Volatinia jacarina*), while parasitized males were found to have a lower frequency of mating displays, this did not translate to mating success as females did not select for unparasitized males (Aguilar et al. 2008). Lastly, while Garamszegi and Møller (2012) found variation in the relationships between haemosporidian parasite infection intensity and different male ornamentation traits across species, this relationship in female birds is less understood.

Although less studied, females can also display an honest signal of their parasite resistance. Roulin et al. (2001) found that an ectoparasitic fly, *Carnus hemapterus*, was less abundant in young produced by female barn owls, *Tyto alba*, with a greater abundance of spots on their plumage (Roulin et al. 2001), and spot quantity was positively related to antibody production (Roulin et al. 2000). Further, a meta-analysis in birds found that female ornamentation color is related to immune response, clutch size, and male mate selection and concluded that female ornamentation may adapt with mate selection similar to male ornamentation; however, more studies are needed to support this conclusion (Hernández et al. 2021).

Haemosporidian parasites (order Haemosporida), among other types of parasites, exert a substantial influence on avian health and can greatly impact bird populations. These parasites infect the blood cells of birds and include protozoan parasites from the genera *Haemoproteus*, *Leucocytozoon*, and *Plasmodium*. Haemosporidian parasites can cause infections ranging from asymptomatic (Sato 2021), sublethal infections (Thurber et al. 2014) to severe, life-threatening diseases (la Puente et al. 2010). Studies on haemosporidian parasites have shown that they impact reproductive success (Korpimaki et al. 1993; Chaisi et al. 2019; Dyrcz et al. 2005), survival (la Puente et al. 2010), and body condition (Dyrcz et al. 2005; Knowles, Palinauskas, and Sheldon 2010). Haemosporidian infections may have different effects on males versus females, which may also vary by system; for example, Korpimaki et al. (1993) found decreased reproductive success in females, but not males, whereas Dyrcz et al. (2005) saw reduced body condition and reproductive success only in males with haemosporidian infection. In light of Hamilton and Zuk, haemosporidian infection should then vary by sexual traits as demonstrated by plumage or ornamentation, given that birds select for mates using these traits

to improve fitness outcomes. However, a comprehensive meta-analysis on haemosporidian parasite infection and sexual traits found results to vary by trait of interest and degree of infection (Garamszegi and Møller 2012).

The gut microbiome, here defined as the community of living microorganisms present within the gastrointestinal tract, plays a significant role in an individual's growth, digestion, and immune function (Kohl 2012; Waite and Taylor 2015). While the role and function of microbiota (intestinal microorganisms) are well understood in mammals (constituting over 90% of existing studies), our knowledge about the microbiome-associated with birds, particularly wild birds (Colston and Jackson 2016; Grond et al. 2018), is comparatively limited. Despite recent advancements, the majority of emerging research still focuses on avian species comparisons or general microbiome composition and diversity, with pathogen infection receiving attention in only a single category (Sun et al. 2022). Given the significance of wild birds in the spread of zoonotic pathogens, further research exploring the relationship between the microbiome and pathogen infection can improve our understanding of the utility of the microbiome as an indicator of avian health.

Host movements such as migration or day to day behavior can influence other health indicators such parasite infection while also playing a direct role in an individual's fitness and health. For example, migrants have been thought to help spread pathogens such as avian influenza and West Nile virus (Reed et al. 2003; Hubálek 2004), but also may be more resistant to infection through increased immunity at stopover sites (Eikenaar et al. 2020) and may experience decreased parasite infection prevalence as a result of migratory escape (Altizer et al. 2011; Hallet al. 2014). A study in barn owls found that individuals traveling longer distances daily also had increased microbiome diversity, suggesting movement behavior may

affect host physiology (hormone levels or immune response) and therefore the associated microbiota (Corl et al. 2020). Further, previous work done in male barn owls found home range size to affect breeding success, such that males with smaller home ranges had increased fledge success (Séchaud et al. 2022). Given that movement indices have been linked with parasite infection, microbiome diversity, and reproductive success, monitoring individual movement can act as a potential indicator of the overall health of birds.

Barn owls offer an ideal study system for examining the relationships between sexual traits (plumage characteristics) and indicators of health (movement, parasitism, microbiome diversity), as well as the subsequent effects on fitness (reproductive success). In this species, there has been extensive research on plumage patterns (Roulin et al. 2001; Roulin 1999), haemosporidian parasite infections (Roulin et al. 2007), and the microbiome (Corl et al. 2020), the latter of which has demonstrated sex-specific relationships between microbiome diversity and reproductive success. The objectives of our study were to: (1) determine the prevalence and intensity of haemosporidian parasite infection in a population of barn owls, (2) assess relationships between parasite infection and other potential health indicators including the microbiome and movement, (3) examine whether an individual's plumage melanization extent vary with either parasite infection intensity, microbiome diversity, or movement, while also accounting for sex and mass as predictor variables, and (4) evaluate whether potential indicators of health (parasite infection intensity, microbiome diversity, movement) predict reproductive success (clutch size, fledge success, Julian laying date). The hypothesized relationships are shown in a conceptual framework for reference (Figure 4.1). These results could provide novel insights on host-pathogen dynamics and sexual selection in birds, by linking plumage trait variation to multiple indicators of avian health and fitness.

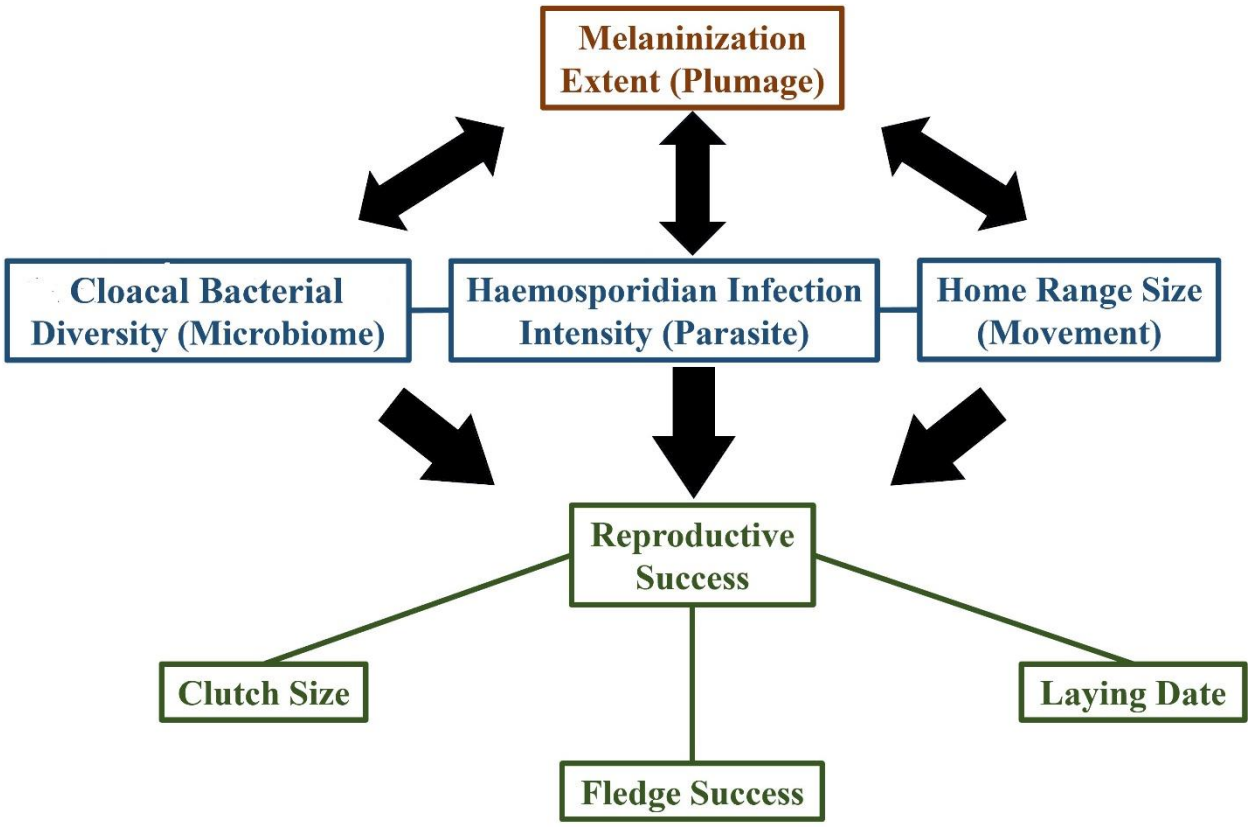


Figure 4.1. Conceptual framework for the hypothesized relationships among plumage traits (red), potential health indicators (blue), and parameters of reproductive success (green), which were examined through the objectives of this study. The arrows indicate the expected directions of the relationships.

Methods

Sample and Data Collection

We collected samples and associated data from 69 adult barn owls actively breeding in the Hula Valley region of northern Israel (Figure 4.2) from April 12th to July 5th, 2017, as previously described in Corl et al. (2020). Briefly, nest boxes were monitored to identify boxes in use and then track reproductive data throughout the duration of the sampling period. To reduce nest abandonment, owls were captured, and swabs taken after at least one nestling hatched either at night from the nest box entrance or during the day while in the nest box. Birds were also fitted

with ATLAS wildlife tags using a Teflon harness to track movement. The following individual characteristics were recorded: *sex*, *clutch size*, laying Julian date of the first egg (*laying date*), proportion of hatchlings successfully fledged (*fledge success*), nest box number (*box*), and two separate total eumelanin-based coloration amount indices: the average number of spots on the breast (*number of spots*) and the average diameter of breast spots (*spot diameter*). For eumelanin coloration assessment, a 60 x 40 mm frame placed on the breast was used to count the total number of spots and measure the diameter for 10 spots within the frame to calculate a mean. We then used these values to calculate a proxy for the energetic cost of eumelanin coloration, which we refer to as *melanization extent,* by taking the average areas of spots (using spot diameter) and multiplying that value by the total number of spots.

A sample for microbiome characterization was collected, as previously described (Corl et al. 2020). Briefly, a cloacal swab was taken from each individual and placed in a 95% EtOH solution, then stored at -20℃ in the field followed by -80℃ in the laboratory until extraction. In addition, to assess haemosporidian parasite status, blood was collected from each individual using a sterile needle and stored on Whatman FTA cards (GE Healthcare, Piscataway, NJ). Cards were stored at room temperature while in the field, then placed in a -80℃ freezer for long-term storage until further processing. All sampling was conducted under the approval of the ethics committee of the Hebrew University of Jerusalem (permit NS-16-14801-2) and University of California Berkeley's IACUC (No. AUP-2016-04-8665-1).

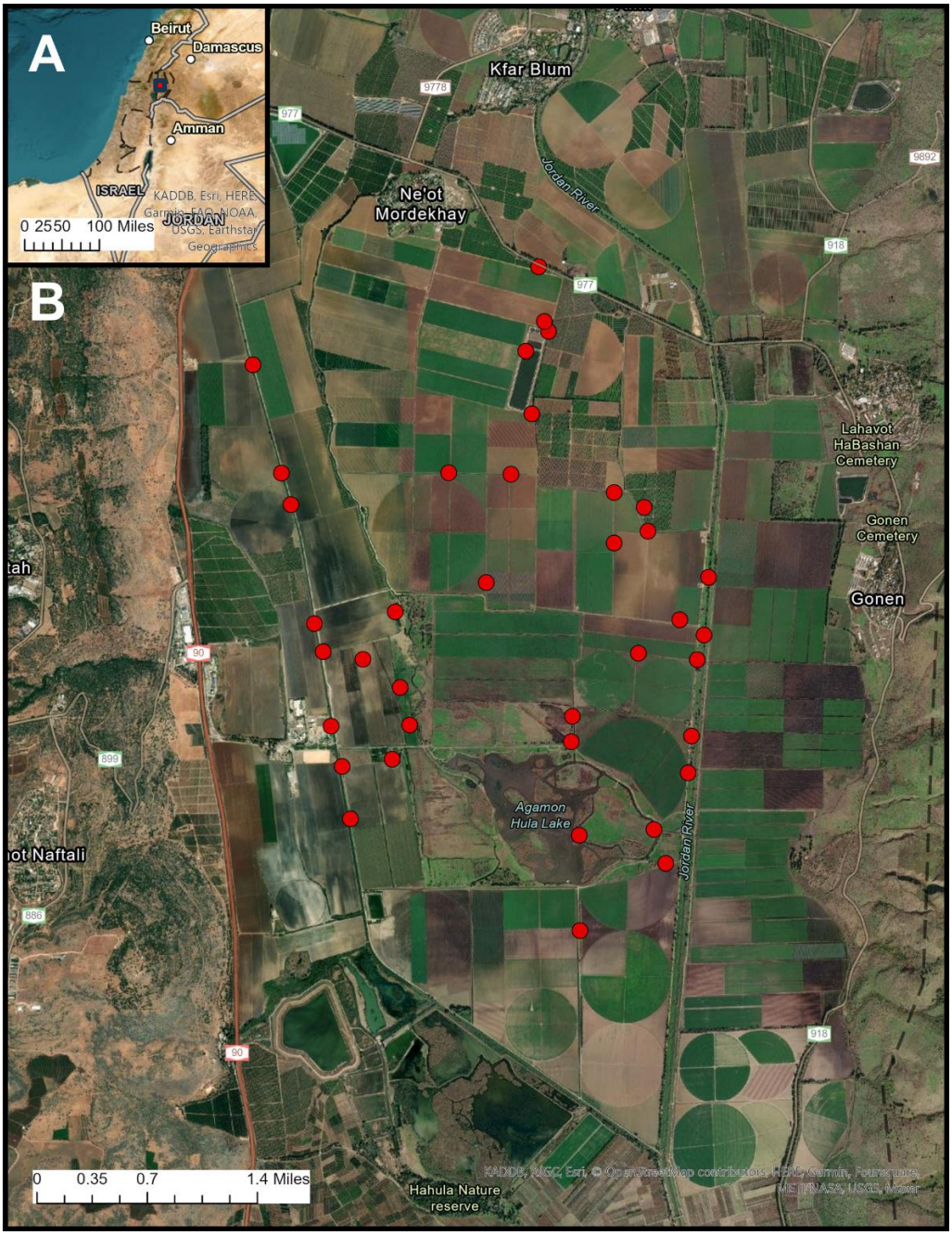


Figure 4.2. A) Map of sampling area in Israel, highlighted in red, with B) a zoomed in panel displaying the Hula Valley. Red points within panel represent individual nest boxes for owls sampled from April 12th to July 5th, 2017.

Movement Data

Movement data was collected from tagged owls using the ATLAS system (Weiser et al. 2016) as described in Corl et al. (2020), which provides location data as often as every 4 seconds, but can result in gaps at points where the signal was obscured. Tagged birds were monitored for movement for a minimum of 11 days and maximum of 15 days post cloacal swab collection. When an owl was within 40 m of its nest, it was considered at the nest, while at any distance >40 m the owl was considered away and either hunting or travelling to and from the nest to hunt. The median distance traveled per day (*median distance*) and *home range area* were calculated in Corl et al. (2020). Briefly, data for median distance was recorded daily for the two-week period and then a mean average was calculated. Home range area was calculated in R (R Core Team 2023) with the adehabitatHR package (Calenge 2006) using the following parameters: ad hoc smoothing method, 2,000 grid size with extent of three, and a 99th percentile level home range.

Microbiome Data Collection

Swabs for these owls were previously extracted in Corl et al. (2020), but samples with low yields were re-extracted and all samples were re-sequenced for this study. Briefly, DNA was extracted from cloacal swab samples using DNeasy PowerLyzer PowerSoil kits (Qiagen Inc., Germantown, MD), with samples arranged and extracted in randomized batches to avoid spatial or temporal batch effects; each batch included a negative control, which was processed identically but without a swab. First, swabs placed in bead tubes were heated at 65°C for 10 minutes prior to bead beating on a PowerLyzer homogenizer (Qiagen Inc., Germantown, MD) for 16 cycles of 30 sec on and 30 sec off at 3,500 rpm. Next, after quantification using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA), samples were concentrated down to 40 µl, half of which was sent to Argonne National Laboratory (Lemont, IL) for sequencing of the

V4 region of the 16S rRNA gene. The primer pair 515F/806R (Caporaso et al. 2012) was used in a reaction mix consisting of 1 µl of DNA, 9.5 µl of MO BIO PCR Water (Certified DNA-Free), 12.5 µl of QuantaBio's AccuStart II PCR ToughMix (QuantaBio, Beverly, MA), and 200 pM of each primer. Thermocycling conditions were as follows: 94°C for 3 min, 35 cycles of 94°C for 45 sec, 50°C for 60 sec, and 72°C for 90 sec, and a final hold at 72°C for 10 min. Reactions were run in triplicate and combined before equimolar amounts of each sample were pooled and sequenced on an Illumina MiSeq to produce 151 bp paired-end sequence reads. We also sequenced nine extraction negative controls (one per batch) and two PCR negative controls, the latter of which were PCR reactions using UltraPure distilled water (Invitrogen, Waltham, MA).

Sequence Quality Control and Filtering

Demultiplexed files were processed through the workflow established by Callahan et al. (2016) using R v4.3.0 (R Core Team 2023). We trimmed the first 10 bases of every read and then used DADA2 (Callahan et al. 2016) to deduce amplicon sequence variants. Forward and reverse reads were merged, and chimeras removed. We classified taxa with the SILVA 138.1 taxonomy database (Quast et al. 2013), aligned sequences with the package DECIPHER (Wright 2015; Wright 2021), and built a maximum likelihood tree with the package phangorn (Schliep 2011). The generated taxonomy table, phylogenetic tree, operational taxonomic unit (OTU) table, and metadata were combined into a single phyloseq object with the package phyloseq (McMurdie and Holmes 2013). We removed 429 sequences identified as contaminants (found within the extraction and PCR negative controls) and removed any sequences that were (1) not classified as bacterial taxa, (2) were unassigned, or (3) identified as mitochondria or chloroplasts. To be conservative, we also removed sequences found only in a single individual, to omit possible sequencing errors or rare contaminants.

After filtering, 9003 OTUs remained for downstream analyses. Samples averaged 62,410 reads with a minimum of 787 and a maximum of 311,453 reads per individual. We removed 8 samples with less than 5,000 reads and then rarefied the data to a sequencing depth of 5,000 reads and a random number seed of 999 to standardize samples (Weiss et al. 2017). Swabs collected from individual recaptured owls were considered duplicates and only the first swab (first capture) was used while data from subsequent swabs were filtered out. We also removed data from initial extractions of re-extracted samples whose first extraction yielded poor sequencing depth and any samples that failed PCR. This left 61 samples, 40 females and 21 males, remaining for downstream statistical analyses.

Haemosporidian Parasite Detection and Quantification

Avian haemosporidian parasite (*Haemoproteus* spp., *Leucocytozoon* spp., and *Plasmodium* spp.) screening was done using quantitative PCR (qPCR) as described in Choi (Chapter 3), which was adapted from Ishtiaq et al. (2017). We used qPCR as it simultaneously detects parasites and measures infection intensity or levels of parasitemia[54,55]. We extracted DNA using DNeasy Blood & Tissue kits (Qiagen, Germantown, MD), following the nucleated blood protocol, from blood collected on Whatman FTA cards and then ran qPCR reactions in duplicate for each sample to test for the presence of avian haemosporidian parasites. The qPCR targeted a 153 bp (base pair) fragment of mitochondrial rRNA using the primer pair 343F/496R (Fallon et al. 2003) that detects all three genera of haemosporidian parasites but does not differentiate between them. For each PCR reaction the following reagents were combined: 2 µl (5-25 ng/µl) of DNA template, 0.2 µM of each primer, 2 µl of nuclease free water, and 5 µl of 2X Luna Universal qPCR Master Mix (New England BioLabs, Ipswich, MA) for a total reaction volume of 10 µl. Thermocycling conditions were as follows: 50°C for 2 min and 95°C for 2 min, followed by 43

cycles of 95°C for 15 sec, 57°C for 45 sec, and 72°C for 30 sec, followed by a melt curve analysis. qPCR reactions were run in 96-well plates that included a negative control (water) and three positive controls representing the three genera of interest; this included a positive *Plasmodium relictum* control from a bird with an active infection and two synthetic positive controls for *Haemoproteus columbae* and *Leucocytozoon majoris* (IDT, Coralville, IA).

Samples were considered positive for haemosporidian parasite infection if both runs yielded a C_T (cycle threshold) of 38 or less (Ishtiaq et al. 2017) and were no more than 2 C_T values apart. Samples with inconsistent results (one negative and one positive or $\Delta C_T > 2$) were repeated and those that remained inconsistent in the second run were removed from downstream analysis to prevent inclusion of false results. We calculated the prevalence (%) of haemosporidians using the number of infected birds divided by the total number of birds and multiplied by 100. For infected birds, the C_T value was used as an index of overall haemosporidian parasite infection intensity as described in Ishtiaq et al. (2017), which demonstrated an inverse relationship between microscopy based-intensity estimates and qPCR C_T values. Thus, lower C_T values are expected to reflect higher levels of infection and vice versa.

Relationships between Haemosporidian Parasite Infection and Potential Indicators of Health

We used R v4.3.0 (R Core Team 2023) for all statistical analyses and the packages phyloseq, ggplot2 (Wickham 2009), and ggpubr (Kassambara 2023) for visualization of data. We first explored the relationship between cloacal microbiome diversity and haemosporidian infection intensity (C_T value) in owls. We measured alpha diversity using the Chao1 estimator, which takes rare and missing species into consideration (Chao 1984; Chao and Shen 2003). We used the Wilcoxon rank sum test (Mann-Whitney U test; Mann and Whitney 1947) to test for differences between infected and uninfected individuals, as our sample size was small and a

Shapiro-Wilk test (Shapiro and Wilk 1965) showed the residuals for our Chao1 data did not follow a normal distribution ($W = 0.90$, $p = 9.12^{-05}$). We then ran a linear model with the package car (Fox and Weisberg 2011) to test whether microbiome Chao1 alpha diversity predicts haemosporidian infection intensity.

We also used phyloseq to generate a principal coordinate analysis (PCoA) to explore beta diversity (i.e., cloacal bacterial community composition) in relation to haemosporidian parasite infection intensity. We used adonis from the vegan package (Oksanen et al. 2020) to calculate permutational multivariate analysis of variance (PERMANOVA) for both the weighted and unweighted UniFrac metrics. UniFrac matches communities using phylogenetic information to estimate distance between samples (similarity matrix) (Lozupone and Knight 2005) with the unweighted UniFrac utilizing only presence/absence data, while the weighted UniFrac integrates relative abundance (Lozupone et al. 2007).

We then examined the relationship between movement and haemosporidian infection intensity (C_T value). We filtered our dataset to include only individuals with movement data, leaving 21 females and 10 males ($n = 31$). We ran a Pearson's correlation matrix to determine whether or not our movement predictor variables (median distance and home range area) were correlated and then a variance inflation factor (VIF) calculation from the package car (Fox and Weisberg 2011). We found median distance and home range area to be correlated ($R^2 = 0.38$, $p = 1.9e^{-4}$) so we only included home range area to align with previous work done in barn owl movement and other health indicators. We then ran a linear model to determine if home range area predicts haemosporidian infection intensity (C_T value).

Relationships between Plumage Energy Costs and Indicators of Health

We used generalized linear models to explore whether barn owl plumage energy costs (melanization extent) relates to indices of owl health (haemosporidian infection intensity, cloacal microbiome bacterial diversity, or movement), while also accounting for sex and mass as predictor variables. We ran three independent model sequences with the following response variables: haemosporidian infection intensity, Chao1 microbiome diversity, and home range area. We included the following variables in each model: melanization extent, sex, and mass. We first ran a Pearson's correlation matrix to identify correlated predictors followed by a variance inflation factor (VIF) calculation from the package car (Fox and Weisberg 2011). Any factors with a VIF greater than 4 were examined to minimize collinearity and considered for removal from the model. We found sex to be highly correlated with mass (Wilcoxon rank sum test, W = 209, $p = 1.22e^{-5}$), thus we removed sex from our models. We then used a backwards model selection algorithm by Akaike information criterion (AIC) (Akaike 1976) with the package AICcmodavg (Mazerolle 2023) to identify the best model. We started with a full model and removed the least significant variable until a significance threshold of $p = 0.05$ or the null model was reached.

Health Indicators as Predictors of Reproductive Success

Lastly, we independently modeled the effects of haemosporidian parasite infection intensity, microbiome Chao1 alpha diversity, or movement as predictors of reproductive success in female barn owls. We used clutch size, fledge success, and laying date as measures of reproductive success (response variables). Given that median distance and home range area are correlated, we only included home range area in our models, as Séchaud et al. (2022) found a significant relationship between home range area and fledge success. We ran 9 separate generalized linear

models for each combination of predictors (infection intensity, Chao1, home range area) and reproductive traits (clutch size, fledge success, laying date) to identify significant (p <0.05) relationships. Sample sizes for the infection intensity and Chao1 models were 40 female owls, while the sample size for home range area was 21 female owls.

Results

Movement but not the Microbiome Predicted Infection Intensity

We found a high prevalence of haemosporidian parasites with 59 of 61 individuals (96.7%) infected and 2 uninfected (one male, one female). The mean infection intensity (C_T value) was 23.6 in this population of barn owls. Given the high prevalence, we used only haemosporidian parasite intensity in subsequent analyses. Microbiome Chao1 alpha diversity did not correlate with haemosporidian infection intensity (C_T value; $R^2 = 0.003$, $p = 0.65$; Supplemental Figure D.1). Similarly, microbiome beta diversity did not differ by haemosporidian parasite infection intensity for either the unweighted UniFrac (C_T value; $F = 0.86$, $p = 0.78$; Supplemental Figure D.2A) or the weighted UniFrac ($F = 1.39$, $p = 0.18$; Supplemental Figure D.2B). As home range area increased, haemosporidian infection intensity decreased ($R^2 = 0.30$, $p = 0.0012$; Figure 4.3).

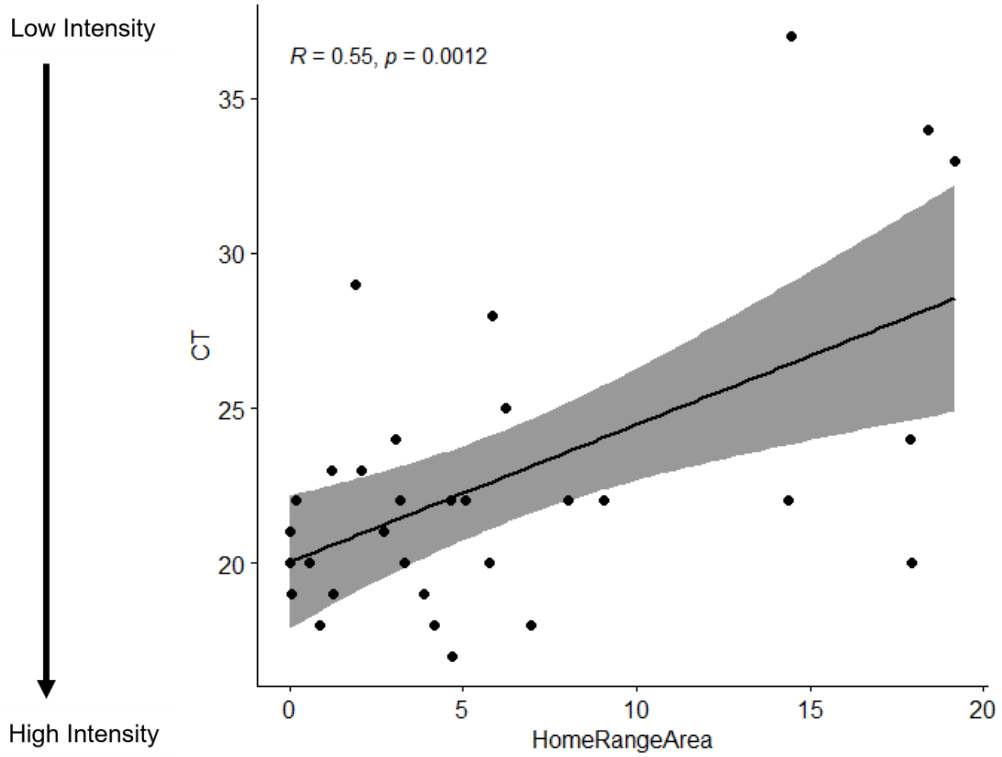


Figure 4.3. Scatter plot of the relationship between home range area and haemosporidian infection intensity (C_T value). Owls with low infection intensity had larger home ranges than owls with high infection intensity ($R^2 = 0.30$, $p = 0.0012$).

Plumage Melanization Extent Predicted Movement

For models assessing the relationships between plumage melanization extent and either haemosporidian parasite infection intensity or Chao1 alpha diversity, the best fit models were the null models (Table D.1). For movement and plumage coloration, the best model included both home range area and mass (Table 4.1). Home range area increased as plumage melanization extent costs and mass increased (Figure 4.4).

Table 4.1. Summary statistics of best model for plumage coloration and movement (home range area) in barn owls.

Predictors	**Beta**	**Std Error**	**Z-value**	***p*-value**
Intercept	19.25	7.60	2.53	0.017
Melanization extent	$4.70e^{-4}$	$1.84e^{-4}$	2.56	0.016
Mass	-0.04	0.02	-2.0	0.050

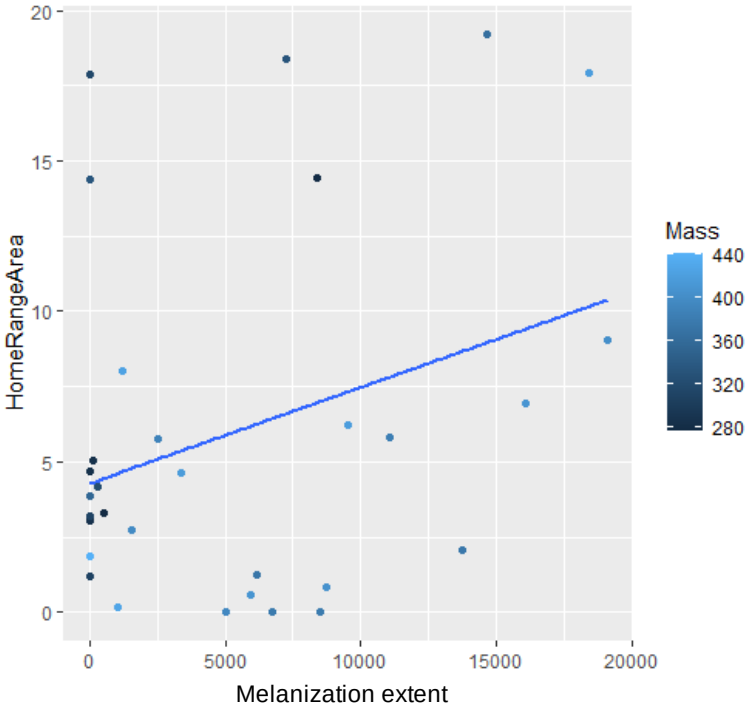


Figure 4.4. Scatter plot of the relationship between plumage melanization extent and home range area in barn owls with dot colors representing mass on a continuous scale from lightest (dark blue) to heaviest (light blue). Home range area increased as plumage melanization extent costs and mass increased.

Movement Predicted Reproductive Success among Barn Owls

There was no relationship between haemosporidian parasite infection intensity and any of the metrics of reproductive success (clutch size, fledge success, or laying date); the null models were the best model for all three (p = 0.92 clutch size; p = 0.64 fledge success; p = 0.73; Table D.2).

We found a significant relationship between microbiome Chao1 alpha diversity and Julian laying date ($p = 8.81e^{-4}$; Table 4.2), such that Chao1 alpha diversity decreased as the egg laying date increased ($R^2 = 0.14$, p = 0.01; Figure 4.5). There was no relationship between clutch size or fledge success and Chao1 alpha diversity (p = 0.62 clutch size; p = 0.33 fledge success; Table D.3).

Table 4.2. Summary statistics of generalized linear model for Chao1 alpha diversity and Julian laying date in barn owls.

	Beta	**Std Error**	**Z-value**	***p*-value**
Intercept	99.56	3.71	26.83	$2.0e^{-16}$
Julian laying date	-0.02	$6.23e^{-3}$	-3.61	$8.81e^{-4}$

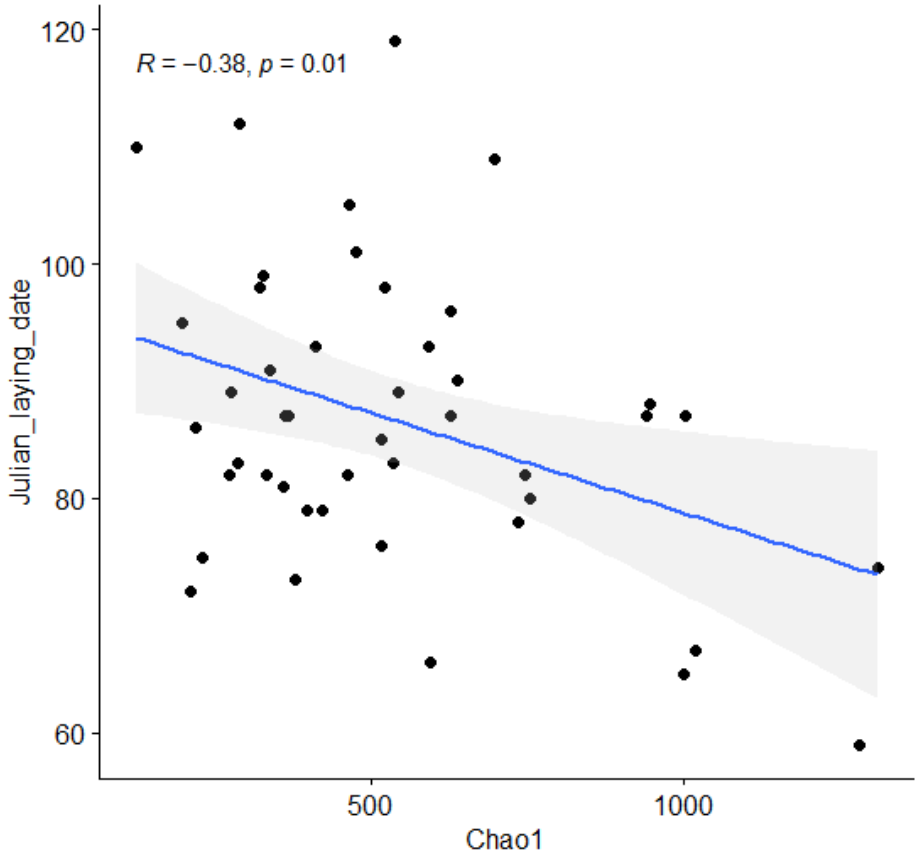


Figure 4.5. Scatter plot of the relationship between Chao1 alpha diversity and Julian laying date in barn owls. Chao1 alpha diversity decreased as laying date increased ($R^2 = 0.14$, $p = 0.01$).

We found a significant relationship between home range area and clutch size ($p = 0.02$; Table 4.3) and a nearly significant relationship between home range area and fledge success ($p = 0.05$; Table 4.4). Clutch size ($R^2 = 0.20$, $p = 0.026$; Figure 4.6) and fledge success ($R^2 = 0.18$, $p = 0.05$; Figure 4.7) both decreased as home range area increased. There was no relationship between laying date and home range area ($p = 0.44$; Table D.4).

Table 4.3. Summary statistics of generalized linear model for home range area and clutch size in barn owls.

	Beta	**Std Error**	**Z-value**	***p*-value**
Intercept	8.25	0.38	21.54	$8.26e^{-15}$
Clutch size	-0.12	0.05	-2.62	0.02

Table 4.4. Summary statistics of generalized linear model for home range area and fledge success in barn owls.

	Beta	**Std Error**	**Z-value**	***p*-value**
Intercept	0.52	0.08	6.15	$6.50e^{-6}$
Fledge success	-0.02	0.01	-2.06	0.05

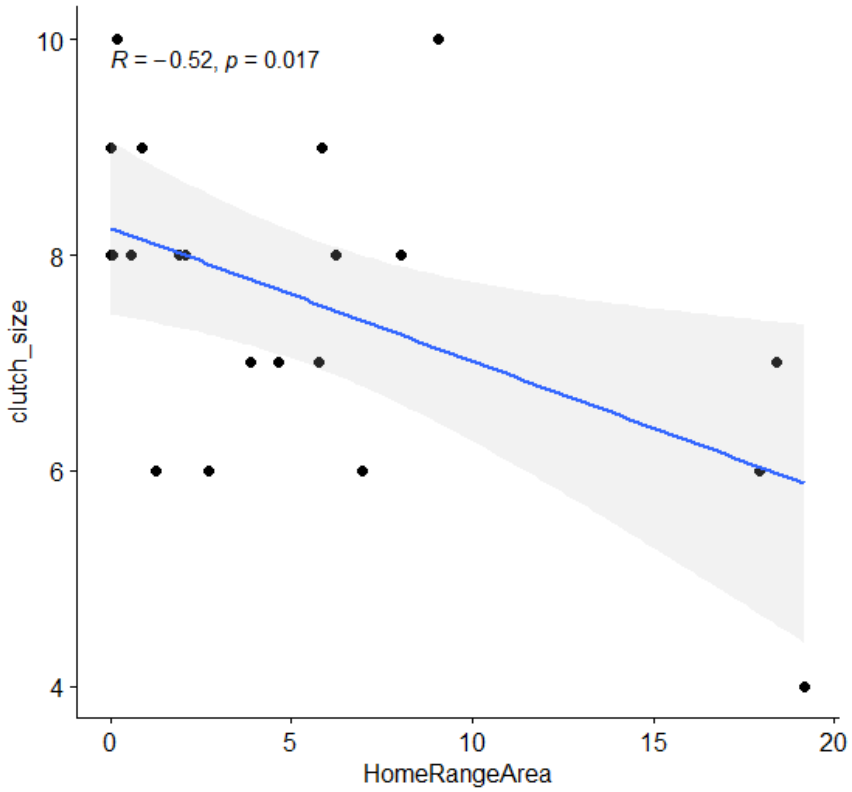


Figure 4.6. Scatter plot of the relationship between home range area and clutch size in barn owls. Home range area increased as clutch size decreased ($R^2 = 0.27$, $p = 0.017$).

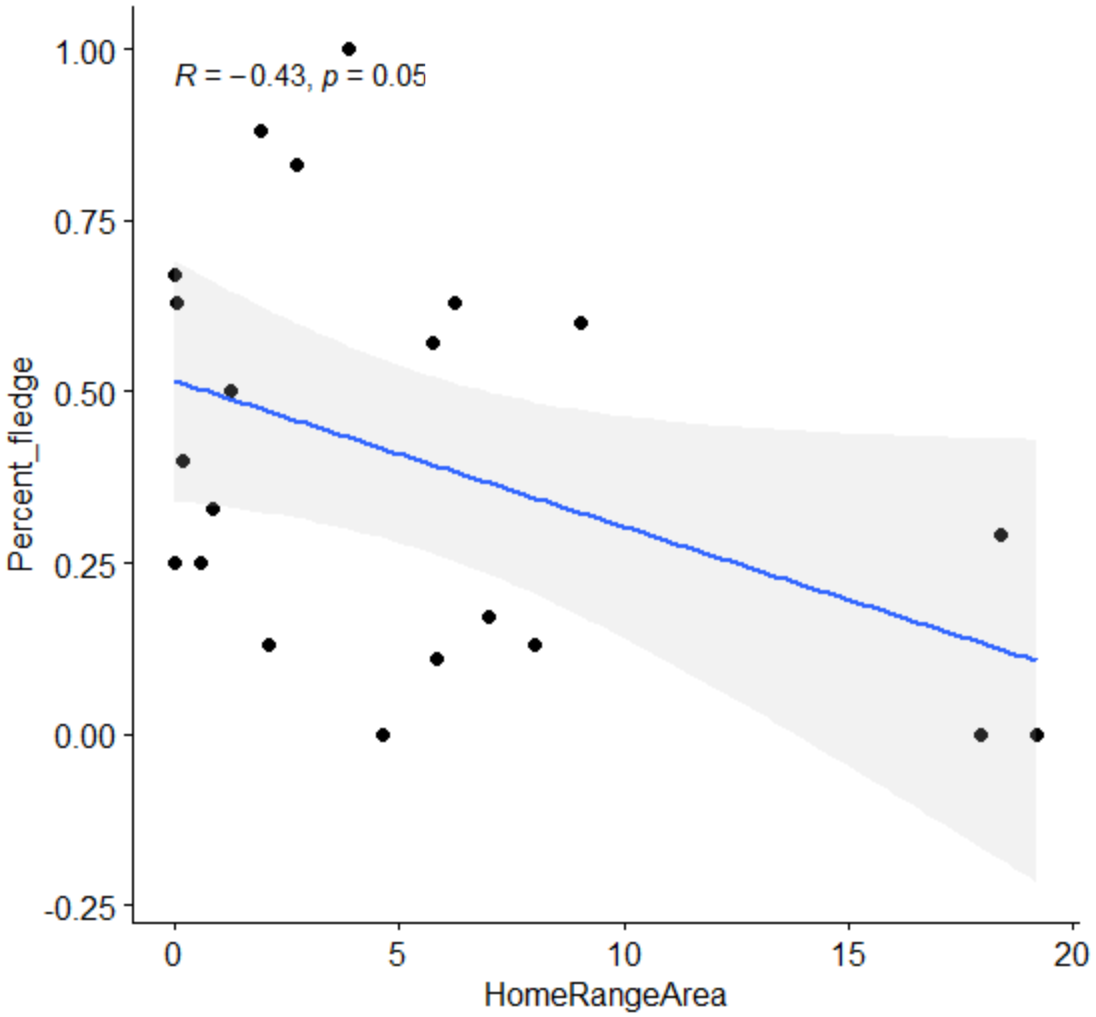


Figure 4.7. Scatter plot of the relationship between home range area and fledge success in barn owls. Home range area increased as fledge success decreased ($R^2 = 0.18$, $p = 0.05$).

Discussion

In this study, we evaluated the Hamilton and Zuk hypothesis by assessing relationships between plumage energy costs and health indicators in barn owls, and then evaluated the impact of those health indicators on fitness, as reflected by reproductive success. The microbiome had no relationship with haemosporidian infection intensity. However, movement may be a potential indicator of individual vigor as haemosporidian infection intensity decreased with home range area. Further, we found that plumage coloration and body mass

predict movement, such that individuals with more plumage coloration, had higher body weights and larger home ranges suggesting a possible indirect relationship between parasite infection and plumage coloration. However, this did not result in enhanced reproductive success, as increased movement was correlated with a reduction in clutch size and fledge success. We also found both microbiome alpha diversity to be a potential indicator of reproductive success; increased microbiome diversity was associated with earlier laying dates.

A possible explanation for the lack of relationship between plumage ornamentation and parasite resistance could be the choice of parasite evaluated in this study. A meta-analysis found that immune function had a stronger effect than parasite load on male ornamentation, which suggests that previous studies finding no relationship between plumage phenotypes and parasite resistance could have focused on parasites generating little to no immune response (Møller et al. 1999). Another possible explanation for a lack of relationship could be due to the timing and duration of sampling in birds; for example, a study on grassquits (Aguilar et al. 2008) found that plumage coloration could be a stronger signal of previous parasite intensities and condition of birds than their current status at time of sampling. Given that we sampled our birds at a single time point we could not factor in previous parasite load and individual condition with plumage ornamentation and thus could be missing long-term effects of infection. Lastly, our results do not align with previous work in barn owls that found female barn owl ornamentation to correlate with immune response (Roulin et al. 2000) and parasite resistance with parasitic flies (Roulin et al. 2001). It is difficult to determine with this study if the lack of a correlation was due to a weaker immune response from haemosporidian infection or due to the timing of our sampling.

The chronic nature of avian haemosporidians (Sato 2021; Thurber et al. 2014) can potentially explain the high prevalence of infection, 96.7%, and lack of relationship between infection and plumage melanization extent cost or reproductive fitness in the Hula Valley population of barn owls (la Puente et al. 2010; Knowles et al. 2010). Previous studies in barn owls have shown little to zero prevalence of avian haemosporidian parasites, for example in Austria and Lithuania (prevalence = 0%; Ilgūnas et al. 2022) and in Thailand (23.8 - 34.8%, Salakij et al. 2018; Pornpanom et al. 2019). In contrast, the study in Austria and Lithuania also found high prevalence in tawny owls (87.2%) and Ural owls (82.8%). Together, these previous works suggest our results may be an anomaly among barn owl populations. This observation of high prevalence in Hula Valley barn owls may be explained by the chronic nature of these parasite infections; although individuals could be easily infected, they may be able to tolerate infection and therefore individuals could have adapted to live with infection while suffering minimal consequences. Thus, if parasites are not applying strong selective pressure on this population, we may not expect to see a relationship between plumage characteristics and haemosporidian parasite resistance or reproductive success.

In our study, we did not differentiate between the three genera of haemosporidian parasites which can subsequently affect our prevalence estimates. The two studies of barn owls in Thailand measured prevalence by genus and found lower prevalence (23.8 - 34.8%, Salakij et al. 2018; Pornpanom et al. 2019); thus our grouping of all 3 genera of haemosporidian parasites together could influence our results by inflating the parasite infection intensity, which may have obscured detection of species-specific effects of infection on reproductive fitness as well as plumage ornamentation in owls. In addition, infection with one parasite can sometimes promote or compete with infection by another parasite (Garcia-

Longoria et al. 2022), and such interactions between haemosporidian parasite species should be accounted for. For reproductive fitness, previous work focused on single genera (Knowles et al. 2010) or found that the effects of haemosporidian parasite infection can vary by parasite species (Korpimaki et al. 1993). Thus, we acknowledge the complex nature of host-parasite interactions and recognize that focusing on different genera or species could alter our results.

Although we found no support for Hamilton and Zuk in terms of parasite resistance, our models found plumage melanization extent costs to increase as home range area increased and that mass decreased as home range area increased. Higher plumage melanization extent costs indicate larger and more numerous spots in our owls suggesting that owls with brighter plumage (more spots) were able to travel further versus owls with lighter plumage. However, this finding does not translate to greater reproductive success, as clutch size, and fledge success decreased with increasing home range size. These contradictory findings here and in other studies (Balenger and Zuk 2014; Brennan 2010), along with differences in methodologies between studies and difficulties in distinguishing between parasite resistance versus lack of exposure (Balenger and Zuk 2014) have led to criticisms of the Hamilton and Zuk hypothesis. These criticisms highlight the complexities of the underlying factors shaping host-parasite evolution.

We found an inverse relationship between movement, as measured by home range area, and parasite infection intensity. This could be because individuals with heavy parasite loads are in poorer condition and thus travel shorter distances. A study in migratory great reed warblers (*Acrocephalus arundinaceus*) found that migration distances decreased with parasite intensity (Emmenegger et al. 2020), indicating that individuals with heavy parasite loads are unable to travel longer distances compared to healthier members of the population. Although

barn owls are not migratory, they could be under similar constraints, such that higher parasite loads could reduce an owl's ability to cover larger distances while hunting for prey. A comparison of the number of hunts completed between low and high intensity owls would shed light on this hypothesis.

We found no relationship between microbiome alpha diversity and plumage melanization extent costs or movement. Previous work in the same system and individuals found a significant relationship between microbiome alpha diversity and movement metrics (Corl et al. 2020), however, our samples were rerun at a higher sequencing depth and rarefied to a higher level (1176 vs 5000 reads) and thus could explain why we found no relationship. The increase in reads could have decreased the number and abundance of rare taxa and thus made the distribution of alpha diversity more even across movement distances. A possible explanation for the lack of relationship between Chao1 alpha diversity and plumage melanization extent costs could be due to the anatomical location of the microbiome characterized. Previous work has found that more iridescent colors of male white-shouldered fairywrens (*Malurus alboscapulatus*) is associated with increased feather microbial diversity than in black matte females and brown fairywrens (Javůrková et al. 2019). Thus, characterizing the feather microbiome of these owls might be a more appropriate target for examining relationships between the microbiome and plumage coloration.

Our results revealing that owls with increased microbiome diversity had earlier laying dates suggest that these owls may be in better condition and thus are able to breed earlier. Previous work in this system found egg laying date to negatively correlate with fledge success (Corl et al. 2020) suggesting that Hula Valley barn owls that breed earlier have a greater probability of chick survival. However, another study in male barn owls found no relationship

between laying date and fledge success (Séchaud et al. 2022) suggesting that this relationship may be population specific.

We found evidence to suggest a negative correlation between movement (as measured by home range area) with metrics of reproductive success, including clutch size and fledge success. Our results do not align with previous work in male barn owls that linked home range area to laying date (Séchaud et al. 2022), but did support a relationship between home range area and fledge success from that same study. However, Séchaud et al. 2022 looked only at male owls while our analysis of reproductive success focused solely on females. At the same time, the owls in our study could be inhabiting a broad range of habitats with variation in food availability, which then impacts reproductive success, movement, and home range. Female owls in our study could be moving further and more often, expending more energy, and leading to less energy to devote to reproduction and an increased amount of time spent away from the nest. Further work examining the different prey captured and the number of hunts per night in relation to reproductive traits and movement would shed light on the mechanism driving the relationship we observed between home range area and reproductive success in barn owls.

Conclusions and Future Directions

While we did not find evidence for a relationship between parasite resistance and plumage coloration, our study supports the Hamilton and Zuk hypothesis when considering movement as an indicator of health. Our results do not align with previous studies done in barn owls which have shown increased ornamentation in females without ectoparasite infection (Roulin et al. 2001) suggesting that studies testing Hamilton and Zuk are parasite-host system specific. Further, considering parasite genus and/or species should be a part of future work in this system

to possibly strengthen existing support for the Hamilton and Zuk hypothesis. Lastly, long-term monitoring and data collection at multiple time points throughout the life of owls could reveal patterns missed when measuring a single time point in the life history of owls. The results of this study provide another layer to the work already in existence regarding barn owl ecology and host-pathogen interactions. Further research may be able to explore whether plumage patterns reflect a variety of individual health traits or a multidimensional metric of health combining multiple indicators that affect reproductive success, and also take into account multiple co-infecting pathogens (O'Brien and Dawson 2011). Our results highlight the importance of movement ecology in shaping host-parasite dynamics and fitness outcomes in non-migratory birds.

REFERENCES

Aguilar, Thais M., Rafael Maia, Eduardo S.A. Santos, and Regina H. Macedo. 2008. "Parasite Levels in Blue-Black Grassquits Correlate with Male Displays but Not Female Mate Preference." *Behavioral Ecology* 19 (2): 292–301.

Akaike, Hirotugu. 1976. "Canonical Correlation Analysis of Time Series and the Use of an Information Criterion." In *Mathematics in Science and Engineering*, edited by Raman K. Mehra and Dimitri G. Lainiotis, 126:27–96. System Identification Advances and Case Studies. Elsevier.

Altizer, Sonia, Rebecca Bartel, and Barbara A. Han. 2011. "Animal Migration and Infectious Disease Risk." *Science* 331 (6015): 296–302.

Ambrosini, Roberto, Margherita Corti, Andrea Franzetti, Manuela Caprioli, et al. 2019. "Cloacal Microbiomes and Ecology of Individual Barn Swallows." *FEMS Microbiology Ecology* 95 (6).

Angeli Dutra, Daniela de, Alan Fecchio, Érika Martins Braga, and Robert Poulin. 2021. "Migratory Birds Have Higher Prevalence and Richness of Avian Haemosporidian Parasites than Residents." *International Journal for Parasitology* 51 (10): 877–82.

Annetti, Kendall, Nelda Rivera, John Andrews, and Nohra Mateus-Pinilla. 2017. "Survey of Haemosporidian Parasites in Resident and Migrant Game Birds of Illinois." *Journal of Fish and Wildlife Management* 8.

Atkinson, Carter T., and Dennis A. LaPointe. 2009. "Introduced Avian Diseases, Climate Change, and the Future of Hawaiian Honeycreepers." *Journal of Avian Medicine and Surgery* 23 (1): 53–64.

Aželytė, Justė, Alejandra Wu-Chuang, Apolline Maitre, Rita Žiegytė, Lourdes Mateos-Hernández, Dasiel Obregón, Vaidas Palinauskas, and Alejandro Cabezas-Cruz. 2023. "Avian Malaria Parasites Modulate Gut Microbiome Assembly in Canaries." *Microorganisms* 11 (3): 563.

Balenger, Susan L., and Marlene Zuk. 2014. "Testing the Hamilton–Zuk Hypothesis: Past, Present, and Future." *Integrative and Comparative Biology* 54 (4): 601–13.

Ayumi Matsuyama-Kato, Khaled Taha-Abdelaziz, Bahram Shojadoost, and Shayan Sharif. 2021. "Gut Microbiota Is Associated with Protection against Marek's Disease Virus Infection in Chickens." *Virology* 553 (January): 122–30.

"BBS - USGS Patuxent Wildlife Research Center." n.d. Accessed May 29, 2023. https://www.pwrc.usgs.gov/bbs/.

Bell, Jeffrey A., Jason D. Weckstein, Alan Fecchio, and Vasyl V. Tkach. 2015. "A New Real-Time PCR Protocol for Detection of Avian Haemosporidians." *Parasites & Vectors* 8 (1): 383.

Biddle, Amy, Lucy Stewart, Jeffrey Blanchard, and Susan Leschine. 2013. "Untangling the Genetic Basis of Fibrolytic Specialization by Lachnospiraceae and Ruminococcaceae in Diverse Gut Communities." *Diversity* 5 (3): 627–40.

Borgia, Gerald, and Ken Collis. 1989. "Female Choice for Parasite-Free Male Satin Bowerbirds and the Evolution of Bright Male Plumage." *Behavioral Ecology and Sociobiology* 25 (6): 445–53.

———. 1990. "Parasites and Bright Male Plumage in the Satin Bowerbird (Ptilonorhynchus Violaceus)1." *American Zoologist* 30 (2): 279–86.

Brennan, Patricia. 2010. “Sexual Selection.” *Nature Education Knowledge* 3 (10): 79.

Brooks, Andrew W., Kevin D. Kohl, Robert M. Brucker, Edward J. van Opstal, and Seth R. Bordenstein. 2016. “Phylosymbiosis: Relationships and Functional Effects of Microbial Communities across Host Evolutionary History.” *PLOS Biology* 14 (11): e2000225.

Calenge, Clément. 2006. “The Package ‘Adehabitat’ for the R Software: A Tool for the Analysis of Space and Habitat Use by Animals.” *Ecological Modelling* 197 (3): 516–19.

Callahan, Ben J., Kris Sankaran, Julia A. Fukuyama, Paul J. McMurdie, and Susan P. Holmes. 2016. “Bioconductor Workflow for Microbiome Data Analysis: From Raw Reads to Community Analyses.” *F1000Research* 5: 1492.

Callahan, Benjamin J., Paul J. McMurdie, Michael J. Rosen, Andrew W. Han, Amy Jo A. Johnson, and Susan P. Holmes. 2016. “DADA2: High-Resolution Sample Inference from Illumina Amplicon Data.” *Nature Methods* 13 (7): 581–83.

Caporaso, J. Gregory, Christian L. Lauber, William A. Walters, Donna Berg-Lyons, James Huntley, Noah Fierer, Sarah M. Owens, et al. 2012. “Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms.” *The ISME Journal* 6 (8): 1621–24.

Carney, Samuel M. 1992. *Species, Age and Sex Identification of Ducks Using Wing Plumage*. Washington, D.C.: US Fish & Wildlife Service.

“CDFW News | Breeding Ducks Increase by 30 Percent in Annual CDFW Waterfowl Breeding Population Survey.” n.d. Accessed September 7, 2023. https://wildlife.ca.gov/News/Archive/2023-waterfowl-breeding-population-survey.

Chaisi, Mamohale E., Samuel T. Osinubi, Desire L. Dalton, and Essa Suleman. 2019. “Occurrence and Diversity of Avian Haemosporidia in Afrotropical Landbirds.” *International Journal for Parasitology: Parasites and Wildlife* 8 (April): 36–44.

Chao, Anne. 1984. “Nonparametric Estimation of the Number of Classes in a Population.” *Scandinavian Journal of Statistics* 11 (4): 265–70.

Chao, Anne, and Tsung-jen Shen. 2003. “Nonparametric Estimation of Shannon’s Index of Diversity When There Are Unseen Species in Sample.” *Environmental and Ecological Statistics* 10 (4): 429–43.

Cho, Hyunjun, and Won Young Lee. 2020. “Interspecific Comparison of the Fecal Microbiota Structure in Three Arctic Migratory Bird Species.” *Ecology and Evolution* 10 (12): 5582–94.

Choi, Olivia N., Ammon Corl, Andrew Wolfenden, Avishai Lublin, Suzanne L. Ishaq, Sondra Turjeman, Wayne M. Getz, Ran Nathan, Rauri C. K. Bowie, and Pauline L. Kamath. 2021. “High-Throughput Sequencing for Examining Salmonella Prevalence and Pathogen—Microbiota Relationships in Barn Swallows.” *Frontiers in Ecology and Evolution* 9 (October): 683183.

Colston, Timothy J., and Colin R. Jackson. 2016. “Microbiome Evolution along Divergent Branches of the Vertebrate Tree of Life: What Is Known and Unknown.” *Molecular Ecology* 25 (16): 3776–3800.

Corl, Ammon, Motti Charter, Gabe Rozman, Sivan Toledo, Sondra Turjeman, Pauline L. Kamath, Wayne M. Getz, Ran Nathan, and Rauri C. K. Bowie. 2020. “Movement Ecology and Sex Are Linked to Barn Owl Microbial Community Composition.” *Molecular Ecology* 29 (7): 1358–71.

Custer, Gordon F., Maya Gans, Linda T. A. van Diepen, Francisco Dini-Andreote, and C. Alex Buerkle. 2023. "Comparative Analysis of Core Microbiome Assignments: Implications for Ecological Synthesis." *MSystems* 8 (1): e01066-22.

Davis, Nicole M., Diana M. Proctor, Susan P. Holmes, David A. Relman, and Benjamin J. Callahan. 2018. "Simple Statistical Identification and Removal of Contaminant Sequences in Marker-Gene and Metagenomics Data." *Microbiome* 6 (1): 226.

Dewar, Meagan L., John P. Y. Arnould, Theo R. Allnutt, Tamsyn Crowley, Lutz Krause, John Reynolds, Peter Dann, and Stuart C. Smith. 2017. "Microbiota of Little Penguins and Short-Tailed Shearwaters during Development." *PloS One* 12 (8): e0183117.

Dietz, Maurine W., Joana F. Salles, Bin-Yan Hsu, Cor Dijkstra, Ton G. G. Groothuis, Marco van der Velde, Yvonne I. Verkuil, and B. Irene Tieleman. 2019. "Prenatal Transfer of Gut Bacteria in Rock Pigeon." *Microorganisms* 8 (1): 61.

Dmytryk, Rebecca. 2012. "Capture, Handling, and Confinement of Wild Birds." In *Wildlife Search and Rescue: A Guide for First Responders*, 71–129. West Sussex, United Kingdom: John Wiley & Sons, Ltd.

Dyrcz, Andrzej, Michael Wink, Andrzej Kruszewicz, and Bernd Leisler. 2005. "Male Reproductive Success Is Correlated with Blood Parasite Levels and Body Condition in the Promiscuous Aquatic Warbler (*Acrocephalus Paludicola*) (El Éxito Reproductivo de Los Machos Está Correlacionado Con Los Niveles de Parásitos En La Sangre y La Condición Corporal En La Especie Promiscua *Acrocephalus Paludicota*)." *The Auk* 122 (2): 558–65.

Eikenaar, Cas, Sven Hessler, and Arne Hegemann. 2020. "Migrating Birds Rapidly Increase Constitutive Immune Function during Stopover." *Royal Society Open Science* 7 (2): 192031.

Emmenegger, Tamara, Staffan Bensch, Steffen Hahn, Dmitry Kishkinev, Petr Procházka, Pavel Zehtindjiev, and Silke Bauer. 2020. "Effects of Blood Parasite Infections on Spatiotemporal Migration Patterns and Activity Budgets in a Long-distance Migratory Passerine." *Ecology and Evolution* 11 (2): 753–62.

Fagerland, Morten W., Stian Lydersen, and Petter Laake. 2013. "The McNemar Test for Binary Matched-Pairs Data: Mid-p and Asymptotic Are Better than Exact Conditional." *BMC Medical Research Methodology* 13 (1): 91.

Fallon, S. M., R. E. Ricklefs, B. L. Swanson, and E. Bermingham. 2003. "Detecting Avian Malaria: An Improved Polymerase Chain Reaction Diagnostic." *The Journal of Parasitology* 89 (5): 1044–47.

Fecchio, Alan, Nicholas J. Clark, Jeffrey A. Bell, Heather R. Skeen, Holly L. Lutz, Gabriel M. De La Torre, Jefferson A. Vaughan, et al. 2021. "Global Drivers of Avian Haemosporidian Infections Vary across Zoogeographical Regions." *Global Ecology and Biogeography* 30 (12): 2393–2406.

Fleskes, Joseph, Andrew Ramey, Andrew Reeves, and Julie Yee. 2017. "Body Mass, Wing Length, and Condition of Wintering Ducks Relative to Hematozoa Infection." *Journal of Fish and Wildlife Management* 8 (February).

Fox, James G., Nancy S. Taylor, Shelly Howe, Michele Tidd, Shilu Xu, Bruce J. Paster, and Floyd E. Dewhirst. 2006. "Helicobacter Anseris Sp. Nov. and Helicobacter Brantae Sp. Nov., Isolated from Feces of Resident Canada Geese in the Greater Boston Area." *Applied and Environmental Microbiology* 72 (7): 4633–37.

Fox, John, and Sanford Weisberg. 2011. *An R Companion to Applied Regression*. SAGE Publications.

Fritzsche McKay, Alexa, and Bethany J. Hoye. 2016. “Are Migratory Animals Superspreaders of Infection?” *Integrative and Comparative Biology* 56 (2): 260–67.

Gaidet, Nicolas, Julien Cappelle, John Y. Takekawa, Diann J. Prosser, Samuel A. Iverson, David C. Douglas, William M. Perry, Taej Mundkur, and Scott H. Newman. 2010. “Potential Spread of Highly Pathogenic Avian Influenza H5N1 by Wildfowl: Dispersal Ranges and Rates Determined from Large-Scale Satellite Telemetry.” *Journal of Applied Ecology* 47 (5): 1147–57.

Gallardo, Pablo, Mariana Izquierdo, Roberto M. Vidal, Nayaret Chamorro-Veloso, Ramon Rosselló-Móra, Miguel O’Ryan, and Mauricio J. Farfán. 2017. “Distinctive Gut Microbiota Is Associated with Diarrheagenic Escherichia Coli Infections in Chilean Children.” *Frontiers in Cellular and Infection Microbiology* 7 (October): 424.

Ganz, Holly H., Ladan Doroud, Alana J. Firl, Sarah M. Hird, Jonathan A. Eisen, and Walter M. Boyce. 2017. “Community-Level Differences in the Microbiome of Healthy Wild Mallards and Those Infected by Influenza A Viruses.” *MSystems* 2 (1).

Garamszegi, László Zsolt, and Anders Pape Møller. 2012. “The Interspecific Relationship between Prevalence of Blood Parasites and Sexual Traits in Birds When Considering Recent Methodological Advancements.” *Behavioral Ecology and Sociobiology* 66 (1): 107–19.

Garant, Dany, Audrey Bourret, Clarence Schmitt, Audrey Turcotte, Fanie Pelletier, and Marc Bélisle. 2018. “Effects of Blood Parasite Infection and Innate Immune Genetic Diversity on Mating Patterns in a Passerine Bird Breeding in Contrasted Habitats.” *PeerJ* 6 (November): e6004.

García-Amado, María Alexandra, Virginia Sanz, Leoncia Margarita Martinez, Mónica Contreras, Miguel Lentino, and Fabian Michelangeli. 2013. “Low Occurrence of Helicobacter DNA in Tropical Wild Birds, Venezuela.” *Journal of Wildlife Diseases* 49 (4): 991–95. https://doi.org/10.7589/2012-09-222.

Garcia-Longoria, Luz, Sergio Magallanes, Xi Huang, Anna Drews, Lars Råberg, Alfonso Marzal, Staffan Bensch, and Helena Westerdahl. 2022a. “Reciprocal Positive Effects on Parasitemia between Coinfecting Haemosporidian Parasites in House Sparrows.” *BMC Ecology and Evolution* 22 (1): 1–11.

Garnham, P. C. C. 1966. *Malaria Parasites and Other Haemosporidia*. Oxford: Blackwell.

“GitHub - FrederickHuangLin/ANCOM-BC2-Code-Archive: Archive: Data, Scripts, and Outputs for the Paper ‘Multi-Group Analysis of Compositions of Microbiomes with Covariate Adjustments and Repeated Measures’. Please Check Our ANCOMBC R Package for the Most up-to-Date ANCOM-BC Function.” n.d. Accessed September 16, 2023. https://github.com/FrederickHuangLin/ANCOM-BC2-Code-Archive/tree/main.

Godfrey, Ralph D., Alan M. Fedynich, and Danny B. Pence. 1987. “Quantification of Hematozoa in Blood Smears.” *Journal of Wildlife Diseases* 23 (4): 558–65.

Godoy-Vitorino, Filipa, Katherine C. Goldfarb, Ulas Karaoz, Sara Leal, Maria A. Garcia-Amado, Philip Hugenholtz, Susannah G. Tringe, Eoin L. Brodie, and Maria Gloria Dominguez-Bello. 2012. “Comparative Analyses of Foregut and Hindgut Bacterial Communities in Hoatzins and Cows.” *The ISME Journal* 6 (3): 531–41.

Góngora, Esteban, Kyle H. Elliott, and Lyle Whyte. 2021. “Gut Microbiome Is Affected by Inter-Sexual and Inter-Seasonal Variation in Diet for Thick-Billed Murres (Uria Lomvia).” *Scientific Reports* 11 (1): 1200.

Greene, Lydia K., Jonathan B. Clayton, Ryan S. Rothman, Brandon P. Semel, Meredith A. Semel, Thomas R. Gillespie, Patricia C. Wright, and Christine M. Drea. 2019. “Local Habitat, Not Phylogenetic Relatedness,

Predicts Gut Microbiota Better within Folivorous than Frugivorous Lemur Lineages." *Biology Letters* 15 (6): 20190028.

Greiner, Ellis C., Gordon F. Bennett, Ellen M. White, and Richard F. Coombs. 1975. "Distribution of the Avian Hematozoa of North America." *Canadian Journal of Zoology* 53 (12): 1762–87.

Grilo, M. L., R. E. T. Vanstreels, R. Wallace, D. García-Párraga, É. M. Braga, J. Chitty, J. L. Catão-Dias, and L. M. Madeira de Carvalho. 2016. "Malaria in Penguins – Current Perceptions." *Avian Pathology* 45 (4): 393–407.

Grond, Kirsten, Brett K. Sandercock, Ari Jumpponen, and Lydia H. Zeglin. 2018. "The Avian Gut Microbiota: Community, Physiology and Function in Wild Birds." *Journal of Avian Biology* 49 (11): e01788.

Hall, Richard J., Sonia Altizer, and Rebecca A. Bartel. 2014. "Greater Migratory Propensity in Hosts Lowers Pathogen Transmission and Impacts." *The Journal of Animal Ecology* 83 (5): 1068–77.

Hamady, Micah, and Rob Knight. 2009. "Microbial Community Profiling for Human Microbiome Projects: Tools, Techniques, and Challenges." *Genome Research* 19 (7): 1141.

Hamilton, William D., and Marlene Zuk. 1982. "Heritable True Fitness and Bright Birds: A Role for Parasites?" *Science* 218 (4570): 384–87.

Hellgren, O., M. J. Wood, J. Waldenström, D. Hasselquist, U. Ottosson, M. Stervander, and S. Bensch. 2013. "Circannual Variation in Blood Parasitism in a Sub-Saharan Migrant Passerine Bird, the Garden Warbler." *Journal of Evolutionary Biology* 26 (5): 1047–59.

Hernández, América, Margarita Martínez-Gómez, René Beamonte-Barrientos, and Bibiana Montoya. 2021. "Colourful Traits in Female Birds Relate to Individual Condition, Reproductive Performance and Male-Mate Preferences: A Meta-Analytic Approach." *Biology Letters* 17 (9): 20210283.

Heusmann, H W. 2017. "Why Are Numbers of Mallards (Anas Platyrhynchos) Declining in the Northeastern United States? The Possible Role of Reduction in Winter Feeding Sites." *The Wilson Journal of Ornithology* 129 (3): 528–34.

Hill, Geoffrey E., and Kristy L. Farmer. 2005. "Carotenoid-Based Plumage Coloration Predicts Resistance to a Novel Parasite in the House Finch." *Naturwissenschaften* 92 (1): 30–34.

Hird, Sarah M., Bryan C. Carstens, Steven W. Cardiff, Donna L. Dittmann, and Robb T. Brumfield. 2014. "Sampling Locality Is More Detectable than Taxonomy or Ecology in the Gut Microbiota of the Brood-Parasitic Brown-Headed Cowbird (Molothrus Ater)." *PeerJ* 2 (March): e321.

Hird, Sarah M., Holly Ganz, Jonathan A. Eisen, and Walter M. Boyce. 2018. "The Cloacal Microbiome of Five Wild Duck Species Varies by Species and Influenza A Virus Infection Status." *MSphere* 3 (5): e00382-18.

Hird, Sarah M., César Sánchez, Bryan C. Carstens, and Robb T. Brumfield. 2015. "Comparative Gut Microbiota of 59 Neotropical Bird Species." *Frontiers in Microbiology* 6 (December).

Holm, Sture. 1979. "A Simple Sequentially Rejective Multiple Test Procedure." *Scandinavian Journal of Statistics* 6 (2): 65–70.

Hubálek, Zdenek. 2004. "An Annotated Checklist of Pathogenic Microorganisms Associated with Migratory Birds." *Journal of Wildlife Diseases* 40 (4): 639–59.

Ilgūnas, Mikas, Tanja Himmel, Josef Harl, Mindaugas Dagys, Gediminas Valkiūnas, and Herbert Weissenböck. 2022. "Exo-Erythrocytic Development of Avian Haemosporidian Parasites in European Owls." *Animals: An Open Access Journal from MDPI* 12 (17): 2212.

Ishtiaq, Farah, Christopher G. R. Bowden, and Yadvendradev V. Jhala. 2017. "Seasonal Dynamics in Mosquito Abundance and Temperature Do Not Influence Avian Malaria Prevalence in the Himalayan Foothills." *Ecology and Evolution* 7 (19): 8040–57.

Ishtiaq, Farah, Megha Rao, Xi Huang, and Staffan Bensch. 2017. "Estimating Prevalence of Avian Haemosporidians in Natural Populations: A Comparative Study on Screening Protocols." *Parasites & Vectors* 10 (1): 127.

Javůrková, Veronika Gvoždíková, Erik D. Enbody, Jakub Kreisinger, Kryštof Chmel, Jakub Mrázek, and Jordan Karubian. 2019. "Plumage Iridescence Is Associated with Distinct Feather Microbiota in a Tropical Passerine." *Scientific Reports* 9 (1): 12921.

Kassambara, Alboukadel. 2023. "_ggpubr: 'ggplot2' Based Publication Ready Plots_." R package version 0.6.0. https://CRAN.R-project.org/package=ggpubr.

Knowles, S. C. L., V. Palinauskas, and B. C. Sheldon. 2010. "Chronic Malaria Infections Increase Family Inequalities and Reduce Parental Fitness: Experimental Evidence from a Wild Bird Population." *Journal of Evolutionary Biology* 23 (3): 557–69.

Kohl, Kevin D. 2012. "Diversity and Function of the Avian Gut Microbiota." *Journal of Comparative Physiology B* 182 (5): 591–602.

Korpimaki, E., H. Hakkarainen, and G. F. Bennett. 1993. "Blood Parasites and Reproductive Success of Tengmalm's Owls: Detrimental Effects on Females but Not on Males?" *Functional Ecology* 7 (4): 420–26.

Koskella, Britt, Lindsay J. Hall, and C. Jessica E. Metcalf. 2017. "The Microbiome beyond the Horizon of Ecological and Evolutionary Theory." *Nature Ecology & Evolution* 1 (11): 1606–15.

Kreisinger, Jakub, Lucie Kropáčková, Adéla Petrželková, Marie Adámková, Oldřich Tomášek, Jean-François Martin, Romana Michálková, and Tomáš Albrecht. 2017. "Temporal Stability and the Effect of Transgenerational Transfer on Fecal Microbiota Structure in a Long Distance Migratory Bird." *Frontiers in Microbiology* 8: 50.

Kruskal, William H., and W. Allen Wallis. 1952. "Use of Ranks in One-Criterion Variance Analysis." *Journal of the American Statistical Association* 47 (260): 583–621.

Lahti, L., and Shetty Sudarshan. 2017. *Tools for Microbiome Analysis in R* (version 1.22.0). R. https://microbiome.github.io/tutorials/.

Larsson, J. 2022. *Eulerr: Area-Proportional Euler and Venn Diagrams with Ellipses* (version 7.0.0). R. https://cran.r-project.org/package=eulerr.

Lin, Huang, Merete Eggesbø, and Shyamal Das Peddada. 2022. "Linear and Nonlinear Correlation Estimators Unveil Undescribed Taxa Interactions in Microbiome Data." *Nature Communications* 13 (August): 4946.

Lin, Huang, and Shyamal Das Peddada. 2020. "Analysis of Compositions of Microbiomes with Bias Correction." *Nature Communications* 11 (1): 3514.

———. 2023. "Multi-Group Analysis of Compositions of Microbiomes with Covariate Adjustments and Repeated Measures." *Research Square*, May, rs.3.rs-2778207.

Liu, Gang, Derong Meng, Minghao Gong, Huixin Li, Wanyu Wen, Yuhang Wang, and Jingying Zhou. 2020. "Effects of Sex and Diet on Gut Microbiota of Farmland-Dependent Wintering Birds." *Frontiers in Microbiology* 11.

Logan, Julie, and Edwards Kirstin. 2009. "Real-Time PCR: Current Technology and Applications." In *Real-Time PCR: Current Technology and Applications*, vii, 284–vii, 284.

Lozupone, Catherine A., Micah Hamady, Scott T. Kelley, and Rob Knight. 2007. "Quantitative and Qualitative Beta Diversity Measures Lead to Different Insights into Factors That Structure Microbial Communities." *Applied and Environmental Microbiology* 73 (5): 1576–85.

Lozupone, Catherine, and Rob Knight. 2005. "UniFrac: A New Phylogenetic Method for Comparing Microbial Communities." *Applied and Environmental Microbiology* 71 (12): 8228–35.

Lu, Zhiyuan, Sisi Li, Min Wang, Can Wang, Derong Meng, and Jingze Liu. 2022. "Comparative Analysis of the Gut Microbiota of Three Sympatric Terrestrial Wild Bird Species Overwintering in Farmland Habitats." *Frontiers in Microbiology* 13.

Lucas, Françoise S., and Philipp Heeb. 2005. "Environmental Factors Shape Cloacal Bacterial Assemblages in Great Tit Parus Major and Blue Tit P. Caeruleus Nestlings." *Journal of Avian Biology* 36 (6): 510–16.

Lumpkin, David C., Troy G. Murphy, and Keith A. Tarvin. 2014. "Blood Parasite Infection Differentially Relates to Carotenoid-Based Plumage and Bill Color in the American Goldfinch." *Ecology and Evolution* 4 (16): 3210–17.

Lundberg, Randi, Christian Scharch, and Dorthe Sandvang. 2021. "The Link between Broiler Flock Heterogeneity and Cecal Microbiome Composition." *Animal Microbiome* 3 (July): 54.

Ma, Xinxin, Qi Wang, Hongmei Li, Chuantian Xu, Ning Cui, and Xiaomin Zhao. 2017. "16S RRNA Genes Illumina Sequencing Revealed Differential Cecal Microbiome in Specific Pathogen Free Chickens Infected with Different Subgroup of Avian Leukosis Viruses." *Veterinary Microbiology* 207 (August): 195–204.

Mann, H. B., and D. R. Whitney. 1947. "On a Test of Whether One of Two Random Variables Is Stochastically Larger than the Other." *The Annals of Mathematical Statistics* 18 (1): 50–60.

Maraci, Öncü, Anna Antonatou-Papaioannou, Sebastian Jünemann, Kathrin Engel, Omar Castillo-Gutiérrez, Tobias Busche, Jörn Kalinowski, and Barbara A. Caspers. 2022. "Timing Matters: Age-Dependent Impacts of the Social Environment and Host Selection on the Avian Gut Microbiota." *Microbiome* 10 (1): 202.

Márcia Barbosa, A., Raimundo Real, A.-Román Muñoz, and Jennifer A. Brown. 2013. "New Measures for Assessing Model Equilibrium and Prediction Mismatch in Species Distribution Models." *Diversity and Distributions* 19 (10): 1333–38.

Mazerolle, Marc J. 2023. *AICcmodavg: Model Selection and Multimodel Inference Based on (Q) AIC(c)* (version 2.3.2). R. https://cran.r-project.org/package=AICcmodavg.

McMurdie, Paul J., and Susan Holmes. 2013. "Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data." *PLoS ONE* 8 (4): e61217.

Meixell, Brandt W., Todd W. Arnold, Mark S. Lindberg, Matthew M. Smith, Jonathan A. Runstadler, and Andrew M. Ramey. 2016. "Detection, Prevalence, and Transmission of Avian Hematozoa in Waterfowl at the Arctic/Sub-Arctic Interface: Co-Infections, Viral Interactions, and Sources of Variation." *Parasites & Vectors* 9 (1): 390.

MerilÄ, Juha, Ben C. Sheldon, and Karin LindstrÖm. 1999. "Plumage Brightness in Relation to Haematozoan Infections in the Greenfinch Carduelis Chloris: Bright Males Are a Good Bet." *Écoscience* 6 (1): 12–18.

Møller, Anders P., P. Christe, and E. Lux. 1999. "Parasitism, Host Immune Function, and Sexual Selection." *The Quarterly Review of Biology* 74 (1): 3–20.

Møller, Anders P. 1990. "Parasites and Sexual Selection: Current Status of the Hamilton and Zuk Hypothesis." *Journal of Evolutionary Biology* 3 (5–6): 319–28.

Morffy Smith, Catherine D., Minghao Gong, Alicer K. Andrew, Brittany N. Russ, Yong Ge, Mojgan Zadeh, Caitlin A. Cooper, Mansour Mohamadzadeh, and Julie M. Moore. 2019. "Composition of the Gut Microbiota Transcends Genetic Determinants of Malaria Infection Severity and Influences Pregnancy Outcome." *EBioMedicine* 44 (June): 639–55.

Muletz Wolz, Carly R., Stephanie A. Yarwood, Evan H. Campbell Grant, Robert C. Fleischer, and Karen R. Lips. 2018. "Effects of Host Species and Environment on the Skin Microbiome of Plethodontid Salamanders." *Journal of Animal Ecology* 87 (2): 341–53.

Murray, Maureen H., Emily W. Lankau, Anjelika D. Kidd, Catharine N. Welch, Taylor Ellison, Henry C. Adams, Erin K. Lipp, and Sonia M. Hernandez. 2020. "Gut Microbiome Shifts with Urbanization and Potentially Facilitates a Zoonotic Pathogen in a Wading Bird." *PLoS ONE* 15 (3): e0220926.

Nearing, Jacob T., André M. Comeau, and Morgan G. I. Langille. 2021. "Identifying Biases and Their Potential Solutions in Human Microbiome Studies." *Microbiome* 9 (1): 113.

Neu, Alexander T., Eric E. Allen, and Kaustuv Roy. 2021. "Defining and Quantifying the Core Microbiome: Challenges and Prospects." *Proceedings of the National Academy of Sciences* 118 (51): e2104429118.

North, Norman R., and Randy J. Hicks. 2017. "The North American Banders' Manual for Waterfowl (Family - Anatidae)." North American Banding Council. http://www.nabanding.net/wp-content/uploads/2012/04/North-American-Banders-Manual-for-Waterfowl.pdf.

O'Brien, Erin L., and Russell D. Dawson. 2011. "Plumage Color and Food Availability Affect Male Reproductive Success in a Socially Monogamous Bird." *Behavioral Ecology* 22 (1): 66–72.

Obrochta, Sean, Maria Luisa Savo Sardaro, Katherine R. Amato, and Maureen H. Murray. 2022. "Relationships Between Migration and Microbiome Composition and Diversity in Urban Canada Geese." *Frontiers in Ecology and Evolution* 10. https://www.frontiersin.org/articles/10.3389/fevo.2022.742369.

Oksanen, Jari, F. Guillaume Blanchet, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R. Minchin, et al. 2020. *Vegan: Community Ecology Package* (version 2.5-7). https://CRAN.R-project.org/package=vegan.

Owen, Jennifer C., and Frank R. Moore. 2008. "Swainson's Thrushes in Migratory Disposition Exhibit Reduced Immune Function." *Journal of Ethology* 26 (3): 383–88. https://doi.org/10.1007/s10164-008-0092-1.

Palinauskas, Vaidas, Lourdes Mateos-Hernandez, Alejandra Wu-Chuang, José de la Fuente, Justė Aželytė, Dasiel Obregon, and Alejandro Cabezas-Cruz. 2022. "Exploring the Ecological Implications of Microbiota Diversity in Birds: Natural Barriers Against Avian Malaria." *Frontiers in Immunology* 13 (February): 807682.

Pearse, Aaron T., Richard M. Kaminski, Kenneth J. Reinecke, and Stephen J. Dinsmore. 2012. "Local and Landscape Associations Between Wintering Dabbling Ducks and Wetland Complexes in Mississippi." *Wetlands* 32 (5): 859–69.

Pekarsky, Sasha, Ammon Corl, Sondra Turjeman, Pauline L. Kamath, Wayne M. Getz, Rauri C. K. Bowie, Yuri Markin, and Ran Nathan. 2021. "Drivers of Change and Stability in the Gut Microbiota of an Omnivorous Avian Migrant Exposed to Artificial Food Supplementation." *Molecular Ecology* 30 (19): 4723–39.

Pornpanom, Pornchai, Carolina Chagas, Preeda Lertwatcharasarakul, Chaiyan Kasorndorkbua, Gediminas Valkiunas, and Chaleow Salakij. 2019. "Molecular Prevalence and Phylogenetic Relationship of Haemoproteus and Plasmodium Parasites of Owls in Thailand: Data from a Rehabilitation Centre." *International Journal for Parasitology: Parasites and Wildlife* 9.

Puente, Josué Martínez-de la, Santiago Merino, Gustavo Tomás, Juan Moreno, Judith Morales, Elisa Lobato, Sonia García-Fraile, and Eduardo Jorge Belda. 2010. "The Blood Parasite Haemoproteus Reduces Survival in a Wild Bird: A Medication Experiment." *Biology Letters* 6 (5): 663–65.

Pulgarín-R, Paulo C., Camila Gómez, Nicholas J. Bayly, Staffan Bensch, Alyssa M. FitzGerald, Naima Starkloff, Jeremy J. Kirchman, et al. 2019. "Migratory Birds as Vehicles for Parasite Dispersal? Infection by Avian Haemosporidians over the Year and throughout the Range of a Long-Distance Migrant." *Journal of Biogeography* 46 (1): 83–96.

Quast, Christian, Elmar Pruesse, Pelin Yilmaz, Jan Gerken, Timmy Schweer, Pablo Yarza, Jörg Peplies, and Frank Oliver Glöckner. 2013. "The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools." *Nucleic Acids Research* 41 (D1): D590–96.

R Core Team. 2023. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org.

Reeves, Andrew B., Mathew M. Smith, Brandt W. Meixell, Joseph P. Fleskes, and Andrew M. Ramey. 2015. "Genetic Diversity and Host Specificity Varies across Three Genera of Blood Parasites in Ducks of the Pacific Americas Flyway." *PLoS ONE* 10 (2): e0116661.

Reinoso-Pérez, María Teresa, Julio César Canales-Delgadillo, Leonardo Chapa-Vargas, and Lina Riego-Ruiz. 2016. "Haemosporidian Parasite Prevalence, Parasitemia, and Diversity in Three Resident Bird Species at a Shrubland Dominated Landscape of the Mexican Highland Plateau." *Parasites & Vectors* 9 (1): 1–12.

Ringelman, Kevin M., Christopher K. Williams, Patrick K. Devers, John M. Coluccy, Paul M. Castelli, Kurt A. Anderson, Jacob L. Bowman, et al. 2015. "A Meta-Analysis of American Black Duck Winter Habitat Use along the Atlantic Coast." *The Journal of Wildlife Management* 79 (8): 1298–1307.

Risely, Alice. 2020. "Applying the Core Microbiome to Understand Host–Microbe Systems." *Journal of Animal Ecology* 89 (7): 1549–58.

Risely, Alice, Mark A. F. Gillingham, Arnaud Béchet, Stefan Brändel, Alexander C. Heni, Marco Heurich, Sebastian Menke, et al. 2021. "Phylogeny- and Abundance-Based Metrics Allow for the Consistent Comparison of Core Gut Microbiome Diversity Indices Across Host Species." *Frontiers in Microbiology* 12.

Roggenbuck, Michael, Ida Bærholm Schnell, Nikolaj Blom, Jacob Bælum, Mads Frost Bertelsen, Thomas Sicheritz-Pontén, Søren Johannes Sørensen, M. Thomas P. Gilbert, Gary R. Graves, and Lars H. Hansen. 2014. "The Microbiome of New World Vultures." *Nature Communications* 5 (1): 5498.

Rohrer, Sage D., Briana Q. Robertson, Lon M. Chubiz, and Patricia G. Parker. 2023. "Gut Microbiome Composition Associated with Plasmodium Infection in the Eurasian Tree Sparrow." *Journal of Avian Biology* 2023 (1–2): e03027.

Roth, Christoph, Tanja Sims, Markus Rodehutscord, Jana Seifert, and Amélia Camarinha-Silva. 2022. "The Active Core Microbiota of Two High-Yielding Laying Hen Breeds Fed with Different Levels of Calcium and Phosphorus." *Frontiers in Physiology* 13 (September): 951350.

Roulin, Alexandre., Cor Dijkstra, C. Riols, and A.-L. Ducrest. 2001. "Female- and Male-Specific Signals of Quality in the Barn Owl." *Journal of Evolutionary Biology* 14 (2): 255–66.

Roulin, Alexandre. 1999. "Nonrandom Pairing by Male Barn Owls (Tyto Alba) with Respect to a Female Plumage Trait." *Behavioral Ecology* 10 (6): 688–95. https://doi.org/10.1093/beheco/10.6.688.

Roulin, Alexandre, Philippe Christe, Cor Dijkstra, Anne-Lyse Ducrest, and Thomas W. Jungi. 2007. "Origin-Related, Environmental, Sex, and Age Determinants of Immunocompetence, Susceptibility to Ectoparasites, and Disease Symptoms in the Barn Owl." *Biological Journal of the Linnean Society* 90 (4): 703–18.

Roulin, Alexandre, Thomas W. Jungi, Hedi Pfister, and Cor Dijkstra. 2000. "Female Barn Owls (Tyto Alba) Advertise Good Genes." *Proceedings of the Royal Society of London. Series B: Biological Sciences* 267 (1446): 937–41.

Roulin, Alexandre, Christian Riols, Cor Dijkstra, and Anne-Lyse Ducrest. 2001. "Female Plumage Spottiness Signals Parasite Resistance in the Barn Owl (Tyto Alba)." *Behavioral Ecology* 12 (1): 103–10.

Saino, Nicola, Michele Incagli, Roberta Martinelli, and Anders Pape Møller. 2002. "Immune Response of Male Barn Swallows in Relation to Parental Effort, Corticosterone Plasma Levels, and Sexual Ornamentation." *Behavioral Ecology* 13 (2): 169–74.

Salakij, Chaleow, Pornchai Pornpanom, Preeda Lertwatcharasarakul, Chaiyan Kasorndorkbua, and Jarernsak Salakij. 2018. "Haemoproteus in Barn and Collared Scops Owls from Thailand." *Journal of Veterinary Science* 19 (2): 280–89.

Sato, Shigeharu. 2021. "Plasmodium—a Brief Introduction to the Parasites Causing Human Malaria and Their Basic Biology." *Journal of Physiological Anthropology* 40 (January): 1.

Schliep, Klaus Peter. 2011. "Phangorn: Phylogenetic Analysis in R." *Bioinformatics* 27 (4): 592–93.

Schumm, Yvonne R., Dimitris Bakaloudis, Christos Barboutis, Jacopo G. Cecere, Cyril Eraud, Dominik Fischer, Jens Hering, et al. 2021. "Prevalence and Genetic Diversity of Avian Haemosporidian Parasites in Wild Bird Species of the Order Columbiformes." *Parasitology Research* 120 (4): 1405–20.

Sebastian, Chris. 2023. "Understanding Waterfowl: Bringing Back the Black Duck | Ducks Unlimited." 2023. https://www.ducks.org/conservation/waterfowl-research-science/understanding-waterfowl-bringing-back-the-black-duck.

Séchaud, Robin, Kim Schalcher, Bettina Almasi, Roman Bühler, Kamran Safi, Andrea Romano, and Alexandre Roulin. 2022. "Home Range Size and Habitat Quality Affect Breeding Success but Not Parental Investment in Barn Owl Males." *Scientific Reports* 12 (April): 6516.

Shade, Ashley, and Jo Handelsman. 2012. "Beyond the Venn Diagram: The Hunt for a Core Microbiome." *Environmental Microbiology* 14 (1): 4–12.

Shapiro, S. S., and M. B. Wilk. 1965. “An Analysis of Variance Test for Normality (Complete Samples)†.” *Biometrika* 52 (3–4): 591–611.

Sharpton, Thomas J. 2018. “Role of the Gut Microbiome in Vertebrate Evolution.” *MSystems* 3 (2): 10.1128/msystems.00174-17.

Shihirai, Hadoram, Ehud Davrat, and David A. Christie. 1996. *The Birds of Israel*. London: Academic Press.

Skeen, Heather R., Nathan W. Cooper, Shannon J. Hackett, John M. Bates, and Peter P. Marra. 2021. “Repeated Sampling of Individuals Reveals Impact of Tropical and Temperate Habitats on Microbiota of a Migratory Bird.” *Molecular Ecology* 30 (22): 5900–5916.

Song, Se Jin, Jon G. Sanders, Frédéric Delsuc, Jessica Metcalf, Katherine Amato, Michael W. Taylor, Florent Mazel, et al. 2020. “Comparative Analyses of Vertebrate Gut Microbiomes Reveal Convergence between Birds and Bats.” *MBio* 11 (1): 10.1128/mbio.02901-19.

Stough, Joshua M. A., Stephen P. Dearth, Joshua E. Denny, Gary R. LeCleir, Nathan W. Schmidt, Shawn R. Campagna, and Steven W. Wilhelm. 2016. “Functional Characteristics of the Gut Microbiome in C57BL/6 Mice Differentially Susceptible to Plasmodium Yoelii.” *Frontiers in Microbiology* 7.

Sun, Fengfei, Junfeng Chen, Kai Liu, Meizhen Tang, and Yuewei Yang. 2022. “The Avian Gut Microbiota: Diversity, Influencing Factors, and Future Directions.” *Frontiers in Microbiology* 13 (August): 934272.

SUNY College of Environmental Science and Forestry. n.d. “U.S. Population of Eastern Mallards Has Dropped by 50 Percent.” Accessed May 29, 2023. https://www.newswise.com/articles/u-s-population-of-eastern-mallards-has-dropped-by-50-percent.

Teitelbaum, Claire S., Shan Huang, Richard J. Hall, and Sonia Altizer. 2018. “Migratory Behaviour Predicts Greater Parasite Diversity in Ungulates.” *Proceedings of the Royal Society B: Biological Sciences* 285 (1875): 20180089.

The Global Consortium for H5N8 and Related Influenza Viruses. 2016. “Role for Migratory Wild Birds in the Global Spread of Avian Influenza H5N8.” *Science* 354 (6309): 213–17.

Thie, Nikki, Ammon Corl, Sondra Turjeman, Ron Efrat, Pauline L. Kamath, Wayne M. Getz, Rauri C. K. Bowie, and Ran Nathan. 2022. “Linking Migration and Microbiota at a Major Stopover Site in a Long-Distance Avian Migrant.” *Movement Ecology* 10 (1): 46.

Thurber, Mary Irene, Kathryn C. Gamble, Bethany Krebs, and Tony L. Goldberg. 2014. “Molecular Detection of *Plasmodium* in Free-Ranging Birds and Captive Flamingos (*Phoenicopterus chilensis*) in Chicago.” *Journal of Zoo and Wildlife Medicine* 45 (4): 749–54.

Tian, Jiashen, Jing Du, Shengjiu Zhang, Yanqiu Li, Xianggang Gao, Jiabo Han, and Zhichuang Lu. 2021. “Age-associated Variation in the Gut Microbiota of Chinstrap Penguins (Pygoscelis Antarctica) Reveals Differences in Food Metabolism.” *Microbiology Open* 10 (2): e1190.

Turjeman, Sondra, Ammon Corl, Andrew Wolfenden, Miriam Tsalyuk, Avishai Lublin, Olivia Choi, Pauline L. Kamath, Wayne M. Getz, Rauri C. K. Bowie, and Ran Nathan. 2020. “Migration, Pathogens and the Avian Microbiome: A Comparative Study in Sympatric Migrants and Residents.” *Molecular Ecology* 29 (23): 4706–20.

Turnbaugh, Peter J., Ruth E. Ley, Micah Hamady, Claire M. Fraser-Liggett, Rob Knight, and Jeffrey I. Gordon. 2007. "The Human Microbiome Project." *Nature* 449 (7164): 804–10.

U.S. Fish and Wildlife Service. 2022. "Waterfowl Population Status, 2022." *U.S. Department of the Interior, Washington, D.C. USA*.

Valkiūnas, G. 2005. "*Avian Malaria Parasites and Other Haemosporidia*." CRC Press. Boca Raton, FL.

Valkiunas, Gediminas, Tatjana Iezhova, Asta Križanauskienė, Vaidas Palinauskas, Ravinder Sehgal, and Staffan Bensch. 2008. "A Comparative Analysis of Microscopy and PCR-Based Detection Methods for Blood Parasites." *The Journal of Parasitology* 94 (April): 1395–1401.

Videvall, Elin, Maria Strandh, Anel Engelbrecht, Schalk Cloete, and Charlie K. Cornwallis. 2018. "Measuring the Gut Microbiome in Birds: Comparison of Faecal and Cloacal Sampling." *Molecular Ecology Resources* 18 (3): 424–34.

Villarino, Nicolas F., Gary R. LeCleir, Joshua E. Denny, Stephen P. Dearth, Christopher L. Harding, Sarah S. Sloan, Jennifer L. Gribble, Shawn R. Campagna, Steven W. Wilhelm, and Nathan W. Schmidt. 2016. "Composition of the Gut Microbiota Modulates the Severity of Malaria." *Proceedings of the National Academy of Sciences* 113 (8): 2235–40.

Waite, David, and Mike Taylor. 2015. "Exploring the Avian Gut Microbiota: Current Trends and Future Directions." *Frontiers in Microbiology* 6.

Waite, David W., and Michael W. Taylor. 2014. "Characterizing the Avian Gut Microbiota: Membership, Driving Influences, and Potential Function." *Frontiers in Microbiology* 5.

"Waterfowl: Birds: Species Information: Wildlife: Fish & Wildlife: Maine Dept of Inland Fisheries and Wildlife." n.d. Accessed September 14, 2023. https://www.maine.gov/ifw/fish-wildlife/wildlife/species-information/birds/waterfowl.html.

Weber, Thomas P., and Nikolaos I. Stilianakis. 2007. "Ecologic Immunology of Avian Influenza (H5N1) in Migratory Birds" 13 (8): 1139–43.

Weiser, Adi Weller, Yotam Orchan, Ran Nathan, Motti Charter, Anthony J. Weiss, and Sivan Toledo. 2016. "Characterizing the Accuracy of a Self-Synchronized Reverse-GPS Wildlife Localization System." In *2016 15th ACM/IEEE International Conference on Information Processing in Sensor Networks (IPSN)*, 1–12.

Weiss, Sophie, Zhenjiang Xu, Shyamal Peddada, Amnon Amir, Kyle Bittinger, Antonio González, Catherine Lozupone, et al. 2017. "Normalization and Microbial Differential Abundance Strategies Depend upon Data Characteristics." *Microbiome* 5.

Whary, Mark T., and James G. Fox. 2004. "Natural and Experimental Helicobacter Infections." *Comparative Medicine* 54 (2): 128–58.

Whitworth, D, SH Newman, T Mundkur, and P Harris. 2007. "Wild Bird Capture Techniques." In *Wild Birds and Avian Influenza: An Introduction to Applied Field Research and Disease Sampling Techniques*, 33–72. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO).

Wickham, Hadley. 2009. *Ggplot2: Elegant Graphics for Data Analysis*. Use R! New York: Springer-Verlag.

Wright, Erik. 2021. *DECIPHER: Tools for Curating, Analyzing, and Manipulating Biological Sequences* (version 2.22.0). Bioconductor version: Release (3.14).

Wright, Erik S. 2015. “DECIPHER: Harnessing Local Sequence Context to Improve Protein Multiple Sequence Alignment.” *BMC Bioinformatics* 16 (1): 322.

Zhang, Zheng, Zhisong Yang, and Lifeng Zhu. 2021. “Gut Microbiome of Migratory Shorebirds: Current Status and Future Perspectives.” *Ecology and Evolution* 11 (9): 3737–45.

Zhou, Lei, Xiaona Huo, Boyu Liu, Hui Wu, and Jiang Feng. 2020. “Comparative Analysis of the Gut Microbial Communities of the Eurasian Kestrel (Falco Tinnunculus) at Different Developmental Stages.” *Frontiers in Microbiology* 11.

APPENDICES

APPENDIX A: CHAPTER SUPPLEMENTAL MATERIAL

Figure A.1. Phylogeny of core taxa for California mallards. California mallards had ten core taxa in their cloacal microbiome.

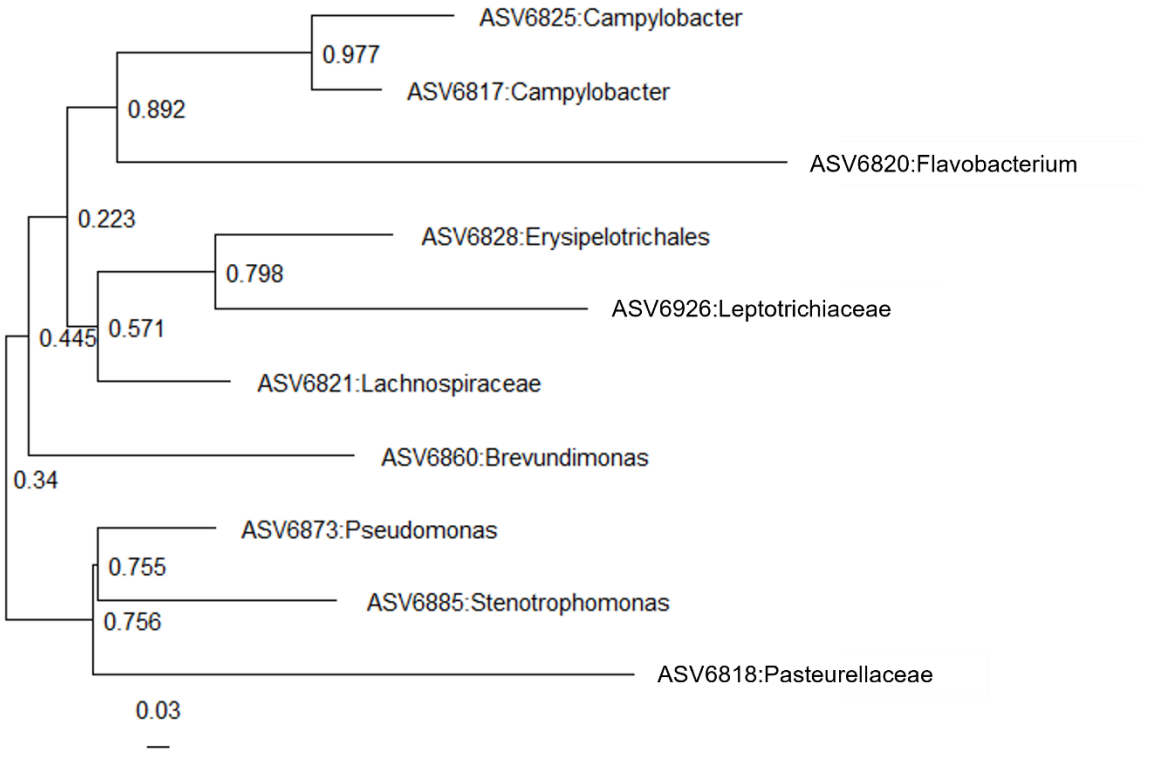
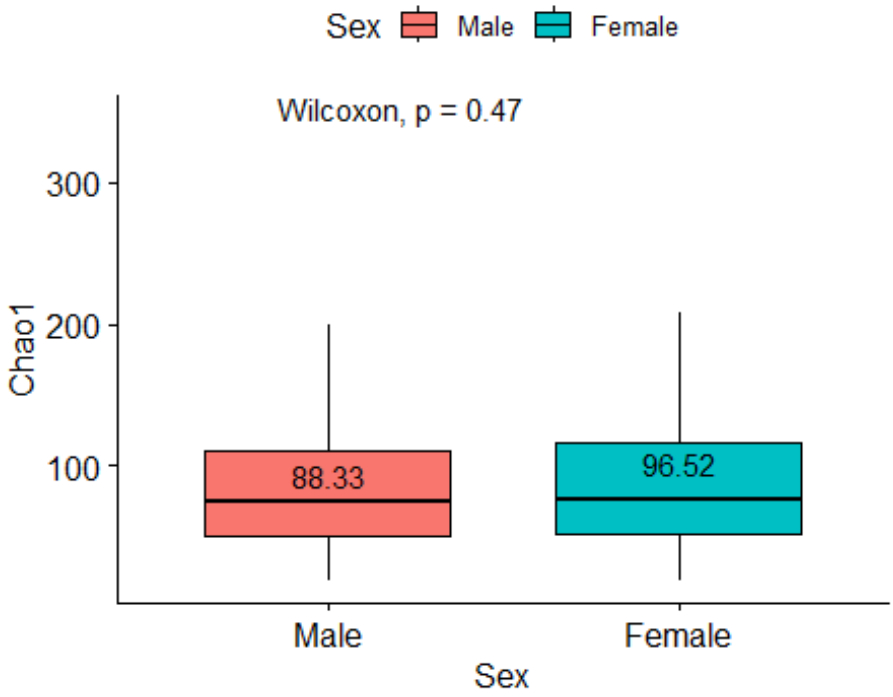


Figure A.2. Boxplot comparison of Chao1 alpha diversity in male (red) and female (blue) mallard ducks. Chao1 alpha diversity did not differ between male and female mallard ducks (W = 17,895, p = 0.47). Males had a mean Chao1 of 88.33 while females had a mean Chao1 of 96.52.



APPENDIX B: CHAPTER 2 SUPPLEMENTAL MATERIAL

Table B.1. Locations, coordinates (Lon/Lat), population names, sample sizes (*N*), number of samples with *Salmonella* present in the microbiome (Pos), proportion positive, and *Salmonella* read statistics (mean, standard deviation, maximum and minimum reads) for all barn swallows (*Hirundo rustica*, $n = 108$) sampled in Israel. These data were used in the analyses and to generate the map (Fig. 1). Coordinates are reported for the first location listed in each population.

Location	**Lon (°E)**	**Lat (°N)**	**Population**	***N***	**Pos**	**Proportion Positive**	**Mean *Sal* Reads**	**Standard Deviation**	**Max *Sal* Reads**	**Min *Sal* Reads**
Beit_ha_shita, Beit_She'an_mall, Tel-Saharon_Alfalfa_Field	35.438	32.551	Beit_shean	38	7	0.18	53.3	68.4	199	2
Levahot_habashan_fish_ponds, Agamon_ringing_station, Rosh_Pina_mall	35.642	33.138	Hula	34	10	0.29	4.7	2.4	8	3
Shafyaim_parking_lot	34.828	32.222	Shefayim	15	8	0.53	1.9	1.5	6	1
Zichron_mall, Ma'agan_Michael_fish_ponds	34.932988	32.569222	Hof_hacarmel	21	3	0.14	3.3	2.3	7	1

Figure B.1. Rarefaction curve showing mean Chao1 estimates of bacterial alpha diversity by 16S rRNA gene sequencing depth in barn swallow (*Hirundo rustica*) fecal samples collected in Israel. The error bars represent standard deviation.

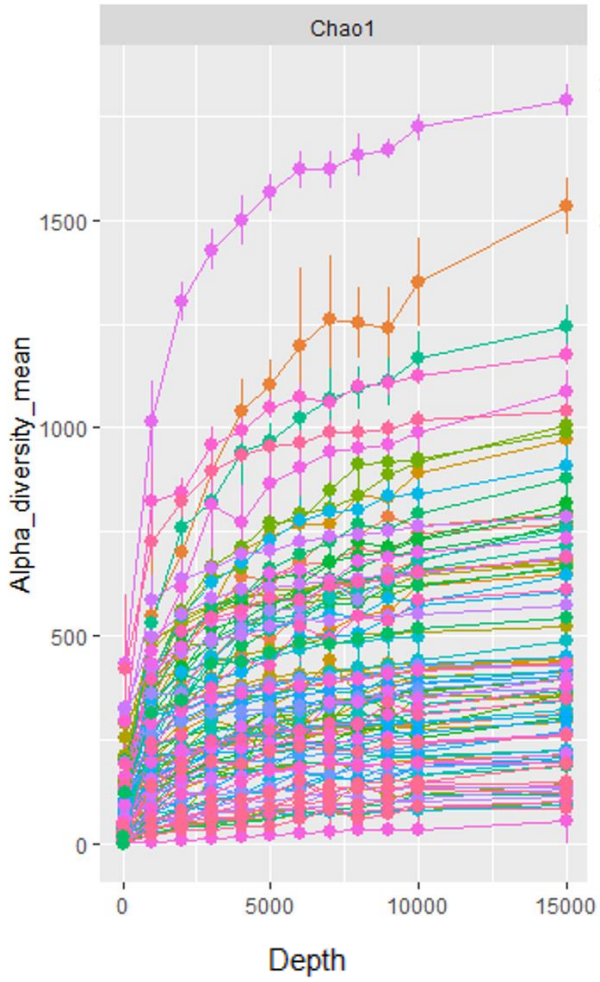


Figure B.2. Total number of reads in samples absent (black) and present (red) for the presence of *Salmonella* from barn swallow (*Hirundo rustica*) fecal samples collected in Israel. *Salmonella*-present samples had significantly higher numbers of 16S rRNA gene sequence reads than negative samples ($p = 0.002$).

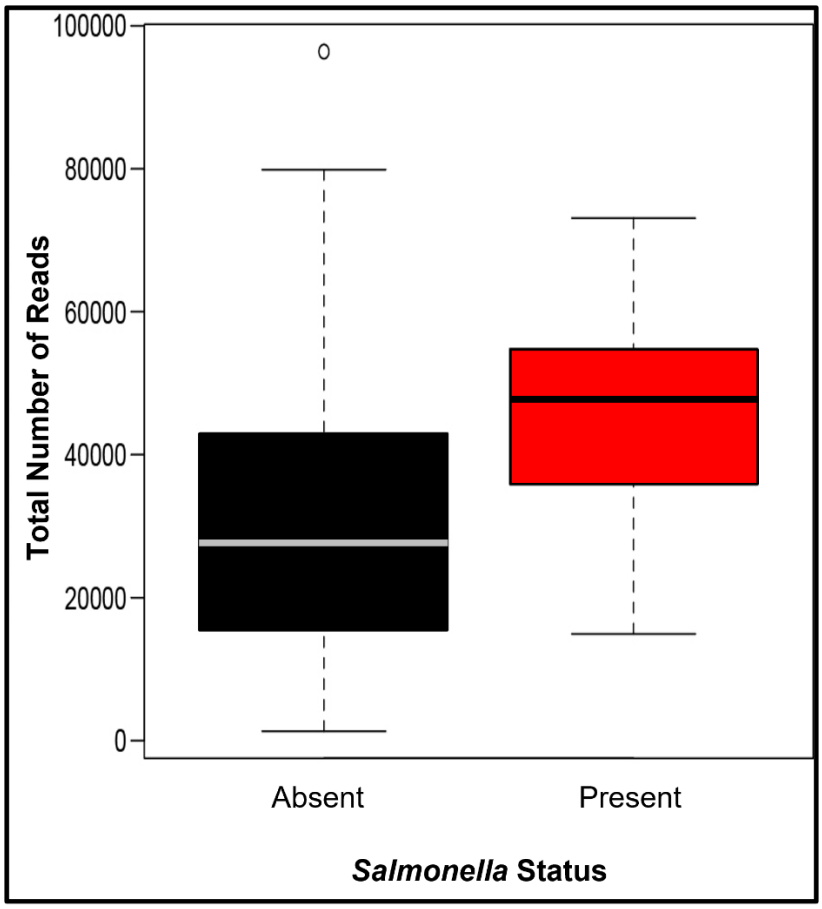


Figure B.3. *Salmonella* absolute abundance by sample ID in barn swallow (*Hirundo rustica*) fecal samples collected in Israel. Abundance was estimated A) before rarefaction, B) after rarefaction at rngseed = 711, C) after rarefaction at rngseed = 33, and D) and after rarefaction at rngseed = 82. Panels B-D provide examples of the stochasticity of detecting *Salmonella* in a sample. Samples marked with an asterisk had abundance levels greater than 15 reads with actual values listed in the bottom right of each panel following the order of the samples.

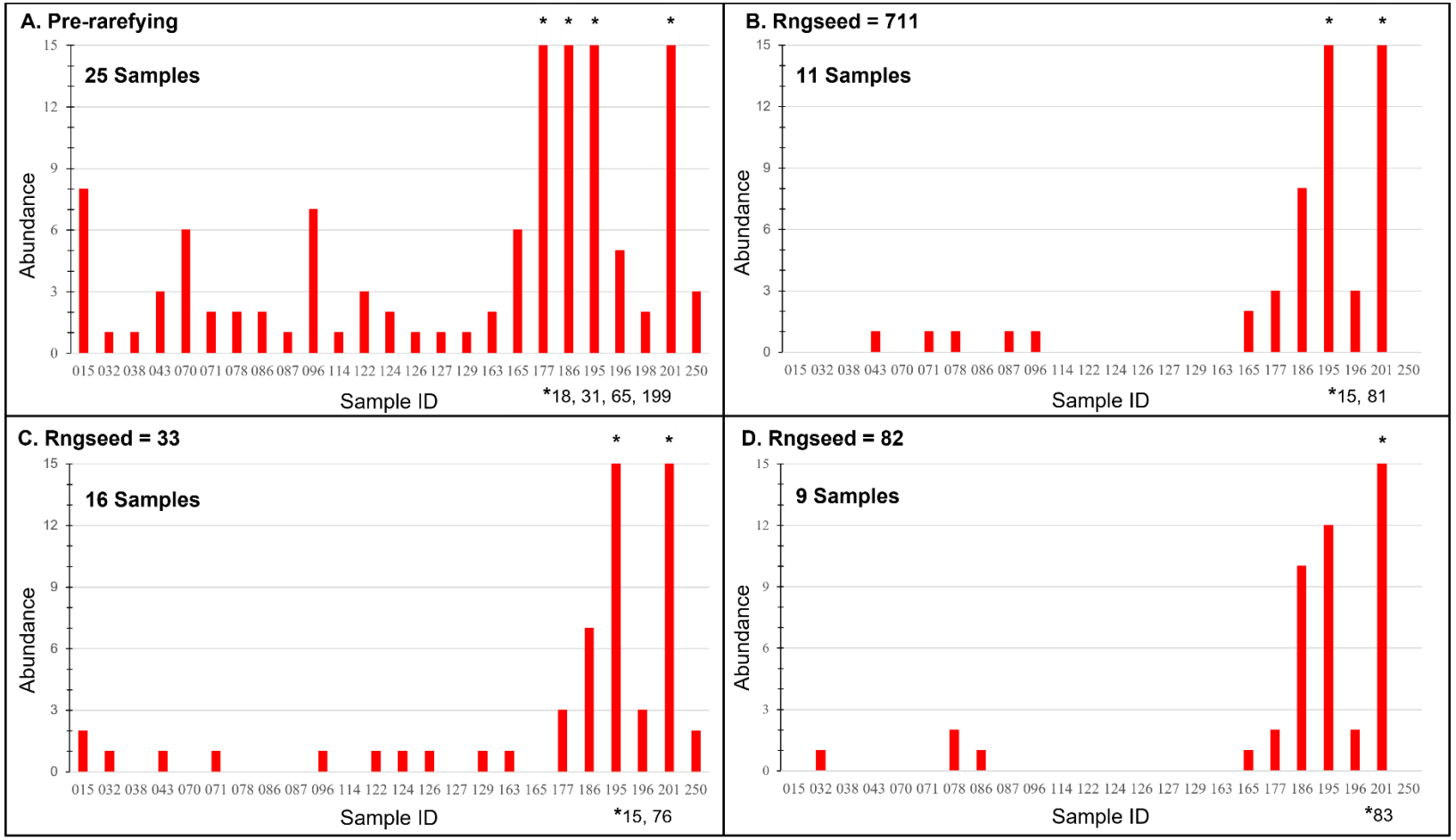


Figure B.4. The percent of barn swallow (*Hirundo rustica*) fecal samples collected in Israel with greater than 45,000 reads (N = 18) that are positive for the presence of *Salmonella* after rarefying at different 16S rRNA gene sequencing depths (5,000-45,000). All these samples were *Salmonella*-positive when all reads were considered.

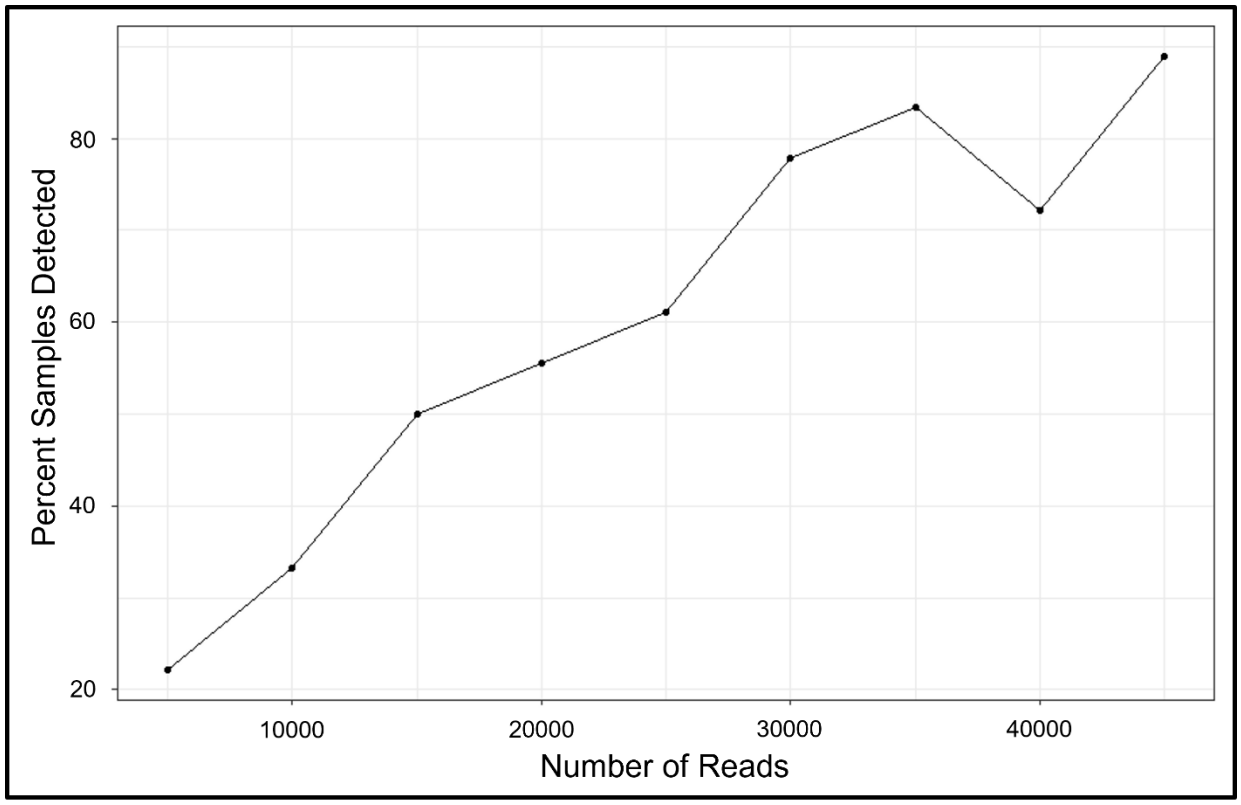


Figure B.5. Bacterial alpha diversity, as estimated by the Chao1 statistic, by *Salmonella* spp. status (absent, present) in barn swallow (*Hirundo rustica*) fecal samples collected in Israel when using a *Salmonella* detection threshold of two reads. *Salmonella* absent samples had significantly lower diversity than positive samples (Mann-Whitney-Wilcoxon test, $p = 0.003$).

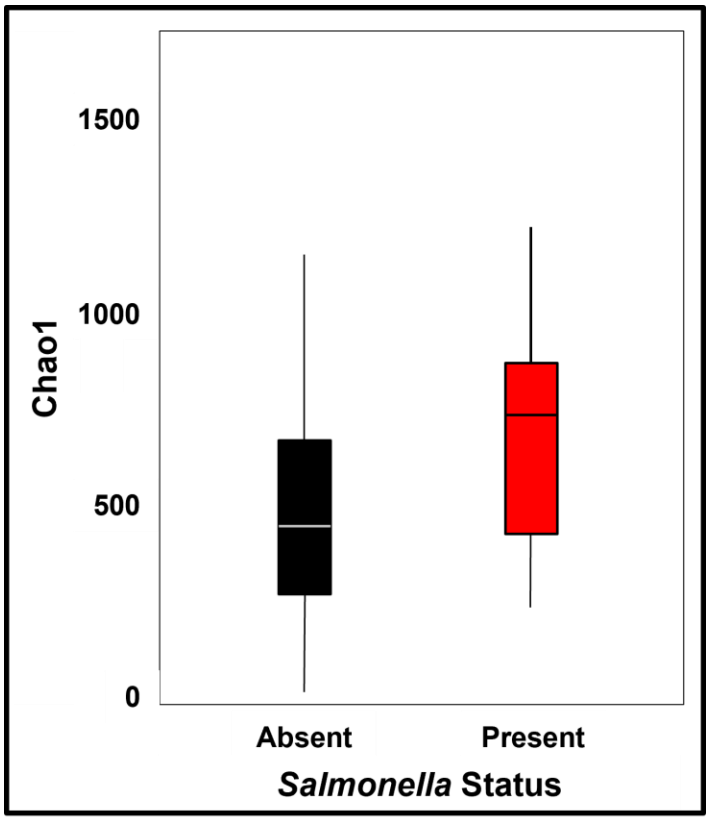


Figure B.6. Proportional abundance of the 10 most abundant bacterial phyla (colors), with all other phyla lumped together (white), showing both *Salmonella* absent (left) and present (right) barn swallows (*Hirundo rustica*) collected in Israel for a *Salmonella* detection threshold of A) one and B) two reads.

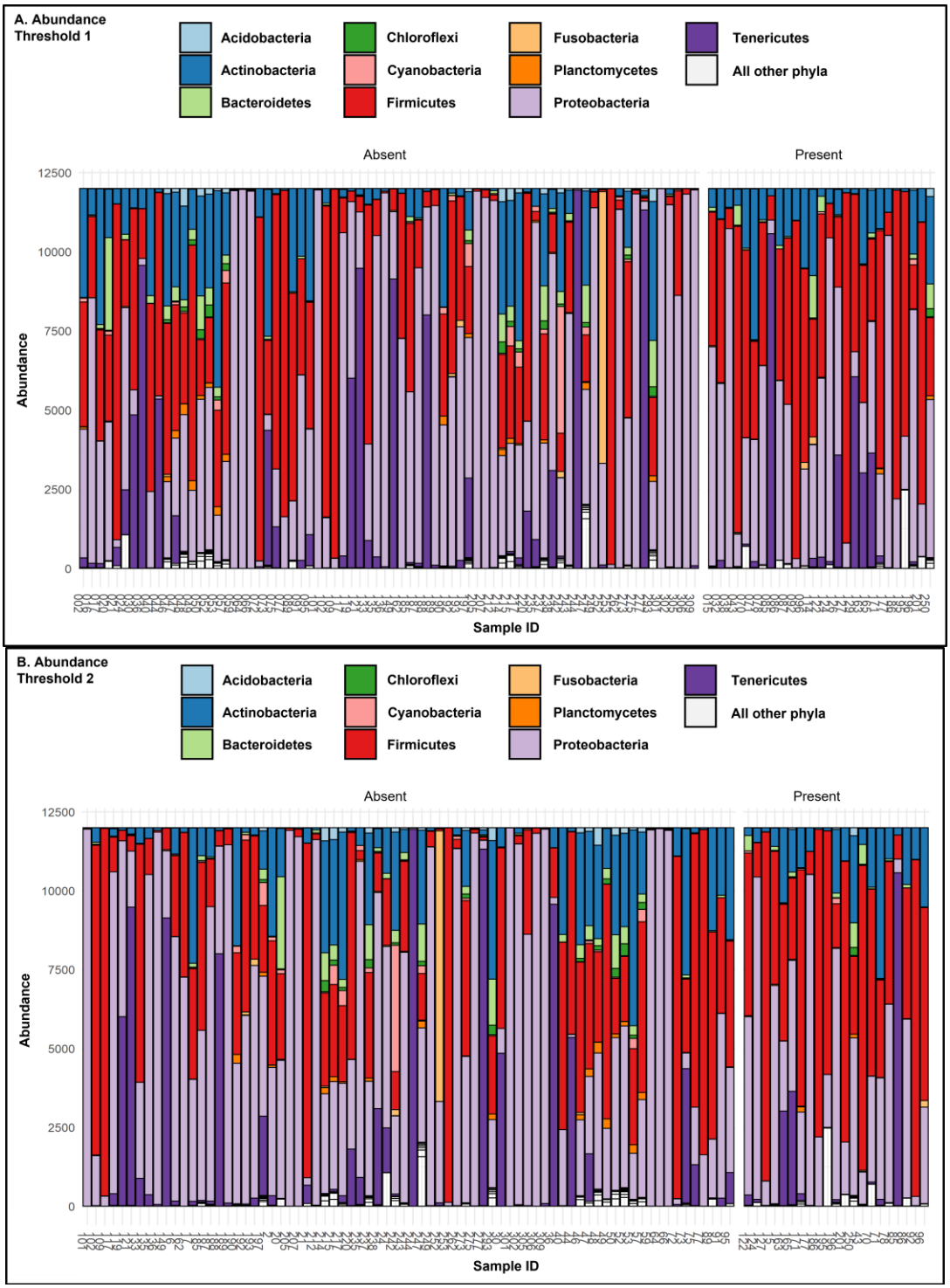
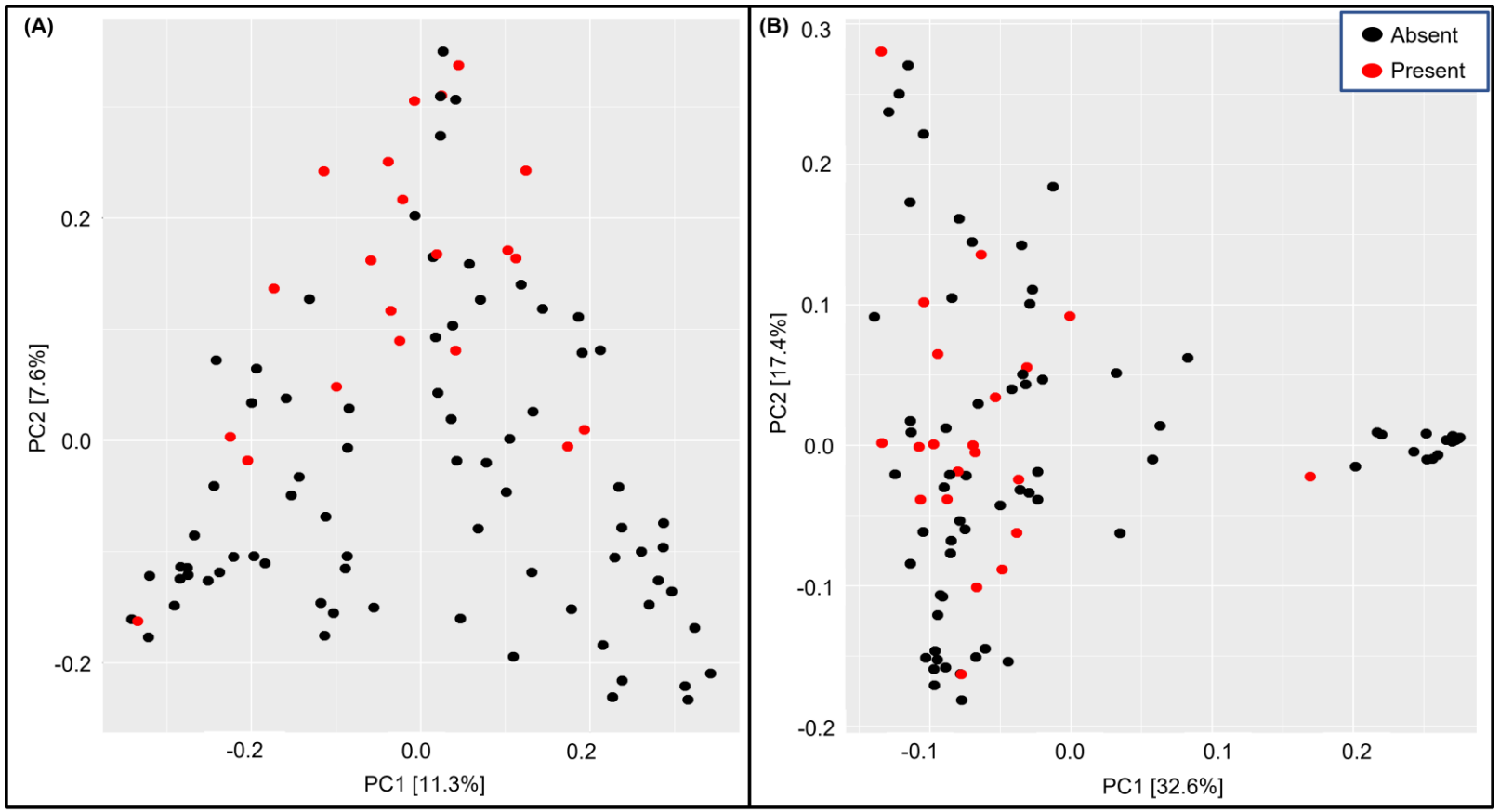


Figure B.7. Principal coordinate analysis plots showing the bacterial communities in barn swallow (*Hirundo rustica*) fecal samples with *Salmonella* (present: red dots) and without (absent: black dots) by (A) unweighted UniFrac and (B) weighted UniFrac metrics for a *Salmonella* detection threshold of two reads. The amount of the variation explained by each axis is in brackets. Bacterial beta diversity significantly differed for both metrics (unweighted: $p = 0.002$, weighted: $p = 0.013$) between *Salmonella*-present and -absent birds. The homogeneity of dispersion was significant for both the unweighted ($p = 0.001$) and the weighted ($p = 0.048$) UniFrac metrics.



APPENDIX C: CHAPTER 3 SUPPLEMENTAL MATERIAL

Figure C.1. Rarefaction curves based on mean Chao1 alpha diversity for A) wood ducks, B) green-winged teal, C) mallards, D) black ducks, E) all species. Rarefaction depths were determined by the points at which all samples, represented by individual curves, plateaued for each plot.

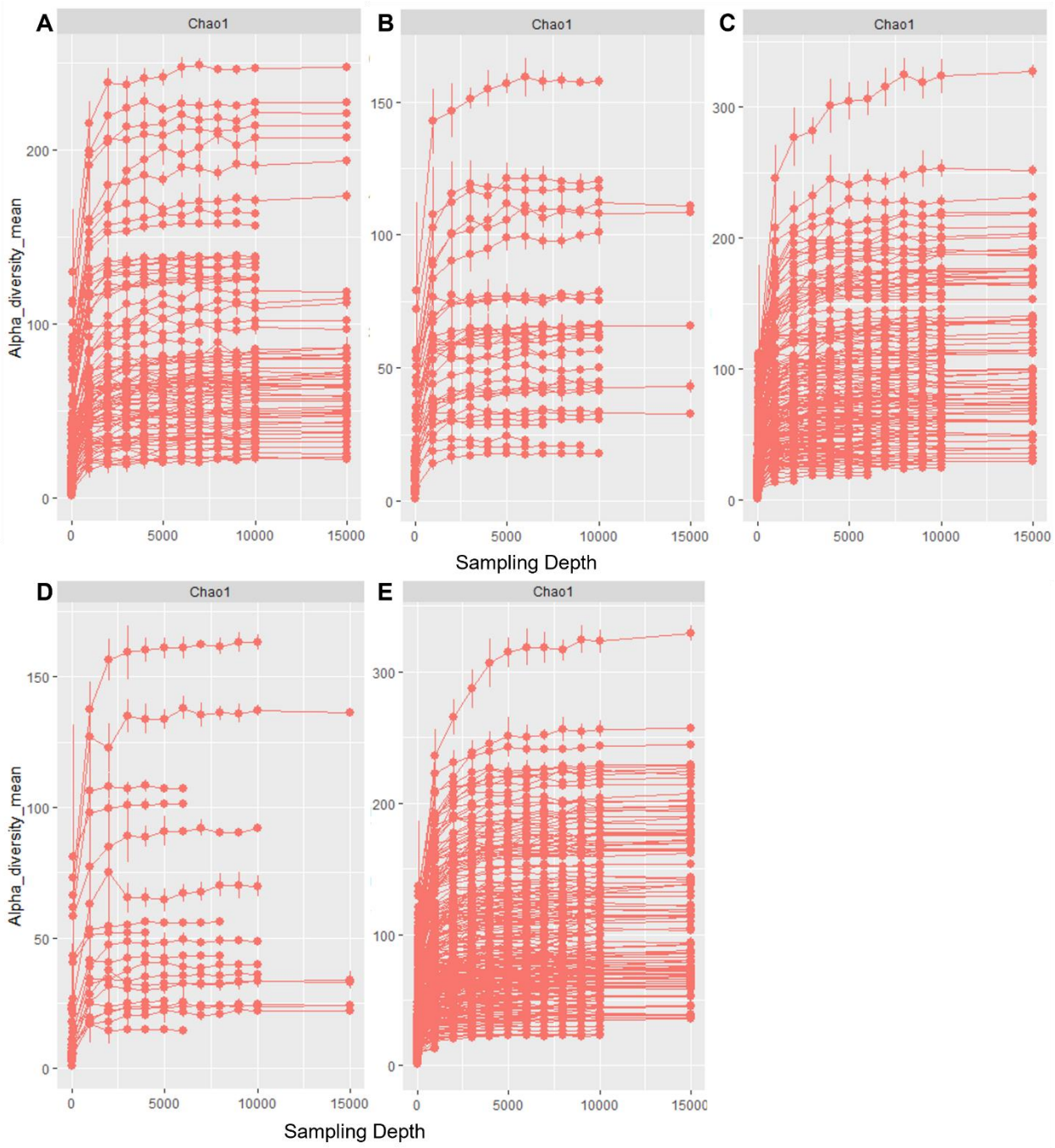


Table C.1. AIC selection of generalized linear models to identify variables associated with haemosporidian parasite infection intensity in black ducks.

Predictor Variables	AIC	ΔAIC
Age + Sex + Weight + Location	87.3	0.0
Sex + Weight + Location	89.0	1.7
Age + Sex + Weight + Wing Length + Location	89.2	1.9
Sex + Weight + Wing Length + Location	90.7	3.4
Weight + Wing Length + Location	93.5	6.2
Age + Weight + Wing Length + Location	95.4	8.0
Weight + Location	99.2	11.9
Age + Weight + Location	100.6	13.3
Weight	120.7	33.3
Sex + Weight + Wing Length	120.7	33.4
Sex + Weight	121.3	34.0
Age + Sex + Weight + Wing Length	121.3	34.0
Age + Weight	122.1	34.7
Weight + Wing Length	122.6	35.3
Age + Sex + Weight	123.3	36.0
Age + Weight + Wing Length	124.0	36.6
Sex + Location	152.8	65.5
Age + Sex + Location	153.0	65.7
Sex + Wing Length + Location	154.6	67.3
Age + Sex + Wing Length + Location	154.6	67.3
Age + Location	154.9	67.5
Location	156.4	69.1
Age + Wing Length + Location	156.8	69.5
Wing Length + Location	158.1	70.7
Age	182.2	94.9
Age + Wing Length	184.2	96.8
Age + Sex	184.2	96.8
Age + Sex + Wing Length	186.1	98.8
Null Model	186.2	98.9
Sex	187.9	100.5
Wing Length	188.2	100.8
Sex + Wing Length	189.9	102.5

Figure C.2. Scatter plot of weight and haemosporidian infection intensity (C_T value) for black ducks. There was not a significant relationship between weight and infection intensity (C_T value; $p = 0.36$).

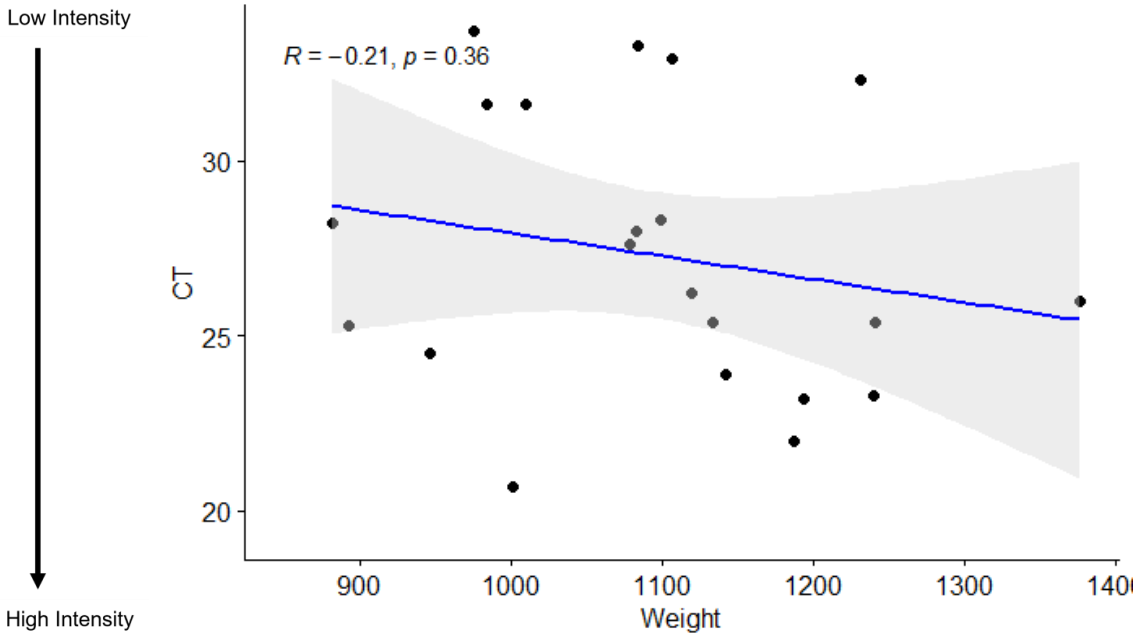


Figure C.3. Boxplot comparison of haemosporidian parasite intensity (C_T value) for female and male black ducks. A Wilcoxon rank sum test revealed no difference in parasite intensity between female (red) and male (blue) black ducks (W = 93, p = 0.29).

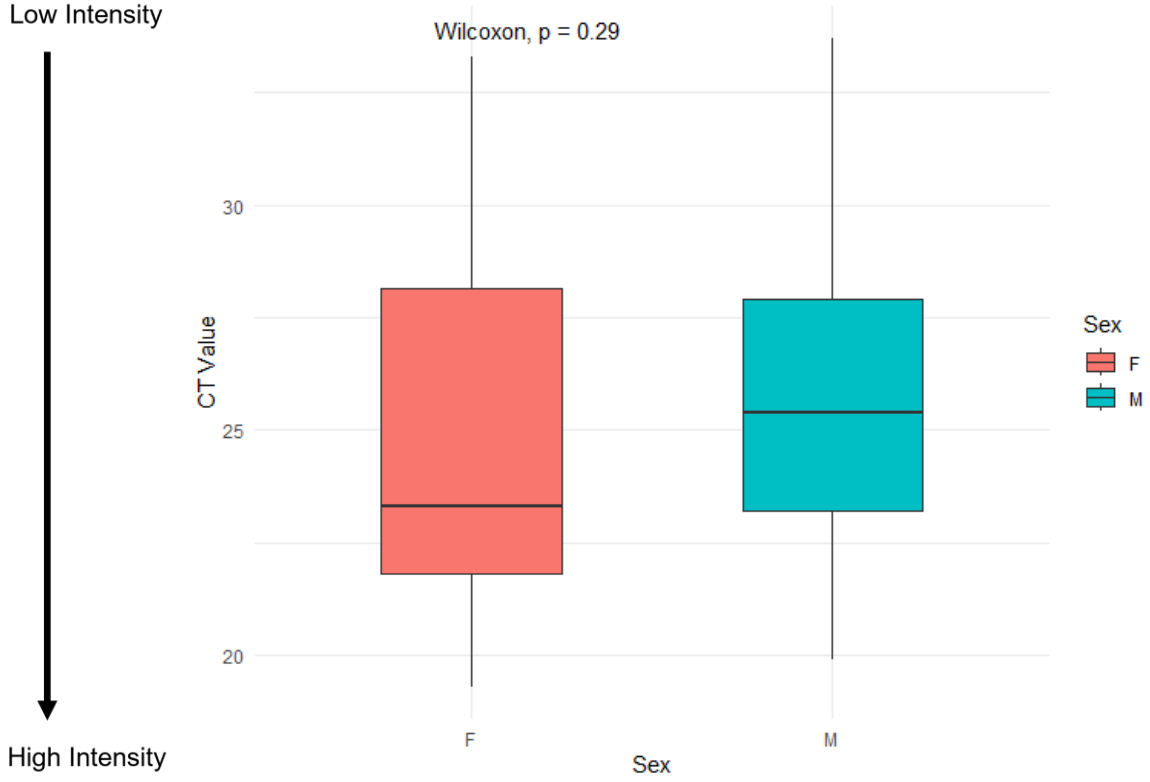


Table C.2. AIC selection of generalized linear models to identify variables associated with haemosporidian parasite infection intensity in green-winged teals.

Predictor Variables	AIC	ΔAIC
Weight + Wing Length + Location	74.0	0.0
Age + Weight + Wing Length + Location	75.2	1.1
Age + Weight	75.5	1.5
Weight + Location	75.6	1.6
Age + Weight + Location	75.9	1.8
Sex + Weight + Wing Length + Location	76.0	2.0
Age + Weight + Wing Length	76.2	2.2
Age + Sex + Weight	76.9	2.8
Age + Sex + Weight + Wing Length + Location	77.1	3.1
Sex + Weight + Location	77.1	3.1
Age + Sex + Weight + Location	77.3	3.2
Age + Sex + Weight + Wing Length	78.1	4.0
Weight	82.6	8.6
Weight + Wing Length	83.7	9.6
Sex + Weight	84.2	10.2
Sex + Weight + Wing Length	85.6	11.6
Location	96.6	22.6
Wing Length + Location	97.5	23.5
Age + Location	98.5	24.5
Sex + Location	98.6	24.6
Sex + Wing Length + Location	99.2	25.2
Age + Wing Length + Location	99.5	25.5
Age + Sex + Location	100.5	26.5
Age	101.0	27.0
Age + Sex + Wing Length + Location	101.2	27.1
Sex	101.7	27.7
Wing Length	101.9	27.8
Age + Sex	102.8	28.8
Age + Wing Length	103.0	29.0
Sex + Wing Length	103.7	29.7
Age + Sex + Wing Length	104.8	30.8

Figure C.4. Scatter plot of wing length and C_T value (haemosporidian infection intensity) for green-winged teals. There was no significant relationship between the two variables ($R^2 = 0.002$, $p = 0.85$).

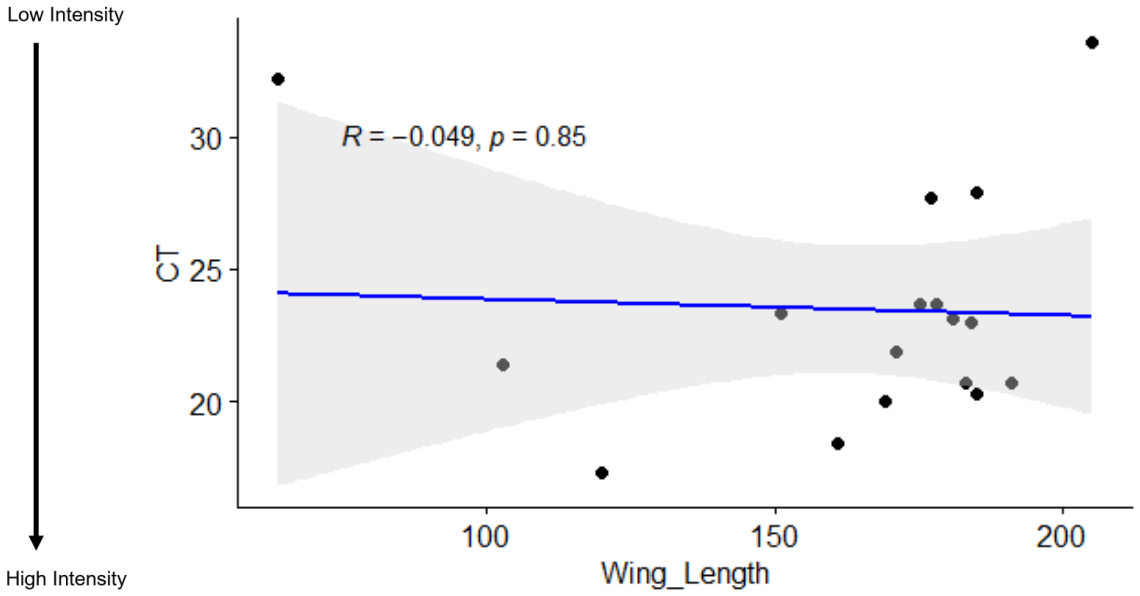


Figure C.5. Scatter plot of weight and C_T value (haemosporidian infection intensity) for green-winged teals. There was no significant relationship between the two variables ($R^2 = 0.03$, $p = 0.57$).

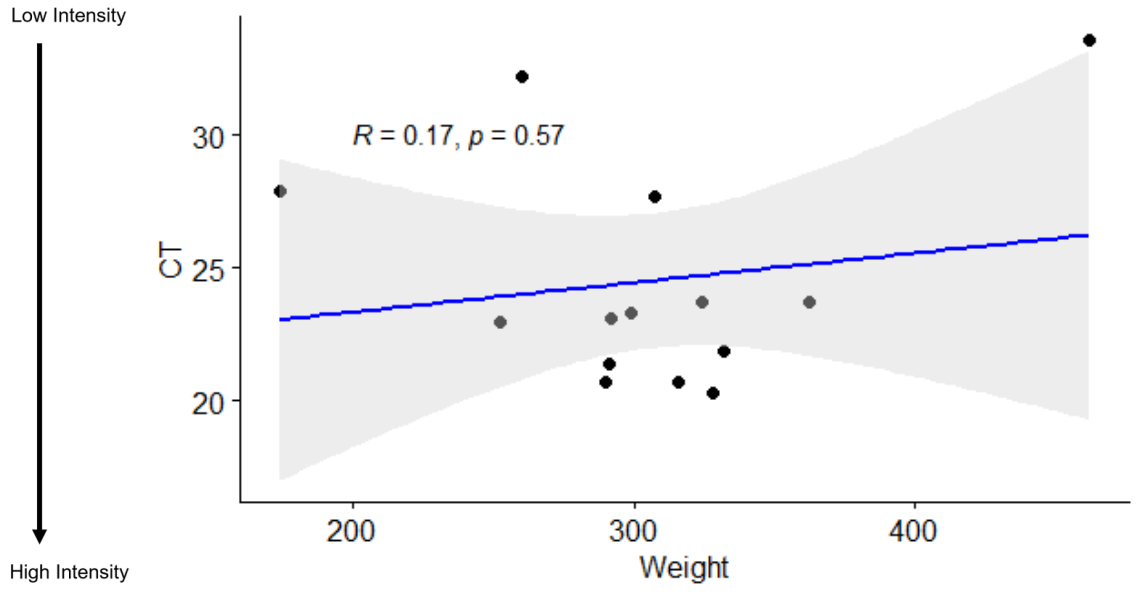


Table C.3. AIC selection of generalized linear models to identify variables associated with haemosporidian parasite infection intensity in mallard ducks.

Predictor Variables	AIC	ΔAIC
Wing Length + Location	884.7	0.0
Location	884.8	0.1
Age + Location	885.3	0.5
Age + Wing Length + Location	885.9	1.2
Sex + Wing Length + Location	886.6	1.9
Sex + Location	886.8	2.1
Age + Sex + Location	887.2	2.5
Age + Sex + Wing Length + Location	887.7	3.0
Age	897.2	12.5
Null Model	897.8	13.1
Age + Wing Length	899.2	14.4
Age + Sex	899.2	14.5
Wing Length	899.5	14.8
Sex	899.8	15.1
Age + Sex + Wing Length	901.1	16.4
Sex + Wing Length	901.5	16.8

Figure C.6. Scatter plot of wing length and C_T value (haemosporidian infection intensity) for mallard ducks. There was no significant difference between C_T values in mallards ($R^2 = 0.002$, $p = 0.6$).

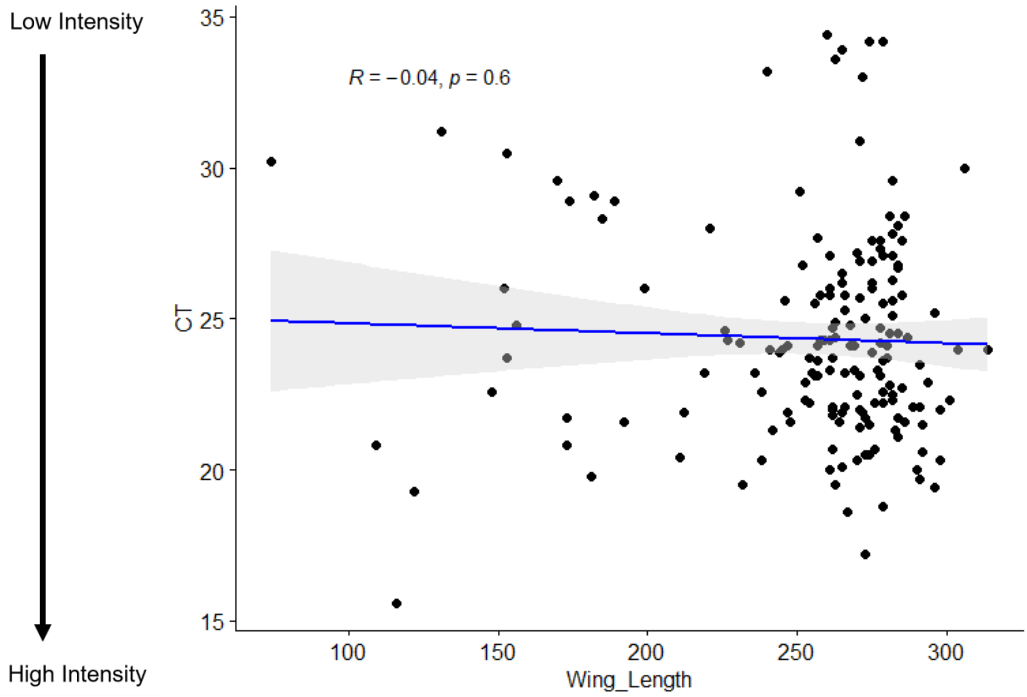


Table C.4. AIC selection of generalized linear models to identify variables associated with haemosporidian parasite infection intensity in wood ducks.

Predictor Variables	AIC	ΔAIC
Location	402.4	0.0
Wing Length + Location	402.6	0.2
Age + Wing Length + Location	402.9	0.5
Age + Sex + Location	403.0	0.5
Sex + Location	403.0	0.5
Age + Location	403.4	0.9
Age + Sex + Wing Length + Location	403.6	1.2
Sex + Wing Length + Location	403.9	1.5
Sex	418.4	16.0
Null Model	419.2	16.8
Age + Sex	419.5	17.1
Sex + Wing Length	420.4	18.0
Wing Length	420.7	18.3
Age	420.9	18.5
Age + Sex + Wing Length	421.5	19.0
Age + Wing Length	422.4	19.9

Figure C.7. Scatter plot of C_T value (haemosporidian infection intensity) and Chao1 alpha diversity for all ducks. There is no significant relationship between the two variables ($R^2 = 0.005$, $p = 0.24$), suggesting that haemosporidian infection intensity does not affect cloacal bacterial species diversity in all ducks.

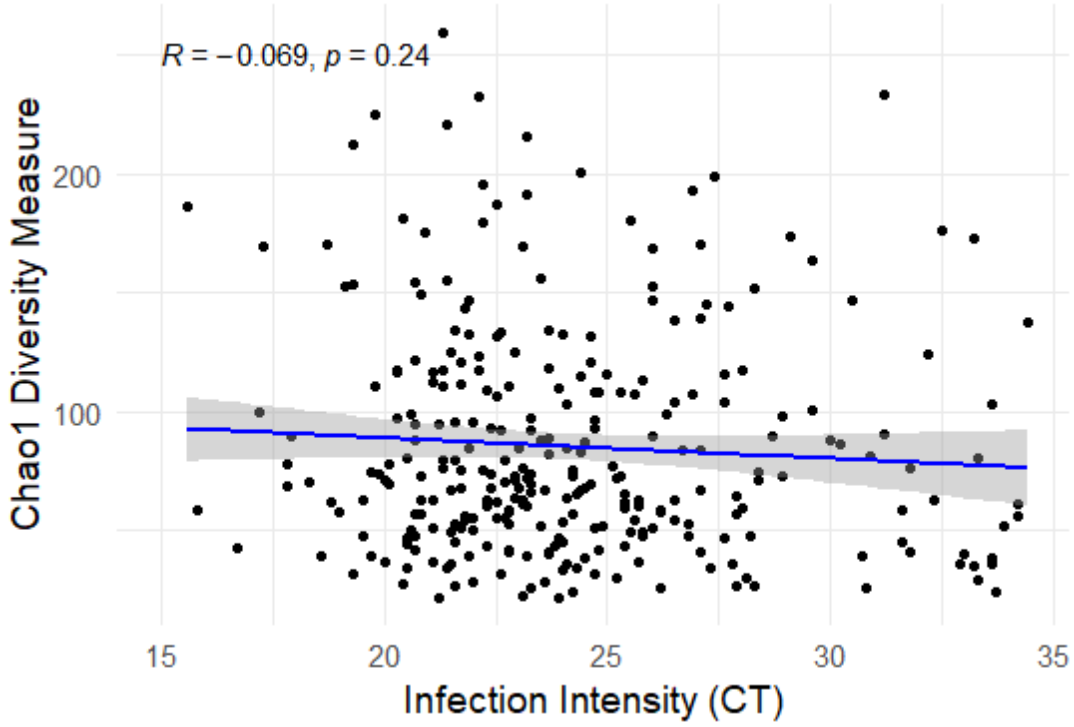


Figure C.8. Scatter plot of C_T value (haemosporidian infection intensity) and Chao1 alpha diversity by host species black ducks, green-winged teals, mallard ducks, wood ducks. There were no significant relationships observed ($R^2 = 0.04$, $p = 0.25$ for black ducks, $R^2 = 0.0002$, $p = 0.97$ for green-winged teals, $R^2 = 0.0081$, $p = 0.25$ for mallard ducks, $R^2 = 0.0006$, $p = 0.83$ for wood ducks) suggesting that haemosporidian infection intensity does not affect cloacal bacterial species diversity in all ducks at the species level.

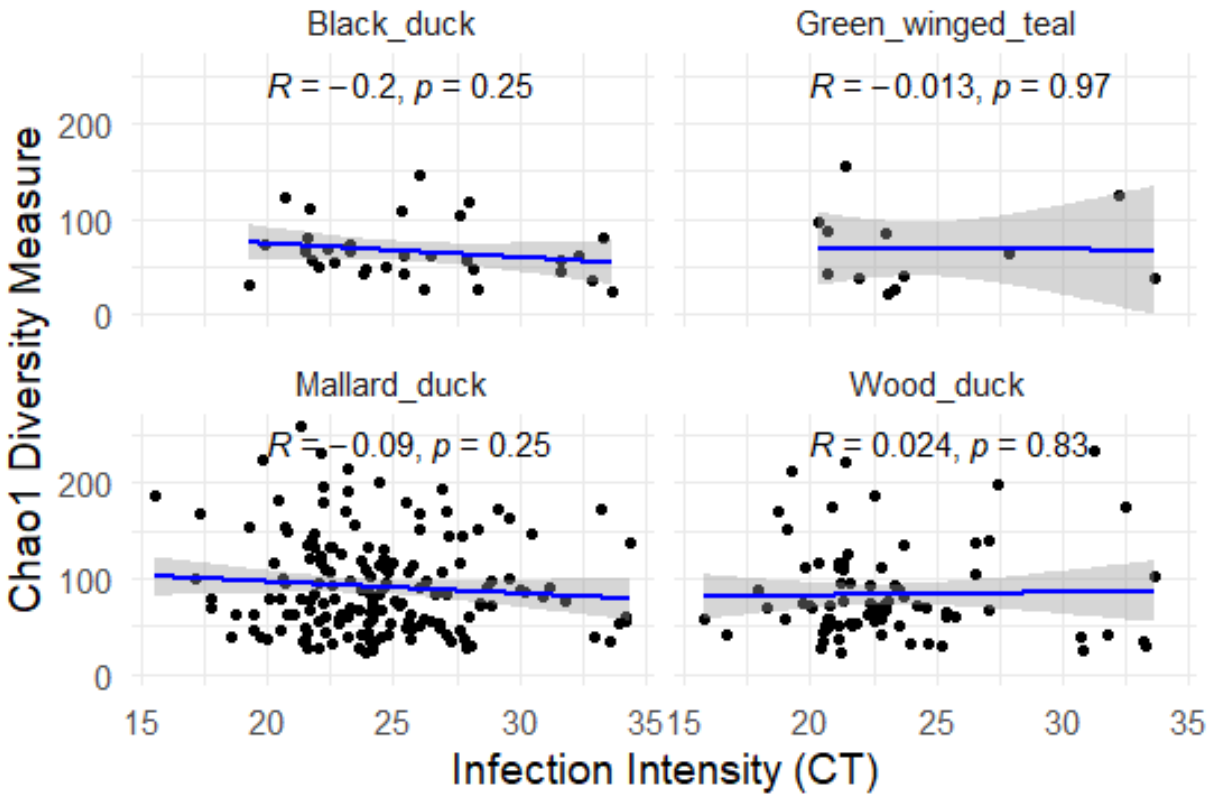


Figure C.9. PCoA of beta diversity colored by haemosporidian infection intensity (C_T values) for black ducks based on the A) Unweighted and B) Weighted UniFrac measures. Neither measure was statistically significant (F = 0.997, p = 0.42 unweighted, F = 0.282 p = 0.96 weighted) suggesting that parasite infection intensity does not affect the cloacal bacterial community composition in black ducks.

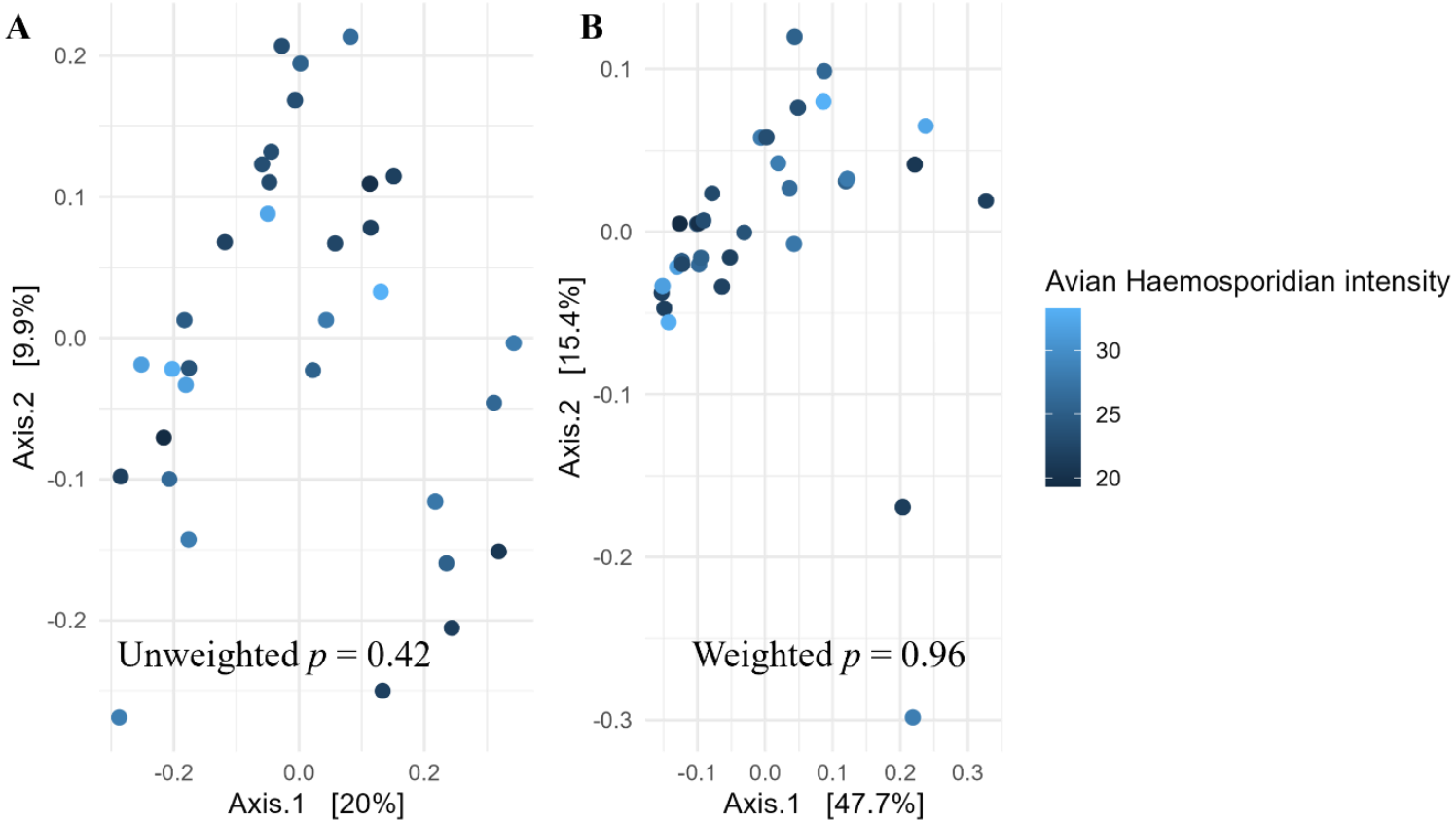


Figure C.10. PCoA of beta diversity colored by haemosporidian infection intensity (C_T values) for green-winged teals based on the A) Unweighted and B) Weighted UniFrac measures. Neither measure was statistically significant (F = 0.651, p = 0.47 unweighted, F = 0.651 p = 0.46 weighted) suggesting that parasite infection intensity does not affect the cloacal bacterial community composition in green-winged teal.

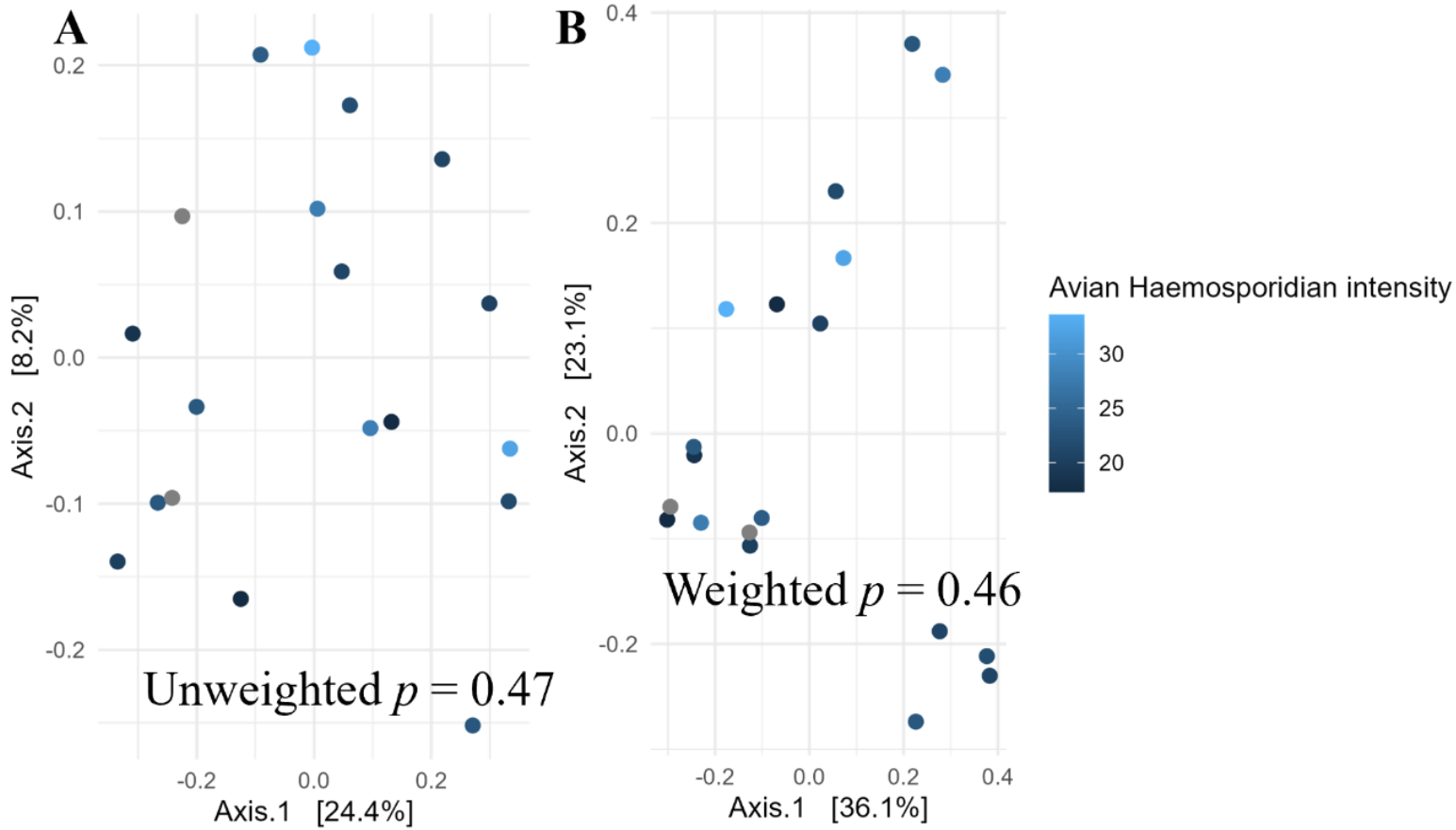


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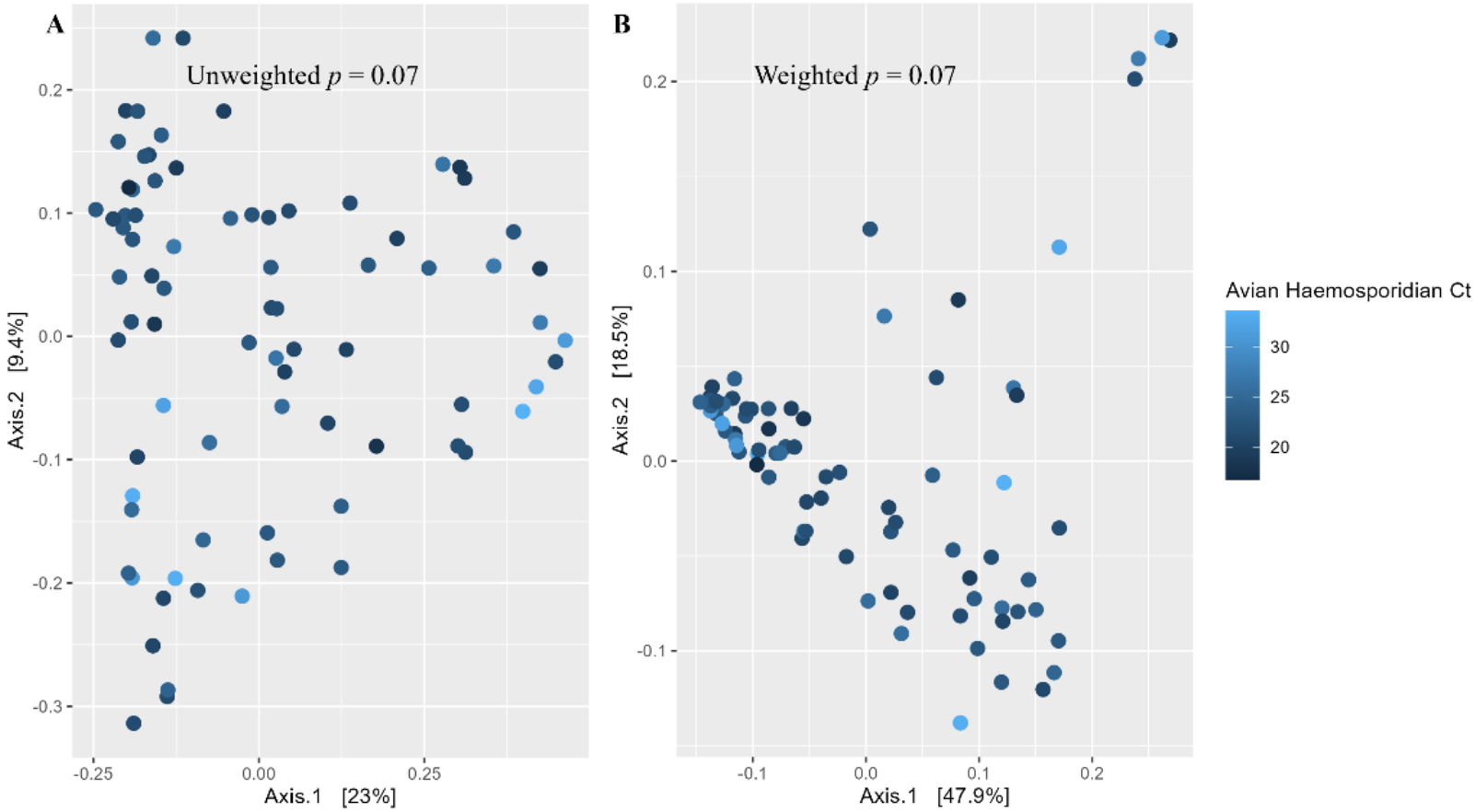
Figure C.11. PCoA of beta diversity colored by haemosporidian infection intensity (C_T values) for wood ducks based on the A) Unweighted and B) Weighted UniFrac measures. Neither measure was statistically significant (F = 1.56, p = 0.07 for both) suggesting that parasite infection intensity does not affect the cloacal bacterial community composition in wood ducks.



APPENDIX D: CHAPTER 4 SUPPLEMENTAL MATERIAL

Figure D.1. Scatter plot of haemosporidian infection intensity (C_T value) and Chao1 alpha diversity. There was no significant relationship between infection intensity and Chao1 alpha diversity (C_T value; $R^2 = 0.003$, $p = 0.65$).

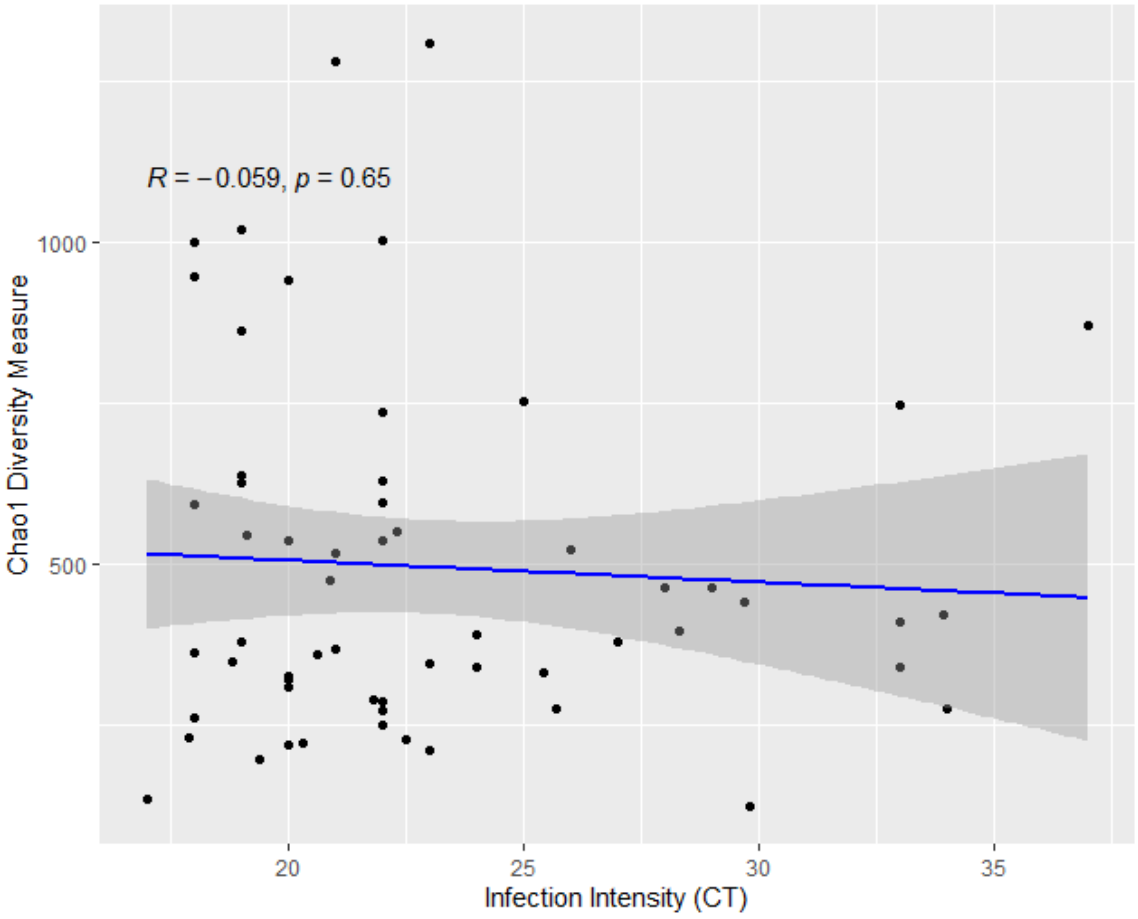


Figure D.2. PCoA of beta diversity colored by haemosporidian parasite infection intensity (C_T value) for owls based on the A) Unweighted and B) Weighted UniFrac measures. Bacterial community composition did not vary significantly with host infection intensity for either the unweighted (F = 0.86, p = 0.78) or weighted UniFrac measure (F = 1.39, p = 0.18).

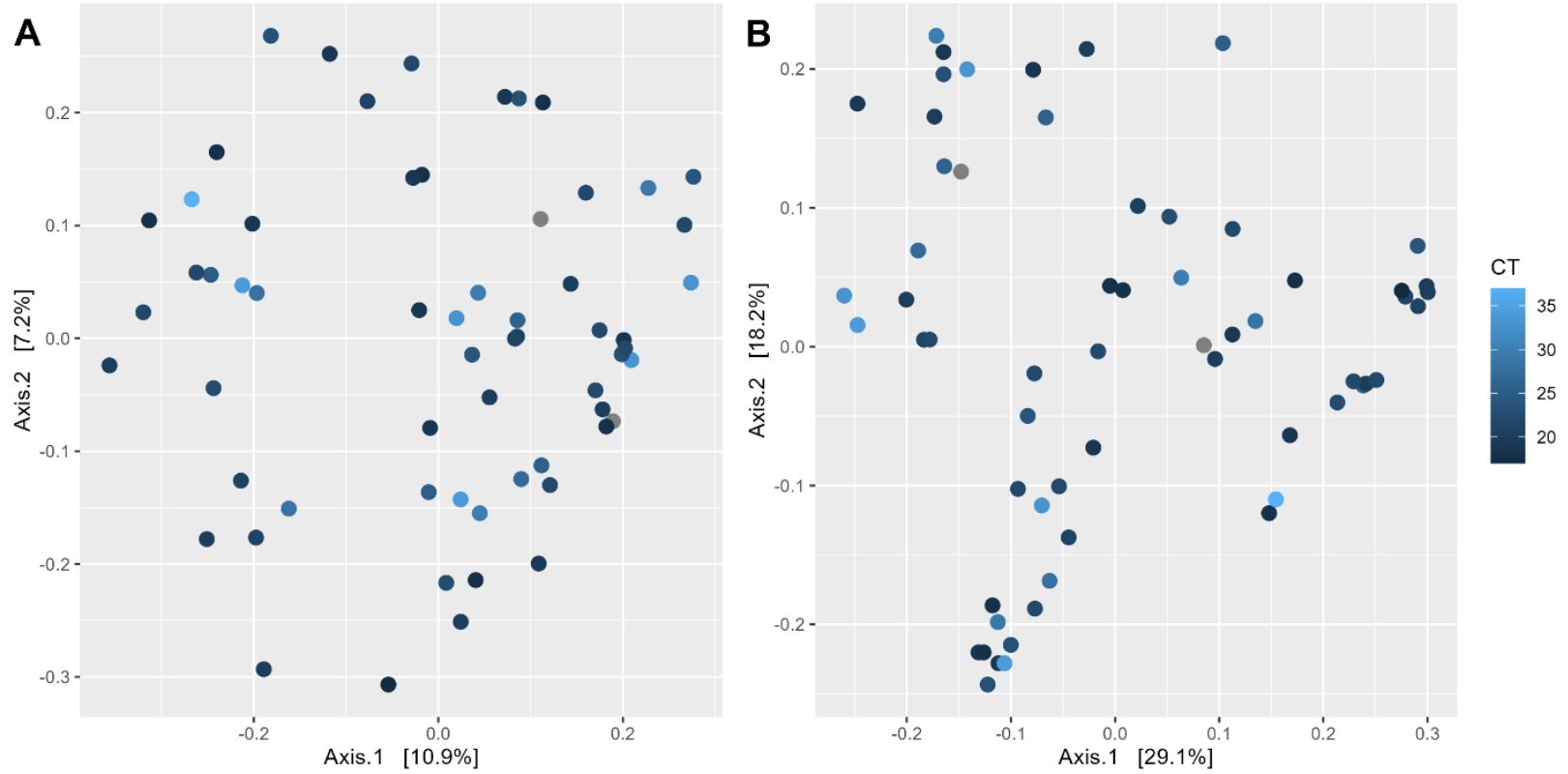


Table D.1. Summary statistics for best models for infection intensity or Chao1 alpha diversity and plumage coloration in barn owls.

Model – Predictors	**Beta**	**Std Error**	**Z-value**	***p*-value**
Intensity – intercept	22.71	0.87	26.17	$2.0e^{-16}$
Chao1 – intercept	528.1	46.14	11.45	$1.8e^{-12}$

Table D.2. Summary statistics for generalized linear models for infection intensity with reproductive success in barn owls.

Model	**Beta**	**Std Error**	**Z-value**	***p*-value**
C_T value ~ Clutch size	$4.40e^{-3}$	0.45	0.1	0.92
C_T value ~ Fledge success	$-5.40e^{-3}$	0.01	-0.47	0.64
C_T value ~ Julian laying date	0.14	0.40	0.35	0.73

Table D.3. Summary statistics for generalized linear models for Chao1 alpha diversity with reproductive success in barn owls.

Model	**Beta**	**Std Error**	**Z-value**	***p*-value**
Chao1 ~ Clutch size	$4.09e^{-4}$	$8.17e^{-4}$	-0.50	0.62
Chao1 ~ Fledge success	$1.72e^{-4}$	$1.74e^{-4}$	0.99	0.33

Table D.4. Summary statistics for generalized linear model for home range area with Julian laying date in barn owls.

Predictor	**Beta**	**Std Error**	**Z-value**	***p*-value**
Intercept	86.32	3.80	22.75	$3.04e^{-15}$
Julian laying date	0.37	0.47	0.78	0.44

BIOGRAPHY OF THE AUTHOR

Olivia Nahrie Choi was born in Niles, Illinois on January 24, 1987. She was raised in Glenview, Illinois and graduated from Glenbrook South High School in 2005. She attended Illinois Wesleyan University in Bloomington, Illinois and graduated in 2009 with a Bachelor's degree in English - Literature. She returned to undergrad at Northeastern Illinois University in Chicago, Illinois and graduated in 2014 with a Bachelor's degree in Biology. She then entered the Biology graduate program at the same university and graduated in 2017. She then moved to Maine and entered The University of Maine's Ecology and Environmental Science program in the fall of 2017. After receiving her degree, Olivia will be joining the University of Maine Cooperative Extension as a Postdoctoral Research Assistant. Olivia is a candidate for the Doctor of Philosophy degree in Ecology and Environmental Sciences from the University of Maine December 2023.