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The University of Southern Mississippi

GUT MICROBIOTA OF MIGRATORY
PASSERINES AT STOPOVER

by

William Bagley Lewis

A Thesis

Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

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ABSTRACT
GUT MICROBIOTA OF MIGRATORY
PASSERINES AT STOPOVER

by William Bagley Lewis

December 2015

Although the gut microbiota provides many beneficial functions to animal hosts, relatively little is known about the gut microbiota of passerines. It is likely that gut microbes are especially important during the migratory phase; however gut atrophy experienced during prolonged migratory flight may cause disruptions of the stable microbiota. Fecal samples were collected from several species of passerine after crossing the Gulf of Mexico during spring migration and before crossing during fall, and microbiota communities were analyzed using next-generation sequencing. Despite showing large inter-individual variation, a core microbiota composed largely of Firmicutes and Proteobacteria was identified in all birds. Microbiota profiles were not related to the energetic condition of birds in either season, therefore microbiota communities do not seem to change based on energetic demand experienced during prolonged migratory flight. Spring and fall migrants showed clear differences in microbiota communities, though only fall migrants showed species-specific profiles. These season and species differences likely reflect the differing conditions and environments experienced by migrants in each stage. Many spring birds recaptured on subsequent days showed distinct shifts in community composition towards a more similar microbiota; with the degree of change in microbiota seemingly related to changes in

energetic condition at stopover. Plastid DNA was found to be abundant in the feces of many birds, correlating to seasonal patterns of frugivory in migrants. Taken together, these results suggest that the gut microbiota of migratory passerines is a diverse and dynamic system which is highly impacted by environmental variables.

DEDICATION

I dedicate this thesis to my family. I thank my parents, Gil Lewis and Susan Bagley, who, from an early age, instilled in me a love for birds, science, and the outdoors. They have been a constant source of inspiration and encouragement throughout my life. Most importantly, I thank my girlfriend, Jessica Trieskey. She has always believed in me, and without her support and encouragement I would not be here today. Thank you: I love you.

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CHAPTER I

INTRODUCTION

Nearctic-Neotropical Migration

Each year billions of birds make a twice-annual migration between breeding grounds in the United States and Canada and wintering areas in the Caribbean and Central and South America. Approximately one third of the annual cycle of passerines is spent on migration with birds alternating short bouts of flight with longer bouts of resting and feeding during stopover. Migration allows for the exploitation of seasonal food abundances; however it is a period of high energetic demand and is believed to incur the highest rates of mortality throughout the year, as Sillit and Holmes (2002) estimated that 85% of annual mortality occurred during migration in Black-throated Blue Warblers (*Setophaga caerulescens*).

During spring and fall migration many birds will migrate directly across the 1000km Gulf of Mexico (GOM) in a non-stop flight instead of taking the longer route around it through Mexico. Birds arriving along the Louisiana coast in spring after crossing the GOM will preferentially overfly the immediate coast and instead land in extensive forest 30-50km inland (Gauthreaux Jr 1971); however birds which have depleted their energy reserves during crossing are forced to stop at the first available stopping habitat after making landfall on the coast. Inclement weather can also force birds of all conditions to land along the coast, leading to “fallouts” where thousands of birds may land at a single coastal stopover site.

Coastal cheniers are the first available stopping habitats for migrants arriving across the GOM in southwest Louisiana. Cheniers are oak-dominated forests which run

along east-west ridges across the Gulf Coast. The slight increase in elevation along the ridges allows for the growth of trees and thus cheniers represent “islands” of suitable stopping habitat surrounded by marsh and the Gulf. Birds stopping at these coastal habitats generally use them for only one or two days before departing to more continuous forest further inland (Moore and Kerlinger 1987, Buler and Moore 2011).

The Gut Microbiota

Understanding the complex interactions between animals and the vast array of microbes living in their gastrointestinal tract, referred to as gut microbiota, has emerged as an important area of scientific research. The gut microbiota is the largest community of microbes in the body and, in humans, may number as high as 100 trillion (Bäckhed et al. 2005). Bacteria generally dominate the gut microbiota but Archaea and Eukarya are present as well (Eckburg et al. 2005, Scanlan and Marchesi 2008). Though characterized by high species richness, many of the microbial species are present only at low abundance and instead the microbiota is dominated by a few types of microbes.

Though members of the gut microbiota may be commensal or pathogenic, research is increasingly suggesting that a multitude of gut microbes experience a mutualistic relationship with their host. The host provides a stable environment and reliable food for the gut microbes, which in turn provide a wide variety of beneficial functions for their hosts. Gut microbes play an important role in extracting nutrients from food and breaking down otherwise indigestible carbohydrates into short-chain fatty acids, which are used as a major energy source by the gut epithelium (Tremaroli and Bäckhed 2012). The gut microbiota has been shown to regulate metabolism and promote deposition of fat in adipose tissue (Bäckhed et al. 2004). A stable microbiota is key to

host pathogen defense, as the resident microbes can prevent pathogens from becoming established through competition for resources and promote the release of antimicrobial peptides from epithelial cells (Cash et al. 2006, Kamada et al. 2012). Moreover, gut microbes are important for proper development of the immune system and can stimulate both innate and active immune responses to infection (Kamada et al. 2013). The microbiota of birds has also been suggested to play a role in nitrogen and water conservation through breaking down uric acid refluxed into the gut through retrograde peristalsis (Mead 1989).

Animals are believed to be born sterile and are quickly colonized by microbes from the environment and/or parents after birth. The microbiota of young individuals is typically variable and characterized by a high number of transient species before shifting to a more stable adult community (van Dongen et al. 2013). This adult community is not static, however, and can be influenced by many factors. One of the major influences on microbiota community structure is diet, which determines the nutrient environment in the gut and thus the types of microbes living there. The influence of diet can be seen at a broad scale, as Ley et al. (2008) found that the microbiota of mammals from 60 species clustered strongly based on whether the host was carnivorous, herbivorous, or omnivorous. Changes in diet can cause shifts in community structure; for example David et al. (2014) showed that switching to animal-based diets high in fats caused dramatic changes in the phylum-level microbiota of humans. Host genetics play a role in structuring gut microbiota (Khachatryan et al. 2008) as well as the host immune system. Gut microbiota is important for proper development and function of the immune system; however the host can conversely regulate microbes in the gut through the action of

(among others) the interleukin IL-22 and antibody IgA (Roberts et al. 2014). Other factors such as stress (Bailey et al. 2011) and changes in gut morphology (Kohl et al. 2013) have also been implicated in determining microbiota community structure.

Much of our knowledge about gut microbiota comes from research performed on humans and laboratory animals such as mice and rats, however much less is known about the gut microbiota of birds. As an adaptation for flight, birds have very short passage times for food through the gut (average of 1 hour in mid-sized passerines); this contrasts with the extended retention time averaging 2-3 days in humans (Cummings et al. 1976, Levey and Karasov 1994). The short retention times of birds mean that food is not sitting in the gut for microbes to feed and grow on; therefore it is unclear if or to what extent the microbiota play a role in the digestion and fat metabolism of birds. Diverse gut microbiota communities have been found in a wide range of bird species (Dewar et al. 2014, Mirón et al. 2014, Ryu et al. 2014), though in contrast to mammals the gut microbiota of birds is generally characterized by lower Bacteroidetes and increased Proteobacteria. Birds also show differing immune function from mammals and produce differing mucins from the gut epithelium which can reduce the virulence of certain pathogens (Brisbin et al. 2008). The bursa of Fabricius, the main source of B cells in birds, is an offshoot of the GI tract and is colonized by gut microbes, though the exact role that this plays in B cell production and specification is currently unknown (Kohl 2012).

The research that has been performed on birds has largely focused on chickens due to the importance of the poultry industry. Probiotics have been shown to positively influence chicken food conversion ratios and growth (Mountzouris et al. 2010); however

the chicken gastrointestinal tract contains paired ceca which allow for food to sit and be fermented by microbes. In passerines, the ceca is either vestigial or absent and thus not likely to act as a microbial fermentation tank. In contrast to chickens kept in controlled and sterile conditions, passerines in the wild are exposed to a variety of differing microbes and environmental factors. This is especially true for migrants, which stop at a variety of habitats during the migratory journey and thus are constantly exposed to novel foods, environments, and microbes.

Research Justification

Though well studied in mammals, little is known about the gut microbiota of passerines. Most of the studies that have been performed on passerines have focused exclusively on pathogens (e.g. Waldenström et al. 2002) or used older molecular analytical techniques which greatly underestimate biodiversity (Maul et al. 2005, Klomp et al. 2008). Only a few studies have used next-generation sequencing to investigate the gut microbiota of passerines (Maeda et al. 2013, Hird et al. 2014, Mirón et al. 2014), but no study to date has looked at the gut microbiota of passerines during migration.

Given the benefits provided by gut microbiota to their hosts, and given that migrating birds encounter a wide variety of environments and foods, it is important to characterize the microbiota of migratory birds and to determine what factors influence community composition. Birds during migration are exposed to frequent changes in food and nutrient intake, novel and potentially pathogenic microbes at each stopover site, suppressed immune function, and cycles of gut atrophy and hypertrophy, all of which may influence the microbiota community assemblage (Piersma et al. 1999, Owen and Moore 2008). A disruption of the stable gut microbiota (referred to as dysbiosis) may

cause a loss of competitive exclusion and exacerbate the reduced immune function experienced during migration, thereby leaving birds more susceptible to pathogens. Migratory birds have been linked to the spread of a variety of pathogens (Reed et al. 2003), therefore disruptions of the stable gut microbiota in migrants may aid in disease transmission. Birds experiencing a decrease in the abundance of beneficial microbes during migration may also be affected by decreased digestive efficiency and fat deposition rates. Reduced refueling capabilities would likely decrease a bird's foraging efficiency and increase stopover duration, thereby increasing overall migration time. Survival and breeding success have been correlated with increased refueling efficiency at stopover sites (Baker et al. 2004) and earlier arrival on the breeding grounds (Møller 1994 Smith and Moore 2005); therefore changes in the gut microbiota may have long-term consequences for migrant fitness.

This study provides the first community-scale investigation of the gut microbiota of migratory passerines at stopover. The second chapter provides a description of the microbial composition of the gut microbiota of migratory passerines and the degree of variation between seasons and species, and also tests the hypothesis that energetic demand experienced during crossing of the Gulf of Mexico will be reflected in the gut microbiota. The third chapter shows changes in the microbiota of passerines at stopover and discusses possible environmental and physical causes. The fourth chapter examines seasonal and between-species variation in plastid DNA in the feces of migratory passerines in relation to frugivory.

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CHAPTER II
CHARACTERIZATION OF THE GUT MICROBIOTA OF MIGRATORY
PASSERINES IN RELATION TO THE GULF OF MEXICO

Introduction

Animals host a large and diverse community of microbes in their gastrointestinal tracts, referred to as the gut microbiota, which can number up to 100 trillion in humans (Bäckhed et al. 2005). Gut microbes have numerous and complex relationships with their hosts, and have been found to provide many beneficial functions such as aiding in the digestion of otherwise indigestible substances (Stevens and Hume 1998), promoting fat metabolism and deposition (Bäckhed et al. 2004), recycling nitrogen and water (Mead 1989), developing and stimulating the immune system (Hooper et al. 2012), and preventing pathogens from becoming established through competitive exclusion (Kamada et al. 2012). Any disruption of the normal microbiota community could result in a loss of these beneficial functions provided to the host.

The gut microbiota has been more extensively studied in humans and laboratory animals, however far less is known about avian gut microbiota with much of the experimental work on avian microbiota being performed on domestic poultry (Kohl 2012). It is unclear how applicable some of these results may be to wild passerines, which have differing gut retention times, diets, gut morphologies, and life histories. For example, most work on chickens has focused on the microbiota of the ceca, which are paired intestinal structures that act as microbial fermentation tanks, however ceca are either vestigial or absent in passerines and thus not likely to influence gut microbiota (Clench 1999). Studies on passerine gut microbiota have traditionally focused

specifically on pathogens (Brittingham et al. 1988) or used older analytical methods (Lucas and Heeb 2005, Klomp et al. 2008) which greatly underestimate biodiversity (Kohl 2012). Recent studies are starting to use next-generation analytical methods on avian gut microbiota (Dewar et al. 2014, Hird et al. 2014) and have identified correlates between avian microbiota and annual survival (Benskin et al 2015); however we still know very little about gut microbiota in passerines, especially during migration.

The migratory phase is a period of high energetic demand for Nearctic-Neotropical passerines and so they may be especially dependent on their microbiota to help meet the physiological demands of migration. Birds during migration show reduced immune function (Owen and Moore 2006, 2008a), thus the ability of gut bacteria to competitively exclude pathogens may be especially important during this phase of the annual cycle to prevent infection. Most birds cannot migrate non-stop from their wintering to breeding grounds, and instead alternate short bouts of flight with longer periods of refueling during stopover (Newton 2008). Birds at stopover must forage efficiently and deposit fat for the next migratory flight (Moore et al. 2005), which may be aided by the microbiota's effects on digestion and metabolism. Moreover, the microbiota may play an important role in maintaining digestive efficiency despite changes in types and qualities of foods experienced at subsequent stopover areas (McWilliams and Karasov 2001). Avian gut microbiota may thus help to increase fat deposition rates at stopover and increase the rate of migration. Migrants arriving earlier on breeding grounds have been found to settle on better quality territories and achieve greater reproductive success (Møller 1994, Smith and Moore 2005). Given the potential importance of gut

microbes to migrant fitness, it is important to characterize the gut microbiota of migratory passerines and determine the factors influencing community composition.

Each spring, millions of Nearctic-Neotropical migrants cross the Gulf of Mexico (GOM) in a single flight. Though many migrants may be able to cross with energy reserves to spare (Moore and Kerlinger 1987, Yong and Moore 1997), migrants must fly the entire 1000km non-stop and thus crossing the GOM is potentially a very energetically-expensive stage of migration. Several condition-dependent factors associated with migration likely impact the gut microbiota of passerines. Avian gastrointestinal tracts are flexible during migration and undergo cycles of atrophy during flight and hypertrophy on landing and feeding (Piersma et al. 1999, Bauchinger et al. 2005). Birds running low on fat reserves en route will further atrophy digestive organs to provide protein for fuel, with Great Knots (*Calidris tenuirostris*) showing a 40% reduction in intestine mass post-migratory flight (Battley et al. 2000, Schilch et al. 2002). Karasov et al. (2004) showed that gut atrophy is associated with fraying of the villi and shedding of the mucosal epithelium into the lumen, therefore the degree of gut atrophy that a bird experiences during migratory flight likely determines the chemical and structural environment of the gut and thus the types of microbes able to survive. The strength of the immune system is also condition dependent, with birds in poorer condition showing weaker immune responses than birds in better condition (Owen and Moore 2008b). The immune system plays an important role in regulating the gut microbiota (Macpherson et al. 2012, Roberts et al. 2014) and so any change in immune function potentially could influence microbiota structure.

The objectives of this study were to: 1) characterize the structure and makeup of the gut microbiota of two species of passerines during spring and fall migration, and 2) determine the relationship between gut microbiota and energetic condition of passerines after arriving across the GOM. It was hypothesized that a bird's arrival condition, representative of the degree of energetic demand experienced en route, would be reflected in its gut microbiota. If energetic demand experienced during crossing of the GOM influences gut microbiota, then birds arriving across the GOM in spring in poorer condition should show differing microbiota compared to birds arriving in better condition. Additionally, post-crossing birds in spring should show a relationship between arrival condition and microbiota while birds in fall which have not crossed the GOM should not show a relationship between condition and microbiota. If energetic demand influences the microbiota, then the combination of disrupted community structure and weaker immune system of birds in poorer condition likely would allow for pathogenic or opportunistically pathogenic bacteria to increase at the expense of beneficial bacteria. Birds arriving in poor condition were therefore predicted to show lower abundance of beneficial bacteria and higher abundance of pathogenic bacteria.

Methods

Spring Migration

Data was collected at the University of Southern Mississippi's long-term research site at Johnson's Bayou, LA. The study site (29°45'N, 93°37'W) is a 3.25 ha portion of coastal chenier (oak-dominated ridge forest) that is situated 0.8mi inland from the coast. Coastal woodlands represent the first-available stopover habitat for migratory birds after crossing the Gulf of Mexico.

During spring migration 2014 (mid March – mid May), birds were captured using a series of 24.5 lengths of mist net (12 x 2.6m and 6x2.6m nets, 30mm mesh). Six nets were canopy nets, consisting of one or two nets on top of each other which could be raised and lowered to the canopy. Nets were operated each day (weather permitting) between 07:30 and 17:00 during the study season. On very hot days, nets were operated until the early afternoon and then again in the late evening (16:00-19:15). Migratory birds typically arrive across the GOM from the mid-morning through the afternoon (Gauthreaux Jr 1971); therefore birds were classified as new arrivals if they were captured between 10:00 - 17:00 on days with a noticeable increase in birds on the site throughout the day. Occasionally large flocks of birds were observed to arrive late in the afternoon, leading to a substantial increase in birds at the site, and on these days birds were classified as new arrivals if captured after the flock arrived. Birds were banded with an aluminum USGS leg band and physical measurements, including subcutaneous fat, body mass, and unflattened wing chord, were recorded. Subcutaneous fat deposits were visibly scored based on Helms and Drury (1960). The species, age, and sex of each bird was determined based on plumage as in Pyle (1977).

Fecal samples were collected from migrant passerines, as they are widely used as proxies for investigating the gut microbiota and are non-invasive (De Filippo et al. 2010, Degnan et al. 2012, Amato et al. 2013, Ryu et al. 2014). Two focal species were chosen for analysis: Gray Catbird (GRCA, *Dumetella carolinensis*) and Swainson's Thrush (SWTH, *Catharus ustulatus*). These species were selected because they are abundant migrants at the study site, thereby providing high capture rates, and are large enough to provide adequate quantities of feces. GRCA were not sampled until after April 15th to

ensure that birds were migrants and not wintering at the site. Upon capture, focal birds were placed in individual compartments of holding boxes atop clean foil-wrapped cardboard trays. Birds were left in the compartments for up to 30 minutes to allow for defecation to occur; once a bird defecated it was removed to be processed and the feces were transferred into a 2mL sterile tube. Materials were not re-used between birds. Filled tubes were placed in a cooler with ice until the end of the day when they were frozen at -20°C until the DNA was extracted. Repeat freeze-thaw cycles were avoided.

Energetic condition was assessed as a measure of subcutaneous fat, unflattened wing chord, and body mass (Ellegren 1989, 1992, Owen and Moore 2006). For each focal species, data from birds captured of that species at the Johnson's Bayou study site from 1993-2013 were used for analysis. Birds were divided into similar wing chord increments (rounded up to the nearest millimeter) and, for each increment, mass was regressed on the fat score of birds. Wing chord increments with less than 25 individuals were excluded. The intercept from the first regressions (corresponding to average fat-free mass for that wing chord) were then regressed against their corresponding wing chord increments. The resulting equation was applied to each bird sampled during spring 2014 to get a measure of its size-corrected fat-free mass, and each bird's energetic condition was calculated by subtracting its expected fat-free mass from its actual mass.

Fall Migration

Fecal samples were collected from GRCA and SWTH during fall migration (August – October 2014) at Bon Secour National Wildlife Refuge along the Fort Morgan Peninsula in coastal Alabama ($30^{\circ}10'N$, $88^{\circ}00'W$). The study site, composed of a mixture of pine forest and oak scrub, can serve as the last stopover habitat for many birds

departing over the Gulf of Mexico (Smolinsky et al. 2013). An array of 32 lengths of mist net (12 x 2.6m and 6x2.6m nets, 30mm mesh) was used to capture birds. Nets were opened daily 30 minutes before sunrise and generally closed in the late morning or early afternoon due to heat. Procedures for banding and processing birds and collecting and storing fecal samples were the same as those described for spring birds in Louisiana. Energetic condition was calculated for each bird similar to the method described above for spring, except using equations derived from a dataset of GRCA and SWTH sampled during fall migration 1990-2013 at Fort Morgan.

DNA Extraction

DNA was extracted from fecal samples using the PowerLyzer® PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA) with a few modifications. Approximately 0.15g of feces were added to the extraction kit if possible; however most samples were below this mass and so all of the feces was added to the kit. DNA was eluted with 50µL of the elution buffer in the final step.

Next-generation Sequencing

16S rRNA gene sequencing was performed by MR DNA Molecular Research LP (Shallowater, TX). Samples were individually barcoded (bTEFAP®) and the V1-V3 region (primers 27F-518R) was amplified using HotStarTaq Plus Master Mix Kit (Qiagen, USA) with an initial denaturing step at 94°C for 3 minutes, then 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute, with a final elongation step of 72°C for 5 minutes. The V1-V3 region was chosen for analysis as it has been shown to be effective at discriminating taxonomic differences between bacteria (Chakravorty et al. 2007). Amplicons from all samples were pooled and purified, and

300bp paired-end sequencing was performed on the Illumina MiSeq platform according to manufacturer protocols using standard Illumina MiSeq sequencing controls.

Processing of sequences was performed using the MR DNA analysis pipeline (Shallowater, TX). Paired-end reads were joined, barcodes and primers were removed, and sequences with <150bp, ambiguous base calls, or homopolymer runs >6bp were removed. Sequences were denoised, clustered into Operational Taxonomic Units (OTUs) at 97% similarity, chimeras were removed, and taxonomy was assigned using BLASTn against a database derived from GreenGenes, RDP11 and NCBI. Non-bacterial OTUs were removed from the dataset. OTUs with total abundance of less than 10 reads and present in less than 5 samples were considered rare and removed from the dataset (Stanley et al. 2013). Taxonomic identity of OTUs was assigned based on percent identity to known sequences, with >95% similarity classified to genus, 90-95% classified to family, 85-90% classified to order, 80-85% classified to class, and 77-80% classified to phylum (Ishak et al. 2011). Using the phyloseq package in R (McMurdie and Holmes 2013), samples were rarefied to the minimum read count among birds in the study 100 times and averaged together (Stanley et al. 2013).

Statistical Analysis

Analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria). Sequencing data was visualized with non-metric multidimensional scaling (NMDS) using the Bray-Curtis dissimilarity index. Species and season differences were tested using a Permutational Multivariate Analysis of Variance (PERMANOVA) with 10000 permutations. Effects of condition and sampling time on the microbiota were tested using Mantel tests with 10000 permutations. All NMDS,

PERMANOVA, and Mantel tests were implemented using the *vegan* function (Oksanen et al. 2014). Phylogenetic trees were constructed via UPGMA in MEGA6 (Tamura et al. 2013) and a heatmap displaying the abundance of select OTUs in spring and fall birds was created using the *gplots* package in R (Warnes et al. 2015). Kruskal-Wallis tests were used to compare the abundance of potentially pathogenic bacteria between species and seasons. Post-hoc Dunn's Tests using the built-in Bonferroni correction, implemented with package *dunn.test* (Dinno 2015), were performed with significant Kruskal-Wallis results. Significance of correlations between energetic condition and the abundance of potentially pathogenic and beneficial bacteria was assessed via Spearman correlations. P-values when running multiple tests were adjusted using a Holm-Bonferroni correction for multiple comparisons (Holm 1979).

Results

Microbiota Composition

Fecal samples from 21 GRCA (13 spring, 8 fall) and 18 SWTH (11 spring, 7 fall) were analyzed with Illumina sequencing. A total of 2,478,194 good-quality non-rare reads were obtained from the 39 birds, averaging 63,543.4 reads/bird (range: 3889-221,440). 3691 OTUs were included in the final analysis, averaging 702.4 OTUs/bird (range: 208-1522).

Though highly variable between birds, the phylum-level microbiota of the two species was generally dominated by Proteobacteria, Firmicutes, and (to a lesser extent) Actinobacteria (Figure 1). Bacteroidetes and other phyla were relatively rare, however one spring GRCA was dominated by Tennericutes. The most prevalent bacterial families

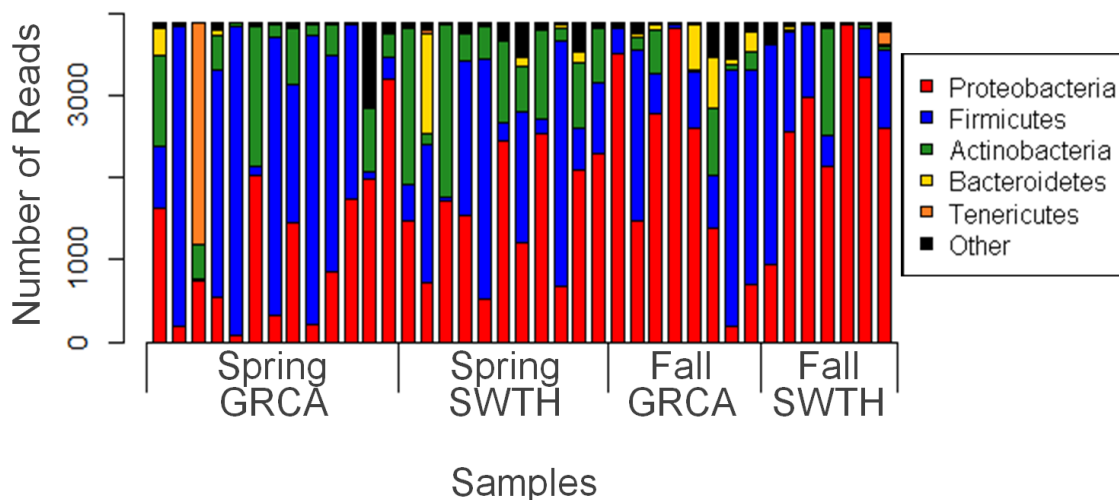


Figure 1. Phylum level microbiota communities of two species of songbird captured at stopover during both spring and fall migration along the northern Gulf Coast.

in spring were *Rhodobacteraceae* (mostly of the genus *Paracoccus*), *Lactobacillaceae* (largely *Lactobacillus*), unclassified Lactobacillales, *Enterobacteriaceae* (largely *Escherichia/Shigella*), and *Enterococcaceae* (unclassified *Enterococcaceae* and *Enterococcus*), with lesser numbers of *Microbacteriaceae*, *Nocardioideaceae* (largely *Marmoricola*), and *Dermacoccaceae*. In fall, migrants were dominated by *Enterobacteriaceae*, predominantly of the genus *Escherichia/Shigella*, *Yersinia*, *Raoultella*, and *Lonsdalea*. Fall birds also showed lower numbers of *Enterococcaceae* (mostly *Enterococcus*), *Lactobacillaceae* (largely *Lactobacillus*), and *Leuconostocaceae*. Figure 2 shows a heatmap of the top 50 most abundant OTUs in migrants.

Despite the high degree of inter-individual variation, a core microbiota was identified in the passerine migrants. All birds across both seasons shared 30 OTUs (0.81% of non-rare OTUs), with the shared OTUs composing an average of 54% of reads/bird (SD=29%). Shared OTUs were members of four phyla (Table 1): Actinobacteria (Class Actinobacteria), Proteobacteria (Class Alphaproteobacteria and Gammaproteobacteria), Firmicutes (Class Bacilli), and one OTU from Tenericutes

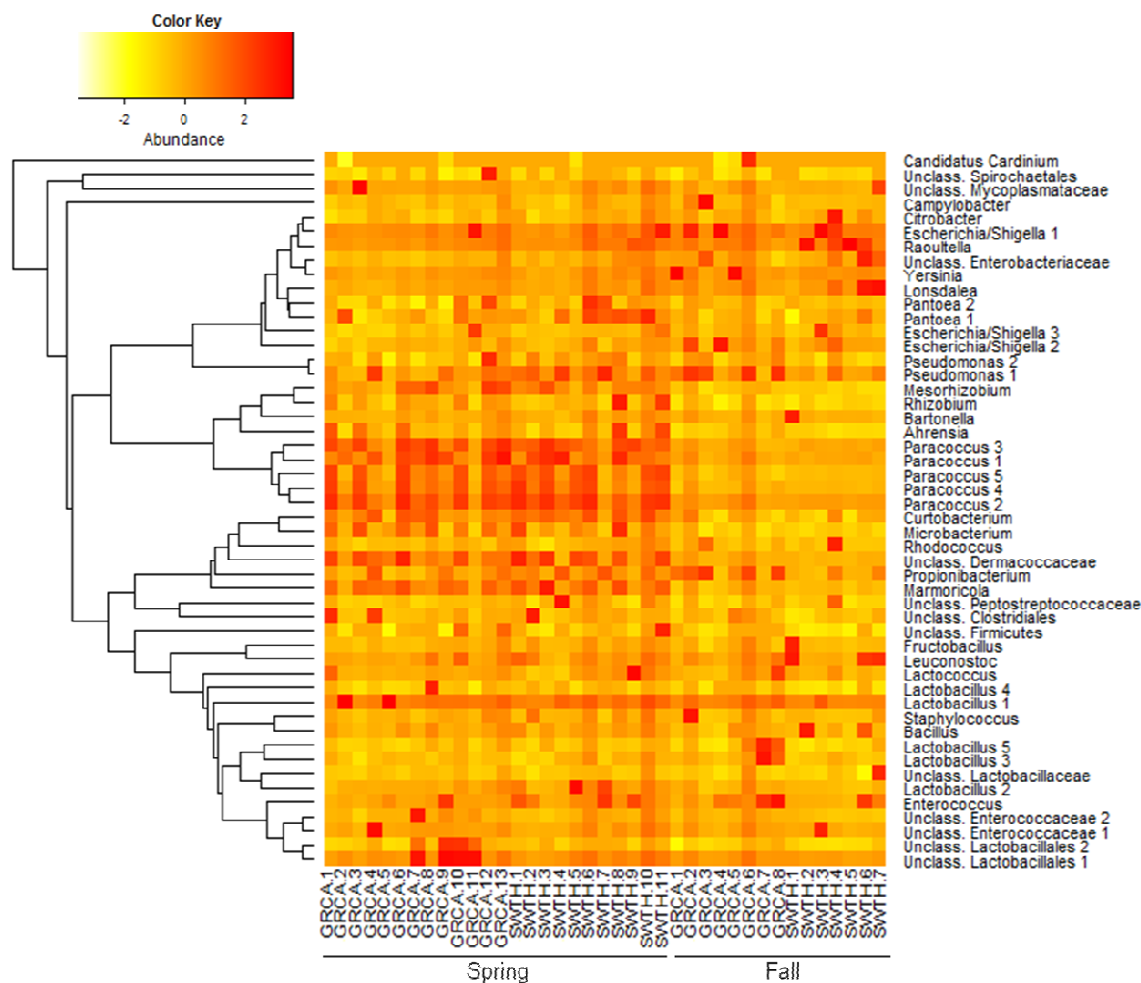


Figure 2. Heatmap showing the abundance of the top 50 OTUs identified from spring and fall migrants. Abundance values are log₁₀-transformed normalized read counts.

(Class Mollicutes). Within the core microbiota were potentially beneficial bacteria, largely *Lactobacillus*, as well as potentially pathogenic bacteria such as *Escherichia/Shigella*, *Yersinia*, and *Pseudomonas*. The 9 most abundant types of bacteria found in the core microbiota were also the most common bacteria in spring and/or fall birds.

A variety of potentially pathogenic bacteria were identified within the microbiota of spring and fall birds. Bacteria belonging to the *Escherichia/Shigella* group were the most prevalent, composing an average of 3.3% of sequences/bird in spring (SD=9.8%)

Table 1

Identities and abundance of all OTUs shared by all GRCA and SWTH during both spring and fall migration.

Taxa	Percent Composition
<i>Paracoccus</i>	15.94
<i>Lactobacillus</i>	12.98
<i>Escherichia/Shigella</i>	12.33
Unclassified Lactobacillales	9.94
<i>Raoultella</i>	7.29
<i>Yersinia</i>	7.10
Unclassified <i>Enterococcaceae</i>	3.97
<i>Lactococcus</i>	3.70
<i>Lonsdalea</i>	3.65
Unclassified <i>Mycoplasmataceae</i>	3.55
Unclassified <i>Dermacoccaceae</i>	3.14
<i>Pseudomonas</i>	3.05
<i>Leuconostoc</i>	2.45
<i>Staphylococcus</i>	1.90
<i>Marmoricola</i>	1.80
<i>Bacillus</i>	1.37
<i>Bartonella</i>	1.19
<i>Propionibacterium</i>	1.17
<i>Microbacterium</i>	1.07
<i>Fructobacillus</i>	1.05
Unclassified <i>Lactobacillaceae</i>	0.65
<i>Bradyrhizobium</i>	0.41
<i>Enterococcus</i>	0.31

Note. Percent composition refers to the percentage of total reads mapped to OTUs shared between all birds which were aligned to a particular genus. Multiple OTUs were aligned to unclassified *Enterococcaceae* (2), *Escherichia/Shigella* (2), *Lactobacillus* (2), and *Paracoccus* (5).

and 13.9% of sequences/bird in fall (SD=30.2%). *Pseudomonas* was also relatively common in both spring (mean=2.3% of reads/bird, SD=4.4%) and fall (mean=2.8% of reads/bird, SD=4.2%). *Campylobacter*, *Staphylococcus*, *Yersinia*, *Enterobacter*, and

Klebsiella were often found at low abundance (frequently <0.5% of reads/bird), though 3 fall GRCA were dominated by these bacteria (2 by *Yersinia* and 1 by *Campylobacter*) while another showed >30% abundance of *Staphylococcus*. *Clostridium* and *Salmonella* were each identified in less than half of the birds and found at very low abundance, so they were not included in further analysis. Abundance of *Klebsiella* was significantly higher in fall SWTH than in spring GRCA, though this was not significant after the correction factor (Kruskal-Wallis Test: $\chi^2 = 10.49$, $df=3$, $p=0.015$, adjusted $\alpha=0.0026$). Similarly, *Yersinia* showed a trend towards higher abundance in fall (Kruskal-Wallis Test: $\chi^2 = 4.32$, $df=1$, $p=0.038$, adjusted $\alpha=0.0028$).

Hatch-year (HY) birds in the fall showed a higher abundance and prevalence of enteropathogens than did after hatch-year (AHY) birds. No AHY bird had >3% abundance of a potential pathogen, however all 11 HY birds had >3% abundance of a least one potential pathogen with 5 of these birds showing >50% abundance. Birds which could be reliably aged in spring were more uniform in the distribution of pathogens, as 1 out of 3 second-year (SY) and 4 out of 17 after second-year (ASY) birds showed >3% abundance of any one pathogen. No spring bird showed >50% abundance of any one pathogen. Statistical tests were not performed due to the low sample sizes for AHY (1 GRCA, 3 SWTH) in fall and SY (2 GRCA, 1 SWTH) in spring.

Season and Species Differences

The microbiota of migratory passerines was characterized by a high degree of inter-individual variation. As can be seen in Figure 3, the microbiota communities showed strong differences between seasons (Pseudo-F=3.43, $df=1$, $p<0.001$) but did not

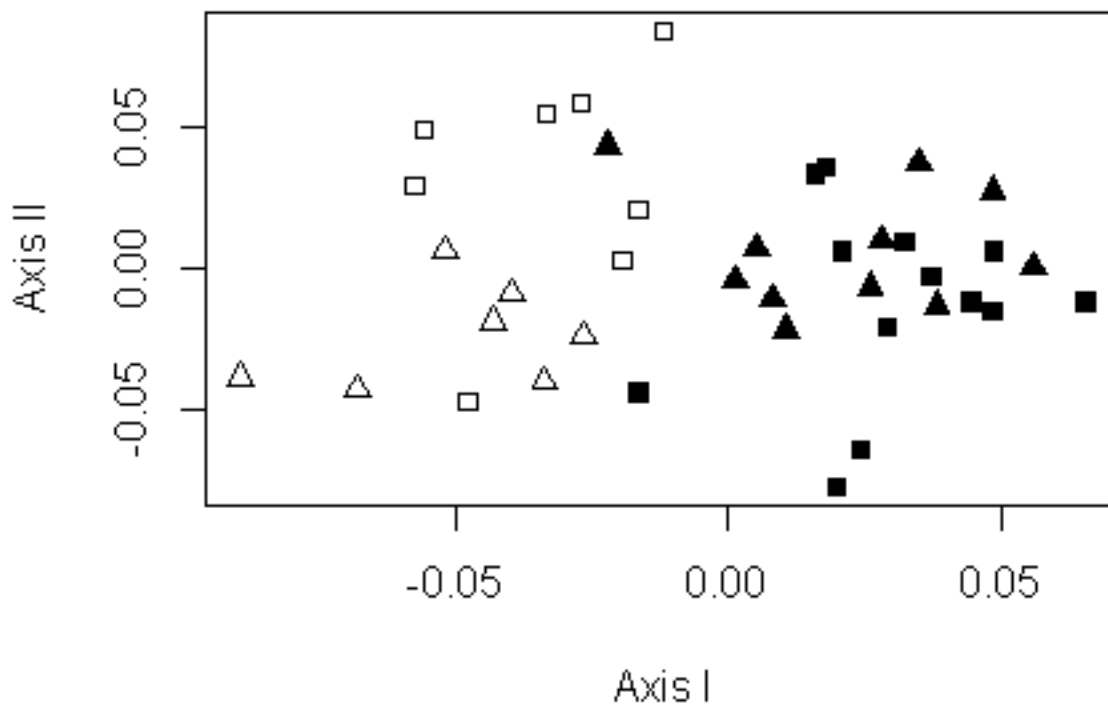


Figure 3. First two NMDS axes ($k=3$, stress=0.17) of microbiota profiles from GRCA (squares) and SWTH (triangles) during spring (filled) and fall (open) migration along the Gulf Coast.

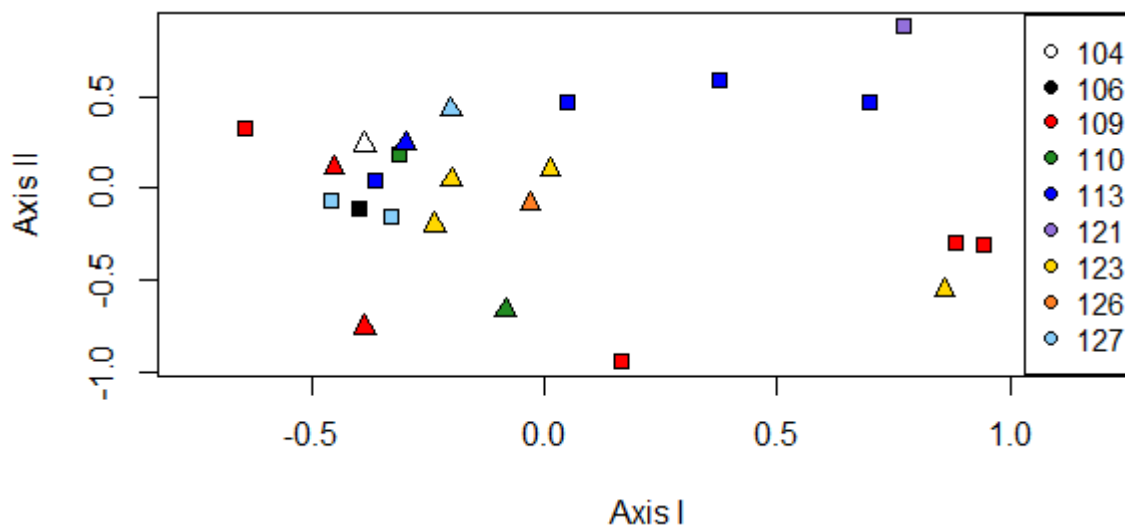


Figure 4. First two NMDS axes ($k=3$, stress=0.14) showing microbiota profiles of GRCA (squares) and SWTH (triangles) after crossing the GOM during spring migration. Points of similar color represent birds sampled on the same date.

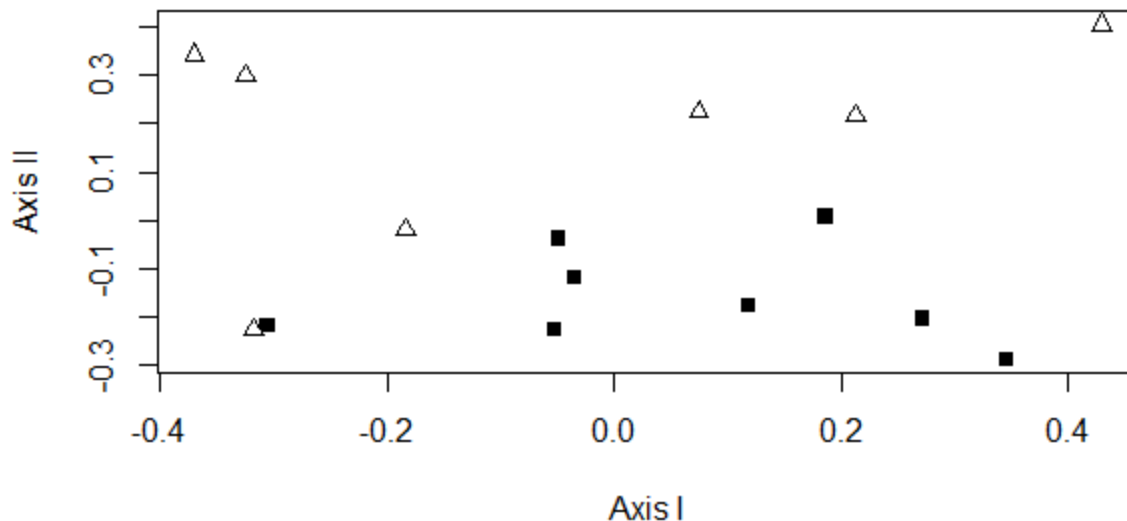


Figure 5. First two NMDS axes ($k=3$, stress=0.11) showing microbiota profiles of GRCA (filled squares) and SWTH (open triangles) at stopover along the northern GOM during fall migration.

show overarching species-specific profiles (Pseudo-F=1.24, $df=1$, $p=0.15$). The microbiota of SWTH and GRCA did not separate into distinct species groups in spring (Pseudo-F=1.32, $df=1$, $p=0.13$), and in fact did not cluster strongly based on any factor measured. There seemed to be a minor influence of sampling date on microbiota communities in spring migrants (Figure 4). Birds from the two days with the highest sample totals (days 109 and 113) showed significant differences in microbiota profiles along the 2nd NMDS axis (two-tailed t-test: $t=2.84$, $df=9$, $p=0.02$). Sample sizes for other days in spring were low. In contrast, fall birds showed clear-species specific profiles (Pseudo-F=1.58, $df=1$, $p=0.024$, Figure 5). No clear patterns were observed with overall community structure and bird age in either season, though sample sizes may have been too low to detect patterns.

Condition

Sampled birds showed variable energetic condition in both spring (mean: 2.65, $SD=3.12$) and fall (mean: 1.90, $SD=4.78$). Energetic condition did not vary significantly

by season (Kruskal-Wallis Test: $\chi^2= 1.61$, $df=1$, $p=0.20$), species (Kruskal-Wallis Test: $\chi^2= 0.26$, $df=1$, $p=0.61$), or the species and season interaction (Kruskal-Wallis Test: $\chi^2= 1.98$, $df=3$, $p=0.58$). Spring migrants sampled later in the day, which would have been on the site longer and had more time to feed, didn't show differing microbiota compared to birds sampled earlier in the day (Mantel $r=0.004$, $p=0.047$), therefore the sampled microbiota seem to be representative of arrival microbiota. Microbiota profiles did not differ between birds of either species based on energetic condition after arriving across the GOM in spring (Mantel $r=0.03$, $p=0.36$). Similarly, gut microbiota was not related to condition during fall migration (Mantel $r=0.01$, $p=0.43$).

After applying the Holm-Bonferroni correction, no significant correlations were found in either season between the condition of migrants and the abundance of potential pathogens (identified above) or bacteria with potentially beneficial functions (*Lactobacillus* and *Lactococcus*). Without the correction factor, *Escherichia/Shigella* (S=1078, Spearman's Rho=0.53, $p=0.008$, adjusted $\alpha=0.0028$), *Campylobacter* (S=1231, Spearman's Rho=0.46, $p=0.02$, adjusted $\alpha=0.0029$), *Lactococcus* (S=1271, Spearman's Rho=0.45, $p=0.03$, adjusted $\alpha=0.0031$), *Yersinia* (S=1282, Spearman's Rho=0.44, $p=0.03$, adjusted $\alpha=0.0033$), and *Enterobacter* (S=1376, Spearman's Rho=0.40, $p=0.05$, adjusted $\alpha=0.0036$) showed significant positive correlations to condition in spring. The results obtained for *Escherichia/Shigella* and *Lactococcus* were driven largely by one or two birds with high levels of the bacteria and good condition; however *Yersinia*, *Enterobacter*, and *Campylobacter* showed a weak trend towards higher loads in better condition birds. No significant correlations between bacterial abundance and condition were observed in fall.

Energetic condition may not be linearly related to gut morphology. Migrants remove protein from the gut throughout flight, however this process is greatly accelerated once fat reserves drop below 5-10% of body mass (Schwilch et al. 2002). A crude method of determining the percent of fat (%fat) on each bird was calculated by dividing the energetic condition by the mass, with birds falling below 5% classified as “lean” and birds falling above 10% classified as “fat”. Individuals falling in the 5-10% range (3 GRCA, 4 SWTH in spring) were not included in the analysis. PERMANOVAs found no significant relationship between %fat and microbiota communities in either spring (Pseudo-F=0.88, df=1, p=0.62) or fall (Pseudo-F=0.71, df=1, p=0.86). Mann-Whitney U-tests were implemented to test the abundance of potentially pathogenic and beneficial bacteria in fat and lean birds. Results were similar to the linear correlation analysis, though fall migrants showed a trend towards higher *Lactobacillus* abundance in lean birds (W=8, p=0.04, adjusted α =0.0028).

Discussion

Microbiota Composition

This study provides the first community-scale characterization of the gut microbiota of passerines during migration. Gray Catbirds and Swainson’s Thrushes during both spring and fall showed a high degree of inter-individual variation in terms of overall microbiota community structure as well as dominant types of bacteria. This variability is consistent with many studies performed on non-laboratory animals (De Filippo et al. 2010, Osei-Poku et al. 2012, Hird et al. 2014, Ryu et al. 2014, Baxter et al. 2015), and likely reflects the multitude of environmental and physiological factors which can influence microbial assemblages. In the current study a variety of factors which could

not be controlled likely influenced the observed inter-individual variation. Birds were sampled midway through migration and may have originated from different areas of the breeding and wintering range. Moreover, birds likely used differing stopover habitats in Central America before crossing the GOM and so would have been exposed to a wide variety of differing foods and environments before arriving at the site. Diet and patch effects are major determinants of microbiota composition, so it is unsurprising that birds utilizing differing areas and habitats prior to arrival at the site would show differing microbiota (Klomp et al. 2008, Ley et al. 2008). In addition, age, sex, and genetic differences among birds may have influenced community structure (Benson et al. 2010, Bolnick et al. 2014, Waite et al. 2014).

Despite this variability, a core microbiota composed of 30 OTUs comprising over half of the total number of reads was common to all birds sampled. Moreover, the most abundant taxa composing the core microbiota (*Paracoccus*, *Lactobacillus*, *Escherichia/Shigella*, Unclassified Lactobacillales, *Raoultella*, and *Yersinia*) were the most abundant taxa in at least one season, which suggests that the observed variation between individuals is more attributable to changes in the abundance of core microbes than to differences in the types of bacteria present. Rerunning the analyses using only the 30 core OTUs yielded very similar results to the whole community dataset in terms of species, condition, and seasonal effects (data not shown), further supporting this notion. Different types of birds seem to have differing core microbiota; Bennett et al. (2013) identified a core in Emus (*Dromaius novaehollandiae*) dominated by *Bacteroides*, *Escherichia*, and *Fusobacterium* while Ryu et al. (2013) identified a core dominated by *Bacillus* in shorebirds. The core microbiota identified in this study appears to be

temporally and geographically stable as it was detected during spring in Louisiana and fall in Alabama. The fact that the core microbes were identified in such widely varying birds of two species implies that they are normal colonizers of the gut of these two species, and may be adapted to the high throughput times showed by passerines (Levey and Karasov 1994). Further study is needed to determine how widespread these core microbes are in the guts of other species of passerine as well as the mechanisms behind their colonization and persistence in avian guts.

In general, the microbiota of the two species was dominated by Proteobacteria and Firmicutes with very low levels of Bacteroidetes. This is in direct contrast to the microbiota dominated by Firmicutes and Bacteroidetes in mammals (Ley et al. 2008) and reptiles (Hong et al. 2011), though it is consistent with findings from shorebirds (Ryu et al. 2014), Kakapos (*Strigops habroptilus*, Waite et al. 2014), and other passerines (Hird et al. 2014, Mirón et al. 2014). The abundance of Alphaproteobacteria (largely *Paracoccus*) was surprising, as these bacteria are generally associated with sediment (Siller et al. 1996) and rarely identified in gut communities (Shulzhenko et al. 2011). It is unlikely that these are merely transient bacteria due to their overall abundance and presence in the core microbiota. High to moderate abundance of Alphaproteobacteria have also been identified in certain species of shorebirds (Santos et al. 2012, Grond et al. 2014) and kittiwakes (van Dongen et al. 2013), with Santos et al. (2013) identifying *Paracoccus* as a major component of the microbiota of Icelandic Black-tailed Godwits (*Limosa limosa*). Alphaproteobacteria may therefore serve some as-of-yet unknown role in the avian gut microbiota.

Bacteria of the genus *Lactobacillus* were common in both spring and fall, and were one of the most abundant microbes in the core microbiota. *Lactobacillus* is widely used as a probiotic, and has been found to promote growth in chickens, inhibit the growth of pathogens, and prevent their attachment to epithelial cells (Jin et al. 1996, Johnson-Henry et al. 2007, Million et al. 2012). Bacteria aligning to genus *Lactococcus*, which are known to stimulate the growth of beneficial bacteria and reduce pathogen abundance (Sun et al. 2012), were also identified in all birds sampled. Though there was no direct correlation between these two taxa and energetic condition, it is still possible that these bacteria have some beneficial functions for migratory passerines. Passerines show reduced immune function during migration (Owen and Moore 2006) and are frequently exposed to novel habitats, therefore beneficial bacteria such as *Lactobacillus* and *Lactococcus* may be especially important during this phase of the annual cycle to prevent the proliferation of pathogenic bacteria.

Several potentially pathogenic bacteria were found in the gut microbiota, with the most abundant being *Escherichia/Shigella*. It is important to note that the virulence of bacterial strains was not investigated in this study so it cannot be conclusively stated that the bacteria were pathogenic or might be influencing bird fitness. For example, Duriez et al. (2001) found that commensal strains of *Escherichia coli* were widespread in human populations while virulence factors were rare. Similarly, most types of *Yersinia* identified in migratory birds in Sweden were non-pathogenic (Niskanen et al. 2003). *Campylobacter jejuni* largely acts as a commensal in chickens, though it can still be transferred and pathogenic to other organisms (Hendrixson and DiRitta 2004). Even though the virulence of bacteria was unknown, the abundance of enteropathogens

identified in this study indicates that migratory passerines have the potential to act as vectors for long-distance disease transmission (Reed et al. 2003).

Relationship between Energetic Condition and Microbiota

The energetic condition of migrant passerines was not related to the community structure of their microbiota after crossing the GOM during spring, nor was it related to microbiota in fall, thus rejecting the hypothesis that energetic demand experienced during prolonged migratory flight will be reflected in the gut microbiota. There are several possible explanations as to why energetic condition was not related to microbiota communities. Poorer condition migrants after crossing the GOM show reduced immune profiles (Owen and Moore 2008b); however sampling birds immediately after the 12-35 flight across the GOM may not be enough time to see downstream effects of the reduced immune function on the microbiota. A second explanation is that energetic condition on arrival does not adequately reflect the energetic demand experienced during crossing or the degree of resulting gut atrophy. Finally, it is possible that energetic demand experienced during migration did influence microbiota; however changes were not stereotypic across individuals. Individually different responses of the gut microbiota to the same stimulus have been found in humans (Dethlefsen and Relman 2011), and so responses to energetic demand during crossing may have varied between birds depending on what microbes were already present in the gastrointestinal tract. Significant seasonal differences were observed in gut microbiota, which may have been partly influenced crossing the GOM in spring but also likely influenced by differing habitats and diets of birds before reaching the study sites in each season. Fall migrants, therefore, may not serve as an accurate representation of pre-crossing microbiota. To adequately assess the

influence of prolonged migratory flight on passerine gut microbiota, birds should be sampled within the same season both after crossing the GOM and before crossing in Central America.

Neither fall nor spring migrants showed the expected negative relationship between abundance of pathogens and energetic condition, and spring migrants actually showed weak trends towards higher loads of *Yersinia*, *Enterobacter*, and *Campylobacter* in better condition birds. These results are in contrast to the findings of Garvin et al. (2006) that many species of Nearctic-Neotropical migrants show a negative relationship between physical condition and blood parasites at stopover. The enteropathogens identified in this study may not be pathogenic and/or their pathogenicity may be mitigated by the other microbes in the gut community.

Seasonal Differences

The microbiota of GRCA and SWTH differed significantly between seasons. The same birds were not sampled in both spring and fall and, though it was unknown where exactly the birds were coming from, birds in different seasons possibly could have come from different populations or differing parts of the breeding range between seasons. In spring, birds had finished wintering in the Neotropics and had crossed the GOM before being sampled, while fall birds had only migrated south through North America. Though the sampled migrants in each season likely used differing stopover areas and ate differing foods, it is nevertheless possible that birds migrating north through the Neotropics may have broadly different experiences and food resources available compared to southbound migrants through the US. The microbiota of fall migrants may reflect conditions in North America while the microbiota of spring birds may reflect conditions in Central and South

America. Additionally, the common experience in spring of crossing the GOM may have influenced the observed seasonal differences in microbiota.

Fall HY birds consistently showed higher abundances of potential pathogens than did fall AHY birds, and these age-related differences explain why several types of enteropathogens showed trends towards higher abundance in fall. This is consistent with age-related differences in blood parasites of Rock Pigeons (*Columba livia*, Sol et al. 2003) and Great Tits (*Parus major*, Allander and Bennett 1994). These differences between age classes may be attributable to a maturation of the immune system in older birds. Additionally, young of many species, including birds, show highly transient microbiota before developing into a more stable adult community later in life (Kohl 2012, Lozupone et al. 2012). Although it is unknown exactly how long it takes for passerines to reach the more stable adult microbiota, it is possible that the microbiota of young birds during fall migration may still be transient thus more susceptible to colonization and proliferation of enteropathogens. A more transient microbiota in young fall birds that has stabilized by the following spring could explain why spring migrants did not show the same age-related effects on enteropathogen load. Finally, it is possible that birds with large populations of enteropathogens did not survive the winter. The fact that no relationship was found between enteropathogen load and energetic condition suggests that the abundance of these bacteria in HY birds may not negatively impact their fitness; however longer-term monitoring is needed to determine what impact these high enteropathogens loads may have on annual survival.

The microbiota of spring migrants showed some differences among birds migrating through the site on different days of the year. The density of migrants at the

site was influenced by weather patterns, with the majority of migrants overflying the site to more continuous forest further inland when experiencing tailwinds and large numbers stopping at the site only if birds experienced inclement weather or headwinds en route (Gauthreaux Jr. 1971), therefore the distribution of collected samples was biased towards particular days. Specifically, birds sampled on the two highest capture days during the season (days 109 and 113) showed significant differences in their microbiota. Densities of migrant passerines in a particular area are influenced by winds aloft (Richardson 1971) and so it is likely that winds experienced during crossing on a particular day may bring birds from different source areas in Central America. Birds in similar environments before crossing the GOM are likely to show similar microbiota (Klomp et al. 2008, Amato et al. 2013, Chapter III), so it is possible that migrants arriving across the GOM which have come from broadly similar areas may have similar microbiota.

Though strong seasonal differences were observed in the microbiota of GRCA and SWTH, no overarching species-specific microbiota profiles were detected. Studies have found conflicting results on the influence of host species on avian gut microbiota, with species-specific profiles being identified in seabirds on the breeding grounds (Dewar et al. 2014) but not in shorebirds during migration (Santos et al. 2012, Ryu et al. 2014). The strong seasonal differences in microbiota observed in this study imply that SWTH and GRCA do not show innate species profiles due to differing gut morphology and physiology, and instead their microbiota is more strongly influenced by environmental conditions experienced during each season changing the abundance of core microbes (Hird et al. 2014).

Despite not showing immutable species-specific microbiota profiles, GRCA and SWTH did exhibit species-specific profiles during fall but not during spring migration. One explanation for this result is that the differential microbiota between species in the fall is a reflection of the different breeding ranges between the two species. The majority of SWTH stopping at the Fort Morgan study site breed in the boreal forests of Central Canada while the majority of GRCA breed in shrubby edge habitats in the Southeastern US (Mack and Yong 2000, Hobson et al. 2007, Smith et al. 2011). In addition, SWTH on the breeding grounds eat predominantly invertebrates while GRCA feed on both invertebrates and fruit (Graber et al. 1970, Holmes and Robinson 1988). Although microbiota communities may change based on conditions at stopover before reaching the study sites (Dimitriu et al. 2013, Chapter III), microbes characteristic of one habitat can persist in the gut over long periods of time despite subsequent changes in habitat (Degnan et al. 2012). The differences between species in fall may thus reflect the broadly different habitats and foods of the breeding grounds.

Spring migrants, in contrast, may show stronger carry-over effects from the wintering grounds. Wintering migrants of the two species consume both fruit and insects and may winter in a more variable array of habitats than in the breeding season (Orejuela et al. 1980, Rappole and Warner 1980, Willis 1980, Smith et al. 2011). The microbiota of wintering migrants would be predicted to show greater intra-species and reduced inter-species variation. Carry-over effects from the temperate breeding and Neotropical wintering areas may account for the strong seasonal differences in microbiota, as well as the lack of species-specific profiles observed in spring.

The common influence of crossing the GOM during spring may have also contributed to the lack of species-specific profiles observed during this season. Although energetic demand experienced en route doesn't seem to influence microbiota, both species of migrant are required to fast during the 12-35 hour flight across the GOM. Fasting for 24 hours has been shown to increase microbial variability between birds (Thompson et al. 2008); therefore the influence of crossing the GOM in spring may have increased variability in microbiota and masked any signal from the different diets and habitats of the two species which were observable during the fall. These explanations are speculative, and future research is necessary to determine the causative agents behind the observed differences between seasons and species. Regardless of the mechanisms involved, seasonal effects were found to have a stronger influence on the microbiota of GRCA and SWTH than did host species.

Summary and Areas of Future Work

This study provides the first community-scale examination of the gut microbiota of passerines during migration and broadens our understanding of the makeup and community structure of avian gut microbiota. Though showing a high degree of inter-individual variability, a core set of microbes was identified as being present in all birds regardless of species and season. At the phylum level, the microbiota was generally dominated by Proteobacteria and Firmicutes, with lesser numbers of Actinobacteria. Bacteria with known beneficial functions, such as *Lactobacillus*, and potential pathogens such as *Escherichia/Shigella* were common and widespread. Future experimental work would be valuable to specifically test the impact that these bacteria have on passerine fitness.

No significant relationships were observed between energetic condition and gut microbiota, thus rejecting the original hypothesis. Migrants showed strong seasonal differences in microbiota which seemed to override any species-specific differences, as the two species separated only during fall migration. The exact mechanisms driving the observed differences between species in fall but not in spring are currently unknown; however one explanation is that the microbiota of fall and spring migrants reflects carry-over effects from the breeding and wintering grounds, respectively. Future research should focus on sampling birds from other locations during migration, notably before crossing the GOM in spring, and from breeding and wintering areas in order to further elucidate how passerine gut microbiota changes throughout the annual cycle and in response to migratory flight across an ecological barrier.

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CHAPTER III
CHANGES IN GUT MICROBIOTA OF MIGRATORY SONGBIRDS AT STOPOVER
AFTER CROSSING AN ECOLOGICAL BARRIER

Introduction

The community of microbes residing in the gastrointestinal tract of animals, referred to as the gut microbiota, is known to provide such a multitude of beneficial functions to the host that it has been termed the “forgotten organ” (O’Hara and Shanahan 2006). Gut microbes have been found to play an important role in host digestion (Stevens and Hume 1998), fat metabolism (Bäckhed et al. 2004), stimulation of the immune system (Kamada et al. 2013), and competitive exclusion of pathogens (Kamada et al. 2012). The gut microbiota can be a highly diverse and complex system with community structure determined by a wide variety of factors such as diet, environment, and immune function (Turnbaugh et al. 2009, Benson et al. 2010, Dimitriu et al. 2013, Roberts et al. 2014). Any change in the external or internal environment of a host organism could influence microbiota composition.

Most studies tracking intra-individual variation in gut microbiota have focused on humans or animals in controlled captive settings, with relatively few studies performed on animals in the wild (e.g. Frey et al. 2006, Degnan et al. 2012, Waite et al. 2014). Controlled studies provide valuable information about the determinants and functions of microbiota; however they do not provide insight into the dynamics of gut microbiota in a natural environment. For example, Benskin et al. (2010) found temporally stable gut microbiota in captive Zebra Finches (*Taeniopygia guttata*); however these birds were fed similar diets and held in constant conditions. Within-or-between year changes in

individual microbiota have been identified in seabirds (White et al. 2010, Dewar et al. 2014), Kakapos (Waite et al. 2014), and nestling passerines (Mills et al. 1999), but not in non-migratory Blue Tits (*Cyanistes caeruleus*, Benskin et al. 2015). The studies on passerines focused on plating of select culturable bacteria and older analytical techniques, and so there is a dearth of information about the temporal patterns of the entire gut microbiota community of wild passerines, especially during migration.

Migration offers an attractive scenario for investigating temporal patterns and the influence of changing habitats on the gut microbiota of wild passerines. Most birds cannot fly non-stop between wintering and breeding grounds, and thus must periodically suspend flight to replenish energy reserves at stopover sites (Newton 2008). Birds at stopover during spring migration likely come from differing wintering habitats and areas of the breeding range (Ruegg et al. 2014, Paxton and Moore 2015). Moreover, individual migrants can show variable migratory routes and use of stopover sites (Lemke et al. 2013), and so birds at stopover along the northern coast of the Gulf of Mexico in spring likely utilized differing arrays of novel stopover habitats in the Neotropics. These migrants at stopover are likely to have been exposed to varying habitats, conditions, and food resources before arriving at the site, which likely explains the high variability in gut microbiota exhibited by migratory passerines recently arrived to a stopover site after crossing the Gulf of Mexico (Chapter II).

In contrast to the diverse habitats experienced by birds during the preceding portion of their migratory journeys, birds at the same stopover site are exposed to the same habitat and array of food types. Diet is one of the strongest factors influencing the makeup of the gut microbiota (Ley et al. 2008, Amato et al. 2013, David et al. 2014).

Birds at a shared stopover site are likely to consume similar types of foods, and so would likely show less inter-individual variability in gut microbiota through stopover compared to at arrival.

Ryu et al. (2014) found that three species of shorebird at stopover in Delaware Bay shared a core microbiota; however they were not able to state definitely that this was due to the shared stopover habitat. Repeat sampling of the same individual through stopover allows for the investigation of temporal dynamics as well as the influence of a shared habitat on the gut microbiota. It is likely that temporal variation in gut microbiota within an individual at stopover will be less than the degree of inter-individual variation (Costello et al. 2009), but if the shared habitat associated with stopover influences microbiota structure then the microbiota of migrants should become more similar or show similar trends between birds throughout stopover. Additionally, birds which are at the same site for longer periods of time may show stronger changes in their microbiota compared to birds at the site for shorter periods, as prolonged exposure to a shared habitat and associated food resources would likely produce stronger impacts on the gut microbiota.

Methods

Gray Catbirds (GRCA, *Dumetella carolinensis*), Swainson's Thrushes (SWTH, *Catharus ustulatus*), and Wood Thrushes (WOTH, *Hylocichla mustelina*) were sampled during spring migration 2014 (mid-March through mid-May) at the University of Southern Mississippi's long-term research station at Johnson's Bayou, LA (29°45'N, 93°37'W). Birds were captured using 24.5 lengths of mist net (12 x 2.6m and 5x2.6m nets, 30mm mesh) operated every day from 07:30 to 17:00. Captured birds were banded

with USGS aluminum leg bands and energetic condition was calculated based upon subcutaneous fat (measured via Helms and Drury 1960), unflattened wing chord, and body mass (Ellegren 1989, 1992, Owen and Moore 2006).

Upon capture, a focal bird was placed atop a clean aluminum foil-wrapped cardboard tray in a holding compartment. Birds were allowed to sit for up to thirty minutes, and if they defecated during this time the bird was removed for processing and feces were transferred to a sterile 2mL collection tube. Fecal samples are commonly used as proxies for gut microbiota and allow repeat sampling from the same individual (Benskin et al. 2010, De Filippo et al. 2010, Degnan et al. 2012, Dimitriu et al. 2013). Feces were stored in a cooler on ice until the end of the day when they were frozen at -20°C until DNA was extracted. Repeat freeze-thaw cycles were avoided. Repeat fecal samples were collected from the same bird if it was re-caught later during stopover (at least the next calendar day) and the bird's energetic condition was reassessed. Shifts in gut microbiota have been found within the same individual in less than 24 hours (Bailey et al. 2011, David et al. 2013), so this timeframe is adequate for investigating stopover-related changes in microbiota.

DNA was extracted from fecal samples using the PowerLyzer® DNA Isolation Kit (MO BIO, Carlsbad, CA) with the following modifications: up to 0.15g of feces was used for the extractions and DNA was eluted in the final step with 50µL of elution buffer. The hypervariable V1-V3 region of the bacterial 16S gene was amplified, as it has been found to provide optimal power to discriminate between bacteria down to the genus level (Chakravorty et al. 2007), and sequenced with the Illumina MiSeq platform by MR DNA Molecular Research LP (Shallowater, TX). 16S amplification was performed using

barcoded 27F forward primer (bTEFAP®), 518 reverse primer, and HotStarTaq Plus Master Mix Kit (Qiagen, USA) with an initial step of 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute, and finished with a final elongation step for 5 minutes at 72°C. PCR products were pooled, purified, and 300bp paired-end sequencing was performed with the Illumina MiSeq platform following manufacturer protocols using standard MiSeq sequencing controls.

Sequence processing was performed using the MR DNA analysis pipeline (Shallowater, TX). Paired-end reads were joined and barcodes and primers were removed. Sequences which were short (<150bp), had ambiguous base calls, or had homopolymer repeats (>6bp) were removed. Sequences were denoised and grouped into Operational Taxonomic Units (OTUs) at a 97% similarity threshold. Chimeras were removed from the dataset and taxonomy of OTUs was assigned using BLASTn against a curated database derived from GreenGenes, RDP11 and NCBI. Non-bacterial OTUs and OTUs classified as rare (occurring with <10 total reads and in <5 birds) were removed (Stanley et al. 2013). OTUs were classified to genus level based on >95% similarity to reference sequences, family from 90-95%, order from 85-90%, class from 80-85%, and phylum from 77-80% (Ishak et al. 2011). Read counts were rarefied 100 times to the lowest read count among all birds using the phyloseq package for R (McMurdie and Holmes 2013) and averaged as in Stanley et al. (2013).

All analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria). Changes in microbiota communities were visualized using non-metric multidimensional scaling (NMDS) with the Bray-Curtis dissimilarity index and significance was tested using Permutational Multivariate Analysis of Variance

(PERMANOVA) with 10,000 permutations (vegan package, Oksanen et al. 2014). Significance of the relationship between the change in energetic condition and the change in microbiota community structure during stopover, measured as the distance moved in the NMDS plot, was assessed via Spearman's correlation. Spearman's correlation was implemented as it is possible that gut microbes may influence digestive efficiency and metabolism (Stevens and Hume 1998, Bäckhed et al. 2004) while modulation of gut morphology (McWilliams et al. 2004) and feeding during stopover may influence microbiota structure (Costello et al. 2010, David et al. 2013). Similarly, distance moved in the plot was regressed against the time between initial and recapture sampling. Indicator Species Analysis was implemented with package *indicspecies* (Cáceres and Legendre 2009) to identify types of bacteria which characterized newly-arrived birds as well as bacteria which characterized recaptured birds later during stopover. An alpha of 0.05 was used for all statistical tests.

Results

Initial and recapture samples were collected from 4 WOTH, 3 GRCA, and 1 SWTH. This corresponds to only 6% (WOTH) and 3% (GRCA and SWTH) of the total number of birds of each species which had initial fecal samples collected. All birds except for one increased in condition between initial and recapture sampling (average increase: 1.5g, range: 0.4-3.5g, Table 2). The one bird which decreased in condition between sampling lost 4.3g, corresponding to 11.3% of its body mass at initial sampling. The Illumina sequencing generated 827,054 good quality non-rare reads (mean: 51,690.9 reads/bird, min: 16,090 reads, max: 136,767 reads). A total of 2578 OTUs were included in the final analysis, averaging 1190.0 OTUs/bird (range: 417-1569). The microbiota of

Table 2

Capture dates and changes in energetic condition between initial and recapture sampling.

Individual	Species	Sampling Date			Energetic Condition (g)		
		Initial	Recapture	Time (hrs)	Initial	Recapture	Change
G1	GRCA	113	114	20	1.82	4.22	2.4
G2	GRCA	109	110	25	-0.90	-0.30	0.6
G3	GRCA	111	112	28	-0.38	0.92	1.3
S1	SWTH	119	121	45	0.62	1.12	0.5
W1	WOTH	106	108	45	10.31	10.71	0.4
W2	WOTH	106	107	16	1.86	3.36	1.5
W3	WOTH	107	108	20	-0.14	-4.44	-4.3
W4	WOTH	108	110	47	-1.79	1.71	3.5

Note. Sampling date refers to day of year. Time refers to number of hours (rounded) between initial and recapture sampling.

all three species was generally dominated by Proteobacteria, Firmicutes, and Actinobacteria, though G1 was initially dominated by Tenericutes (Figure 6). Birds at stopover showed varying degrees of change in their phylum-level microbiota between initial and recapture sampling, as some birds (such as W3) did not change very much

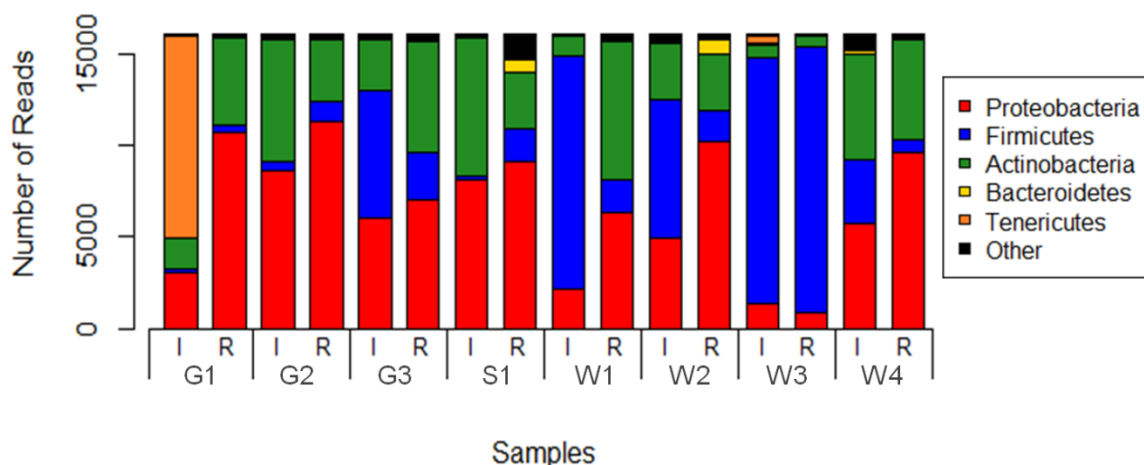


Figure 6. Changes in the phylum-level microbiota of three species of passerines throughout stopover after crossing the GOM during spring migration. Columns marked “I” represent microbiota at initial sampling and columns marked “R” represent microbiota at recapture sampling.

while others (G1 and W2) showed large differences (Figure 6). Microbiota communities of recaptured birds during stopover were not significantly different from when initially sampled (Pseudo-F=1.03, df=1, p=0.44). Despite this, the NMDS plot of the initial and recapture microbiota shows that five birds (G1, G3, S1, W1, and W4) all moved towards a similar area in the NMDS plot (Figure 7). Birds which plot closer together with NMDS have more similar microbiota communities than do birds which plot further apart, therefore the microbiota of these 5 birds became more similar during stopover. The more similar recapture microbiota of these five birds was characterized by higher levels of *Microbacterium*, Unclassified *Dermacoccaceae*, *Paracoccus*, *Yersinia*, and *Campylobacter*. Birds G2 and W2 also plotted in this same region of Figure 7; however no obvious pattern was observed with the measured factors (time between sampling, initial condition, change in condition, sampling date) that would explain why the

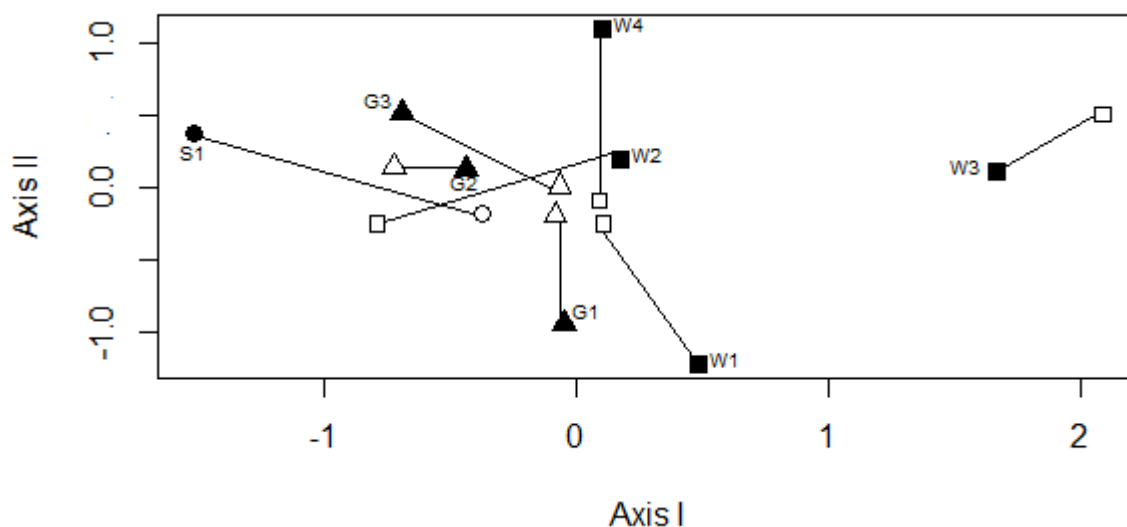


Figure 7. NMDS (k=2, stress=0.12) showing initial (filled) and recapture (open) microbiota from GRCA (triangles), SWTH (circles), and WOTH (squares) during stopover at coastal Louisiana. Connected points represent initial and recapture microbiota profiles from the same bird. Bird ID is labeled next to each initial sample.

microbiota of these two birds would not become more similar to the other birds through stopover. The microbiota of the one bird which lost condition at the site (W3) was initially distinct from the microbiota of other birds and became increasingly dissimilar during stopover. The microbiota of this bird was characterized by high initial loads of *Enterococcus*, which increased throughout stopover.

The degree of change in microbiota community structure was not significantly correlated to the change in energetic condition between sampling ($r=0.45$, $df=6$, $p=0.27$). In contrast, the degree of change in microbiota showed a significant positive linear relationship with the time between initial and recapture sampling ($F_{1,6}=15.4$, $p=0.008$, $R^2=0.72$). Birds which were at the site for longer periods of time between sampling thus showed greater changes in microbiota structure than did birds with less time between sampling (Figure 8).

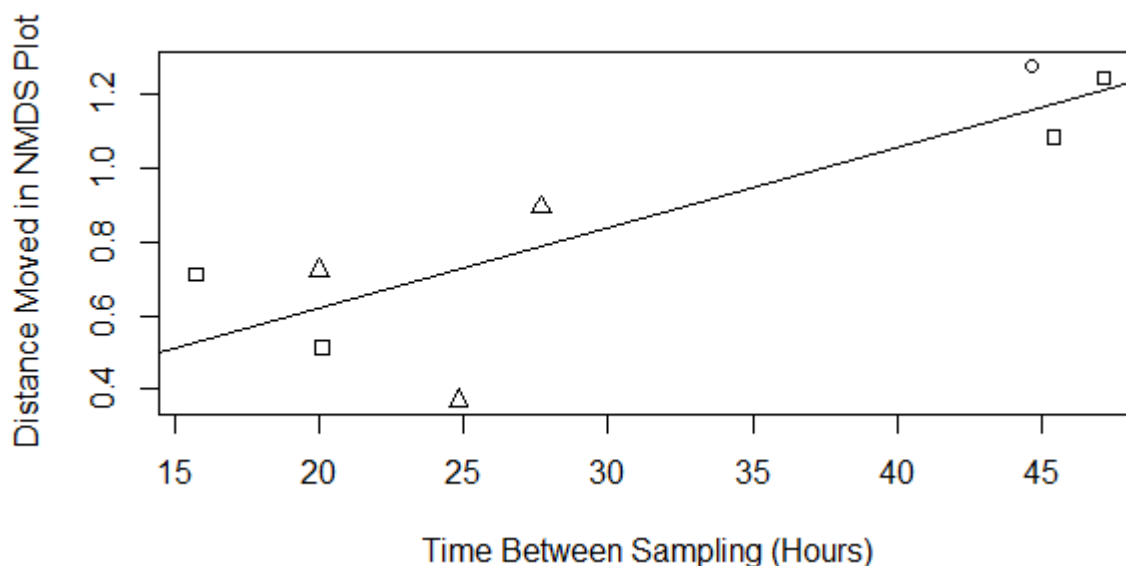


Figure 8. Plot of time between sampling and change in the microbiota of GRCA (triangles), SWTH (circle), and WOTH (squares) during stopover at coastal Louisiana. $R^2=0.72$.

None of the OTUs identified through the indicator species analysis increased in all birds or decreased in all birds. Among the OTUs representative of recapture samples, three of the highest indicator value OTUs mapped to *Lactobacillus* and composed a combined 1% of total reads. Within each bird the pattern of increase or decrease in abundance between sampling was similar between these three OTUs, and so they were combined for analysis. All birds which gained condition between sampling also showed a significant increase in the abundance of these types of *Lactobacillus* (one-tailed paired t-test: $t=2.43$, $df=6$, $p=0.026$), while the one bird which lost condition between sampling showed reduced abundance (Figure 9).

Discussion

Despite collecting initial fecal samples from 212 birds of three species, only 8 birds were re-sampled on a subsequent day. The very low number of next-day recaptures

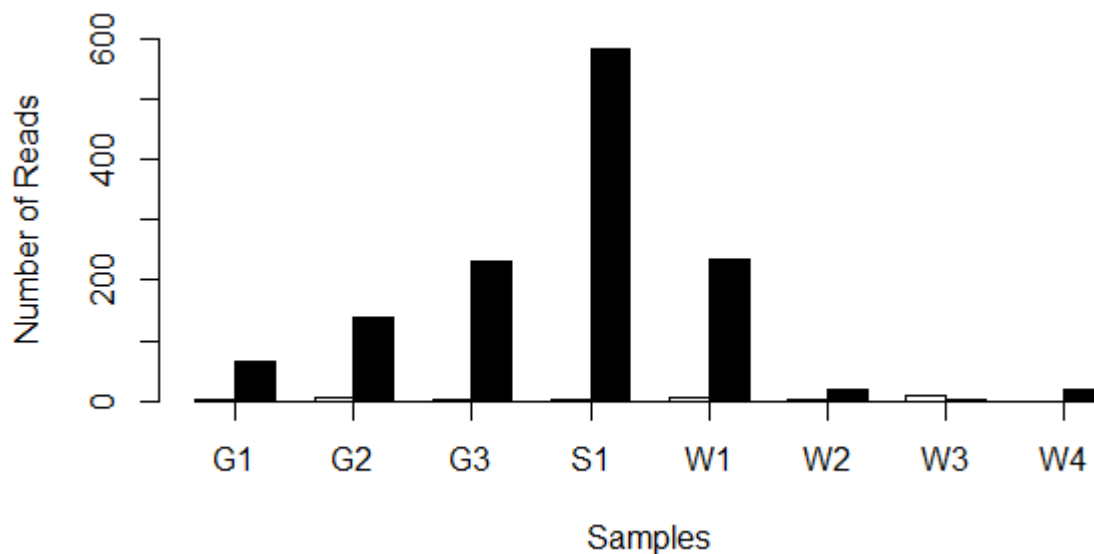


Figure 9. Abundance of three OTUs with the highest indicator values from indicator species analysis, all of genus *Lactobacillus*, in birds after arrival at the study site (open bars) and later during stopover (filled bars). All birds gained condition except for W3.

is consistent with previous findings from the same area of coastal Louisiana (Moore and Kerlinger 1987, Yong and Moore 1997), and implies that most migrants stopping at the site depart for more continuous forests further inland on the night of arrival (Moore and Simons 1995, Buler and Moore 2011). Nevertheless, most (7 of 8) birds which were re-sampled on a subsequent day did show an increase in energetic condition and so the study site may serve as suitable stopover habitat for at least some migrants (Moore and Kerlinger 1987, Yong and Moore 1997).

Similar to findings from shorebirds and other species of passerines (Grond et al. 2014, Hird et al. 2014, Mirón et al. 2014), the gut microbiota of the GRCA, SWTH, and WOTH both at initial and recapture sampling were generally dominated by Proteobacteria, Firmicutes, and Actinobacteria. One GRCA was dominated by Tenericutes, predominantly of the family *Mycoplasmataceae*, on initial sampling however when it was recaptured later during stopover the abundance of these bacteria was greatly reduced in favor of proportional increases in Proteobacteria and Actinobacteria. Bacteria belonging to *Mycoplasmataceae* are known to be pathogenic in humans (Sasaki et al. 2002) and so it is possible that the high loads observed initially reflect some form of disturbed state for the microbiota (Kamada et al. 2013), potentially related to gut atrophy or fasting experienced by this bird while crossing the GOM (Biebach 1998, Thompson et al. 2008). If this is the case, then the reduced levels of *Mycoplasmataceae* at recapture may reflect a return to a more normal microbiota during stopover when the bird had the chance to rest and feed (Bailey et al. 2011, David et al. 2014). One other bird showed a similar reduction in *Mycoplasmataceae* through stopover, further supporting this notion.

The microbiota of birds at initial sampling was not significantly different from that at recapture sampling, nor did the microbiota of all birds at stopover show specific directional responses. Nevertheless, the microbiota of five birds (2 GRCA, 1 SWTH, 2 WOTH) became more similar through stopover. Birds did not show obvious species-specific changes in microbiota during stopover, though sample sizes were low for each species. The fact that many birds showed very similar microbiota later during stopover provides support for the hypothesis that passerine microbiota at stopover, which is initially highly variable, will become more similar due to the shared habitat. Numerous studies have noted the effect of a shared habitat on the microbiota (Klomp et al. 2008, Benson et al. 2010, Amato et al. 2013, Stanley et al. 2013), which is likely driven by similarities in food consumed and exposure to common microbes. Moreover, microbiota communities changed more the longer the bird stayed a stopover.

These results show that the microbiota of migrants can show abrupt changes during short bouts at stopover, thus much of the variation in microbiota of newly-arrived initial samples may be attributable to differing habitat use before arriving at the site. It is unclear why the other two birds which also gained condition during stopover did not move towards the common microbiota. One potential explanation is that these birds might have had a somewhat differing experience at the site, for example some birds may have eaten more insects while others consumed more berries. An alternative, but not mutually-exclusive, explanation is that stopover-induced changes in the gut microbiota are dependent on the types and abundances of bacteria initially present.

This study cannot rule out the possibility that the observed changes in microbiota during stopover are reflective of a recovery period following fasting and prolonged flight

experienced by migrants during the 12-35 hour crossing of the GOM. Feeding after prolonged fast in Burmese Pythons (*Python bivittatus*) has been shown to substantially modify their gut microbiota (Costello et al. 2010), which seems to be driven by an influx of nutrients as well as physiological and morphological changes to the gut environment. Though the migratory birds sampled in this study did not fast for as long as the pythons in Costello et al. (2010), similar results may have occurred. Birds after prolonged migratory flight show atrophied digestive organs which are then rebuilt during stopover (Biebach 1998, Schwilch et al. 2002, Karasov et al. 2004), and it is possible that the observed similarities in recapture microbiota may be driven by a common response to feeding and changing gut morphology after prolonged migratory flight. Birds generally require 1-2 days at stopover before complete recovery of gut function (McWilliams and Karasov 2001, Karasov et al. 2004), which also could explain why birds at the site for 2 days before re-sampling showed more dramatic changes in microbiota than birds at the site for 1 day or less before re-sampling. Retention time of food in the gut also decreases throughout stopover, likely in concert with rebuilding of the intestinal tract (Bauchinger et al. 2009). This gut re-modulation explanation is not mutually-exclusive from the shared site explanation, and it is likely that a combination of both led to the observed changes in microbiota during stopover. Sampling birds at a variety of study sites along the northern Gulf Coast during spring migration would help to further elucidate the influence of site effects and post-GOM recovery on migrant gut microbiota.

Only one bird lost condition between sampling, however it is worth discussing as it showed a differing response to every factor investigated. This bird lost a substantial amount of condition between sampling, implying that it was not able to forage effectively

at the site, while all other birds increased condition at least somewhat and thus likely were able to forage effectively. The bird which lost condition also showed the lowest degree of phylum-level change in the microbiota at stopover, and its microbiota became more dissimilar from the others. Changes in diet can rapidly modulate the gut microbiota (David et al. 2014) and feeding may be required to rebuild the digestive tract (McWilliams and Karasov 2001). Birds which were not able to forage effectively at stopover would thus not be expected to show much in the way of diet-related site effects. These birds may have further atrophied digestive organs to fuel metabolism (Karasov et al. 2004), and so would be expected to show differing changes in microbiota through stopover than would birds which foraged effectively and re-built the gut. Furthermore, increases in *Lactobacillus* have been observed in individuals re-feeding after prolonged fasts (Costello et al. 2010), which is a possible explanation for the observed relationship between changes in condition at stopover and changes in the abundance of the three types of *Lactobacillus* identified in the indicator species analysis. Several species of *Lactobacillus* have been associated with weight gain in mammals and birds (Million et al. 2012), so an initial increase in *Lactobacillus* post-feeding could further promote weight gain in feeding birds at stopover. Future work could focus on increasing the sample size of reduced-condition birds to see if the same patterns hold as well as determining if there is any relationship between a migrant's initial microbiota and its ability to increase condition at stopover.

This study is the first to track temporal changes in the gut microbiota of passerines during migration. All birds during stopover did not show predictable changes in microbiota, with birds instead seeming to show differing responses based on their

ability to forage effectively and increase condition. Despite this, the microbiota of the majority of birds became more similar throughout stopover, though it cannot be conclusively stated how much of this was due to similar food resources at a shared site and how much was attributable to the act of feeding and changes in gut environment after migratory flight. These results suggest that a shared habitat per se does not increase microbiota similarity, however it can increase the likelihood that individuals will have a more similar experience and thereby more similar microbiota community structure.

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CHAPTER IV
SEASONAL VARIATION IN PLASTID-ASSOCIATED DNA IN FECES OF
MIGRANT PASSERINES AND CORRELATES TO FRUGIVORY

Introduction

Migration is a period of high energy demand for passerines moving between Nearctic breeding grounds and wintering areas in the Neotropics. In order to achieve these journeys, migrants at stopover sites deposit substantial fat stores to fuel migratory flight (Newton 2008). Toward this end, many species of passerines show seasonal diet changes towards increased frugivory during migration (Worthington 1989, Bairlein 2002). Fruits are easy to find and eat, thereby reducing searching and capture energy and time, and are easily digested so that large quantities can be eaten at a time (Bairlein 2002). Indeed, consumption of fruit during migration has been shown to promote fattening over strict insectivory (Bairlein and Gwinner 1994, Parrish 1997). Most work on seasonal frugivory has focused on fall migration due to the abundance of fruit during this season (e.g. Parrish 1997, Lepczyk et al. 2000, Smith and McWilliams 2010); however spring migrants may also consume fruit if locally available (Fry et al. 1970, Blake and Loiselle 1992).

If birds are eating substantial amounts of fruit during migration, evidence of this frugivory may be identified through analysis of fecal DNA. The bacterial 16S gene has been widely used to analyze bacterial communities present in fecal samples (De Filippo et al. 2010, Amato et al. 2013, Dewar et al. 2014); however plastids evolved through endosymbiosis with Cyanobacteria and so also contain the 16S gene (Neefs et al. 1990, Dyall et al. 2004). Fruits contain large numbers of plastids, primarily chromoplasts in

mature fruits (Bouvier and Camara 2006), and so frugivorous migrants may show substantial amplification of plastid DNA during 16S-based PCR of fecal DNA. This study uses fecal DNA to infer patterns of frugivory in passerine migrants during spring and fall migration. It should be noted that this was not the primary purpose for collecting the fecal samples, and instead the collection and sequencing methods were designed to investigate gut microbiota communities in migratory passerines (see Chapters II and III for results on microbiota of passerines during migration).

Methods

Spring Migration

Gray Catbirds (GRCA, *Dumetella carolinensis*), Swainson's Thrushes (SWTH, *Catharus ustulatus*), and Wood Thrushes (WOTH, *Hylocichla mustelina*) were sampled from mid-March through mid-May 2014 at the University of Southern Mississippi's long-term banding station outside of Johnson's Bayou, LA (29°45'N, 93°37'W). The study site is a 3.25ha portion of oak-dominated chenier forest, situated less than 1 mile inland from the coast, and represents the first available stopover habitat for migrants after having crossed the Gulf of Mexico in spring. *Morus rubra* (Red Mulberry) and *Rubus* sp. are the main plants at the site bearing fruit during spring migration (Barrow et al. 2000). An array of 24.5 lengths of mist net (12 x 2.6m and 5x2.6m nets, 30mm mesh) was implemented to capture birds, after which they were extracted and placed in a holding compartment atop a clean aluminum foil-wrapped tray. Birds were given up to 30 minutes to defecate on the tray, after which they were removed, banded with a USGS aluminum leg band, and physical measurements were recorded. Feces were transferred to a sterile 2mL tube and kept on ice until they were frozen at -20°C at the end of the day.

Eight birds (3 GRCA, 1 SWTH, 4 WOTH) were re-captured and re-sampled on a subsequent day.

Fall Migration

Additional samples were collected during fall migration 2014 (mid-August through October) at the University of Southern Mississippi's long-term banding station at the Bon Secour National Wildlife Refuge along the Fort Morgan Peninsula in coastal Alabama (30°10'N, 88°00'W). The study site is composed of pine forest intermixed with pine scrub, with Redbay (*Persea borbonia*), Yaupon (*Ilex vomitoria*), and Gallberry (*Ilex glabra*) being the most prevalent fruiting plants (see Zenzal et al. 2013 for a further description of the site). GRCA and SWTH were sampled during the fall, however too few WOTH were sampled during this season to include in analysis. A series of 32 lengths of mist net (12 x 2.6m and 6x2.6m nets, 30mm mesh) was used to capture birds, and fecal samples were collected via the same method as in spring in Louisiana.

DNA Sequencing and Analysis

Fecal DNA was extracted with the PowerLyzer® PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA), using up to 0.15g of sample and eluting with 50uL of the elution buffer. Paired-end 300bp sequencing was performed by MR DNA Molecular Research LP (Shallowater, TX). The V1-V3 region of the 16S rRNA gene was amplified using a barcoded 27F forward primer (bTEFAP®), 518 reverse primer, and HotStarTaq Plus Master Mix Kit (Qiagen, USA). The following PCR program was implemented for amplification: initial step of 94°C (3 minutes), a series of 28 cycles of 94°C (30 seconds), 53°C (40 seconds), and 72°C (1 minute), and final elongation at 72°C (5 minutes). Pooled products were purified and sequenced on the Illumina MiSeq platform according to

manufacturer's instructions. The MR DNA analysis pipeline was used to perform sequencing processing. Paired reads were joined, barcodes removed, and sequences <150bp and sequences with ambiguous base calls were removed from the dataset. After denoising, OTU generation was performed through clustering at 97% similarity and chimeras were removed. Taxonomy of OTUs was assigned with a BLASTn search against GreenGenes, RDP11, and NCBI. Non-bacterial and non-plastid OTUs were removed from the dataset. OTUs mapping to angiosperms composed over 99% of all plastid-associated reads, and so plastid OTUs mapping to other sources were discarded.

Statistical Analysis

The proportion of total reads in each bird which mapped to angiosperm plastids was calculated and these values were used for analyses. All analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria). Differential relative abundance of plastid DNA between species and seasons, as well as differential relative abundance by capture date within the spring, was assessed via Kruskal-Wallis tests. Post-hoc Dunn's Tests (package `dunn.test`, Dinno 2015) were implemented when significant differences were found with the Kruskal-Wallis tests. An alpha of 0.05 was used for all statistical tests.

Results

Fecal samples were analyzed from 30 spring (14 GRCA, 12 SWTH, 4 WOTH) and 13 fall (8 GRCA, 7 SWTH) migrants. Illumina sequencing generated 3,787,106 good-quality reads from 8851 OTUs. 182 of these OTUs mapped to angiosperm plastids, though the percentage of plastid reads per sample varied widely (mean: 19.5%, SD: 30.4%, range: 0.2%-92.6%). 3 plastid OTUs were identified in all birds sampled and

contained over 98% of all plastid reads in the dataset. The closest taxonomic matches to these OTUs were mulberry (*Morus*) and strawberry (*Fragaria*); however the BLASTn search returned a multitude of hits of varying taxonomic relatedness. Classification of the plastid DNA may not be reliable at lower taxonomic levels. This lack of taxonomic resolution is similar to the findings of Mishler et al. (1992) that 16S rRNA alone was not able to definitively resolve taxonomy in bryophytes.

The proportion of plastid reads in migrants was found to vary significantly by species and season ($\chi^2=9.90$, $df=4$, $p=0.042$). Post-hoc tests showed that the proportion of plastid reads was significantly lower in Spring WOTH and Fall SWTH than in Spring SWTH and Fall GRCA (Figure 10). Spring GRCA did not show significantly different plastid relative abundance from spring or fall migrants of any species. In spring, the proportion of plastid DNA in fecal samples was found to increase during the course of the season (Figure 11). Specifically, birds sampled before day of year 113 (April 23) showed significantly lower relative abundance of plastid DNA than

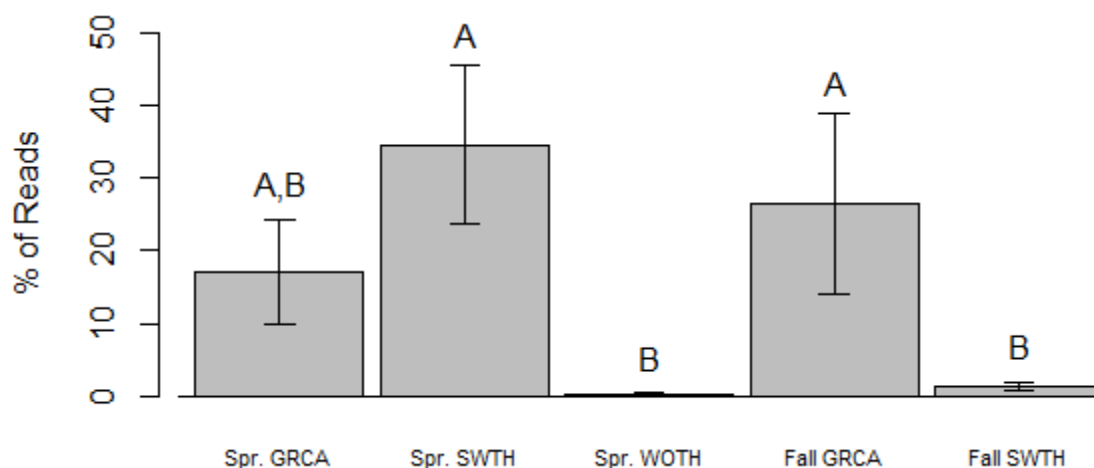


Figure 10. Percentage of total reads/bird mapping to plastid DNA from passerine migrants at stopover along the Northern Gulf Coast during spring and fall migration. Bars represent ± 1 SE away from the mean. Letters above bars represent statistical groups as determined by post-hoc Dunn's test.

birds sampled on and after day 113 ($\chi^2 = 22.61$, $df=2$, $p < 0.001$). The proportion of plastid DNA in birds seemed to start increasing on day 113 and peak in birds captured at the end of the season, with later birds averaging 56.9% of their total reads mapping to plastid DNA (SD=29.2%). A similar pattern was observed in the eight birds which were re-sampled throughout stopover, as the 6 birds recaptured before day 113 (4 WOTH, 2 GRCA) showed an average change in plastid relative abundance of -0.04% of total reads (SD=0.13%). In contrast, the one GRCA which was sampled on day 113 and again the next day increased in plastid relative abundance by 2.88% of total reads and the SWTH sampled on days 119 and 121 increased plastid relative abundance by 30.10% of total reads between sampling. The strong seasonal differences drove the observed differences

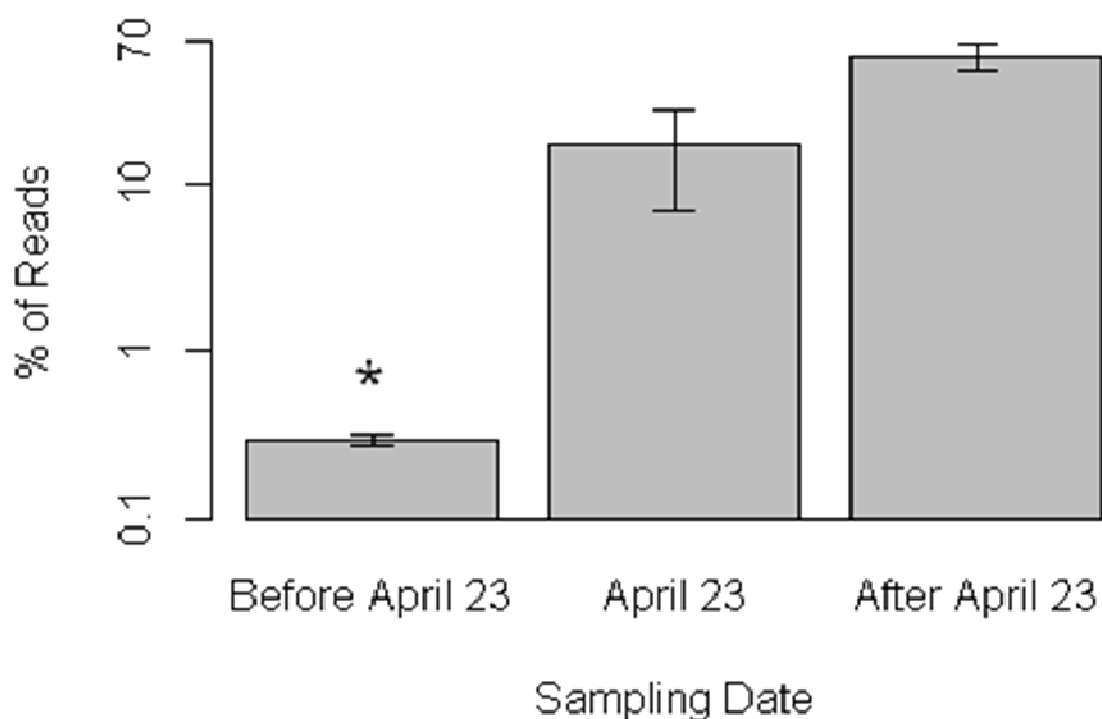


Figure 11. Percentage of total reads/bird mapping to plastid DNA from three species of spring passerine migrants at stopover sampled before, on, and after day of year 113. Note that y-axis is on a logarithmic scale. Bars represent ± 1 SE away from the mean. * denotes significance at $\alpha=0.05$.

between species in spring, as all of the WOTH were sampled during the early season, the GRCA were spread throughout the season, and 2/3 of the SWTH were sampled on or after day 113. No influence of sampling date was observed in fall.

Discussion

Many migrants showed substantial loads of plastid DNA in fecal samples, composing up to 92% of the fecal DNA from some birds. It is possible that some plastid DNA may have been inadvertently ingested through the gut of consumed herbivorous arthropods (Hanshaw et al. 2013); however the dominance of plastid DNA in many birds could likely only have been achieved if it was consumed directly. None of the three study species are herbivorous, though they all are at least partially frugivorous during migration (Mack and Yong 2000, Evans et al. 2011, Smith et al. 2011). Birds eating fruit show rapid gut throughput times (<1 hour), which seems to be an adaptation allowing for large quantities of fruit to be consumed and processed in a short amount of time (Worthington 1989, Levey and Karasov 1992). Frugivorous migrants thus would be consuming high amounts of fruit, and passing it quickly through their gut, which would explain the observed abundance of plastid DNA in the feces of many birds.

In spring, the abundance of plastid DNA was found to sharply increase in late April. This observation of increased plastid DNA in late spring coincides with the fruiting phenology of mulberry and *Rubus* sp., both at the study site (personal observation) and elsewhere in the Southeastern US (Johnson and Landers 1978, Skeate 1987). Migrants have been reported to consume fruit at stopover along the Northern Gulf Coast during spring migration (Barrow et al. 2000). Additionally, large numbers of migrants at the site, including the three species, have anecdotally been observed to consume large quantities

of fruit after they have ripened in late April (personal observation). Moreover, captured birds showed evidence of frugivory, such as purple staining on the faces and bills from consuming berries and seeds and purple coloration of feces, predominantly during the later portion of the season (personal observation). The observed seasonal patterns of plastid DNA in fecal samples during spring seem reflective of the observed seasonal patterns in frugivory, with birds sampled early in the season (before fruit ripening) showing low plastid abundance, birds sampled on day 113 (when fruit was starting to ripen) showing intermediate plastid abundance, and birds sampled late in the season (when fruit was ripe and available for consumption) showing very high plastid abundance. Profiling of the 16S DNA in fecal samples therefore seems to be a reliable measure of the degree of frugivory in birds.

In contrast to spring migrants, fall migrants did not show strong seasonal patterns in plastid abundance. This result is likely tied to the fact that fruits are available for consumption throughout the entire season at the fall site (F. R. Moore unpublished data) while in spring they do not ripen until the latter portion of the season. Instead, fall GRCA showed significantly higher relative abundance of plastid DNA than did fall SWTH. This result is surprising, as both species consume fruit during fall migration (Parrish 1997); however these differences in fecal plastid loads may reflect differing behaviors of the two species at the study site. SWTH generally depart from the study site on the night of banding (Smolinsky et al. 2013) and anecdotal observations on radio-tracked SWTH indicates that they may not be actively foraging at the site (L. Schofield personal communication). Radio-tracked GRCA on the site, in contrast, actively associated with fruiting plants (J. Farrington and F. R. Moore unpublished data). Furthermore, 7 of the 8

fecal samples from GRCA analyzed in the current study showed evidence of fruit (seeds and/or fruit skin) in the feces while only 2 of 7 samples from SWTH showed presence of fruit. Taken together, these results suggest that GRCA actively feed on fruit at the site, while SWTH at the site may instead depart the site as quickly as possible. More detailed studies are needed to explicitly test the dietary habitats and migratory strategies of GRCA and SWTH at this site during fall migration.

As an aside, this study underscores the importance of performing sequencing when analyzing microbiota communities. The community structure of spring samples was additionally analyzed using Denaturing Gradient Gel Electrophoresis (DGGE, data not shown). DGGE provides a community fingerprint for each sample by separating DNA on a gel based on G-C content; however it cannot provide information about the taxonomic identities of DNA in a sample (Muyzer and Smalla 1998). Results of community-scale analyses of the samples were similar for both DGGE and sequencing data of total amplified fecal DNA (combining bacterial and plastid DNA) The results of the DGGE and the sequencing of total DNA were driven by the late-season abundance of fecal plastid DNA, as both showed clear differences between early and late birds. When re-running the analyses only using the bacterial reads, however, no strong seasonal influence was observed (Chapter II). DGGE thus serves as a reliable indicator of the overall community structure of DNA in a sample; however it cannot distinguish between bacterial and plastid DNA and may produce misleading results if solely relied upon. This study shows that gut microbiota research, especially if studying frugivorous or herbivorous subjects, should be aware of potential amplification of plastid DNA (Hanshew et al. 2013, Shelomi et al. 2013).

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