Development and dynamics of gut microbial communities of migratory shorebirds in the Western Hemisphere

by

Kirsten Grond

B.S., University of Groningen, 2007
M.S., University of Groningen, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
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Abstract

Gastrointestinal microbiota play a vital role in maintaining organismal health, through facilitating nutrient uptake, detoxification and interactions with the immune system. Shorebirds vary widely in life-history characteristics, such as habitat, migration and breeding system, but the dynamics of their gut microbial communities are unknown. In my dissertation, I investigated composition and dynamics of gut microbiota in migratory shorebirds from embryos to 10 day old chicks, and determined environment and host-related factors affecting gut microbial communities of adults. First, I tested whether precocial chicks from three species of arctic-breeding shorebirds acquire gut microbiota before or after hatching using next-generation sequencing techniques. In addition, I documented the rate and compositional dynamics of gut microbial establishment. I showed that gut microbiota were absent in shorebird embryos before hatching, but that stable gut communities rapidly established within the first three days after hatching. In addition, gut microbiota of young shorebird chicks were more similar to the environmental microbiome than later in life, suggesting that the environment is a likely source for microbial recruitment. After reaching adulthood, shorebirds migrate long distances between breeding and non-breeding sites, potentially exposing them to a wide range of microorganisms. Host phylogeny and environmental factors have both been identified as drivers of gut microbiota composition in birds in previous studies. The second part of my project aimed to identify host and environmental factors that underlie variation in gut microbiota composition in eight species of migratory shorebirds sampled across the North American Arctic. I found that sampling site was the main driver of variation in gut microbiota of Arctic-breeding shorebirds, and that site-related variation in gut microbiota of shorebirds was a result of differences in core bacterial taxa. A relatively large influence of local environment on gut microbiota composition of migratory shorebird chicks and adults leads to the question: how are shorebirds affected by local pathogen communities? Migratory behavior has been hypothesized to have evolved as a response to variation in climatic conditions and food availability, to avoid predation, and to reduce risk of exposure to pathogens. The *migratory escape* hypothesis predicts avoidance of high disease prevalence areas through migration, and has been proposed as one of the main reasons that many bird species migrate to the Arctic for breeding.

To test the migratory escape hypothesis in shorebirds, I screened for prevalence of seven known avian pathogens in shorebirds at different stages of migration. I did not detect the majority of pathogens we tested for, with the exception of *Campylobacter jejuni* and *C. coli*. Prevalence of *C. jejuni* in shorebirds was linked to sampling sites but not shorebird species. My dissertation is the first comprehensive study of the gut microbial characteristics of migratory shorebirds. Overall, local environment emerged as an important factor in shaping microbiota composition in Arctic-breeding shorebirds throughout my dissertation research. The role of local environment in shaping gut microbiota invites future investigations of the interactions among shorebirds and the microorganisms present in their environment, as well as the functions gut microbiota perform within their shorebird hosts.

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I would like to start this section with acknowledging my academic family. One very important person who made my PhD possible was of course my (Canadian) advisor, Brett Sandercock. Brett took a chance when hiring this strange Dutch person with an unnatural interest in microbes, and, by doing so, allowed me to pursue my research dream. He has been nothing but supportive throughout all the adventures and sometimes hardships that my PhD research presented over the years, which included lots of grant applications, travel to exotic places, rejected manuscripts, wasting 4K on the wrong primers (still ashamed ☹) and some truly mediocre writing. I could not have asked for a better advisor. Thank you, Brett! I can only hope that you do not regret your choice of student(s) too much, but I hope that large quantities of stroopwafels made up for some of the pain. I wish you all the best on your Norwegian adventure and hopefully we will be able to meet up sometime in the future.

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In 2012 I moved to Manhattan with my best friend, Bram. Now, 5 years later, a lot has changed in our personal and work lives, but I can luckily still call you my best friend. Together we have traveled the world, adopted two crazy felines, Bette and Steve, and somehow managed to both finish our PhDs on time (you will too next month, I promise). Now comes the time that we will live in different places for the first time in 13 years, which is not something I look forward to. That said, I know we will stay in touch

and I wish you and Jackie all of the amazing things in life, including awesome jobs, unicorns, piles of hutspot, kittens (=happiness), the day they develop Age of Empires into a virtual reality game, lots of diet coke (no ice!), and of course many more Royal Rumbles and other events that defy comprehension.

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And now my family. You all deserve much more than a few words here, but I'll do my best. My parents, Heine and Joris, have supported me in every way possible throughout my life and writing this proefschrift. They patiently read all of my chapters, listened to me complain over Skype, and helped me through many moments of 'backflow'. I would also like to thank Rinske and Joost, the best sister and brother any middle sister can wish for. Thank you for patiently putting up with all the times I was stressed, too busy to talk, or time difference got in the way. I love you more than anything!

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Dedication

To Hanne van der Iest

21 years my friend

Chapter 1 - INTRODUCTION

"*Alles is overal*: maar, *het milieu selecteert*"

"*Everything is everywhere*, but, *the environment selects*"

Lourens G. M. Baas Becking (1895-1963)

One of the most famous quotes in microbiology, Baas Becking's hypothesis referred to the relationship between the geographic distribution of microorganisms over the earth and the metabolic functions they can perform (Baas Becking 1934; de Wit and Bouvier 2006). Almost a century later, the first part of the Baas-Becking hypothesis ("Everything is everywhere") has been disputed widely due to a better understanding of limitations in dispersal abilities of microorganisms and the use of deep sequencing techniques to investigate whole microbial communities (Thurber 2009; Aguilar *et al.* 2014). However, the selective power of the environment on shaping microbial communities still holds, true and the general concept contributed to the foundation of the niche concept in ecology (de Wit and Bouvier 2006).

Few environments pose a greater selective pressure on their microbial communities than the gastrointestinal tract. The intestinal environment is characterized by a narrow range in pH, absence of oxygen, and, in endotherms, a specific temperature range, which results in colonization by specialist microorganisms adapted to these conditions. Despite presenting a narrow and specific environment, the animal gut contains a large host-associated microbial community, and is essential for maintaining organismal health (Lin and Zhang 2017).

Microbiology of the avian gastrointestinal tract is a relatively new field, and is still in its infancy compared to mammalian and human studies. The first use of sequence-based techniques to identify gut microbiota in birds emerged in the 1990's, but were aimed at detection of single pathogens and did not address the gut microbiota on a community level. Whole community sequencing of avian microbiota did

not occur until 2010 (Day *et al.* 2010); almost a decade after next-generation sequencing techniques became publicly available. Since then, studies on avian gut microbiota have focused on domesticated species, such as poultry and pets, and have rarely been used to examine the role of gut microbiota in free-living birds under natural conditions.

The overarching goal of my PhD was to characterize bacterial communities of free-living shorebirds throughout their life-cycle. To summarize previous studies on wild birds, I first conducted an in-depth review of current knowledge on avian gut microbiota. In **Chapter 2**, I show that microbiome research is an emerging field in ecology and evolutionary biology, and wild birds are an interesting group to study due to their wide variation in phylogenetic history, physiology and life-history characteristics, and their global distributions. In my review, I investigated broad-scale patterns in gut microbial community and function in wild birds, and assessed genetic and environmental factors that shape gut microbiota of birds under natural conditions. In addition, I described gut microbiota composition in functionally distinct sections of the avian gastro-intestinal tract, and considered the functional ecology of host-microbiome interactions, including the microbial role in nutrient uptake, detoxification of chemicals, and interactions with the avian immune system. I identify a suite of gaps in our current knowledge in the field of avian gut microbiota research and suggest avenues for future research.

One of the current assumptions in avian research is that the internal environment of developing egg is sterile within the chorion membrane, resulting in chicks hatching without gut microbiota (van der Wielen *et al.* 2002; Kohl 2012). The assumption of egg sterility has been reiterated widely, but has not been tested. In **Chapter 3**, I provide the first evidence that wild birds hatch without a gut microbiome in place. I also show that guts of chicks are colonized by bacteria within a day of hatching, and that gut microbial communities stabilize in abundance and composition after only three days of age for precocial chicks at an Arctic site.

Arctic-breeding shorebirds migrate long distances between their breeding and non-breeding sites. As described in **Chapter 2**, gut microbiota interacts closely with host health, but factors that determine

gut microbial composition can differ widely among host taxa. In mammals, host phylogeny has been identified as the main driver of gut microbiota, which has been attributed to vertical transfer of microbiota during birth. Past studies of birds have found equivalent support for host phylogeny, but also environmental factors as drivers of gut microbiota composition. In **Chapter 4**, I aimed to identify host and environmental factors that underlie variation gut microbiota composition in eight species of migratory shorebird in the North American Arctic. I characterized bacterial communities from fecal samples collected from adult shorebirds at nine breeding sites across a wide geographic area of Alaska and Canada, and found that breeding site was the main driver of variation in gut microbiota of Arctic-breeding shorebirds instead of host species. Subsequently, the potential importance of local environment as a driver of major shifts in gut microbiota composition of Arctic-breeding shorebirds could have implications with respect to changes in the environmental microbiome resulting from climate change. that threatens the majority of shorebird species.

In **Chapter 5**, I expanded my sampling efforts to include non-breeding sites in North and South America across the Western Hemisphere. Migratory shorebirds encounter different environments throughout their annual cycle. Different environments contain different pathogens, and risk of infection can differ among sites. The *migratory escape* hypothesis describes the avoidance of high disease prevalence areas through migration, and has been suggested as one of the mechanisms underlying migratory behavior. I tested the migratory escape hypothesis through assessing the prevalence of seven known avian pathogens in shorebirds at different stages of migration across North and South America.

In **Chapter 6**, I conclude my dissertation with a synthesis of my review and discussion of my empirical results. I summarize the novel results of my dissertation research and identify useful areas for future research.

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Chapter 2 - THE AVIAN GUT MICROBIOTA: COMMUNITY, PHYSIOLOGY AND FUNCTION IN WILD BIRDS

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ABSTRACT

Gastrointestinal microbiota play a vital role in maintaining organismal health, through facilitating nutrient uptake, detoxification and interactions with the immune system. The gastrointestinal microbiota of birds has been poorly studied, especially in wild species under natural conditions. Studies on avian gut microbiota are outnumbered ten to one by studies of mammals, and are dominated by research on domestic poultry. Unlike domestic poultry, wild birds vary widely in environmental conditions, physiology, and life-history characteristics, such as migration and mating strategy, resulting in a vast diversity in gut microbiota composition and function. Avian life-history characteristics pose different selection pressures on the gut microbiota, and ultimately affect health of the host. In this paper, we review current knowledge on gut microbiota of wild birds, including: partitioning of digestive function and microbiota among different gastrointestinal compartments, microbial diversity and function in the context of host diet, energetics and behavior, and the intrinsic and extrinsic factors impacting aspects of gut microbiota in free-living birds. The shared core microbiota of wild birds was dominated by members of the Phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*. However, microbial community composition showed inter- and intra-specific variation within and among gastrointestinal tract sections. We conclude our review by identifying key research areas that require further investigation: 1) expanding the range of avian host taxa investigated, 2) identifying function of avian gut microbiota, and 3) complementing current exploratory studies with experimental manipulations to identify key determinants of gut microbiota composition.

Keywords (5-10): 16S rRNA gene, bacteria, environment, gastrointestinal tract, metagenomics, microbial diversity

I. INTRODUCTION

The microbiota of the gastrointestinal (GI) tract and interactions with host organisms are emerging topics in microbiology, ecology and medicine. Intestinal microbiota strongly influence an organism's health, and in humans consist of $>10^9$ bacterial cells per gram of intestinal content (Stevens & Hume, 1998). Rapid advances in molecular methodologies in the past decade, combined with the appreciation of the large impact gut microbiota have on health, have resulted in an exponential increase in studies, mainly targeting mammalian systems (Fig. 2.1a). Despite their great diversity and ecological significance, research on gut microbiota of birds has lagged behind. Studies on avian gut microbiota are outnumbered 10:1 by mammalian studies, and are dominated by studies of domestic poultry (Fig. 2.1b). Over 1,300 publications on microbiota of poultry have been published since 1948, and focus mainly on the influence of diet on microbiota, the role of microbiota in meat production, and the reaction of microbiota to antibiotics and probiotics. Wild birds remain understudied despite their relevance for pathogen transmission, and for understanding diet and environmental influences on GI microbial structure and function.

Microbial gut communities of birds have received some attention because wild birds are a source of a number of human and animal diseases through direct transmission, or by acting as vectors for zoonotic pathogens (Tsiodras *et al.*, 2008). Migratory bird species are especially suitable for long-distance pathogen transmission. Pathogen transmission through fecal pollution by wild birds has been studied widely, but has predominantly focused on one or several pathogens. Also, these studies have mainly focused on human health aspects. A large proportion of wild bird populations are declining (Vickery *et al.*, 2014), but what role animal health plays in ongoing declines is unknown.

Birds are an exciting group to investigate because the lineage comprises over 10,000 species, and are a diverse group of organisms that vary in life-history characteristics such as migratory behavior, the ability to fly, diet, mating systems, longevity and physiology, all of which may impact GI microbiota structure and function. Still, wild bird gut microbiota and its interactions with the host have received little attention. Kohl (2012) wrote an early review of the avian gut microbiota, and only identified eight studies

that had used 16S rRNA gene sequencing techniques to investigate gut microbial communities in wild birds. Since then, our knowledge of wild bird microbiota has increased through new applications of high-throughput sequencing and first use of predictive metagenome function analysis (Waite & Taylor, 2014). Waite and Taylor pointed out that new molecular techniques have increased the number of studies focusing on diversity of gut microbiota. Identifying microbial diversity of the host gut is a necessary first step, but provides limited information on functional aspects of the community.

Gut microbiota and microbial interactions with the animal host provide valuable information on organismal health. However, a majority of past studies have been conducted on humans or animal model systems, such as rodents and chickens. The aim of our review is to summarize current knowledge of gut microbiota of wild birds, identify current knowledge gaps, and suggest future research directions. We cover the topics of microbial diversity and function in different parts of the GI tract, and discuss intrinsic and extrinsic factors affecting gut microbial communities in wild birds. Gut microbiota are predominantly comprised of bacteria, although efforts to further our understanding of the importance of archaea and fungi are growing. We focus on the bacterial avian gut microbiota in this review, with the exception of sections discussing microbial function for which the presence or absence of individual genes are of importance. Our review focuses on wild bird microbiota, but we reference mammalian and poultry studies where information on wild birds is sparse or absent, if such information provides insights that can potentially be extrapolated to wild birds under natural conditions. The terms *microbiota* and *microbiome* are often used interchangeably, but have different meanings. Here, we use microbiota to refer to the collection of microbes within a given environment and microbiome to refer to all genomes of these microorganisms combined (Waite & Taylor, 2014).

Gut microbiota of poultry and other domesticated birds have been recently reviewed in depth by Oakley *et al.* (2014) and Stanley, Hughes, & Moore (2014). Domestication and controlled environments likely change gut microbiota through inbreeding, non-natural and homogeneous diets, controlled high density living conditions and the use of antibiotics. Extrapolating results, such as composition and function of gut microbiota, from model organisms to wild animals should be done with caution.

Additionally, avian pathogens have been well studied (Reed *et al.*, 2003; Benskin *et al.*, 2009). We will not review this field, with the exception of a few examples of pathogens affecting gut microbial function.

Understanding wild bird gut microbiota dynamics will provide us with novel insights into the interactions between free-living organisms and their environment, and will contribute to our interpretation of basic and applied aspects of microbial community structure and function.

II. LITERATURE REVIEW

To assess the current state of gut microbiota research, we compared publication records of studies on gut microbiota of all mammals, humans, all birds and wild birds, as recorded in the Web of Science database. We used search terms based on changing terminology over the search period, which covered 1900-2016. The search terms used for mammals and humans were: gut microbiota/microbiome/gut bacteria/gastrointestinal microbiota + mammal/human. Search terms used for wild and domestic birds were: gut microbiota/microbiome/gut bacteria + wild bird/bird/chicken/broiler/poultry. Results of our search terms for mammalian and human studies were too abundant to assess individually. We sorted results by publication date and scanned abstracts of publications on the first 50 results. We assessed the proportion of publications that were on topic, and estimated the total number of relevant publications. Our publication numbers for mammals and humans are therefore estimates and do not reflect exact numbers. We found a total of ~19,300 and 7,400 publications for mammals and humans, and 32 and 1,246 publications for wild and domestic birds from 1942-2015. Due to the low number of publications (0-2 per year) in the period 1900-1980, we present search results from 1980-2016 (Fig. 2.1).

We include information from culture-based studies in our review, but we focus on sequence-based studies for direct comparisons of the complete gut microbiota among wild birds. Culture-based studies are generally targeting specific microorganisms, whereas we aim to broadly understand the entire resident bacterial community of the gut.

III. METHODS FOR STUDYING GUT MICROBIOTA

Which bacteria are identified in microbiota studies depends strongly on the choice of research methods and tools. Methods can be broadly divided into *culture-dependent methods*, which use selective culture media for detection, and *culture-independent* molecular methods, which often involve target gene sequencing of *in situ* microbial communities. Advantages and biases of both categories, and of different methods within these categories have been reviewed (Hirsch, Mauchline, & Clark, 2010; Stewart, 2012; Faure & Joly, 2015). Here, we provide only a short synopsis of the culture-dependent and independent methods.

1. Culture-dependent methods

Until the widespread availability of DNA sequencing techniques, gut microbial communities were mainly studied through use of culture-dependent techniques where microorganisms were grown in suitable media. As identification of microorganisms was only possible through use of selective and differential media, the majority of studies focused on or few microbial target taxa. The limitations of culturing are well understood, and include the inability to grow a majority of the earth's microbes in pure culture. In the human gut, 80% of resident bacteria have not been cultured, or at present cannot be cultured at all (Turnbaugh *et al.*, 2007). A particular disadvantage of using culture-based methods is the difficulty of culturing microorganisms in the domain Archaea. Archaea can have distinct functions within the GI tract, such as involvement in carbohydrate metabolism (Hoffmann *et al.*, 2013). Omitting the Archaea means ignoring one of the three microbial domains and likely results in an incomplete picture of gut microbial communities and their function in host physiology. Also, generally, culturing can be a time-consuming and therefore costly method.

On the other hand, the ability to culture 20% of gut microbiota is high compared to other systems, such as soil or aquatic environments, and results from our accumulated knowledge on the gut environmental conditions and high culturing effort. One advantage of culture-dependent techniques is the ability to collect direct evidence of the physiological attributes and environmental tolerances of bacteria

under controlled conditions, which cannot be investigated with most culture-independent techniques. From taxonomic marker gene sequence data, functional characteristics such as preferred growth substrates and nutrient requirements can only be inferred from comparison to related taxa, or from previous culturing studies. Another advantage is continuity with historical studies, particularly on defined members of the GI tract microbiota. In avian studies, targeted approaches based on culturing have been used to identify known pathogens, such as *Campylobacter* spp. (Waldenström *et al.*, 2002; Keller *et al.*, 2011).

2. Culture-independent methods

Molecular tools used for microbial community analysis and identification have undergone a rapid development over the past two decades, and have led to a more detailed dissection of the communities as well as an increase in publications on gut microbiota. 16S and 18S ribosomal RNA genes were first employed as bacterial identification markers as early as 1977, when they were used in a phylogenetic analysis of Eubacteria, Archaea and Eukarya (Woese & Fox, 1977). Early culture-independent avian microbiota studies were predominantly coarsely compared microbial communities, and, due to high sequencing costs, with lesser focus on identification of individual microorganisms. Several community fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) and automated ribosomal intergenic spacer analysis (ARISA) were applied to describing communities in avian gut microbiota. Fingerprinting techniques provide a measure of how many variants of a gene, for example the 16S rRNA gene, are present, but do not provide information on abundance or identity of the microbial community. Early sequencing mainly consisted of constructing clone libraries directly from PCR amplicons, or indirectly through cloning informative fragments extracted from gels used in fingerprinting techniques. Each cloned gene was subsequently sequenced individually using Sanger sequencing. Both fingerprinting and cloning-sequencing techniques are relatively time-consuming with low data yield compared to high-throughput sequencing (HTS) methods. A more taxonomically informative method with respect to community composition is the use of high-density oligonucleotide microarrays, like the

PhyloChip microarray. The microarray chip contains multiple probes for each of more than 59,000 known microbial 16S rRNA gene phylotypes (Brodie *et al.*, 2006).

Development of HTS techniques, such as 454 pyrosequencing and the Illumina sequencing platform, have provided us with the opportunity to profile microbial communities with a relatively low cost per base pair (Roesch *et al.*, 2007; Faure & Joly, 2015). The rapid decrease in sequencing cost has allowed for these next-generation techniques to be used widely, although costs are still relatively high for small research facilities. Also, HTS techniques produce enormous data volumes that require computationally intensive analysis. Lowering sequencing costs and development of broadly applicable sequence library preparation methods and informatics tools have made these techniques widely accessible (Caporaso *et al.*, 2012). A remaining challenge for interpreting large datasets is the relatively low coverage of phylogenetically well-resolved bacteria and archaea in the available gene reference databases (Pompanon & Samadi, 2015), resulting in an inability to reliably annotate many phylotypes from environmental microbial sequencing libraries.

A powerful tool yet to be employed in the study of avian microbiota is *metagenomic sequencing* (Riesenfeld, Schloss, & Handelsman, 2004), which constitutes the complete interrogation of the gene content in the microbiome. Metagenomic sequencing relies on shotgun sequencing of all DNA fragments in the sample, and subsequently assigning the relevant sequences to known microorganisms and protein functional groups. Due to the high data coverage needed to identify many genes from these DNA fragments, greater sequencing depth is necessary for metagenomic analyses than for marker gene sequencing, which increases cost and computational challenges in the downstream analyses. Gut microbial function has been increasingly studied using metagenomics in humans and other mammals, and provides a substantial baseline of known microbial function for avian studies. However, functions may differ among mammalian and avian gut microbiota, and caution is required when extrapolating from mammalian to avian systems. Still, a growing literature illustrates that metagenomics approaches have a great potential to address functional aspects of avian gut microbiota, particularly for comparison among host taxa with different dietary, physiological and life-history characteristics.

IV. THE CORE MICROBIOTA

Organisms can house thousands of microbial Operational Taxonomic Units (OTUs), an heuristic analog to "species", within their GI tract, with a large percentage of these microbes unique to the individual host. To assess the composition of commonly shared microbial OTUs among individuals in an environment or under defined circumstances (for example, within the GI tracts of all vertebrates, mammals, or wild birds), the concept of a core microbiota can be used. The definition of a 'core' microbiota depends strongly on the ecological question asked, and can include shared microbial presence, function, and lineages among two or more microbial communities of interest (Turnbaugh *et al.*, 2009; Shade & Handelsman, 2012). Comparisons among core microbiota across studies generally occurs at deep phylogenetic levels such as Phylum, as the enormous diversity on shallower phylogenetic levels often results in incomplete coverage in host species or taxa. We determined core microbiota of wild birds based on shared microbial presence and function on a Phylum level (Fig. 2.2). We were interested in broad patterns across species, and limited data on shallower phylogenetic levels did not allow for more in depth comparisons.

Phylum is a relatively coarse level of classification, and differences in gut microbiota communities between birds and mammals become more apparent at the finer Class, Genus and OTU levels. An OTU is a classification strategy widely used in the study of microbiota based on similarity of the taxonomic marker 16S rRNA gene, and is often set to define the finest ("species") level of bacterial taxonomic classification at 97% sequence similarity (Stackebrandt & Goebel, 1994). Due to large within-host-taxon variation in microbiota composition, it is challenging to define a bird and mammal 'core' microbiota at a scale finer than that of a Phylum. Large within-host-taxon variation in microbiota composition can be attributed to many factors, both genetic and environmental (section VII).

Several potential challenges arise with defining a "core microbiota" for comparison of host taxa. The number of different bacterial Phyla or, at the finest scale, OTUs detected in a sample is directly

proportional to research methods and effort. Higher sequencing depth increases the probability of detecting rare OTUs (Schloss & Handelsman, 2005), but may concurrently increase the number of artefactual OTUs included in the analyses. If sequencing depth is insufficient, the exclusion of rare taxa from analyses can miss clinically relevant minority bacterial populations, which is referred to as the 'Depth Bias' (Lagier *et al.*, 2012). The functional importance of rare OTUs may be high (Sogin *et al.*, 2006; Jones & Lennon, 2010), but is often poorly understood, and inclusion of rare taxa in a "core microbiota" definition may obscure the patterns in the more abundant and possibly functionally dominant OTUs. On the other hand, phylogenetically distantly related microorganisms may be functionally redundant, and fulfill similar physiological functions within the gut. Differences among gut microbiota at deep phylogenetic level such as Phylum do not necessarily imply differences in broad function. Martiny et al. (2015) conducted a meta-review summarizing the phylogenetic basis of microbial traits. The authors conclude that simple traits, such as nutrient acquisition, may be conserved at shallower phylogenetic levels but then display functional redundancy at a deeper Phylum level. Last, comparing core microbiota among individuals or host species derived from different studies presents the problem of biases generated by use of different pre-sequencing preparation methods, sequencing platforms, and/or analysis pipelines.

Despite the caveats of interpreting gut microbiota composition at Phylum level, it does enable us to make broad inferences about microbial community and functional dynamics associated with the diverse set of life history characteristics found in wild birds.

At a Phylum level, *Firmicutes* and *Bacteroidetes* dominate the mammalian gut microbiota (Leser & Mølbak, 2009). Our compilation of studies from 1980-present suggest some differences between the avian and mammalian microbiomes: the avian gut microbiota overall was dominated by *Firmicutes*, *Proteobacteria*, with lower *Bacteriodetes* and *Actinobacteria* abundances (Fig. 2.2). *Firmicutes* are predominantly gram-positive bacteria, and include the obligate or facultative anaerobe Classes of *Bacilli*, *Clostridia* and *Mollicutes*, each of which are commonly found in GI tracts. *Firmicutes* that inhabit the GI tract are involved in fermentation of organic molecules and have been positively linked to obesity in

humans, and to weight gain in mammalian and avian models (Turnbaugh *et al.*, 2006; Angelakis & Raoult, 2010; Clemente *et al.*, 2012).

Birds carry a higher proportion of *Proteobacteria* in their GI tract compared to mammals. The Phylum *Proteobacteria* includes five classes (α, β, γ, δ, ε) that vary widely in occurrence and function within and outside the GI tract. *Proteobacteria* are gram-negative and represent 34% or all known bacteria. The *Proteobacteria* include functionally diverse bacteria including phototrophs, nitrifiers and enterics, and are found in most environments (Garrity, 2005). *Proteobacteria* also include a range of pathogens, such as *Campylobacter, Escherichia, Helicobacter, Rickettsia, Salmonella* and *Vibrio*, most of which have been isolated from birds (Keller *et al.*, 2011; Wallménius *et al.*, 2014; Ryu *et al.*, 2014; Diakou *et al.*, 2016).

Bacteroidetes are gram-negative bacteria that vary from strict aerobes to obligate anaerobes and occur throughout the entire GI tract. These bacteria are believed to have a mutualistic relationship with their host, and are involved in biopolymer degradation, immune function, pathogen exclusion and GI tract development (Thomas *et al.* 2011). Outside the GI tract, *Bacteroidetes* are common micorbes of soils, and marine and freshwater environments.

Actinobacteria are gram-positive bacteria inhabiting a wide range of environments, including soils, gastrointestinal tracts and freshwater and marine waters (Janssen, 2006; Barka *et al.*, 2016). The *Actinobacteria* include pathogens, such as *Corynebacterium*, *Mycobacterium* and *Nocardia* species. Within gastrointestinal tracts, the Genus *Bifidobacterium* includes commensals (Barka *et al.*, 2016), and has been used as probiotic (Kailasapathy & Chin, 2000).

The Phyla discussed above are common in a wide range of habitats. However, it is important to keep in mind that Phylum is a broad category of classification. On shallower phylogenetic levels such as an OTU, microbial communities associated with different habitats will be comprised of different OTUs with potentially different functions. Comparison of microbiota on a Phylum level among different environments will therefore present interpretation challenges. However, despite the limitations of using core microbiota, it can be a useful tool for broad investigations of and comparisons among gut microbiota.

V. FUNCTION OF GUT MICROBES

The core gut microbiota of wild birds predominantly consist of *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* (Fig. 2.2). Here, we discuss the general functions of gut microbiota in general, and functions of these four Phyla, and their potential role in the gut environment.

1. General functions of gut microbiota

a. Nutritional uptake

Gut microbiota are broadly involved in digestion of food products, facilitating the breakdown of dietary polymers to compounds that can be used by the host. The extent to which gut microbiota aid in nutritional uptake likely depends on host physiology and diet. For example, a well-developed crop as found in the hoatzin (*Opisthocomus hoazin*) aids digestion of the complex polymers found in the species' folivorous diet. Foregut fermentation, as occurs in the Hoatzin crop, pre-digests food and allows the birds to use otherwise indigestible food sources.

At another extreme are nectarivores, which rely largely on simple sugars for their metabolic energy. Nectarivores, such as hummingbirds, did not appear distinct in their microbiota at a Phylum level (Hird *et al.*, 2015). However, Preest, Folk, & Beuchat (2003) identified potential involvement of the hummingbird gut microbiota in nitrogen recycling through the process of urate decomposition, which is advantageous due to the low-nitrogen content of nectar.

b. Detoxification

The avian gut microbiota, and specifically microbiota associated with the crop and ceca, may be involved in detoxification of food compounds. Different dietary compounds, such as phenols, resins and saponins associated with plant defenses against herbivory, can be toxic to birds but are common in diets of herbivorous birds. Limited evidence suggests that bird species subjected to high concentrations of dietary toxins employ GI bacteria to detoxify these compounds. High tannin concentrations in the

folivorous diet of hoatzins are potentially remediated by a community of crop bacteria that also includes members of the known cellulose-degrading Classes *Actinobacteria* and *Clostridia* (Garcia-Amado *et al.*, 2007).

Grouse (Family: *Tetraonidae*) have a winter diet of buds and catkins high in resins and phenolics, which are likely detoxified in the ceca. It has been suggested that grouse cecal bacteria undergo a gradual shift at the start of winter, to facilitate the change towards a higher resinous content in their winter diet (Bryant & Kuropat, 1980). The bacteria that may detoxify the resin compounds remain to be identified.

The crop and ceca are currently the only regions of the GI tract presently known to contain detoxifying bacteria. The crop is the first GI structure encountered by incoming food compounds, and therefore a logical reservoir for detoxifying bacteria in foregut fermenters like the Hoatzin. The ceca are located at the end of the GI tract, which raises the question of how the birds are affected by toxic compounds before they reach the ceca. In the case of grouse, resins have strong antimicrobial properties (Himejima *et al.*, 1992; Dığrak, İlçim, & Alma, 1999), and may thus impact the downstream microbiota. It is possible that mitigation of the anti-microbial effects of resins occur in the crops of grouse, but this has not yet been confirmed.

As an alternative to detoxification by gut microbes, several bird species may seek dietary supplements. For example, South American parrot species regularly use clay licks to gain micronutrients, and as a hypothesized detoxification mechanism (Lee *et al.*, 2014; Costa-Pereira *et al.*, 2015). The extent to which ingestion of clay aids in detoxification, or replaces the role of GI microbiota has been debated (Lee *et al.*, 2010).

c. Immune function

Interactions among gut microbiota and the avian immune system are poorly understood and have not been investigated in wild birds. In contrast to mammals, the development of pathogen specific antibody-producing B-lymphocytes in young birds occurs in the Bursa of Fabricius (or "bursa"), which is an offshoot of the intestines located near the cloaca. The bursa is responsible for B-lymphocyte

production until sexual maturity, after which production occurs predominantly in the blood marrow (Madej *et al.*, 2013). After reaching maturity the bursa atrophies and loses its function. Bacteria colonize the bursa immediately after hatch (Kimura, Yoshikane, & Kobayashi, 1986), and could potentially play a role in development of the adaptive immune response.

The mammalian gut microbiota likely plays an important role in maintaining the balance among different pathogen-detecting T-lymphocytes (Mazmanian *et al.*, 2005). The antigen recognition spectrum of T-lymphocytes narrows over time, which can likely be attributed to interactions with commensal microbiota. Immune reactions to desired host microbiota as well as to diet-associated microbiota have to be minimized, to avoid unnecessary investment and potential detrimental immune responses (Kohl, 2012). *Bacteroidetes* have been associated with host health through involvement in T-lymphocyte response activation and competitive exclusion of pathogens in mammals (Thomas *et al.*, 2011). In addition, *Firmicutes* could be involved in T-lymphocyte immunity. In chickens, antibiotic treatment early in life reduced the relative abundance of *Firmicutes*, but increased *Proteobacteria* (Simon *et al.*, 2016). Also, lower T-lymphocytes dependent antibody titers were observed later in life in the antibiotic treatment group than in untreated chicks, which could indicate a role of these bacterial Phyla in adaptive immune function.

d. Competitive exclusion of pathogens

Gut microbiota can play an important role in maintaining health of the host through interactions with pathogens (Servin, 2004). Commensal microbiota can interact indirectly with pathogens through stimulating or suppressing immune function, and directly through competitive exclusion and production of antimicrobial compounds, such as bacteriocins and other toxins (Hammami *et al.*, 2013; Kamada *et al.*, 2013). Antimicrobial compounds are usually specific to the pathogenic or undesired bacterial taxon, and do not affect the commensal community itself. In humans, competitive exclusion can occur through competition for space and nutrients among commensal bacteria and pathogens (Kamada *et al.*, 2013). No

one has investigated commensal-pathogen interactions in the avian GI tract, but the mechanisms of competitive exclusion and antimicrobial properties are likely similar in birds as in mammals.

2. Functions of core Phyla

a. Firmicutes

Firmicutes produce short-chain fatty acids as byproducts of fermentation, which can be directly absorbed by the host gut wall as an energy source (den Besten *et al.*, 2013). *Firmicutes* in mammalian guts are associated with carbohydrate fermentation and degradation of polysaccharides (Flint *et al.*, 2012). We found no studies attempting to infer *Firmicutes* function in wild birds, but in chickens, several studies show a positive relationship between *Firmicutes* abundance and mass gain and immune function, indicating similar roles of *Firmicutes* in mammals and birds (Liao *et al.*, 2012; Zhang *et al.*, 2015). Further, supplementing chicken diets with *Firmicutes* as probiotics, particularly *Bacillus subtilis* and *Enterococcus faecium* can increase nutrient uptake and general metabolic efficiency (Li *et al.*, 2016; Zheng *et al.*, 2016).

Relative abundance of *Firmicutes* is lower on average among wild birds compared to domestic chickens (Fig. 2.2). Differences in abundance are unsurprising as chickens are bred for optimized mass gain as fat, while wild birds generally maintain low body fat levels, except for the time preceding migration when some bird species can double their body mass. A rapid increase in lipid deposits that can occur in birds prior to migration raises the question whether *Firmicutes* abundance increases during this time period to facilitate weight gain. No field studies had investigated changes in gut microbial communities in relation to pre-migratory fattening.

b. Proteobacteria

The function of *Proteobacteria* in the avian digestive tract has not been investigated. One difference among between animal host taxa is the high abundance of *Proteobacteria* in wild birds compared to mammals and domestic chickens. Within the *Proteobacteria* classes, *α-Proteobacteria* are

relatively abundant (45%) in wild birds, in contrast to only 15% relative abundance in mammalian hosts (Ley *et al.*, 2008). *α-Proteobacteria* are abundant in marine bacterioplankton communities and soil rhizospheres (Cottrell & Kirchman, 2000; Fierer & Jackson, 2006). In the studies we summarized in this review, *α-Proteobacteria* appeared slightly more abundant in wild birds that are associated with marine environments (57.5%) than terrestrial environments (45.5%). Alternatively, *α-Proteobacteria* were found to be the most important group involved in degradation of an active acid herbicide (Liu *et al.*, 2011), which could indicate a possible role for detoxification in the GI tract.

c. Bacteroidetes

Bacteroidetes have a mutualistic relationship with their host, and their abundance in humans has been observed to be inversely related to abundance of *Firmicutes* (Turnbaugh *et al.*, 2007). In mammals, *Bacteroidetes* degrade complex biopolymers, and one of their functions in the GI tract is the degradation of polysaccharides such as carbohydrates and plant cell wall components (Thomas *et al.*, 2011). *Bacteroidetes* relative total abundance in birds is ~ 10%, which is lower than in non-human mammals (16%) and humans (46%). Lower average abundances of *Bacteroidetes* in birds compared to mammals can potentially be attributed to dietary differences, and the wide dietary range among different bird species allows for further investigation into this relationship. It is possible that the polymer degrading function of *Bacteroidetes* in birds is fulfilled by other microorganisms, or that these processes occur in defined sections of the GI tract. In birds, the ceca consist of two elongated, blind sacs extending into the body cavity from where the ileum and colon join, and are involved in fermentation of complex polymers (Section VII.4). Higher abundance of *Bacteroidetes* were found in the ceca of Japanese quail (*Coturnix coturnix*), emu (*Dromaius novaehollandiae*) and ostrich (*Struthio camelus*) (Fig. 2.3.; Matsui *et al.*, 2010; Bennett *et al.*, 2013; Kohl *et al.*, 2014), which supports the hypothesis that *Bacteroidetes* are localized in abundance and function within the avian GI tract.

c. Actinobacteria

The fourth most abundant Phylum of microorganisms in the avian GI tract is the *Actinobacteria*. Abundance of *Actinobacteria* in humans is positively correlated to fiber intake (Dominianni *et al.*, 2015) and associated with carbohydrate breakdown in honey bees (Lee *et al.*, 2015), indicating a potential commensal or mutualistic relationship with the animal host. No studies have investigated the function of *Actinobacteria* in wild or domestic birds.

VI. GASTRO-INTESTINAL PHYSIOLOGY AND MICROBIOTA

The avian gastrointestinal tract includes the oral cavity, esophagus, crop, proventriculus, gizzard, small intestine, ceca, large intestine, and the cloaca. These nine components of the GI tract represent discrete anatomical sections with specific digestive functions and putatively differentiated microbiota (Fig. 2.3). The cloaca does not have a digestive function, but receives products from both the digestive and urogenital systems. We included the cloaca in our review, as it was the most sampled environment within wild birds. The esophagus and crop are food storage structures in most bird species. The proventriculus chemically digests food, whereas the gizzard is a muscular stomach that degrades food physically. Different components of the GI tract vary in their biochemical properties such as oxygen content and pH, which can pose a selective pressure on the microbial community. The esophagus, crop and cloaca are considered semi-oxic environments, facilitating communities of aerobes, micro-aerobes and facultative anaerobes, including members of the α, β, and γ-*Proteobacteria*. The sections of the GI tract located between the crop and cloaca are dominated by obligate or facultative anaerobes, including members of the *Firmicutes* and *Proteobacteria* (Hird *et al.*, 2015; Waite & Taylor, 2015).

We focus on discussing microbiota of the crop, intestines, ceca and cloaca, and summarized our findings from the current literature (Fig. 2.3). We located no studies that addressed stomach or colon microbiota separately. Intestinal microbial communities have predominantly been inferred from analyzing fecal samples and cloacal swabs, two non-invasive techniques appropriate for sampling wild birds.

1. Crop & Esophagus

The crop consists of a pouch below the esophagus at the beginning of the GI tract in the majority of bird species, and is used as a temporary holding structure for quick ingestion of food. Long-term retention of food in the crop allows birds to utilize food sources that are difficult to digest, such as high cellulosic plant material. Although the majority of bird species have crops, the only bird species known with a well-developed fermenting crop system is the hoatzin, a tropical bird with an exclusively folivorous diet (Godoy-Vitorino *et al.*, 2008). The crop of the Hoatzin contains a microbial community functionally analogous to the bovine rumen, and includes methanogenic archaea, rumen bacteria and eukaryotic protozoa (Godoy-Vitorino *et al.*, 2012). Waite, Deines, & Taylor (2012) collected crop samples from a flightless herbivorous parrot, the kakapo (*Strigops habroptila)*, and observed dissimilar bacterial and archaeal communities compared to those collected using similar approaches from the hoatzin. The low OTU richness in the kakapo crop suggests that foregut fermentation is less important in this species (Fig. 2.3). Different subphyla of *Proteobacteria*, specifically *α*, and *γ-Proteobacteria*, dominate crops of the kakapo and hoatzin. A second study of Hoatzin showed the highest proportions belonging to bacteria from Classes *Bacteroidia* and *Clostridia*.

2. Proventriculus and Gizzard

The avian stomach consists of two parts: the proventriculus and the gizzard. The proventriculus, or stomach, is a small, glandular organ where food and enzymes are mixed before the food particles enter the gizzard. The gizzard is a muscular compartment with a textured (koilin) lining where food is mechanically ground up and digested. The proventriculus is usually acidic, likely biasing the resident microbiota towards acidophiles. Acidity of the proventriculus likely poses the first strong selection on microorganisms entering the digestive tract (Beasley *et al.*, 2015). Stomach acidity varies among bird species, with carrion-eating birds such as vultures notably having most acidic stomachs, suggesting a possible role of diet in shaping acidity (Roggenbuck *et al.*, 2014). No wild bird studies have investigated microbial communities and function in the proventriculus and gizzard, and even poultry research is

limited on this topic. Chicken gizzard microbiota were highly similar to crop microbiota (Sekelja *et al.*, 2012). *Lactobacilli* comprised 43% of the gizzard microbiota in the domestic chicken GI tract (Gong *et al.*, 2007). *Lactobacilli* are expected in the gizzard because these bacteria tolerate acidic environments, and also produce acids.

3. Small Intestine

The small intestine consists of three sections that show little differentiation in the avian gut (the duodenum, jejunum and ileum) and is located between the gizzard and ceca. The functions of the avian small intestine are similar to the mammalian small intestine, and include nutrient absorption, and further processing of food using enzymes and bile excreted from the pancreas and liver. Identification of gut microbiota from the small intestine is especially challenging due to its location in the GI tract, which precludes non-lethal sampling. Limited data for the gut microbiota of the small intestine are available for domestic poultry and indicate that the different sections of the small intestine possess similar microbiota and are dominated by *Lactobacilli* and *Clostridia* (Amit-Romach, Sklan & Uni, 2004).

4. Ceca

The majority of bird species possess ceca as part of the digestive tract. The size and width of the ceca vary considerably among and within bird species, and are generally longer in herbivorous birds. In willow ptarmigan (*Lagopus lagopus)* and broiler chickens, different dietary components, such as fiber content or fermentable content of food, can result in morphological change of the ceca (Pulliainen & Tunkkari, 1983; Józefiak *et al.*, 2006; Rehman *et al.*, 2007). Morphological changes in the ceca as a result of shifts in diet indicate that the function of the ceca includes fermentation of dietary compounds (Svihus, Choct, & Classen, 2013). In addition, ceca play an important role in electrolyte and water reabsorption (Thomas, 1982). Ceca are emptied regularly, but at a slower rate than normal defecation occurs. Depending on the bird species, cecal material is generally retained 3-4 times longer than fecal material (Duke, 1986).

Microbial communities of the ceca are distinct from the rest of the GI tract (Waite, Eason, & Taylor, 2014; Han *et al.*, 2016), and may vary based on host dietary status. In Japanese quail, cecal microbiota were relatively stable during a period of fasting (Kohl *et al.*, 2014), with the exception of an increase in *Verrucomicrobia* and slight decrease in *Bacteroidetes*. Cecal microbiota of the northern bobwhite (*Colinus virginianus*) were dominated in abundance by *Firmicutes*, followed by *Proteobacteria* and *Actinobacteria* (Su *et al.*, 2014), and these microbial profiles differed substantially between the microbiota of Japanese quail and capercaillie (*Tetrao urogallus*; Fig. 2.3). However, bobwhite samples were cultured opposed to the use of direct sequencing approaches, which may introduce a potential culture bias.

Ceca are absent in most bird species with protein and sugar rich diets, such as carnivores, piscivores, nectarivores or frugivores, despite of these various diet preferences covering a broad range of fiber content (Clench & Mathias, 1995). Absence of ceca and cecal-affiliated microbiota implies that birds either have broader host and microbial functions in other areas of the GI tract to fill the role of the ceca, or that ceca and cecal microbiota specifically involved in digestion of food components are absent in the host diets. The absence of ceca in host species with large variation in diet suggests that the functions of cecal bacteria in these birds are fulfilled in other areas of the GI tract, which should be reflected in their microbial communities.

Cecal bacterial classes shared among all bird species are *Clostridia* and *Bacteroidia*, followed by *Actinobacteria*. In one exception to the pattern, *Actinobacteria* were not detected in Ostriches which belong to a flightless, primitive group of ratite birds (Matsui *et al.*, 2010). Despite harboring several well-known pathogenic microorganisms, most members of the Class *Clostridia* are considered commensals and, in mammals, are involved in fermentation and maintaining gut homeostasis (Lopetuso *et al.*, 2013). Functions of *Clostridia* in birds are likely similar to function in mammals.

5. Colon

The large intestine of birds is relatively short and the avian colon is located between the ceca and the cloaca. In birds, its main function is water and electrolyte reabsorption. Unlike the fermentative role of the colon in mammals, fermentation in the avian digestive tract predominantly occurs in the ceca and, in the case of the hoatzin, the crop (Lei *et al.*, 2012). The colon in domesticated Japanese quail was dominated by the Genera *Lactobacillus*, *Bacteroides*, *Ruminococcus* and *Clostridium*, although the majority of sequences remained unclassified (Wilkinson *et al.*, 2016). Generally, fecal bacteria are assumed to reflect the colon microbiota. However, quail fecal microbiota consisted of microorganisms of both colon and cecal origin, indicating mixing of fecal microbiota from the different sections of the GI tract (Wilkinson *et al.*, 2016).

6. Cloaca

The cloaca has no digestive function, but serves as the exit cavity for the digestive and urogenital systems, and may have a unique microbiome due to its selective semi-aerobic environment. The cloaca is exposed to fecal bacteria, sexually transmitted bacteria, and possibly environmental bacteria associated with the eggshell, nesting material or environmental components such as water and soil. Cloacal swabbing has been the most used method for studying gut microbial diversity in wild birds with 15 published studies, although a majority of these studies relied on culturing and fingerprinting techniques. The cloacal microbial community in wild birds was dominated by *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Fig. 2.3). *Firmicutes* has highest relative abundance in three Procellariiform seabird species (Dewar *et al.*, 2014a), three shorebird species (Santos *et al.*, 2012), and two penguin species (Banks, Cary, & Hogg, 2009; Dewar *et al.*, 2014b), followed by *Proteobacteria*. In contrast, in the insectivorous barn swallow (*Hirundo rustica*), showed the opposite pattern, with *Proteobacteria* outnumbering *Firmicutes* by a 2:1 ratio. Although the Phyla are the same as found in fecal microbiota, communities likely differ on shallower phylogenetic levels.

Cloacal samples may fail to detect low abundance bacteria, and consequently rare taxa might be overlooked. Cloacal samples are collected using sterile cotton swabs, which can result in low biomass samples. The cloacal microbiota is expected to reflect the microbiota associated with waste products from the avian digestive system. However, the semi-aerobic conditions in the cloacal area likely select against obligate anaerobes that make up part of the intestinal microbiota. While the cloacal microbiota is presumed to contain a representation of upstream microorganisms from other sections of the GI tract, how well cloacal microbiota reflect the gut microbiota of wild birds remains still untested, since the only comparative studies have been conducted in domestic poultry.

Salmonella spp. were detected in 92% of fecal samples, but only in 4% of cloacal swabs collected from laying hens (García *et al.*, 2011). Samples were not collected from the same individuals, but were obtained from the same production facility hall, and indicate that cloacal swabbing only seems to capture a fraction of the gut microbial community. Ingresa-Capaccioni *et al.* (2014) compared detection of *Campylobacter* spp. from fecal and cloacal samples collected from chickens in production facilities, and detected *Campylobacter* spp. in 61.9% of cloacal, and 69.1% of fecal samples. *Campylobacter* is a common genus in the avian GI tract (Waldenström *et al.*, 2002, 2007; Keller *et al.*, 2011), whereas *Salmonella* is comparatively rare. A discrepancy in detection rates between *Salmonella* and *Campylobacter* may therefore potentially be contributed to differences in abundance.

7. Feces

Fecal samples are advantageous as a noninvasive approach for sampling the microbial gut communities of wild birds. Fecal bacteria have been widely used as a proxy for intestinal microbiota, because many investigators consider the sacrifice of wild birds to investigate intestinal bacteria undesirable. One of the challenges of studying feces instead of the actual GI microbiota is that fecal microbiota may be comprised of a mixture of bacteria from different sections of the GI tract. Also, fecal sampling can be sensitive to environmental contamination, depending on collection method A study comparing microbial communities in different segments of the GI tract of domestic pigs showed that fecal

microbiota was most similar to microbiota in the large intestine, with 75% similarity compared to 38% similarity with the small intestine (Zhao *et al.*, 2015).

In birds, the only study comparing fecal microbiota with microbiota of other GI components was conducted in domestic chickens. Fecal microbiota did not cluster with any of the components (crop, gizzard, ileum and colon) in particular, but rather consisted of a mixture of bacteria from all components (Sekelja *et al.*, 2012). Short-term fluctuations in fecal microbial communities were attributed to differential representation of the different GI components over time. Fecal microbiota in mammals is considered relatively stable over time (Claesson *et al.*, 2011; Newman *et al.*, 2012; Martinez, Muller, & Walter, 2013; Becker *et al.*, 2015). If fecal microbiota composition in wild birds is comparable to chickens, the variable contribution of microorganisms of different parts of the GI tract could obscure temporal relationships and internal turnover.

Hird et al. (2015) described colon microbiota of 59 Neotropical bird species and found a core microbiota among all host species consisting of *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. Host species was the most important factor for determining the finer-scale taxonomic composition of gut microbiota, suggesting some host specificity in the occurrence of unique microbial OTUs. Our literature search confirmed *Firmicutes* and *Proteobacteria* as the most abundant Phyla present in fecal microbiota of a wide range of wild bird species, although abundance and occurrence of additional Phyla were variable (Fig. 42.3.

VII. FACTORS AFFECTING GUT MICROBIOTA

Gut microbiota composition and dynamics are determined by a range of intrinsic and extrinsic factors (Fig. 2.4). *Intrinsic* factors are inherent to the host organism, and include genetic makeup, age, sex, and health, whereas *extrinsic* factors include host diet, social interactions, and the pool of environmental microbial inocula. Intrinsic and extrinsic factors are closely connected in their influence on gut microbial communities. For example, preferential diet of a bird may be species-specific and could be considered to be an intrinsic factor. However, food-associated microbial communities may vary by

location, and food quality can pose a differential selection pressure on the gut microbiota, both extrinsic factors. Foraging location during long-distance movements has an intrinsic component related to migratory behavior, but every foraging environment includes extrinsic pressures, such as variability in food choices based on availability and competition.

Disentangling the different intrinsic and extrinsic factors that affect avian gut microbial composition is challenging because of the wide range of bird life-histories. Bird species range from strict diet and habitat specialists, like some species of hummingbirds, to generalists like gull species that have a broad dietary range and occupy a wide variety of environments. We consider intrinsic and extrinsic factors separately for organizational purposes, but recognize that these effects on gut microbiota are often interconnected and interactive.

1. Extrinsic factors

a. Diet

In a majority of studies, changes in diet have been identified as the underlying cause of change in gut microbial communities. Broad dietary preferences, such as frugivory or insectivory, are intrinsic factors, while the extrinsic component of diet includes the ingestion of microorganisms associated with dietary food sources. Different geographic locations provide different microbial environments, and birds in different locations may ingest different food-associated microorganisms. Ingestion of microorganisms with food is likely one of the major pathways of microbial colonization of the avian gastrointestinal tract because eggs of birds are hatched outside of the parent, unlike mammals which can be first inoculated through the birthing process. To what extent ingested microorganisms contribute to or affect the mature gut microbiota in wild birds is unknown. Studies of domestic chickens found effects of different diets on establishment of the gut microbiota composition (Wise & Siragusa, 2007; Stanley *et al.*, 2012, 2014), but these studies cannot discern the selective effects of different dietary nutrient and fiber contents versus input of microbiota from different food-associated microbial communities.

Microbial communities of herbivorous bird guts are often dominated by members of the *Bacteroidetes* Phylum. *Bacteroidetes* are thought to assist with decomposition of polysaccharides, cell wall components, and other complex polymers (Thomas *et al.*, 2011). Carnivorous bird species have broad diets, ranging from carrion to marine invertebrates and have gut microbiota dominated by *Proteobacteria* and *Firmicutes* (Grond *et al.*, 2014; Ryu *et al.*, 2014; Blanco, 2014). Vultures have unique adaptations to their carrion-based diet, including extreme stomach acidity and a gut microbiota dominated by toxin-producing *Clostridia* ((Roggenbuck *et al.*, 2014).

Some species of birds experience large seasonal shifts in diet. However, no studies investigating seasonal in bird gut microbiota variation in relation to dietary change exist as of present. Seasonal variation in gut microbiota is common and has been observed in humans (Zhang *et al.*, 2014; Hisada, Endoh, & Kuriki, 2015), several primate species (Sun *et al.*, 2016), wood mice (*Apodemus sylvaticus*; Maurice *et al.*, 2015), American bison (*Bison bison;* Bergmann *et al.*, 2015) and giant pandas (*Ailuropoda melanoleuca*; Xue *et al.*, 2015). Migratory birds switch diets, and in addition to potentially different microbial requirements to aid digestion, these birds also likely ingest a wide variety of microorganisms associated with their different food sources. How these different microbial exposures affect nutrient uptake and bird health is remains unknown.

b. Environment

Environmental microbial communities are extremely variable due to heterogeneity in biotic and abiotic factors. Birds are exposed to different microbes, all potential gut inocula, through shifts in environmental conditions, including diet, water, soil, nesting environments, and social interactions.

Surprisingly, sampling location was not a major determinant in gut microbial composition in adults of 59 species of Neotropical birds (Hird *et al.*, 2015). However, swainson's thrushes (*Catharus ustulatus*) and gray catbirds (*Dumetella carolinensis*) shared similar shifts in gut microbiota composition between spring and fall stopover, indicating environmental influence (Lewis, Moore, & Wang, 2016). In addition, when investigating passerine gut microbiota, environmental factors such as location and diet

were the main factors affecting gut microbiota (Hird *et al.*, 2014). Variation in importance of site may be due to the age of sampled individuals, as young birds may have not yet developed a stable gut microbiota. To determine influence of environmental microorganisms on avian gut microbiota, we need simultaneous sampling of both birds and their local environments, such as sediment and water at foraging or nesting sites, and nesting materials. In addition, we need to identify successional trajectories in gut microbiota of young birds to determine when birds acquire their mature and stable microbiota.

c. Behavior: migration & social interactions

Resident birds may spend their whole lives in one geographical area. Compared to birds with large home ranges and migratory birds, residents are exposed to far less diverse inocula from which to recruit their gut microbiota. No studies have compared gut microbiota of resident and migratory individuals of the same or related species. However, somewhat counterintuitively, plumage microbiota were more diverse in resident birds than in migratory birds (Bisson *et al.*, 2009). The authors attributed differences among birds to higher microbial exposure during ground-foraging behavior, the dominant foraging strategy among resident species. Migratory species predominantly foraged in the shrub and canopy layer, and had little exposure to the soil microbiome. To test whether microbial exposure differs between resident and migratory birds, as for any comparative experiment, controlling for similar non-migratory behavior is needed.

Actual environmental microbial exposure may not necessarily be greater in migratory than resident birds, depending on their site fidelity and patterns of space use. Many migratory shorebird species return to the same sites during the breeding and non-breeding seasons, which effectively results in the use of many small habitat patches over a geographical gradient (Leyrer *et al.*, 2006; Merkel *et al.*, 2006; Johnson *et al.*, 2010). Also, retention time of local microbiota in the avian gut is not known. Variation in gut microbiota turnover associated with different stages of the migratory cycle therefore may not reflect sampling time and location in gut microbiota.

Exposure to different microbial environments during migration may also be influenced by the formation of large, potentially mixed-species flocks in relatively small areas. Avian interactions can facilitate transfer of microorganisms through close contact and involuntary coprophagy, which has been identified as the potential mechanism for spread of gull fecal indicator bacteria, *Catellicoccus marimammalium*, to shorebirds (Grond *et al.*, 2014; Ryu *et al.*, 2014). Large aggregations of birds as found in colonial breeding species could also result in the spread of gut microbiota among conspecifics, and potentially play a role in inoculation of chicks and juveniles.

2. Intrinsic factors

a. Phylogenetic history

Host species and evolutionary history strongly determine the gut microbiota of mammals (Ley *et al.*, 2008), with microbial gut communities more similar among closely related species. Coevolution in animals that have the potential for vertical transmission of whole microbial communities through the process of birth has been hypothesized as one of the reasons for the importance of phylogeny in microbiota structuring (Ley, Peterson, & Gordon, 2006). Several studies tested for similar patterns among birds, although these relationships appear less distinct. Hird et al. (2015) evaluated the influence of 18 categorical variables including host species, diet and geographical location on wild bird gut microbiota, and found that variables associated with host taxonomy were the strongest determinant of gut microbial community. Waite & Taylor (2014) also observed phylogeny as the main determinant of gut microbiota in the meta-analysis they conducted on a suite of avian species.

The gut microbiota of young of magpies (*Pica pica*) and parasitic young of great spotted cuckoos (*Clamator glandarius*) differed from that of their nest mates (Ruiz-Rodríguez *et al.*, 2009), despite being raised in the same nest environment. On the other hand, gut microbiota were more similar between siblings of the great tit (*Parus major*) within the same nest than among conspecific young in other nests (Lucas & Heeb, 2005). Further work is needed to determine whether the genetic relatedness or the similarity in diet provided by the parents lead to these differences. For example, raising chicks from bird

species with varying phylogenetic relatedness under similar environmental and dietary conditions would allow us to isolate genetic effects on gut microbial composition.

b. Age & Sex

Microbial colonization of bird guts is hypothesized to occur after hatching of the egg (van der Wielen *et al.*, 2002; Kohl, 2012). However, sampling of the microbiota during late incubation has documented the presence of several species of microbes at low abundance within the embryo gut (Kizerwetter-Świda & Binek, 2008). Infection of the reproductive tract in chickens can cause pathogenic bacteria to penetrate the egg shell from the outside environment, as well as to enter the egg during egg formation (Gantois *et al.*, 2009; Martelli & Davies, 2012; Cox *et al.*, 2012). If active transfer of maternal gut bacteria into the egg during egg formation was possible, chicks could hatch with gut microbiota present. Early recruitment could give the chicks a head start with respect to digestion and nutrient uptake, and ultimately could benefit growth. However, when GI tracts of wild birds are colonized, through whichever mechanisms, and by which microorganisms, remains unknown. Sampling wild bird embryos before hatch would help to elucidate the presence or absence of microbiota, and the potential for maternal control over her offspring's gut microbiota.

Microbial recruitment to young bird guts may occur through various routes. Altricial birds are dependent on their parents for food, which enables parents to influence their offspring's gut microbiota through prey selection and transfer of saliva. Precocial young leave the nest soon after hatch and often forage independently, limiting a direct role for parental influence on gut microbiota. Precocial parents can potentially influence their chicks' gut microbiota through leading them to foraging areas with beneficial prey, or brooding and preening of the chicks. In addition, coprophagy by chicks could accelerate the microbial recruitment and establishment processes.

Gut bacterial communities during early life in altricial birds are highly variable in diversity and abundance (González-Braojos *et al.*, 2011) and are markedly different from gut microbiota of conspecific adults (van Dongen *et al.*, 2013; Waite *et al.*, 2014). Once hosts reach maturity, their gut microbiota are

often assumed to be relatively stable (Faith *et al.*, 2013), but long time-series with monitoring of adult bird microbiota are lacking. Waite et al. (2014) found that fecal microbial communities of adult kakapos that were collected one year apart were markedly different. However, whether the intra-individual variation in microbiota composition is a result of increasing age, or within the natural range of variation we cannot yet say, as only two samples were collected per individual. Long-term, repeated sampling of individual birds is needed to determine the natural variation in their gut microbiota and to identify consistent age effects.

Males and females differ in reproductive physiology and behavior, which may manifest as different gut microbial profiles. Male and female northern bobwhites differed in three of the eight most common bacterial genera: *Enterococcus*, *Rothia* and *Streptococcus* (Su *et al.*, 2014). Despite the well-studied interactions between hormones and the human gut microbiota (summarized in Neuman *et al.*, 2015), little information is available for birds. Studies investigating hormones in birds have focused predominantly on the immunosuppressive effects of testosterone (Alonso-Alvarez *et al.*, 2009), but connections to gut microbiota have not been investigated. The only study on hormone-microbiota interactions has been conducted in commercial broiler chicks: supplementation of melatonin in broiler food increased growth hormone levels in plasma, as well as *Lactobacillus* abundance, broiler health and growth (Akbarian *et al.*, 2014).

c. Reproduction

In addition to the innate physiological differences associated with sex, there are reproductive behaviors specific to each sex that would affect gut microbiota composition, such as mating system. Sexually monogamous bird species are exposed to fewer microorganisms during mating than species with multiple sexual partners. Studies that have investigated sexual transfer of microbiota in wild birds and other vertebrates have predominantly used the cloacal swabbing method to investigate the affected microbial community. Lizards with multiple partners were shown to have a more diverse cloacal microbiota than sexually monogamous species (White *et al.*, 2011), a pattern that has yet to be

investigated in birds. The effects of mating on the cloacal microbiota in birds were shown to be transient (White *et al.*, 2010), and perhaps unlikely to have a lasting effect on the avian microbiota. However, different mating systems could result in differential pathogen transfer which can affect the partners' health.

Breeding can affect gut microbiota directly through transfer of microorganisms during the mating process and indirectly through increased close contact between mates during incubation and chick provisioning. No differences were found in cloacal microbiota between male and female barn swallows (*Hirundo rustica*), when birds were sampled during the breeding season (Kreisinger *et al.*, 2015). In addition, many bird species are known to geographically segregate by sex outside of the breeding season (Cristol, Baker, & Carbone, 1999; Alves *et al.*, 2013), which would expose males and females to different diets and habitats. How differential migration affects microbial exposure for males and females is not known, but could be investigated using localized sampling efforts during the non-breeding season.

d. Physiology: GI-tract morphology

Animal behavior can affect their microbiota and vice versa (Ezenwa *et al.*, 2012). In birds, migration is one of the main behaviors that can influence gut microbial composition. In addition to the extrinsic effects of migration on gut microbiota, there may be intrinsic physiological effects due to plasticity in bird gut morphology and function during migration. Migratory shorebirds shrink their GI tract in anticipation of long-distance movements, resulting in an average 30% reduction in GI tract length (Battley *et al.*, 2000). Effects of change in gut morphology on microbiota are unknown, but the lack of nutrient input during flight, combined with the microbial habitat alteration could lead to an impoverished microbiota. Many migratory birds frequent one or more staging sites during their migrations, which are usually food-rich locations that birds use as mid-migration fueling sites. Intensive foraging at staging sites results in ingestion of large quantities of local food-associated microorganisms, potentially inoculating impoverished communities with local microbes.

During spring migration at Delaware Bay (DE, USA), shorebirds use the same habitat and food source, consisting of eggs of horseshoe crabs (*Limulus polyphemus*; Clark, Niles, & Burger, 1993). Three shorebird species at this site, the red knot (*Calidris canutus*), ruddy turnstone (*Arenaria interpres*) and semipalmated sandpiper (*C. pusilla*), differed in their fecal microbial communities, despite experiencing a similar foraging environment (Grond *et al.*, 2014; Ryu *et al.*, 2014). Interspecific differences support the potential importance of phylogeny as a determinant of gut microbial communities. Similarly, shorebirds staging in the Tagus estuary in Portugal used the same habitat and resources, but showed distinct communities among species (Santos *et al.*, 2012). One explanation for these results is that even in reduced form, the microbiota present in the GI tract can outcompete incoming food-associated microorganisms. Alternatively, retention time of food in shorebirds during stopover may be too short to allow settlement of food-associated bacteria.

To investigate how migratory movements affect gut microbiota, it would be necessary to collect samples from birds prior to migration, immediately upon arrival at staging sites, and then during the staging period. Timing of migration and physiological adaptations to migration are intrinsic traits, which are expressed under captive conditions. Pre-migratory fattening and GI tract shrinkage can be observed in captive wild birds, which, in combination with wind tunnel experimental flights, could be valuable tools for assessing the effect of migration on gut microbiota.

e. Health & Fitness

The influence of the gut microbiota on health has been studied intensively in humans and, to a lesser extent, in domestic poultry. How gut microbiota affects health in wild birds is unknown, due to the difficulties of determining health status for free-living birds and low recovery rates of dead animals.

Gut microbiota can improve health through aiding nutrient uptake and through interactions with the immune system. A study investigating the effect of immune supplementation and challenge on the cloacal microbiota in homing pigeons revealed effects on evenness of the microbial community, but not on richness or diversity (Matson *et al.*, 2015). The authors did not identify microbial communities, and

we therefore do not know which bacteria specifically were affected by the experimental manipulations. To gain understanding of how gut communities interact with the immune system under normal and immunologically challenging conditions, will require extensive surveys combining health measurements, immunological assays and assessment of the gut microbiota in wild bird populations.

Gut microbiota composition could positively affect host fitness through its involvement in host health. The hologenome theory argues that organisms evolve together with their microbiomes, and that the microbial community can increase fitness parameters, such as survival, phenotypic plasticity and reproductive performance, of hosts when environmentally stressed (Zilber-Rosenberg & Rosenberg, 2008). Changes in gut microbiota in termites (*Zootermopsis angusticollis* & *Reticulitermes flavipes*) and the honey bee (*Apis mellifera*) decreased fecundity (Hamdi *et al.*, 2011; Rosengaus *et al.*, 2011). Water fleas (*Daphnia magna*) raised under sterile conditions showed severe decreases in fitness parameters. Fecundity in birds is a relatively easy parameter to monitor, especially in captive populations or in wild cavity nesting species. To understand how gut microbiota contribute to host fitness in birds, we need to manipulate gut microbial communities under field conditions, and then monitor fitness parameters, such as fecundity.

VIII. DISCUSSION & FUTURE DIRECTIONS

In our review, we have highlighted the many opportunities provided by wild bird GI tract microbiome research to learn how the environment and host interact to determine the composition and function of the gut microbiota. Two main topics require future extensive research effort: i) increase understanding of baseline, or "core", gut microbial diversity within and among avian taxa, ii) use controlled empirical experiments to identify the functional importance of gut microbiota to the host, and ii) identify function of avian gut microbiota using meta-omics tools.

At present, we are limited in the broad inferences we can make from gut microbial diversity, as studies on wild birds are relatively new, and to date, have mainly focused on one or a few species. Expanding the range of avian species studied will increase our understanding of variation in inter-specific

gut microbial diversity, which is a necessary first step in addressing the knowledge gaps in the areas we identified.

Sample collection from a large variety of bird species is challenging, but collaborations among different fields and research groups or organizations could offset some of the challenges. Hird et al. (2015) were able to investigate gut microbial communities in 59 neotropical bird species from 14 different orders by collaborating with the Museum of Natural Science at Louisiana State University during specimen collection. Alternatively, international collaborations have the potential for sample collection over large geographical ranges. In addition to increasing the variety of species studied, it is important to investigate the within-individual variation in the microbiota in different parts of the GI tract. Collecting fecal and cloacal samples as proxies for gut microbiota is a common approach. It is not clear whether or not the microbiota in upstream sections of the GI tract are accurately reflected in these samples, which affects the relevance of these samples to understanding links with digestive function. However, investigating upstream sections of the avian GI tract requires sacrifice of the host, which may be undesirable for some populations of wild birds.

Function of gut microbiota in birds remains poorly understood. Birds are fundamentally distinct from mammals. As a result, assigning functions to avian gut bacteria based on their equivalent function in the mammalian gut microbiota should be done with considerable caution. To assess broad functional patterns in gut microbial diversity, identifying a "core" microbiota of different hosts can be a good starting point. Identifying core taxa could aid in a better, targeted selection of focal taxa for physiological or genome studies. However, we also want to stress the importance of identifying the limitations associated with inference at coarse phylogenetic levels. Host-specificity of gut microbiota and their physiological function likely become more apparent at finer phylogenetic levels, and we encourage investigation at lower taxonomic levels than at the Phylum level.

One challenge of studying wild birds under natural conditions is disentangling the large number of intrinsic and extrinsic factors that potentially influence host microbial communities. Statistical methods are available to disentangle the importance of different factors on gut microbial communities, but require

extensive sampling of a wide range of intrinsic and extrinsic factors. Capturing wild birds changes their direct environment, diet and social interactions from natural conditions thus complicating the interpretation of experimental results. In the future, controlled experimental studies between paired free-living and captive populations should be used to address questions regarding microbial function, and responses to extrinsic and intrinsic factors. However, one must bear in mind the biases associated with studying captive, and especially captive-raised birds. Gut microbial communities differ between wild and domesticated birds and attempts to extrapolate from model to wild organisms requires considerable caution. That said, on a Phylum level we observed substantial similarity between domestic chickens and wild birds (Fig. 2.2), indicating that model organisms serve as an important tool and starting point to form hypotheses on the microbiota responses to changes, such as changes in diet and habitat.

Metagenomic sequencing will help to elucidate the bacterial genes present in gut communities, but only has limited use for inferring bacterial function because it relies on known gene function for annotation. To address the function of the avian gut microbiota, it is essential to detect the genes that are expressed, and thus functionally active at the time of sampling. Metatranscriptomics is a deep sequencing approach that can provide information on realized function through relative expression quantitation and annotation of the transcribed functional genes present in the sample. Comparing microbiome gene transcription profiles among host species that differ in life-history characteristics, such as diet, sex or age, will provide valuable insights into the variation in gut microbiota function in birds. Both metagenomic and metatranscriptomic sequencing rely on existing data for gene and transcript annotation. Construction of a carefully annotated set of reference genomes from a variety of bird species would greatly improve the inferences we can draw from these sequencing methods.

IX. CONCLUSIONS

1) Gut microbial communities of wild birds are highly diverse and show large within and among species variation.

2) Sections of the gastrointestinal tract differ widely in their microbial communities, and commonly used sampling methods like cloaca or fecal samples may not reflect specific gut components.

3) Phylogenetic history is a main determinant of gut microbial composition in birds. However, several studies show an influence of local environment on avian gut microbiota, indicating potential sensitivity to environmental change.

4) We identified three important future research areas, including broadening the range of avian taxa investigated, identifying function of avian gut microbiota using meta-omics tools, and using experimental manipulations to identify key determinants of gut microbiota composition.

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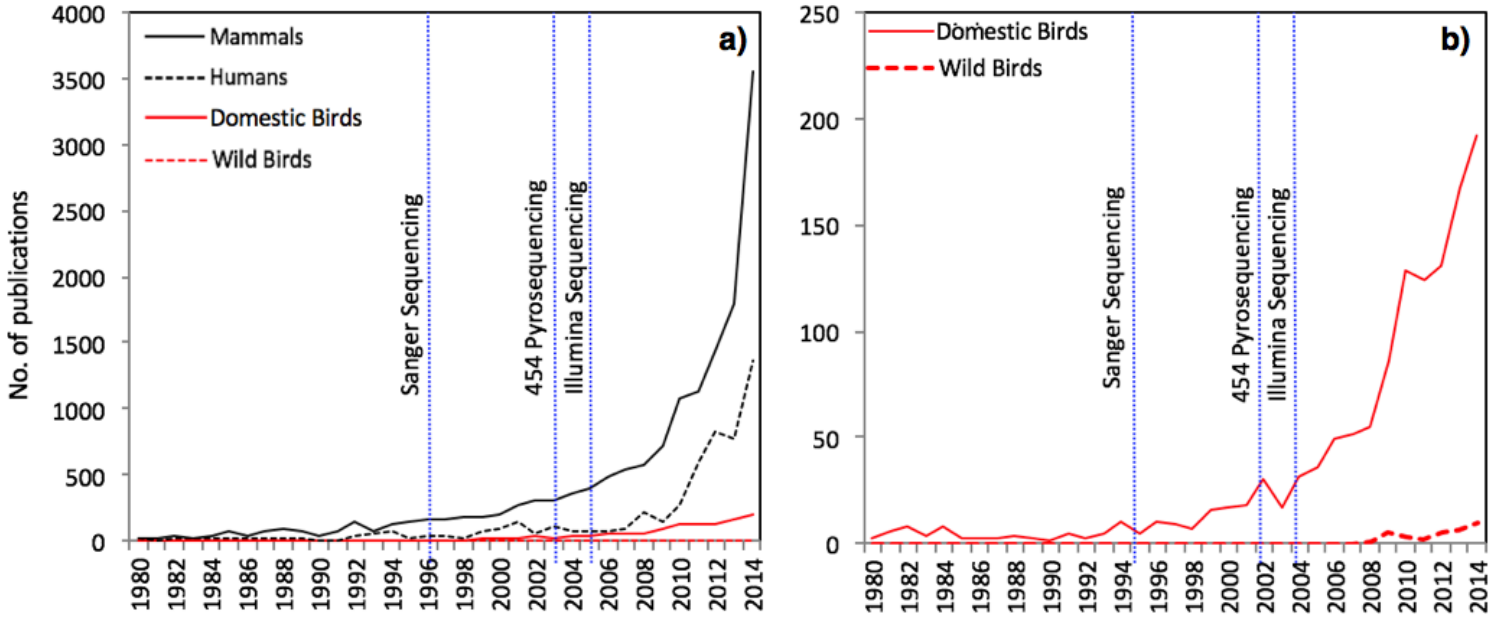

Figure 2.1 Studies on gut microbiota of birds and mammals (a), and wild and domestic birds (b) as indexed in Web of Science. Dashed vertical lines represent the first use of the respective sequencing technique in gut microbiota research. Data include studies using culture-dependent and culture-independent methods.

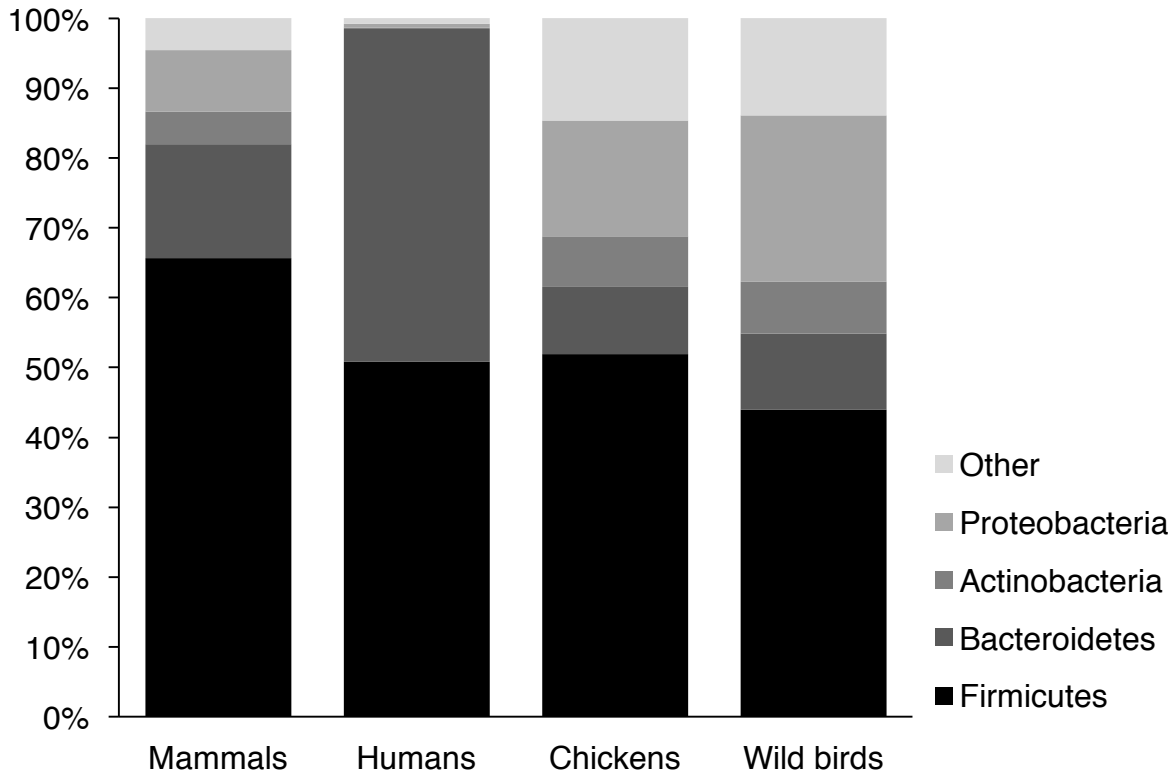

Figure 2.2 Core microbiota of mammals (domestic & wild; Ley et al. 2008), humans (Eckburg et al. 2005), domestic chickens (Waite and Taylor 2015) and wild birds (This study).

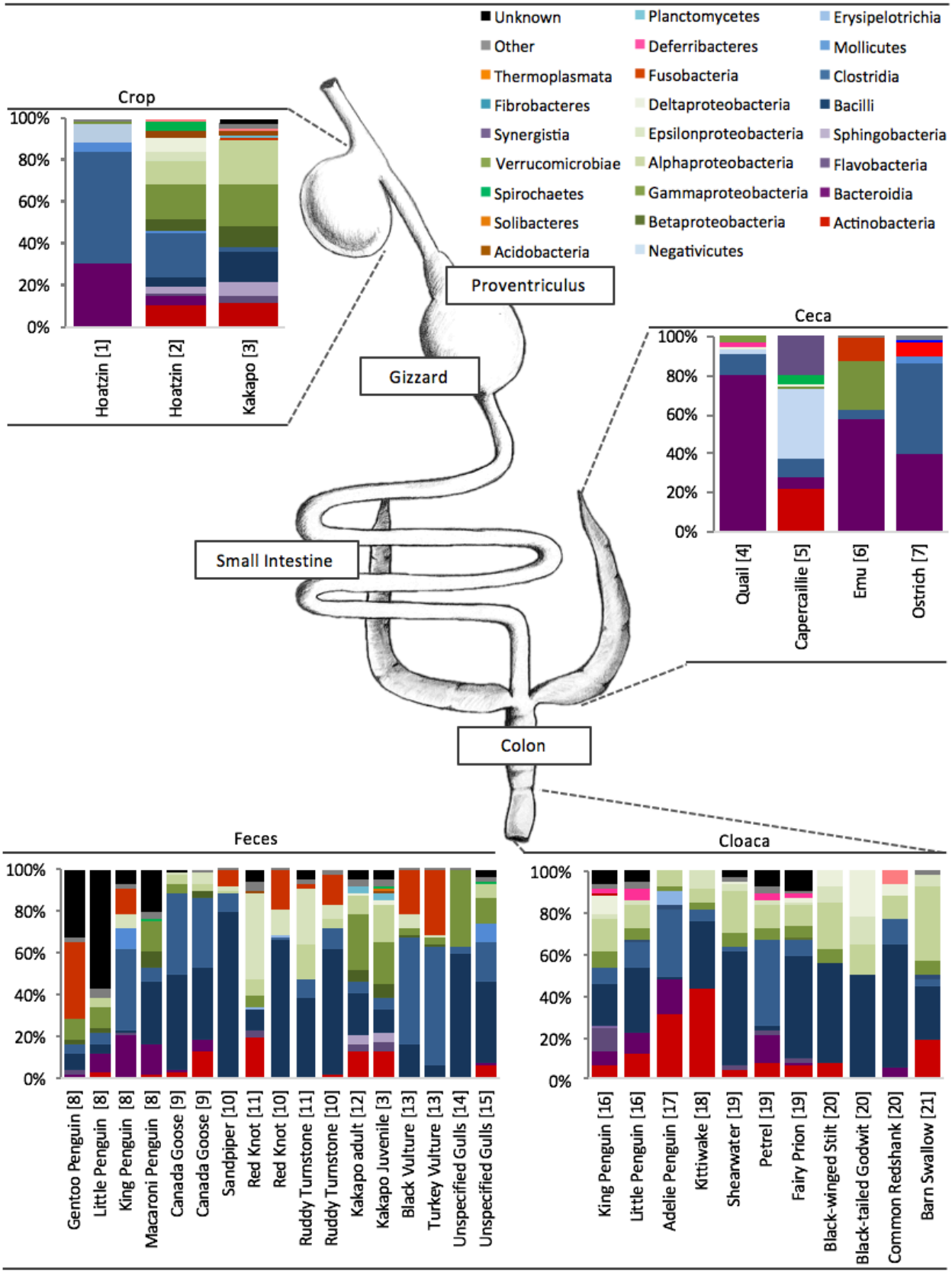
Unknown
Other
Thermoplasmata
Fibrobacteres
Synergistia
Verrucomicrobiae
Spirochaetes
Solibacteres
Acidobacteria
Planctomycetes
Deferribacteres
Fusobacteria
Deltaproteobacteria
Epsilonproteobacteria
Alphaproteobacteria
Gammaproteobacteria
Betaproteobacteria
Negativicutes
Erysipelotrichia
Mollicutes
Clostridia
Bacilli
Sphingobacteria
Flavobacteria
Bacteroidia
Actinobacteria
Crop
100%
80%
60%
40%
20%
0%
Hoatzin [1]
Hoatzin [2]
Kakapo [3]
Proventriculus
Gizzard
Small Intestine
Colon
Ceca
Quail [4]
Capercaillie [5]
Emu [6]
Ostrich [7]
Feces
Gentoo Penguin [8]
Little Penguin [8]
King Penguin [8]
Macaroni Penguin [8]
Canada Goose [9]
Canada Goose [9]
Sandpiper [10]
Red Knot [11]
Red Knot [10]
Ruddy Turnstone [11]
Ruddy Turnstone [10]
Kakapo adult [12]
Kakapo Juvenile [3]
Black Vulture [13]
Turkey Vulture [13]
Unspecified Gulls [14]
Unspecified Gulls [15]
Cloaca
King Penguin [16]
Little Penguin [16]
Adelie Penguin [17]
Kittiwake [18]
Shearwater [19]
Petrel [19]
Fairy Prion [19]
Black-winged Stilt [20]
Black-tailed Godwit [20]
Common Redshank [20]
Barn Swallow [21]

Figure 2.3 Bacterial diversity in different sections of the avian gastrointestinal tract. Adapted from the Ruffed Grouse digestive tract by Stevens & Hume (1998). We only included studies that used methods to characterize gut microbial communities, to avoid potential selective bias of culture-dependent techniques. References: [1] Godoy-Vitorino et al. 2008, [2] Godoy-Vitorino et al. 2012, [3] Waite et al. 2014, [4] Kohl et al. 2014, [5] Wienemann et al. 2011, [6] Bennett et al. 2013, [7] Matsui et al. 2010, [8] Dewar et al. 2013, [9] Lu et al. 2009, [10] Ryu et al. 2014, [11] Grond et al. 2014, [12] Waite et al. 2014, [13] Roggenbuck et al. 2014, [15] Koskey et al. 2014, [15] Lu et al. 2008, Dewar et al. 2014, [17] Banks et al. 2009, [18] van Dongen et al. 2013, [19] Dewar et al. 2014, [20] Santos et al. 2012, [21] Kreissinger et al. 2015.

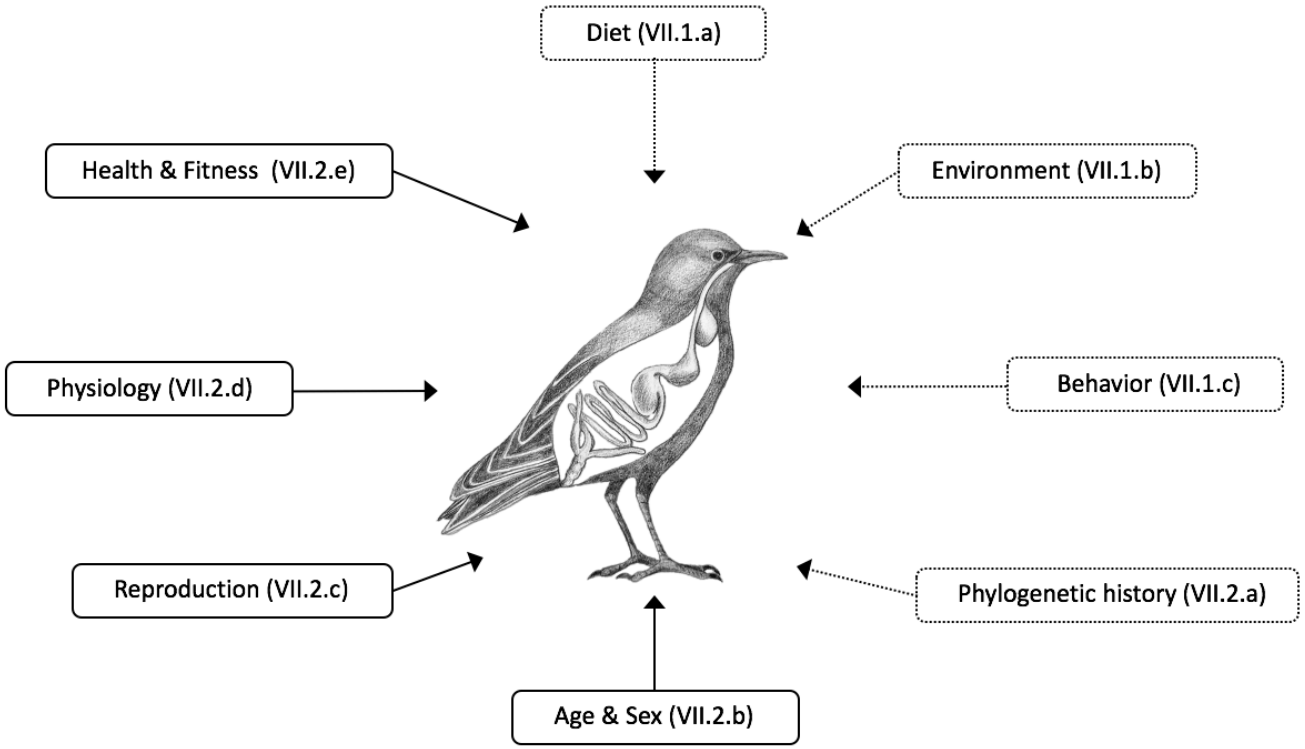

Figure 2.4 Extrinsic and intrinsic factors affecting the avian gut microbiota. Extrinsic factors are boxed in solid lines, and intrinsic factors in dashed lines. Numbers represent sections of this review describing the different factors.

Chapter 3 - RECRUITMENT AND ESTABLISHMENT OF THE GUT MICROBIOME IN ARCTIC SHOREBIRDS

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ABSTRACT

Gut microbiota play a key role in host health. Mammals acquire gut microbiota during birth, but timing of gut microbial recruitment in birds is unknown. We evaluated whether precocial chicks from three species of arctic-breeding shorebirds acquire gut microbiota before or after hatching, and then documented the rate and compositional dynamics of accumulation of gut microbiota. Contrary to earlier reports of microbial recruitment before hatching in chickens, quantitative PCR and Illumina sequence data indicated absent or negligible microbiota in the guts of shorebird embryos before hatching. Analyses of chick feces indicated an exponential increase in bacterial abundance of guts 0-2 days post-hatch, followed by stabilization. Gut communities were characterized by stochastic recruitment and convergence towards a community dominated by Clostridia and Gammaproteobacteria. We conclude that guts of shorebird chicks are void of microbiota prior to hatch, but that a stable gut microbiome establishes as early as 3 days of age, probably from environmental inocula.

Keywords: 16S rRNA gene, bacteria, *Calidris,* gut microbiota, qPCR, precocial young

INTRODUCTION

Gut microbiota contribute to maintaining organismal health through nutrient uptake (Leser and Mølbak 2009), detoxification of digestive byproducts (Kohl 2012), energy and fat metabolism (Velagapudi *et al.* 2010), and interactions with the host immune system (Rescigno 2014). Environmental exposure early in life can shape the gut microbiota, and aid in defending against pathogens while the immune system is immature (Bar-Shira *et al.* 2003). In mammals, maternal vaginal and fecal microbe transmission are crucial in the recruitment and establishment of microbiota in the digestive tract of a neonate (Palmer *et al.* 2007). Thus, maternal effects strongly control the initial composition of gut microbiota in mammalian offspring (Stevens and Hume 1998).

It remains unclear whether birds can acquire gut microbiota while inside the egg, and how microbial communities accumulates after initial recruitment. Bacterial colonization can theoretically occur before or after hatching, and we propose two alternative hypotheses to describe the process of microbial colonization: the *head start* and the *sterile egg* hypothesis. The *head start* hypothesis posits that microbes enter the gastrointestinal tract of avian embryos through transovarian transmission during oogenesis as a maternal effect, or possibly from the environment by penetration through eggshell pores and embryonic membranes after egg-laying (Gantois *et al.* 2009; Cox *et al.* 2012; Martelli and Davies 2012). The *sterile egg hypothesis* predicts that microbiota recruitment in the avian gut occurs after hatch, because the chorion membrane maintains a sterile environment within the egg (van der Wielen *et al.* 2002; Kohl 2012). The physiology of egg formation supports the latter hypothesis, but enteric bacteria have been cultured from the guts of embryos of domestic chicken (*Gallus gallus domesticus*; Kizerwetter-Świda and Binek, 2008), and vertical transfer of pathogenic *Salmonella enterica* has been documented between mother and embryo in domestic chickens (Guard-Petter 2001; De Buck *et al.* 2004). The two hypotheses differ in the maternal contribution to gut microbiota of offspring, and in the source of inoculum. The *head start* hypothesis predicts that maternal or environmental inoculation occurs before hatching, possibly resulting in metabolic and immunological advantages at hatching. In contrast, if gut

microbiota are absent in embryos, as posited by the *sterile egg* hypothesis, chicks must recruit microbiota from the environment after hatch.

Developmental strategies after hatching vary within birds and range from chicks hatching with complete dependence on their parent(s), like most altricial species, to superprecocial species that are independent of parental care after hatching. Altricial and precocial birds likely differ in the degree of maternal influence on microbial inoculation of their chicks. Altricial birds have undeveloped young that require parental feeding and brooding until fledging, whereas precocial chicks hatch fully developed with invaginated yolk sacs, leave the nest immediately, forage independently, but rely on parental brooding and defense until able to thermoregulate (Sibly *et al.* 2012). As a result, altricial birds have higher potential for parental influence on gut microbiota through salivary transfer and a longer period of nest occupation, whereas precocial parents have little direct influence on their offspring's microbiota aside from exposure to bacteria on feathers during brooding, and leading young to brood-rearing areas.

In addition to uncertainty about the timing of initial recruitment of microbiota, the early successional dynamics of the avian gut microbiota are unclear. For example, it remains unknown how rapidly chick guts are colonized, in what order bacterial taxa become established, and when convergence towards an adult gut microbiome occurs. Precocial young of most Arctic-breeding shorebirds are nidifugous, self-feeding and dependent on parents for thermoregulation, and could benefit from recruiting gut microbiota before hatching, because symbionts can promote nutritional uptake and growth immediately after hatch (Angelakis and Raoult 2010). Efficient use of nutrients may be particularly important in the Arctic because a short and synchronous breeding season requires rapid chick development (Schekkerman *et al.* 2003), favoring the recruitment of microbiota via the *head start* hypothesis.

We investigated the ontogeny of the gut microbial community in three species of Arctic-breeding shorebirds. Our objectives were to: 1) test the *head start* and *sterile egg* hypotheses and determine if precocial chicks of arctic shorebirds recruit gut microbiota before or after hatching, and 2) assess successional trajectories in the gut microbiota of shorebird chicks after hatching. To address our two

objectives, we combined quantitative PCR (qPCR) and Illumina MiSeq techniques to analyze the gut microbiota of embryos prior to hatch and in fecal samples collected from shorebird chicks from hatch to up to 10 days of age.

MATERIALS AND METHODS

Ethics statement

All animal work, including handling of birds, collection of viable eggs and chick transmitter application, was approved by the Institutional Animal Care and Use Committee at Kansas State University (Permit no. 3261). Field research with wildlife was conducted under the federal US Fish and Wildlife scientific collection permit (No. 778151-3) and the State of Alaska Department of Fish and Game scientific permit (No. 13-106) issued to KG, RBL and BKS.

Sample collection and preparation

We collected 27 eggs from seven clutches of dunlin (*Calidris alpina*) and 18 eggs from five clutches of semipalmated sandpipers (*Calidris pusilla*), at Utqiaġvik (formerly Barrow), Alaska (71º17'26"N, 156º47'19"W) in June-July 2013. We estimated incubation stage via changes in egg buoyancy that occur with embryonic development (Liebezeit *et al.* 2007) and collected eggs 1-3 days before the predicted hatch date to ensure near complete embryo development. After collection, embryos were removed from eggs, euthanized, and kept frozen at -20°C until dissection. Before dissection, embryos were washed in a weak solution of 0.006% sodium hypochlorite (10% bleach) to minimize contamination by external microorganisms. We aseptically removed the lower intestinal tract between the gizzard and cloaca and stored samples at –80°C. We also collected the invaginated yolk sacs from the embryos of five dunlin and four semipalmated sandpipers.

To investigate gut microbiota of mobile broods after hatching, we selected dunlin and red phalaropes (*Phalaropus fulicarius*) as study species, because dunlin use mesic terrestrial habitat, whereas

red phalaropes use freshwater habitat (Cunningham *et al.* 2016). Different habitats and associated microbiomes could affect recruitment of gut microbiota in chicks. We fit the attending adults with 1.55 g VHF radios, and, for dunlin, one chick per brood with 0.26 g VHF radios (Holohil Systems Ltd., Carp, ON, Canada; Supplementary Figure 3.1). Red phalarope chicks were too small at hatch to apply transmitters so we located young by tracking the attending male. We attempted to locate mobile broods every three days, using radio telemetry and by searching areas where broods were last seen. Once broods were located, chicks were captured and feces were collected by placing the chicks in individual compartments in an insulated, thermally heated bag, lined with sterile wax paper. Chicks were held for < 5 min to minimize stress and then released together. At several capture sites, we also collected a mix of water and soil in a 1.5ml Eppendorf tube using a flame-sterilized spatula. All samples were stored in 100% ethanol at -20°C until further analyses.

To remove ethanol from fecal samples, we centrifuged samples for 10 min at 10,000 rpm and discarded the supernatant. We repeated this step twice with 1 ml of RNase/DNA free molecular grade water to minimize ethanol contamination (Grond *et al.* 2014; Ryu *et al.* 2014). We shredded embryonic gastro-intestinal tissues, and extracted DNA from embryo and chick fecal samples using the MoBio Power Lyzer/Power Soil kit following the manufacturer's instructions (Mo Bio Laboratory, Carlsbad, CA, USA), except for replacing the bead beating step with 15 min vortexing at high velocity for tissue homogenization. Yield of genomic DNA was determined using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA).

Conventional PCR

To test for the presence of bacteria in the embryonic gut, we PCR-amplified total bacterial communities using general bacterial primers 515F and 806R to generate 16S rRNA gene amplicons (Caporaso *et al.* 2012). Primer sequences are provided in the Supplemental Materials (Supplementary Table 3.1). PCR reactions were conducted in a 25 µl reaction volume, using AmpliTaq Gold DNA polymerase (Applied Biosystems, Waltham, MA, USA) and 5 µl of DNA template (5ng-DNA/µl). PCR

conditions consisted of 30 cycles of: 15s at 95°C, 30s at 55°C and 30s at 72°C, preceded by an initial denaturing step of 10 min at 95°C, and followed by a final extension step of 5 min at 72°C.

To ensure that the bacterial DNA within the embryo lower GI tract was not degraded by our disinfection procedures, we amplified avian DNA from 24 random samples with primers 2550F and 2718R designed for molecular sexing of non-ratite birds, following PCR conditions of Fridolfsson and Ellegren (1999). Had the sodium hypochlorite compromised the DNA, we should not have been able to acquire avian or bacterial amplicons. Validation tests confirmed the integrity of the lab procedure and our ability to amplify the bacterial DNA.

Quantitative PCR

We estimated 16S rRNA gene copy numbers in embryo gut, yolk, and fecal samples in triplicate qPCR reactions on a BioRad CFX96 Touch Real-Time PCR thermocycler (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). We used a TaqMan® qPCR assay (5'–CTTGTACACACCGCCCGTC–3'; Applied Biosystems, Waltham, MA, USA) targeting the bacterial 16S rRNA gene. in combination with 2X TaqMan® Gene Expression Master Mix with two general bacterial primers at 100nM final concentrations (F_Bact1369 and R_Prok1492**;** Furet *et al.* 2009; primer sequences can be found in Supplementary Table 3.1). Cocktails included 4 µl of DNA template (5ng-DNA/µl) and conditions consisted of 2 min at 50°C and an initial denaturing for 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The negative control was RNase/DNA free molecular grade water and the positive control was Semipalmated Sandpiper fecal DNA at 5ng-DNA/µl. Standard curves were generated using 2 to 2 x 10^6 16S rRNA gene copies of *Staphylococcus aureus* subsp. *aureus* (efficiency = 105-116%, R^2 = 0.984–0.990). To control for the possibility of PCR inhibition and to establish minimum detection levels, we spiked a subset of samples (n = 15) with a dilution series of the *Staphylococcus aureus* rRNA gene ranging from 10^0-10^6 copies.

16S rRNA gene sequencing and sequence analyses

We used general bacterial 515F and uniquely barcoded 806R primers to generate multiplexed 16S rRNA gene amplicons from embryo guts and fecal samples, following protocols of the Earth Microbiome Project (Caporaso *et al.* 2012). PCR reactions were performed in a 25 µl reaction volume, using AmpliTaq Gold DNA polymerase (Applied Biosystems, Waltham, MA, USA) and 5 µl of DNA template (5ng-DNA/µl), and were run using 25 cycles, opposed to the 35 cycles described in the EMP protocols. We removed primers from our PCR product using the Agencourt AMpure XP PCR purification system (Beckman Coulter, Brea, CA) following manufacturer's instructions except AMpure volume ratio was adjusted to 1:1 and the ethanol washes were repeated three times instead of two. We sequenced the V4 region in 2 x 250 bp paired-end runs using the Illumina MiSeq platform. Each Illumina run included a 15% PhiX spike. Embryo samples, and fecal samples from chicks of 0-3 days old were sequenced twice, because of low sequence yields.

Sequences of bacterial rRNA were quality filtered, contiged and demultiplexed using the QIIME Program (Caporaso *et al.* 2010), and aligned against the GreenGenes 16S rRNA gene reference database (v.13_8; DeSantis *et al.*, 2006). We identified chimeras – artifacts that combine multiple different sequences – using CHIMERASLAYER (Haas *et al.* 2011). Chimeras, singletons and non-aligned sequences were removed from the dataset, and the remaining sequences were clustered to Operational Taxonomic Units (OTUs) at 97% sequence similarity, and assigned to taxa using the Naive Bayesian Classifier with an RDP reference (Wang *et al.* 2007). After taxonomy assignment, we identified and removed non-target sequences of Archaea, chloroplasts, and mitochondria.

Prior to subsequent analyses, we rarefied chick samples to 5,000 sequences per sample due to low sequence yields in samples of chicks from 0-2 days of age. All embryo samples yielded far fewer than 5,000 sequences, and were analyzed without rarefication.

Statistical analysis

Statistical analyses were conducted in R (ver. 3.3.1, R Development Core Team, 2016). To test for differences in 16S rRNA gene copy numbers between embryos and chicks, we performed a one-way analysis of variance and assessed pairwise differences using a post-hoc Tukey HSD test with functions of the base Program R. To determine whether dunlin and red phalarope chicks differed in gut microbiota, or if samples could be pooled for further analyses, we analyzed homogeneity of variance using the *betadisper* function from the 'vegan' package (Oksanen *et al.* 2016). To test for pairwise differences in variance between young and old chicks, and environmental samples, we performed a permutation F test using the *permutes* function in the 'vegan' package.

To look for natural breaks in bacterial abundance in our chick qPCR data, we used the *segmented* function from the 'Segmented' package (Muggeo 2008). Large changes in the slope represent changes in the accumulation of 16S rRNA copies in the chick gut and were used to determine whether we could pool our chick microbiota data into naturally occurring, broader age classes. To test for significant differences among slopes in bacterial abundance versus chick age, we ran a Davies test using the *davies.test* function from the 'Segmented' package.

We calculated diversity indices (observed number of OTUs, Simpson's (1-D) and Shannon's H) using the QIIME alpha_diversity.py script. In addition, we calculated evenness (Pielou's J) using the *diversity result* function in the 'BiodiversityR' package (Kindt and Coe 2005). We estimated beta diversity, to compare variance between age groups (0-2 days versus 3-10 days) and environment. We tested for pairwise differences among age groups, using post-hoc Tukey HSD tests. We determined shared OTUs among young and old chicks and environmental samples, and constructed a scaled Venn diagram using EulerAPE (Micallef and Rodgers 2014).

To investigate potential differences among microbial communities in chicks and the environment, we applied non-metric multidimensional scaling (NMDS) of Bray-Curtis distance matrices using the *metaMDS* function in the 'vegan' package. We tested for treatment differences through an analysis of similarity using the *anosim* function in the 'vegan' package. Means are shown ± standard error.

Data accessibility

The nucleotide sequences and metadata will be made available through Figshare.

RESULTS

1. Timing of Recruitment

Conventional and Quantitative PCR

Conventional PCR assays yielded no visible 16S rRNA gene amplification from gut or yolk samples of shorebird embryos collected 1-3 days before hatching. Consistently, the high fidelity and high sensitivity TaqMan qPCR assays suggested extremely low copy numbers of 16S rRNA genes in the intestinal tract and yolk sac of embryos. The qPCR detected bacterial amplification in our positive control after 12.6 ± 1.1SE cycles, compared to 33.8 ± 0.2 and 33.2 ± 0.3 cycles for the guts of dunlin and semipalmated sandpiper embryos, 34.1 ± 0.2 and 33.7 ± 0.2 cycles for yolk samples, and 33.9 ± 0.5 cycles for our negative control. We detected negligible copy numbers of the bacterial 16S rRNA gene in all embryo samples, and we pooled samples from semipalmated sandpipers and dunlin for statistical analyses. Our gut and yolk samples did not differ from the negative controls in either threshold cycle or copy numbers (Tukey HSD; $p > 0.22$; Fig. 3.1a), and all samples and negative controls had higher threshold cycle and lower copy numbers than our positive controls (Tukey HSD; $p < 0.001$).

We successfully amplified a subset of samples spiked with 10 copies of *S. aureus* within 15 cycles, confirming our ability to detect low copy numbers of bacterial 16S rRNA and demonstrating an absence of PCR inhibitors in our samples. Additionally, avian DNA from all 24 gut samples amplified strongly in conventional PCR, further confirming that disinfecting the embryo's external surfaces did not compromise DNA within the GI tract of shorebird embryos. Thus, our conventional and qPCR assays indicate that fewer than 10 copies of bacterial rRNA copies - or fewer than one to five bacterial cells - were present in our embryo samples, which represented the entire gastrointestinal system from the gizzard to cloaca.

Sequencing

Illumina MiSeq libraries acquired from embryo samples contained an average of 108.6 ± 15.9 sequences (range 0-427), whereas the positive control contained over 7 million sequences. A second sequence library generated from the gastro-intestinal tracts and yolk samples from shorebird embryos resulted in similarly low yields. Bacterial sequences detected in the embryo samples were indistinguishable from the negative controls (Tukey HSD; $p > 0.99$). The microbial communities in embryo gut samples did not differ from yolk samples ($F_{2,38} = 101.6$, $p = 0.13$). Overall, our sequence data indicated that minimal or no microbiota were present in the gastrointestinal systems or yolk sacs of shorebird embryos before hatching of the eggs.

2. Establishment of the Microbiota

We collected 45 fecal samples from 1-10 day old chicks of dunlin and red phalaropes at Utqiaġvik, AK, in 2013. Despite the different habitats used by dunlin and red phalaropes to rear their chicks, fecal samples of chicks did not differ in the abundance and variance of their microbiota ($F_{2,27} = 5.5$, $p = 0.16$). We pooled samples for further analyses to increase sample sizes.

Abundance

We observed an increase in 16S rRNA gene copy numbers from age 0-2 days. After 3 days post-hatch, copy numbers stabilized at on average 395,852 ± 98,048 per sample (Fig. 3.1b). Our broken-stick regression indicated a natural break at 3 days post-hatch, and the slopes between 0-2 and 3-10 days post-hatch differed significantly (Davies' test, $p < 0.001$; Supplementary Figure 3.2). The slope from day 3-10 did not differ from 0, suggesting stability with no change in microbial abundance over this period ($t = 0.095$, $p = 0.925$). As a result, we pooled our samples into a "young" (0-2 day age) and "old" (3-10 day age) group for further analyses.

Richness and Diversity

After quality filtering, we retained 1,826,091 high quality sequences for further analyses. After rarefaction, we included five samples of 0-2 day old chicks, 19 samples from 3-10 day old chicks, and six environmental samples.

Overall, environmental samples were highest in bacterial richness, followed by young chicks, and old chicks. We identified a total of 36 different bacterial Phyla in our samples. Environmental samples had the highest number of Phyla (34), followed by young chicks (23), and old chicks (19). OTUs were distributed across a total of 723 bacterial genera. The environmental samples had the highest richness with 568 genera. Samples from young chicks were richer than samples from old chicks, with 432 genera compared to 379 genera. Similarly, total unique OTU numbers were highest in environmental samples, followed by young and old chicks (Fig. 3.2). The environmental samples contained 1,060.5 ± 122.7 OTUs per sample, young chick samples had 308.4 ± 80.1 OTUs, and old chick samples had 105.7 ± 7.7 OTUs (Supplementary Figure 3.3). Observed OTU richness was lower in samples from old chicks than in samples from young chicks (Tukey HSD, $p = 0.03$), consistent with environmental/habitat filtering occurring as chicks aged. In both young and old chicks, the observed OTU richness was lower than in the environmental samples (Tukey HSD, $p < 0.001$). Simpson's (1-D) and Shannon's (H) diversity indices indicated that environmental samples had the highest diversity scores (0.98 ± 0.01; 8.10 ± 0.39), and old chicks the lowest (0.56 ± 0.04; 1.98 ± 0.14). In addition, evenness of bacterial communities in the gut was highest in environmental samples (0.81 ± 0.03), followed by young chicks (0.53 ± 0.11), and old chicks (0.30 ± 0.02; Supplementary Figure 3.3).

At a community level, our NMDS ($k = 3$, stress = 0.083) distinguished environmental from chick samples (ANOSIM, $R = 0.853$, $p = 0.003$; Fig. 3.3), and young chick samples were more similar to environmental samples (ANOSIM, $R = -0.052$, $p = 0.599$), than old chick samples. The gut microbiota composition of chicks was dynamic over time. Relative abundance of *Firmicutes* increased from 37.7 ± 14.2% in young to 73.4 ± 5.9% in old chicks (Fig. 3.4). The increase was mainly driven by increases in abundance of the Classes *Clostridia* and *Bacilli* (Fig. 3.5). Within the Genus *Clostridia*, *Clostridium*

colinum – an opportunistic avian pathogen - was responsible for 73.6 ± 8.6% of sequences among young chicks, and 88.1 ± 4.1% in old chicks. The second most abundant OTU within the *Clostridia* belonged to the Genus *Candidatus* Arthromitus with a relative abundance of 6.1 ± 2.7% in young, and 10.3 ± 4.1% in old chicks. In contrast to *Firmicutes*, *Proteobacteria* relative abundance decreased from 45.5 ± 14.4% in young to 22.3 ± 6.0% in old chicks. Within *Proteobacteria*, 43% of sequences belonged to the Genus *Rickettsiella* within the *Gammaproteobacteria*. The relative abundance of *Actinobacteria* and *Bacteroidetes* declined by 50-80% from young to old chicks (*Actinobacteria*: 4.1 ± 1.5% to 0.3 ± 0.1%; *Bacteroidetes*: 5.0 ± 2.2% to 0.1 ± 0.02%; Fig. 3.5).

Beta diversity of bacterial OTUs was greater among old chicks than among the environmental samples ($F_{2,27} = 3.347$, $p = 0.03$), but did not differ between young chicks and the environment ($F_{2,27} = 3.347$, $p = 0.26$), and only marginally between young and old chicks ($F_{2,27} = 3.347$, $p = 0.07$).

DISCUSSION

1. Timing of Recruitment

Our data strongly supported the *sterile egg hypothesis*, which posits that embryos hatch from eggs with a sterile gut. Our conventional and qPCR assays consistently indicated low copy numbers of 16S rRNA genes in the embryo and yolk samples that were either below detection level (conventional PCR) or indistinguishable from negative controls to which no DNA template had been added. The low copy numbers detected in the negative controls likely represent primer dimers or other artifacts, as products appeared late in the amplification process after > 30 cycles (Arikawa *et al.* 2008). Further, samples spiked with as few as 10 copies of *S. aureus* amplified after 15 cycles or less, confirming our ability to detect low copy numbers if bacteria had been present.

Similarly to the PCR assays, we documented little evidence of bacteria in our embryo samples or the negative controls in the Illumina MiSeq sequencing analyses. The detection of any sequences in our

negative controls might be attributed to several potential causes. Carlsen *et al.* (2012) demonstrated tag switching in 0.7-1.6% of sequences during pyrosequencing, resulting in erroneous assignment of sequences to samples. In fact, the total number of sequences in our embryos and negative controls comprised only 0.06% of all sequences, a proportion far below error rates estimated by Carlsen *et al.* (2012). Similarly, barcode switching in Illumina sequencing studies can present a substantial source of error. Sinclair *et al.* (2015) used both forward and reverse barcoded primers and estimated that barcode switching could represent up to 22% of sequences in the Illumina platform. We used only reverse-barcoded primers and were unable to estimate or detect barcode switching. Sequences in negative controls (or samples without adequate template) may also originate earlier in the library preparation process. Last, DNA extraction kits and PCR reagents may contain some bacterial DNA, leading to false detections in low template samples like our embryos (Salter *et al.* 2014).

The apparent absence of bacteria in the guts of shorebird embryos collected under natural conditions contrasts with past reports from domestic chickens. Kizerwetter-Świda and Binek (2008) cultured a variety of enteric bacteria from chicken embryos obtained from a local hatcher. However, if the gut microbiota recruitment and establishment were similar in shorebirds and chickens, we should have detected low levels of bacterial with our culture-independent methods that targeted the entire spectrum of gut bacteria. Differences in environmental conditions between domestic chickens in a commercial-scale production facility versus Arctic-breeding birds under field conditions might lead to different routes for bacterial transmission. For example, *Salmonella enteritidis* can infect chicken embryos via infection of the female chicken's reproductive organs, resulting in incorporation of bacteria into the egg during oogenesis (Gantois *et al.* 2009). The likelihood of a similar infection in wild birds may be low and possibly the reason for no evidence of bacteria in our wild embryos. Overall, our field data indicate that gut microbiota become established after chicks hatch and are exposed to the environmental microbiome.

2. Establishment of the Microbiota

Based on our qPCR data, the bacterial abundance increased exponentially in chick guts during the first three days after hatching. Precocial chicks of Arctic shorebirds leave the nest within a day of hatching and feed independently, and we have observed chicks feeding as early as 1 day of age (personal communication: D.E. Gerik). Since parents do not provision precocial chicks in Arctic-breeding shorebirds, chicks likely acquire gut microbiota from ingestion of prey-associated microbiota. An inoculation route via diet was supported by our ordination results which showed that the microbiota of young chicks were more similar to the environmental microbiota in community composition than old chicks.

A rapid increase in the microbial abundance within the first three days after hatching may be enhanced by the supply of yolk to the chick gastro-intestinal tract. During the first 2-3 days after hatch, precocial chicks use an invaginated yolk sac for nutrients while they learn to forage (Forsythe 1973; Schekkerman and Boele 2009). The yolk mainly consists of fat, protein and carbohydrates, and contains vitamins and trace elements (Vieira 2007). Combined with the relatively high body temperature of the chick, yolk provides a rich substrate for bacterial growth, and is commonly used in bacterial pure culture media (Carter 1960; Westblom *et al.* 1991; Byrne *et al.* 2008).

We observed higher richness at a Phylum, Genus and OTU level among gut microbes in young chicks than old chicks. OTU richness was three times higher in young chicks than old chicks for some bacterial Phyla, indicating either selective recruitment or host-controlled environmental filtering prior to microbiota stabilization at 3-10 days of age. Community filtering for specific, high abundance gut symbionts was supported by a decrease in evenness, which suggested a rapid increase in abundance of dominant bacterial taxa with age. Depletion of the yolk sac after 2-3 days and the associated decrease in available nutrients to the chick gut microbiota might also explain the decrease in diversity. After nutrients are depleted, only bacteria that use nutrients derived from the chick diet, or those produced by other gastro-intestinal bacteria, would be sustained. After 3 days post-hatch, we observed establishment of a stable abundance of gut microbiota. Bacterial abundance did not change over the rest of our sampling

period, but then differed by two orders of magnitude between 10-day-old chicks and adult birds. We were unable to sample young birds after ten days of age, because chick mortality and enhanced mobility made locating chicks challenging.

As chicks aged from 0 to 10 days old, their gut microbiota became dominated by bacteria within the Phyla *Firmicutes* and *Proteobacteria*. Increasing abundance of *Clostridia* was the main driver of the rise in *Firmicutes* abundance. *Clostridia* also are early colonizers of the human infant gastro-intestinal tract and essential in maintaining gut homeostasis (Lopetuso *et al.* 2013). The prevalence and natural occurrence of *Clostridia* in the gastro-intestinal tract in other wild birds is unknown, but their high abundance in the guts of shorebird chicks suggests a role for commensalism or potentially mutualism.

Within the Class *Clostridia*, *Clostridium colinum* was the most abundant species with a relative abundance of 70%. *C. colinum* is an opportunistic avian pathogen and can cause ulcerative enteritis in chickens and quail. *C. colinum* infects a variety of birds, including domestic poultry, northern bobwhites (*Colinus virginianus*), American robins (*Turdus migratorius*), western bluebirds (*Sialia mexicana*), and lories (*Trichoglossus* sp. and *Eos* sp.; Winterfield and Berkhoff 1977; Porter 1998; Bildfell *et al.* 2001; Pizarro *et al.* 2005; Beltran-Alcrudo *et al.* 2008). All previous detections of *C. colinum* have been associated with disease or death of the host, but the occurrence of *C. colinum* in shorebird chicks could present evidence of alternative functions in the avian gut. *C. colinum* was not detected in the environmental samples we sequenced, suggesting that chicks may acquire this bacterium from their diet or another source.

Proteobacteria were the second most abundant Phylum within the chick gut; their relative abundance decreased from young to old chicks. Similar to *Clostridia*, human infants experience an early increase in the Class *Gammaproteobacteria* (La Rosa 2014). *Gammaproteobacteria* represent a variety of species, but their functions have not been identified in avian systems. Within the *Proteobacteria*, we observed a shift from an evenly structured community including all Classes of *Proteobacteria* to a community dominated by *Gammaproteobacteria*. *Rickettsiella* was the dominant Genus within the *Gammaproteobacteria*. *Rickettsiella* spp. are associated with microbiota of terrestrial arthropods

(Bouchon *et al.* 2016; Duron *et al.* 2016), including several pathogens and endosymbionts (Leclerque and Kleespies 2008; Tsuchida *et al.* 2010).

Based on differences in gut microbiota of chicks and adults, it is likely that chicks continue to acquire more bacteria as they grow. Perhaps the largest expansion of the microbial community occurs when birds migrate for the first time and are exposed to new, diverse environments across a large geographic area. Exposure to new microbial communities in the environment during migration may increase gut microbial abundance, when bacteria occupy previously unused niches, but this question requires further study.

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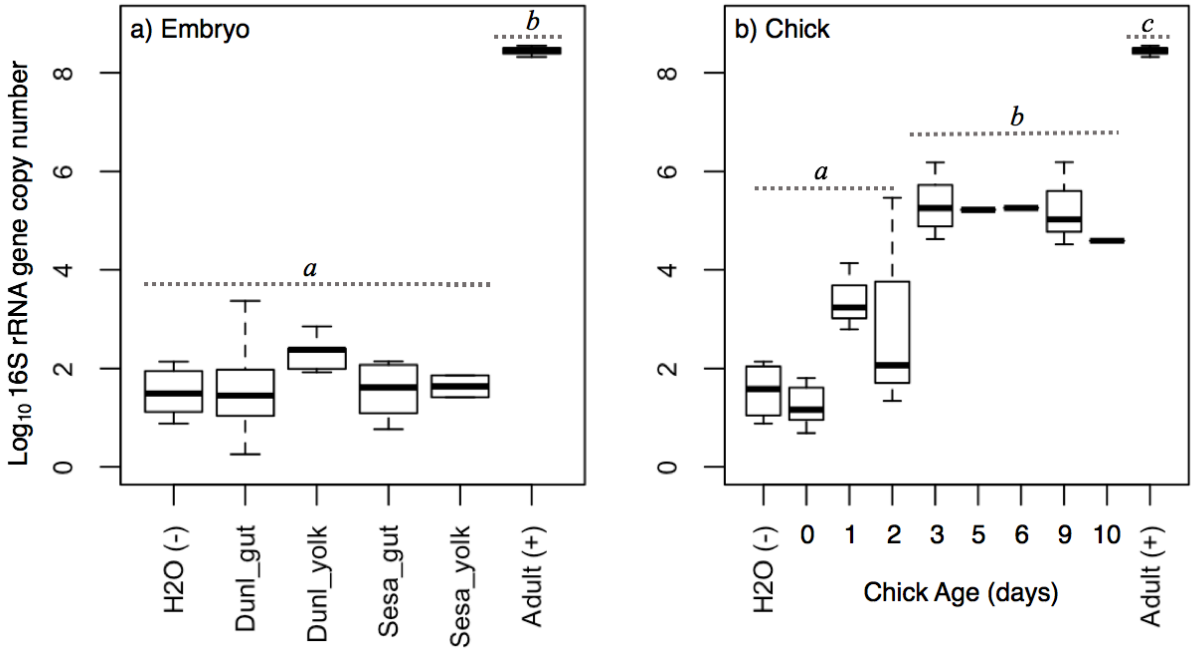


Figure 3.1 Median (thick, black line), 25% and 75% quartiles (boxes), and 90% confidence intervals (whiskers) for the $\log_{10}$ 16S rRNA gene copy numbers. (a) Samples included lower gastrointestinal tracts (gut) and yolk sacs from developed embryos of dunlin (DUNL) and semipalmated sandpipers (SESA) at Utqiaġvik, Alaska, 2013. The negative control was RNase/DNA free molecular grade water, whereas the positive control was a fecal sample from an adult SESA (Adult (+); 5 ng-DNA/µl). Dashed lines and letters above boxes represent significant differences in 16S rRNA copy number (Tukey HSD). We did not include the microbial community from our positive control in further analyses, but show it here for reference purposes. (b) 16S rRNA gene copy numbers in fecal samples from REPH and DUNL chicks from 0-10 days after hatch.

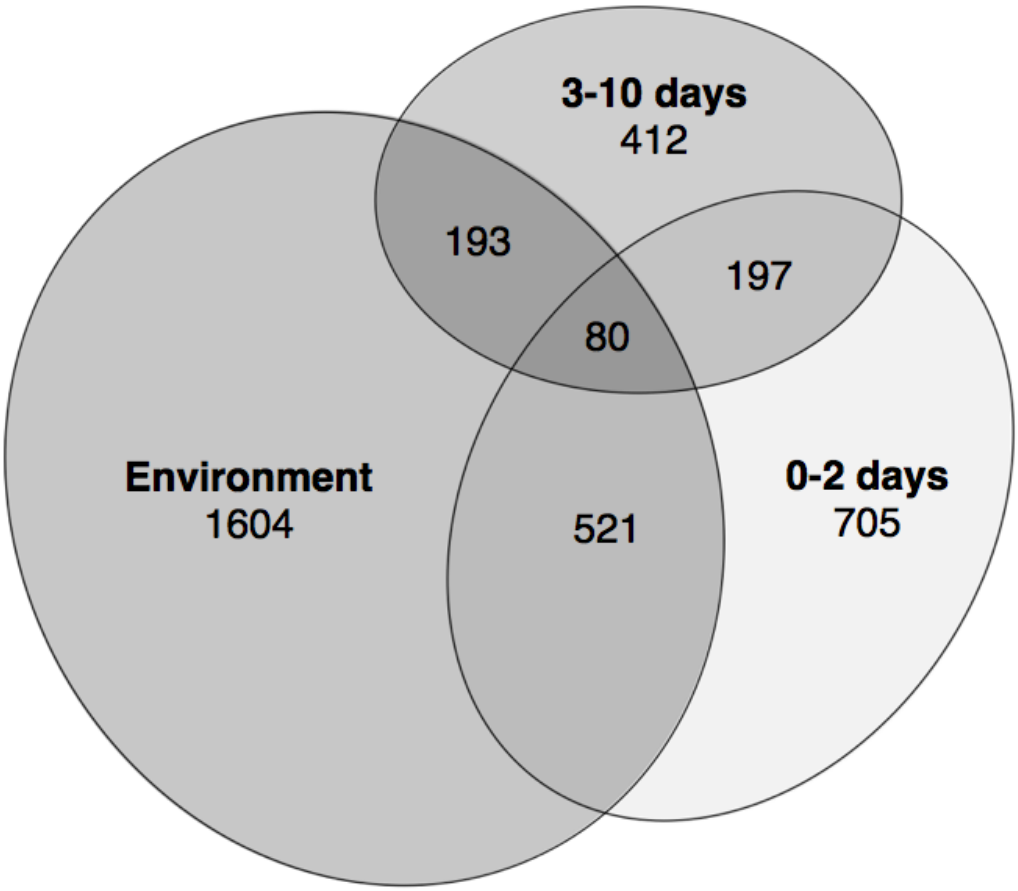


Figure 3.2 Scaled Venn diagram of the bacterial OTUs isolated from fecal samples of young (0-2 days) and old (3-10 days) dunlin and red phalarope chicks, as well as environmental samples of water and soil. Numbers represent numbers of unique or shared OTUs.

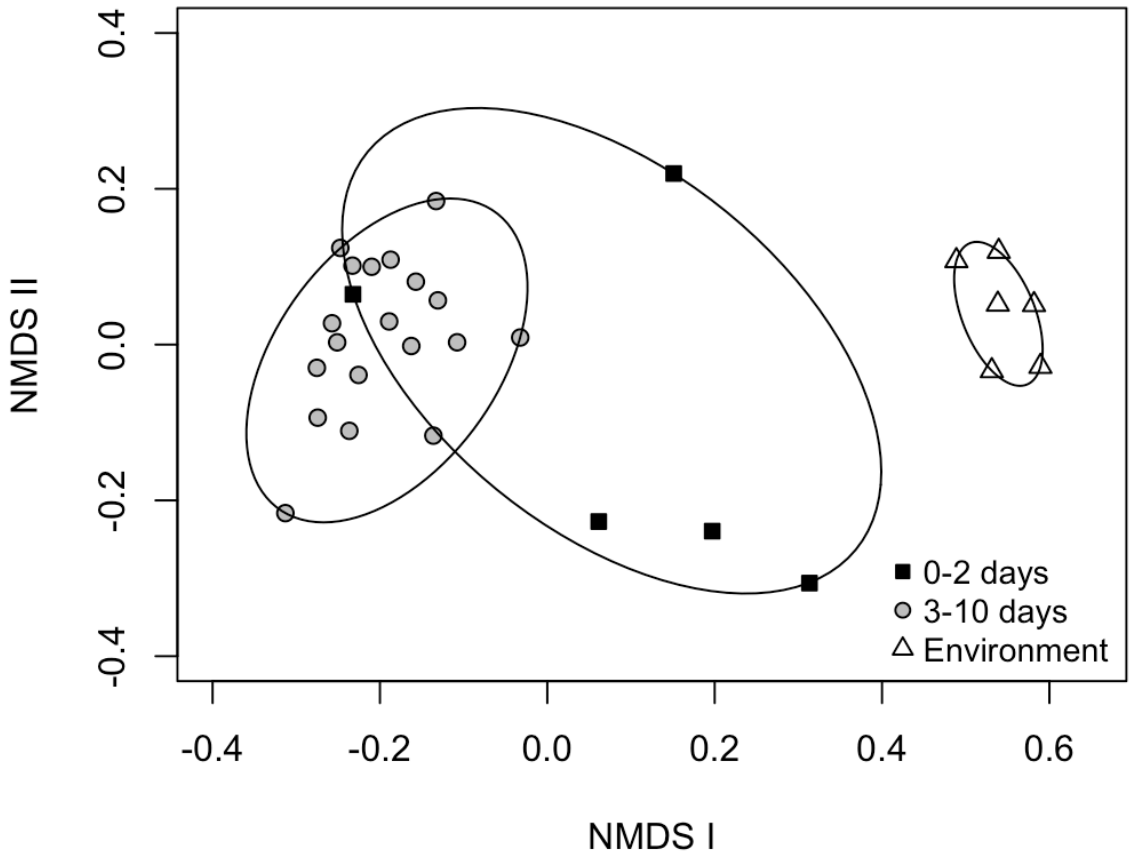


Figure 3.3 Non-metric multi-dimensional scaling (NMDS) of the OTUs in the fecal samples of young chicks (0-2 days, n = 5) and old chicks (3-10 days, n = 19) of dunlin and red phalaropes, and environmental samples (n = 6), at Utqiaġvik, Alaska, 2013.

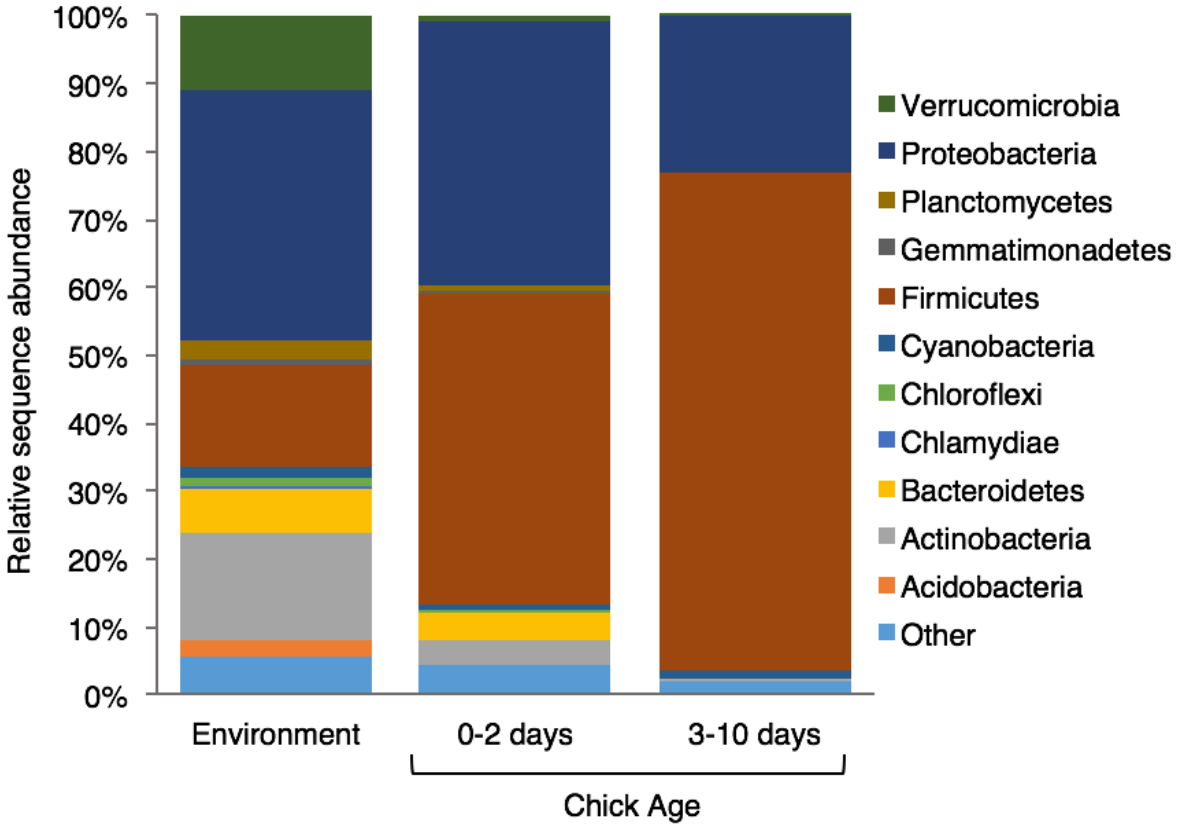


Figure 3.4 Relative abundance of bacterial Phyla in environmental samples and fecal samples of young (0-2 days) and old (3-10 days) chicks of dunlin and red phalaropes at Utqiaġvik, Alaska, 2013. Day 0 represents day of hatching.

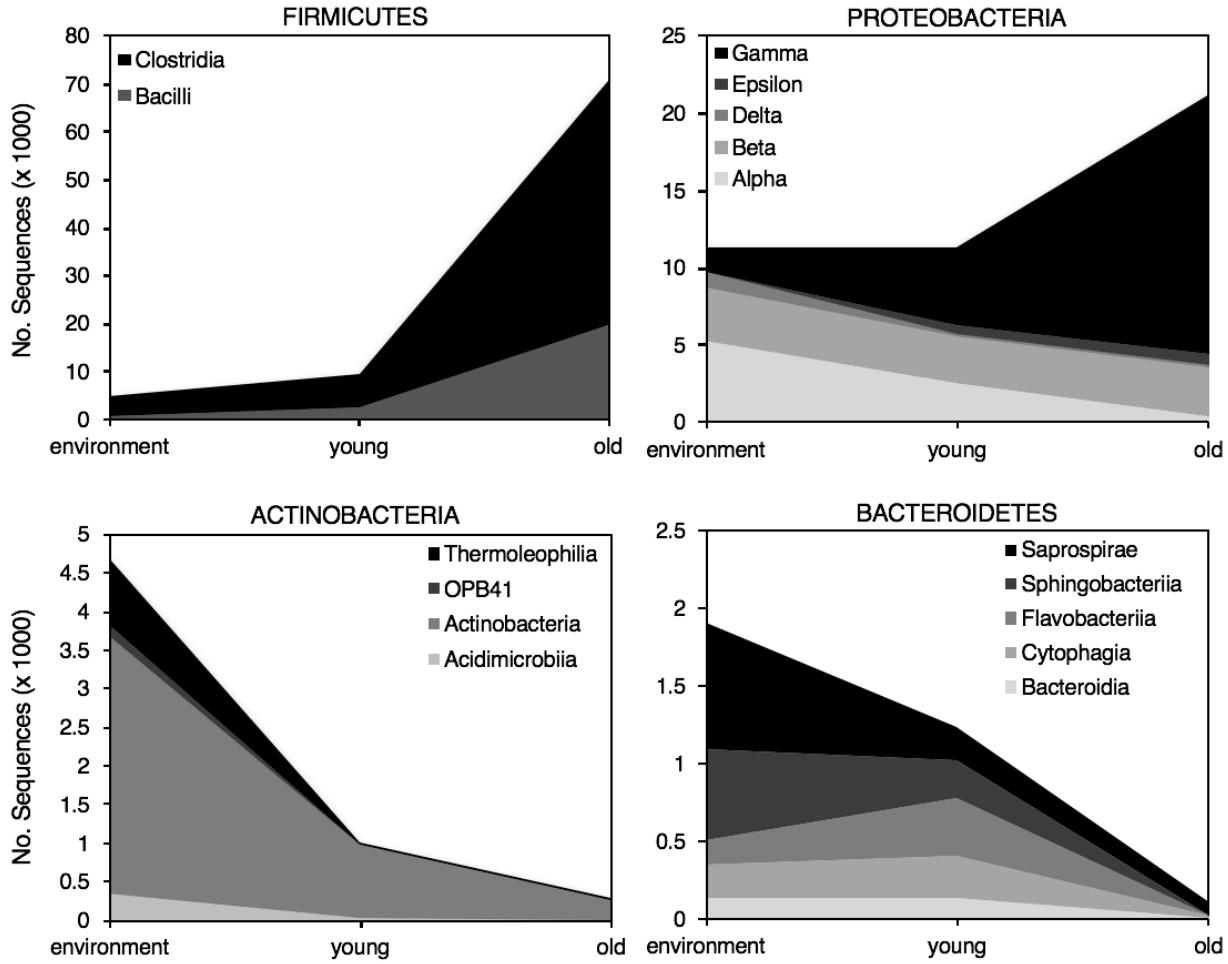


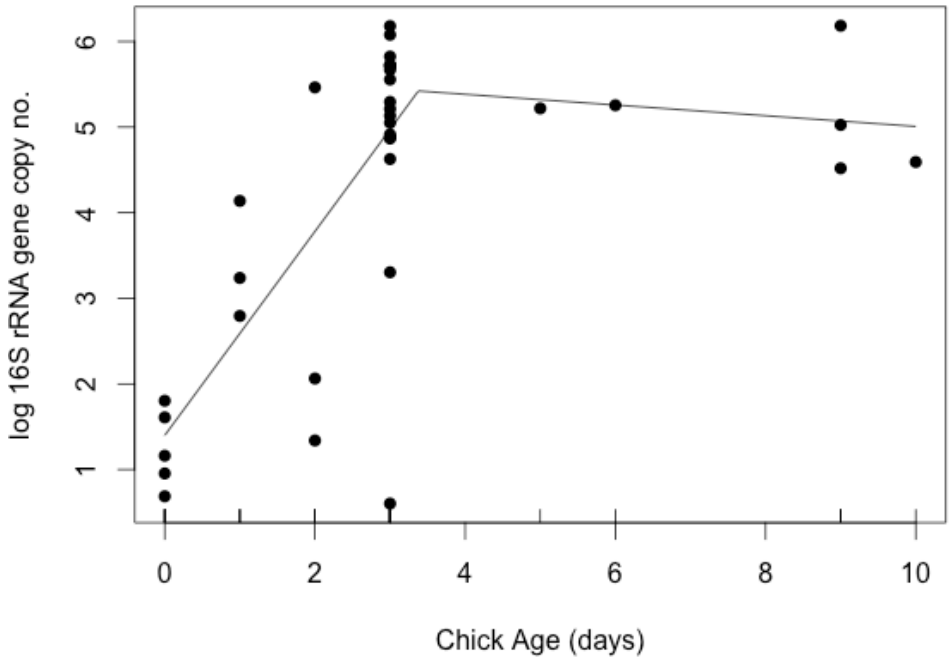
Figure 3.5 Sequence abundance per bacterial Class for the four main Phyla found in environmental samples of water and soil, and young (0-2 days) and old (3-10 days) chick fecal samples collected from dunlin and red phalaropes at Utqiaġvik, Alaska, 2013.

SUPPLEMENTARY TABLES & FIGURES

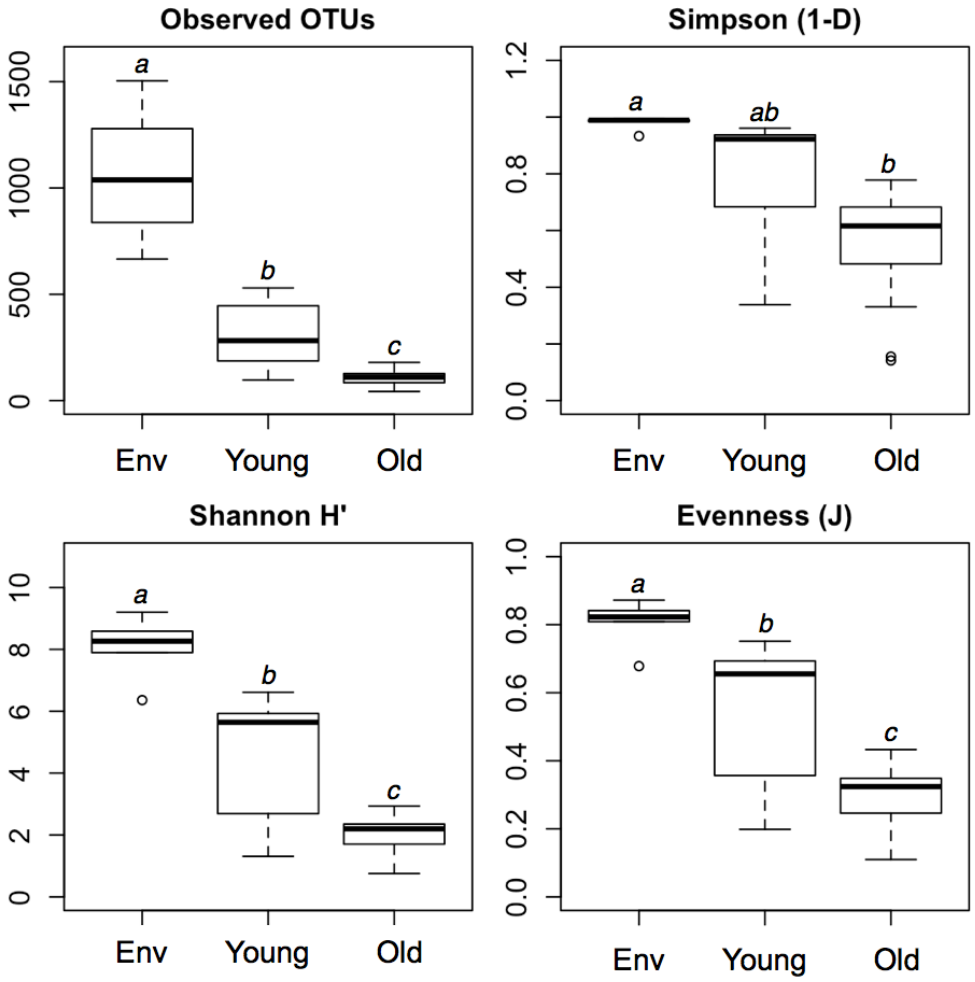
Supplementary Table 3.1 Primers used for conventional and quantitative PCR.

Primer	Sequence	Source
515F	5'-GTGCCAGCMGCCGCGGTAA-3'	Caporaso *et al.,* 2012
806R	5'-GGACTACHVGGGTWTCTAAT-3'	Caporaso *et al.,* 2012
2550F	5'-GTTACTGATTCGTCTACGAGA-3'	Fridolfsson and Ellegren, 1999
2718R	5'-ATTGAAATGATCCAGTGCTT-3'	Fridolfsson and Ellegren, 1999
F_Bact1369	5'-CGGTGAATACGTTCCCGGTAC-3'	Furet *et al.*, 2009
R_Prok1492	5'-TACGGCTACCTTGTTACGACT T-3'	Furet *et al.*, 2009

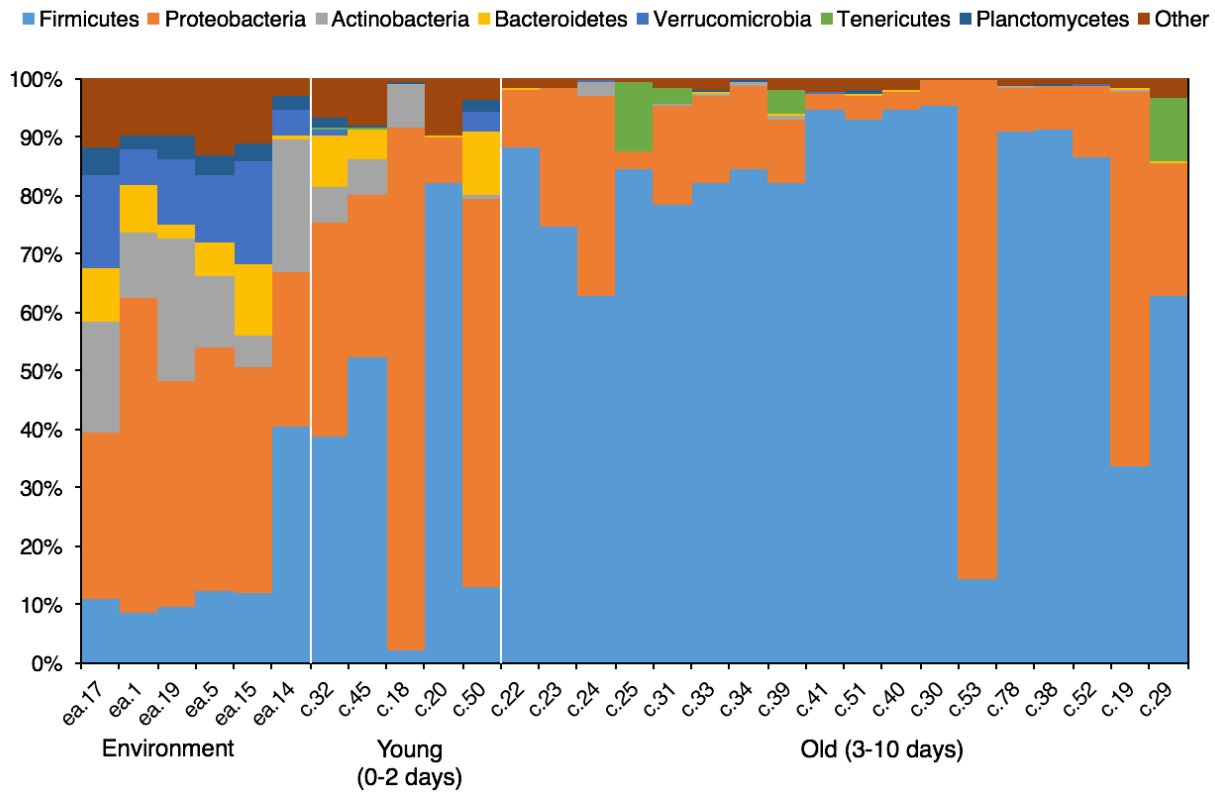
Supplementary Figure 3.1 Dunlin chick with transmitter with antenna glued onto its back. Photo: Kirsten Grond.



Supplementary Figure 3.2 Broken stick regression fitted to qPCR data of chicks of different ages.



Supplementary Figure 3.3 Richness and evenness of gut microbial community in fecal samples collected from chicks of ages 0-2 days (Young), 3-10 days (Old), and environmental samples (Env). Significance is shown by italic letters above boxes (Tukey HSD).



Supplementary Figure 3.4 Phylum level bacterial diversity of fecal samples from young chicks (0-2 days), old chicks (3-10 days) and environmental samples (soil and water). Chick fecal samples were collected from red phalarope and dunlin chicks at Utqiaġvik, AK, in 2013.

Chapter 4 - CHARACTERIZING COMMUNITY AND DRIVERS OF GUT MICROBIOTA IN ARCTIC-BREEDING SHOREBIRDS

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ABSTRACT

Gut microbiota interacts closely with host health, but factors that determine gut microbial composition can differ widely among host taxa. In mammals, host phylogeny has been identified as the main driver of gut microbiota, a result of vertical transfer of microbiota during birth. Past studies of birds have found equal support for host phylogeny, but also environmental factors as drivers of gut microbiota composition. In our field study, we aimed to identify host and environmental factors that underlie variation in gut microbiota composition in eight species of migratory shorebird in the North American Arctic. We characterized bacterial communities from 375 fecal samples collected from adult shorebirds at nine breeding sites in Alaska and Canada, by sequencing the V4 variable region of the bacterial 16S rRNA gene. The gut microbiota of shorebirds was dominated by Firmicutes (55.4%), Proteobacteria (13.8%), Fusobacteria (10.2%) and Bacteroidetes (8.1%), which was consistent with earlier studies. The relatively high abundance of Fusobacteria in the shorebird gut resulted from dominance of the genera *Fusobacterium* and *Cetobacterium* across all sampling sites. Site-related variation in gut microbiota of shorebirds was a result of differences in the common, high abundance (core) bacterial taxa, whereas variation related to host species was driven by differences in the rare, low-abundance taxa. Sampling site was the main driver of variation in gut microbiota of Arctic-breeding shorebirds (R^2 = 11.6%), followed by host species (R^2 = 1.8%), and sampling year (R^2 = 0.9%). Our study is the first to highlight the potential importance of local environment as a driver gut microbiota composition in Arctic-breeding shorebirds.

Keywords: 16S rRNA gene, host, environment, gut microbiome

INTRODUCTION

The importance of gut microbiota in maintaining gut homeostasis, and its contributions to organismal health have received increasing attention over the past decades. Microorganisms in the gastro-intestinal tract outnumber host cells by a ten-fold difference (Turnbaugh *et al.* 2007), and play a major role in nutrient uptake and immune function (Leser and Mølbak 2009; Hooper, Littman and Macpherson 2012). Mammals acquire their gut microbiota at birth (Leser and Mølbak 2009), but bird gut microbiota establish from environmental inocula after hatch (Grond *et al.*). After gut microbial communities reach an adult microbial community, composition can be affected by a number of intrinsic and extrinsic factors, including host phylogeny, age and diet (Ley *et al.* 2008; Goodrich *et al.* 2014; Hird *et al.* 2015).

Evolutionary history, or host phylogeny, is the dominant factor that contributes to gut microbiota in mammals, including humans (Ley *et al.* 2008; Goodrich *et al.* 2014). However, factors that shape the gut microbiota of birds are less clearly defined. In neotropical birds, factors associated with host phylogeny contributed most to shaping the gut microbiota, followed closely by several ecological variables such as sampling and foraging location (Hird *et al.* 2015). Similarly, host species was ranked above ecological factors as a driver of gut microbiota composition in a meta-analysis that included a range of phylogenetically distinct hosts, including the flightless Emu (*Dromaius novaehollandiae*) and Kakapo (*Strigops habroptilus*), the strictly folivorous Hoatzin (*Opisthocomus hoazin*), several penguin species and domestic chickens (Waite and Taylor 2014).

In contrast, several other studies found ecological factors as the main contributing factors to gut microbiota (Hird *et al.* 2014; Barbosa *et al.* 2016; Lewis, Moore and Wang 2016). A study investigating factors affecting microbial communities in the gut of parasitic Brown-headed Cowbirds (*Moluthrus ater*) and its passerine hosts showed that environmental factors, such as sampling locality and diet, were stronger determinants than factors associated with host taxonomy (Waite and Taylor 2014). Staging environment was also important for gut microbial composition of several passerine species during migration (Lewis, Moore and Wang 2016), and nest environment influenced gut microbiota of chicks, but not adults in Chin-strap Penguin (*Pygoscelis antarcticus*; Barbosa *et al.* 2016).

A central challenge in the field of host-microbiome research is decoupling the multitude of different factors that may simultaneously affect gut microbiota composition and dynamics. To address phylogenetic and environmental influences, many conspecific or closely related individuals must be sampled at multiple sites, a task that may often be difficult or impossible. We attempted to investigate the influence of environment- and host-related factors on gut microbiota of shorebirds; a diverse family that include over 200 species and many that are long-distance migrants. The range of phylogenetic relatedness and the wide variety in life-history characteristics among shorebird species allow for identifying the predominant factors that contribute to community dynamics in gut microbiota. In addition, many shorebird species share breeding sites, thus allowing for simultaneous sampling of multiple species within a local environment.

We investigated the contribution of site- and host-related factors to gut microbiota composition of migratory shorebirds at breeding sites across the North-American Arctic. Specifically, we were interested in 1) characterizing the bacterial community of the shorebird gut during the breeding season in the Arctic, 2) testing which factors were the main drivers of shorebird gut microbiota composition, and 3) assessing the relative contribution of host and environmental factors among host-species or breeding sites. We collected fecal samples from eight shorebird species at nine Arctic breeding sites in Alaska and Canada, and used high-throughput sequencing to assess the contribution of seven site- and host-related factors to variation in gut microbiota in shorebirds.

METHODS

Sample collection

Our field studies were conducted as part of the Arctic Shorebird Demographics Network (ASDN). The ASDN is a large, collaborative research network that consists of 17 Arctic field sites in Russia, Alaska, and Canada, and was established in 2010 with the goal to conduct standardized demographic analyses on Arctic-breeding shorebird species (Brown *et al.* 2013). Collaboration with ASDN partners enabled us to sample shorebirds across a large geographical area.

We collected fecal samples from eight species of shorebirds at nine sites in the Alaskan and Canadian Arctic from 2011-2013 (Table 4.1 & 4.2; Figure 4.1). Birds were trapped at their nest after day 7 of incubation using walk-in traps and bow nets. After capture, birds were placed in a darkened, plastic box for up to 5 min. Prior to each new individual, boxes were sterilized with bleach wipes, and the bottom of the box was lined with a clean sheet of wax paper. After defecating, birds were banded and biometric measurements collected. Birds were released within 30 min of capture. Fecal samples were removed from the wax paper using a sterile tongue depressor to transfer samples to a 1.5 ml sterile Eppendorf tube. All handling of the wax paper was conducted while wearing sterilized latex gloves. All fecal samples were preserved in 100% ethanol at collection, and stored frozen at -20°C until further analyses.

Molecular Analyses

DNA extraction. – We removed ethanol from fecal samples, by centrifuging the fecal samples for 10 min at 10,000 rpm and removing supernatant. We repeated this cleaning step twice with 1 ml of RNase/DNA free molecular grade water to minimize ethanol in the sample (Grond et al. 2014; Ryu et al. 2014). We extracted DNA from fecal samples using the MoBio Power Lyzer/Power Soil kit as per the manufacturer's instructions (Mo Bio Laboratory, Carlsbad, CA, USA), except for replacing the bead beating step with 15 min high velocity vortexing. Genomic DNA yields were determined using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA).

PCR. – We generated multiplexed 16S rRNA gene libraries from fecal samples using bacterial primers 515F and 806R (Caporaso *et al.* 2012), latter of which was uniquely barcoded. We performed PCR reactions in triplicate in a 25 µl reaction volume, using TaqMan® Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA) and 5 µl of DNA template (5 ng DNA/µl). PCR conditions consisted of 25 cycles of: 15 s at 95°C, 30 s at 55°C and 30 s at 72°C, preceded by an initial denaturing step of 10 min at 95°C, and followed by a final extension step of 5 min at 72°C. We removed residual primers from our PCR product using the Agencourt AMpure XP PCR purification system (Beckman Coulter, Brea, CA)

following manufacturer's instructions with the following modifications: we adjusted the template:AMpure volume ratio to 1:1, and repeated the ethanol wash step three times instead of two.

Sequence analyses. –We sequenced the V4 region of the 16S rRNA gene in 2 x 250bp paired-end runs using the Illumina MiSeq platform. Each Illumina run included a 15% PhiX spike as a control library. Sequence quality filtering, contiging and demultiplexing were performed using QIIME (Caporaso *et al.* 2010). We aligned sequences with the GreenGenes 16S rRNA gene reference database (v.13_8) (DeSantis *et al.* 2006). We identified chimeras using CHIMERASLAYER (Haas *et al.* 2011), and we removed chimeric sequences, singletons and non-aligned sequences from our dataset. We assigned sequences to OTUs at 97% sequence similarity, and assigned them to taxon affinities using the RDP classifier (Wang *et al.* 2007). After assigning taxonomy, we identified non-target archaeal, chloroplast and mitochondrial sequences, and removed them from the dataset. Prior to downstream analyses, we rarefied our samples to 10,000 sequences per sample.

Data Analyses

Richness & Evenness. – We calculated alpha diversity indices of OTU composition of our samples (observed number of OTUs, Simpson's (1-D), and equitability (evenness) using the QIIME alpha_diversity.py script . We compared richness and evenness indices using a one-way ANOVA and assessed pairwise differences using a post-hoc Tukey HSD test in R (ver. 3.3.1) (R Development Core Team 2016).

Community & Distance relationships. – We generated weighted and unweighted UNIFRAC distance matrices (UDM) for microbial communities in fecal samples at an OTU level (Lozupone and Knight 2005). Weighted UDMs take OTU abundance into account, whereas unweighted UDMs only account for presence/absence of OTUs within a sample. To assess whether community similarity decreased with distance between sampling sites, we used weighted UDMs to calculate Distance-Decay Relationships

(DDRs) through pair-wise community distances between fecal samples of five shorebird species that occurred at multiple sites: Semipalmated Sandpipers (*Calidris pusilla*), Dunlin (*Calidris alpina*), Western Sandpiper (*Calidris mauri*), Red Phalarope (*Phalaropus fulicarius*) and Red-necked Phalarope (*Phalaropus lobatus*). We log10 transformed the UDMs after adding 0.01 to all 0 values in the distance matrix. We calculated geographic distances (km) between pairs of sampling sites, and regressed similarity of gut microbial communities against geographic distance. We fit linear models for each host species, and estimated whether slopes significantly differed from 0. In addition, we regressed richness indices against sampling latitude and fitted linear models to test whether microbial richness of the shorebird gut microbiome declined with increasing latitude.

Data sets. – To assess factors affecting gut microbiota composition in shorebirds at different environment and host-related levels, we selected subsets of our samples for further analyses:

I. All fecal samples of Arctic-breeding shorebirds (n = 375)

II. High-arctic sites (n = 249). Samples from sites situated on the North Slope in the high Arctic (Utqiaġvik (formerly known as Barrow), Ikpikpuk River, Colville River, Canning River, MacKenzie River Delta).

III. Low-arctic sites (n = 126). Samples from sites situated in the subarctic (Cold Bay, Yukon Delta, Nome, Cape Krusenstern).

IV. Calidrine sandpipers (n = 302). All samples from shorebird species from the Genus *Calidris*, which include Pectoral Sandpiper (n = 16), Dunlin (n = 110), Semipalmated Sandpiper (n = 130) and Western Sandpiper (n = 43).

V. Phalaropes (n = 50). All samples from species from the Genus *Phalaropus*, which included Red Phalarope (n = 18) and Red-necked Phalarope (n = 32).

VI. Dunlin (n = 110). Our Dunlin samples included two subspecies: *C. alpina arcticola* (n = 61) and *C. alpina pacific* with different breeding areas and migratory strategies (n = 49). Dunlin

were sampled at six different sites (Utqiaġvik, Ikpikpuk River, Canning River, Cape Krusenstern, Cold Bay, Yukon Delta).

VII. Semipalmated Sandpipers (n = 130). Semipalmated Sandpipers were widely distributed and sampled at six sites (Cape Krusenstern, Canning River, Colville River, Ikpikpuk River, MacKenzie River Delta, Nome).

Variable selection. – We tested eight variables for significant contributions to variation in gut microbiota composition in our data subsets (Table 4.3). We selected three hierarchical variables associated with levels of host phylogeny (Family, Genus and Species), four variables associated with sampling site and habitat (Site, Biome, Habitat, Latitude), and sampling Year. We divided sampling sites into sub-Arctic and high-Arctic in our Biome variable. Bird species were assigned to one of four Habitat categories that they used during the breeding season: Terrestrial (T), Mesic (M), Terrestrial/Mesic (TM), and Aquatic (A) (Cunningham, Kesler and Lanctot 2016).

Variable Significance and Contribution. – Statistical analyses were conducted in R and QIIME (Caporaso et al. 2010). We tested for significance of the selected variables using ANOSIM and Adonis in QIIME in weighted and unweighted UDMs. After determining which variables significantly contributed to the variation in our datasets, we determined the relative contributions of each variable with a multifactorial permutational multivariate analyses of variance (PERMANOVA), using the adonis function in the 'vegan' package in R (Oksanen et al. 2016). We permuted the order of the variables in our multifactorial PERMANOVA, to test whether variable order affected significance and relative contribution.

To investigate potential differences among microbial communities within our different datasets, we applied non-metric multidimensional scaling (NMDS) of Bray-Curtis distance matrices using the metaMDS function with k = 3 dimensions in the 'vegan' package. In addition, to assess contribution of our explanatory variables to the variation in the NMDS, we fitted the variables to the ordination using the envfit function in the 'vegan' package.

Data accessibility. – The nucleotide sequences and metadata will be made available through Figshare.

RESULTS

After rarefaction to 10,000 sequences per sample, our total dataset included microbial communities of 375 fecal samples from nine shorebird species collected at eight arctic breeding sites (Table 4.4). Our analyses of gut microbial communities were based on these rarified datasets.

Bacterial composition of the shorebird gut microbiota

Richness and Evenness. – In our full dataset of 375 samples, we detected 34 bacterial Phyla, representing a total of 24,944 unique OTUs. On average, we detected 12.0 ± 0.18 Phyla, and 684.9 ± 16.1 OTUs per sample. Richness and evenness indices differed among the nine field sites (Fig. 4.2; Observed OTUs: $F_{8,366} = 10.4$, $p < 0.001$; Simpson 1-D: $F_{8,366} = 2.96$, $p = 0.003$; Evenness J: $F_{8,366} = 2.53$, $p = 0.011$). The differences were driven by a higher OTU richness at our Ikpikpuk River site (Tukey HSD, Observed OTUs: $p = 0.007 \pm 0.005$) and lower richness and evenness at the MacKenzie River Delta (Tukey HSD, Simpsons 1-D: $p = 0.013 \pm 0.011$; Evenness J: $p = 0.001$).

Community & Distance relationships. – Overall, we did not detect strong Distance-Decay relationships in our data (Supplementary Figure 4.1). We found no evidence of DDRs in three of our five host species investigated (Semipalmated Sandpipers: $F_{1,8383} = 2.40$, adj. $R^2 = 0.0002$, $p = 0.121$; Red Phalarope: $F_{1,151} = 0.52$, adj. $R^2 = -0.0031$, $p = 0.470$; Red-necked Phalarope: $F_{1,494} = 2.19$, adj. $R^2 = 0.0024$, $p = 0.141$). Dunlin and Western Sandpiper linear models had slopes that significantly differed from zero, but explanatory power of models was low with coefficients of determination lower than 0.01 (Dunlin: $F_{1,5993} = 15.30$, adj. $R^2 = 0.0024$, $p < 0.001$; Western Sandpiper: $F_{1,901} = 9.56$, adj. $R^2 = 0.0094$, $p = 0.002$).

We found no evidence for a decrease in bacterial richness with increasing latitude (Supplementary Figure 4.2). Richness indices did not significantly differ among samples collected at different latitudes (Observed OTUs: $F_{1,373} = 0.01$, adj. $R^2 = -0.0027$, $p = 0.966$; Simpson: $F_{1,373} = 0.21$, adj. $R^2 = -0.0021$, $p = 0.640$), and evenness was similar among latitudes ($F_{1,373} = 1.09$, adj. $R^2 = 0.0003$, $p = 0.299$).

Taxon Diversity. – Firmicutes (55.4% ± 1.4), Proteobacteria (13.8% ± 0.9), Fusobacteria (10.2% ± 0.9) and Bacteroidetes (8.1% ± 0.7) dominated in our samples at a phylum level. Fusobacteria were relatively abundant in shorebirds, resulting from the high occurrence of Fusobacteria in all samples collected at Cape Krusenstern (Fig. 4.1, 4.4a). Gut microbiota of shorebirds sampled at Cape Krusenstern (31.2 ± 3.6%) included two genera that comprised > 98% of all Fusobacteria: *Fusobacterium* spp. (60.5%) and *Cetobacterium* spp. (37.5%). The relative abundance of these genera within the Fusobacteria was similar among most sites, despite the overall higher Fusobacteria abundance at Cape Krusenstern (Supplementary Figure 4.4, 4.5). The most abundant Classes within the Firmicutes were the *Bacilli* (43.0%) and *Clostridia* (14.7%; Supplementary Figure 4.4). *Bacilli* were dominated by species within the Order *Lactobacillales*, and the genus *Lactobacillu*s. The closest relative at a species level was *Lactobacillus ruminis* (99.3% sequence similarity), a common inhabitant of the gut environment and associated with the degradation of cellulose (Liu *et al.* 2016)

Core microbiota. – We defined core microbiota as the community of OTUs that were present in > 50% of our 375 samples. Core microbiota contained a total of 67 OTUs, which was 0.3% of the total number of OTUs detected.. Core OTUs differed among sites (envfit; $R^2 = 0.19$, $p < 0.001$), but not among host species (envfit; $R^2 = 0.03$, $p = 0.074$). The most abundant core OTUs belonged to the Phylum Firmicutes (66.3%), and specifically to the Order Lactobacillales (45.9%). The known avian pathogen *Clostridium colinum* comprised 4.5% of all sequences. After Firmicutes, Fusobacteria were most abundant with 14.1%, followed by Bacteroidetes (8.9%) and Proteobacteria (7.4%).

Drivers of the shorebird gut microbiota

Site explained the most variation in the OTU composition of the gut microbiota in adult shorebirds during the breeding season (R^2 = 12.4%, $p < 0.001$). The NMDS showed that microbial communities were less similar if grouped by sampling site than by species, and site explained more variation in than species (Fig. 4.3). Our multifactorial PERMANOVA showed Site as the dominant contributing variable in all our data sets (R^2 = 7.0-13.1%, $p < 0.001$; Table 4.5). For our full dataset, Species and Year also contributed significantly to the variation in our data, but to a lesser extent than Site (R^2 = 1.0-2.7, p = 0.001-0.006; Table 4.5). Permuting the order of the variables did not change our results. Weighted UDMs explained on average 0.5% more variation than the unweighted UDMs, which was due to the large number of low abundance OTUs being overrepresented in unweighted UDMs. In our single-factor PERMANOVAs, Site was again the dominant explanatory factor in all datasets, with exception of the High Arctic (Supplementary Table 4.1). At our High Arctic sampling sites, Species and Genus both explained 6.8% of variation in gut microbial communities ($p < 0.001$) of the weighted UDM, opposed to 5.5% for site ($p < 0.001$).

In our two single-species datasets for Dunlin and Semipalmated Sandpipers, Site contributed 12.7% and 12.1% to the overall variation in microbial community, respectively, which was over four times higher than the other significant variables: Biome (Dunlin) and Year (Semipalmated Sandpiper). Our Phalarope subset included two species: the Red Phalarope and the Red-necked Phalarope, and the two species did not differ in gut microbiota in any of our analyses. Phalarope samples collected in different years differed significantly from each other in our ANOSIM analyses (R = 0.496-0.792, $p < 0.001$; Supplementary Table 4.1), and contributed 8.3-8.6% to the difference in OTU composition in our single variable PERMANOVA (weighted UDM; Supplementary Table 4.1). Year was a significant contributing variable in our multifactorial permanova ($p < 0.001$) but contributed only 0.9-2.6% to the variation in gut microbiota.

DISCUSSION

Drivers of the shorebird gut microbiota

Of the factors we tested, breeding site contributed most to variation in gut microbiota of Arctic-breeding shorebirds, followed by host species. We did not find support for climate-associated variables, such as latitude and location within the low- or high-Arctic, indicating that environmental variables are likely important in explaining variation in the gut microbiota in shorebirds. Our findings contrast with results from several large-scale comparative studies of avian microbiomes (Waite and Taylor 2014; Hird *et al.* 2015), which identified host species as the main determinant of microbial community in the gut. Concurrent with other avian and mammalian studies (Waite and Taylor 2014; Hird *et al.* 2015; Avena *et al.* 2016), a large part of the variation in gut microbiota of Arctic-breeding shorebirds (87%) remained unexplained in our models. We detected ~ 25,000 unique OTUs in our 375 samples, but only detected 684.9 ± 16.1 OTUs per sample. Variation in low-abundance OTUs among individuals likely contributed to the unexplained variation we observed.

A majority of studies that identified host phylogeny as the main driver of microbial diversity focused on non-migratory species. In contrast, focusing on migratory passerines, Lewis *et al.* (2016) found evidence for a larger influence of environment on gut communities than host species. Migratory birds are exposed to many new environments during their annual cycle, potentially associated with turnover in gut microbiota throughout the year. Diet of migratory birds can vary widely throughout the year. For example, several of our study species switch from a diet of terrestrial arthropods during the breeding season to diets that consist of marine copepods, shellfish and even bacterial biofilm during the non-breeding season (Quinn and Hamilton 2012; Jardine *et al.* 2015). Two exceptions of species that maintain a terrestrial diets include American Golden Plovers and Pectoral Sandpipers, which forage in crop fields and freshwater marshes (Isacch, Darrieu and Martínez 2005; Smith *et al.* 2012). Changes in gut microbiota throughout the year could benefit migratory birds, as locally acquired microbiota could aid in digestion of these local prey items. In addition, migratory birds could benefit from greater flexibility in their gut microbial communities if encountering different pathogen communities along their migration.

Migratory birds can harbor a larger variety of pathogens, and have higher infection intensity (Koprivnikar and Leung 2015; Clark, Clegg and Klaassen 2016; Leung, Koprivnikar and Eizaguirre 2016). Acquisition of local microorganisms may result in gut microbiota that are better able to outcompete local pathogens, and thus indirectly aid the host.

High latitude environments, such as the Arctic, often have lower microbial richness than low latitude sites (Fuhrman *et al.* 2008; Sul *et al.* 2013; Andam *et al.* 2016). However, available studies were conducted on soils, which are subjected to edaphic and climate influences year-round. We found no correlation between latitude and microbial richness in shorebird guts, nor did we find evidence for bacterial communities decreasing in similarity with increasing distance between sampling sites. The absence of a latitudinal effect on gut microbiota of shorebirds is unsurprising, as shorebirds are highly mobile throughout the year and their temporary stay at the breeding sites may be too short to establish latitudinal gradients in gut bacterial communities on the scale we examined.

One of the remaining questions is what happens to gut microbiota composition of birds at a larger geographic scale, for example during their long-distance movements. Migrating to a richer microbial environment and exposure to local environmental microbiomes could increase the contribution of site to composition of the avian gut microbiota. Comparison of gut communities of shorebirds across a larger latitudinal range, for example between breeding and non-breeding sites, could elucidate these potential patterns.

Diet is an important driver of gut microbial diversity in mammals (Ley *et al.* 2008), but is less important in birds (Waite and Taylor 2014; Hird *et al.* 2015). We did not include diet as a potential explanatory variable in our models, as all shorebird species we investigated forage on arthropods during the breeding season. Microorganisms associated with arthropod communities at different sampling sites could differentially shape the microbial community of the gut, but arthropod communities have not yet been sampled. Broadly, our shorebird species could be assigned to different habitats within the breeding sites, which allowed us to indirectly infer whether terrestrial and aquatic prey communities were of importance by including our habitat variable.

Site and species-specific effects

Site remained a significant driver of gut microbiota composition at a core microbiota level, suggesting that differences among sites were driven by differences in high abundance taxa, opposed to the high number of peripheral, low abundance OTUs. Shifts in core microbiota during the breeding season could reflect functional shifts in microbiota caused by different nutrient requirements per site. However, it is more likely that shifts in core microbiota are associated with site-specific differences in prey-associated microbiomes, as shorebirds have similar diets and behaviors throughout their breeding range. Host species effects were not significant when investigating core microbiota only, suggesting that effects associated with host species are driven by rare OTUs.

One interesting observation was the high relative abundance of *Fusobacteria* across all individuals sampled at the Cape Krusenstern site. Cape Krusenstern is located less than 200 miles of the Nome site, but the relative abundance of *Fusobacteria* in Nome was lower, and comparable to our other sampling sites. Fusobacteria are a common member of the gastro-intestinal microbiota in birds (Bennett *et al.* 2013; Dewar *et al.* 2014; Roggenbuck *et al.* 2014; Hird *et al.* 2015; Barbosa *et al.* 2016), and *Cetobacterium* spp. were previously detected in shorebirds (Grond *et al.* 2014; Ryu *et al.* 2014). In contrasts to other sites, shorebirds at Cape Krusenstern were observed to forage on saline and brackish mud flat areas (pers. comm. M. Boldenow). Although most commonly isolated from freshwater fishes (Tsuchiya, Sakata and Sugita 2008; Larsen, Mohammed and Arias 2014; Liu *et al.* 2016), *Cetobacterium* spp. have also been detected in guts of sea mammals (Foster *et al.* 1995), and a high abundance of Fusobacteria in shorebirds at Cape Krusenstern could result from differences in foraging site and diet-associated microbiota.

Conclusion

We showed that of the variation explained by our models, breeding site was the dominant factor contributing to variation in gut microbiomes of migratory shorebirds. However, we still explained only a

relatively small fraction of the variability in gut microbiota with our selected variables, which suggests that either we did not include an important driver of shorebird microbiota, or that innate large variability in microbial communities exists within the shorebird gastro-intestinal tract. Also, to determine whether contributing factors and gut microbial composition are stable between breeding and non-breeding sites, we suggest extended sampling of migratory shorebirds throughout the annual cycle.

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TABLES & FIGURES

Table 4.1 Shorebird species investigated in our study. Habitat categories consist of Terrestrial (T), Terrestrial/Mesic (TM), Mesic (M), and Aquatic (A).

Species	Scientific name	Abbreviation	Habitat	Status*
American Golden Plover	*Pluvialis dominica*	AMGP	T	LC
Long-billed Dowitcher	*Limnodromus scolopaceus*	LBDO	M	LC
Pectoral Sandpiper	*Calidris melanotos*	PESA	TM	LC
Dunlin	*Calidris alpina*	DUNL	TM	LC
Semipalmated Sandpiper	*Calidris pusilla*	SESA	TM	NT
Western Sandpiper	*Calidris mauri*	WESA	T	LC
Red Phalarope	*Phalaropus fulicarius*	REPH	A	LC
Red-necked Phalarope	*Phalaropus lobatus*	RNPH	A	LC

*Population Status as listed on the IUCN Red List. Least Concern (LC) and Near Threatened (NT)

Table 4.2 Locations and sampling years of field sites in the Arctic Shorebird Demographics Network.

Site		Abbreviation	Latitude (ºN)	Longitude (ºW)	Years sampled
Cold Bay	AK, USA	COBA	55.204500	-162.718400	2011
Yukon Delta	AK, USA	YUDE	61.368900	-163.716100	2011
Nome	AK, USA	NOME	64.497934	-165.408204	2011, 2013, 2014
Cape Krusenstern	AK, USA	CAKR	67.417246	-163.874238	2013, 2014
Utqiaġvik	AK, USA	UTQI	71.292646	-156.782563	2011
Ikpikpuk River	AK, USA	IKRI	70.814400	-154.405300	2011, 2013
Colville River	AK, USA	CORI	70.384028	-150.806197	2011, 2013
Canning River	AK, USA	CARI	69.945375	-145.098152	2011, 2013
MacKenzie River	NW Territories, Canada	MARI	68.815927	-137.090836	2011

Table 4.3 Host and site variables used to test for contributions to variation in gut microbiota composition in fecal samples from Arctic-breeding shorebirds collected from 2011-2014.

Variable	Description	Levels	Used in data Subset
Site	Sampling site	COBA, YUDE, NOME, CAKR, UTQI, IKRI, CORI, CARI, MARI*	1-7
Biome	Broad habitat category of sampling locations	Sub-Arctic, High Arctic	1,4-7
Habitat	Local habitat used by host species	Terrestrial (T), Terrestrial/Mesic (TM), Mesic (M), Aquatic (A)	1-4
Latitude		Continuous	1-7
Family	Host Family	*Charadriidae, Scolopacidae*	1-3
Genus	Host Genus	*Pluvialis, Calidris, Limnodromus, Phalaropus*	1-3
Species	Host species	AMGP, LBDO, PESA, SESA, WESA, DUNL, RNPH, REPH**	1-5
Year	Sampling year	2011, 2013, 2014	1-7

* Full site names shown in Table 2.

** Full species names shown in Table 1.

Table 4.4 Sample sizes per site per species after rarefaction. For species abbreviations see Table 1.

Site	AMGP	LBDO	PESA	DUNL	SESA	WESA	RNPH	REPH	**Total**
Cold Bay				19					19
Yukon Delta				16					16
Nome					25	23	9		57
Cape Krusenstern				13	6	14	1		34
Utqiaġvik	5	21		23	2	5	1	1	58
Ikpikpuk River				19	50		5	9	83
Colville River			2	11	15	1	4	3	36
Canning River			10	9	24		5	5	53
MacKenzie River			4		8		7		19
Total	5	21	16	110	130	43	32	18	375

Table 4.5 Multifactorial perMANOVA tests for significance and relative contribution of seven environmental and host-related factors to variation in weighted and unweighted UNIFRAC Distance Matrices constructed from shorebird fecal communities.

		Weighted UDM		Unweighted UDM	
Dataset	Variable	R^2	p	R^2	p
All samples	Site	**12.4**	<0.001	**8.7**	<0.001
	Biome	0.3	0.229	0.4	0.028
	Habitat	1.4	<0.001	0.5	0.002
	Latitude	0.4	0.151	0.4	0.029
	Family	0.4	0.091	0.4	0.006
	Genus	1.8	0.002	1.9	0.001
	Species	2.7	0.006	1.7	<0.001
	Year	1.0	<0.001	0.4	0.013
High-arctic	Site	**7.0**	<0.001	**6.1**	<0.001
	Habitat	0.9	0.033	0.4	0.226
	Family	0.7	0.109	0.7	0.009
	Genus	2.9	0.004	2.5	0.001
	Species	3.0	0.211	3.4	0.004
	Year	0.2	0.809	0.4	0.274

Sub-arctic	Site	**10.4**	<0.001	**6.3**	<0.001
	Habitat	0.9	0.232	1.0	0.108
	Genus	3.3	0.006	2.0	<0.001
	Species	2.7	0.215	3.1	0.012
	Year	1.6	0.052	1.1	0.036
Calidrids	Site	**13.1**	<0.001	**9.4**	<0.001
	Biome	0.5	0.078	0.6	0.003
	Habitat	0.3	0.367	0.4	0.108
	Latitude	0.3	0.149	0.6	0.007
	Species	1.2	0.111	1.1	0.040
	Year	0.9	0.007	0.6	0.004
Phalaropes	Site	**15.9**	0.098	**14.5**	0.016
	Biome	1.1	0.813	2.0	0.431
	Latitude	2.3	0.300	1.6	0.833
	Species	1.2	0.773	1.8	0.663
	Year	0.9	0.831	2.6	0.069
Dunlin	Site	**12.7**	0.003	**11.7**	<0.001
	Biome	1.9	0.034	1.7	0.003
	Latitude	1.1	0.241	1.2	0.059

	Year	0.6	0.629	0.9	0.407
Semipalmated Sandpiper	Site	**12.1**	0.002	**10.6**	<0.001
	Biome	5.0	<0.001	4.0	<0.001
	Latitude	1.5	0.054	1.2	0.013
	Year	2.5	0.007	0.8	0.189

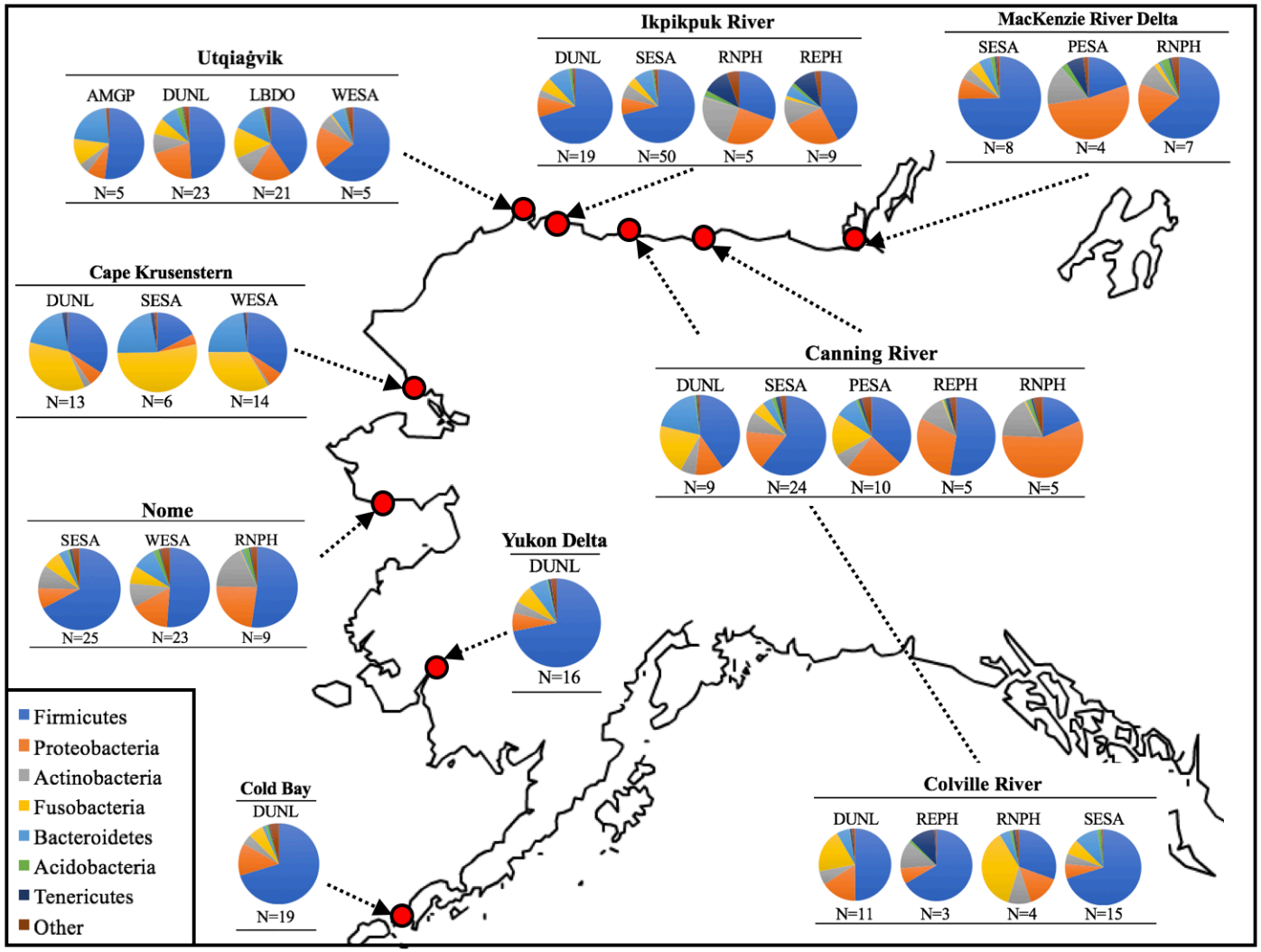

Utqiaġvik
AMGP
DUNL
LBDO
WESA
N=5
N=23
N=21
N=5
Ikpikpuk River
DUNL
SESA
RNPH
REPH
N=19
N=50
N=5
N=9
MacKenzie River Delta
SESA
PESA
RNPH
N=8
N=4
N=7
Cape Krusenstern
DUNL
SESA
WESA
N=13
N=6
N=14
Canning River
DUNL
SESA
PESA
REPH
RNPH
N=9
N=24
N=10
N=5
N=5
Nome
SESA
WESA
RNPH
N=25
N=23
N=9
Yukon Delta
DUNL
N=16
Cold Bay
DUNL
N=19
Colville River
DUNL
REPH
RNPH
SESA
N=11
N=3
N=4
N=15
Firmicutes
Proteobacteria
Actinobacteria
Fusobacteria
Bacteroidetes
Acidobacteria
Tenericutes
Other

Figure 4.1 Sampling sites with bacterial communities shown per shorebird species as isolated from fecal samples collected from 2011-2014 in Alaska and Canada (MacKenzie River Delta). N represents sample sizes, and bacterial composition is depicted on the Phylum level. Full species names can be found in

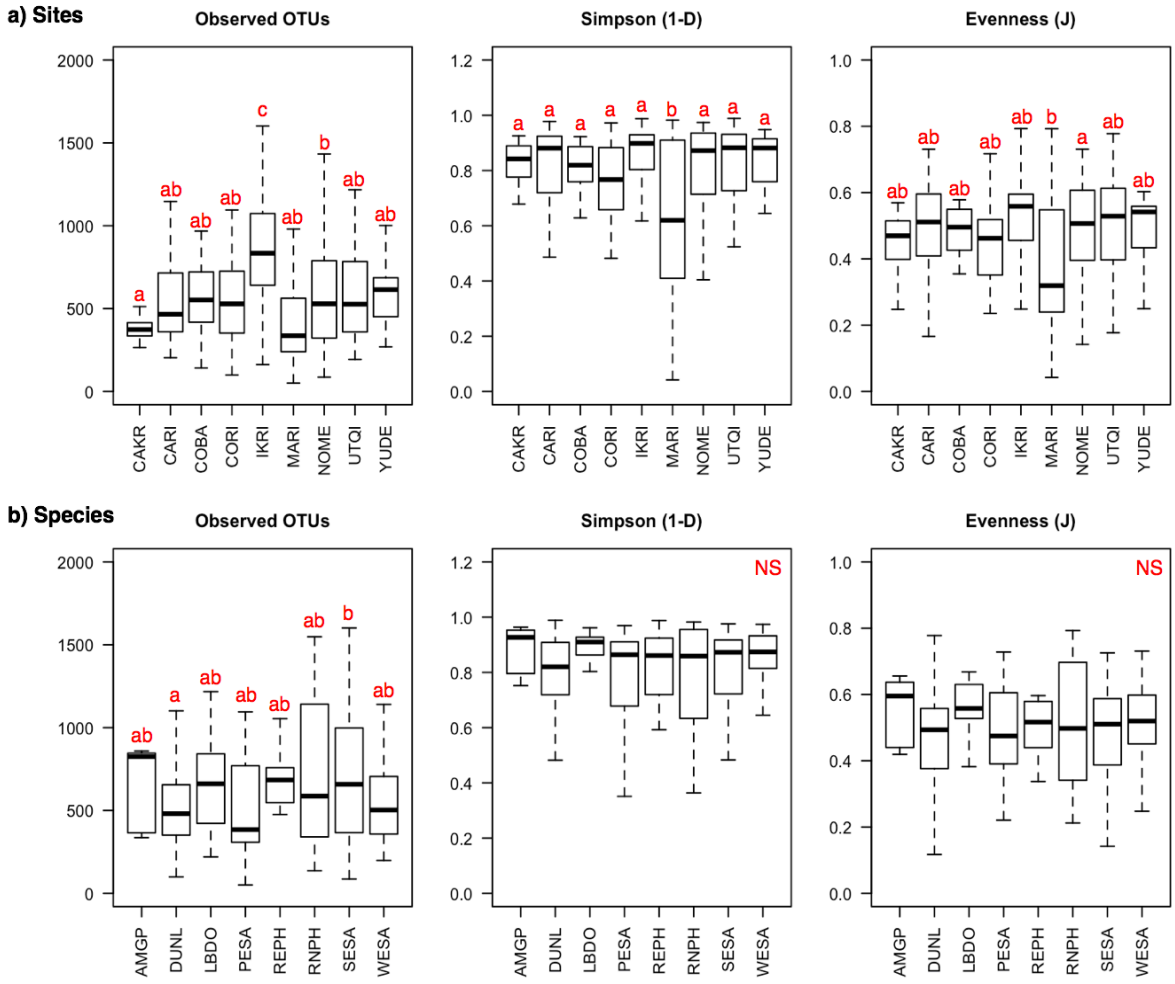


Figure 4.2 Bacterial OTU richness and evenness depicted per sampling site (a) and host species (b) of fecal samples collected from eight species of shorebird at eight arctic breeding sites. Letters represent pair-wise significance (TukeyHSD). Site and host species abbreviations can be found in Table 1 and 2

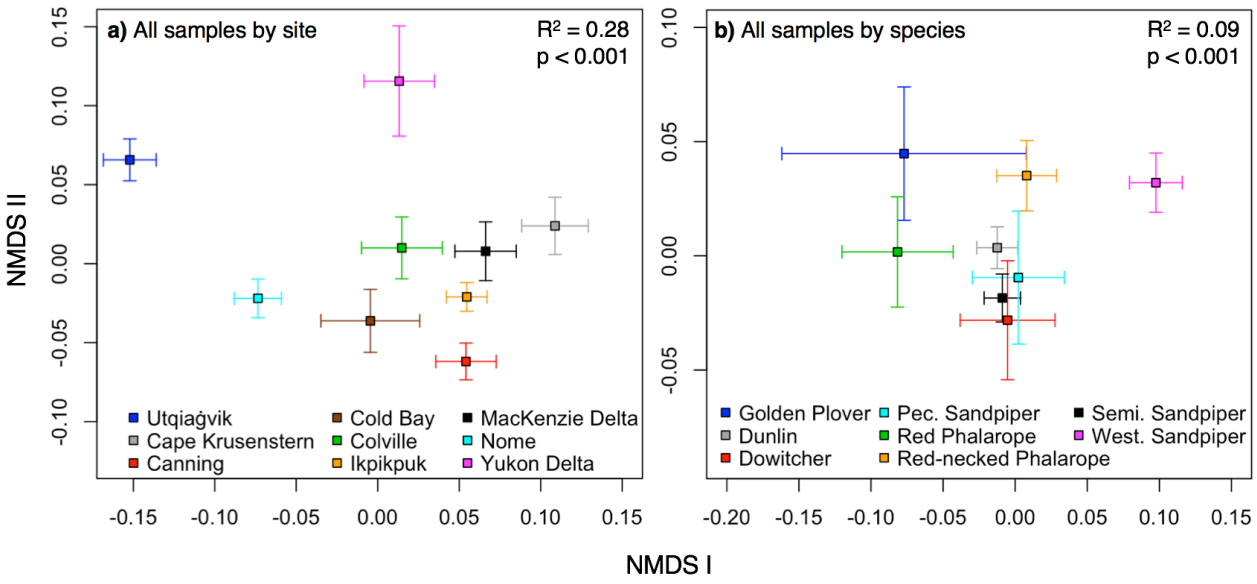


Figure 4.3 Non-metric Multidimensional Scaling of the contribution of: a) sampling site, and b) host species to fecal microbiota composition of Arctic-breeding shorebirds in 2011-2014. Squares represent centroids, and bars are standard error. Significance was set at $\alpha = 0.05$.

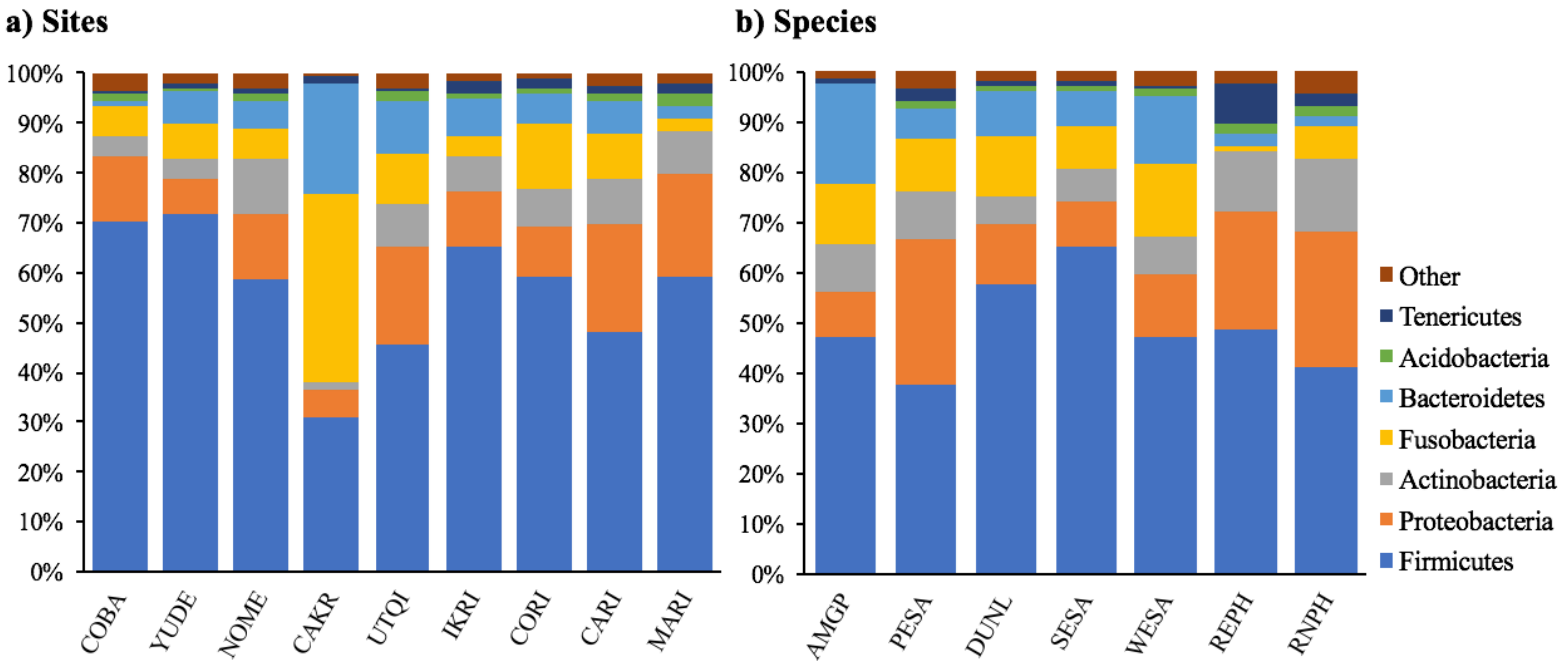


Figure 4.4 Community composition of fecal microbiota collected from arctic shorebirds from 2011-2013 averaged per site (a) and per species (b).

Site and species abbreviations can be found in Table 1 and 2.

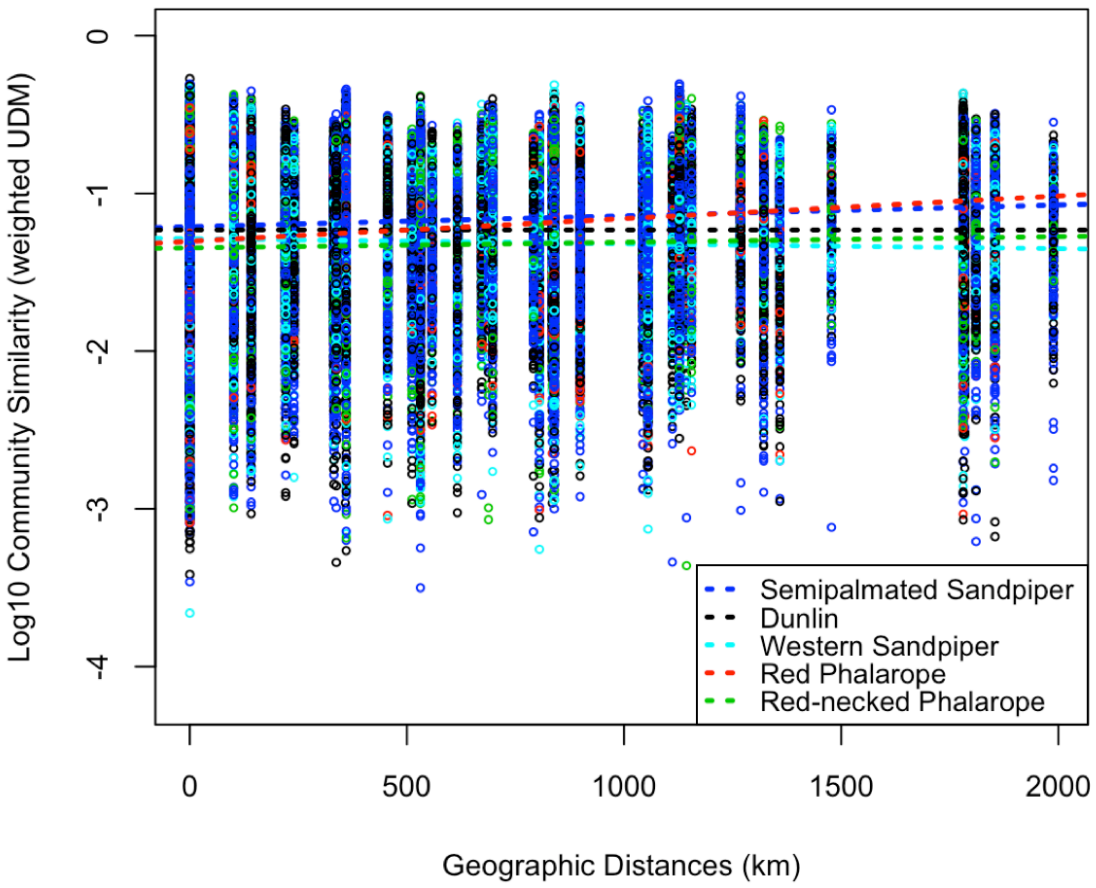
SUPPLEMENTARY TABLES & FIGURES

Supplementary Table 4.1 ANOSIM and adonis tests for significance and relative contribution of seven environmental and host-related factors to variation in weighted and unweighted UNIFRAC Distance Matrices constructed from shorebird fecal communities. Highest relative contributions per dataset and distance matrix are shown in bold.

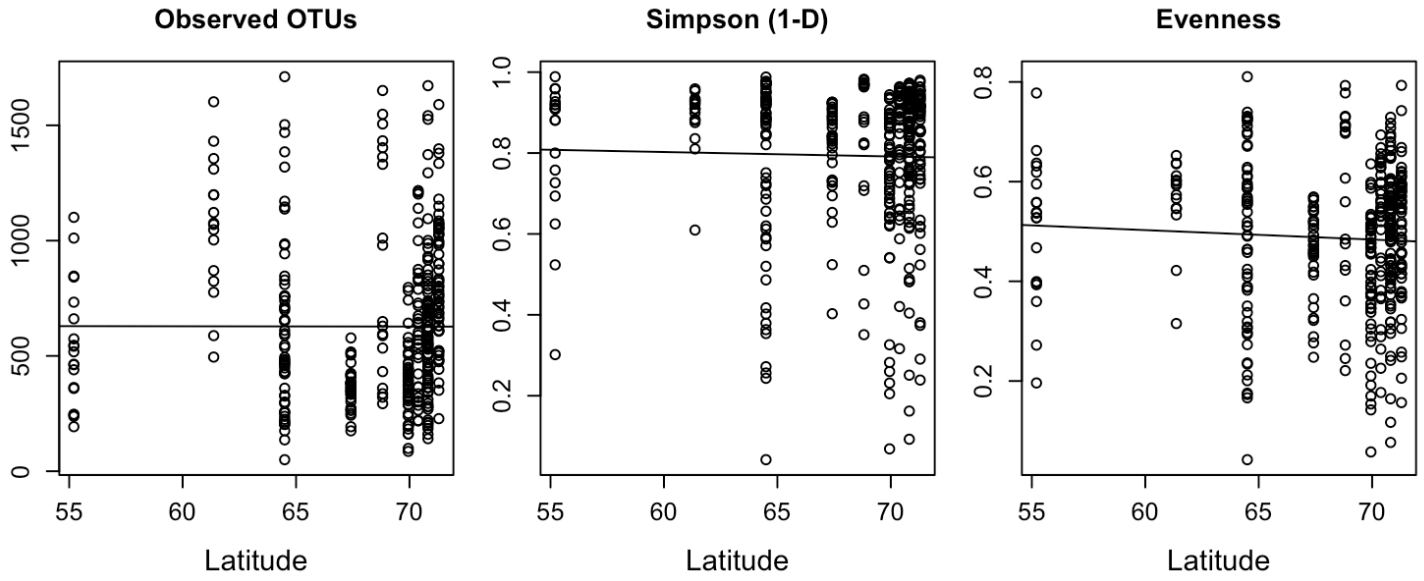
		ANOSIM				**ADONIS**			
		weighted		**unweighted**		**weighted**		**unweighted**	
Dataset	**Variable**	R	p	R	p	R^2	p	R^2	p
All (n=382)	Site	0.164	<0.001	0.259	<0.001	**13.5**	<0.001	**9.2**	<0.001
	Species	0.188	<0.001	0.120	<0.001	8.0	<0.001	5.3	<0.001
	Biome	0.052	0.015	0.137	<0.001	1.6	<0.001	1.5	<0.001
	Year	0.188	<0.001	**0.290**	<0.001	9.6	<0.001	4.1	<0.001
	Family	0.083	0.003	0.083	0.161	0.8	0.012	0.4	0.002
	Genus	**0.211**	<0.001	0.121	<0.001	5.5	<0.001	3.2	<0.001
	Habitat	0.095	<0.001	0.086	<0.001	5.1	<0.001	3.3	<0.001
	Latitude	0.165	<0.001	0.261	<0.001	0.7	0.016	0.9	<0.001
High arctic (n=256)	Site	0.141	<0.001	**0.229**	<0.001	5.5	<0.001	**11.0**	<0.001
	Species	**0.276**	<0.001	0.223	<0.001	**6.8**	<0.001	5.1	<0.001
	Genus	0.236	<0.001	0.156	<0.001	**6.8**	<0.001	4.4	<0.001
	Latitude	0.140	<0.001	0.227	<0.001	1.5	0.004	1.5	<0.001

Low arctic (n=126)	Site	0.243	<0.001	0.259	<0.001	**24.6**	<0.001	**6.9**	<0.001
	Species	0.099	<0.001	0.158	<0.001	7.1	0.002	2.8	0.046
	Genus	**0.289**	0.003	0.240	0.002	4.1	0.003	1.7	0.003
	Latitude	0.244	<0.001	0.260	<0.001	7.6	<0.001	3.5	<0.001
Calidrids (n=302)	Site	0.236	<0.001	0.334	<0.001	**16.3**	<0.001	**10.7**	<0.001
	Species	0.124	<0.001	0.077	<0.001	2.9	<0.001	2.2	<0.001
	Biome	0.071	<0.001	0.137	<0.001	2.1	<0.001	1.7	<0.001
	Year	0.221	<0.001	0.314	<0.001	10.4	<0.001	4.4	<0.001
	Habitat	0.024	0.122	0.030	0.040	0.6	0.067	0.7	0.002
	Latitude	**0.243**	<0.001	**0.337**	<0.001	1.0	0.005	1.0	<0.001
Phalaropes (n=50)	Site	0.150	0.002	0.228	<0.001	**17.1**	<0.001	**17.4**	<0.001
	Species	0.000	0.918	0.000	0.813	2.4	0.287	2.7	0.051
	Biome	0.180	0.042	0.296	0.014	3.0	0.005	3.1	0.022
	Year	**0.496**	<0.001	**0.792**	<0.001	8.6	<0.001	8.3	<0.001
	Fat	0.074	0.067	0.046	0.212	3.4	0.102	2.2	0.273
	Latitude	0.150	0.011	0.228	0.002	2.8	0.185	3.3	0.017
Dunlin (n=109)	Site	0.167	<0.001	**0.352**	<0.001	**17.2**	<0.001	**13.5**	<0.001
	Biome	0.032	0.059	0.106	<0.001	3.0	0.005	2.5	<0.001
	Year	**0.217**	0.003	0.266	<0.001	8.6	<0.001	4.2	<0.001

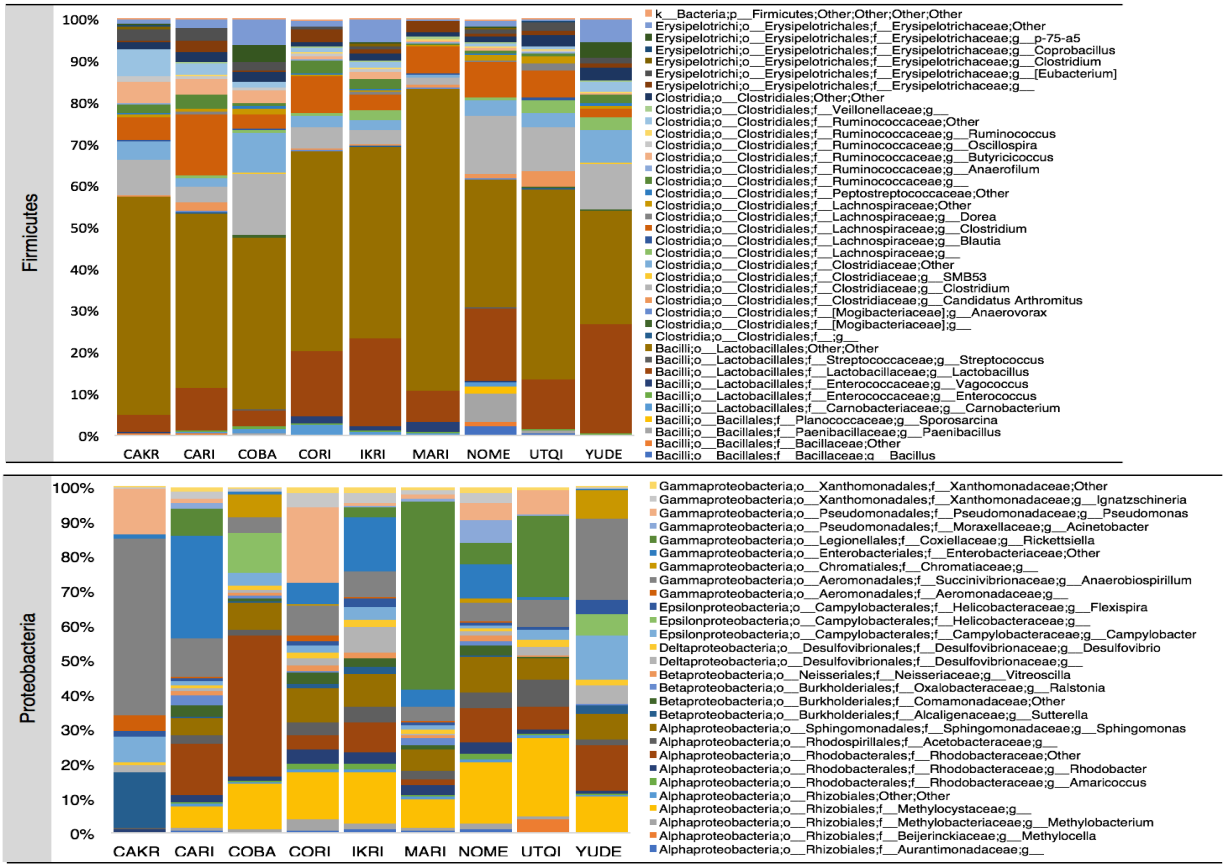
	Fat	0.102	0.006	0.083	0.004	8.2	0.003	6.6	<0.001
	Latitude	0.168	<0.001	**0.352**	<0.001	3.3	0.003	2.4	<0.001
Semipalmated Sandpiper (n=132)	Site	0.324	<0.001	**0.488**	<0.001	**20.5**	<0.001	**13.9**	<0.001
	Biome	0.246	0.002	0.454	<0.001	3.2	0.003	3.6	<0.001
	Year	0.263	<0.001	0.476	<0.001	8.5	<0.001	5.3	<0.001
	Fat	-0.06	0.965	-0.02	0.779	3.9	0.960	6.6	0.054
	Latitude	**0.337**	<0.001	0.487	<0.001	3.1	<0.001	4.4	<0.001



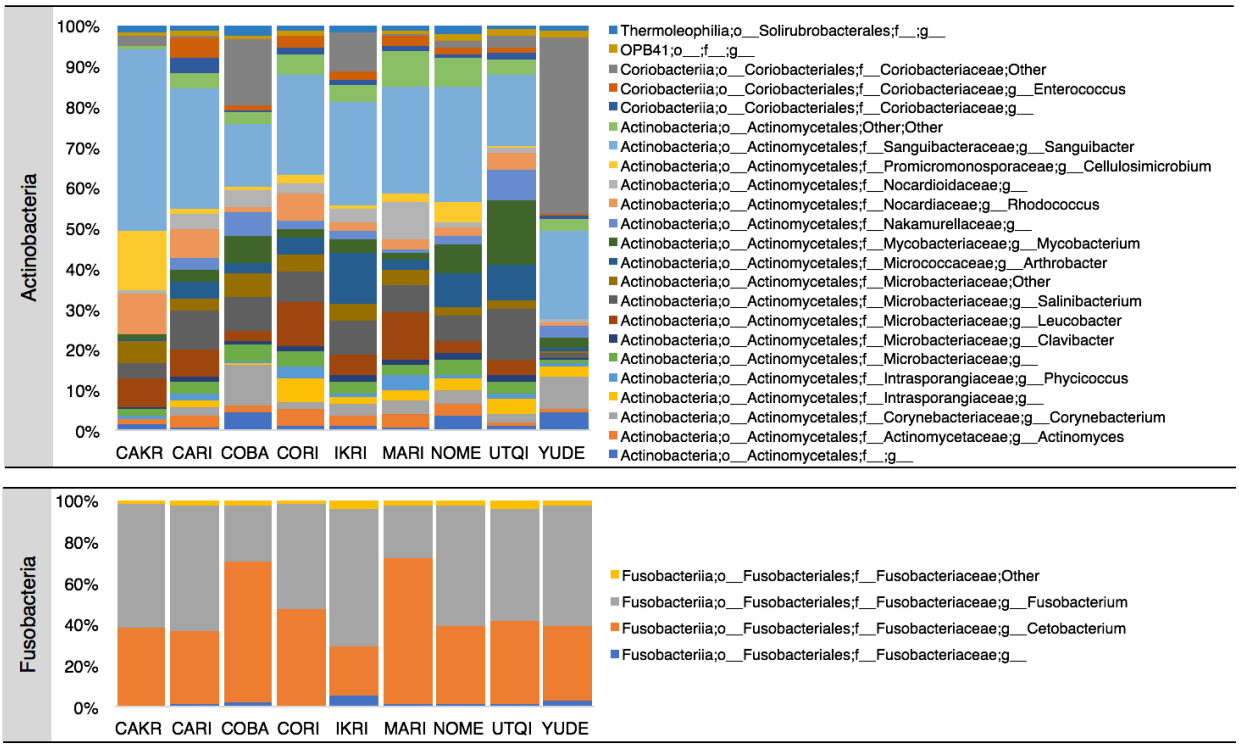
Supplementary Figure 4.1 Distance-decay plot of fecal community similarity and distance between sampling sites (km) of. Community similarity is represented as the $\log_{10}$ of the weighted Unifrac Distance Matrix of OTU table. Dashed lines represent the linear relationship between community similarity per species, and the distance between sampling sites.



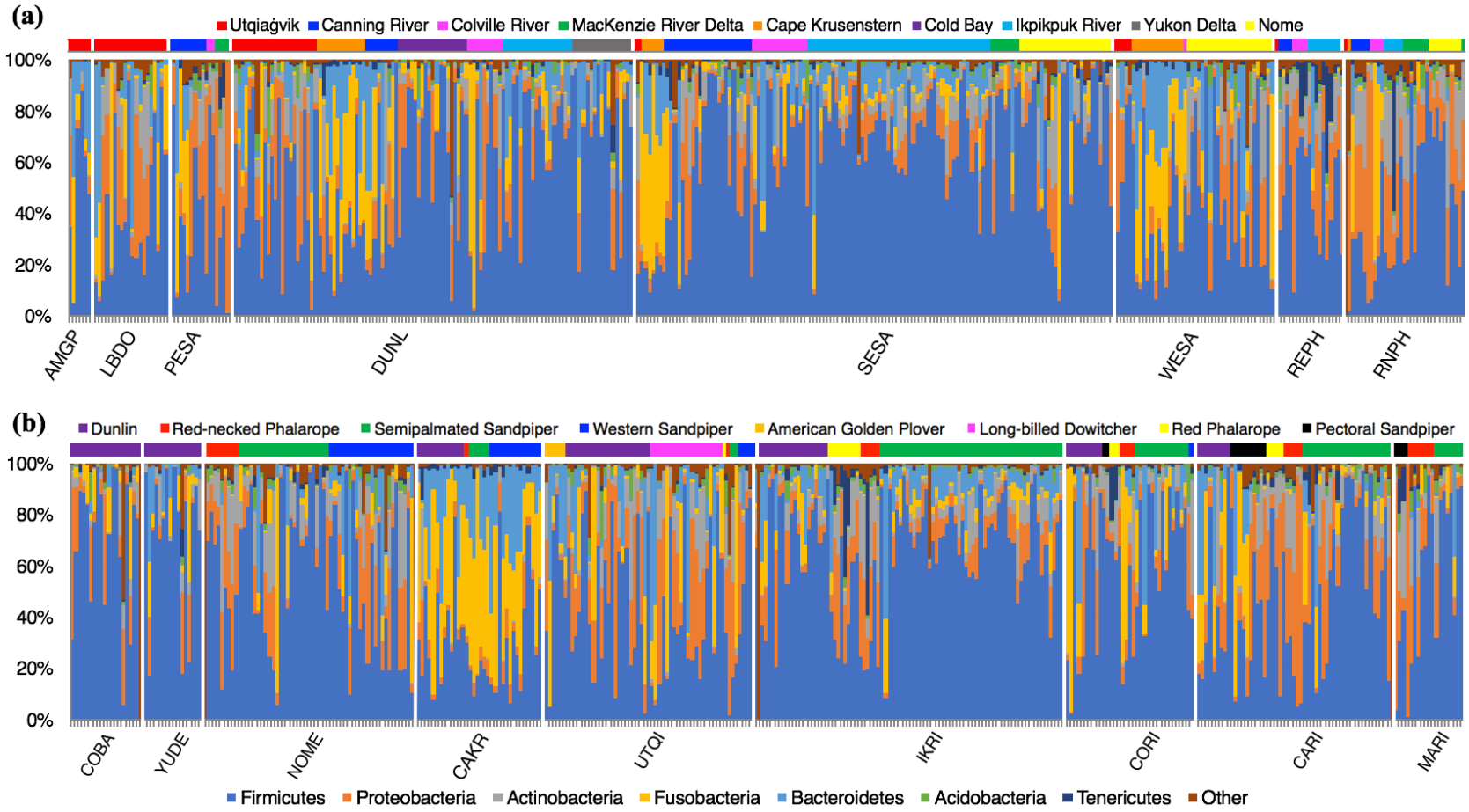
Supplementary Figure 4.2 Richness indices of fecal samples collected from eight species of shorebirds at different latitudes in the North-American and Canadian Arctic. Lines represent linear models fitted to the indices.


Firmicutes
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CAKR
CARI
COBA
CORI
IKRI
MARI
NOME
UTQI
YUDE
k__Bacteria;p__Firmicutes;Other;Other;Other;Other
Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;Other
Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__p-75-a5
Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Coprobacillus
Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Clostridium
Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__[Eubacterium]
Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__
Clostridia;o__Clostridiales;Other;Other
Clostridia;o__Clostridiales;f__Veillonellaceae;g__
Clostridia;o__Clostridiales;f__Ruminococcaceae;Other
Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus
Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira
Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Butyricicoccus
Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Anaerofilum
Clostridia;o__Clostridiales;f__Ruminococcaceae;g__
Clostridia;o__Clostridiales;f__Peptostreptococcaceae;Other
Clostridia;o__Clostridiales;f__Lachnospiraceae;Other
Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Dorea
Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Clostridium
Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia
Clostridia;o__Clostridiales;f__Lachnospiraceae;g__
Clostridia;o__Clostridiales;f__Clostridiaceae;Other
Clostridia;o__Clostridiales;f__Clostridiaceae;g__SMB53
Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium
Clostridia;o__Clostridiales;f__Clostridiaceae;g__Candidatus Arthromitus
Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__Anaerovorax
Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__
Clostridia;o__Clostridiales;f__;g__
Bacilli;o__Lactobacillales;Other;Other
Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus
Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus
Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Vagococcus
Bacilli;o__Lactobacillales;f__Enterococcaceae;g__Enterococcus
Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__Carnobacterium
Bacilli;o__Bacillales;f__Planococcaceae;g__Sporosarcina
Bacilli;o__Bacillales;f__Paenibacillaceae;g__Paenibacillus
Bacilli;o__Bacillales;f__Bacillaceae;Other
Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus

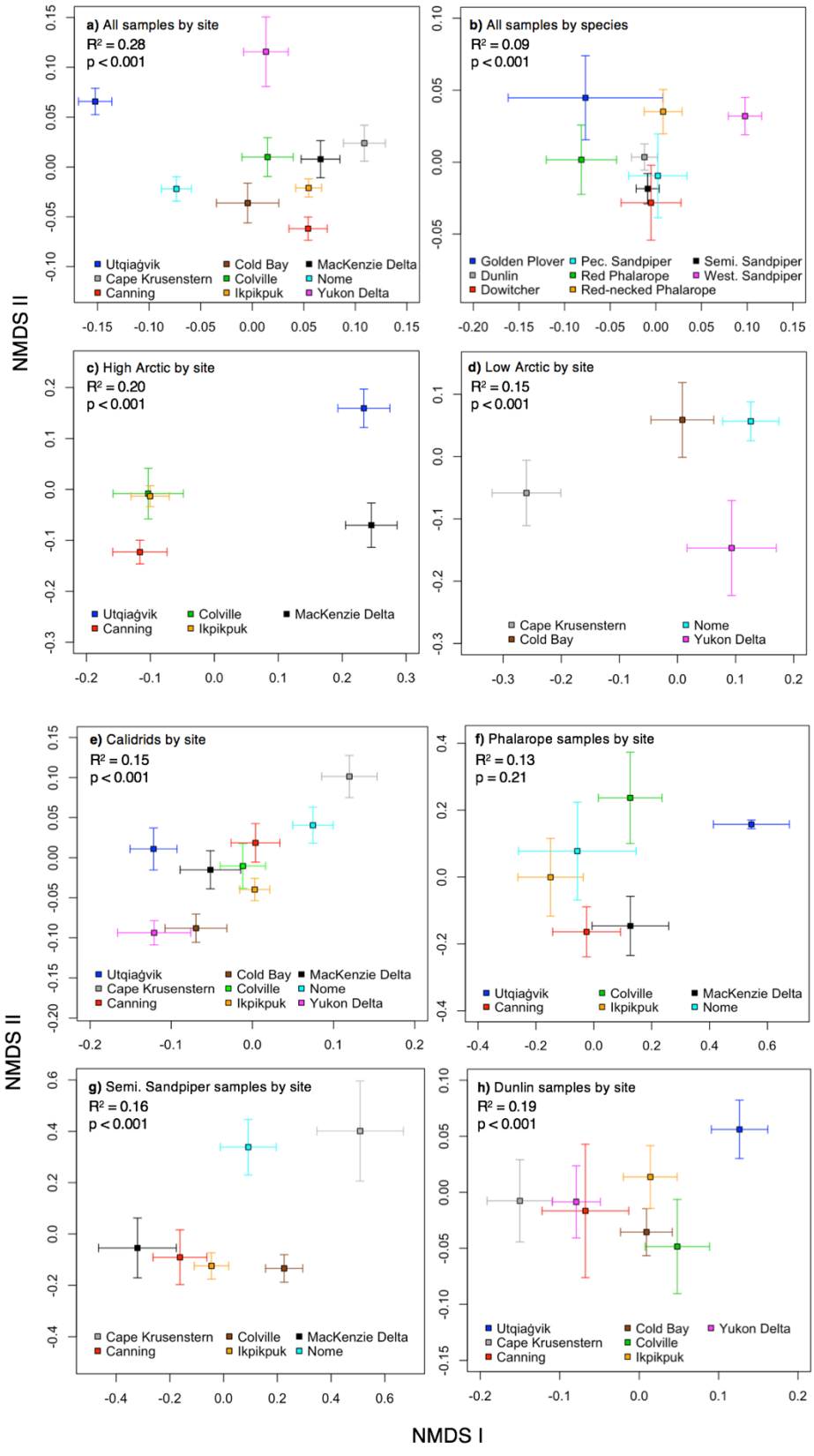
Proteobacteria
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CAKR
CARI
COBA
CORI
IKRI
MARI
NOME
UTQI
YUDE
Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;Other
Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Ignatzschineria
Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas
Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter
Gammaproteobacteria;o__Legionellales;f__Coxiellaceae;g__Rickettsiella
Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;Other
Gammaproteobacteria;o__Chromatiales;f__Chromatiaceae;g__
Gammaproteobacteria;o__Aeromonadales;f__Succinivibrionaceae;g__Anaerobiospirillum
Gammaproteobacteria;o__Aeromonadales;f__Aeromonadaceae;g__
Epsilonproteobacteria;o__Campylobacterales;f__Helicobacteraceae;g__Flexispira
Epsilonproteobacteria;o__Campylobacterales;f__Helicobacteraceae;g__
Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__Campylobacter
Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__Desulfovibrio
Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae;g__
Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Vitreoscilla
Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Ralstonia
Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;Other
Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__Sutterella
Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas
Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae;g__
Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;Other
Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobacter
Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Amaricoccus
Alphaproteobacteria;o__Rhizobiales;Other;Other
Alphaproteobacteria;o__Rhizobiales;f__Methylocystaceae;g__
Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium
Alphaproteobacteria;o__Rhizobiales;f__Beijerinckiaceae;g__Methylocella
Alphaproteobacteria;o__Rhizobiales;f__Aurantimonadaceae;g__



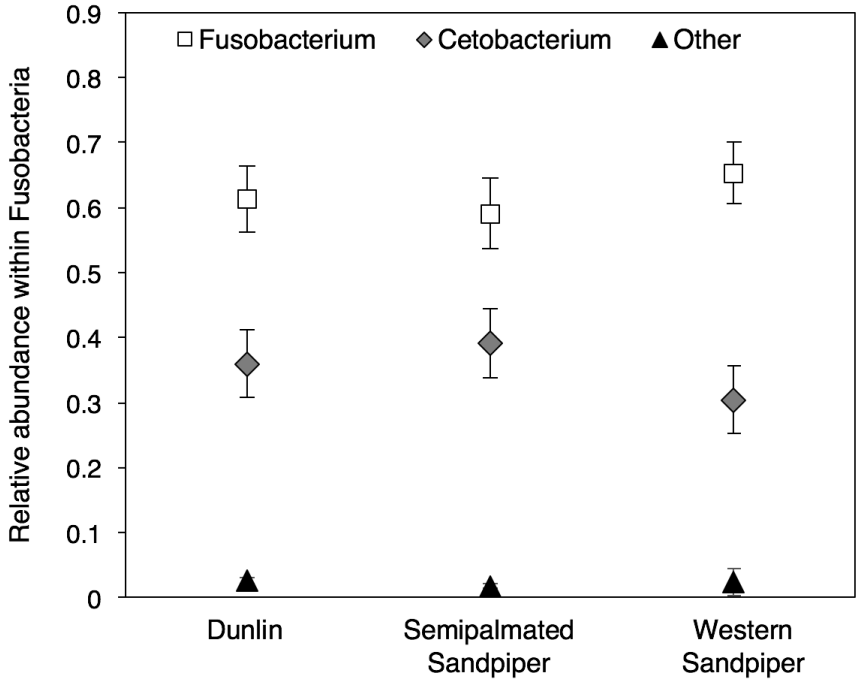
Supplementary Figure 4.3 Relative abundance of bacterial Classes within the four major Phyla found in arctic breeding shorebirds: *Firmicutes*, *Proteobacteria*, *Fusobacteria* and *Actinobacteria*. For site name abbreviations, see Table 4.2.


(a)
Utqiaġvik
Canning River
Colville River
MacKenzie River Delta
Cape Krusenstern
Cold Bay
Ikpikpuk River
Yukon Delta
Nome
100%
80%
60%
40%
20%
0%
AMGP
LBDO
PESA
DUNL
SESA
WESA
REPH
RNPH
(b)
Dunlin
Red-necked Phalarope
Semipalmated Sandpiper
Western Sandpiper
American Golden Plover
Long-billed Dowitcher
Red Phalarope
Pectoral Sandpiper
100%
80%
60%
40%
20%
0%
COBA
YUDE
NOME
CAKR
UTQI
IKRI
CORI
CARI
MARI
Firmicutes
Proteobacteria
Actinobacteria
Fusobacteria
Bacteroidetes
Acidobacteria
Tenericutes
Other

Supplementary Figure 4.4 a) Relative abundance of bacterial Phyla found in fecal samples collected from eight shorebird species collected at nine arctic sites from 2011-2014. Species distribution per site is shown above the graph. Abbreviations of sites displayed on the x-axis are shown in Table 4.1. b) Figure 3b. Relative abundance of bacterial Phyla found in fecal samples collected from eight shorebird species collected at nine arctic sites from 2011-2014. Site distribution per species is shown above the graph. Abbreviations of species names displayed on the x-axis are shown in Table 1.


a) All samples by site
R² = 0.28
p < 0.001
Utqiaġvik
Cape Krusenstern
Canning
Cold Bay
Colville
Ikpikpuk
MacKenzie Delta
Nome
Yukon Delta
b) All samples by species
R² = 0.09
p < 0.001
Golden Plover
Dunlin
Dowitcher
Pec. Sandpiper
Red Phalarope
Red-necked Phalarope
Semi. Sandpiper
West. Sandpiper
c) High Arctic by site
R² = 0.20
p < 0.001
Utqiaġvik
Canning
Colville
Ikpikpuk
MacKenzie Delta
d) Low Arctic by site
R² = 0.15
p < 0.001
Cape Krusenstern
Cold Bay
Nome
Yukon Delta
e) Calidrids by site
R² = 0.15
p < 0.001
Utqiaġvik
Cape Krusenstern
Canning
Cold Bay
Colville
Ikpikpuk
MacKenzie Delta
Nome
Yukon Delta
f) Phalarope samples by site
R² = 0.13
p = 0.21
Utqiaġvik
Canning
Colville
Ikpikpuk
MacKenzie Delta
Nome
g) Semi. Sandpiper samples by site
R² = 0.16
p < 0.001
Cape Krusenstern
Canning
Colville
Ikpikpuk
MacKenzie Delta
Nome
h) Dunlin samples by site
R² = 0.19
p < 0.001
Utqiaġvik
Cape Krusenstern
Canning
Cold Bay
Colville
Ikpikpuk
Yukon Delta
NMDS I
NMDS II

Supplementary Figure 4.5 Non-metric Multidimensional Scaling of the contribution of: a) sampling site, and b) host species to fecal microbiota composition of Arctic-breeding shorebirds in 2011-2014. Squares represent centroids, and bars are standard error. Significance was set at $\alpha = 0.05$.



Supplementary Figure 4.6 Relative abundance of genera within the Fusobacteria Phylum in fecal samples collected from Dunlin, Semipalmated Sandpipers and Western Sandpipers at Cape Krusenstern, AK, from 2011-2014. Error bars represent standard error.

Chapter 5 - PREVALENCE OF BACTERIAL PATHOGENS IN SHOREBIRDS IN THE WESTERN HEMISPHERE

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ABSTRACT

Migratory shorebirds encounter different environments during their annual cycle. Different environments contain different pathogens, and risk of infection may vary among sites. The *migratory escape* hypothesis argues that migratory animals avoid high disease prevalence areas through movements, and has been suggested as one of the mechanisms underlying migratory behavior. The goals of our study were to test the migratory escape hypothesis by assessing the prevalence of seven known avian pathogens in shorebirds at different stages of migration, and to explore potential patterns of pathogen co-occurrence in infected hosts. We collected 226 fecal samples from nine migratory and one resident shorebird species at four widely distributed sites in the Western Hemisphere, including one breeding site, two staging sites and one non-breeding site. We screened our samples for seven avian bacterial pathogens (*Campylobacter jejuni*, *C. coli*, *C. lari*, *Pasteurella multocida*, *Clostridium perfringens*, *Salmonella typhymurium*, and *Eryspelothrix rhusopathia*) in fecal samples of wild birds with targeted PCR assays. With the exception of *Campylobacter jejuni* and *C. coli*, we did not detect five of our seven pathogens in shorebird fecal samples. Prevalence of *C. jejuni* were highest among birds sampled in Argentina (99.0 ± 0.9%; n = 79) and Alaska (96.3 ± 2.2%; n = 51), followed by Washington (65.5%; n = 51) and Delaware (56.1 ± 3.9%; n = 62). We detected *C. coli* in 0.9% of samples, which did not allow us to address co-infection dynamics. We did not detect the majority of our avian pathogens in migratory shorebirds throughout the Western Hemisphere, and our data showed site but not species-related variation in prevalence of *C. jejuni*.

Keywords: *Campylobacter jejuni*, *Campylobacter coli*, disease, migration, PCR, Scolopacidae

INTRODUCTION

Migration is a common phenomenon across many animal taxa, including invertebrates, fish, birds and mammals. Migratory behavior has been hypothesized to have evolved as a response to variation in climatic conditions and food availability (Durant et al. 2007), to avoid predation (McKinnon et al. 2010), and to reduce risk of exposure to pathogens (Hall et al. 2014, Johns and Shaw 2016).

The *migratory escape* hypothesis predicts avoidance of high disease prevalence areas through migration, and has been described in Monarch butterflies (Satterfield et al. 2015), fish (Poulin et al. 2012), and Lesser Black-backed Gulls (Arriero et al. 2015). Migratory escape has been proposed as one of the main reasons that many bird species migrate to the high-Arctic for breeding, because pathogen exposure is hypothesized to decrease with increasing latitude (Piersma 1997). Indeed, shorebirds had a higher infection occurrence of avian influenza on their northbound than southbound migrations, suggesting higher exposure or susceptibility at their non-breeding sites at low latitudes (Krauss et al. 2004). Also, non-breeding site was found to drive the prevalence of avian malaria in shorebirds in Australia (Clark et al. 2016), supporting the *migratory escape* hypothesis.

However, migratory birds can have higher parasite prevalence and infection intensity than migratory birds (Leung et al. 2016), which does not support the mechanisms of pathogen avoidance hypothesized to underlie migration. A large-scale study comparing intestinal nematode richness between over 200 migratory and resident bird species showed a higher richness of nematode species in migratory birds (Koprivnikar and Leung 2015). In waterfowl, diversity and prevalence of haematozoan parasites were positively related to migration distance, suggesting that migratory birds could actually be exposed to a more diverse pathogen array (Figuerola and Green 2000).

Wild birds are known vectors of a number of infectious diseases. Viral and protozoan pathogens distributed by wild birds include avian influenza virus (Krauss et al. 2004), West Nile virus (Dusek et al. 2009), reovirus (Sandercock et al. 2008), and the causative agents of avian malaria (Yohannes et al. 2008). Bacterial diseases, such as avian botulism and avian cholera, are generally studied after mass

mortality events associated with disease outbreaks (Friend et al. 2001, Adams et al. 2003, Włodarczyk et al. 2014). As a result, we know little about the natural dynamics of bacterial pathogens in wild birds.

Shorebirds include over 200 species, many of which are migratory, and Arctic-breeding shorebirds have among the longest migrations found in birds and mammals. The majority of migratory shorebird species are rapidly declining in numbers, predominantly due to anthropogenic habitat loss (Kentie et al. 2015, Melville et al. 2016, Piersma et al. 2016). As migratory animals can experience an increase in pathogen pressure alongside population declines (Satterfield et al. 2015), it is necessary to understand dynamics in pathogen prevalence in shorebirds throughout their annual cycle.

To test the *migratory escape* hypothesis for shorebirds, we investigated pathogen prevalence throughout the migratory cycle in the Western Hemisphere. The objectives of our study were to: 1) test the *migratory escape* hypothesis through assessing the prevalence of seven bacterial pathogens in shorebirds at different stages of migration, and 2) explore potential patterns in pathogen co-occurrence. We predict that pathogen prevalence is lower at higher latitudes, and prevalence will be lowest in birds sampled during the Arctic breeding season. To test our objectives, we collected fecal samples from ten shorebird species at four sites in North and South America, and used targeted PCR assays to determine pathogen prevalence.

METHODS

Sample collection. – We collected fecal samples from 10 species of shorebirds at one breeding site in Alaska (2013; Utqiaġvik, formerly Barrow), two spring-migration staging sites in Delaware (2015; Slaughter Beach) and Argentina (2015; Bahía San Antonio), and at two non-breeding site in Washington (2015; Ocean Park) and Argentina (2015; Bahía San Antonio; non-breeding site for Two-banded Plover) (Table 5.1; Figure 5.1). Birds were captured with nest traps during the breeding season, and with mist nets and cannon nets at staging and non-breeding sites. Immediately after capture, birds were placed in a darkened plastic box for up to 10 min. For each new individual, boxes were sterilized with bleach wipes, and the bottom of the box was lined with a clean sheet of wax paper. After defecating, birds were banded

and biometric measurements were collected. Fecal samples were transferred to 1.5ml sterile Eppendorf tubes. All handling of the wax paper was conducted while wearing sterile latex gloves. All fecal samples were preserved in 100% ethanol at collection, and stored frozen at -20°C for further analyses. Samples collected in Argentina were shipped at room temperature (3 days), due to logistical challenges for international shipments with dry ice. Samples were stored frozen at -20°C upon arrival in the USA.

DNA extraction. – To remove ethanol from fecal samples, we centrifuged samples for 10 min at 10,000 rpm and removed supernatant. We repeated the cleaning step twice with 1 ml of RNase/DNA free molecular grade water (Grond *et al.* 2014; Ryu *et al.* 2014). DNA was extracted from fecal samples using the MoBio Power Lyzer/Power Soil kit as per the manufacturer's instructions (Mo Bio Laboratory, Carlsbad, CA, USA), except we used 15 min high velocity vortexing instead of a bead beating step. Genomic DNA yields were determined using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA).

PCR . – We used conventional PCR to test for prevalence of seven bacterial pathogens (*Campylobacter jejuni*, *C. coli*, *C. lari*, *Pasteurella multocida*, *Clostridium perfringens*, *Salmonella typhymurium*, and *Eryspelothrix rhusopathia*). To ensure that our assays were valid, we included positive controls in duplicate for each pathogen in our PCR runs. Positive controls consisted of DNA extracted from pure cultures of our selected pathogens, which we obtained from the Kansas State College of Veterinary Medicine. Controls were extracted using the same method and DNA was diluted to match the same concentration (5 ng/µl) as our samples. We also included negative controls consisting of RNase/DNA free molecular grade water in duplicate for each PCR run. Amplification reactions were performed in 25 µl reaction volumes, containing 25 ng (5 µl at 5 ng/µl) of template, 100 nM of primers, 12.5 µl TaqMan™ Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Primers for

amplification of bacterial pathogens were obtained from literature (Table 5.2), and we followed cycling conditions from the referenced literature.

RESULTS AND DISCUSSION

To ensure broad taxonomic and geographic coverage, we selected a total of 226 fecal samples from ten shorebird species that were captured at four sites in North and South America (Table 5.1). We determined prevalence of seven known avian pathogens using targeted PCR assays (Table 5.2). With the exception of *Campylobacter jejuni* and *C. coli*, we did not detect any of the other five pathogens in shorebird fecal samples. One of the pathogens we screened for, *Pasteurella multocida*, is the causative agent for avian cholera and has been documented to have expanded into the Arctic in Common Eiders (*Somateria mollisima*) and various seabirds (Descamps et al. 2012, Bodenstein et al. 2015). However, we failed to detect *P. multocida* in shorebirds, possibly indicating the absence of an inter-species transmission route. Prevalence of *C. jejuni* was highest among shorebirds sampled in Argentina (99.0 ± 0.9%) and Alaska (96.3 ± 2.2%), followed by Washington (65.5%) and Delaware (56.1 ± 3.9%; Figure 1). Prevalence of *C. coli* was low, with only one detection in a Ruddy Turnstone (*Arenaria interpres*) in Delaware (n = 20; 5.0%) and two detections in Two-banded Plovers (*Charadrius falklandicus*) in Argentina (n = 34; 6.1%).

Prevalence of *C. jejuni* was similar among different species captured at the same site, but differed among sites (Figure 5.1). In Delaware Bay, prevalence of *C. jejuni* was the lowest of our sampling sites with a range of 41.7%-61.7% across shorebird species. Shorebirds have been implicated as vectors for avian influenza (Krauss et al. 2010), especially when migrating through the Delaware Bay in spring. Delaware Bay is thought to be a disease hotspot due to high densities of birds aggregating along the Bay coast during spring migration (Krauss et al. 2010). Shorebirds congregate to feed on the eggs of Horseshoe Crabs (*Limulus polyphemus*) and potential for pathogen transmission is high as shorebirds forage in large, mixed-species flocks. Although prevalence of *C. jejuni* in Delaware Bay was low

compared to our other sites, prevalence was still higher than has been reported in previous studies of wild birds (Keller et al. 2011; 7.2%, Keller and Shriver 2014; 8.1%, but see Pacha et al. 1987;5-81%). For example, Keller *et al.* (2014) used a combination of culturing, PCR and sequencing to evaluate prevalence of *C. jejuni*, *C. lari* and *C. coli* in 269 shorebirds from four species in Delaware Bay and detected *C. jejuni* in 24.2% of samples, which was two times lower than we detected.

Shorebirds have the potential to spread pathogens over long distance. We found relatively high prevalence of *C. jejuni*, but otherwise we did not find evidence for shorebirds acting as potential disease vectors. Birds are considered natural reservoirs of *C. jejuni* (Pacha et al. 1988, Sahin et al. 2015). *C. jejuni* has been detected across a wide range of bird taxa (Waldenström et al. 2007, Lu et al. 2011, Hermans et al. 2012, Keller and Shriver 2014), but no cases of a *C. jejuni* infection resulting in Campylobacteriosis have been observed in wild birds. A potential absence of pathogenicity of *C. jejuni* to wild birds could explain the presence of *C. jejuni* in the majority of our samples, without any observations of clinical signs of disease in wild-caught birds.

We detected *C. coli* in 1.3% of our samples and failed to detect *C. lari* in our study, which matched the absence of both pathogens in shorebirds sampled by Keller et al. (2011). Interestingly, *C. lari* was detected in 10.1-38.4% of fecal samples collected from Ruddy Turnstones, Red Knots, and Semipalmated Sandpipers collected in Delaware Bay for a previous cloning study in 2011 (Ryu et al. 2014), but *C. jejuni* and *C. coli* were not detected. Differences in prevalence of the three *Campylobacter* spp. could indicate temporal variation in infection patterns, or could potentially result from use of different molecular methods.

The pathogen species or genera we selected for our study were all isolated from wild birds in previous studies (Friend et al. 2001, Santos et al. 2012, D'Amico et al. 2014, Keller and Shriver 2014). Our failure to detect five of these pathogens in a large sample of >200 shorebird fecal samples across the Western Hemisphere might have one of three explanations. First, our negative results could have been artifacts of our choice of primers and detection ability. However, we successfully amplified our positive

controls at relatively low concentrations, supporting the validity of our methods. Moreover, we did not detect contamination in any of our negative controls.

A second explanation could be that shorebirds might have low exposure to pathogens at our field sites, with low prevalence as a result. Low pathogen exposure at our sites would support the *migratory escape* hypothesis, if it was a result of pathogen avoidance due to migratory behavior. We were unable to test whether shorebirds had low exposure to pathogens, as we did not measure any indicators of infection such as pathogen-specific antibodies. Surveying antibody levels could inform us on exposure rates, because detection in healthy individuals would indicate successful recovery from infection.

Last, it is possible that our sampling was biased towards healthy birds, despite sampling birds during different times of year using multiple capture techniques. Elsewhere, House Finches (*Haemorhous mexicanus*) infected with *Mycoplasma gallisepticum* had a lower encounter rate than healthy birds (Faustino et al. 2004), which was attributed to behavioral changes associated with infection. However, Mallards (*Anas platyrhynchos*) that were infected with Avian Influenza had higher recapture probabilities than uninfected Mallards (Avril et al. 2016). Other than behavioral changes, migratory culling could also result in sampling biased towards healthy individuals. Migratory culling constitutes the rapid disappearing of diseased individuals from the population, and has been confirmed in different taxa of migratory animals (Senar and Conroy 2004, Poulin et al. 2012, Satterfield et al. 2015). Linking behavioral differences to infection status is challenging in free-living birds. Observation and sampling of birds during disease outbreaks would allow testing for a capture bias, but where and when disease outbreaks occur is challenging to predict. Alternatively, experimentally infecting wild birds taken into captivity could elucidate potential changes in behavior of infected individuals.

Overall, we did not detect the majority of pathogens we screened for, with the exception of *C. jejuni* and *C. coli*. Prevalence of *C. jejuni* in shorebirds was relatively high compared to literature estimates, and differed among sampling sites but not among species. Infection with *C. jejuni* did not appear to affect shorebird health, and investigating the possible interactions between *C. jejuni* and its wild

bird hosts, as well as expanding the range of pathogens tested, would be logical next steps in assessing disease risk in migratory shorebirds.

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TABLES & FIGURES

Table 5.1 Study species of migratory shorebirds and four sites where fecal samples were collected from. Sample sizes represent samples scanned for the presence of seven avian pathogens. Avian pathogens are described in Table 2.

Species		Alaska	Delaware	Washington	Argentina
Two-banded Plover	*Charadrius falklandicus*				33
Ruddy Turnstone	*Arenaria interpres*		21		
Red Knot	*Calidris canutus*		20		30
Sanderling	*Calidris alba*			30	15
Dunlin	*Calidris alpina*	13	13		
Semipalmated Sandpiper	*Calidris pusilla*	15	8		
Western Sandpiper	*Calidris mauri*	12			
Short-billed Dowitcher	*Limnodromus griseus*		4		
Red Phalarope	*Phalaropus fulicarius*	12			

Table 5.2 Primers used for seven avian pathogens that fecal samples were scanned for. Primers were obtained from literature, and cycling conditions from reference papers were applied.

Pathogen	**Disease**	**Primer**	**Sequence(5'-3')**	**Source**
Campylobacter jejuni	Gastroenteritis	10F	AGAGTTTGATCCTGGCTNAG	Denis et al. 1999
		804R	GACTACCNGGGTATCTAATCC	
Campylobacter coli	Gastroenteritis	CcF	GTAAAACCAAAGCTTATCGTG	Abdulrahman et al. 2015
		CcR	TCCAGCAATGTGTGCAATG	
Campylobacter lari	Gastroenteritis	ClF	TAGAGAGATAGCAAAAGAGA	Abdulrahman et al. 2015
		ClR	TACACATAATAATCCCACCC	
Pasteurella multocida	Avian cholera	KMT1T7-F	ATCCGCTATTTACCCAGTGG	Varte et al. 2014
		KMT1SP6-R	GCTGTAAACGAACTCGCCAC	
Clostridium perfringens	Necrotic enteritis	CPAlphaF	GCTAATGTTACTGCCGTTGA	Park et al. 2015
		CPAlphaR	CCTCTGATACATCGTGTAAG	
Salmonella typhimurium	Salmonellosis	spyF	TTGTTCACTTTTTACCCCTGAA	Nair et al. 2015
		spyR	CCCTGACAGCCGTTAGATATT	

Eryspelithrix	Erysipelas	MO101F	AGATGCCATAGAAACTGGTA	Makino et al. 1994
rhusopathiae		MO101R	CTGTATCCGCCATAACTA	

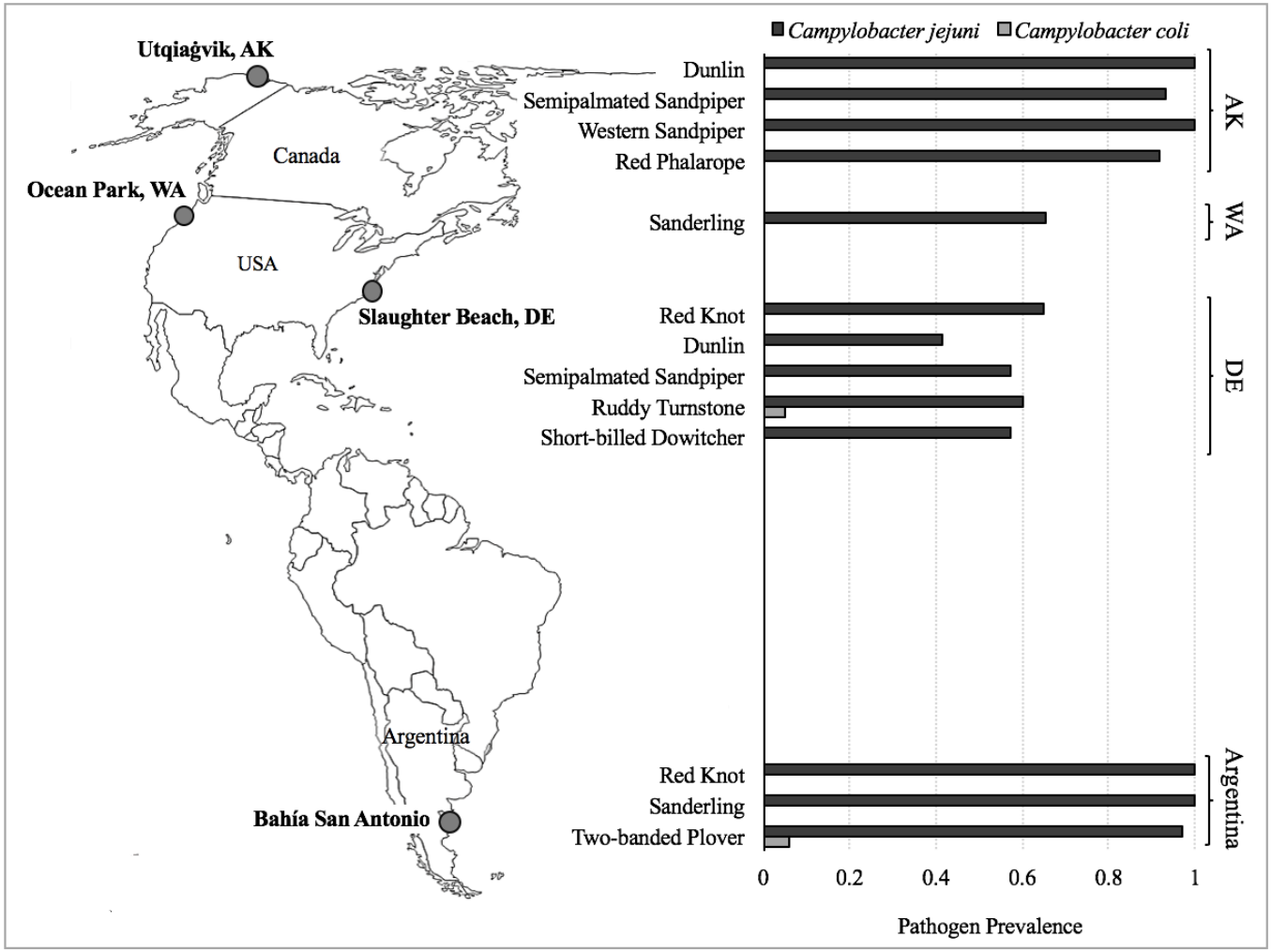

Table 5.3 Sampling sites and prevalence of *Campylobacter jejuni* and *C. coli* in fecal samples of migratory shorebirds captured in 2013 at Utqiaġvik, and 2015 at Ocean Park, Slaughter Beach and Bahía San Antonio.

Chapter 6 - CONCLUSIONS AND FUTURE IMPLICATIONS

Host-microbiome research has been rapidly expanding over the past decade due to and increasing recognition of links between gut microbiota and organismal health, and the increasing availability of high-throughput sequencing techniques. However, the dynamics and drivers of gut microbiota in birds have been poorly studied, especially in wild species under natural conditions. With my PhD research, I have made several new contributions to the emerging field of avian microbiome research.

First, I reviewed the existing literature on gut microbiota of wild and domestic birds to determine the current state of knowledge of the field. I show that the number of publications on avian gut microbiota have increased over the past decade due to developments in molecular techniques, but that a majority of these studies have focused on domestic poultry. Gut communities of wild birds show large variation among different avian taxa, and differences were related to a number of intrinsic and extrinsic factors, such as diet, age, phylogeny and environment. My field study on migratory shorebirds contributes valuable new data to the relatively small number of studies that have been conducted on wild birds up to present.

Second, I tested the assumption of embryo sterility in wild birds under field conditions, and found the first support that embryos of wild, precocial birds indeed not have gut microbiota until after hatching. Past research on gut microbiomes focused mainly on mammalian hosts and the role of birth in establishing of the gut microbiome, and our understanding of avian systems has lagged behind. My finding that embryos of shorebirds are sterile up until hatch combined with the independent, self-feeding lifestyle of precocial chicks limits the potential for maternal control over their offspring's gut microbiota. In addition, my work is the first field study to document recruitment and establishment of the gut microbiota in free-living, precocial chicks under natural conditions. I showed that chicks acquire their microbiota rapidly, emphasizing the importance of the first few days of life and the environment they are exposed to for establishing a gut microbiota in chicks.

Third, I characterized the microbial communities for eight species of migratory shorebirds over a wide geographic range of the North American Arctic, which adds substantially to the growing body of literature on gut microbiota of wild birds that I reviewed in Chapter 2. To understand how gut microbiota and the environment affect shorebird health, a first step was to identify members of the gut community and document baseline microbial community composition across a range of bird shorebird species.

Similar to chicks, local environment was an important driver of gut microbiota composition in adult shorebirds. The Arctic is one of the regions that is strongest affected by climate change, which has resulted in high relative temperature increases over the past decades (IPCC 2014). Increasing Arctic temperatures were shown to result in large shifts and reduced evenness in soil microbial communities (Deslippe *et al.* 2012) as well as resulted in northwards range shifts of avian malaria (Loiseau *et al.* 2012) and macro-parasites such as ticks and parasitic nematodes (Kutz *et al.* 2009).

Last, a subset of avian gut microbes can be pathogenic and affect human health. I contributed to our knowledge of pathogen prevalence in shorebirds with my fifth chapter. Surveying pathogen occurrence in migratory shorebirds provided baseline data for future monitoring efforts and can help in predicting potential disease outbreaks. I did not detect the majority of pathogens I screened for, and future work needs to expand our range of pathogens to include viral, protozoan and a wider array of bacterial pathogens. I observed site, but not species-related differences in prevalence of *C. jejuni* at our four sampling sites. In the future, broader geographical monitoring could be used to identify hotspots for specific pathogens.

A recurring finding throughout my dissertation was the importance of local environment in shaping gut microbiota of shorebirds at different stages of life. In chicks, gut microbiota was recruited from the environment after hatching, whereas in adults, breeding site was the main driver of variation in microbial communities, and pathogen prevalence differed among sampling sites. The potential importance of local environment as a driver of microbial composition of shorebird guts highlights the need for collecting baseline community data over a wide range of species and sites. In addition, a majority of shorebirds are affected by and habitat degradation (Melville, Chen and Ma 2016; Piersma *et al.* 2016),

which necessitates improving our understanding of the interactions between shorebirds and their microbial environment.

Future directions

The interpretation of gut microbial data can be challenging due to its enormous diversity and complexity (Shetty *et al.* 2017). To reveal patterns and test hypotheses using microbiome data, we need large sample sizes, which are often challenging or impossible to obtain under field conditions. International collaborations among organizations and field sites can provide a solution for meeting sample size requirements and provide infrastructure for large-scale sampling efforts in the future. In collaboration with the Arctic Shorebird Demographics Network in the US and Canada, the US Fish and Wildlife Service in Washington, and Delaware and CENPAT-CONICET in Argentina, I was able to investigate drivers of gut microbiota composition and pathogen distribution by using samples from shorebird species collected over a wide geographical range. As another example, Hird et al. (2015) collaborated with the Museum of Natural Science at Louisiana State University to investigate gut microbial communities in 59 neotropical bird species from 14 different orders; an effort that would not have been achievable without logistical support from the museum collectors. Due to the large diversity we have observed in gut microbiota across bird taxa (Chapter 2), international collaborations will be a valuable approach for the advancement of our knowledge of wild bird microbiomes and what drives these communities.

For my PhD research, I focused on characterizing members of the avian gut microbiota and broad drivers of community variation and diversity. A logical next step in avian microbial research is to expand our studies to investigating the functional ecology of microbiota in birds. Addressing microbial function in complex communities is challenging as it requires knowledge of gene function in a wide array of microorganisms. The gut is one of the best characterized systems with respect to gene function, due to a strong research focus on health implications of gut microbiota in humans (Rutayisire *et al.* 2016; Lin and Zhang 2017). Birds carry different microbial communities than mammals, but several studies have been

able to generate putative functional profiles of avian gut microbiota using gene function prediction programs (Langille *et al.* 2013; Oulas *et al.* 2015).

One of the challenges of inferring gene function from 16S rRNA genes and metagenomic sequencing is that no distinction is made between expressed and non-expressed genes. Transcriptomics is a technique that sequences transcribed genes only, which means that only genes that are expressed at the time of sampling will be sequenced. Sequencing of the total gene pool versus the active gene community will provide us with more accurate functional profiles of the gut microbiota in birds under different ecological conditions (Abram 2015).

Following function of gut microbiota, we need to investigate how changes in functional profiles affect the host itself. The link between gut microbiota composition and health has been well established in humans, but we are lacking knowledge on the host-microbiota interaction in birds. Studies on poultry have shown that dietary changes can result in shifts in gut microbiota, but whether the changes in gut microbial communities are beneficial for bird health is uncertain. As I discussed in Chapter 5, it is challenging to investigate avian health in wild populations as diseases can result in behavioral changes and rapid loss of diseased individuals from the population. In addition, there is no consensus on what physiological parameters constitute a healthy individual in wild birds.

To quantify bird health, studies have generally focused on collecting either immune measurements, or microbiome data. The combination of microbiology and eco-immunology applied to wild birds could help establish baseline health parameters, but would require experimental manipulation of gut microbial communities to determine effect on the birds' immune function, and vice versa. As it is challenging to perform experiments on free-living birds due to the large potential for confounding factors associated with the outside environment, future studies should investigate the microbiota-health relationship in wild birds temporarily brought into captivity and maintained in common garden or reciprocal transplant experiments.

Overall, my studies have shed light on dynamics and drivers of gut microbiota in shorebirds throughout their life, but many questions remain. Future studies investigating microbial function in the wild bird gut, as well as mapping the interactions between gut microbiota and avian health, will allow us to bridge the gap in knowledge that currently exists among avian and mammalian studies.

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