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Physiology and Genetics Shape the Microbiome of a Seabird Species (*Oceanodroma Leucorhoa*) More than Environmental and Social Factors

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PHYSIOLOGY AND GENETICS SHAPE THE MICROBIOME OF A SEABIRD SPECIES (*OCEANODROMA LEUCORHOA*) MORE THAN ENVIRONMENTAL AND SOCIAL FACTORS

by

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The microbiome provides multiple benefits to animal hosts that can profoundly impact health and behavior. Microbiomes are well-characterized in humans and animals in controlled settings, yet assessments of wild bird microbial communities remain vastly understudied. This study examines the multiple factors that affect the microbiome of a burrow-nesting *Procellariiform* bird species, Leach's storm-petrel. 16S rRNA-based Illumina Mi-Seq analyses are used to assess the composition and structure of bird and burrow-associated bacterial communities. Results indicate that sex and skin site contribute to bird-associated bacterial community variation, and MHC heterozygosity impacts these bacterial assemblages in a sex and site-specific manner, potentially having implications on odor-mediated mate selection. Environmental and social factors only minimally influence bird-associated bacterial assemblages, although environmental impact is sex and site-specific. While other studies have examined factors that impact the avian microbiome, most focus on microbial assemblages in terrestrial bird species, which differ substantially from seabirds in their life histories. Here, individual physiological and genetic influences outweigh environmental and social factors on microbiome composition, suggesting a dependence on individual genetics in mate selection potentially through microbiome-mediated odor cues for this species. This is the first study to examine multiple factors that affect the surface microbiome of a seabird.

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BACKGROUND AND LITERATURE REVIEW

Introduction

All animal bodies are inhabited by a collection of symbiotic microbial communities known as the microbiome. This multifunctional microbial network outnumbers somatic cells ten to one and adds over eight million genes to an already extensive eukaryotic genetic repertoire (Ezenwa et al., 2012; Funkhouser and Bordenstein, 2013). The microbiome affords multiple internal and external benefits to an animal host. For example, nutrients and energy from normally indigestible material are made usable by gut bacteria in the phyla *Bacteroidetes* and *Firmicutes* (Org et al., 2015; Zhang et al., 2015), and symbiotic gut microbes stimulate the production of anti-microbial and pro-inflammatory factors to fight tissue-destroying *Enterococcus faecium* and *Clostridium difficile* infections (Buffie and Pamer, 2013). On the skin, elevated levels of *Propionibacterium* markedly reduce harmful *Haemophilus ducreyi* infections (Rensburg et al., 2015), and commensal *Staphylococcus epidermis* produces compounds that selectively inhibit pathogenic *Staphylococcus aureus* (Grice and Segre, 2011).

A great majority of microbiome studies have been performed in mammalian species, including humans, captive zoo animals, and domesticated pets (Caporaso et al., 2011; Ley et al., 2008; Suchodolski et al., 2015). Yet symbiotic microbial communities confer many benefits to mammalian and non-mammalian species alike, including insects and birds. For example, gut symbionts of the desert locust *Schistocerca gregaria* protect against invasion by pathogenic *Serratia marcescens* (Dillon et al., 2005), and honeybees (*Bombus terrestris*) exposed to the fecal matter of conspecifics are protected from the gut parasite *Crithidia bombi* (Koch and Schmid-Hempel, 2011). Uropygial gland-associated bacteria protect European hoopoes (*Upupa epops*) from feather-degrading *Bacillus licheniformis* (Ruiz-Rodríguez et al., 2009) and members of *Clostridia* and *Fusobacteria* found in

the hindgut of Turkey vultures (*Cathartes aura*) allow these scavengers to digest carrion and tolerate bacterial toxins (Roggenbuck et al., 2015). While a fully functioning and stable microbiome can afford these and other benefits to animal hosts, imbalances can be highly detrimental. For example, humans afflicted with genetically determined immunodeficiency abnormalities harbor anomalous skin microbiota and become susceptible to fungal and microbial pathogens (Smeekens et al., 2013), and atypical intestinal microbiota lead to autoimmunity in patients with type I diabetes (Giongo et al., 2011). In mice, experimentally induced colitis leads to dysbiosis of oral microflora (Rautava et al., 2015), and in arthropods, microbiome imbalances are linked to reduced health, fitness, and mate competitiveness, as seen in the medfly *Ceratitis capitata* (Hamdi et al., 2011).

Over the past few years, knowledge of the animal microbiome has become plentiful. Technological advances, such as next-generation 16S rRNA gene sequencing, have allowed scientists to detect a wide array of bacterial communities, most of which cannot be grown using standard culture techniques. The vast majority of microbiome studies has been performed in controlled environments, leaving the wild animal microbiome comparatively under evaluated. It is critical to understand this "second genome" of wild animals to unravel host-microbe co-evolutionary relationships (Amato, 2013), developmental and genomic interactions (McFall-Ngai et al., 2013), and animal behaviors (Penn and Potts, 1998; Theis et al., 2013; Tung et al., 2015). Such complex explorations are critical to bolster our understanding of wild animal populations, communities, and ecosystems, which have been historically dominated by studies involving mammals and even further underrepresented by those involving birds (Waite and Taylor, 2015). Birds maintain ecosystem balance by pollinating plants, dispersing seeds, scavenging carcasses, stabilizing complex food webs, and recycling nutrients (Beasley et al., 2012; Clout and Hay, 1989). Although birds are critical contributors to the earth's ecosystem, little is known about their microbiomes. Advances have been

made in understanding the factors that affect the symbiotic microbial communities of the avian gut (Roggenbuck et al., 2015; Waite and Taylor, 2014).

Examinations of avian microbiomes are relatively new and exciting, and this knowledge base is expanding from existing studies involving birds and other vertebrates alike. Multiple important questions should be posed pertaining to these microbial networks and how they interact with animals and their environments. For example, to what extent is the microbiome controlled by host genetics? How much of an impact does the environment have on the composition and/or structure of these microbial communities? And how could interactions between mates and conspecifics affect these communities? This review explores recent literature on the avian microbiome, focusing primarily on birds and including work performed on non-avian species, including mammals, insects, and fish, where appropriate. Also included in this review is a discussion of avian olfaction, its importance in the lives of birds, and how it may be linked to individual recognition and mate selection. The final section will focus on data that implicate microbial communities and the major histocompatibility complex as influences on chemical signaling involved in mate recognition.

Factors Impacting Avian Microbiomes

Body Site Location

Multiple physiological, genetic, environmental, and social factors can impact the composition and structure of the animal microbiome. Human skin colonization depends on topographical location and endogenous factors such as age and sex (Grice and Segre, 2011). *Corynebacteria* spp. grow well in high humidity axillary regions of the human body, while drier arm and leg skin sites support fewer microorganisms (Grice and Segre, 2011). Haired regions of dog skin contain higher bacterial diversity and richness than mucosal sites (Hoffmann et al., 2014). The skin and feathers of birds are also topographically diverse. Birds preen their feathers, and bare skin exposed after brood patch development comes into direct contact with the egg during incubation. Recent interest in

studying the avian skin microbiome was sparked by examinations of the avian uropygial gland and the antimicrobial properties of the preen oil it produces, particularly in passerines. The uropygial gland is the only avian exocrine gland and is located dorsally at the base of the tail of most avian species (Montalti et al., 2005). It secretes oily sebum and waxy esters, which birds apply to feathers through preening to clean and waterproof their feathers and to make them more flexible (Martínez-García et al., 2015). The antimicrobial effects of preen oil are augmented by specialized antimicrobial symbiotic bacteria residing in the uropygial gland, which are well-characterized by multiple studies involving European hoopoes (*Upupa epops*). Uropygial glands of European hoopoes experimentally inoculated with antibiotics stop producing antimicrobial volatile compounds, suggesting that uropygial gland symbionts, particularly *Enteroccus* spp., are involved in producing these antimicrobial compounds (Martín-Vivaldi et al., 2009; Soler et al., 2008). In addition to protecting feathers from the destructive bacteria *Bacillus licheniformis* (Williams et al., 1990), preen oil has been implicated in affecting other aspects of the avian life history. Hoopoes inoculate their eggs with preen oil to protect them from infection with bacteria known to be pathogenic to embryos, including multiple *Micrococcus* spp., *Escherichia coli*, and *Staphylococcus* spp. (Martínez-García et al., 2015; Soler et al., 2014).

Studies of the avian skin are dominated by, but not limited to, studies of the uropygial gland. Other bird body sites have only recently been explored. For example, the face microbiome of Turkey vultures (*Cathartes aura*) and Black vultures (*Coragyps atratus*) differs substantially from the microbiome of the anaerobic hindgut (Roggenbuck et al., 2015), and multiple human pathogens, including *Campylobacter, Clostridium,* and *Salmonella* spp. can be isolated from the skin of production chickens in evaluations of food safety for humans (Oakley et al., 2013). Interestingly, the scientific literature appears to be completely devoid of examinations of the avian brood patch. The brood patch, sometimes referred to as the incubation patch, is a featherless, highly vascularized patch of skin located on the ventral abdomen that is characterized by edema and hyperplasia during

development (Jones, 1971). One or both parents develop a brood patch during the egg incubation stage that subsequently comes into direct contact with the egg to regulate its temperature. Brood patch formation, hypervascularization, and edema are due to increases in estrogen and prolactin (Jones, 1971). Some bacteria are sensitive to hormones (Garcia-Gomez, Elizabeth et al., 2012), and seasonal hormonal and physiological changes may affect the bacterial species present on the brood patch. It is possible that the composition and structure of the brood patch microbiome is not only different from other body sites, but due to reproductive hormone fluctuations, is sex-specific. Scientific literature pertaining to the brood patch microbiome appears to be lacking, leaving the microbial communities of a critical avian body site open for exploration. Given these physiological differences, microbiota at these sites are likely both highly distinctive and highly influenced by individual bird characteristics, yet no studies to date have conducted comprehensive analyses of multiple avian surface body sites.

Avian Genetics

Another factor that can influence the microbiome is host genetics (Org et al., 2015). Genetic influence on the microbiome is often studied in the context of sometimes uncontrollable environmental factors, making the direct effect of inheritability difficult to ascertain. However, when controlling for environment, genetic factors can strongly affect the abundance of core microbiota (Org et al., 2015). Family members share more microbial communities than non-family (Spor et al., 2011) and monozygotic twins have more similar gut microbiota than dizygotic twins (Goodrich et al., 2014). In mice, the composition of intestinal microbiota can be predicted by genetic markers such as quantitative trait loci (Benson et al., 2010). Genetic influences on avian microbial community composition and structure are just starting to emerge. For example, when controlling for environmental factors, relatedness significantly impacts cloacal microbiota in broiler chickens, primarily represented by differential abundances of *Lactobacillus* spp. between males and females

(Zhao et al., 2013). In Adelie penguins (*Pygoscelis adeliae*), genetic distance between conspecifics significantly correlates with fecal microbiota and outweighs the effects of physical distance (Banks et al., 2009). However, in some bird species, particularly passerines, genetic factors do not impact symbiotic microbial communities. In a recent study conducted by Whittaker et al. (2016), male and female Dark-eyed juncos had similar bacterial community profiles. Similarly, in studies conducted on Barn swallows (*Hirundu rustica*) and Zebra finches (*Taeniopygia guttata*) environmental and social interactions greatly impacted the cloacal microbiome, but effects of sex and relatedness were not detected (Kreisinger et al., 2015; Kulkarni and Heeb, 2007) These results suggest that genetic factors may impact the microbiomes of some bird species more than others. These disparities may be attributed to differences in species-specific life histories, but future studies are needed to further explore these ideas. Another genetic factor that impacts the animal microbiome is the major histocompatibility complex, or MHC, and has been demonstrated in mouse models. In congenic mice, MHC genotype influences antibody responses against commensal gut bacteria, which leads to the establishment of unique microbial communities (Kubinak et al., 2015), and some animals choose mates based on MHC genotype through a variety of phenotypic selection mechanisms (Milinski, 2006). A potential link between the MHC, the microbiome, and animal communication will be discussed further in section 4.

Environmental and Social Sources of Microbial Diversity

While individual host genetic factors can impact the microbiome, environmental and social interactions can facilitate the exchange of symbiotic bacterial communities between hosts and their environments (Ezenwa et al., 2012). Such examinations are particularly well-documented in studies involving humans and indoor environments. In a study performed by Lax et al. 2014, indoor environments were quickly colonized by the microbiota of their human inhabitants, and each individual home could be identified by a microbial signature specific to its occupants. Interestingly,

microbial community transfer was predominantly one-way, marked by significantly more pronounced microbial community transfer from humans to abiotic surfaces (Lax et al., 2014).

While assessing environmental impact, background genetic interference should be minimized, which can be accomplished using genetically similar mice. For example, weaned isogenic mice allocated to the same cage showed markedly more similar altered Schaedler flora than mice housed among multiple cages (Deloris Alexander, A. et al., 2006), and isogenic mice reared in separate research institutions carried drastically different gut microflora (Friswell et al., 2010). Recently, studies exploring the effects of environmental factors on the microbiome in birds have been conducted, primarily focusing on gut microbiota. These studies all demonstrated a strong effect of environment on host-associated microbiota that outweighed genetic influence. For example, bacteria experimentally inoculated on the feathers of Zebra finches were transferred from one bird to another via allopreening and subsequently isolated from the cloacal microbiota, signifying an oral-fecal-genital transmission pattern in this species (Kulkarni and Heeb, 2007). The gut microbiome of the Brown-headed cowbird *Molothrus ater*, a brood parasite, is strongly influenced by environmental factors, which greatly outweigh individual genetic effects and host parental influence on gut microbiota (Hird et al., 2014). Sibling Great tits (*Parus major*) reared in the same nest shared more similar cloacal microbial communities than those reared in separate nests (Lucas and Heeb, 2005), and genetically similar hoopoes reared in the same nest shared more uropygial gland bacteria than those reared in separate nests (Ruiz-Rodríguez et al., 2014). Direct contact with nest material can also affect the avian microbiome in some species. Nest-associated microbial communities contribute to the composition of the eggshell microbiome in hoopoes (Martínez-García et al., 2016) and Reed warblers (*Phragmites australis*) (Brandl et al., 2014), signifying a strong influence of direct contact with nest material on bird-associated microbiota. This result contrasts with findings from studies on humans, who were the primary source of microbial communities

shared between host and abiotic surface (Lax et al., 2014; Wood et al., 2015). These differences are likely attributed to the stark contrasts between indoor built human environments and the naturally-occurring microbe-heavy nest environments of birds, but future studies to test this hypothesis are required.

The impact of environment on host-associated microbial communities is further characterized by social interactions between and among conspecifics and mates. Examinations of social factors that affect the host microbiome have been demonstrated in numerous vertebrate species. In wild baboons, social network is the primary driver explaining gut bacteria composition (Tung et al., 2015). Individuals transfer bacteria to one another through direct physical contact, and the effect of social network remains strong even after controlling for kinship, diet, and shared environments (Tung et al., 2015). In mice, physical interactions among cage members are responsible for the transfer of gut microbiota between individuals (Turnbaugh et al., 2009). A similar pattern has also been demonstrated in some bird species. Breeding pairs of free-living Barn swallows share similar gut-associated microbiota (Kreisinger et al., 2015; Tung et al., 2015) and cloacal bacteria are transferred via sexual contact in wild kittawakes (*Rissa tridactyla*) (White et al., 2010). Exploration of the effects of social interactions on the avian microbiome have been limited to examinations of gut microbioa, and only a few have explored the effects social interactions on the composition and structure of the skin microbiome (e.g. Whittaker et al., 2016). The effects of social interactions on the avian skin microbiome are still relatively unknown, and warrant further investigation through studies on species within multiple bird orders.

Further examinations of avian skin-associated microbial communities in wild bird species will help elucidate the effects of multiple factors that could shape the avian microbiome. While the microbiome provides critical symbiotic benefits to each individual host, its functions extend even

further into the realm of inter-specific communication, and may have substantial impacts on mate selection and overall species fitness, as discussed in the following sections.

Microbial Impacts on Animal Behavior

Bacteria and Olfactory Mammals

In addition to providing multiple benefits to individuals, the microbiome has been implicated in dramatically influencing host behavior (Archie and Theis, 2011; Ezenwa et al., 2012). Many mammals rely heavily on odor to recognize conspecifics (Bonadonna and Nevitt, 2004; Karlsson et al., 2010). They mark substrates with urine, feces, or products of specialized glands to relay information about individual identity, genotype and group membership (Archie and Theis, 2011; Leclaire et al., 2014b). Mammals that communicate by odor cues possess specialized scent glands that are found in multiple warm, moist, and nutrient-rich locations of the body (Archie and Theis, 2011). Bacteria exist in glands and secretions involved in chemical communication, and their role in influencing scent cues has long been suggested (Leclaire et al., 2014a; Theis et al., 2013). Microbial communities can influence individual animal odor profiles by directly producing odorants or by metabolizing existing endogenous organic compounds (Leclaire et al., 2014a). These communities may also be responsible for producing group or kin signatures, and their compositions are influenced by sex in Meerkats (*Suricata suricatta*), Greater Sac-winged bats (*Saccopteryx bilineata*), and White-tailed deer (*Odocoileus virginianus*) (Leclaire et al., 2014a). In hyenas, the structure of microbial communities covaries with the composition of volatile compounds found in scent secretions, suggesting that symbiotic bacteria influence odor composition (Theis et al., 2013).

A bacterial role in mediating chemical communication was first proposed through the fermentation hypothesis, which states that bacteria ferment non-odorous mammalian substrates into volatile, odorous compounds that the host then uses to communicate with conspecifics (Albone and Perry, 1976). Underlying bacterial communities are expected to covary with individual odor profiles

(Theis et al., 2013). The fermentation hypothesis is supported in mammals, but this hypothesis has yet to be demonstrated in any avian species.

Bacteria and Olfactory Birds.

Although multiple studies have demonstrated a bacterial influence on odor profiles in mammals (Archie and Theis, 2011), no studies have yet convincingly shown that bacteria influence the odor signatures of birds. Recently, Whittaker et al. (2016) demonstrated that the bacterial odor profiles of the Dark-eyed junco do not covary, suggesting that odor profiles are not affected by bacterial communities. However, it is possible that Dark-eye juncos, like other *Passeriiformes*, are not highly dependent on odor cues for critical life activities as highly olfactory birds, such as seabirds and vultures. *Passeriiformes* have much smaller olfactory anatomy than, for example, highly olfactory seabirds that depend on odor cues for critical life activities. Another possibility is birds do not have a dedicated scent gland. The closest analog to a dedicated scent gland in birds is the uropygial gland, which serves multiple functions, and as a result may confound bird studies (Whittaker et al., 2016).

Multiple seabird species belonging to the order *Procellariiformes*, such as albatrosses, shearwaters, and petrels, possess large olfactory bulbs that represent up to one third of the total brain (Bang and Cobb, 1968). These birds are highly dependent on odor cues for multiple critical life activities, including foraging, burrow relocation, and individual recognition (Bonadonna and Nevitt, 2004). Scent-marking behaviors were historically thought to be absent among birds, although recent findings have demonstrated that this is a critical behavior for certain bird species (Roper, 1999; Whittaker et al., 2014). In addition to possessing highly developed olfactory anatomy, petrels have a noticeable musky scent, and analysis of petrel feather odors shows that they have endogenously produced personal odor that is distinctive from other individuals (Célérier et al., 2011; O'Dwyer and Nevitt, 2009).

Scent plays an important role in the lives of petrels starting at an early age. European Storm-petrel chicks (*Hydrobates pelagicus*) require an intact sense of smell to relocate their nests after being displaced short distances, and are attracted to the smell of their own body odor over that of a conspecific (Bonadonna and Nevitt, 2004; O'Dwyer et al., 2008). They nest in burrows buried deep in rocky structures, yet these burrows open up to a wide-open common area where chicks tend to wander. The ability of petrel chicks to smell their own body odor and nesting material allows them to find their way back home in a cluster of very similar-looking homes (O'Dwyer et al., 2008). As petrels age, their reliance on olfactory cues changes to accommodate changes in life history. In choice experiments, adult petrels can discriminate between the scent of their own nest versus that of a conspecific (Bonadonna and Nevitt, 2004). However, in the presence of conspecifics, adults show an aversion to their own personal odor, likely as a mechanism to avoid inbreeding (Bonadonna and Nevitt, 2004; Célérier et al., 2011; Hagelin and Jones, 2007; Mardon and Bonadonna, 2009). These findings suggest that odor-mediated mate selection may have evolved in petrels to promote genetic compatibility (Mardon and Bonadonna, 2009).

MHC Genetics, Odor Profiles and the Microbiome

For many bird species, olfaction is critical for basic self versus non-self discrimination (Bonadonna and Nevitt, 2004). However, individual odor signatures may be influenced by a more complex underlying genetic component. For years, it has been hypothesized that the major histocompatibility complex, or MHC, influences the individual odor profiles of many species (Charpentier et al., 2008; Wedekind et al., 1995). The major histocompatibility complex is a diverse gene cluster involved in vertebrate adaptive immunity that mediates recognition of self and foreign antigens, including host pathogens (Penn and Potts, 1998; Yamazaki et al., 1979; Zelano and Edwards, 2002). Rodents, humans, and lizards prefer the scent of individuals with dissimilar MHC genes (Leclaire et al., 2014b; Wedekind et al., 1995). In many animal species, the choosy sex prefers a

mate that is either heterozygous or harbors a dissimilar set of MHC alleles, which would confer optimal pathogen resistance to the next generation (Milinski, 2006). That choice is mediated by one or more mechanisms, including color discrimination in the three-spined stickleback fish *Gasterosteus aculeatus* (Aeschlimann et al., 2003), spur length in the Ring-necked pheasant *Phasianus colchicus* (Schantz et al., 1996), and odor in humans and mammals (Ezenwa and Williams, 2014; Theis et al., 2012; Wedekind et al., 1995).

Some animals maintain MHC diversity through disassortative mating, while others rely on alternative mechanisms. For example, some bird species diversify genetics through extra-pair fertilizations, which introduce genetic variability. The Seychelles warbler (*Acrocephalus sechellensis*) and the Scarlet rosefinch (*Carpodacus erythinus*) increase MHC diversity through extra-pair fertilizations, and highly diverse individuals tend to live longer than those with less genetic diversity (Brouwer et al., 2010; Winternitz et al., 2014). Female house sparrows (*Passer domesticus*) display no preference for MHC-dissimilar males, and instead mate with males with numerous MHC alleles when their own allele counts are low (Griggio et al., 2011). Some non-passerine species show no overt preference for MHC-dissimilar mates. For example, the Megallanic penguin *Spheniscus magellanicus* exhibits no such preference, and associations of MHC genotype and fitness in this species suggest that pathogen resistance plays a stronger role in maintaining MHC diversity than does mate choice (Knafler et al., 2012). In chickens, MHC variation strongly correlates with disease resistance, suggesting a link between MHC genes, pathogen resistance, and mate choice in natural bird populations (Zelano and Edwards, 2002). Multiple mechanisms to maintain MHC diversity exist, and appear to vary among bird orders.

While MHC genes have been implicated in influencing individual animal profiles and potentially affecting mate choice, the underlying mechanisms are not entirely clear (Leclaire et al., 2012). However, multiple hypotheses have been made to explain how the MHC could influence

odor (Boehm and Zufall, 2006; Penn and Potts, 1998). In mouse models, peptide ligands from MHC molecules alone can activate subsets of neurons found in the main olfactory epithelium of mice, suggesting a direct role of MHC-associated peptides in odor production (Boehm and Zufall, 2006). However, birds lack a vomeronasal organ (Keverne, 1999), and need to rely on production of volatile compounds that could activate the avian main olfactory epithelium. In the context of the fermentation hypothesis, bacteria could ferment MHC gene products themselves or MHC-associated peptides into volatile, odorous compounds (Penn and Potts, 1998), which would then be leveraged by the choosy sex during mate selection.

Recently, odor has been implicated as a mechanism that some bird species rely on to recognize conspecifics and potential mates on the basis of their MHC genotype. Mated pairs of Blue petrels (*Halobaena caerulea*) are more dissimilar at MHC type II loci than expected by chance, potentially attributed to MHC-mediated odor cues (Strandh et al., 2012). Black-legged kittawakes (*Rissa tridactyla*) prefer to mate with those that are genetically dissimilar, and do not rely on audible or visual cues in mate selection, implicating odor as a mechanism for mate choice. Additionally, heterozygosity correlates with odor profile in Black-legged kittawakes, and chemicals found in their uropygial gland secretions correlate positively with MHC relatedness in males and females alike (Leclaire et al., 2011). Although MHC genotype appears to have an effect on avian odor profiles, a link between the major histocompatibility complex, the avian microbiome, and mate selection has yet to be established. Only recently has an attempt been made to forge this link in a study involving Dark-eyed juncos. While individual bacterial community profiles in this study did not covary with odor profiles (Whittaker et al., 2016), the possibility of a link between individual genetics, the microbiome, and odor profiles still exists, potentially waiting to be discovered in future studies involving bird orders that are more dependent on their olfactory systems for critical life activities.

Future studies applying this conceptual framework will ultimately advance what is known about olfaction and its role in avian communication.

Concluding Remarks and Future Directions

The vast majority of microbiome studies have relied on mammalian models and only recently have examinations of the avian microbiome begun to surface. Birds represent 30% of all vertebrate species and are critical in maintaining healthy ecosystems. The few studies that have focused on avian microbiota have primarily involved terrestrial birds (Martín-Vivaldi et al., 2009; Ruiz-Rodríguez et al., 2009; Whittaker et al., 2016), and even fewer have focused on seabirds, which represent 3.5% of all avian species (BirdLife International, 2012).

Seabirds provide important ecosystem services as pioneer species that enable the process of ecological succession by dispersing seeds and nutrients to island and coastal habitats (Sekercioglu, 2006). In this context, seabirds may also serve as critical vectors for microbial biogeographical distribution, yet little is known about what factors affect the microbiomes of seabirds, including members of the order *Procellariiformes*. Given the life history characteristics of *Procellariiform* species, a variety of biotic and abiotic variables may work in conjunction to define the microbiome of these unique birds. Members of *Procellariiformes* vary greatly in size and conservation status, so it is important to examine a species that can sustain investigator disturbances. Some species within *Procellariiformes*, such as the Wandering albatross (*Diomedea exulans*), are difficult to study due to their immense size, and others, such as *Pseudobulweria* spp., are on the brink of extinction (Gangloff et al., 2012). Leach's storm-petrel is an exceptional model to study the interactions of the microbiome, the major histocompatibility complex, and odor in the context of mate selection. This seabird is small, easily handled, and is not negatively affected by investigator disturbances (Blackmer et al., 2004). Leach's storm-petrels have long-term monogamous partnerships, are long-lived, and lay only a single egg per season, and therefore would be expected to benefit from a system chemical communication

system that could impact offspring fitness. Because Leach's Storm-petrels partake in extra-pair fertilizations only very infrequently (Huntington et al., 1996), it is likely that these birds leverage an alternative mechanism, such as odor cues, to maintain genetic diversity and ultimately achieve fitness in the face of an ever-changing pathogenic landscape. However, the mechanism that drives genetic diversity in this species is not known, as is the role of bacteria in chemical signaling. Future studies may reveal a unique perspective to add to this knowledge base to further ascertain if seabird microbiota are determined by fundamentally different processes than terrestrial bird microbiota. Determinations of the impact of symbiotic bacteria on chemical communication in olfactory bird species could have an enormous impact on further understanding the link between the microbiome, its influence on chemical sensing, and mate selection.

CHAPTER II

PHYSIOLOGY AND GENETICS SHAPE THE MICROBIOME OF A SEABIRD SPECIES (*OCEANODROMA LEUCORHOA*) MORE THAN ENVIRONMENTAL AND SOCIAL FACTORS

Introduction

The microbiome provides multiple benefits to animal hosts that can profoundly impact health and behavior (Org et al., 2015; Ruiz-Rodríguez et al., 2009). Microbiomes are well-characterized in humans and animals in controlled settings, yet the relationship between symbiotic microorganisms and wild animals remains vastly understudied (Colston and Jackson, 2016). Understanding this "second genome" of wild animals is a critical step toward unravelling host-microbe co-evolutionary relationships (Amato, 2013), developmental and genomic interactions (McFall-Ngai et al., 2013), and animal behaviors, including mate choice and self-recognition (Penn and Potts, 1998; Theis et al., 2013; Tung et al., 2015). Wild animal microbiota have been examined primarily in mammalian models, and a crucial knowledge gap exists in understanding the role of the microbiome in birds. Avian species represent over 30% of all vertebrate species (The World Conservation Union, 2014). The few studies that have focused on avian microbiota involve terrestrial birds, yet very few have focused on seabirds which represent 3.5% of all avian species (BirdLife International, 2012). Seabirds provide important ecosystem services as pioneer species that enable ecological succession by dispersing seeds and nutrients to island and coastal habitats (Sekercioglu, 2006). In this context, seabirds may also serve as important vectors for microbial biogeographical distribution, yet little is known about what factors affect seabird microbiomes.

Seabird models differ from other organisms, including terrestrial birds, that have been the focus of previous microbiome studies in several distinct ways. In particular, members of the avian order *Procellariiformes* are long-lived philopatric colonial nesters that form monogamous pairs and are

primarily pelagic (Warham, 1996). They lay a single egg per breeding season that is incubated by one parent while the other forages at sea, for up to six days (Blackmer et al., 2005). Many also undertake annual trans-equatorial migrations (González-Solís et al., 2007) which may lead to greater exposure to oceanic microbial diversity than terrestrial birds. Like other seabirds, *Procellariiformes* species possess relatively large uropygial glands which produce sebum and waxy esters that, through the actions of preening, make feathers flexible and waterproof (Mardon et al., 2011). Finally, *Procellariiformes* are highly dependent on olfactory communication and possess large olfactory bulbs compared to other bird orders (Bang and Cobb, 1968). It is thought that the odors they detect and produce may result from individual-specific microorganisms, thus allowing them to discriminate between conspecifics and potential mates (Bonadonna and Nevitt, 2004; Penn and Potts, 1998). Given these life history characteristics, the microbiome of seabirds and particularly *Procellariiformes,* may be influenced by fundamentally different biotic and abiotic variables than terrestrial birds.

Physiological, genetic, environmental, and social factors all influence host-microbiome interactions, and likely work to shape the microbiome of seabirds as well. In humans, microbial colonization on the skin depends on topographical location and endogenous factors such as age and sex (Grice and Segre, 2011). For example, high humidity in human axillary regions promotes the growth of *Corynebacteria* spp., compared to drier arm and leg skin sites that support fewer microorganisms (Grice and Segre, 2011). In dogs, haired skin sites contain more diverse microorganisms than mucosal sites (Hoffmann et al., 2014). Avian body surfaces have diverse ecological niches as well, which likely support distinct microbial communities at different sites. The uropygial gland secretes sebum and waxy esters that birds use in preening (Montalti et al., 2005), and several studies of terrestrial birds indicate that the environment has a strong influence on the microbiota that are present at this site (Martínez-García et al., 2016; Ruiz-Rodríguez et al., 2014). Conversely, the brood patch is a highly vascularized body site, which enables temperature regulation

in eggs (Jones, 1971), and is most likely to transfer microbial communities to eggs and chicks. Given these physiological differences, the uropgyial gland and brood patch sites likely carry distinctive microbial communities, but no studies to date have examined what factors influence microbial composition across multiple surface site locations in any avian species.

One factor that demonstrably shapes the microbiome in animals is host genetics (Org et al., 2015). Sex is one of the most important genetic differences, and has been shown to impact bacterial communities in humans (Fierer et al., 2008), mammals (Leclaire et al., 2014a; Theis et al., 2013), and birds (Saag et al., 2011). Genetic relatedness can also yield more similar microbiomes. For example, humans in the same family share more gut-associated microbial communities than non-relatives (Spor et al., 2011), and human gut microbiota are more similar between monozygotic twins than dizygotic twins (Goodrich et al., 2014). In mice, multiple genetic markers strongly predict the composition of intestinal microbial communities (Benson et al., 2010). Finally, immunological genetic variation and its effect on the microbiome is an understudied area in wild animals, but may be the most important factor in determining microbiome individuality (Milinski, 2006). For example, polymorphisms of the major histocompatibility complex (MHC) genes influence gut-associated microbiota in three-spined stickleback fish (*Gasterosteus aculeatus*) (Bolnick et al., 2014) and congenic laboratory mice (Kubinak et al., 2015; Toivanen et al., 2001). In birds, MHC genotype can be influenced either by disassortative mating or by pathogen pressure (Dearborn et al., 2015; Knafler et al., 2012), but no studies to date have investigated which of these factors affect avian microbiomes.

While individual variation plays a role in shaping host microbiota, environmental factors can also greatly impact bacterial communities. For example, goat intestinal microbiota inoculated into bedding quickly colonize the intestinal tracts of inbred mice (Bai et al., 2016). Several studies of genetically similar terrestrial birds indicate that the cloacal microbiomes are strongly impacted by nest membership (Lucas and Heeb, 2005; Whittaker et al., 2016). Additionally, microbial

communities found on nest material contribute to the composition of the eggshell microbiome in hoopoes (*Upupa epops*) (Martínez-García et al., 2016) and Reed warblers (*Phragmites australis*) (Brandl et al., 2014), signifying a strong influence of environmental contact on bird-associated microbiota. The environment can also include social interactions with other members of the population. In wild baboons, bacterial communities are transferred among social group members through direct physical contact, and the effect of social network remains strong even after controlling for genetic relatedness and habitat (Tung et al., 2015). In mice, physical interactions are responsible for the transfer of gut microbiota between individuals (Turnbaugh et al., 2009). Within some terrestrial bird species, cloacal bacteria are transferred by allopreening (Kulkarni and Heeb, 2007) and gut-associated microbiota are more similar between mated pairs (Kreisinger et al., 2015). External host-associated communities can also be influenced by the social interactions. For example, genetically similar hoopoes reared in the same nest shared more uropygial gland bacteria than those reared in separate nests (Ruiz-Rodríguez et al., 2014), further demonstrating that social interactions can impact skin-associated microflora. However, seabirds, including members of *Procellariiformes*, have vastly different life history characteristics than the terrestrial birds in which these studies have been conducted, and their microbiomes may be influenced by other environmental determinants.

In this study, the physiological, genetic, environmental and social factors that shape the microbiome of a pelagic seabird, Leach's storm-petrel (*Oceanodroma leucorhoa* or hereafter LESPs), were examined. This wild species is within the order *Procellariiformes*, and is an excellent model for microbiome studies. The birds are small and easily handled (Hedd and Montevecchi, 2006), are long-lived (Blackmer et al., 2004), do not participate in extrapair copulation (Dearborn et al., 2015), nest in dense clusters of burrows (Lormee et al., 2012), and return to the same site each breeding season if reproductively successful the previous season (O'Dwyer et al., 2008). Finally, LESPs are characteristic of other *Procellariiformes* in that they rely on olfactory cues for behaviors such as

foraging, burrow relocation, and recognition of individuals and conspecifics (Nevitt, 2008), and these may be mediated by individually-specific microbiota (Penn and Potts, 1998). In this study, four specific hypotheses were tested with respect to the microbiome of LESPs: 1) Uropygial gland and brood patch sites will be colonized by significantly different bacterial communities, 2) Genetic diversity, with respect to sex and MHC zygosity, will influence the composition of bacterial communities at these sites, 3) Birds will share more bacterial communities with their own home burrow than with a random burrow, and 4) Mated pairs of birds will share more bacterial communities with each other than with a randomly-selected non-mate. This is the first study to provide a comprehensive examination of multiple factors that shape the microbiome of a *Procellariiformes* bird species.

Methods

Sample Collection

All samples were obtained from an established study colony of LESPs (*Oceanodroma leucorhoa*) located on Bon Portage Island, Nova Scotia, Canada, July 18-20, 2013, after females had laid their eggs. For this study, sampling was focused at a bird colony location on the southeast side of the island (43.460082, -65.743648). Samples were collected from the northeast and southwest sections of this colony. Experienced bird handlers removed individual birds from their ground burrows and held the birds in place while a research assistant collected swab samples. The external surfaces of uropygial glands and brood patches of 8 male and 13 female birds were swabbed for 30 seconds with sterile cotton swabs (Medline Part#MDS202000). The cotton tip was aseptically broken into a 1.2 ml microfuge tube containing 1 ml of sterile phosphate buffered saline. Swab samples were immediately placed on ice until the end of the field day. Within 8 hours of collection, samples were vortexed at high speed for 30 seconds and centrifuged at 13,000 RPM for 30 minutes in a microcentrifuge (Eppendorf 5452), and then frozen at -20^{o}C. Swab samples were kept frozen during

transportation to Western Michigan University and were stored at -80° C until DNA extraction could be conducted. While the bird was in-hand, blood samples were also collected to determine sex and DAB1/DAB2 genotype (MHC type I and II loci) and morphological measurements including bird mass, tarsal length, and wing chord length were recorded.

While the bird was held out of each burrow, soil samples were collected using sterilized 1 cm corers from three locations: deep within the burrow, the entrance of the burrow, and 1 foot away from the burrow entrance (hereafter within burrow, at entrance, and outside burrow, respectively). The time of collection, soil and air temperature outside the burrow and soil and air temperature inside the burrow were also collected at this time. Forty g of soil was collected from each of 26 burrows, 7 of which were unoccupied, and subsampled into two Ziploc bags containing 10 g and 30 g of soil each. All soil samples were placed on ice in the field. Ten g samples were frozen within 8 hours of collection, kept frozen during transport to Western Michigan University and placed in a -80° C freezer for storage until DNA extraction could be conducted. Thirty g samples were kept on ice during transport, and were used to measure soil abiotic characteristics within 5 days of collection.

Once the swabs were obtained from the bird occupying the burrow, soil samples were collected, and the bird was placed back in the burrow. The sampling team then placed a "lattice" of small twigs in front of a burrow that had been sampled. Researchers returned on subsequent days to see if the lattice had been knocked down, indicating that the sampled bird had left the burrow and its mate had returned. In these instances, the mate was also removed from the burrow, identified by band number, and physiological, blood and swab samples were collected. In total, 2 swabs were collected from 8 individual males and 13 individual females, from which 5 male/female mated pairs were identified. Finally, 3 soil samples from each of 26 burrows were taken to complete the dataset.

Soil Characteristics

For each soil sample collected, pH, percent soil moisture, nitrate (NO_3^-) and ammonium (NH_4^+) concentrations were measured on unfrozen soils. Large plant roots were removed from all soils prior to taking any measurements. pH was measured by mixing 5 g of field fresh soil with 10 mL of distilled deionized (DDI) water with a stir bar, and recording stable pH using a laboratory meter (Fisher Accumet). Percent soil moisture was determined by placing 10 g of field fresh soil into an aluminum tin and determining the change in mass of soil before and after drying at 65° C for 1 week. To measure NO_3^- and NH_4^+ concentrations, 10 g of fresh soil were shaken at 150 rpm in acid-washed centrifuge tubes with 50 mL of 2M KCl for 1 h, then centrifuged at 3400 rpm for 5 minutes and finally filtered through a GF/F filter (Whatman). NO_3^- and NH_4^+ concentrations were measured from extracts using the 96-well plate protocols described by Rhine et al 1998.

Genomic DNA Purification

Samples were thawed on ice on the day of DNA extraction. DNA was purified using the PureLink Genomic DNA Mini Kit (Life Technologies, Grand Island, New York) following the manufacturer's instructions for processing gram positive bacteria. DNA extracted from swabs was eluted in a final volume of 25 μL of solution elution buffer. DNA from soil microbial communities was purified using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, California) following the manufacturer's instructions. DNA extracted from soil was eluted in a final volume of 75 μL of elution buffer. Two blank extractions were conducted using each kit to control for contaminant DNA associated with the extractions. DNA concentrations were determined using both a Qubit dsDNA HS assay kit (Life Technologies Q32854) and a Qubit 2.0 quantitation system. DNA concentrations ranged from <0.5 – 2,890 ng mL^{-1} for swab extracts and 1.57 x 10^3-1.8 x 10^4 ng mL^{-1} for soil extracts. All DNA extracts were stored at -80° C prior to library preparation and sequencing.

Sequence Processing

Amplicon preparation and Mi-Seq (Illumina, San Diego, CA) sequencing was conducted at Michigan State University Genomics Core Facility. Bacterial 16S rRNA genes were PCR amplified using primers specific for the V4 hypervariable region (Kozich et al., 2013). A subset of PCR products was analyzed on a 1% agarose gel stained with ethidium bromide to ensure that samples contained sufficient DNA for amplification procedures. DNA libraries were normalized using the SequalPrep Normalization Plate Kit, 96-well (Thermo Fisher Scientific, Waltham, MA), and samples from each replicate plate were pooled into single wells. Pooled samples were quantified using a Kapa Biosystems qPCR kit (Kapa Biosystems, Inc., Wilmington, MA), and samples were normalized to an equal concentration. Each sample pool was loaded on an Illumina Mi-Seq flow cell v2 and sequenced using a 500 cycle (PE250) reagent kit. Bases were called using Real Time Analysis (RTA) software v1.18.54, and RTA output was demultiplexed and converted to fastq files using Illumina Bc12Fastq v1.8.4.

Steps for primer sequence removal, quality filtering and merging forward and reverse reads were performed using PANDAseq version 2.8 (Masella et al., 2012). Sequences were excluded from analysis if they contained ambiguous base calls, runs of greater than eight identical bases, quality scores of less than 0.9 in a sliding scale of 0 to 1, fewer than 247 bases, more than 275 bases, or sequence overlap of less than 47 bases. After these steps a total of 8,176,816 high-quality reads remained in the dataset. 518,815 chimeric sequences were identified and filtered with QIIME v.1.9.1 (Caporaso et al., 2010) using the USearch 6.1 algorithm (Edgar, 2010). The remaining 7,658,001 sequences were clustered into operational taxonomic units (OTUs) using the pick_open_reference_otus.py script in QIIME, which selected open-reference OTUs via the USearch 6.1 algorithm and removed singleton sequences. Taxonomy was assigned using the Ribosomal Database Project classifier (Wang et al., 2007) against the Silva version 119 reference

database (Quast et al., 2013). 1,007 OTUs were identified in the blank control samples and were removed from the dataset. 300 OTUs were identified as associated with Archaea, chloroplasts and mitochondria, which were also removed from the dataset. After splitting the OTU table by sample type, the resulting swab and soil OTU tables were rarefied to 9,000 and 30,000 sequences per sample, respectively. A total of 42 swab samples and 78 soil samples were retained in the final dataset after rarefying. Rarefied datasets were used to conduct downstream comparisons within swab or soil sample types. The entire unrarefied dataset of swab and soil samples combined was used to determine OTUs shared between swab and soil samples.

Statistical Analyses

Observed OTUs were used as a measure of community richness to calculate α diversity based on the Shannon-Weaver index (Chao and Shen, 2003) using R version 3.3.0 (R Development Core Team, 2016), implemented through R Studio version 0.99.902 (RStudio Team, 2016), and vegan version 2.3-5 (Oksanen et al., 2015). Shannon diversity group differences were determined by the Wilcoxon rank sum test using the wilcox.test function. Between-sample β-diversity (n = 42 for birds) was calculated by generating unweighted and weighted UniFrac distance matrices and visualizing the data from the first two axes of a principal coordinates analysis (PCoA) using the distance and plot_ordination functions in PhyloSeq version 1.16.2 (McMurdie and Holmes, 2013). Group significance (sex, swab site, DAB2 homozygous/heterozygous, soil depth, and burrow occupancy) within multivariate community data was determined using the adonis function (Permutational MANOVA, or PERMANOVA) in vegan. Between-group comparisons of bacterial community relative abundance (female uropygial gland, female brood patch, male uropygial gland, male brood patch) were performed on appropriately transformed datasets using one-way ANOVA (Casella, 2008), and orthogonal contrasts were performed on groups of interest using the package Phia (Helios De Rosario-Martinez, 2015). OTUs responsible for between-group differences were

determined by simper (similarity percentage) analysis using the sim command (Clarke, 1993) in vegan, which included species that contributed to at least 70% of the differences between groups of interest. The envfit function in vegan was used to overlay morphological and environmental vectors on appropriate PCoA plots, and differences in percent shared OTUs between birds and burrow soil sites were determined by one-way ANOVA. For envfit analyses, samples lacking any one or more data points (e.g. wing chord, tarsus, weight, DAB2 genotype) were excluded. Distance matrices based on burrow coordinates were generated using the spDists command in the sp v1.2-3 package (Pebesma and Bivand, 2005), and mantel tests were used to determine correlations between UniFrac and burrow distance matrices using the mantel function in vegan (Legendre and Legendre, 2012). Welch's two-sample t-tests (Moser and Stevens, 1992) were used to compare percent shared OTUs between birds in pairs using the t.test function.

Results

Influence of Sex, Morphology and Genetics

Within each sex, observed OTU richness was similar between the uropygial gland and brood patch body locations. The same four phyla represented the greatest relative abundance in all swab communities (*Proteobacteria, Actinobacteria, Bacteroidetes,* and *Firmicute*s, Figure 1A).

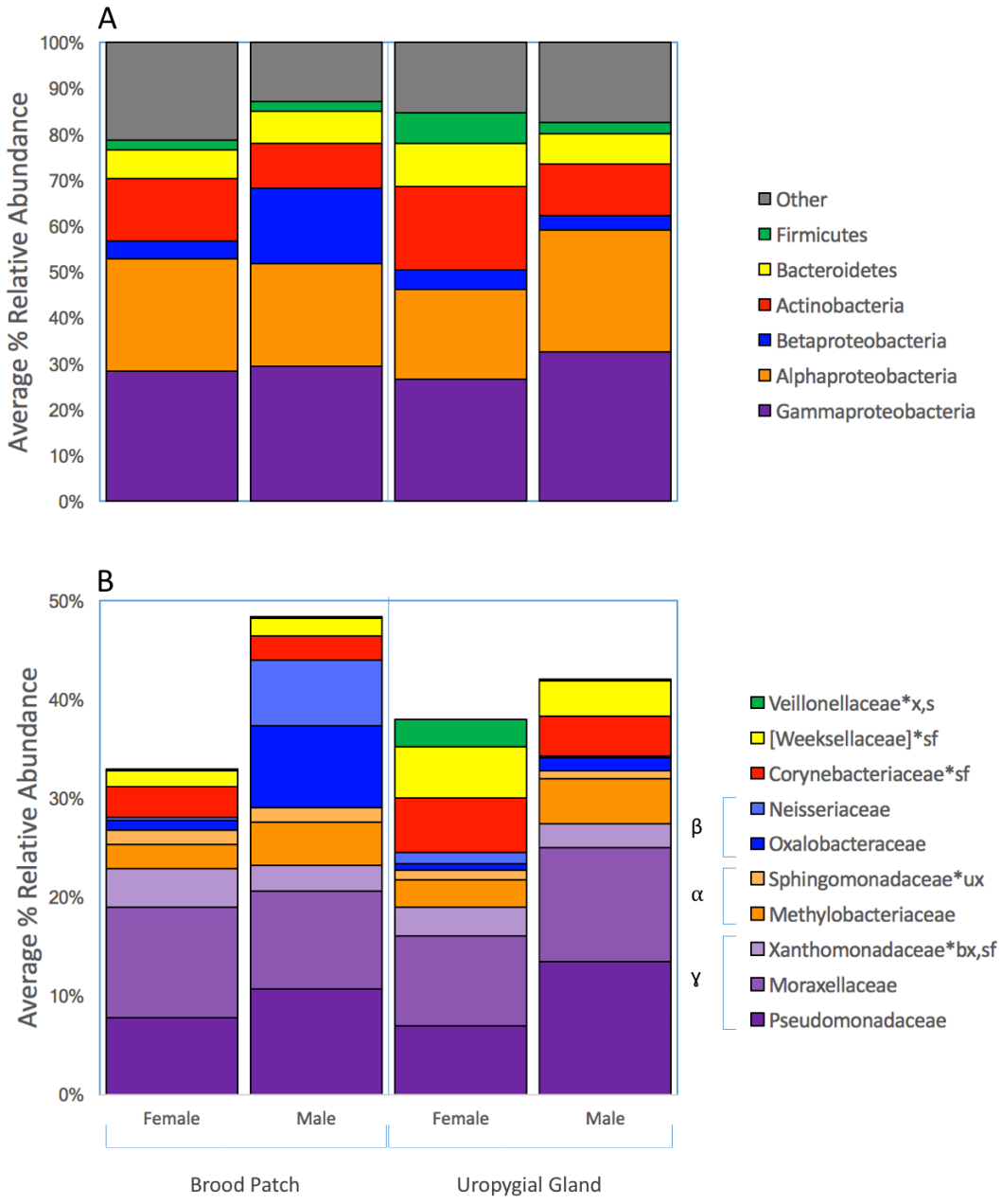

Figure 1 – Relative abundance of bird-associated bacterial communities. Communities ranked by phylum (A) and most abundant families (B). Both body sites were characterized by highly abundant *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. The relative abundance of the families *Corynebacteriaceae*, *Sphingomonadaceae*, *Veillonellaceae*, *Weeksellaceae*, and *Xanthomonadaceae* differed significantly among swab samples (marked with *), resulting in a significant overall difference between uropygial gland and brood patch communities ($p < 0.002$). Colors of the families in B correspond to the phyla represented in A. *Proteobacteria* are marked as (β) *Betaproteobacteria*, (α) *Alphaproteobacteria*, and (γ) *Gammaproteobacteria*. Community differences between groups are marked as follows: ux: uropygial gland communities differ by sex, bx: brood patch communities differ by sex, sf: female skin sites differ, x: both sexes differ by skin site, s: both skin sites differ by sex.

However, relative abundances of *Veillonellaceae, Weeksellaceae, Corynebacteriaceae, Sphingomonadaceae,* and *Xanthomonadaceae* differed significantly between body sites ($p < 0.002$, Figure 1B). Total within-sample alpha diversity including both swab locations was higher in female birds (Shannon index, 4.66 +/- 0.181) than male birds (Shannon index 4.17 +/- 0.356). This difference was driven by significantly higher diversity at female brood patches (Wilcoxon rank sum test, Shannon index, $W = 79$, $p = 0.025$) than the male brood patches, but uropygial sites had similar diversity in both sexes ($W = 72$, $p = 0.162$). Male and female birds carried structurally different microbial communities at their brood patch sites (pseudo-$F(1,19) = 1.770$, $p = 0.015$, Figure 2A) and uropygial glands (pseudo-$F(1,19) = 2.332$, $p = 0.005$, Figure 2B). Simper analysis revealed that OTUs within the families *Pseudomonadaceae*, *Moraxellaceae*, *Corynebacteriaceae*, *Methylobacteriaceae*, and *Sphingomonadaceae* were most responsible the structural differences observed in uropygial gland communities between males and females, and that bacteria within the families *Neisseriaceae*, *Pseudomonadaceae*, *Methylobacteriaceae*, *Oxalobacteraceae*, and *Moraxellaceae* drove differences between male and female brood patch communities (Table 1). However, the composition (presence/absence) of OTUs was the same between the two sexes at the two body sites (uropygial gland pseudo-$F(1,19) = 1.25$, $p = 0.07$, brood patch pseudo-$F(1,19) = 0.88$, $p = 0.81$). Because communities varied by both swab location and sex of the bird, all analyses were broken down categorically to avoid confounding results. Categories are: female brood patch swabs, female uropygial gland swabs, male brood patch swabs, and male uropygial swabs, as described in Figure 1.

Table 1
OTUs identified using SIMPER analyses

Comparison	Five most influential OTUs	Represented Family	% contribution to difference	% average abundance (Female)	% average abundance (Male)
Female vs. Male, Uropygial Gland	KC358339.1.1270	*Pseudomonadaceae*	3.1	4.82 ± 0.011	8.37 ± 0.015
	FJ612285.1.1489	*Moraxellaceae*	2.4	1.99 ± 0.018	5.20 ± 0.025
	CP001809.1856259.1857766	*Corynebacteriaceae*	2.1	5.04 ± 0.008	4.04 ± 0.011
	JF222412.1.1310	*Methylobacteriaceae*	1.7	2.48 ± 0.009	4.06 ± 0.013
	FJ891018.1.1343	*Sphingomonadaceae*	1.7	2.14 ± 0.005	4.69 ± 0.007
Female vs. Male, Brood Patch	JQ191134.1.1362	*Neisseriaceae*	3.2	0.16 ± 0.039	6.23 ± 0.056
	KC358339.1.1270	*Pseudomonadaceae*	2.7	4.79 ± 0.011	4.94 ± 0.016
	JF222412.1.1310	*Methylobacteriaceae*	2.0	1.97 ± 0.014	3.97 ± 0.020
	JQ316675.1.1495	*Oxalobacteraceae*	1.9	0.06 ± 0.020	3.85 ± 0.028
	FJ612285.1.1489	*Moraxellaceae*	1.8	2.43 ± 0.010	3.34 ± 0.013

In this bird population, several morphological parameters varied by sex (female mass (g), *M* = 48.51 g, *SD* = 4.37, male mass (g), *M* = 50.47, *SD* = 3.88; female tarsus (mm), *M* = 24.42, *SD* = 0.77, male tarsus (mm), *M* = 24.58, *SD* = 0.73; female wing chord (mm), *M* = 162.72, *SD* = 2.93, male wing chord (mm), *M* = 159.59, *SD* = 3.12). Female birds had longer wing chords than males (t = -2.657, p = 0.02), but did not differ by mass (t = -1.138, p = 0.268). Wing chord length explained 32.3% of the variation in brood patch bacterial community structure (R^2 = 0.323, p = 0.018, n = 19), indicating that birds with longer wing chords carried more similar microbiota (Figure 2B). After separating samples by sex, wing chord length explained 53.2% of the variation in female brood patch community structure (R^2 = 0.532, p = 0.05, n = 12), but did not explain variation in male brood patch communities (R^2 = 0.211, p = 0.783, n = 5). No other morphological parameters measured explained a significant portion of the variation in brood patch communities in either males or females.

While wing chord length explained significant variation in the female petrel brood patch microbiome, genetic factors explained variation at the male uropygial site (Figure 2B).

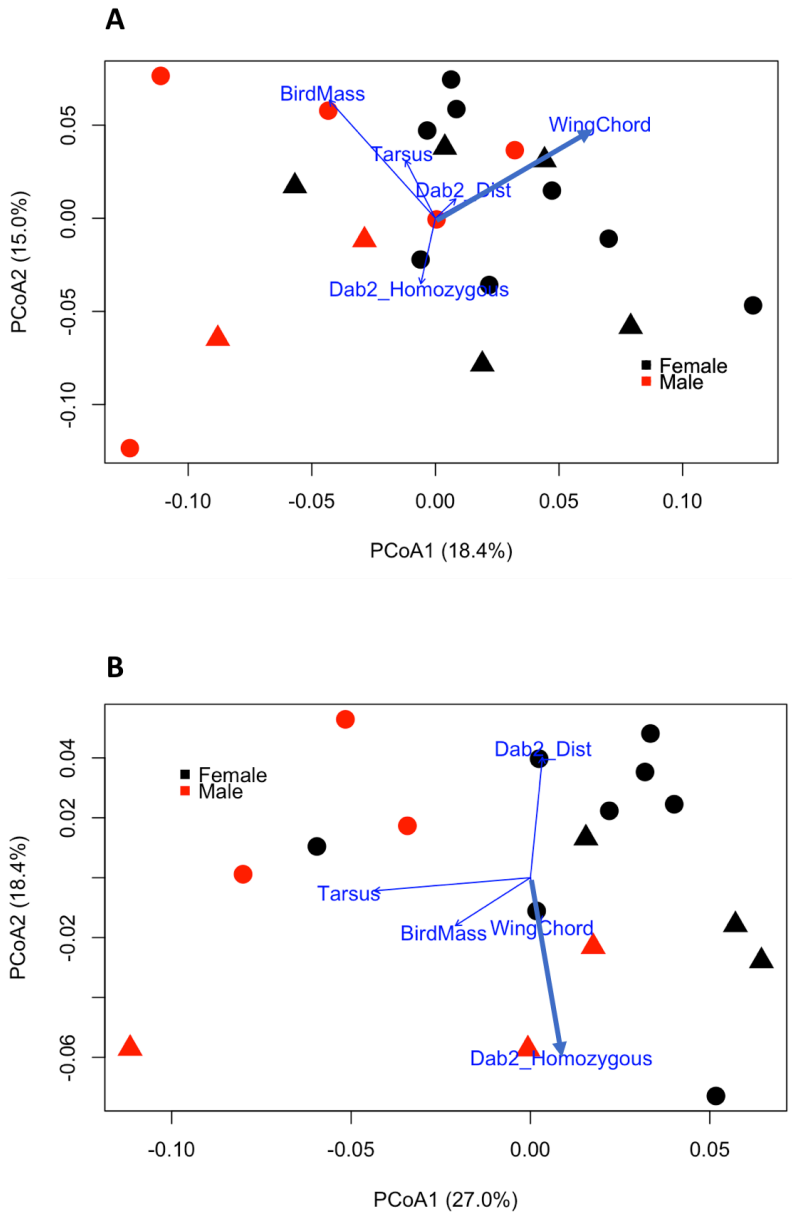

Figure 2- PCoA of brood patch (A) and uropygial gland (B) bacterial communities based on weighted UniFrac dissimilarity metric. Colors represent female (black) and male (red) birds. Triangles are DAB2 homozygous and circles are DAB2 heterozygous. Female and male bacterial communities were significantly different at both swab sites (uropygial gland, pseudo-F(1,19) = 2.768, p = 0.004, n = 21; brood patch, pseudo-F(1,19) = 1.818, p = 0.009, n = 21). Morphological and genetic factors are represented by arrows, and the length of each arrow is proportional to the explanatory power of each variable. In addition to sex, wing chord partially explained differences in brood patch bacterial community structure (R^2 = 0.323, p = 0.018). DAB2 homozygosity explained a marginally significant amount of variation in uropygial gland community structure (R^2 = 0.363, p = 0.046).

In LESPs, the DAB2 gene expresses an MHC Class II antigen which aids in immune system function. Bacterial community structure at the uropygial gland differed between males that were homozygous and heterozygous at this gene locus (weighted UniFrac pseudo-$F(1,6) = 1.859$, $p = 0.015$, $n = 8$). However, in females, allele identity at DAB2 did not influence bacterial community structure at the uropygial gland. Additionally, DAB2 zygosity did not explain any variation in bacterial community structure at the brood patch location in either sex ($p > 0.319$, Figure 2A). Finally, uropygial gland and brood patch bacterial communities were similar among all birds regardless of maximum amino acid distance between loci at DAB2 ($p > 0.378$). While relatedness did not influence the microbiota carried by these birds, MHC genetics affected bacterial community structure in a sex-specific manner.

Environmental Factors: Influence of Home Burrow

Although individual factors explained important variations in the LESP microbiome, this species builds and inhabits an underground burrow, where pairs individually nest, and may acquire microbiota from the environment. Soil samples were collected from three locations of each petrel burrow. Bacterial communities collected from within, at entrance and outside burrows did not differ between those that were occupied by a bird and those that were currently unoccupied during the sampling season ($p = 0.262$, $n = 26$, Figure 3).

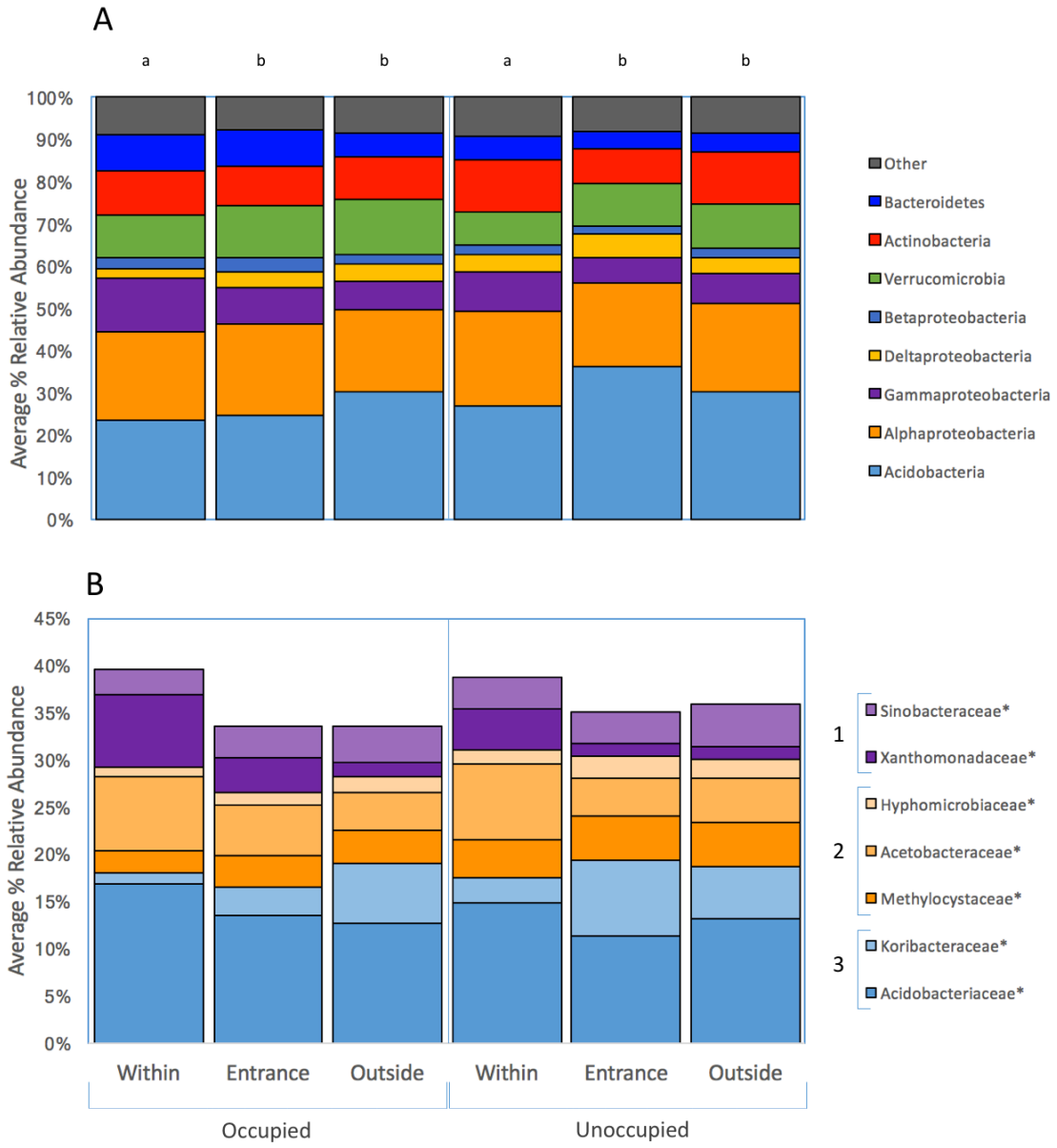

Figure 3- Relative abundance of soil bacterial communities. All OTUs ranked by phylum (A) and family (B) among occupied and unoccupied within burrow, at entrance, and outside burrow. Burrow occupancy had no effect on bacterial community composition or structure and did not interact with burrow soil site, but burrow communities were significantly different based on burrow soil site (pseudo-$F(2,75) = 2.796$, $p < 0.001$, $n = 75$). Families that differed significantly among within, entrance, and outside burrow soil are marked with an asterisk (*). Phyla or class levels in (B) are represented as 1) *Gammaproteobacteria*, 2) *Alphaproteobacteria*, and 3) *Acidobacteria*.

Additionally, soil pH, NH_4^+ and moisture measurements were similar between occupied and unoccupied burrows at each of the three burrow soil sites (Figures 4A-4C).

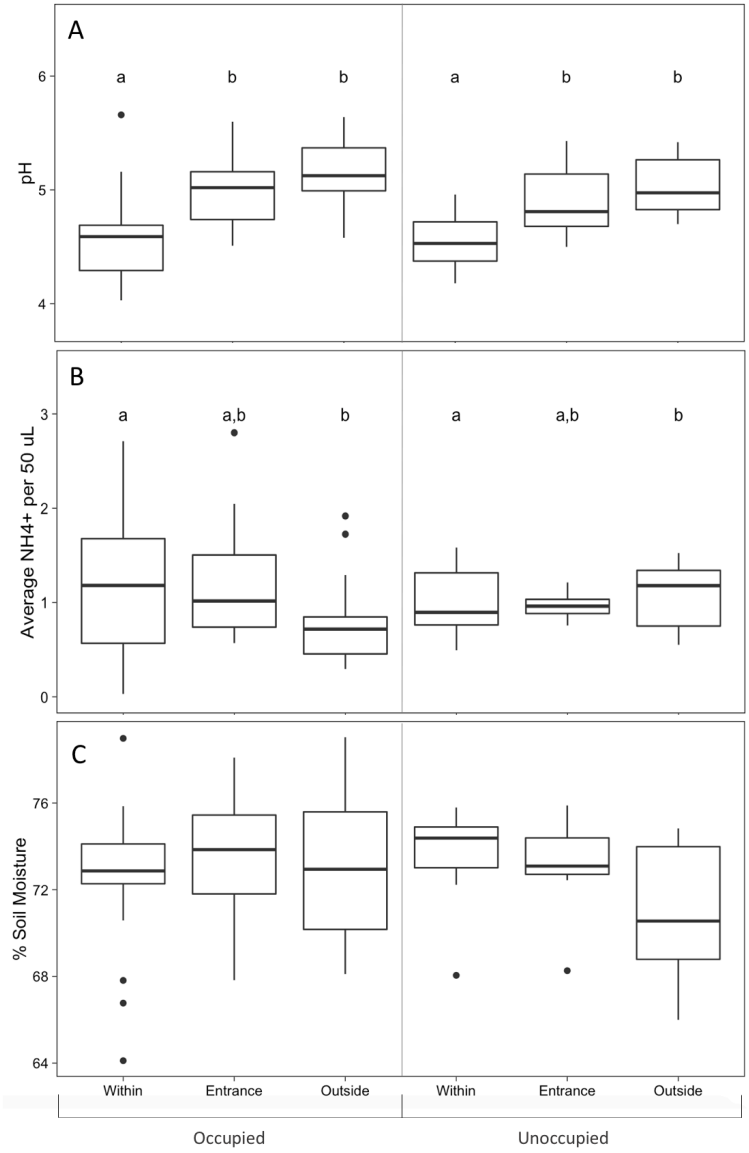

Figure 4- Abiotic soil characteristics. Soil pH (A), NH_4^+ (B), and moisture (C) between occupied and unoccupied burrows within the burrow, at the entrance, and outside the burrow represented by bar and whisker plots. Boxes represent upper and lower quartiles, whiskers depict maximum and minimum values, and points are outliers. Horizontal bars within each box represent the median. Soil pH was significantly lower within the burrow ($F = 19.120$, $p < 0.001$). NH_4^+ content was significantly higher within the burrow compared to outside the burrow ($p = 0.02$). Soil moisture was similar between occupied and unoccupied burrows, and was similar at all soil locations. Burrow occupancy had no effect on soil pH ($p = 0.929$), NH_4^+ ($p = 0.469$), or soil moisture ($p = 0.541$).

Birds shared the same amount of OTUs with their home burrows as with a randomly selected non-home burrow, regardless of sex or body site location (Table 2). On average, 3.8% (± 0.8) of the OTUs found at both body sites were also observed in soil from within the burrow, where the nest is located. Birds shared more OTUs with soil from within the burrow than with soil collected at the burrow entrance or outside the burrow ($p = 0.008$, $n = 21$).

Table 2
OTUs shared between birds and their burrow environments

Comparison	Burrow Soil Site	Test Statistic t	P Value	Burrow	Mean % Shared OTUs	95% CI	*n*
Female uropygial gland	Within	-1.2905	0.2117	Home	4.46	1.98	13
				Away	4.22	1.75	13
	Entrance	-1.5503	0.1335	Home	3.06	1.10	13
				Away	3.11	1.12	13
	Outside	-1.3029	0.2051	Home	2.94	1.07	13
				Away	3.06	1.24	13
Female brood patch	Within	1.4306	0.1656	Home	5.22	1.61	13
				Away	5.17	2.19	13
	Entrance	1.5879	0.1262	Home	3.97	1.36	13
				Away	3.84	1.20	13
	Outside	1.0098	0.3219	Home	3.66	1.10	13
				Away	3.73	1.31	13
Male uropygial gland	Within	0.0446	0.9648	Home	2.56	1.07	8
				Away	2.56	1.27	8
	Entrance	-0.3348	0.7419	Home	2.11	1.14	8
				Away	2.13	1.16	8
	Outside	0.0031	0.9976	Home	1.80	0.84	8
				Away	1.80	0.88	8
Male brood patch	Within	0.3141	0.7565	Home	2.98	1.16	8
				Away	2.74	0.97	8
	Entrance	0.2613	0.7963	Home	2.14	0.89	8
				Away	2.20	0.92	8
	Outside	-0.2947	0.7710	Home	1.83	0.62	8
				Away	1.97	0.72	8

Finally, female birds shared a greater number of brood patch-associated OTUs with soil from within the burrow than male birds ($t = 2.443$, $p = 0.02$), suggesting that burrow-associated microorganisms contribute more to the female petrel microbiome than the males (Figure 5).

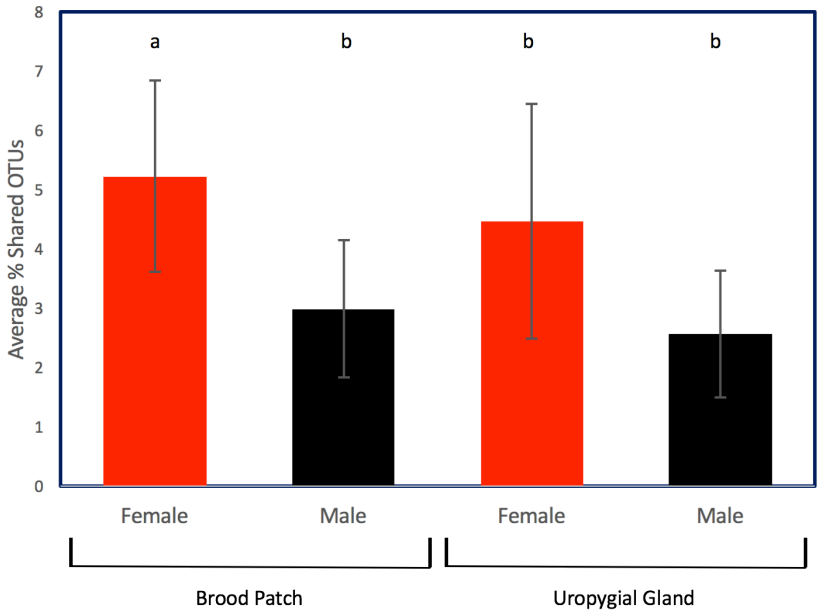

Figure 5- Average percent shared OTUs between birds and home burrow soil. Female brood patches shared significantly more OTUs with soil from within the burrow ($p = 0.02$, $n = 13$). Additionally, female brood patch bacterial community composition covaried with burrow geo-location (unweighted Unifrac, Mantel $R = 0.340$, $p = 0.006$), lending further support that burrow microbiota play a greater role in shaping female brood patch microbiomes than male microbiomes at either skin site.

The geographic location of the burrow entrance within the southeast portion of the island had an effect on both soil bacterial community structure and petrel-associated microbiota. Burrow bacterial community structure differed significantly by burrow geo-location at all depths (Mantel $R = 0.12$, $P = 0.053$), mid-burrow (Mantel $R = 0.24$, $p = 0.003$) and surface soil (Mantel $R = 0.23$, $P = 0.004$). Similarly, female brood patch community composition, which shared the most OTUs with burrow soils, also varied by burrow geo-location (unweighted Unifrac, Mantel $R = 0.340$, $p = 0.006$). Together, these results suggest that, while birds have little specific influence on their burrow

environment, burrow microbiota play a greater role in shaping female brood patch communities than for males.

Social Effects: Influence of Mate Microbiota

Sex-specific effects related to morphology, genetics and environment all play a role in explaining variation in the LESP microbiome, but it was hypothesized that social interactions between mated pairs of birds would provide limited contributions to the microbiome due to their relatively isolated lifestyle. In this population, bacterial communities collected from female birds shared the same amount of OTUs with their male burrow mates as with non-paired males ($t = -1.767$, $p = 0.100$, Table 3), regardless of body site.

Table 3
OTUs shared between birds and their burrow mates

Swab Type	Test Statistic t	P Value	Bird	Mean % Shared OTUs	95% CI	*n*
Female All Samples	- 1.767	0.100	Burrow Mate	10.57	1.03	9
			Random	12.06	1.65	9
Male All Samples	0.323	0.752	Burrow Mate	10.56	1.19	7
			Random	10.23	1.72	7
Female Uropygial Gland	- 0.916	0.403	Burrow Mate	11.13	2.12	4
			Random	12.32	3.54	4
Female Brood Patch	-1.457	0.194	Burrow Mate	10.11	1.60	5
			Random	11.85	2.88	5
Male Uropygial Gland	-0.535	0.634	Burrow Mate	11.29	3.94	3
			Random	11.83	1.69	3
Male Brood Patch	0.713	0.500	Burrow Mate	10.11	1.60	5
			Random	9.34	2.58	5

Discussion

In this study, 16S rRNA amplicon data were used to explore the factors that shape the microbiome of LESPs. While several terrestrial bird microbiomes have been characterized, this is the first examination of the microbiome of a seabird, and the first study to include the avian brood patch as a site of investigation. The main result of this study was that bacterial communities were body site and sex specific, with male and female brood patches and uropygial glands harboring different bacterial communities from each other. Feathers and skin are particularly important sites for investigation because they are the first barrier between an animal body and the environment. Therefore, interactions between birds, their conspecifics and their nest environments are likely to influence the composition of the microbiome (Kulkarni and Heeb, 2007; Whittaker et al., 2016). While defining "healthy core microbiota" across body sites has been demonstrated in human microbiome studies (Caporaso et al., 2011; Ursell et al., 2012), little information is available about what defines the healthy microbiome in wild animal populations, including wild birds. This phenomenon may play as crucial a role in the next generation of wildlife disease protection, as it will for human health (Amato, 2013; McFall-Ngai et al., 2013). Wild bird microbiome monitoring could, for example, unveil periodic increases in bird-specific pathogens, such as feather-destroying *Bacillis licheniformis*. However, the skin microbiome of birds has been under-evaluated, and is supported by only a handful of studies that examined differences in microbial communities across body sites (Nawrot et al., 2009; Roggenbuck et al., 2015; Whittaker et al., 2016).

Petrel Microbiota Differ by Body Site and Sex

Core taxa colonizing the uropygial gland and brood patch sites of Leach's storm petrels belonged to the phyla *Proteobacteria, Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. At this taxonomic level, these results are similar to previous studies investigating the microbiomes other birds, including Brown-headed cowbirds (*Molothrus ater*, Hird et al., 2014) and Dark-eyed Juncos (*Junco hyemalis*,

Whittaker et al., 2016). However, when examined at a higher level of resolution, uropygial gland and brood patch bacterial communities were very different. These two body sites are physiologically and topographically different, which leads to differences in bacterial community structure in animals (e.g. Grice and Segre, 2011). In humans, sebum production, pH, and humidity differ among skin sites in humans and support different bacterial community subsets, typically supporting more potentially pathogenic taxa, such as *Staphylococcus*, at dry body sites (Grice and Segre, 2011). Additionally, human skin sites with greater sweat and sebum production recover from microbial invasion more quickly than sites with fewer glands (Harder and Schröder, 2005). Body site microbial topography has also been shown to influence microbiomes in other animals. In Rainbow trout (*Oncorhynchus mykiss*), there are marked differences between skin and gill bacterial communities (Lowrey et al., 2015). In dogs, haired skin sites support higher bacterial diversity and richness than mucosal skin sites (Hoffmann et al., 2014). In birds, the waxy, sebaceous microenvironment of the uropygial gland and the seasonally bare and warm environment of the brood patch (Stettenheim, 2000) provide fundamentally different ecological niches for bird-associated microbiota. The uropygial gland secretes lipids and sebum that birds spread over their plumage during preening (Bonadonna and Sanz-Aguilar, 2012; Montalti et al., 2005). In the LESPs examined in this study, *Corynebacteriaceae* were particularly abundant in the uropygial glands of both sexes, although relative abundance was higher in females. This family of bacteria is known to metabolize apocrine sweat to produce volatile organic compounds (VOCs) on human bodies to produce odor (Callewaert et al., 2013; Grice and Segre, 2011). In addition, bacteria within *Alphaproteobacteria* and *Gammaproteobacteria*, including ecologically important *Pseudomonadaceae* and *Methylobacteriaceae*, were predominant community members at the uropygial glands of both sexes, though males carried more. *Pseudomonas* are known odor producers, capable of using oils as substrates to produce VOCs (Rasmussen et al., 2016), and members of *Methylobacteriaceae,* common in the environment, are associated with human foot odor (Wood and Kelly, 2010). The presence of

these two known odor-producing bacterial families at the uropygial gland site suggests that sex-specific bacterial production of VOCs may play an important role in olfactory communication in LESPs.

Brood patch sites were significantly more diverse in females than in males. There are physiological differences between male and female brood patches, even in species where both sexes contribute to incubation activities. The brood patch is a small, hypervascularized portion of skin that comes into direct contact with the egg to regulate appropriate incubating temperatures (Bailey, 1952; Jones, 1971). In Zebra finches (*Taeniopygia guttata*), the female brood patch transfers more heat to the egg than the male brood patch (Hill et al., 2014). Male Reed warblers (*Acrocephalus scirpaceus)* increase egg temperature during incubation at a faster rate than females (Kleindorfer et al., 1995), and male Yellow-eyed penguins (*Megadyptes antipodes*) have higher brood patch temperatures than females (Massaro et al., 2006). Variation in temperature ranges between male and female brood patches is likely a large physiological contributor to microbial variation at that location. In LESPs, males and females carried the same core taxa at brood patch sites, but they varied in relative abundance. Male brood patches harbored relatively more *Pseudomonadaceae* and *Methylobacteriaceae*, while females carried more *Moraxellaceae*. It is possible that the relative abundances of these bacterial families were additionally influenced by sex-specific temperature differences at this site. While the relationship between brood patch temperatures, physiologies, and microbiota have not been investigated in any bird species, results from this study provide evidence that support this hypothesis.

Sex-specific variation in uropygial gland and brood patch bacterial communities can also be influenced by reproductive hormones. Sex differences in skin microbiota exist in other animals, including humans (Brotman et al., 2014; Jensen et al., 1981; Koren et al., 2012; Markle et al., 2013), meerkats (*Suricata suricatta*) (Leclaire et al., 2014a), spotted hyenas (*Crocuta crocuta*) (Theis et al., 2013), and Barn swallows (*Hirundo rustica*) (Kreisinger et al., 2015). Fluctuations in sex-specific reproductive

hormones and timing with respect to reproductive state can have drastic effects on bacterial community structure in vertebrates, which may also apply to birds. In meerkats, host sex differences in bacterial communities are only apparent after reaching adulthood (Leclaire et al., 2014a), and in hyenas, bacterial profiles vary with female reproductive state (Theis et al., 2013b). Hormone fluctuations, particularly decreased estradiol production in females and lower testosterone production in males, associated with post-laying reproductive state have been shown to vary drastically in other avian species (Blas et al., 2010; Ottinger and Bakst, 1995; Paster, 1991). In Black kites (*Milvus migrans*), estradiol in females and testosterone in males peak prior to egg laying and then sharply decreases during the incubation stage (Blas et al., 2010). A similar decrease in estradiol occurs post-laying in Canvasback ducks (*Aythya* valisineria) (Bluhm et al., 1983). In this study, female petrels had recently laid their eggs and most of the nests sampled contained eggs at the time of sampling, and estradiol levels in female LESPs were likely reduced compared to pre-laying levels. In experimentally inoculated mice, estradiol level correlates with *Pseudomonas aeruginosa* bacterial load (Guilbault et al., 2002). Female LESPs carried fewer *Pseudomonas* spp. than males, potentially due to a post-laying estradiol reduction. In humans, females carry more *Moraxellaceae* than men (Cundell, 2016), likely due to sex-specific differences in sebum production, which can be influenced by estradiol (Giltay and Gooren, 2000). This could explain why female LESPs harbored more *Moraxellaceae* at the brood patch than males.

A recent study demonstrated that female and male Dark-eyed juncos shared similar cloacal and uropygial microbial communities (Whittaker et al., 2016). However, those birds were sampled close to the time nestlings fledged, likely allowing hormone levels adequate time to return to post-reproductive levels, which may have altered bacterial community structure. Thus, in petrels, recent egg production may have led to greater differences in hormone levels between females and males at the time sampling was conducted than at other time points throughout their annual reproductive

cycle, which could be reflected in the microbiome. Future studies could be conducted to determine a correlation between sex-specific hormone levels and bacterial community composition in LESPs.

Sex-specific behaviors could also explain the differences between female and male bacterial community structure (Leclaire et al., 2014a). Male and female behavior differs in many bird species. The closely-related European Storm-petrel exhibits sex-specific migratory patterns (Medeiros et al., 2012) which may have implications for host exposure to the environment, food intake, and subsequent effects on microbial community structure. Female Wilson's Storm-petrels take longer trips and provide heavier meals to chicks during times of food scarcity, which can increase environmental exposure to more diverse microorganisms in females (Gladbach et al., 2009). Male Great tits invest less energy in providing food than their female counterparts, thus allowing more time for preening that could lead to differences in bacterial community structure (Saag et al., 2011). In this study, LESPs males harbored more *Oxalobacteraceae* and *Methylobacterium* spp. than females. Members of both families are commonly found in the environment, often associated with the plant phyllosphere, rhizosphere, and soil (Green et al., 2007; Kelly et al., 2014). The differential abundances of these bacterial families on male and female birds suggests that LESPs interact with the environment in a sex-specific manner which contributes to bacterial community structure variation the sexes.

MHC Genotype Influences the Microbiome in a Site- and Sex-Specific Manner

While sex-specific differences impacted bacterial community structure at multiple body sites, other genetic factors associated with MHC genotype also explained variation in bacterial communities. The results show that male petrels carrying homozygous DAB2 genotypes had significantly different uropygial gland bacterial communities than heterozygous males. Additionally, male LESPs carried more *Pseudomonadaceae* and *Methylobacteriaceae* at both skin sites than females, possibly attributed to MHC genotype. MHC genes are widely diverse and are a necessary component

for adaptive immunity, providing the host organism with defense against a wide range of pathogens (Milinski, 2006). Some animals maintain MHC diversity by mating disassortatively, while others rely on alternative mechanisms. For example, female house sparrows (*Passer domesticus*) display no preference for MHC-dissimilar males, and instead preferentially mate with males with numerous MHC alleles (Griggio et al., 2011). The Megallanic penguin *Spheniscus magellanicus*, which has a similar ecology and life history as the LESP, exhibits no disassortative mating preference, and associations of MHC genotype and fitness suggest that pathogen resistance plays a stronger role in maintaining MHC diversity than does mate choice (Knafler et al., 2012). Additionally, microbiomes have been shown to differ by MHC genotypes in some animals, including the three-spine stickleback fish (*Gasterosteus aculeatus*) and inbred laboratory mice (Bolnick et al., 2014; Toivanen et al., 2001). In LESPs, male-specific microbiota and a linked olfactory mate-selection mechanism may contribute to mate selection by females (Hoover, personal communication). Peptides bound by MHC proteins are non-volatile, while MHC-associated odors are volatile (Penn and Potts, 1998). If MHC is involved in chemical communication, then some mechanism is likely required to volatilize these peptides. Bacteria are capable of these chemical conversions, and are hypothesized to be responsible for volatilization of MHC peptides in other animals (James et al., 2013; Stevens et al., 2014). Individual bacterial and odor profiles co-vary in some animals, including the spotted hyena (*Crocuta crocuta)* (Theis et al., 2013b), but not in others, such as the Dark-eyed junco (Whittaker et al., 2016). Whether this covariation between bacterial community structure and VOC profile exists in LESPs was not the focus of this study, though the identification of several volatile-producing families of bacteria (e.g. *Pseduomonadaceae* and *Corynebacteriacae*) provides evidence to support this hypothesis.

Petrel Microbiomes Are Influenced by Sex-Dependent Burrow Interactions

Although individual-specific variation impacted bacterial community structure, LESPs spend considerable time in the burrow during egg incubation, leading to specific environmental effects of the burrow on bird-associated microbiota. However, the results demonstrate that the burrow environment did not provide a large component of the LESP microbiome, except for female-specific brood patch communities. Females shared a greater number of bacterial OTUs with their burrows than males, though LESP males typically spend more time in the burrow during egg incubation than females (Blackmer et al., 2005; Montevecchi et al., 1992). Additionally, females tend to be absent from the burrow for approximately ten days prior to egg laying (Rayner et al., 2014). However, while the two sexes had the same body mass on average, females had longer wing chord length, indicating that females had larger overall body sizes. The larger-sized body females may simply have greater overlapping body space with the burrow than the smaller males, leading to more shared OTUs with deep burrow soils. Multiple species within *Acidobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia*, including OTUs from the families *Acidobacteriaceae*, *Hyphomicrobiaceae*, candidate family *auto67_4W*, *Koribacteraceae, Sinobacteraceae,* and *Xanthomonadaceae* were the most abundant communities shared between the female brood patch and deep burrow soil. Members of the families *Acidobacteriaceae* and *Koribacteraceae* are ubiquitously found in soil (Pershina et al., 2015), and members of families *Hyphomicrobiaceae*, belonging to the highly diverse *Alphaproteobacteria*, are found in both soil and bird samples (Bao et al., 2014; Hwang and Cho, 2008). Members of the family *Xanthomonadaceae* are common environmental bacteria (Jacques et al., 2016), and some members are known to be plant pathogens (Mhedbi-Hajri et al., 2011). In particular, denitrifying *Rhodanobacter* spp. bacteria within *Xanthomonadaceae*, were more abundant in deep burrow soils, related to higher levels of NH_4^+ found at this burrow location in both occupied and

unoccupied burrows. Similar bacterial communities were shared between the male brood patch and the burrow soil environment as were observed in females, though in significantly smaller quantities.

Contrary to studies that associated humans to their home environments, LESPs did not have a microbial impact on their burrows. Bacterial communities found in human indoor environments overlap with their inhabitants by as much as seventy-five percent, a stark contrast to what was observed in this study, suggesting that the human skin microbiome is much more likely to resemble the immediate environment of its host (Lax et al., 2014). Burrow-nesting birds, like LESPs, keep their habitats clean and free of excrement (Huntington et al., 1996; Soler et al., 2008), resulting in little effect on burrow soil nutrient status, as seen in the results comparing occupied and unoccupied burrows. Additionally, the antimicrobial effects of preen oil can create a barrier between birds and their burrow environments. For example, the oily secretions produced by the uropygial glands of female European hoopoes (*Upupa epops*) prevent pathogenic bacteria from colonizing feathers and egg shells (Martínez-García et al., 2015) and inhibit the growth of several feather-degrading bacteria in wild house-finches (*Carpodacus mexicanus*) (Shawkey et al., 2003). In addition to its physiological functions, preen oil in seabirds specifically may act as a barrier to microbial invasion from environmental sources to the microbiome.

The lack of an environmental effect on LESP-associated microbiota is contrary to findings in other studies investigating microbiomes of terrestrial birds, and provides a unique insight into the differing deterministic factors that shape the microbiome between *Passeriformes* and *Procellariformes* avian orders. For example, bacterial assemblages of Dark-eyed juncos are highly influenced by nest communities (Whittaker et al., 2016), and the cloacal microbiota of Great tit nestlings raised in the same nest are more similar than those reared in separate nests (Lucas and Heeb, 2005). Conversely, LESP pairs take individual trips out to sea during egg incubation that can last up to six days, at

which time their bacterial communities could become homogenized by air and waterborne microorganisms, strengthening internally-driven sex-specific effects on the microbiome.

Petrel Microbiomes Are Not Influenced by Social Interactions

The birds in this study did not share a high percentage of bacterial communities with their burrow environments, yet it was still possible that they shared bacterial communities with one another in a social context. Socially monogamous pairs of LESPs share a common burrow environment, lending support to the idea that birds in a mated pair would be expected share bacterial communities. However, in this study, LESPs shared the same amount of OTUs with their burrow mates as with randomly chosen non-mates. This result contradicts findings in several studies that have shown a strong effect of social interactions in other bird species. For example, heterospecific Great tits raised in the same nest had more similar cloacal microbiomes than biological siblings reared in separate nests (Lucas and Heeb, 2005), and Dark-eyed junco nestlings had more cloacal microbial communities in common with their mothers than with their fathers due to frequency of physical contact (Whittaker et al., 2016). There are several possible reasons for these disparate observations. First, in contrast to many other bird species, Leach's storm petrel mates rarely occupy the burrow at the same time. While one bird remains in the burrow with the egg, its mate spends several days away foraging for food at sea (Huntington et al., 1996). As a result, few bacteria may be shared between the two birds because they have limited physical contact. Second, female petrels had more OTUs in common with other females, and male petrels had more in common with other males than either sex had with its mate. Most studies evaluating the composition of microbiomes shared between animal hosts rely on examinations of gut microbiota (Kreisinger et al., 2015; Tung et al., 2015) It is possible that LESP pairs share similar gut microbiota, which can be evaluated in future studies. This suggests that sex, and other factors determined by sex,

such as hormone levels and body size, play more of a role in determining the petrel microbiome than contact with its mate.

Conclusions

This investigation is the first study to provide information about the factors that influence the microbiome of a migratory seabird. The results demonstrate that sex and body site play the most important roles in defining the LESP microbiome. MHC genotype and burrow environment were important only as sex-specific effects, and mate interaction did not influence LESP microbiota. Lack of effect of environmental and social factors in these birds is likely indicative of their lifestyle, as these effects have demonstrable importance in shaping the microbiome of other terrestrial birds. As LESPs spend little time roosting together in a burrow and travel over 1000 km per trip to forage for food (Pollet et al., 2014), individual bird microbiota are much more driven by individualistic factors than other bird species. Examinations of wildlife-associated microbiota are important for understanding animal health, preservation, and behavior. The results of this study add a unique perspective to this knowledge base, demonstrating that seabird microbiota are determined by fundamentally different processes than terrestrial bird microbiota. Future studies that determine the impact of symbiotic bacteria on chemical communication in olfactory bird species could have an enormous impact on further understanding the link between the microbiome, its influence on chemical sensing, and mate selection.

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APPENDIX

IACUC Approval Letter

UNIVERSITY OF CALIFORNIA, DAVIS

BERKELEY · DAVIS · IRVINE · LOS ANGELES · MERCED · RIVERSIDE · SAN DIEGO · SAN FRANCISCO

SANTA BARBARA · SANTA CRUZ

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Davis, CA 95616-8545
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April 14, 2015

To Whom it May Concern:

The following application was reviewed and approved by the UC Davis IACUC on April 09, 2015. Active protocols are reviewed annually.

Title: **Collaborative Proposal: A New Model For Chemical Ecology: Integrating Chemistry, Genetics and Behavior to Understand the Role of Individual Scent in a Colonial Nesting Seabird.**
Principal Investigator: **Gabrielle A. Nevitt**
Protocol #: **17588**
Institution: **University of California, Davis**

This institution is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW). The Assurance Number is A3433-01.

The IACUC is constituted in accordance with U.S. Public Health Service (PHS) Animal Welfare Policy and includes a member of the public and a non-scientist.

Alan Ekstrand
IACUC Administrator
IACUC Office
University of California, Davis