The causes and consequences of variation in the cloacal microbiome of tree swallows (*Tachycineta bicolor*)

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ACADEMIC ABSTRACT

Animals are ecological landscapes that host communities of microbes often referred to as microbiomes. These microbes can be transferred between individuals when they come into contact, such as during mating. Microbes that reside in or on any aspect of a host that becomes exposed to the reproductive tract or gametes comprise the reproductive microbiome. These microbes within the reproductive microbiome are important to overall host biology because they can influence host reproductive function, and thus play a role in shaping host ecology, evolution, and fitness. Though previous work has revealed much about the impact of beneficial and pathogenic microbes within the reproductive tract, much is left to be learned from describing the dynamic nature of the reproductive microbiome, and ultimately, how it affects host fitness. For my dissertation, I asked questions regarding how and why reproductive microbiome diversity varies among individuals. For instance, does reproductive microbiome diversity vary with respect to the number of mates or mating activity? Does reproductive microbiome diversity vary with host age or breeding stage? Are there fitness consequences associated with differences in reproductive microbiome diversity? To explore these questions, I studied tree swallows (*Tachycineta bicolor*), a socially monogamous bird in which both females and males engage in extra-pair mating activity. I focused on the cloacal microbiome as it is the site of contact during mating, and thus where microbes can be sexually transferred between individuals. I found that social partners did not have more similar cloacal microbiome diversity compared to other individuals in the same population, and that cloacal microbiome diversity was similar between sexes (Chapter II). By combining an observational approach with a hormone implant manipulation, I found that neither the number of sires per brood nor the increased mating activity of females significantly influenced cloacal microbiome richness or community structure. However, female age and breeding stage did significantly correlate with cloacal microbiome richness and community structure (Chapter III). Based on these findings, I hypothesized that the effect of mating activity on variation in the cloacal microbiome may only be detectable over a female's lifetime, and not within a single breeding season. In addition, I found evidence for a relationship between lay date and cloacal microbiome structure, after controlling for age. And I found that older females lay earlier in the season compared to younger, first-time breeding females (Chapter IV). These results provide support for a relationship between lay date and the cloacal microbiome and highlight the importance of age to this relationship. Lastly, I discussed future steps that can be taken to extend the framework established by my dissertation research, and thereby gain further insight into the factors shaping the reproductive microbiome (Chapter V).

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GENERAL AUDIENCE ABSTRACT

Animals host communities of microbes often referred to as microbiomes, and these microbes can be transferred between individuals when they come into contact, such as during mating. Microbes that reside in or on any part of a host that becomes exposed to the reproductive tract make up the reproductive microbiome. These microbes within the reproductive microbiome are important to an animal's biology because they can influence reproductive function, such as fertilization and pregnancy success. Though previous work has revealed much about the impact of beneficial and pathogenic microbes within the reproductive tract, much is left to be learned from describing the dynamic nature of the reproductive microbiome as a whole and how it affects an animal's reproductive success. For my dissertation, I sought to understand how and why reproductive microbiome diversity varies among individuals, especially in relation to mating. To explore these questions, I studied tree swallows (*Tachycineta bicolor*), a species of bird in which females and males will pair up to breed and rear young, yet both may mate with individuals other than their partner. I focused on the microbiome within the cloaca of birds as it is where contact is made during mating, and thus where microbes can be sexually transferred between individuals. I found that social partners did not have more similar cloacal microbiome diversity compared to other individuals in the same population, and that cloacal microbiome diversity was similar between females and males (Chapter II). Since tree swallows frequently mate with multiple partners and it is possible for bacteria to be acquired though each mating attempt, I proposed that it is important to consider the number of mates per individual when assessing the diversity of the cloacal microbiome. I then performed observational and experimental studies where I assessed the number of mates per female and manipulated female sexual activity with hormone implants. I found that neither the number of sires per brood nor the increased mating activity of females influenced cloacal microbiome diversity, however, female age and breeding stage were significantly associated with cloacal microbiome diversity (Chapter III). Based on these findings, I hypothesized that the effect of mating activity on variation in the cloacal microbiome may only be detectable over a female's lifetime, and not within a single breeding season. In addition, I found evidence for a relationship between lay date and cloacal microbiome structure, after controlling for age. And I found that older females lay earlier in the season compared to younger, first-time breeding females (Chapter IV). Taken together, these results provide support for a relationship between lay date and the cloacal microbiome and highlight the importance of age to this relationship. Lastly, I discussed future steps that can be taken to extend the framework established by my dissertation research and gain further insight into factors shaping the reproductive microbiome (Chapter V).

Dedication

To my parents
&
a four-year old Jess who wished she could go to school forever.

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CHAPTER I: Introduction

Variation in extra-pair sexual activity

Our understanding of animal behavior, mate choice, and ultimately, the evolution of mating systems completely changed in the 1980s when molecular techniques revealed high rates of extra-pair mating in populations of species previously thought to be monogamous. Since then, it has become clear that socially monogamous species that engage in extra-pair sexual activity (e.g., solicitations, copulations, and fertilizations) are in fact common throughout the animal kingdom. While evidence of extra-pair sexual activity (i.e., extra-pair offspring) in socially monogamous species has been documented in insects (e.g., Dillard 2017), fish (e.g., Avise et al. 2002), amphibians (e.g., Liebgold et al. 2006), reptiles (e.g., Uller & Olsson 2008), and mammals (e.g., Cohas & Allainé 2009), birds are arguably the best studied (e.g., Griffith et al. 2002), and the focus of this dissertation. Indeed, the large body of literature describing extra-pair sexual activity in birds has found genetic polygyny and/or polyandry to be prevalent across the ~80% of bird species that are socially monogamous and highly variable within populations (Brouwer & Griffith 2019). Furthermore, while previous work has extensively studied the occurrence and tradeoffs of extra-pair sexual activity in socially monogamous systems from the male perspective, female birds in some socially monogamous species also solicit and secure copulations from males other than their social partner (Brouwer & Griffith 2019, Forstmeier et al. 2014, Reichard & Boesch 2003).

Understanding why some females of socially monogamous species engage in extra-pair copulations has been a goal of ornithologists and behavioral ecologists alike (Forstmeier et al 2014). The prevalence of extra-pair copulations is intriguing from an evolutionary perspective because individuals, particularly females, should be balancing the costs and benefits of having multiple sexual partners. For females, some of the proposed benefits of having multiple partners include fertility insurance of their mate and increasing genetic diversity of their young. Proposed costs of having multiple partners for females include potential loss of paternal care of the young and harassment by the female's social partner (see Table 1 in Forstmeier et al. 2014). However, general support for these hypothesized benefits and costs is minimal or circumstantial (Forstmeier et al. 2014).

For this dissertation, I focus on a potential trade-off of extra-pair copulations to female birds that has yet to be adequately studied but could prove to be substantial: sexually transmitted bacteria. Socially monogamous, yet sexually polygamous species, are opportune systems in which to study how variation in mating behavior influences both the transmission of sexually transmitted bacteria between individuals and the maintenance of these bacteria within the reproductive microbiome of individuals. In such species, individuals vary in the number of mates that they have during the breeding season and thus may vary in their acquisition of sexually transmitted bacteria and in the community composition of their reproductive microbiome. Previous work has explored the similarity of select microbiota or the reproductive microbiome as a whole between pair-bonded social partners in both truly monogamous systems (White et al. 2010) and socially monogamous, but sexually polygamous systems (Hupton et al. 2003, Kreisinger et al. 2015, Lombardo & Thorpe 2000, Lombardo et al. 1996, Stewart & Rambo 2000). In addition, comparisons of reproductive microbiome diversity and composition among species with divergent mating systems (e.g., monogamous vs. polygamous: Poiani & Gwozdz 2002, Poiani & Wilks 2000a, Poiani & Wilks 2000b) have also been described. This dissertation builds upon this work by investigating how variation in sexual activity and number of sexual partners – in addition to variation in life history and physiology – contribute to variation in the reproductive microbiome.

Reproductive microbiome

The reproductive microbiome is the community of microorganisms (e.g., bacteria, fungi, viruses, protozoans) residing in or on any aspect of a host that becomes exposed to the reproductive tract or gametes of another individual (Rowe et al. 2020). This dissertation focuses on the reproductive microbiome specific to birds, which can generally be referred to as the cloacal microbiome. In birds—as well as amphibians, reptiles, cartilaginous fishes, and monotremes—the cloaca is the terminus for the digestive and reproductive tracts, and the site of contact and ejaculate transfer during copulation. As such, the cloaca is the reproductive structure in both females and males through which communities of beneficial, commensal, and pathogenic microbes can be transmitted sexually (e.g., White et al. 2011, Kulkarni & Heeb 2007). This complex and highly dynamic community of microbes can be shaped by a suite of factors, including host behavior, social environment, life history, and physiology (Rowe et al. 2020).

Variation in cloacal microbiome with respect to host behavior & social environment

There is an interconnected, bidirectional relationship between animal hosts and their associated microbes, whereby host-associated microbes can influence host behavior and vice versa (Archie & Tung 2015, Ezenwa et al. 2012, Hawley et al. 2020, Münger et al. 2018). For instance, host-associated microbes can mediate intra-species signaling (e.g., scent gland microbes produce chemicals used in animal interactions, Theis et al. 2012), inter-species signaling (e.g., skin microbes produce chemicals that attract mosquitos, Verhulst et al. 2011), and mate choice (e.g., gut microbes influence animal mating preferences, Sharon et al. 2012). Focusing on the former, previous work has identified animal host behaviors that significantly vary with host-associated microbiomes. For example, behaviors such as social group membership, cohabitation, social interactions (e.g., allogrooming, cleaning, regurgitation, huddling, licking), parental care, migration, and coprophagy are associated with skin and/or gut microbiomes (Cook et al. 2005, Cooper 2004, Koch & Schmid-Hempel 2011, Kyle & Kyle 1993, Lax et al. 2014, Raulo et al., 2021, Risely et al. 2018, Song et al. 2013, Tung et al. 2015, Xavier et al. 2019, Zhang et al. 2018). Animal movement ecology and mating have been found to correlate with variation in the cloacal microbiome specifically (Bellinvia et al. 2020, Corl et al. 2020, Kulkarni & Heeb 2007, White et al. 2010, White et al. 2011). Most of this previous work investigating variation in the cloacal microbiome has assessed these behaviors from a broad perspective, with respect to differences between sexes and mating systems more generally (monogamous versus polygamous). To begin to reconstruct the *causative* behavioral factors influencing variation in the cloacal microbiome, experiments incorporating fine-scale individual variation in mating behavior, for example, especially in free-living animals, would prove particularly informative.

Variation in cloacal microbiome with respect to host life history & physiology

Recent work has sought to characterize if and how variation in the host-associated cloacal microbiome tracks changes in host life history and physiology. Numerous studies using community-based approaches to assess the cloacal microbiome (e.g., 16S rRNA, ARISA) have documented that the cloacal microbiome varies with age, especially when considering offspring and adults (Ambrosini et al. 2019, Barbosa et al. 2016, van Dongen et al. 2013, but see Klomp et al. 2008). In adults, documented changes in cloacal bacterial diversity between life history stages suggest that physiology and overall breeding condition may play a role in shaping the cloacal

microbiome. For example, in male rufous-collared sparrows (*Zonotrichia capensis*), the within-individual phylogenetic diversity of cloacal bacteria changed between breeding and non-breeding stages (Escallón et al. 2019). In this same study population, the phylogenetic diversity of cloacal bacteria positively correlated with circulating testosterone concentrations (Escallón et al. 2017). The cloacal microbiome has also been found to differ by sex during the breeding season in some species (*Tyto alba*, Corl et al. 2020, *Zonotrichia capensis*, Escallón et al. 2019, *Hirundo rustica*, Ambrosini et al. 2019, but see Kreisinger et al. 2015). These studies suggest that differences in host physiology and behavior during the breeding season may play a role in shaping the acquisition and maintenance of cloacal bacterial communities of both sexes. Overall, differences in host life history and physiology, coupled with differences in cloacal microbiome diversity and structure, have the potential to profoundly influence the reproductive success of individuals and scale up to impact ecological and evolutionary processes at the population level.

Reproductive microbiome-fitness relationship

Microbes within the reproductive tract of animals can impact host reproductive function and survival, and thus may play a role in influencing host fitness outcomes. For example, *E. coli* in reproductive tracts of females can attach to sperm cells and inhibit mobility, and in some cases rupture the sperm plasma membrane and render the gamete completely immobile and unable to function properly (Bonet et al. 2018, Haines et al. 2013). Microbes within the reproductive tracts of females can have significant implications for pregnancy outcomes (Heil et al. 2019, Younes et al. 2018). *Lactobacilli* has been found to be beneficial in maintaining a healthy vaginal microbiome in humans and fighting pathogens. In contrast, microbes such as *Gardnerella*, *Mycoplasma*, and *Prevotella* have been found to be associated with a number of reproductive tract infections, some of which result in premature births and low birth weight. Lastly, the microbial seeding of babies can be very important in minimizing infection risk (Shao et al. 2019). Babies that ingest microbes from the reproductive tract of their mothers on their way out of the womb are less susceptible to infection by opportunistic pathogens. Importantly, previous work has focused on either beneficial or pathogenic microbes within the reproductive tract of animals, but particularly humans, and largely ignored the reproductive microbiome.

Current dissertation

For my dissertation, I focused on understanding the causes and consequences of variation in the reproductive microbiome of free-living birds. I addressed the following questions:

- Does variation in the cloacal microbiome correlate with sex? Do pair-bonded social partners have similar cloacal microbiomes?
- How does sexual activity, breeding stage, and age influence the cloacal microbiome?
- Can variation in the cloacal microbiome predict host-associated fitness?

To address these questions, I studied a population of free-living tree swallows (*Tachycineta bicolor*) during the breeding season in Virginia, USA. Tree swallows are classically described as socially monogamous, yet both females and males engage in extra-pair sexual activity that may include extra-pair solicitations, copulations, and fertilizations (Dunn et al. 2009, Lifjeld et al. 1993). In previously studied populations of tree swallows, the proportion of broods containing extra-pair young ranged from 68-87% and the proportion of extra-pair young in the population ranged from 51-53% (see Table 4 in Conrad et al. 2001; Dunn et al. 1994a, 1994b, Lifjeld et al. 1993; Kempenaers et al. 1999).

I assessed the causes and consequences of variation in the cloacal microbiome from an ecological perspective. The considerable amount of previous work that has documented the variation in, and high rates of, extra-pair activity in tree swallows has helped establish the behavioral and ecological framework for my dissertation. By investigating the relationships between the host-associated reproductive microbiome and both the host's social and physical environment, my research has identified potential extrinsic and intrinsic factors that are associated with and shape the reproductive microbiome of a free-living bird.

I conducted three empirical studies:

1) In Chapter II, I used an observational approach to investigate whether social monogamy or sex explain variation in the cloacal microbiome of tree swallows.
2) In Chapter III, I used both observational and experimental studies to assess age, breeding stage, and mating activity as drivers of variation in the cloacal microbiome of female tree swallows.

3) In Chapter IV, I used an observational approach to gain insight into the relationship between a fitness-associated trait and both variation in cloacal microbiome diversity and female age.
4) In Chapter V, I briefly summarize the framework established by my dissertation research and discuss future steps that can be taken to extend this framework and refine our understanding of the factors shaping the reproductive microbiome more broadly.

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CHAPTER II: Cloacal bacterial communities of tree swallows (*Tachycineta bicolor*): Similarity within a population, but not between pair-bonded social partners

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Abstract

Host-associated microbial communities can influence the overall health of their animal hosts, and many factors, including behavior and physiology, can impact the formation of these complex communities. Bacteria within these communities can be transmitted socially between individuals via indirect (e.g., shared environments) or direct (e.g., physical contact) pathways. Limited research has been done to investigate how social interactions that occur in the context of mating shape host-associated microbial communities. To gain a better understanding of these interactions and, more specifically, to assess how mating behavior shapes an animal's microbiome, we studied the cloacal bacterial communities of a socially monogamous yet genetically polygynous songbird, the North American tree swallow (*Tachycineta bicolor*). We address two questions: (1) do the cloacal bacterial communities differ between female and male tree swallows within a population? and (2) do pair-bonded social partners exhibit more similar cloacal bacterial communities than expected by chance? To answer these questions, we sampled the cloacal microbiome of adults during the breeding season and then used culture-independent, 16S rRNA gene amplicon sequencing to assess bacterial communities. Overall, we found that the cloacal bacterial communities of females and males were similar, and that the communities of pair-bonded social partners were not more similar than expected by chance. As social partners were not assessed at the same time, it is possible that breeding stage differences masked social effects on bacterial community diversity and structure. Further, given that tree swallows exhibit high variation in rates of extra-pair activity, considering extra-pair activity when assessing cloacal microbial

communities may be important for understanding how these bacterial communities are shaped. Further insight into how bacterial communities are shaped will ultimately shed light on potential tradeoffs associated with alternative behavioral strategies and socially-transmitted microbes.

Introduction

Host-associated microbial communities can contribute to the overall health of their animal hosts (Archie & Tung 2015, Bäckhed et al. 2005, Fraune & Bosch 2010, McFall-Ngai et al. 2013), and many factors, including behavior and physiology, can impact the composition of these complex communities (Adair & Douglas 2017, Archie & Theis 2011, Archie & Tung 2015, Ezenwa et al. 2012). Across animal taxa, for example, social behaviors, such as allopreening or allogrooming, cohabitation, group membership, and mating, play a role in shaping the diversity and structure of an animal's microbial communities (Banning et al. 2008, Koch & Schmid-Hempel 2011, Kulkarni & Heeb 2007, Lax et al. 2014, Tung et al. 2015, White et al. 2010). While microbes line most body surfaces, and distinct eco-evolutionary pressures may be imposed on each microbial community (e.g., those associated with the skin, feathers, oral, gut, cloaca) (van Veelen et al. 2017), the vast majority of studies relating social behavior and host-associated microbial communities have focused on the gut and skin (Archie & Tung 2015).

In reptiles, amphibians, birds, and a few mammals the cloaca is the terminus for the digestive and reproductive tracts, and thus, cloacal microbial communities are impacted by both diet and reproductive behavior (Kulkarni & Heeb 2007, Maul et al. 2005). Given that cloacal contact during copulation can result in the transmission of microbes between individuals (Kulkarni & Heeb 2007) and that pair-bonded social partners maintain a relatively close relationship throughout the breeding season, it has been hypothesized that pair-bonded social partners share more similarity in their cloacal microbial communities compared to others in the same population (White et al. 2010, Kreisinger et al. 2015, Ambrosini et al. 2019, Lombardo et al. 1996, Stewart & Rambo 2000). This hypothesis has been tested in several systems and has generally been supported. For example, using culture-dependent bacterial assessment methods, Lombardo et al. (1996) and Stewart and Rambo (2000) showed similarity in cloacal bacterial communities between social partners in tree swallows (*Tachycineta bicolor*) and house sparrows (*Passer domesticus*). More recent work using culture-independent methods has revealed that pair-bonded social partners

experimentally blocked from making cloacal contact have significantly less similar cloacal bacterial communities compared to control pairs in black-legged kittiwakes (*Rissa tridactyla*) (White et al. 2010). In addition, two observational studies on barn swallows (*Hirundo rustica*) in Europe found that cloacal bacterial communities of pair-bonded social partners were more similar to each other than to other individuals in the population or than expected by chance (Kreisinger et al. 2015, Ambrosini et al. 2019); however, the effect size was small in one of the studies (Kreisinger et al. 2015).

To gain a more complete picture of the effects of social partnerships during the breeding season on cloacal bacterial community diversity and structure, and to follow up on prior studies in tree swallows (Lombardo et al. 1996) and barn swallows (Kreisinger et al. 2015, Ambrosini et al. 2019), we used culture-independent, 16S rRNA gene amplicon sequencing to study the cloacal bacterial communities of breeding North American tree swallows. This is the first study to apply culture-independent methods to study the cloacal microbiome of tree swallows. We asked two main questions: (1) do the cloacal bacterial communities differ between female and male tree swallows within a population? and (2) do pair-bonded social partners share more similar cloacal bacterial communities than expected by chance? For the first question, we hypothesized that the cloacal bacterial communities of females and males would differ due to physiological differences that exist between the sexes during the breeding season, such as differences in hormonal profiles (Adkins-Regan 2005) and immune activity (McGraw & Ardia 2005). For the second question, based on previous research examining the similarity of cloacal bacterial communities between social partners (White et al. 2010, Kreisinger et al. 2015, Ambrosini et al. 2019, Lombardo et al. 1996), we hypothesized that cloacal bacterial communities between pair-bonded social partners would be more similar than expected by chance because pair-bonded partners would interact more frequently with each other and with the same environment (i.e., the nest) than with other individuals in the population. Overall, this research used a sequencing-based approach to provide a more comprehensive assessment of the bacteria present within the cloacae of birds, as well as deepened our understanding of how social interactions, specifically mating partnerships, shape cloacal bacterial communities.

Methods

Study site and species

We studied a breeding population of free-living tree swallows (*Tachycineta bicolor*) at Kentland Farm, a 350-acre mosaic of fields owned by Virginia Tech, in Montgomery County, Virginia, U.S.A. (37°11'53.6 N, 80°34'58.0 W; 520 m.a.s.l.). Tree swallows are small, migratory, aerial insectivorous passerines (Winkler et al. 2011). They are socially monogamous and thus form pair-bonds during the breeding season that can extend across multiple years. Nevertheless, tree swallows exhibit high rates of extra-pair sexual activity (~50% of nestlings are extra-pair offspring), with both females and males engaging in extra-pair solicitations, copulations, and fertilizations (Dunn et al. 2009, Lifjeld et al. 1993). In our study population, tree swallows breed from late March to early August, and use man-made nest boxes set up along fence and tree lines that border open agricultural fields. The average distance between boxes occupied by tree swallows is approximately 25 meters. Within our study population, ~ 54% of the tree swallow young are from extra-pair fertilizations, with 84% of broods containing one or more extra-pair young (J. Hernandez unpublished data, based on 256 young and 55 broods).

Sample collection

We captured adult tree swallows at nest boxes during the 2016 and 2017 breeding seasons from May to June. We caught the birds by hand when possible, otherwise we used a trap door to capture the birds when they entered the nest box. Upon capture, we banded each bird with a uniquely numbered aluminum U.S. Fish and Wildlife bird band. We also banded each female with a lime green color band and each male with a pink color band for visual identification from a distance. We identified females by the presence of a brood patch or, in the case of females in their first breeding season, by brown plumage. We identified males by the presence of an enlarged cloacal protuberance and the absence of a brood patch. By denoting the sex of each bird with a color band, we were able to target our capturing of each sex more effectively. Upon capture, we also measured the right wing and mass of each bird for subsequent host body condition analyses. To determine the pair-bonded social pair of a nest, we conducted observational surveys whereby we noted the female found to be incubating the eggs and the male seen to be feeding the subsequently hatched nestlings. We then sampled females during incubation and most males two to three weeks later during nestling provisioning. There was only one pair of the 13 total pairs we sampled in which

the female and the male were sampled at the same time, since they were in the box copulating at the time of capture (J. Hernandez, personal observation). To collect cloacal bacteria, we gently inserted a sterile swab (PurFlock®, Puritan, USA) ~4 mm into the cloaca and revolved it once (Escallón et al. 2017). Swab samples were stored in sterile 1.5 mL tubes on ice in the field and later frozen in a -80°C freezer upon return to the lab (< 5 hours post-collection). We used gloves when handling and swabbing the birds, and changed gloves between individuals. The Virginia Tech Institutional Animal Care and Use Committee (IACUC) approved of this research.

DNA extraction, amplification, and sequencing

To extract DNA from the cloacal swabs, we used the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA). We followed the manufacturer's protocol for the purification of total DNA from animal tissues, including the pretreatment for gram-positive bacteria, for all swab samples. The pretreatment incorporates an additional incubation with lysozyme to most effectively lyse the cell walls of gram-positive bacteria prior to total DNA purification. We then targeted the hypervariable region 4 (V4) of the 16S rRNA gene for amplification using the primers 515F and 806R (Caporaso et al. 2011). The reverse primer contained a GOLAY error-correcting 12bp barcode to tag individual samples. Similar to Costello et al. (2009), we performed PCR in triplicate using 12 µL DNA-free PCR water (MO BIO, Carlsbad, CA, USA), 10 µL Hot MasterMix (5 PRIME, Gaithersburg, MD, USA), 0.5 µL of each forward and reverse primer, and 2 µL template DNA. We ran negative controls without template DNA for each sample. The thermal cycling conditions were as follows: 3 min at 94°C, 35 cycles with 45 s at 94°C, 1 min at 50°C, 1.5 min at 72°C, and 10 min at 72°C. Triplicate reactions were then pooled for each sample and visualized on a 1% agarose gel. We quantified the amplified bacterial DNA in each sample using a Qubit™ 2.0 fluorometer and Qubit™ dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA). Lastly, we pooled 200 ng DNA of each sample and purified the final pool (QIAquick PCR Purification kit, Qiagen, Valencia, CA, USA). This final pool was sent to the Dana Farber Cancer Institute of Harvard University to be sequenced on an Illumina MiSeq instrument, as described by Caporaso et al. (2012), using a 250bp single-end strategy that captured most of the V4 region, which is 291bp. Samples collected from the 2016 and 2017 breeding seasons were extracted, amplified, and sequenced using identical protocols in August 2016 and 2017, respectively.

Sequence data processing

Amplicon sequences for the 2016 and 2017 sequencing runs were combined for downstream data processing. Forward reads of raw Illumina 16S rRNA amplicon sequences were de-multiplexed and quality filtered using the Quantitative Insights Into Microbial Ecology (QIIME) 1.9 bioinformatics pipeline (Caporaso et al. 2010). In QIIME, we removed sequences that contained errors in the barcode, and set the minimum number of consecutive high-quality base calls to include a read to 0.50 fraction of the input read length (default is 0.75). We also specified the minimum acceptable Phred quality score as 20. In other words, we kept the reads whose Phred score was equal to or above 20 to be of high quality and removed any reads below that threshold. Using the UCLUST method (Edgar 2010) and a 97% sequence similarity threshold, we then clustered sequences into operational taxonomic units (OTUs). We selected the most abundant sequence from each cluster to represent each OTU. We used PyNAST to search representative sequences against the Greengenes 13.8 database (Caporaso et al. 2009, DeSantis et al. 2006). The RDP classifier (Wang et al. 2007) was then used to assign the Greengenes taxonomy of each OTU using the default minimum confidence value of 0.5. OTUs with fewer than 0.01% of the total number of reads (i.e., fewer than 224 reads in our dataset) were removed (Bokulich et al. 2013). Also, given that the sequencing depth per sample ranged from 9,360 to 119,903 reads per sample, we rarefied samples to 9,300 reads prior to analyses. Normalizing a dataset using rarefaction is appropriate, particularly when the sequencing depth across samples is highly variable and uneven, as is the case with our data set (Weiss et al. 2017). There was a 13-fold difference (i.e., highly uneven distribution) between the samples with the highest and lowest sequencing depths in our study.

Statistical analyses

All analyses were performed in R (v. 3.4.1) (R Core Team, 2017). To determine whether cloacal bacterial communities differed between adult female and male tree swallows, we assessed shared and unique OTUs between the sexes. In addition, we determined the most abundant OTUs in the cloacae of sampled birds using a relative abundance cut off of 5%. Abundant OTUs were identified to the minimum possible taxonomic level and the distribution of their relative abundance across samples was visualized using a heatmap (‘gplots’ package: function *heatmap.2*). To assess the bacterial diversity for each bird sampled (i.e., alpha diversity), we calculated OTU richness, the

Shannon Index, and Faith's phylogenetic distance in QIIME. Alpha diversity metrics of cloacal bacterial communities for both sexes were visualized using boxplots and generalized linear models were fitted to the diversity metrics to test for statistical differences between sexes. We used a negative binomial distribution with the log link function ('MASS' package: function *glm.nb*) for OTU richness to account for overdispersion ('AER': *dispersiontest*). We used a gamma distribution ('MASS': *glm*, family = Gamma) for the Shannon Index and Faith's phylogenetic distance. In the generalized linear models, we included "Sex", "Julian Date", "Year", and "Host Body Condition" as explanatory variables. To assess host body condition, we calculated a scaled-mass index based on a bird's mass and wing length (Peig & Green 2009).

To assess whether variation in the structure of cloacal bacterial communities differed between females and males (i.e., beta diversity) in our population, we calculated non-parametric, permutational multivariate analyses of variance (PERMANOVA; 'vegan': *adonis*) based on 999 permutations. The results presented are based on Bray-Curtis and Jaccard metrics, because there were no apparent differences between results using Bray-Curtis or weighted UniFrac, or between Jaccard and unweighted UniFrac. Bray-Curtis and weighted UniFrac consider relative abundances, while Jaccard and unweighted UniFrac consider presence-absence. Bray-Curtis considers count-based data, while UniFrac metrics consider phylogenetic-based distances. Dissimilarity distances between females and males were then visualized using a non-metric multidimensional scaling (NMDS) plot. To compare the multivariate spread of the data between the sexes, we tested the multivariate homogeneity of group dispersions ('vegan': *betadisper*) using a permutation test ('vegan': *permutest*) for females and males.

To determine if the cloacal bacterial communities of pair-bonded social partners were more similar to each other compared to other randomly selected pairs, we compared the frequency distributions of sampled and randomized pairs within our study population. We manually calculated the difference between the OTU relative abundances of all possible combinations of hypothetical, randomly assigned female-male pairs. We took the absolute value of the OTU relative abundance differences and then generated a density plot to visualize the distribution of these differences for randomized female-male pairs. We repeated these steps to generate a density plot of the distribution of differences for sampled, pair-bonded female-male pairs. In a density plot,

distributions that overlay each other generally indicate that the two distributions are not significantly different. To statistically compare the distributions of random and sampled pairs, and thus determine if sampled pairs were more similar to each other than expected by random chance, we performed a permutation test ('coin': *independence_test*). Further, we performed a hierarchical, polythetic, agglomerative cluster analysis based on Bray-Curtis and Jaccard distance metrics. This method clusters together individuals with more similar cloacal bacterial communities; therefore, if pair-bonded social partners are clustered closer together, we can deduce that they have more similar cloacal bacterial communities compared to other birds in the study population.

Results

We sampled 13 social pairs (n=4 pairs in 2016, 9 pairs in 2017), as well as an additional three females and four males for which we did not collect cloacal swab samples from their social partners (total n=7 in 2016). Each bird was sampled only once. The average (± standard deviation) time between the sampling of female and male social partners was 14.23 (± 5.92) days, with the lowest and highest sampling time differences being 0 and 21 days, respectively.

We identified 594 OTUs across the 33 individual bird cloacal samples. The bacterial phyla with the highest relative abundances were Actinobacteria, Firmicutes, and Proteobacteria (Fig 1). The most prevalent family, Corynebacteriaceae (phylum Actinobacteria), was present in 100% of individuals, followed by the families Microbacteriaceae (phylum Actinobacteria), Rhizobiaceae (phylum Proteobacteria), and Enterococcaceae (phylum Firmicutes), which were present across at least 90% of individuals. Only OTUs from the genus Corynebacterium (phylum Actinobacteria, class Actinobacteria, order Actinomycetales, family Corynebacteriaceae) comprised the core microbiome, which were present in the cloacae of 100% of birds sampled and occurred at a relative abundance of ~40% across individuals (Fig 2). OTUs from the families Enterobacteriaceae (phylum Proteobacteria, class Gammaproteobacteria, order Enterobacteriales) and Micrococcaceae (phylum Actinobacteria, class Actinobacteria, order Actinomycetales) were the second and third most abundant and were each present across individuals at mean relative abundances of ~7.5 and 4.7%, respectively (Fig 2).

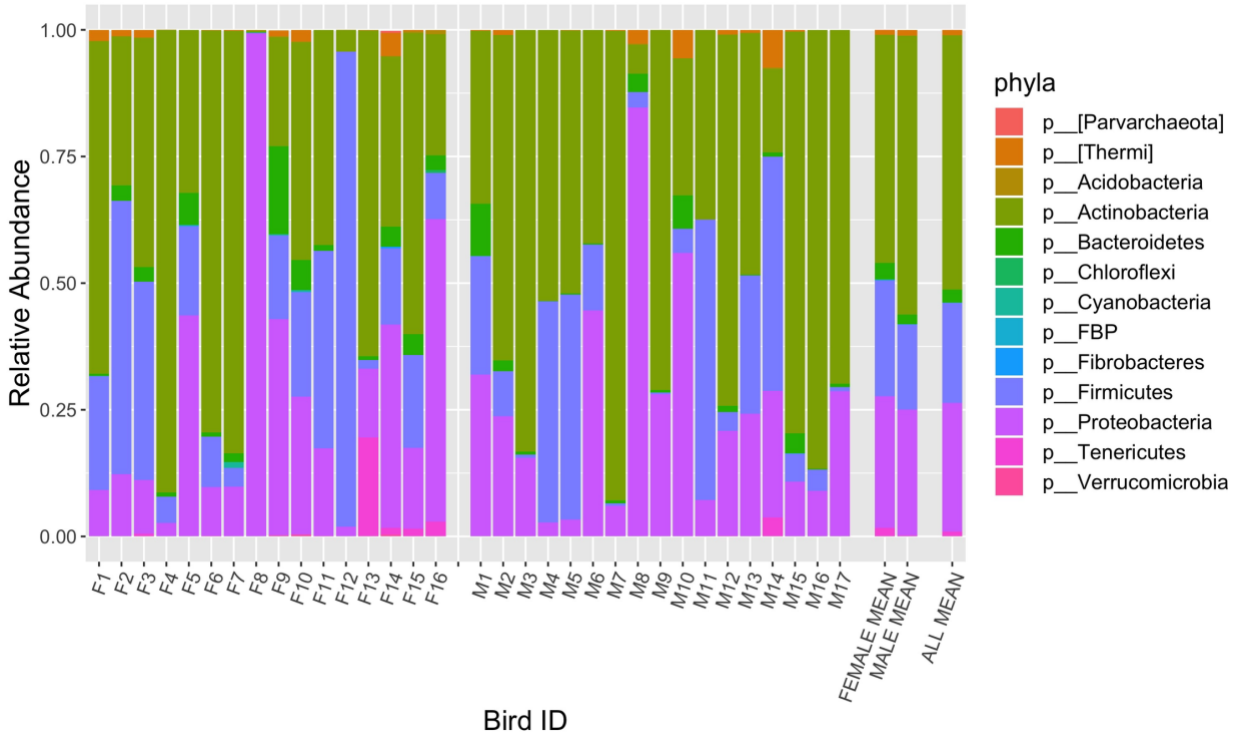

Figure 1. Mean relative abundance of bacterial phyla present in the cloacae of sampled adult tree swallows. Each column depicts the phyla represented within the cloaca of an individual bird. 'F' and 'M' refer to individually sampled females and males. 'Female Mean' and 'Male Mean' depict the average phyla represented per sex. 'All Mean' depicts the average phyla represented across sexes.

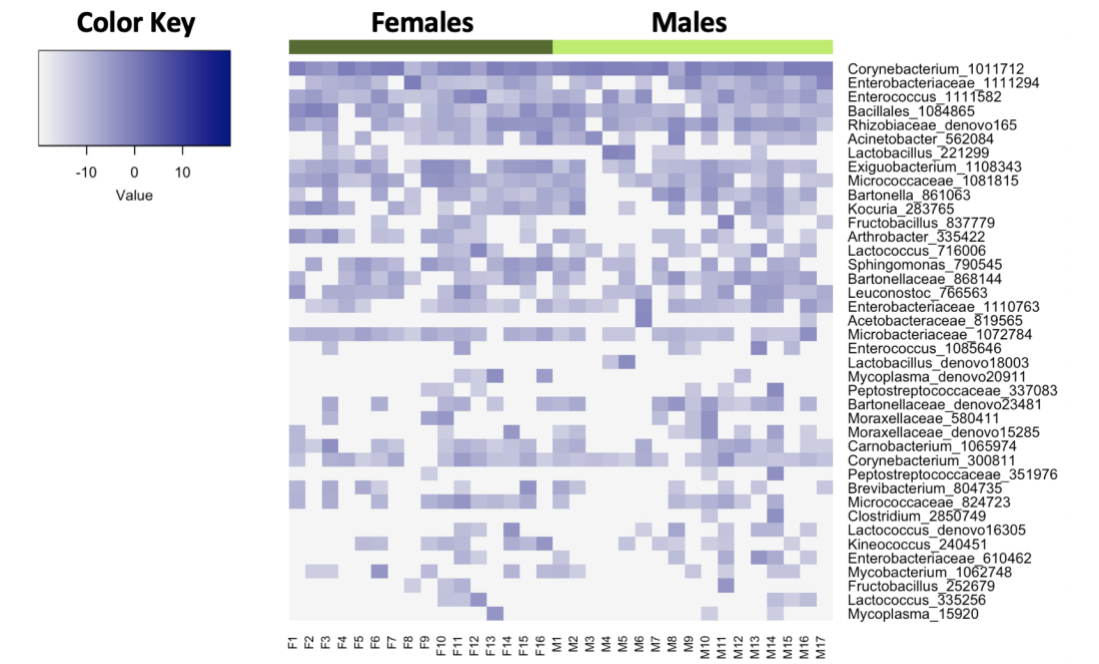

Figure 2. Heatmap of relative abundances (log-transformed) of the most abundant OTUs present in the cloacae of adult tree swallows. Each column depicts individual birds sampled. 'F' and 'M' refer to individually sampled females and males, respectively. The dark green bar at the top highlights sampled females, while the light green bar highlights sampled males. Each row depicts a bacterial taxon present at a relative abundance greater than 5% in the sampled population. Bacterial taxa are labeled to the lowest possible level of taxonomic classification.

Females and males had similar OTU richness (*glm.nb*, b = 0.041, SE = 0.19, $z_{(29)}$ = 0.21, p = 0.83), with an average (± standard deviation) of 179 ± 67 OTUs in females and 155 ± 63 OTUs in males. There was also not a significant difference between the bacterial communities of females and males in terms of the Shannon Index, which considers both richness and evenness of the community (*glm*, b = 0.031, SE = 0.072, $t_{(29)}$ = 0.42, p = 0.68) or Faith's phylogenetic diversity (*glm*, b = 0.0065, SE = 0.012, $t_{(29)}$ = 0.55, p = 0.59) (Fig 3). Additionally, there were no differences in cloacal bacterial community diversity within seasons (*glm.nb*, OTU richness: b = -0.012, SE = 0.0077, $z_{(30)}$ = -1.5, p = 0.13, *glm*, Shannon: b = -0.055, SE = 0.034, $t_{(30)}$ = -1.6, p = 0.12, *glm*, Faith's: b = -0.084, SE = 0.070, $t_{(30)}$ = -1.2, p = 0.24) or between seasons (*glm.nb*, OTU richness: b = 0.19, SE = 0.13, $z_{(30)}$ = 1.4, p = 0.15, *glm*, Shannon: b = 0.55, SE = 0.59, $t_{(30)}$ = 0.94, p = 0.36), with one exception (i.e., *glm*, Faith's: b = 2.6, SE = 1.2, $t_{(30)}$ = 2.1, p = 0.040). There was not a significant relationship between cloacal bacterial community diversity and host body condition (*glm.nb*, OTU

richness: b = 0.024, SE = 0.049, $z_{(30)}$ = 0.50, p = 0.62, *glm*, Shannon: b = -0.025, SE = 0.017, $t_{(30)}$ = -1.5, p = 0.15, *glm*, Faith's: b = -0.0017, SE = 0.0028, $t_{(30)}$ = -0.61, p = 0.55). Further, females and males did not significantly differ in cloacal bacterial community structure (*adonis*, Bray-Curtis: pseudo-$F_{(1,31)}$ = 0.77, R^2 = 0.024, p = 0.68; Jaccard: pseudo-$F_{(1,31)}$ = 0.84, R^2 = 0.026, p = 0.66) (Fig 4). The multidimensional spread of female and male cloacal bacterial communities was not significantly different (*permutest*, Bray-Curtis: $F_{(1,31)}$ = 1.9, p = 0.18; Jaccard: $F_{(1,31)}$ = 1.8, p = 0.18); thus, there was no statistical evidence for a difference in dispersion between the bacterial communities of each sex.

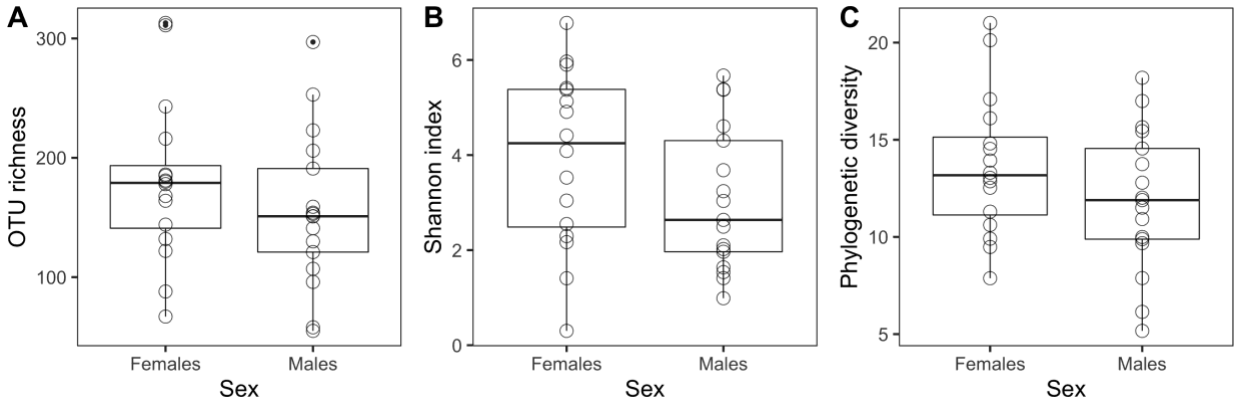

Figure 3. Alpha diversity metrics for bacterial OTUs sampled from the cloacae of adult tree swallows. (A) OTU richness (i.e., observed OTUs), (B), Shannon Index, and (C) Faith's phylogenetic diversity indices were calculated. The circles represent individuals sampled of each sex.

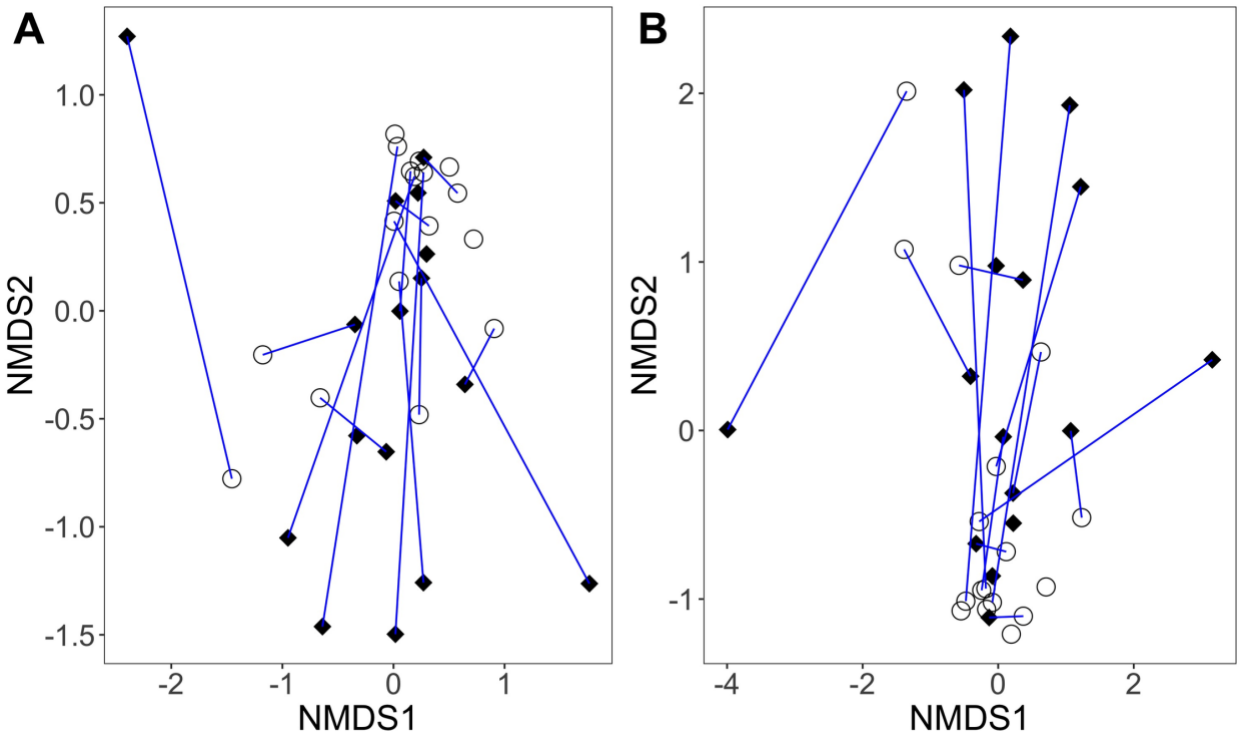

Figure 4. Cloacal bacterial community structure of adult tree swallows. Non-metric multidimensional scaling (NMDS) ordinations are based on (A) Bray-Curtis and (B) Jaccard distance metrics. Each symbol represents one individual (black diamonds = females, open circles = males). Pair-bonded social partners are connected by a blue line. Points closer together exhibit individuals with more similar cloacal bacterial community structure.

The difference in cloacal bacterial composition between sampled pair-bonded female-male social pairs was not statistically different from the difference in cloacal bacterial composition between randomly paired female-male pairs within our study population (*independence_test*, Z = 0.039, p = 0.97) (Fig 5). Additionally, based on the composition of their cloacal bacterial communities, pair-bonded social partners did not cluster more closely than other sampled individuals (Fig 4, partners are connected by a blue line; Fig 6). In other words, social partners did not have more similar cloacal bacterial communities than other sampled individuals. Only the one social pair that we sampled at the same time exhibited cloacal bacterial communities more similar to each other than to other individuals in the population (Fig 6, see 'F11' and 'M11').

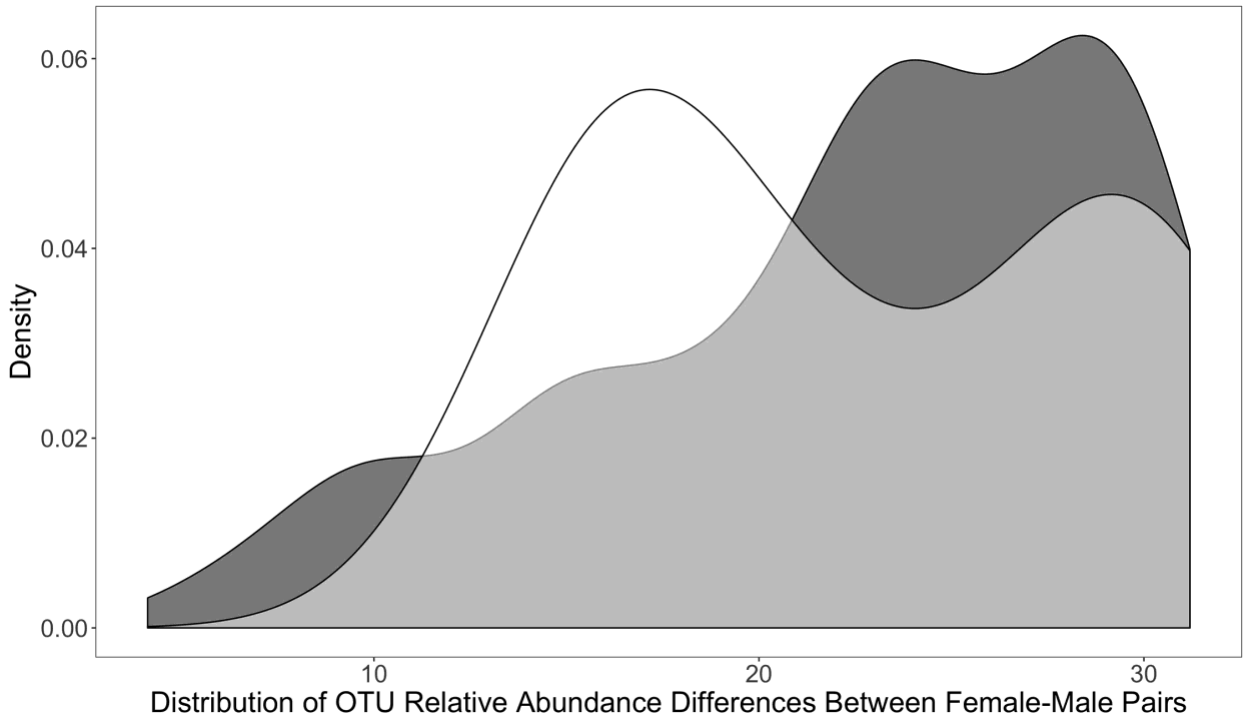

Figure 5. OTU relative abundance differences for randomized (dark gray) and sampled (white) female-male pairs. Samples on the left side of the distribution are more similar, while samples on the right side are less similar and thus exhibit greater differences.

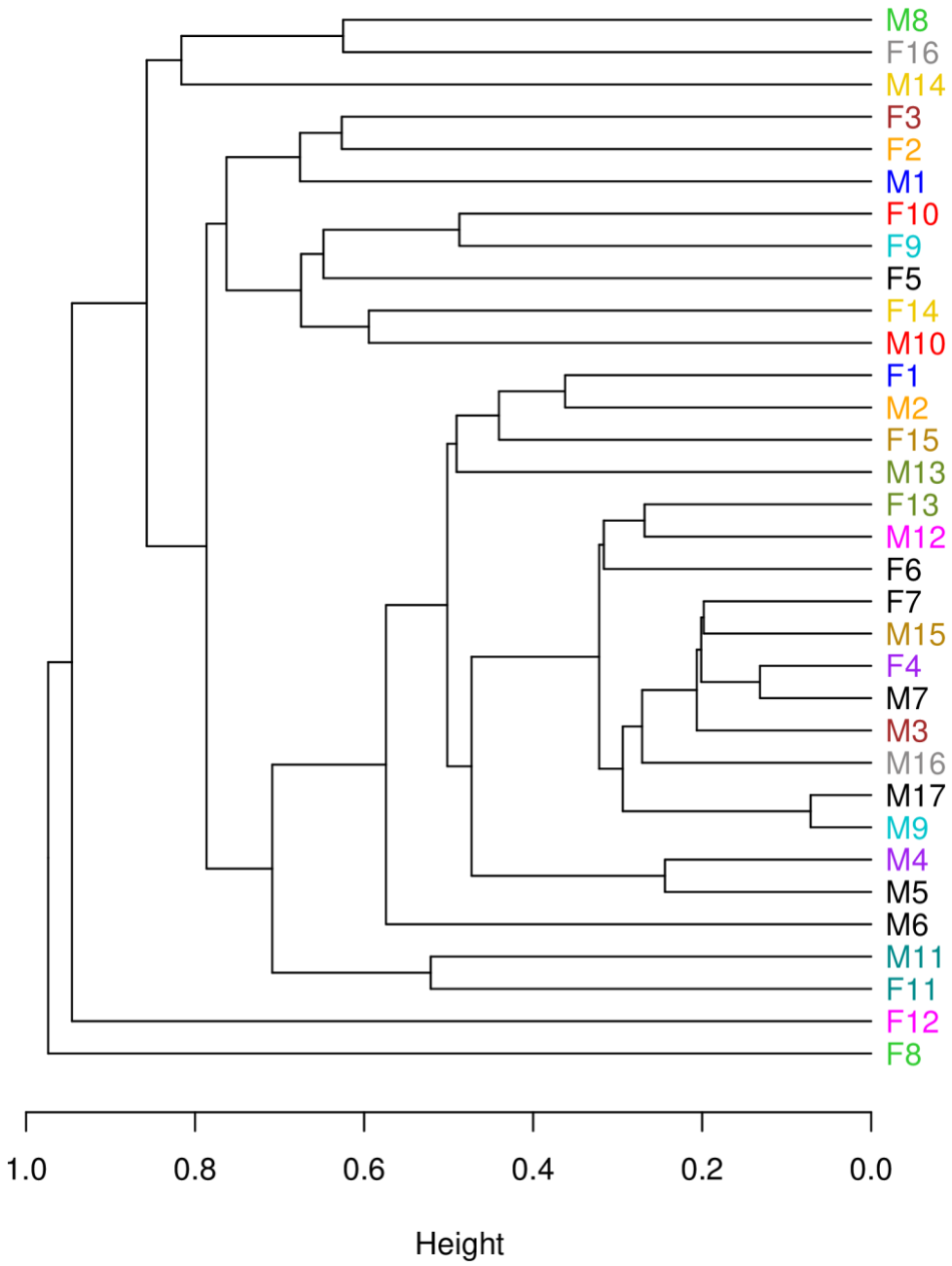

Figure 6. Hierarchical cluster analysis (based on Bray-Curtis dissimilarity) showing the overall level of similarity of cloacal bacterial communities for sampled females and males. The cluster analysis using the Jaccard distance metric was visually indistinguishable from the Bray-Curtis based analysis, so we only show the latter here. 'F' = female, 'M' = male, similar color = social pair, black = individual was sampled but not its social partner.

Discussion

In this study, we assessed (1) whether the cloacal bacterial communities differed between female and male tree swallows within a population, and (2) whether pair-bonded social partners shared more similar cloacal bacterial communities than expected by chance. Using 16S rRNA gene amplicon sequencing, we found that cloacal bacterial communities were generally similar between female and male tree swallows. We also found that the cloacal bacterial communities of pair-bonded social partners were not more similar than expected by chance. That is, cloacal bacterial community diversity and structure did not vary based on sex or social pair bond.

Neither of our hypotheses were supported and this could be due to rates of extra-pair copulations and/or diet. First, birds transmit bacteria via copulations through both cloacal contact and ejaculate transfer (Kulkarni & Heeb 2007, Westneat & Rambo 2000) and high rates of extra-pair copulations could serve to homogenize the cloacal bacterial communities across the population (Kreisinger et al. 2015). Second, it is important to bear in mind that the cloaca functions as the terminus for both the reproductive and the digestive tract in birds. The role that diet plays in shaping the cloacal bacterial communities of birds is currently unclear, however, previous research has found that diet shapes the bacterial communities anterior to the cloaca (e.g., the crop, small and large intestines, and cecum) across bird species (Waite & Taylor 2014). Previous work on breeding tree swallows has found that adults forage near their breeding site and tend to consistently select similar prey items (McCarty & Winkler 1999). Given that the tree swallows sampled in this study likely forage in the same general area on the same available prey items, we would expect diet composition to be similar across birds in our study population. Thus, future studies trying to understand the factors that shape cloacal bacterial communities should primarily consider rates of extra-pair copulations.

Among birds, the behavior and physiology of both sexes change as the breeding season progresses (e.g., Bech et al. 1999, Nelson 2015, Williams 2012), and these behavioral and physiological changes may also influence the diversity and structure of cloacal bacterial communities. In this study, we sampled pair-bonded female and male social partners during different stages of the breeding season (i.e., females during incubation and most males several weeks later during nestling provisioning). There was only one pair in which the female and the male were sampled at the same time, since they were in the box copulating at the time of capture. Interestingly, only this pair-

bonded pair exhibited cloacal bacterial communities that were more similar to each other than to other individuals in the population or than expected by chance. Therefore, it is possible that partners exhibit similarity in their cloacal bacterial communities, but detecting this similarity requires sampling of social partners at the same time or, at the very least, during the same breeding stage. For example, in previous studies focused on socially monogamous yet genetically polygynous birds, both females and males were sampled during nestling provisioning and found to have similar cloacal bacterial communities (Kreisinger et al. 2015, Lombardo et al. 1996, Stewart & Rambo 2000). However, of these previous studies two (Lombardo et al. 1996, Stewart & Rambo 2000) were culture-dependent studies that focused on a limited amount of pre-selected bacteria, and the other (Kreisinger et al. 2015) found a weak effect with regard to females and males having similar cloacal bacterial communities. In a study focused on a truly monogamous bird, the black-legged kittiwake (*Rissa tridactyla*), females and males of a social pair were sampled during incubation and nestling provisioning, respectively, and were nevertheless found to have similar cloacal bacterial communities (White et al. 2010). Given that black-legged kittiwakes exhibit true monogamy, such high fidelity between social partners may have maintained high similarity between the cloacal bacterial communities of pairs, regardless of females and males being sampled during different stages of the breeding season. Overall, to account for any changes in behavior and physiology across the breeding season and to most effectively assess similarity in cloacal bacterial communities between social partners, the sampling of both partners should be coordinated to occur at the same time.

Previous work examining the similarity of the cloacal bacterial communities of social partners in tree swallows used culture-dependent methods (Lombardo et al. 1996). The isolates cultured and studied by Lombardo et al. [see Table 1] included Lactobacilli, *Staphylococcus* spp., *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp. In our study, we used culture-independent, next-generation sequencing. We did not find that any of the cloacal bacteria Lombardo et al. (1996) cultured were members of the core bacterial communities of birds within our study population. While *Staphylococcus* spp. and Lactobacilli were detected, their relative abundances were <0.001%. This suggests that culturing of cloacal bacteria may not consistently result in isolation of the most dominant bacteria in the system. It is also possible that the bacteria cultured by

Lombardo et al. (1996) are rare in our study population and these bacteria were not retained during sample processing or sequence quality filtering.

In conclusion, our results suggest that the cloacal bacterial communities of female and male tree swallows are similar within our study population and that pair-bonded social partners do not share more similar cloacal bacterial communities than expected by chance. Given that tree swallows exhibit high variation in rates of extra-pair activity, we argue that considering rates of extra-pair activity or the number of sexual partners per bird when assessing cloacal bacterial communities may be important for understanding how cloacal bacterial communities are structured. Also, since cloacal bacterial communities comprise bacteria derived from both the reproductive and digestive tract, diet should also be considered when assessing cloacal bacterial communities. Lastly, we suggest that pair-bonded social partners should be sampled within the same breeding stage to control for any temporal shifts in individual behavior and physiology that may influence shifts in cloacal bacterial community structure across breeding stages.

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CHAPTER III: Assessing age, breeding stage, and mating activity as drivers of variation in the reproductive microbiome of female tree swallows

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Abstract

Sexually transmitted microbes are hypothesized to influence the evolution of reproductive strategies. Though frequently discussed in this context, our understanding of the reproductive microbiome is quite nascent. Indeed, testing this hypothesis first requires establishing a baseline understanding of the temporal dynamics of the reproductive microbiome and of how individual variation in reproductive behavior and age influence the assembly and maintenance of the reproductive microbiome as a whole. Here, we ask how mating activity, breeding stage, and age influence the reproductive microbiome. We use observational and experimental approaches to explain variation in the cloacal microbiome of free-living, female tree swallows (*Tachycineta bicolor*). Using microsatellite-based parentage analyses, we determined the number of sires per brood (a proxy for female mating activity). We experimentally increased female sexual activity by administering exogenous 17ß-estradiol. Lastly, we used bacterial 16S rRNA amplicon sequencing to characterize the cloacal microbiome. Neither the number of sires per brood nor increased sexual activity of females significantly influenced female cloacal microbiome richness or community structure. Female age, however, was positively correlated with cloacal microbiome richness and influenced overall community structure. A hypothesis to explain these patterns is that the effect of sexual activity and the number of partners on variation in the cloacal microbiome manifests over an individual's lifetime. Additionally, we found that cloacal microbiome diversity (Shannon Index, Faith's phylogenetic distance) decreased and community structure shifted between breeding stages. This is one of few studies to document within-individual changes and age-related differences in the cloacal microbiome across successive breeding stages. More broadly, our results

contribute to our understanding of the role that host life history and behavior play in shaping the cloacal microbiomes of wild birds.

Introduction

The social and reproductive behavior of animals can shape the diverse microbial communities ('microbiomes') that live in and on their bodies (Archie & Tung 2015, Dugatkin 2020, Ezenwa et al. 2012, Münger et al. 2018, Westneat 1987). Animal mating behaviors, for instance, can both transmit and acquire microbes that become incorporated into the host-associated reproductive microbiome. Considerable theoretical work has explored the potential transmission of microbes through mating (Boots & Knell 2002, Graves & Duvall 1995; Hamilton 1990, Kokko et al. 2002, Lockhart et al. 1996, Loehle 1995, Lombardo 1998, Lombardo et al. 1999, Poiani 2010, Shelldon 1993, Thrall et al. 1997, Thrall et al. 2000), but relatively few empirical studies focusing on the reproductive microbiome of wild animals exist. Further, though this theoretical work has advanced our understanding of the transmission and potential consequences of pathogenic microbes, it has largely ignored the reproductive microbiome as a whole. Microbes in the reproductive tract have the potential to considerably impact host reproductive function and thus may play a significant role in shaping the evolution of mate choice, sexual conflict, reproductive isolation, and mating systems more broadly (reviewed in Rowe et al. 2020).

In birds, amphibians, reptiles, cartilaginous fishes (i.e., chondrichthyans), and some mammals (i.e., monotremes), the cloaca is the reproductive structure of both sexes. The cloaca harbors a diverse community of microbes (hereafter the 'cloacal microbiome') as the terminus for the digestive and reproductive tracts and the site of contact and ejaculate transfer during copulation (e.g., White et al. 2011, Kulkarni & Heeb 2007, Westneat & Rambo 2000). The cloacal microbiome consists of a combination of beneficial, commensal, and pathogenic microbes, and can be shaped by a variety of factors, including host life history, environment, and behavior (Rowe et al. 2020). Within-individual changes in the phylogenetic diversity of male cloacal bacteria between breeding and non-breeding stages suggests that physiology and overall breeding condition may also play a role in shaping the cloacal microbiome (Escallón et al. 2019).

Socially monogamous species that engage in extra-pair copulations exhibit individual differences in the number of mates that they have during the breeding season and thus likely vary in their exposure to sexually transmitted microbes. While evidence of extra-pair copulations in socially monogamous species has been documented in insects (e.g., Dillard 2017), fish (e.g., Avise et al. 2002), amphibians (e.g., Liebgold et al. 2006), reptiles (e.g., Uller & Olsson 2008), and mammals (e.g., Cohas & Allainé 2009), birds are arguably the most well studied (e.g., Griffith et al. 2002) and the focus of this study. In birds, most studies examining the impact of mating behavior on reproductive microbiomes have selectively targeted a limited set of bacteria in social partners (Hupton et al. 2003, Lombardo & Thorpe 2000, Stewart & Rambo 2000) or have focused on interspecific comparisons of species with divergent mating systems using culture-dependent approaches (e.g., monogamous vs. polygamous: Poiani & Gwozdz 2002, Poiani & Wilks 2000a, Poiani & Wilks 2000b). Culture-independent work exploring how the reproductive microbiome is shaped with respect to mating has focused on genetically monogamous systems and/or pair-bonded social partners (Ambrosini et al. 2019, Hernandez et al. 2020, Kreisinger et al. 2015, White et al. 2010). For instance, pairs of genetically monogamous black-legged kittiwakes (*Rissa tridactyla*) exhibit similar cloacal bacterial communities when allowed to copulate, and this similarity was reduced when pairs were experimentally blocked from mating (White et al. 2010). Moreover, in barn swallows (*Hirundo rustica*), a socially monogamous species that exhibits extra-pair mating activity, social partners exhibited more similar cloacal bacterial communities than expected by chance, though one study acknowledged a low effect size with regard to this pattern (Kreisinger et al. 2015, Ambrosini et al. 2019). In contrast, the cloacal bacterial communities of tree swallow (*Tachycineta bicolor*) social partners were not more similar than expected by chance (Hernandez et al. 2020). However, these studies have not focused on variation in sexual activity and the number of sexual partners, two factors that are hypothesized to contribute to variation in cloacal microbiome community structure.

Here, we test the hypothesis that mating activity influences reproductive microbiome richness and community structure using both an observational and an experimental approach. First, we compared natural variation in the number of sires represented in a brood (a proxy for female mating activity) and the diversity of the cloacal microbiome of females in a population of tree swallows (*Tachycineta bicolor*). We predicted that females with more sires within their brood would have

increased cloacal bacterial richness and that the number of sires would explain variation in the structure of the bacterial community. Similarly, we predicted that older females would have higher cloacal bacterial richness compared to younger females, presumably in part due to the increased exposure to bacteria from previous mating opportunities that have occurred over their lifetime. Second, we performed an experimental study administering exogenous 17*ß*-estradiol (hereafter, 'estradiol') using silastic implants to increase female sexual activity and compared cloacal microbiome diversity to control females given blank implants. Exogenously administered estradiol has been consistently found to be positively associated with increased solicitations and copulations in female birds (e.g., Leboucher *et al*. 1998; Moore 1982). We predicted that females implanted with exogenous estradiol would more actively solicit copulations, both from their social mate and from other males, and would thus have higher cloacal microbiome richness compared to control females.

Methods

Study system & study site

We studied a breeding population of free-living tree swallows at Virginia Tech's Kentland Farm in Montgomery County, Virginia (37°11'53.6 N, 80°34'58.0 W; 520 m.a.s.l.; Fig 1). In this population, tree swallows breed from late March to early August and use nest boxes that we have set approximately 25 meters apart bordering agricultural fields. Tree swallows are socially monogamous, however, both females and males may engage in extra-pair solicitations, copulations, and fertilizations (Dunn et al. 2009; Lifjeld et al. 1993). In previous studies of other populations, the proportion of tree swallow broods containing extra-pair young ranged from 68–87% (see Table 4 in Conrad et al. 2001; Dunn et al. 1994a, 1994b, Lifjeld et al. 1993; Kempenaers et al. 1999). Tree swallows are generally considered to be a single-brooded species; however, there is some evidence for double-brooding (e.g., Monroe et al. 2008). In our study population, the majority of females are single-brooded. The few that lay a second clutch each year do so if their first clutch failed, often due to predation or a cold snap. Only one or two females a year will lay a second clutch after having a successful first clutch (Hernandez, *unpublished data*). All methods in this study were approved by the Institutional Animal Care and Use Committee of Virginia Tech.

Figure 1. Tree swallows during the breeding season in southwestern Virginia, USA. Photo captured by Ben J. Vernasco.

Study design

Our goal for the observational study was to assess the relationship between the number of sires per brood and variation in the female's cloacal microbiome. We sampled a total of 388 individuals (71 adult females and 317 nestlings) in 2017 (Table 1). The cloacal bacterial samples from all 71 adult females were used for microbiome analyses. We assessed age based on plumage as first-time breeding females (or females in their second year, hereafter, 'SY' females) had immature brown plumage, while females breeding after their second year (hereafter, 'ASY' females) had mature blue plumage (Hussell 1983). To assess rates of extra-pair fertilizations (a conservative measure of extra-pair copulations), 60 of the 71 sampled females and their putative offspring (i.e., 278 nestlings comprising 60 broods) were genotyped for paternity analyses (via DNA extracted from blood). See Table 1 for sample sizes. Each individual was sampled once.

Our goal for the experimental study was to assess the effects of increased sexual activity on the richness and community structure of the female cloacal microbiome. We experimentally administered exogenous estradiol and subsequently assessed nestling paternity to determine if the treatment affected extra-pair fertilizations, in addition to overall sexual activity. We included 10 females (N = 3 SY, N = 7 ASY) and 41 nestlings from 2018, and 34 females (N = 7 SY, N = 27 ASY) and 112 nestlings from 2019 in this study (Table 1). We were unable to genotype all females and their respective broods due to females abandoning their nests (N = 5 in 2019, 3 from estradiol-implanted and 2 from blank-implanted females) or nests being predated (N = 1 in 2018, N = 1 in 2019, both from SY estradiol-implanted females; Table 1). We considered nests to have been predated if we observed a snake in the box or a ransacked nest, or abandoned if the eggs were present but cold, and never hatched. The blood DNA of 37 females and their putative offspring (i.e., 153 nestlings comprising 37 broods) were genotyped for paternity analyses (Table 1). These included 9 females and 41 offspring comprising 9 broods genotyped in 2018, and 28 females and 112 offspring comprising 28 broods genotyped in 2019. We combined the data for the 2018 and 2019 seasons for subsequent statistical analyses.

Table 1. Number of female tree swallows sampled with respect to treatment group, number of sires per brood, age, and year.

	Observational Study	Experimental Study		
Number of sires per brood	**No implant**	**No implant**	**Blank implant**	**Estradiol implant**
1	12	6	2	1
2	35	3	7	9
3	11	1	5	2
4	2	1	-	-
No data				
Predated	-	-	-	2
Abandoned	-	-	2	3
Age				
SY	26	2	3	4
ASY	45	9	13	13
Year				
2017	71			
2018		3	3	4
2019		8	13	13

Sample collection

For the observational study, we caught adult and nestling tree swallows in nest boxes from March–July, 2017. We determined the pair bonded female and male social pair of a nest through observational surveys. Specifically, we assumed that the female found incubating the eggs and the male seen most frequently feeding the subsequently hatched nestlings were the social pair of each nest. We captured and sampled adult females on day six of incubation, adult males on day three of nestling provisioning, and nestlings on day six post-hatching. In tree swallows, incubation typically lasts 11–20 days and the nestling period lasts 15–25 days (Winkler et al. 2020), with nestling provisioning occurring from the onset of nestlings hatching to just a few days prior to

fledging. To collect cloacal bacteria for microbiome analyses, we gently inserted a sterile swab (PurFlock®, Puritan, USA) approximately 4 mm into the cloaca of adult tree swallows and revolved it once. We used sterile techniques when sampling the cloaca by using new gloves and a new pre-sterilized and pre-sealed swab per bird sampled. To collect blood for paternity analyses and condition assessments, we gently punctured the brachial vein and collected ~100 µL of blood from adults and ~75 µL of blood from nestlings using microhematocrit capillary tubes. Swab and blood samples were stored on ice in the field. Swab samples were frozen upon return to the lab (< 5 hours post-collection) in a -80°C freezer. Blood samples were centrifuged, and then plasma and red blood cells were frozen in separate tubes.

For the experimental study, we caught each adult female twice, first during nest building, which is prior to egg laying, and again on day six of egg incubation (mean time between sampling breeding periods: 23 days; range: 9-66 days) from March–July 2018 and 2019. Upon each capture, we sampled cloacal bacteria using a sterile swab (PurFlock®, Puritan, USA), collected a blood sample, and took morphometric measurements. The first time we caught each female (i.e., during nest building), we randomly assigned her to one of three groups: a no implant control, a blank implant control, or an estradiol implant. Females in the estradiol treatment groups received a silastic implant packed with crystalline estradiol (e.g., Danner et al. 2011), while females in the blank implant group received an empty silastic implant. All implants were ~6 mm in length, sealed at both ends, and were inserted subcutaneously along the female's left flank. We chose to make the first sampling period prior to egg laying to allow enough time for the implant to release exogenous hormone (if the female was given an estradiol implant) and thus alter the sexual behavior of the female before laying. Previous work in a temperate-breeding songbird found that females treated with exogenous estradiol exhibited significantly more solicitation displays compared to control females as soon as 4 days post-implantation (Searcy & Capp 1997) and as long as 80 days post-implantation (Moore 1982). The second time we caught each female, we collected all samples and then removed the implant. We chose to take the second sample on day six of egg incubation to decrease the likelihood that the female would abandon the breeding attempt. Adult male and nestlings were captured and sampled as before.

Body condition estimates

To assess body condition, we calculated both a morphological- and a physiological-based index. For the morphological index, we calculated a scaled-mass index based on a bird's mass and wing length (Peig & Green 2009). For the physiological index, we measured each bird's hematocrit, the ratio of red blood cell volume to total blood volume (Minias 2014 but see: Smith & Barber 2012).

Blood DNA processing

Blood DNA samples were processed in fall 2017 for the observational study and in 2018 and 2019 for the experimental study. To extract DNA from blood samples, we used the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA). We extended the initial 56°C incubation to 24 hours to optimize the lysing of red blood cells. To perform multiplex PCR-based analyses of microsatellites, we used the Type-it Microsatellite PCR Kit (Qiagen Inc., Valencia, CA, USA) and amplified the extracted DNA at eight highly polymorphic microsatellite loci in two multiplexed PCRs (*See Supplemental Information*, Table S1). We amplified the extracted DNA at two additional highly polymorphic microsatellite loci (Tal11 and Tal8) in our multiplexed PCRs for the experimental study (Table S2). Forward primers were fluorescently labeled. We modified the Type-it® Microsatellite PCR Kit protocol to run 12.5 μL reactions. The thermal cycling conditions were as follows: 2 min at 95°C, followed by 28 cycles of 30 s at 95°C, 90 s at 56°C or 58°C, and 30 s at 72°C. A final extension step consisted of 30 min at 60°C. PCR mix A (see Table S1) was run at 56°C, while PCR mix B was run at 58°C. We prepared a 1:10 dilution of each multiplexed PCR product and sent samples to the DNA Analysis Facility on Science Hill at Yale University for genotyping. Genotyping was performed on a 3730xl 96-Capillary Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA), with DNA fragment sizes estimated using GeneScan™ 500 LIZ® size standard.

We scored alleles using Geneious v.10.2.2 (Biomatters) and then determined the observed and expected heterozygosity of loci using CERVUS (v. 3.0.7; Kalinowski et al. 2007; Marshall et al. 1998). Next, we inferred parentage and sibship using COLONY (v. 2.0.6.5; Wang 2004; Jones & Wang 2010). We specified a female and male polygamous mating system, without inbreeding or cloning. We selected a full-likelihood analysis approach, with a medium run length, and medium likelihood precision. We opted not to have the program update allele frequency, scale sibship, or

assume a prior maternal or paternal sibship size distribution. Further, we specified a codominant marker type, 0.001 allelic dropout rate, and 0.001 genotyping error rate for all eight loci. We also allowed for putative mothers to mismatch offspring at only one locus (Ferretti et al. 2016; Fernando et al. 2001). The allele frequency and inbreeding coefficient for the sampled population was estimated in COLONY based on the genotypes of all sampled individuals. From the COLONY output, we first compared the genetic profiles of nestlings and the social female (putative mother) to confirm maternal parentage. Then, based on the genotypes of the sampled nestlings, the genotype of the sampled mother, and the frequency of alleles of the sampled population, COLONY reconstructed the minimum number of sires (based on genotypes) that would need to exist to account for the allelic variation in nestling genotypes within a brood.

Bacterial DNA processing

We processed bacterial DNA samples as in Hernandez et al. (2020) for bacterial DNA extraction, amplification, and sequencing. Briefly, we extracted DNA from cloacal swabs using the DNeasy Blood and Tissue Kit protocol. We used one kit for the observational study samples and one kit for both years of the experimental study samples. We used the primers 515F and 806R to target the hypervariable V4 region of the 16S rRNA gene for amplification. The 806R primers included uniquely indexed adaptors to allow for multiplexing. We performed PCR in triplicate and ran negative controls without template DNA for each sample. We pooled the triplicate reactions for each sample and visualized the PCR products using agarose gel electrophoresis. Then, we quantified the amplified bacterial DNA in each sample using a Qubit 2.0 Fluorometer and a dsDNA High Sensitivity assay kit. Lastly, we pooled 200 ng DNA from each sample into a single library for sequencing. The library was sequenced using 1 x 250bp sequencing on the Illumina MiSeq instrument, as described by Caporaso et al. (2012), at the Dana Farber Cancer Institute of Harvard University. Samples collected in 2017 and 2018 were each sequenced in separate runs (2017: 71 samples, 2018: 20 samples). Samples collected in 2019 were sequenced in two separate runs (34 samples/run), with samples randomly allocated to each run.

We de-multiplexed and quality filtered single end reads of raw 16S rRNA amplicon sequences using the Quantitative Insights Into Microbial Ecology (QIIME) 2 (version 2018.11) pipeline (Bolyen et al. 2019). Reads were error corrected and filtered to remove phiX and chimeric reads

using DADA2 (Callahan et al. 2016). We specified a truncation quality score of 11, with no end trimming, retaining the full 250bp read length across all samples. After DADA2 filtering, reads per sample for the observational study ranged from 31,252 to 168,791, with a total of 71 samples and 14,111 amplicon sequence variants (ASVs) represented. After DADA2 filtering, we merged the three resulting ASV tables for the experimental study together and the reads per sample ranged from 11 to 121,932, with a total of 88 samples and 20,336 ASVs represented. We next removed ASVs with fewer than 0.01% of the total number of reads (i.e., total frequency fewer than 829 reads in the observational study dataset and 747 in the experimental study dataset) (Bokulich et al. 2013). To taxonomically classify our ASVs, we used a Naïve Bayes classifier that was pre-trained on the Silva 132 database 99% ASVs from the 515F/806R region of sequences (Quast et al. 2013) using scikit-learn 0.20.2 (Pedregosa et al. 2011). We filtered out all ASVs that were taxonomically annotated as Mitochondria, Chloroplast, Eukaryota, and Unassigned. Our final sampling depth for the observational study dataset ranged from 25,159 to 159,867. Based on the alpha rarefaction curve, samples were rarefied to 25,000 to standardize sequencing effort. Our final observational study table contained 71 samples and 527 ASVs. Our final sampling depth for the experimental study dataset ranged from 1,380 to 121,359, with 682 ASVs represented. Based on alpha rarefaction curves, we rarefied samples to 7,400. Our final experimental study table contained 88 samples and 682 ASVs.

Hormone assay

We measured the plasma volume for all blood samples (2017 mean: 26.6 μL, 2018 mean: 33.8 μL, 2019 mean: 34.2 μL) and then extracted the sample with dichloromethane. Then, we quantified the total plasma estradiol concentration (ng/mL) using a direct radioimmunoassay (following Moore et al. 2004, Wingfield et al. 1991), adjusted for individual extraction efficiency (2017: 42.1%, 2018: 42.1%, 2019: 46.1%). We ran samples from 2017 and 2018 in the same assay, and samples from 2019 in a separate assay. To maximize the hormone detection probability, we ran samples in singlet in one assay. The order of individual samples within the assay was randomized. The intra-assay coefficient of variation, which estimates the variation among standards within an assay, was 14.6%, 14.6%, and 2.63% for 2017, 2018, and 2019 respectively. The assay limit of detection was ~1.3, 1.3, and 0.5 ng/mL in 2017, 2018, and 2019 and was calculated based on individual plasma volumes and extraction efficiency.

Statistical analyses

Observational Study

We performed all analyses in R (v. 3.6.1) (R Core Team 2018). For the microbiome analyses, we assessed shared and unique ASVs across female cloacal bacterial communities. In addition, we determined the most abundant ASVs in the cloaca of each sampled female bird using a relative abundance cutoff of 5%.

To identify the possible predictors associated with the alpha diversity of female cloacal bacterial communities, we used model selection and multimodal inference approaches. Alpha diversity is an estimate of within-sample diversity (in this case, within an individual bird's cloaca) and is based on ASVs for this study. We calculated three alpha diversity metrics: ASV richness (the total number of taxa), Shannon Index (considers both species abundance and evenness), and Faith's phylogenetic distance (assesses phylogenetic breadth; hereafter, 'Faith's PD'). We generated competing linear models that included cloacal microbiome alpha diversity (ASV richness, Shannon Index, Faith's PD) as the response variable, and number of sires per brood (1, 2, 3+) and female age (SY, ASY) as covariates. We then used Akaike's Information Criterion (AICc) corrected for small sample sizes to compare and rank these competing models (Table S3; Akaike 1973; Burnham & Anderson 2002), selecting models with a ΔAICc of <2 as 'better fit' models. We incorporated covariates from the highest ranked models into our assessments of cloacal bacterial community diversity; summaries of competing 'best fit' models (ΔAICc of <2) are presented in Table S4. Lastly, we used ggplot ('ggplot2':*ggplot*; Wickham 2016) for visualizations.

To assess cloacal bacterial beta diversity or whether the variation in cloacal bacterial community structure differed among females with respect to age and number of sires per brood, we performed non-parametric, permutational multivariate analyses of variance (PERMANOVA; 'vegan': *adonis*) based on 999 permutations (Anderson 2001; McArdle & Anderson 2001). Beta diversity is an estimate of variation in community structure among samples (in this case, among birds) and was computed based on both Bray-Curtis and Jaccard distance metrics. While Bray-Curtis and Jaccard both evaluate count-based data, Bray-Curtis evaluates relative abundances, while Jaccard evaluates presence-absence. We compared the multivariate homogeneity of group dispersions

('vegan': *betadisper*) for sampled females with respect to age and number of sires per brood using a permutation test ('vegan': *permutest*) (Anderson 2006; Anderson et al. 2006). Finally, we used a non-metric multidimensional scaling (NMDS; Kruskal 1964a,b) plot to visualize the dissimilarity distances among females.

To identify the most relevant predictors associated with the body condition indices of females, we used the same model selection and AICc comparisons as for the alpha diversity analyses above. Briefly, we generated linear models that included body condition (scaled-mass index, hematocrit) as the response with both number of sires per brood and female age as covariates, and then used AICc to compare and rank the models (Table S5). We incorporated covariates from the highest ranked models into our assessments of condition. We also generated linear models that included the alpha diversity metric (ASV richness, Shannon Index, or Faith's PD) as the response variable and the host body condition as the predictor variable. To assess whether the variation in cloacal bacterial community structure differed among females with respect to body condition, we performed PERMANOVAs and compared the multivariate homogeneity of group dispersions as before.

To assess if female age or the number of sires per brood had an effect on a female's reproductive success, we assessed average brood mass (i.e., average mass of all nestlings within a brood), hatch success, and fledging success. For each model we set the reproductive success metric as the response variable. For average brood mass, we chose to fit a linear model ('stats': *lm*) with female age (or number of sires per brood) and nestling age at sampling as predictor variables. For hatch success, we fit a generalized linear model with a beta distribution ('betareg': *betareg*) and included female age or number of sires per brood as the predictor variable and weighted the model by the total number of eggs laid (mean: 5 eggs; range: 3-6 eggs). We transformed the response variable (i.e., hatch success) using the transformation recommended by the R documentation for this function, because the data included 0 and 1. The transformation recommended is as follows: $(y \cdot (n-1)+0.5)/n)$), where n is the sample size (Cribari-Neto & Zeileis 2009, Smithson & Verkuilen 2006). For fledging success, we fit a linear model and included female age or number of sires per brood as the predictor variable.

To assess whether the number of sires per brood was influenced by circulating estradiol concentrations in females and female age, we used model selection and multimodal inference statistical approaches. We generated competing linear models that included the number of sires per brood as the response variable and estradiol concentrations (natural log-transformed) and female age as predictor variables (Table S9). We removed 8 samples for which there was less than 10 μL plasma used in the radioimmunoassay, since the volume was too low to effectively quantify estradiol concentration. Then, we used AICc to compare and rank our competing models (Akaike 1973; Burnham & Anderson 2002). We incorporated covariates from the highest ranked models into our assessments of circulating estradiol concentrations.

Experimental Study

We assessed the clutch and brood sizes for all sampled females. To determine whether clutch or brood size varied among females in different treatment groups, we ran ANOVA models with clutch size or brood size as the response and female treatment group as the predictor, followed by a Tukey's post-hoc test to assess significant differences between groups. We also ran an ANOVA model with the number of sires per brood (1,2,3,4) as the response and female treatment group as the predictor. Note that we set the number of sires per brood as a continuous response variable when set as the response but consider the number of sires per brood as a categorical variable when set as a predictor elsewhere.

To confirm whether the administration of exogenous estradiol (via the estradiol implant) had an effect on circulating estradiol concentrations in females, we generated a linear model with estradiol concentrations (natural log-transformed) as the response variable and treatment group (blank implant, estradiol implant), sampling year (2018, 2019), and the number of days a female was implanted (mean: 23 days; range: 9-66 days) as predictor variables. Note that we only included samples taken during incubation for this analysis and that we only consider the blank implant as the true control for the estradiol implant treatment.

We assessed shared and unique ASVs across female cloacal bacterial communities. In addition, we determined the most abundant ASVs in the cloaca of each sampled female using a relative abundance cut off of 5%.

We calculated these three alpha diversity metrics (ASV richness, Shannon Index, and Faith's PD) for each female for both sampling events (i.e., nest building and incubation), and then calculated the change in each metric. To assess if treatment group had an effect on the change in cloacal bacterial diversity, we used model selection and a multimodal inference approach. We generated competing linear models for each diversity metric: ASV richness, Shannon Index, and Faith's PD. For each of the three linear models, we set change in the diversity metric as the response variable (ASV richness, Shannon Index, Faith's PD) and treatment (no implant, blank implant, estradiol implant), year of sampling (2018, 2019), and the number of days a female was implanted (mean: 23 days; range: 9-66 days) as possible explanatory variables (Table S11). We did not include female age in the models because our ability to detect an effect was weak given the low sample sizes (see Table 1). We used ggplot ('ggplot2':*ggplot*; Wickham 2016) for visualizations.

To determine whether there was an overall change in female cloacal bacterial diversity between breeding stages regardless of treatment, we fit linear mixed-effect models and set the diversity metric as the response variable and breeding stage (nest building, incubation) as the explanatory variable with the bird's unique band ID included as a random effect.

To assess whether the variation in the structure of cloacal bacterial communities differed among females, we performed PERMANOVA analyses ('vegan': *adonis*) based on 999 permutations (Anderson 2001; McArdle & Anderson 2001). We set the distance metric (Bray-Curtis, Jaccard) as the response variable and included treatment group, breeding stage, year of sampling, and the number of days implanted as explanatory variables, with an interaction between treatment group and breeding stage. We also controlled for repeated measures in the dataset by including each bird's unique band ID in the 'strata' argument of the PERMANOVA model. We compared the multivariate homogeneity of group dispersions ('vegan': *betadisper*) for sampled females with respect to treatment and sampling event using permutation tests ('vegan': *permutest*) (Anderson 2006; Anderson et al. 2006). We performed permutation tests to assess the homogeneity of group dispersions of cloacal bacterial communities per treatment before and after implantation. Finally, we used a NMDS (Kruskal 1964a,b) plot to visualize the dissimilarity distances between sampled females.

To assess if treatment group had an effect on a female's condition, we fit a linear model for each condition index. For each model, we set the condition metric as the response variable (scaled-mass index, hematocrit) and included treatment group, year of sampling, and the number of days implanted as predictor variables. The variable 'Year' was not included in models for hematocrit because we did not have data on hematocrit for 2018. In the case of treatment significantly varying with a condition metric (in this study, e.g., hematocrit), we performed statistical comparisons among treatments ('emmeans': *contrast*) to compare the hematocrit of females in different treatment groups. Further, to assess whether microbiome diversity and/or community structure varied with host condition, we ran linear models and performed PERMANOVAs as before.

To assess if treatment group had an effect on a female's reproductive success, we assessed average brood mass, hatch success, and fledging success as for the observational study analyses, with a few changes. For all three models, we set female treatment as the main predictor of interest and we performed statistical comparisons among treatments ('emmeans': *contrast*) to compare the reproductive success of females in different treatment groups.

Results

Observational study

The average clutch size was 5.2 ± 0.09 eggs (mean ± SEM, N = 71 clutches, range = 3-6 eggs) and the average brood size was 4.5 ± 0.13 nestlings (mean ± SEM, N = 71 broods, range = 2-6 nestlings) for the sampled population.

We identified 527 ASVs across 71 individual female cloacal bacteria samples. The families with a mean relative abundance >5% included Corynebacteriaceae (relative abundance 23%, phylum Actinobacteria), Enterobacteriaceae (22%, Proteobacteria), Enterococcaceae (7%, Firmicutes), and Micrococcaceae (6%, Actinobacteria). Families within the phyla Bacteroidetes, Chlamydiae, Chloroflexi, Cyanobacteria, Fusobacteria, Deinococcus-Thermus, Epsilonbacteraeota, FBP, Tenericutes, and an unidentified phylum comprised the remaining ~42%. More specifically, the ASVs with the highest relative abundance (all identified down to genus) included

Corynebacterium 1 (22%), *Escherichia-Shigella* (16%), *Enterococcus* (4%), *Rothia* (4%), and *Exiguobacterium* (3%). The most prevalent ASVs (identified down to genus) were present in 90% of females and included *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Corynebacterium 1*, *Escherichia-Shigella*, *Exiguobacterium*, *Rothia*, and *Sphingomonas*. There was an average (± standard deviation) of 126 ± 66 ASVs/female (range: 13-281).

Female age, but not number of sires per brood, was significantly related to ASV richness, the Shannon Index, and Faith's phylogenetic distance (Table S3, S4). More specifically, female age was positively associated with ASV richness (b = 36.2, SE = 15.8, p = 0.03), Shannon Index (b = 0.68, SE = 0.32, p = 0.03), and Faith's PD (b = 3.0, SE = 1.4, p = 0.04; Fig 2).

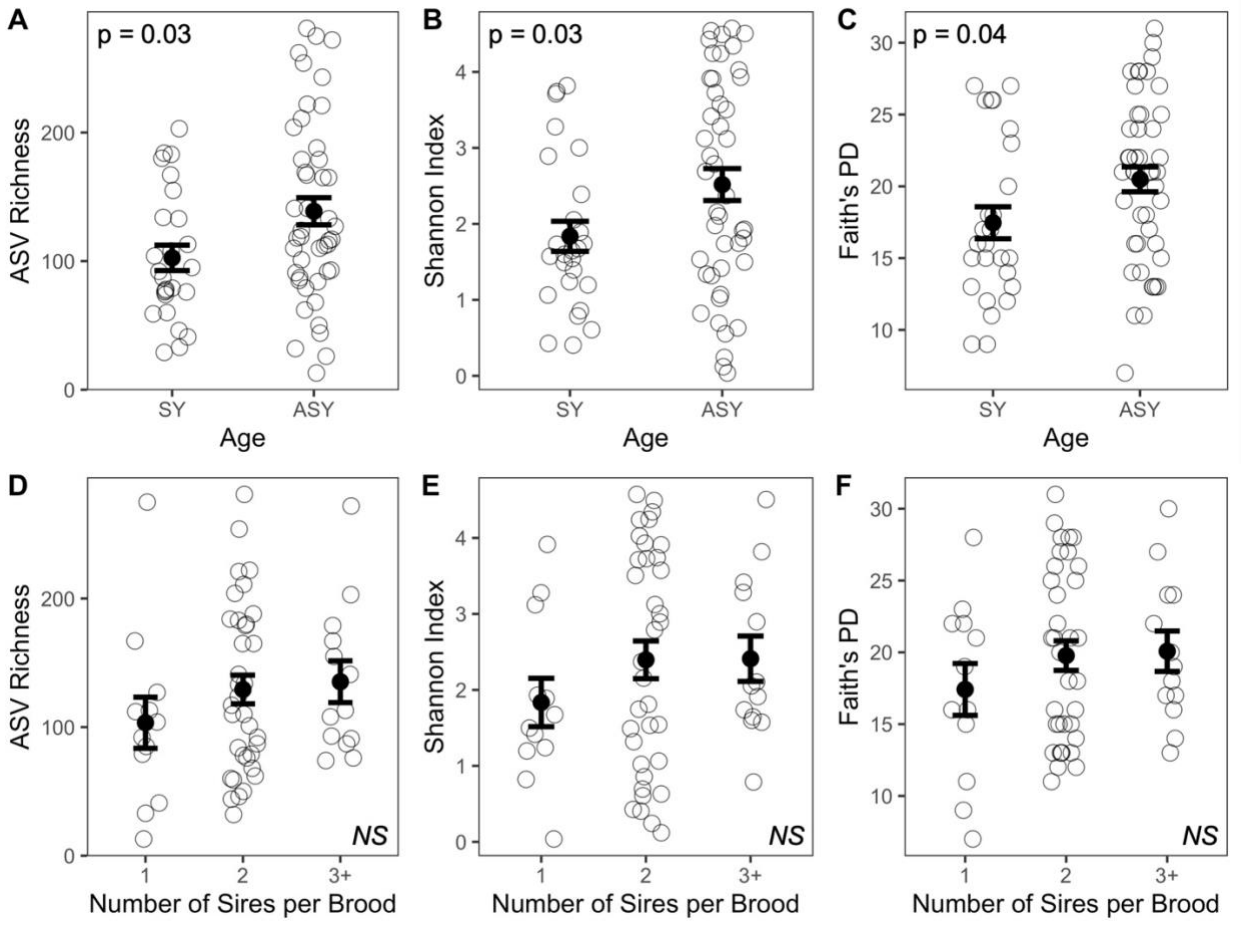

Figure 2. ASV richness (A,D), Shannon Index (B,E), and Faith's phylogenetic distance (C,F) of bacterial amplicon sequence variants (ASVs) sampled from the cloacae of female tree swallows with respect to female age (A-C) and the number of sires (D-F) per brood. 'SY'

refers to Second Year females, i.e., females sampled during their first breeding season, while, 'ASY' refers to After Second Year females, i.e., females sampled after their first breeding season. Each point represents an individual female. Sample sizes include n = 26 (SY) and n = 45 (ASY) females for panels A-C and n = 12 (1 sire), n = 35 (2 sires), and n = 13 (3+ sires) for panels D-F. Mean ± standard error is shown. P-values are based on linear models.

Female age also explained significant variation in the structure of cloacal bacterial communities (Bray-Curtis: *pseudo-*$F_{1,69}$ = 2.3, p = 0.03; Jaccard: *pseudo-*$F_{1,69}$ = 2.0, p = 0.02), though the effect was relatively weak (Bray-Curtis: R^2 = 0.03; Jaccard: R^2 = 0.03) (Fig 3A). The dispersion of female cloacal bacterial communities did not differ significantly based on female age (Bray-Curtis: *pseudo-*$F_{1,69}$ = 0.62, p = 0.43; Jaccard: *pseudo-*$F_{1,69}$ = 0.89, p = 0.35). Females did not significantly differ in the structure of cloacal bacterial communities when considering their number of sires per brood (Bray-Curtis: *pseudo-*$F_{2,57}$ = 0.78, R^2 = 0.03, p = 0.74; Jaccard: *pseudo-*$F_{2,57}$ = 0.83, R^2 = 0.03, p = 0.75) (Fig 3B). The dispersion of female cloacal bacterial communities did not differ significantly based on number of sires (Bray-Curtis: *pseudo-*$F_{2,57}$ = 0.52, p = 0.60; Jaccard: *pseudo-*$F_{2,57}$ = 0.50, p = 0.61)

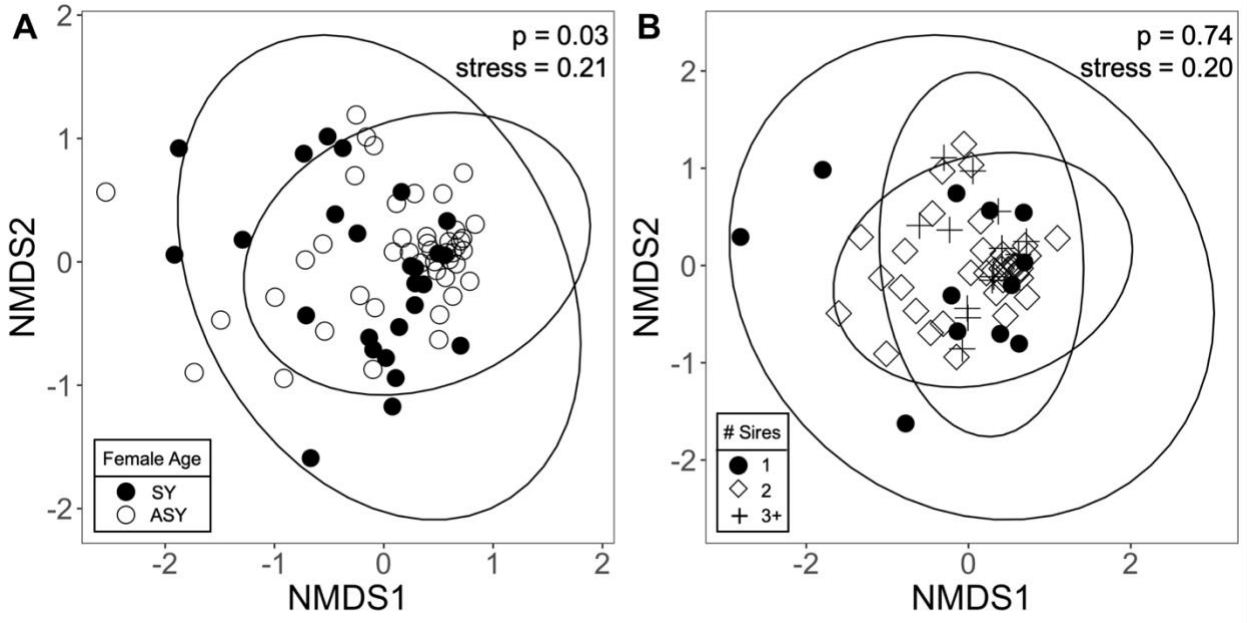

Figure 3. Cloacal bacterial beta diversity (non-metric multidimensional scaling plot, NMDS, based on Bray-Curtis dissimilarity) of adult tree swallows with respect to (A) female age and (B) number of sires per brood. Each point represents the cloacal bacterial community of one

female. In (A), closed circles = SY females, open circles = ASY females. In (B), closed circles = broods with 1 sire, open diamonds = broods with 2 sires, cross = broods with 3+ sires. Sample sizes include $n = 71$ for panel A and $n = 60$ for panel B. NMDS based on Jaccard dissimilarity (not pictured here) is similar. P-values are based on PERMANOVA models.

We found some support for a relationship between scaled-mass index and the number of sires per brood, but not between scaled-mass index and female age (Tables S5, S6, S7). More specifically, the difference in scaled-mass index between females with one sire per brood compared to two sires per brood was significant (1-2: $b = -1.8$, $SE = 0.66$, $p = 0.03$). We also found no evidence for a relationship between hematocrit and either the number of sires per brood or female age (Tables S5, S6, S7).

Additionally, we found no relationship between either scaled-mass index or hematocrit and cloacal microbiome alpha diversity (*SMi*, ASV richness: $b = 3.3$, $SE = 3.9$, $p = 0.40$; Shannon Index: $b = 0.02$, $SE = 0.08$, $p = 0.76$; Faith's PD: $b = 0.16$, $SE = 0.35$, $p = 0.65$; *Hematocrit*, ASV richness: $b = 70.3$, $SE = 260$, $p = 0.79$; Shannon Index: $b = 3.8$, $SE = 4.9$, $p = 0.44$; Faith's PD: $b = 13.0$, $SE = 23.0$, $p = 0.57$). There was no relationship between either scaled-mass index or hematocrit and cloacal microbiome structure (*SMi*, Bray-Curtis: *pseudo*-$F_{1,68} = 0.61$, $R^2 = 0.01$, $p = 0.84$; Jaccard: *pseudo*-$F_{1,68} = 0.66$, $R^2 = 0.01$, $p = 0.90$; *Hematocrit*, Bray-Curtis: *pseudo*-$F_{1,65} = 0.55$, $R^2 = 0.01$, $p = 0.89$; Jaccard: *pseudo*-$F_{1,65} = 0.66$, $R^2 = 0.01$, $p = 0.90$).

Hatch success, but not average brood mass or fledging success, significantly varied with female age (Table S8). More specifically, broods of older females had significantly lower hatch success compared to broods of younger, first-time breeding females ($b = -0.59$, $SE = 0.11$, $p < 0.001$). There were no relationships between any of these three reproductive success proxies and the number of sires per brood (Table S8).

We found no relationship between the number of sires per brood and circulating estradiol concentrations (Table S9, S10), even when considering female age (Table S9, S10).

Experimental study

In 2018, the average clutch size was 5.0 ± 0.15 eggs (mean ± SEM, N = 10 clutches, range = 4-6 eggs) and the average brood size was 4.1 ± 0.50 nestlings (mean ± SEM, N = 10 broods, range = 0-5 nestlings) for the sampled population. In 2019, the average clutch size was 5.1 ± 0.21 eggs (mean ± SEM, N = 34 clutches, range = 2-8 eggs) and the average brood size was 3.3 ± 0.37 nestlings (mean ± SEM, N = 34 broods, range = 0-7 nestlings) for the sampled population. Neither clutch size nor brood size significantly differed among females based on treatment group (*ANOVA*, Clutch size*:* $F_{2,41}$ = 1=0.9, p = 0.41, Brood size: $F_{2,41}$ = 2.2, p = 0.13). The number of sires per brood was not statistically different with respect to treatment group (*ANOVA*, $F_{2,34}$ = 1.3, p = 0.28); the mean number of sires per brood was 1.7 for non-implanted females, 2.2 for blank-implanted females, and 2.1 for estradiol-implanted females.

Exogenous estradiol administration (via the estradiol implant) did have an effect on circulating estradiol concentrations (b = 0.69, SE = 0.29, p = 0.03), with circulating estradiol concentrations higher in estradiol-implanted females compared to blank-implanted females during incubation. Sampling year also had a significant effect on circulating estradiol concentrations (b = -0.97, SE = 0.34, p = 0.01), with females sampled in 2019 exhibiting lower estradiol concentrations compared to females sampled in 2018 during incubation. There was no effect of the number of days a female was implanted on circulating estradiol concentrations (b = -0.002, SE = 0.01, p = 0.86).

We identified 682 ASVs across 88 female cloacal bacteria samples. During nest building, the families with a mean relative abundance >5% included Rhizobiaceae (relative abundance 14%, Proteobacteria), Corynebacteriaceae (14%, phylum Actinobacteria), Sphingomonadaceae (9%, Proteobacteria), Enterobacteriaceae (7%, Proteobacteria), Burkholderiaceae (6%, Proteobacteria), and Mycoplasmataceae (5%, Tenericutes). Families within the phyla Acidobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Dependentiae, FBP, Firmicutes, Opisthokonta, Planctomycetes, Verrucomicrobia, and an unidentified phylum comprised the remaining ~45%. More specifically, the ASVs with the highest relative abundance (all identified down to genus, except for the last one which was identified down to family) included *Corynebacterium 1* (14%), *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (7%), *Escherichia-Shigella* (4%), *Sphingomonas* (3%), and Rhizobiaceae (3%). The most prevalent

ASVs (present in 90% of females and identified down to genus) were *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Flavobacterium*, *Pantoea*, and *Sphingomonas*. There was an average (± standard deviation) of 135 ± 52 ASVs per female (range: 49-274). During incubation, the families with a mean relative abundance >5% included Enterobacteriaceae (20%, Proteobacteria), Corynebacteriaceae (16%, Actinobacteria), Enterococcaceae (7%, Firmicutes), Mycoplasmataceae (7%, Tenericutes), and Rhizobiaceae (6%, Proteobacteria). Families within the phyla Acidobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Dependentiae, FBP, Opisthokonta, Planctomycetes, Verrucomicrobia, and an unidentified phylum comprised the remaining ~44%. More specifically, the ASVs with the highest relative abundance (all identified down to genus) included *Corynebacterium 1* (16%), *Escherichia-Shigella* (14%), *Catellicoccus* (6%), *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (3%), and *Ureaplasma* (3%). The most prevalent ASVs (present in 90% of females and identified down to genus) were *Corynebacterium 1*, *Escherichia-Shigella*, *Flavobacterium*, and *Sphingomonas*. There was an average (± standard deviation) of 116 ± 53 ASVs per female (range: 19-226).

There was no significant relationship between the change in female cloacal bacteria alpha diversity (ASV richness, Shannon Index, or Faith's PD) and treatment group (Tables S11, S12). There was a positive relationship between the number of days a female was implanted (i.e., time between the two sampling periods) and both ASV richness (b = 1.4, SE = 0.58, p = 0.02) and Faith's PD and (b = 0.17, SE = 0.1, p = 0.04). And there was a negative relationship between the change in Faith's PD and sampling year (b = -5.3, SE = 2.5, p = 0.04).

Regardless of treatment, female cloacal bacterial diversity decreased between nest building and incubation (Fig 4). More specifically, there was a significant decrease in Shannon Index (b = -0.83, SE = 0.33, p = 0.02) and in Faith's PD (b = -3.4, SE = 1.6, p = 0.04), and a trending decrease in ASV richness between nest building and incubation (b = -18.8, SE = 11.1, p = 0.10).

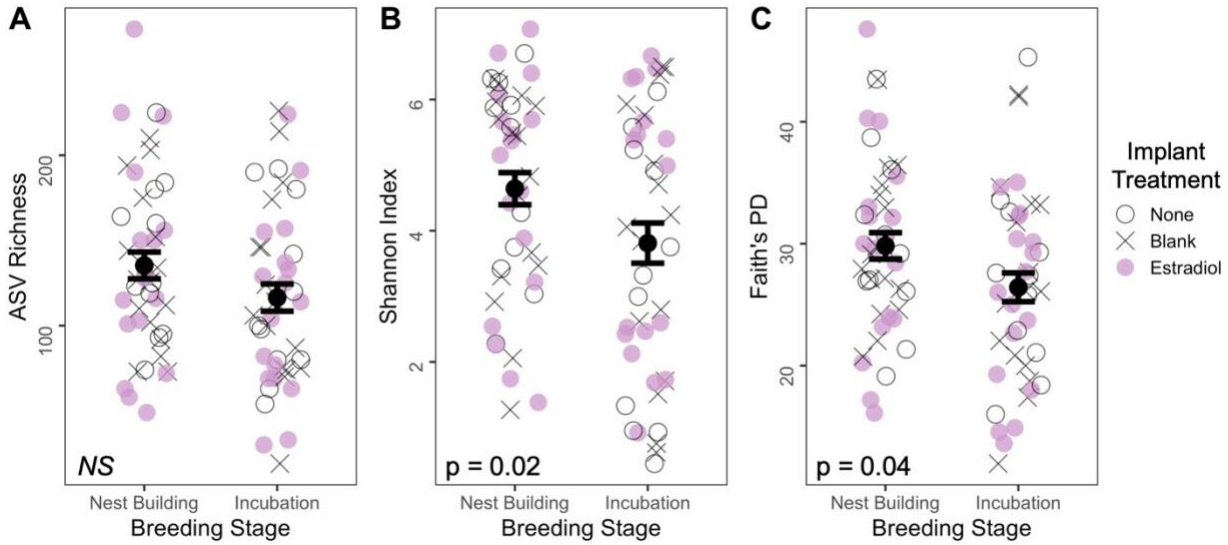

Figure 4. Alpha diversity metrics for bacterial amplicon sequence variants (ASVs) sampled from the cloacae of female tree swallows taken during two breeding stages (Nest Building, Incubation). ASV richness (A), Shannon Index (B), and Faith's phylogenetic diversity (C) indices were calculated. The first sampling time point (Nest Building) refers to initial capture and implantation, while the second sampling time point (Incubation) refers to the second capture event when the implant was removed. Point color and shape represent female treatment group: no implant control = open circle, blank implant = X, estradiol implant = pink filled circle. A total of 44 females were sampled during nest building and the same females were sampled again during incubation. Sample sizes include n = 10 females in 2018 and n = 34 females in 2019. Mean ± SEM are shown. P-values are based on linear mixed-effect models.

There was a significant difference in cloacal bacterial community structure between nest building and incubation (Bray-Curtis: *pseudo*-$F_{1,80}$ = 3.4, R^2 = 0.04, p < 0.001; Jaccard: *pseudo*-$F_{1,80}$ = 2.4, R^2 = 0.03, p < 0.001), though the effect was relatively weak (Fig 5). There was no significant difference in cloacal bacterial community structure when considering treatment group (Bray-Curtis: *pseudo*-$F_{2,80}$ = 1.4, R^2 = 0.03, p = 0.31; Jaccard: *pseudo*-$F_{2,80}$ = 1.2, R^2 = 0.03, p = 0.32) or the number of days a female was implanted (Bray-Curtis: *pseudo*-$F_{1,80}$ = 2.0, R^2 = 0.02, p = 0.16; Jaccard: *pseudo*-$F_{1,80}$ = 1.5, R^2 = 0.02, p = 0.17). There were trends in cloacal bacterial community structure when considering year (Bray-Curtis: *pseudo*-$F_{1,80}$ = 2.1, R^2 = 0.02, p = 0.08; Jaccard: *pseudo*-$F_{1,80}$ = 1.7, R^2 = 0.02, p = 0.07) and the interaction between breeding stage and treatment (Bray-Curtis: *pseudo*-$F_{2,80}$ = 1.2, R^2 = 0.02, p = 0.08; Jaccard: *pseudo*-$F_{2,80}$ = 1.1, R^2 = 0.02, p = 0.08), though the effect sizes were weak. The dispersion of female cloacal bacterial communities was significantly different when considering treatment (Bray-Curtis: *pseudo*-$F_{2,85}$ = 3.7, p = 0.03;

Jaccard: *pseudo-*$F_{2,85}$ = 4.7, p = 0.01), breeding stage (Bray-Curtis: *pseudo-*$F_{1,86}$ = 5.9, p = 0.02; Jaccard: *pseudo-*$F_{1,86}$ = 4.2, p = 0.04), sampling year (Bray-Curtis: *pseudo-*$F_{1,86}$ = 11.2, p = 0.002; Jaccard: *pseudo-*$F_{1,86}$ = 13.4, p < 0.001), and the number of days implanted (Bray-Curtis: *pseudo-*$F_{24,63}$ = 2.4, p = 0.004; Jaccard: *pseudo-*$F_{24,63}$ = 6.1, p < 0.001). Females among treatment groups did not differ in cloacal bacterial community structure during nest building nor during incubation when considered separately (Tables S13, S14).

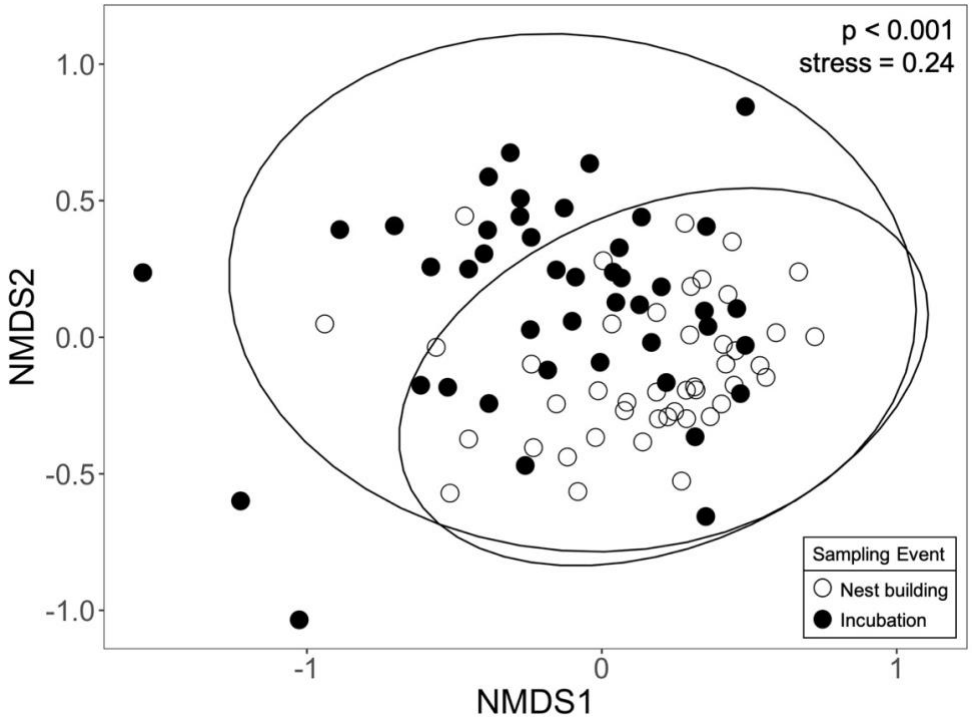

Figure 5. Cloacal bacterial beta diversity (non-metric multidimensional scaling plot, NMDS based on Bray-Curtis dissimilarity) of female tree swallows at two breeding stages (nest building, incubation). Each circle represents an individual female. Open circles = females sampled during nest building, filled circles = females sampled during incubation. Circles closer together indicate individuals with more similar cloacal bacterial community composition. Sample sizes include n = 44 females per sampling period. NMDS based on Jaccard dissimilarity (not pictured here) is similar. P-value is based on a PERMANOVA model. See Fig S1 for an NMDS connecting individual female points.

Estradiol-implanted females had significantly lower hematocrit compared to blank-implanted females (Blank-Estradiol: b = 0.03, SE = 0.01, p = 0.03). The difference in hematocrit of non-

implanted and estradiol-implanted females (None-Estradiol: b = 0.02, SE = 0.01, p = 0.15) or of non-implanted and blank-implanted females (None-Blank: b = -0.01, SE = 0.01, p = 0.85) was not statistically significant. There was also no statistical difference when comparing the hematocrit of females between treatment groups prior to implantation (None-Blank: b = 0.01, SE = 0.01, p = 0.86; None-Estradiol: b = 0.01, SE = 0.01, p = 0.61; Blank-Estradiol: b = 0.01, SE = 0.01, p = 0.87). The difference in female scaled-mass index between sampling years was statistically significant, with females sampled in 2019 exhibiting lower scaled-mass index on average compared to females sampled in 2018 (b = -2.1, SE = 0.84, p = 0.02). There was no relationship between scaled-mass index and treatment (None-Blank: b = 0.48, SE = 0.91, p = 0.60; None-Estradiol: b = 0.74, SE = 0.92, p = 0.43) or the number of days a female was implanted (b = -0.04, SE = 0.03, p = 0.14).

We found no relationship between cloacal microbiome diversity and scaled-mass index (ASV richness: b = -0.62, SE = 3.4, p = 0.86; Shannon Index: b = 0.15, SE = 0.13, p = 0.25; Faith's PD: b = -0.04, SE = 0.50, p = 0.94) or hematocrit (ASV richness: b = 280, SE = 340, p = 0.42; Shannon Index: b = 12, SE = 13, p = 0.38; Faith's PD: b = 67, SE = 47, p = 0.16). Nor was there a relationship between cloacal microbiome structure and scaled-mass index (Bray-Curtis: *pseudo-*$F_{1,42}$ = 1.1, R^2 = 0.03, p = 0.28; Jaccard: *pseudo-*$F_{1,42}$ = 1.1, R^2 = 0.03, p = 0.23) or hematocrit (Bray-Curtis: *pseudo-*$F_{1,30}$ = 0.76, R^2 = 0.02, p = 0.74; Jaccard: *pseudo-*$F_{1,30}$ = 0.82, R^2 = 0.03, p = 0.76).

Hatch success, but not average brood mass or fledging success, varied significantly with female treatment group (Table S15). More specifically, estradiol-implanted females had significantly lower hatch success compared to non-implanted females (None-Estradiol: b = 0.17, SE = 0.05, p = 0.001). There was a trending difference between the hatch success of estradiol- and blank-implanted females (Blank-Estradiol: b = 0.10, SE = 0.05, p = 0.09), but not between non- and blank-implanted females (None-Blank: b = 0.07, SE = 0.05, p = 0.24).

Discussion

In this study, we combined robust observational and experimental approaches to test the hypotheses that the number of sires per brood (observational study) and increased sexual activity of females via experimentally elevated estradiol concentrations (experimental study) significantly

influence the richness and community structure of the female cloacal microbiome. We did not find support for either hypothesis within the context of one breeding season. The lack of a significant effect may be due to (1) the duration of the study being too short to detect a relationship between sexual behavior and the cloacal microbiome, and/or (2) the limitation of using the number of sires per brood as a proxy for the number of mates per female in a system where the rates of extra-pair activity are high. In contrast to these results, our findings that female age positively predicted cloacal microbiome diversity (ASV richness, Shannon Index, Faith's PD) and influenced shifts in bacterial community structure does suggest that, over longer time periods, multiple breeding attempts could alter the cloacal microbiome in distinct ways. In addition, our study is one of the few studies to document within-individual changes in the cloacal microbiome within one breeding season, thus highlighting how dynamic the cloacal microbiome can be on a short timescale. Taken together, our results contribute to our understanding of the role that host life history and behavior play in shaping the cloacal microbiomes of wild birds.

We found support for our prediction that older females would have higher richness and a differently structured cloacal microbiome compared to younger females, which may be due to higher lifetime mating opportunities and/or competitive mating advantages associated with age. Older females have presumably secured more copulations across multiple breeding seasons, which could increase exposure to bacteria as social monogamy is probably only seasonal in this species (Lifjeld et al. 1993), though the same pair may mate in subsequent breeding seasons due to high philopatry rates (*ITM, personal observation*; Winkler et al. 2004). Older females may also have a competitive advantage over first-time breeding females and mate assortatively with older, more experienced males (Bitton et al. 2008, Ferrer & Penteriani 2003, Johnstone et al. 1996, Robertson & Rendell 2001), or be better able to capitalize on extra-pair mating opportunities (Bouwman & Komdeur 2005). If an age-assortative mating scenario is true in this tree swallow system, then we would predict older females to exhibit higher cloacal bacterial richness due to a combination of mating with more males and mating with older males that have in turn mated with more females previously. Though we do not have the necessary age data for males to be able to assess whether the older females we sampled were socially paired to older males, tree swallows in other populations consistently mate assortatively by age (Bitton et al. 2008, Robertson & Rendell 2001). Further, while our study assessed the number of mates per female using the number of sires per

brood as a proxy, we did not consider the number of mates of the socially partnered male. In great tits (*Parus major*), males socially paired to older females secured higher rates of extra-pair paternity (Roth et al. 2019), potentially transmitting bacteria acquired from those extra-pair matings back to their social partners. There is also a positive relationship between male age and extra-pair paternity, as evidenced in two meta-analyses incorporating studies done across passerine species (Cleasby et al. 2012, Hsu et al. 2015). Males with many partners could alter the microbiome of their social partners, but high rates of extra pair copulations could also increase mixing and homogeneity of the cloacal microbiome across the entire population.

Additional non-mating related processes that may explain age-related changes in the microbiome include changes in host life history, behavior, immune function, resource demands, and physiology. Several studies have compared the cloacal microbiome between young and adults and found that the cloacal microbiome, and gastrointestinal tract more broadly, of young is typically comprised of transient microbes, while that of adults is comprised of a relatively more stable community of microbes (e.g. van Dongen et al. 2013, Kohl et al. 2018, but also Burns et al. 2016, Trosvik et al. 2010). It is possible that the increased stability of these communities with aging is due in part to the development of the host immune system and increased selection for particular microbes and microbial communities over time (Burns et al. 2016, Kohl et al. 2018, McFall-Ngai et al. 2013). Investment in immune function can change as host resource demands change over time (Cichón et al. 2003, Lavoie 2006, Lozano & Lank 2003, Saino et al. 2003). For example, a host's investment in survival and reproduction is expected to shift over the course of an animal's life, and such shifts in resource investment may reflect or be reflected by shifts in microbiome diversity and structure (McFall-Ngai et al. 2013). Given that investment into both the immune response, survival overall, and reproduction may be physiologically demanding in different ways, changes in physiological function to meet these diverse investment requirements may also reflect changes in the host-associated microbiome (Ley et al. 2008).

We found a significant shift in within-individual cloacal microbiome richness and community structure between breeding stages, independent of treatment. While a previous study performed in male rufous-collared sparrows (*Zonotrichia capensis*) found similar shifts in the microbiome between and across breeding seasons (Escallón et al. 2019), this is one of the few studies to find

evidence for changes in the microbiome at a shorter timescale—within one breeding attempt—and in females (White et al. 2010). These changes in the cloacal bacterial community may be due to a variety of non-independent host-specific factors that may change across breeding stages, including behavior, physiology, and immunity, as well as other factors, such as environment and diet. Overt behavioral changes occur in female birds across breeding stages as females invest time and energy on courtship solicitations and copulations during nest building and on incubation after egg-laying (Norris & Lopez 2011). Cloacal bacterial communities are more similar between niches that make contact more frequently (e.g., bird cloaca-bird cloaca, bird cloaca-nest; van Veelen et al. 2017). For example, pair bonded social partners experimentally inhibited from making cloacal contact during mating exhibited a decrease in overall cloacal microbiome diversity over time (White et al. 2010), suggesting that a shift from frequent copulations (e.g., during nest building) to less frequent copulations (e.g., during incubation) results in a decrease in cloacal microbiota. These behavioral changes are likely influenced by changes in hormone profiles across breeding stages, specifically with respect to temperate-zone seasonally breeding birds (Wingfield et al. 2000). For instance, circulating estradiol concentrations are consistently found to be highest when female birds are most sexually active (e.g., during nest building: Dawson 1983, Norris & Lopez 2011, Williams 2012). Behavioral and physiological changes across breeding stages also likely influence immunological changes in the host (Milenkaya et al. 2013, Norris & Evans 2000) that may indirectly affect the colonization and/or maintenance of cloacal bacteria. Finally, given that the cloaca is the terminus for the digestive tract in birds, any changes in diet across breeding stages may induce changes in the cloacal microbiome.

There are a number of possible reasons why there was not a short-term effect of number of mates on cloacal microbiome diversity. First, we did not find a difference in the number of sires per brood when comparing estradiol and blank-implanted females, and this may be due to the consistent high rates of extra-pair fertilizations in the population and/or limitations inherent in identifying the number of mates per individual. While we made the assumption that the number of sires per brood was a proxy for the number of mates per female, this assumption may not be valid for this tree swallow system since rates of extra-pair activity are so high (almost 80% of nests have extra-pair young). As evidence of this, the proportion of sires per brood remained consistent (and high) between the females in the observational study and the subsequent experiment, even though

we administered hormone implants to increase the sexual activity of the experimental females. The application of exogenous estradiol hormone is an established method for increasing the frequency of solicitations and copulations in female birds (Moore 1982). Nevertheless, across three breeding seasons, over 50% of sampled female tree swallows had broods sired by two distinct males, and 71–78% of females had broods sired by two or three distinct males. There does not currently exist a method to comprehensively and accurately document the number of copulations secured by a free-living bird. While spatial proximity data loggers, for example, may record how close two individuals are to each other, there is no way to deduce if the birds are mating, allopreening, fighting, or simply perched next to one another (Krause et al. 2013, Ryder et al. 2012). Although technological advances, such as the application of small video cameras in combination with proximity data loggers on free-living birds, have helped contextualize social interactions (Rutz et al. 2007), interactions such as extra-pair copulations can occur at night or just before dawn, when video capture is not possible.

In conclusion, we have shown how dynamic the cloacal microbiome of individuals can be both within and across breeding seasons in a free-living bird species. We found support for a relationship between female age and the cloacal microbiome; we hypothesize that this age-related pattern is due to the effect of sexual activity and the number of partners manifesting over an individual's lifetime. An alternative hypothesis explaining age-related changes in the microbiome may be that host-associated microbiome diversity and structure track changes in a host's changing investment in survival versus reproduction as they age. Further, the significant decrease in cloacal microbiome richness and the shift in overall community structure between nest building and incubation underscore the important role that the breeding stage plays in shaping the cloacal microbiome of a wild bird. Future studies should focus on longitudinally sampling the cloacal microbiome of individuals over the course of their entire life, with sampling occurring at multiple time points within a single breeding season, between breeding and non-breeding stages (but see: Escallón et al. 2019), and across breeding seasons.

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CHAPTER IV: Examining the relationship between lay date and the cloacal microbiome in female tree swallows

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Abstract

The timing of breeding is one of the most important components of host reproductive success and may be influenced by a myriad of direct and indirect factors. Host-associated microbes in the gut and reproductive tracts of animals influence host physiology and behavior, yet whether these microbes, and the microbiome more broadly, are associated with the timing of breeding remains largely unknown. Here, we ask if female lay date (i.e., the date a female laid the first egg in her first clutch for the season) varies with cloacal microbiome alpha and beta diversity in a free-living population of tree swallows (*Tachycineta bicolor*). We measured the natural variation in lay date and cloacal microbiome diversity across a single breeding season. We found support for a relationship between lay date and cloacal microbiome structure (based on Bray-Curtis and Jaccard distance metrics, but not Weighted or Unweighted UniFrac), after controlling for age. Additionally, we found weak support for a relationship between lay date and cloacal microbiome alpha diversity (based on richness, the number of effective species, and phylogenetic distance). We found a negative relationship between lay date and female age, with older females laying earlier in the season compared to younger, first-time breeding females. Taken together, our results provide support for a relationship between lay date and the cloacal microbiome and highlight the importance of age to this relationship.

Introduction

The timing of breeding is an important fitness-related trait determined by a hierarchy of environmental and physiological factors, and early or late breeding can be associated with different costs and benefits (Price et al. 1988, Winkler et al. 2020). In many temperate-breeding birds, females that lay earlier have larger clutch sizes, higher number of offspring fledged, higher recruitment to the natal population, and, in some cases, higher lifetime reproductive success (e.g., Dunn & Winkler 2010, Klomp 1970, Perrins 1970, Verhulst et al. 1995, Winkler et al. 2020). In

general, female reproductive timing is hypothesized to be a condition-dependent trait, and higher individual quality or condition is associated with earlier lay dates (Verhulst & Nilsson 2007, Winkler et al. 2020). In addition to condition, older females tend to lay earlier and have higher reproductive success compared to younger, first-time breeding females (e.g., Coulson & White 1958, Nol & Smith 1987, Martin 1995, Goutte et al. 2010). Understanding which factors are associated with the timing of breeding can shed light on the causes and consequences of variation in host reproductive success.

The reproductive microbiome comprises the dynamic community of microbes that exist within the reproductive tract of an animal (e.g., reproductive fluids, organs, or tissues; Rowe et al. 2020). These microbes can influence reproductive function and directly or indirectly impact the reproductive success of hosts (reviewed in Rowe et al. 2020). For example, *Escherichia coli* in the reproductive tracts of females can attach to sperm cells and, in some cases, rupture the sperm plasma membrane and render the gamete immobile and unable to properly function (Bonet et al. 2018, Haines et al. 2013). Microbes within the reproductive tracts of females can have positive or negative associations with reproductive outcomes and offspring health (Heil et al. 2019, Younes et al. 2018, Rowe et al. 2020, Shao et al. 2019). Overall, previous research has predominately focused on how reproductive function relates to specific pathogenic or beneficial microbes. It is important, however, to bear in mind that microbes within the reproductive tract of animals exist within a *community* of microbes. It is thus imperative to investigate associations with the reproductive microbiome as a whole to better disentangle its relationship with host reproductive function and, ultimately, host reproductive success (Rowe et al. 2020). An initial step in understanding microbiome-fitness relationships is testing for the existence of a relationship between fitness-related traits and the diversity of the reproductive microbiome.

Here, we ask if female reproductive timing varies with cloacal microbiome alpha and beta diversity in a free-living population of a cavity-nesting songbird, the tree swallow (*Tachycineta bicolor*). The day in which female tree swallows lay their first egg (i.e., lay date) is associated with lifetime reproductive success, with earlier breeders having higher lifetime reproductive success (Winkler et al 2020). One hypothesis explaining this relationship is that individuals of higher phenotypic quality or in better condition are able to breed earlier (Verhulst & Nilsson 2007, Winkler et al.

2020). Indeed, older females, which are frequently found to be of higher quality (e.g., Mauck et al. 2004), tend to breed earlier and also have more diverse cloacal microbiomes (Hernandez et al. 2021). Because older females have more diverse cloacal microbiomes, we hypothesize that earlier breeding females will exhibit differences in cloacal microbiome diversity compared to later breeding females, even independent of female age. We focus on the cloacal microbiome, as it is where copulatory contact and the transfer of seminal fluid occur, and is therefore considered a component of the avian reproductive microbiome (Rowe et al. 2021). Overall, this study provides novel insight into the possible relationship between the reproductive microbiome and a fitness-related trait in a free-living population of birds.

Methods

Study system

We studied a breeding population of female tree swallows in Montgomery County, Virginia, USA (37°11'53.6 N, 80°34'58.0 W; 520 m.a.s.l.) in 2017. Tree swallows in this population breed from late March to early August and use nest boxes set up ~25 meters apart along the edges of agricultural fields. Tree swallows live 2.7 years on average, with the oldest recorded tree swallow documented at 11 years of age (Winkler et al. 2011). Though generally classified as single-brooded, some tree swallows are double-brooded (Clapp 1997, Monroe et al. 2008, Robertson et al. 1992).

Sample collection

We captured and sampled adult female tree swallows in nest boxes from April to July in 2017 (*n* = 68). Each female's age was assessed based on plumage: 'Second Year' (hereafter, SY) females are in their first breeding season and had immature brown plumage, while 'After Second Year' (hereafter, ASY) females are in their post-first year breeding season and had mature blue plumage (Hussell 1983). To standardize the reproductive stage in which each female was sampled, we caught each female once on day 6 of incubation. We collected cloacal bacteria for microbiome analyses by inserting a sterile swab (PurFlock®, Puritan, USA) approximately 4 mm into the cloaca and rotating it once. Sterile techniques were used when sampling the cloaca. Cloacal swab samples were stored on ice in the field and frozen in the lab within 5 hours of collection. In addition, we monitored nest boxes daily from April to July to record the date the first egg was laid

(hereafter, lay date), the total number of eggs laid per clutch (hereafter, clutch size), the average brood mass on day 6 post-hatching, and the number of nestlings that fledged (hereafter, fledging success). The birds sampled for this study were also included in a previously published study (Hernandez et al. 2021). All methods used in this study were approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Bacterial DNA processing

We extracted DNA, then amplified and sequenced the cloacal bacterial communities following methods in Hernandez et al. (2021). Samples were prepped for sequencing by a single individual (JH). Briefly, we extracted DNA from cloacal swabs using a Qiagen DNeasy Blood & Tissue Kit and then amplified the hypervariable V4 region of the 16S rRNA gene using the primers 515F and 806R (barcoded). We performed PCR in triplicate with negative controls for each sample, and then pooled the triplicate reactions per sample and visualized the PCR products. After quantifying the amplified bacterial DNA per sample, we pooled 200 ng DNA from each sample into a library for sequencing. The library was sequenced using a 1 x 250bp strategy on an Illumina MiSeq instrument (following Caporaso et al. 2012) at the Molecular Biology Core Facilities of the Dana Farber Cancer Institute of Harvard University. All samples were sequenced in one run.

We used the Quantitative Insights Into Microbial Ecology (QIIME) 2 (version 2019.1) bioinformatics pipeline to de-multiplex and quality filter single-end reads of raw 16S rRNA gene amplicon sequences (Bolyen et al. 2019). DADA2 was used to error correct reads and filter out both phiX and chimeric reads (Callahan et al. 2016). We set the truncation quality score to 11 and specified no end trimming to retain the full 250bp read length across all samples. Our reads, per sample, ranged from 31,252 to 168,791 with 12,591 amplicon sequence variants (ASVs) represented. Next, we removed ASVs with fewer than 0.005% of the total number of reads (i.e., total frequency fewer than 359 reads in this study) (Bokulich et al 2013). Our reads, per sample, then ranged from 26,176 to 162,475 with 986 ASVs. We then taxonomically classified ASVs using a Naïve Bayes classifier pre-trained on the Silva 132 database 99% ASVs from 515F/806R region of sequences (Quast et al. 2013). ASVs taxonomically annotated as Eukaryota, Mitochondria, Chloroplast, or Unassigned were filtered out. The final sampling depth for this dataset range 25,949 to 162,320. Based on alpha rarefaction curves, we rarefied samples to 25,000. The final

table contained 68 samples and 954 ASVs. Next, we constructed a phylogeny based on our sequence data using a *de novo* approach in QIIME2. We created a sequence alignment using MAFFT (Multiple Alignment using Fast Fourier Transform) and masked the alignment to remove ambiguously aligned regions. Then, we inferred a phylogeny using RAxML rapid bootstrapping with 100 replicates and a 'GTRCAT' substitution model, and rooted the phylogeny at the mid-point.

Statistical analyses

All analyses were performed in R (version 4.0.3) (R Core Team 2020). To characterize the cloacal microbiome, we assessed shared and unique ASVs across cloacal swab samples. We also determined the most abundant ASVs present in each sample using a relative abundance cutoff of 5%, and we determined the most abundant ASVs present in 90% of females sampled.

We used model selection and multimodal inference approaches to identify the predictors associated with variation in lay date (Burnham et al. 2011). Lay Date was represented as Julian Date in all models. We built generalized linear models to identify the relationship between lay date and both cloacal bacterial diversity (alpha and beta) and female age. Alpha diversity refers to the within-sample diversity and, in this study, all alpha diversity metrics are based on ASVs. ASV Richness is the total number of ASVs, the Hill Number represents the effective number of species, and Faith's phylogenetic distance (hereafter, Faith's PD) measures biodiversity based on phylogenetic distance among ASVs (Chao et al. 2010, Hill 1973, Jost 2006). We used Bray-Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac distance matrices to calculate beta diversity and then performed principle components analyses ('stats':*princomp*; Mardia et al. 1979, Venables & Ripley 2002). We used the first Principal Component axis (hereafter, PC 1), which explained between 27-55% of the variation, as our measure of beta diversity. Beta diversity describes the variation in community structure among samples. Bray-Curtis and Weighted UniFrac assess relative abundances, while Jaccard and Unweighted UniFrac assess presence-absence. Bray-Curtis and Jaccard consider count-based data, while both UniFrac metrics consider phylogenetic distance. We set lay date as the response variable, cloacal bacterial diversity and female age (SY, ASY) as covariates, and Gamma as the distribution. Then, we compared and ranked competing models using Akaike's Information Criterion (AICc) corrected for small sample sizes (Akaike 1973, Burnham & Anderson 2002). Each model set included four models: (1) a model with no covariates,

(2) a model with a categorical variable denoting female age (i.e., SY, ASY), (3) a model with a continuous variable describing cloacal microbiome alpha or beta diversity, and (4) a model with both the age and microbiome variables. We did not include more than one diversity metric in each model set due to correlations between diversity metrics. We considered models with a ΔAICc of <2 as the 'best fit' models and incorporated covariates from these models when assessing variation in lay date. We used ggplot ('ggplot2':*ggplot*, Wickham 2016) for visualizations.

Results

In all model sets, there was strong support for a relationship between female lay date and age. Older, ASY females initiated egg laying earlier in the breeding season compared to younger, SY females (Figure 1), with ASY females, on average, initiating laying on May 3, 2017 and SY females, on average, initiating laying on May 14, 2017 (Figure 1). In two of the four beta diversity model sets, there was also support for a relationship between lay date and cloacal microbiome structure. Specifically, model sets that included the first Principal Coordinates axis (PC1) from PCO based on Bray-Curtis and Jaccard distance metrics, but not weighted and unweighted UniFrac, received more support than models that only included age (Tables 1). In the model sets that included PC1 from Bray-Curtis and Jaccard distance metrics, there was a negative relationship between lay date and the cloacal microbiome structure (Table 2, Figure 2). Additionally, model selection provided weak support for a relationship between lay date and cloacal microbiome alpha diversity (Figure 2, Tables 3,4). More specifically, the top ranked models of each model set (See Table 3) included the alpha diversity metric and female age, or only included female age. When the top supported model included age and the alpha diversity metric, the beta coefficient for the alpha diversity metric approached significance (Table 4).

We identified 954 ASVs across 68 individual female cloacal bacteria samples. There was an average (± standard deviation) of 165 ± 101 ASVs/female (range: 14 - 426). The families with a mean relative abundance over 5% included Enterobacteriaceae (relative abundance 22%, phylum Proteobacteria), Corynebacteriaceae (21%, Actinobacteria), Enterococcaceae (7%, Firmicutes), and Micrococcaceae (6%, Actinobacteria). Families within the phyla Bacteroidetes, Tenericutes, Deinococcus-Thermus, Chlamydiae, Cyanobacteria, Acidobacteria, FBP, Fusobacteria, Armatimonadetes, Verrucomicrobia, Epsilonbacteraeota, Spriochaetes, Gemmatimonadetes, and

an unidentified phylum made up the remaining ~44%. The most prevalent ASVs (present in at least 90% of females and all identified to genus) were *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Corynebacterium 1*, *Escherichia-Shigella*, *Exiguobacterium*, *Rothia*, and *Sphingomonas*. The ASVs (identified to genus) with the highest relative abundance in the dataset were *Corynebacterium 1* (20%), *Escherichia-Shigella* (17%), *Enterococcus* (4%), *Rothia* (4%), and *Exiguobacterium* (3%).

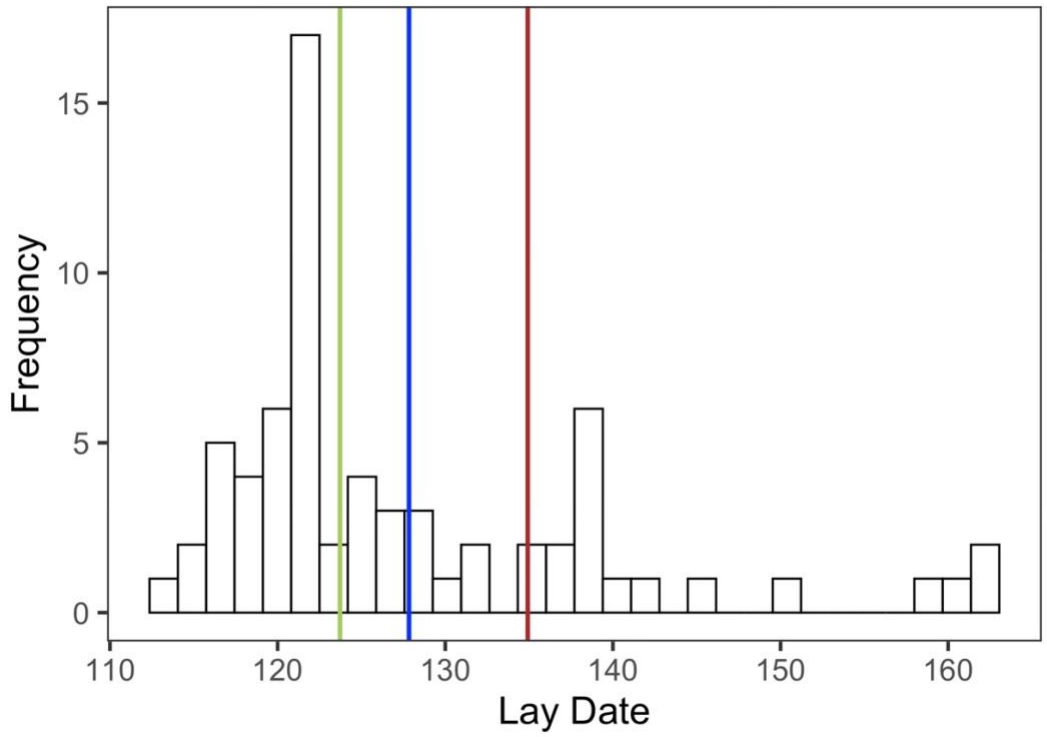

Figure 1. Lay date of sampled female tree swallows in 2017. The blue line depicts the mean lay date for all sampled females (May 7th), the green line depicts the mean lay date for all sampled ASY females (May 3rd), and the brown line depicts the mean lay date for all sampled SY females (May 14th).

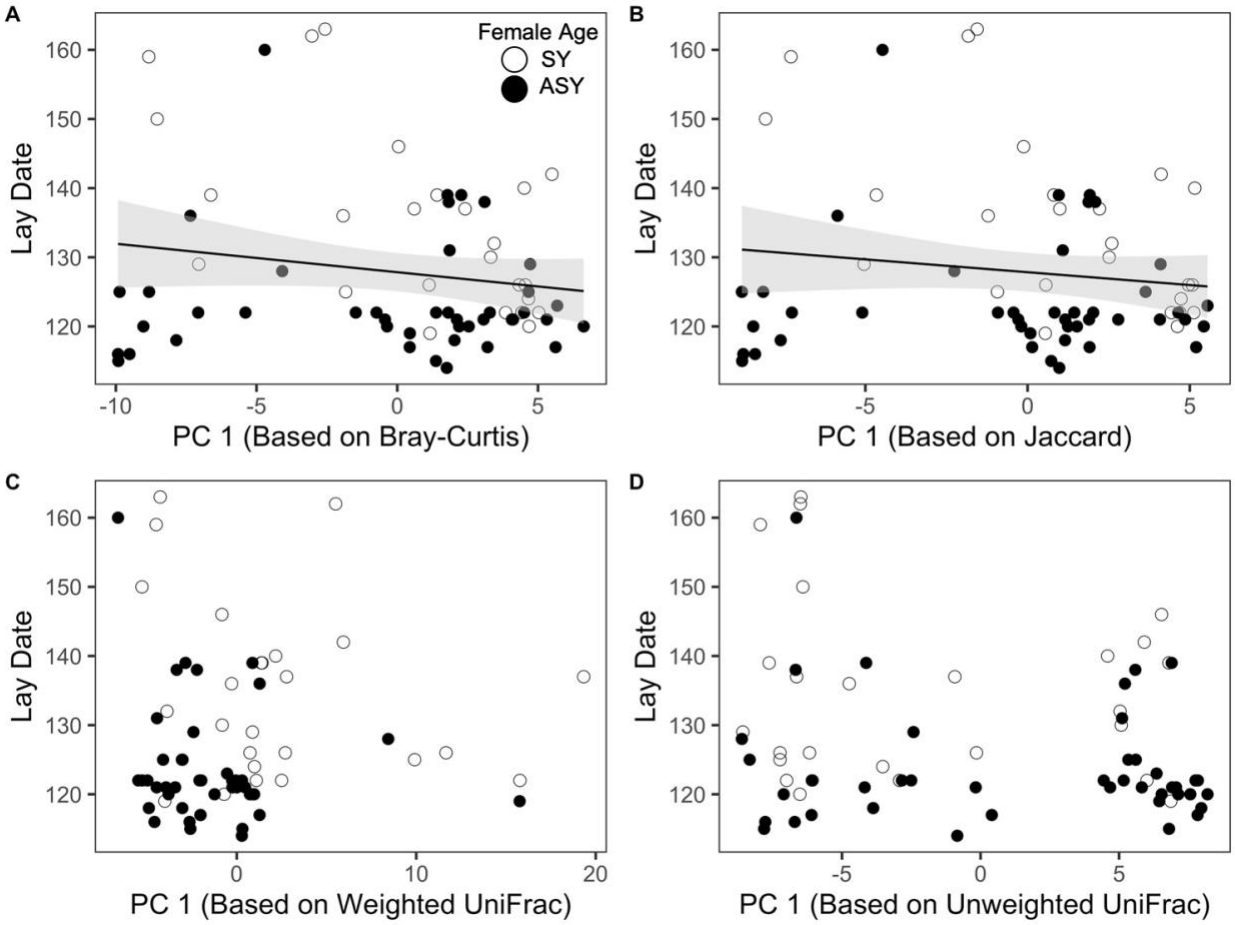

Figure 2. Lay date with respect to cloacal microbiome structure (or beta diversity) based on Bray-Curtis (A), Jaccard (B), Weighted UniFrac (C), and Unweighted UniFrac (D) distance metrics. All beta diversity metrics are based on bacterial amplicon sequence variants (ASVs) sampled from the cloacae of female tree swallows (n = 68). Each point represents the cloacal microbiome of an individual female. Open circles refer to 'Second Year' or 'SY' females in their first breeding season post-hatch year; closed circles refer to 'After Second Year' or 'ASY' females in a breeding season two or more years post-hatch year. Lay date is depicted in Julian date. 'PC 1' refers to the first Principal Component axis.

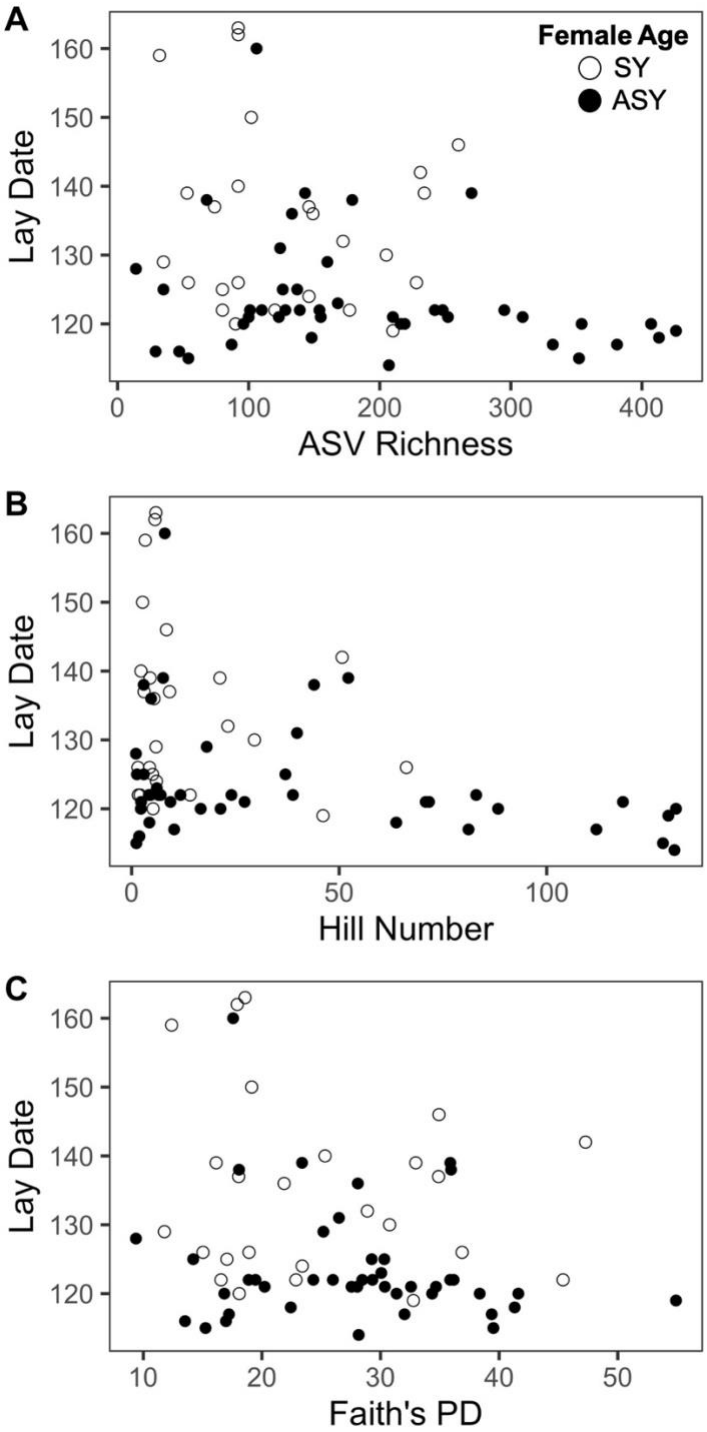

Figure 3. Lay date with respect to ASV richness (A), Hill Number (B), and Faith's phylogenetic distance (C). All alpha diversity metrics are based on bacterial amplicon sequence variants (ASVs) sampled from the cloacae of female tree swallows (n = 68). Each point represents the cloacal microbiome alpha diversity of an individual female. Open circles refer to 'Second Year' or 'SY' females in their first breeding season post-hatch year; closed circles refer to 'After Second Year' or 'ASY' females in a breeding season two or more years post-hatch year. Lay date is depicted in Julian date.

Table 1. AICc-based model selection parameters and output for cloacal microbiome structure (based on Bray-Curtis, Jaccard, weighted UniFrac, and unweighted UniFrac). 'Lay date' refers to the first day an egg was laid per clutch. 'PC 1' refers to the first Principal Component axis. 'Female age' refers to a female bird's age, as determined by plumage scores.

g1 = glm (Lay date ~ PC 1 + Female age)
g2 = glm (Lay date ~ PC 1)
g3 = glm (Lay date ~ Female age)
g4 = glm (Lay date ~ 1)

(A) Model selection for **PC 1** (based on Bray-Curtis)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g1	4	507.91	0.00	0.76	0.76	-249.64
g3	3	510.18	2.27	0.24	1.00	-251.90
g4	2	525.61	17.70	0.00	1.00	-260.71
g2	3	525.63	17.73	0.00	1.00	-259.63

(B) Model selection for **PC 1** (based on Jaccard)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g1	4	507.97	0.00	0.75	0.75	-249.67
g3	3	510.18	2.21	0.25	1.00	-251.90
g4	2	525.61	17.64	0.00	1.00	-260.71
g2	3	526.41	18.44	0.00	1.00	-260.02

(C) Model selection for **PC 1** (based on weighted UniFrac)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g1	4	509.24	0.00	0.62	0.62	-250.30
g3	3	510.18	0.94	0.38	1.00	-251.90
g4	2	525.61	16.37	0.00	1.00	-260.71
g2	3	527.79	18.56	0.00	1.00	-260.71

(D) Model selection for **PC 1** (based on unweighted UniFrac)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g3	3	510.18	0.00	0.64	0.64	-251.90
g1	4	511.31	1.14	0.36	1.00	-251.34
g2	3	524.15	13.98	0.00	1.00	-258.89
g4	2	525.61	15.43	0.00	1.00	-260.71

Table 2. Model summaries for the top ranked models from each model set in Table 1. We include all model summaries. Model summaries in grey refer to models with delta AICc scores > 2.0.

(A) Model summaries for **PC 1** (based on Bray-Curtis)

Response	Covariate	Estimate	SE	t value	*P* value
Lay date	(Intercept)	7.4e-03	1.2e-04	64	**<0.001**
	PC 1	3.2e-05	1.5e-05	2.1	**0.04**
	Female Age	7.0e-04	1.5e-04	4.6	**<0.001**
	(Intercept)	0.01	0.0001	62	**<0.001**
	Female Age	0.001	0.0002	4.3	**<0.001**
	(Intercept)	7.8e-03	8.7e-05	90	**<0.001**
	(Intercept)	7.8e-03	8.6e-05	91	**<0.001**
	PC 1	2.5e-05	1.7e-05	1.4	0.16

(B) Model summaries for **PC 1** (based on Jaccard)

Response	Covariate	Estimate	SE	t value	*P* value
Lay date	(Intercept)	7.4e-03	1.2e-04	63	**<0.001**
	PC 1	3.6e-05	1.7e-05	2.1	**0.04**
	Female Age	7.2e-04	1.5e-04	2.1	**<0.001**
	(Intercept)	0.01	0.0001	62	**<0.001**
	Female Age	0.001	0.0002	4.3	**<0.001**
	(Intercept)	7.8e-03	8.7e-05	90	**<0.001**
	(Intercept)	7.8e-03	8.6e-05	91	**<0.001**
	PC 1	2.5e-05	2.0e-05	1.1	0.26

(C) Model summaries for **PC 1** (based on weighted UniFrac)

Response	Covariate	Estimate	SE	t value	*P* value
Lay date	(Intercept)	7.4e-03	1.2e-04	60	**<0.001**
	PC 1	2.7e-05	1.6e-05	1.7	0.09
	Female Age	7.7e-04	1.6e-04	4.7	**<0.001**
	(Intercept)	0.01	0.001	62	**<0.001**
	Female Age	0.001	0.0002	4.3	**<0.001**
	(Intercept)	7.8e-03	8.7e-05	90	**<0.001**
	(Intercept)	7.8e-03	8.7e-05	89	**<0.001**
	PC 1	4.3e-07	1.7e-05	0.3	0.98

(D) Model summaries for **PC 1** (based on unweighted UniFrac)

Response	Covariate	Estimate	SE	t value	*P* value
Lay date	(Intercept)	0.01	0.0001	62	**<0.001**
	Female Age	0.001	0.0002	4.3	**<0.001**
	(Intercept)	7.4e-03	1.2e-04	61	**<0.001**
	PC 1	1.3e-05	1.3e-05	1.0	0.31
	Female Age	6.3e-04	1.6e-04	3.9	**<0.001**
	(Intercept)	7.8e-03	8.5e-05	92	**<0.001**
	PC 1	2.6e-05	1.4e-05	1.9	0.07
	(Intercept)	7.8e-03	8.7e-05	90	**<0.001**

Table 3. AICc-based model selection parameters and output for (A) amplicon sequence variants (ASV) Richness, (B) Hill Number, and (C) Faith's Phylogenetic Distance. Lay date' is the day the first egg was laid per clutch. 'ASV Richness', 'Hill Number', and 'Faith's PD' are the alpha diversity metrics of interest. 'Female age' refers to a female bird's age, as determined by plumage scores. The reference Female Age group is Second Year or 'SY', which describes females in their first breeding season. '

g1 = glm (Lay date ~ Alpha diversity metric + Female age)
g2 = glm (Lay date ~ Alpha diversity metric)
g3 = glm (Lay date ~ Female age)
g4 = glm (Lay date ~ 1)

(A) Model selection based on AICc for **ASV richness**

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g1	4	509.71	0.00	0.56	0.56	-250.54
g3	3	510.18	0.46	0.44	1.00	-251.90
g2	3	521.54	11.83	0.00	1.00	-257.58
g4	2	525.61	15.89	0.00	1.00	-260.71

(B) Model selection based on AICc for **Hill Number**

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g1	4	508.47	0.00	0.7	0.7	-249.92
g3	3	510.18	1.71	0.3	1.0	-251.90
g2	3	518.96	10.50	0.0	1.0	-256.29
g4	2	525.61	17.14	0.0	1.0	-260.71

(C) Model selection based on AICc for **Faith's PD**

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g3	3	510.18	0.00	0.55	0.55	-251.90
g1	4	510.58	0.41	0.45	1.00	-250.97
g2	3	524.41	14.24	0.00	1.00	-259.71
g4	2	525.61	15.43	0.00	1.00	-260.71

Table 4. Model summaries for the top ranked models from each model set in Table 3. The reference Female Age group is Second Year or 'SY', which describes females in their first breeding season. 'Lay date' is the day the first egg was laid per clutch. 'Female age' refers to a female bird's age, as determined by plumage scores. We include all model summaries. Models are separated by horizontal lines. Model summaries highlighted in grey refer to models with delta AICc scores > 2.0.

Response	Covariate	Estimate	SE	t value	*P* value
Lay date	(Intercept)	7.3e-03	1.6e-04	46	**<0.001**
	ASV Richness	1.3e-06	8.0e-07	1.6	0.12
	Female Age	6.0e-04	1.6e-04	3.8	**<0.001**
	(Intercept)	0.01	0.0001	62	**<0.001**
	Female Age	0.001	0.0002	4.3	**<0.001**
	(Intercept)	7.5e-03	1.6e-04	47	**<0.001**
	ASV Richness	2.1e-06	8.5e-07	2.4	**0.02**
	(Intercept)	7.8e-03	8.7e-05	90	**<0.001**
Lay date	(Intercept)	7.4e-03	1.2e-04	61	**<0.001**
	Hill Number	4.2e-06	2.2e-06	1.9	0.06
	Female Age	5.7e-04	1.6e-04	3.6	**<0.001**
	(Intercept)	0.007	0.0001	62	**<0.001**
	Female Age	0.001	0.0002	4.3	**<0.001**
	(Intercept)	7.6e-03	1.0e-04	75	**<0.001**
	Hill Number	6.6e-06	2.3e-06	2.9	**0.01**
	(Intercept)	7.8e-03	8.7e-05	90	**<0.001**
Lay date	(Intercept)	0.01	0.001	62	**<0.001**
	Female Age	0.001	0.0002	4.3	**<0.001**
	(Intercept)	7.2e-03	2.3e-04	31	**<0.001**
	Faith's PD	1.1e-05	8.1e-06	1.3	0.2
	Female Age	6.4e-04	1.6e-04	4.1	**<0.001**
	(Intercept)	7.4e-03	2.5e-04	29	**<0.001**
	Faith's PD	1.6e-05	9.0e-06	1.8	0.08
	(Intercept)	7.8e-03	8.7e-05	90	**<0.001**

Discussion

The reproductive microbiome is hypothesized to be indirectly or directly associated with host fitness, yet this relationship has yet to be adequately studied in a free-living species. In this study, we used an observational approach to test the hypothesis that earlier breeding, female tree swallows exhibit differences in cloacal microbiome diversity compared to later breeding females. We found support for a relationship between female lay date and cloacal microbiome structure (beta diversity based on Bray-Curtis and Jaccard, but not Weighted or Unweighted UniFrac), after controlling for female age. In addition, we found weak support for a relationship between lay date and cloacal microbiome alpha diversity (ASV Richness, Hill Number, Faith's PD). We found that lay date was negatively correlated with female age, which agrees with the pattern that older females lay earlier in the season compared to younger females (e.g., Coulson & White 1958, Nol & Smith 1987, Martin 1995, Goutte et al. 2010). Because female lay date is hypothesized to be associated with an individual's quality and/or condition, particularly in female tree swallows (Winkler et al. 2020), cloacal microbiome diversity may be associated with or indicative of variation in individual quality or condition. Our results therefore provide support for a relationship between lay date and the cloacal microbiome.

The cloacal microbiome, and the reproductive microbiome more broadly, may be a trait associated with the quality and/or condition of an individual and, by extension, to their timing of breeding. Though the mechanism underlying a direct relationship between the reproductive microbiome and the timing of breeding is unknown, it is clear from previous research that microbes in the reproductive tract and gut of their host can influence physiological and behavioral processes associated with an individual's reproductive success (e.g., Cusick et al. 2021, Ezenwa et al. 2012, Johnson & Foster 2018, Rowe et al. 2020). If a direct relationship exists, our results suggest that it is the structure of the cloacal microbiome, rather than the richness, number of effective species, or phylogenetic distance, that is associated with host reproductive success.

Because we took an observational approach, we cannot rule out the possibility of an indirect relationship between lay date and the cloacal microbiome. In other words, the cloacal microbiome may be correlated with another factor that mediates variation in quality or condition and drives the relationship between lay date and the cloacal microbiome. For instance, our study and previous

studies in birds have identified a relationship between lay date and female age (e.g., Coulson & White 1958, Nol & Smith 1987, Martin 1995, Goutte et al. 2010), and a positive correlation between the cloacal microbiome and female age (Hernandez et al. 2021). In this case, older females are of higher quality or condition and also exhibit more diverse cloacal microbiomes. Age-specific microbiomes may be due to the acquisition of microbes from copulations occurring across multiple breeding seasons, the selective disappearance of females with certain microbiomes, or age-related changes in host life history, behavior, resource demands, and/or physiology (Escallón et al. 2019, Hernandez et al. 2021).

In conclusion, our results provide evidence for a negative correlation between a fitness-related trait (i.e., lay date) and the diversity of the cloacal microbiome, as well as age. Future research can test the hypothesis that the cloacal microbiome is an aspect of an individual's phenotypic quality. By developing experiments with the predictive capability to refine our understanding of *if* and *how* the reproductive microbiome interacts with the host and other host-associated intrinsic (e.g., age, physiology, quality) and extrinsic (e.g., social and physical environment) factors, we can better understand how various factors interact to influence the timing of breeding.

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Chapter V: Conclusions

Summary

This dissertation focused on understanding the causes and consequences of variation in the reproductive microbiome of free-living birds from an ecological perspective. Since microbes within the reproductive tract of animals can impact host reproductive function and survival, these microbes – and the reproductive microbiome more broadly – likely influence host fitness outcomes. In **Chapter I**, I introduced the reproductive microbiome and discussed potential intrinsic and extrinsic factors that may play a role in shaping host-associated communities of microbes. In **Chapter II**, I assessed whether social monogamy or sex explained variation in the cloacal microbiome of tree swallows during the breeding season and found that the cloacal microbiome diversity of pair-bonded social partners was not more similar than expected by chance while the cloacal microbiome diversity of females and males were similar. Given these findings and the high rates of extra-pair activity in tree swallows, I argued that considering extra-pair activity when characterizing cloacal bacterial communities is important for understanding how these communities are structured. In **Chapter III**, I combined an observational and experimental approach to determine if age, breeding stage, and mating behavior explained variation in the cloacal microbiomes of females within the course of one breeding attempt. I found that host age and breeding stage, but not sexual activity or number of mates, play a role in shaping the cloacal microbiome of females. I proposed that the evidence for an effect of female age, but not sexual activity or number of mates, suggests that the effect of sexual activity and number of mates may not become apparent unless quantified over an individual's lifetime breeding tenure. In **Chapter IV**, I investigated whether there was a relationship between the timing of breeding and the cloacal microbiome. I found a relationship between the timing of breeding and cloacal microbiome structure, after controlling for age. Below, I discuss future directions that build upon the framework established by my dissertation research and focus on refining our understanding of factors shaping the reproductive microbiome.

Over the course of my dissertation research, three themes have become apparent:

1) There is not support for a relationship between female mating activity or number of mates and the cloacal microbiome over the course of a single mating season.

2) The effect of mating behavior on reproductive microbiome diversity manifests over an individual's lifetime rather than over a breeding season.
3) The reproductive microbiome can influence an individual's fitness.

Robustly testing for a relationship between mating activity and the cloacal microbiome

From the perspective of microbiome diversity, I robustly tested the hypothesis that female mating activity or number of mates influenced cloacal microbiome diversity and found no empirical support for this relationship within the course of a single mating season. Our finding that the cloacal bacterial communities were similar between the sexes and that the communities between pair-bonded social partners were not more similar than expected by chance (Chapter II), could suggest that cloacal microbiomes of individuals are being homogenized through copulations with multiple mates. Tree swallows across populations have been found to exhibit high rates of extra-pair sexual activity (~50% of nestlings are extra-pair offspring; Dunn et al. 2009, Lifjeld et al. 1993), with approximately 54% of the nestlings in our study population being extra-pair offspring and 84% of broods containing extra-pair offspring (Chapter II). Ultimately, our findings that there was no relationship between the number of sires per brood or increased sexual activity and cloacal microbiome diversity (Chapter III) establishes that there is no support for the hypothesis that female mating activity or number of mates influences cloacal microbiome diversity in our study population, at least over the course of a single breeding season. Future research testing this hypothesis should focus on comparing across tree swallow populations to corroborate the lack of support. Studies designed to distinguish between microbes transmitted sexually versus microbes transmitted either through other modes of horizontal transmission between individuals (e.g., allopreening, shared perches) or microbes originating from the diet will prove particularly fruitful. One method of distinguishing between sexually transmitted microbes and alternative horizontally transmitted microbes is with contraceptive devices that block the contact of reproductive structures and insemination during mating (e.g., White et al. 2010). Parsing out the contribution of diet-based microbes to the reproductive microbiome compared to sexually transmitted microbes is challenging in a free-living population but can be done by studying a species with separate excretory openings for the digestive and reproductive tract. Distinguishing between diet-based and sexually transmitted microbes is more readily feasible in captive populations where food resources can be sterilized or the same for all individuals. By continuing to comprehensively test the

hypothesis that sexually transmitted microbes pose a cost to females that engage in multiple mating, we can further understand why some females are truly monogamous while others are not. Such research into a potentially nonadaptive phenomena will be particularly informative to the field of behavioral ecology, where most research has primarily focused on testing adaptive hypotheses (e.g., good genes hypothesis, fertility insurance hypothesis) to understand why females of socially monogamous species activity participate in extra-pair sexual activity (Forstmeier et al. 2014).

Exploring lifetime breeding activity and reproductive microbiome diversity

Despite finding that neither the number of sires per brood nor sexual activity were significantly associated with cloacal microbiome diversity (Chapter III), I did find a positive relationship between female age and cloacal microbiome diversity. This age-microbiome relationship suggests that there may be a relationship between female mating behavior and cloacal microbiome diversity, but that the duration of the study was too short to detect a relationship. Future research examining the relationship between mating behavior and cloacal microbiome diversity should thus focus on performing longitudinal studies that assess the number of mates (and age of mates) and cloacal microbiome diversity over females' reproductive lifetime, in addition to other potential factors that may shape the microbiome and change over time such as diet, life history, and physiology. By taking a longitudinal approach, it would be possible to determine which factors contribute to variation in the cloacal microbiome and allow us to better track changes in the microbiome with respect to host-associated and environmental shifts over time. With access to cloacal bacterial samples from the same individuals as they age, it would also be possible to gain insight into how the cloacal bacterial diversity changes across breeding seasons. In species where it is feasible to sample the individuals during both breeding and non-breeding seasons across their lifetime, access to repeated samples would prove particularly insightful towards understanding how structural dynamics of cloacal bacterial communities change across life history stages (e.g., Escallón et al. 2019). Another approach to testing the effect of mating activity on cloacal bacterial diversity is to design studies that control for age. For instance, in a captive system, females of the same age could be exposed to one mate (i.e., monogamous control group), while other females of the same age could be exposed to a range of mates (i.e., polyandrous treatment groups). The reproductive microbiome diversity can then be compared between the control and treatment groups. Previous

work has compared the reproductive microbiome of mated versus non-mated individuals of the same age (e.g., *common bedbug*: Bellinvia et al. 2019) and monogamous versus non-monogamous individuals of varying ages (e.g., *common lizard*: White et al. 2011), but no research has compared the reproductive microbiome diversity of non-mated or monogamous individuals to same-aged individuals with a range of mates. By taking a more fine-scale experimental approach that controls for individual age, we can better test whether mating activity plays a direct role in shaping the reproductive microbiome.

Investigating the reproductive microbiome as an aspect of individual quality

There is much interest in understanding whether and how microbiomes inhabiting most every niche of a vertebrate host's body relate to host phenotype and, more importantly and ultimately, host fitness. We know that microbes can influence host development, physiology, and behavior and host behavior and life history can influence microbe richness and microbiome diversity more broadly (e.g., Archie & Tung 2015, Ezenwa et al. 2012, Hawley et al. 2020, Ley et al. 2008, McFall-Ngai et al. 2013). Tying microbiome diversity to fitness in the forms of reproductive success or longevity are important future goals. My findings suggest that older females have more diverse cloacal microbiomes (Chapter III) and initiate breeding earlier than first-time breeding females (Chapter IV), and that earlier breeding females have differently structured cloacal microbiomes compared to later breeding females (Chapter IV). Previous work has also demonstrated that older females have higher reproductive success than younger females (e.g., Coulson & White 1958, Nol & Smith 1987, Martin 1995, Goutte et al. 2010). Based on these patterns, I propose the hypothesis that the cloacal microbiome – or the reproductive microbiome, more broadly – is associated with an individual's quality and/or condition. By 'individual quality', I refer to the "axis of among-individual heterogeneity that is correlated with fitness" (Wilson & Nussey 2010). Measuring individual quality in organisms can be challenging since an individual's fitness, and thus survival and reproductive success, can covary with respect to multiple host-associated intrinsic and extrinsic traits. To test the hypothesis that the reproductive microbiome is an aspect of individual quality, the first step future work should take is to demonstrate that the reproductive microbiome is consistent and repeatable across years. Repeated samples of the same individuals during the breeding cycle over multiple years should be taken to assess the repeatability of reproductive microbiome diversity. To pinpoint the most biologically relevant period at which

a sample should be taken (e.g., nest building versus mating versus incubation), samples will need to be taken at multiple stages of the breeding cycle and their repeatability compared. Being able to demonstrate evidence for a link (direct or indirect) between the reproductive microbiome and host individual quality will provide important insight into how the reproductive microbiome helps shape host fitness from an ecological perspective (Fig 1).

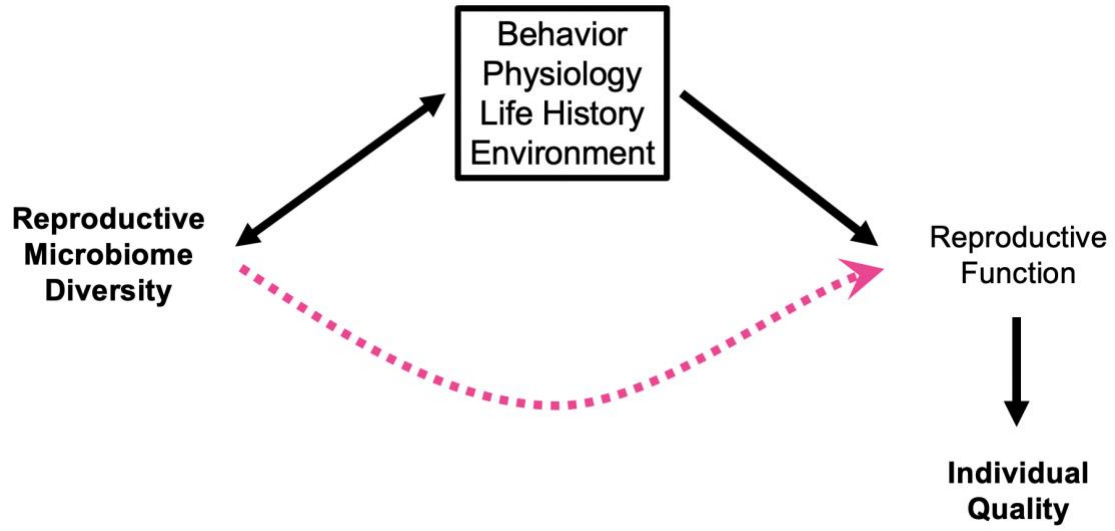

Figure 1. Conceptual figure of the proposed relationship between the reproductive microbiome, host-associated intrinsic and extrinsic traits, and individual quality. Solid arrows depict established relationships, while the pink dotted arrow depicts a predicted relationship. The reproductive microbiome can indirectly (black lines) or directly (pink line) influence reproductive function and thus individual quality.

Conclusion

The reproductive microbiome can significantly impact host reproductive function and performance. Identifying the factors influencing the acquisition and maintenance of the microbes within the reproductive tract will contribute to our understanding of variation within the reproductive microbiome. By connecting reproductive microbiome diversity to fitness-related traits such as reproductive success and survival, we can gain critical insight into the evolutionary implications of variation in the reproductive microbiome with respect to sexual selection, sexual conflict, mating systems, and reproductive isolation. The findings in my dissertation suggest that mating behavior may influence variation in the reproductive microbiome over an individual's lifetime and that the reproductive microbiome may be associated with an individual's quality

and/or condition. This dissertation also revealed promising avenues of future research into the relationship between reproductive microbiome diversity and host life history, physiology, and both the social and physical environment that are ripe for study. My hope is that the framework established by this dissertation serves as a helpful foundation as we move toward further refining our understanding of the dynamics shaping (and being shaped by) the reproductive microbiome.

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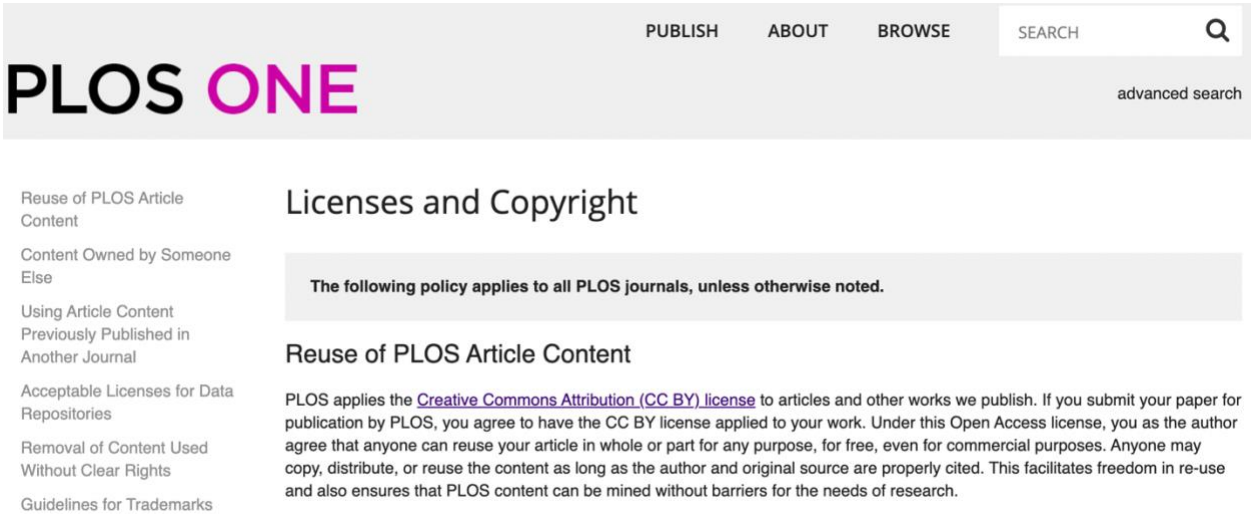

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APPENDIX C: CHAPTER III SUPPLEMENTAL DOCUMENT

Table S1. Characteristics of the eight microsatellite loci multiplexed in the observational study. Primers for all eight microsatellite loci were previously cross-amplified in tree swallows (*Tachycineta bicolor*) in Makarewich et al. (2009). Loci were split into two PCR mixes (A or B) with respect to their annealing temperature. N = number of individuals genotyped. N_A = number of alleles. H_O = observed heterozygosity. H_E = expected heterozygosity.

Locus	Fluorescent Label	Allele size range	Annealing temperature (°C)	N	N_A	H_O	H_E	PCR Mix
Tle19	6-FAM	140-174	56	365	15	0.7918	0.8391	A
Tle16	PET	227-249	56	365	10	0.6703	0.6767	A
TaBi4	VIC	243-295	56	365	12	0.8959	0.8530	A
TaBi8	PET	301-339	56	365	14	0.8324	0.8191	A
TaBi1	NED	299-343	56	365	12	0.7041	0.7331	A
TaBi25	PET	117-177	58	365	17	0.9836	0.7768	B
TaBi34	6-FAM	172-212	58	365	20	0.9699	0.8883	B
Tal6	VIC	326-348	58	365	7	0.4740	0.6247	B

Table S2. Characteristics of the ten microsatellite loci multiplexed in the experiment. Primers for all ten microsatellite loci were previously cross-amplified in tree swallows (*Tachycineta bicolor*) in Makarewich et al. (2009). Loci were split into two PCR mixes (A or B) with respect to their annealing temperature. N = number of individuals genotyped. N_A = number of alleles. H_O = observed heterozygosity. H_E = expected heterozygosity.

Locus	Fluorescent Label	Allele size range	Annealing temperature (°C)	N	N_A	H_O	H_E	PCR Mix
Tle19	6-FAM	146-174	56	266	12	0.808	0.868	A
Tle16	PET	227-251	56	266	8	0.707	0.802	A
TaBi4	VIC	244-296	56	266	12	0.891	0.867	A
TaBi8	PET	306-341	56	266	14	0.816	0.849	A
TaBi1	NED	306-342	56	266	10	0.741	0.738	A
TaBi25	PET	119-177	58	266	15	0.748	0.665	B
TaBi34	6-FAM	171-211	58	266	16	0.917	0.894	B
Tal6	VIC	337-348	58	266	6	0.492	0.618	B
Tal11	NED	195-215	58	266	10	0.650	0.627	B
Tal8	6-FAM	272-340	58	266	29	0.951	0.936	B

Table S3. AICc-based model selection parameters and output for (A) amplicon sequence variants (ASV) Richness, (B) Shannon Index, and (C) Faith's Phylogenetic Distance. The reference age group is 'SY', which describes first-time breeding females.

(A) Model selection based on AICc for **ASV richness**.

g1 = lm(Richness ~ Number of sires per brood *Female age)
g2 = lm(Richness ~ Number of sires per brood +Female age)
g3 = lm(Richness ~ Female age)
g4 = lm(Richness ~ Number of sires per brood)
g5 = lm(Richness ~ 1)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g3	3	796.82	0.00	0.76	0.76	-395.23
g5	2	799.85	3.03	0.17	0.92	-397.84
g2	6	801.92	5.10	0.06	0.98	-394.31
g4	5	804.81	7.99	0.01	1.00	-396.94
g1	9	808.81	11.98	0.00	1.00	-393.93

(B) Model selection based on AICc for **Shannon Index**.

g1 = lm(Shannon ~ Number of sires per brood *Female age)
g2 = lm(Shannon ~ Number of sires per brood +Female age)
g3 = lm(Shannon ~ Female age)
g4 = lm(Shannon ~ Number of sires per brood)
g5 = lm(Shannon ~ 1)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g3	3	240.76	0.00	0.72	0.72	-117.20
g5	2	243.24	2.48	0.21	0.92	-119.53
g2	6	245.88	5.12	0.06	0.98	-116.28
g4	5	248.08	7.31	0.02	1.00	-118.58
g1	9	252.21	11.44	0.00	1.00	-115.63

(C) Model selection based on AICc for **Faith's Phylogenetic Distance**.

g1 = lm(Faith's ~ Number of sires per brood*Female age)
g2 = lm(Faith's ~ Number of sires per brood +Female age)
g3 = lm(Faith's ~ Female age)
g4 = lm(Faith's ~ Number of sires per brood)
g5 = lm(Faith's ~ 1)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g3	3	454.53	0.00	0.71	0.71	-224.08
g5	2	456.88	2.35	0.22	0.93	-226.35
g2	6	459.79	5.26	0.05	0.98	-223.24
g4	5	461.92	7.39	0.02	1.00	-225.50
g1	9	467.11	12.58	0.00	1.00	-223.08

Table S4. Model summaries for the top ranked models from each model set in Table S3. The response variables are bolded in the first column and the independent covariates specified in the top-ranking model(s) are in the second column. The reference age group is 'SY', which describes first-time breeding females. We include all top model summaries for which the delta AICc score is < 2.0 and below the null model. The 'Intercept' is included as a covariate if the null model was ranked highest.

Response	**Model covariates**	**Estimate**	**SE**	**t value**	***P* value**
ASV Richness	(Intercept)	102.5	12.6	8.1	**<0.001**
	Female Age	36.2	15.8	2.3	**0.03**
Shannon	(Intercept)	1.8	0.25	7.3	**<0.001**
	Female Age	0.68	0.32	2.2	**0.03**
Faith's	(Intercept)	17.5	1.1	15.4	**<0.001**
	Female Age	3.0	1.4	2.1	**0.04**

Table S5. AICc-based model selection parameters and output for (A) scaled-mass index and (B) hematocrit. 'SMi' refers to scaled-mass index, a morphological condition metric (See Methods of Main Text). 'Number of sires' refers to the number of sires per brood, based on paternity analyses. 'Female age' refers to a female's age, based on plumage scores.

Model selection based on AICc for **scaled-mass index**.

g1 = lm(Scaled-mass index ~ Number of sires per brood *Female age)
g2 = lm(Scaled-mass index ~ Number of sires per brood +Female age)
g3 = lm(Scaled-mass index ~ Female age)
g4 = lm(Scaled-mass index ~ Number of sires per brood)
g5 = lm(Scaled-mass index ~ 1)

Model	K	AICc	Delta_AICc	AICc Wt	Cum.Wt	LL
g4	4	252.56	0.00	0.58	0.58	-121.91
g2	5	254.92	2.36	0.18	0.76	-121.90
g5	2	255.22	2.66	0.15	0.92	-125.50
g3	3	257.33	4.77	0.05	0.97	-125.45
g1	7	258.65	6.09	0.03	1.00	-121.23

Model selection based on AICc for **hematocrit**.

g1 = lm(Hematocrit ~ Number of sires per brood *Female age)
g2 = lm(Hematocrit ~ Number of sires per brood +Female age)
g3 = lm(Hematocrit ~ Female age)
g4 = lm(Hematocrit ~ Number of sires per brood)
g5 = lm(Hematocrit ~ 1)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g4	4	-230.43	0.00	0.36	0.36	119.61
g5	2	-230.34	0.09	0.35	0.71	117.28
g3	3	-228.58	1.84	0.14	0.86	117.52
g2	5	-228.37	2.06	0.13	0.99	119.78
g1	7	-223.71	6.72	0.01	1.00	120.02

Table S6. Model summaries for the best performing model per body condition metric in Table S5. The response variables are bolded in the first column and the independent covariates specified in the top-ranking model(s) are in the second column. The reference age group is 'SY', which describes first-time breeding females, and 'Number of sires per brood 1', which describes broods with one sire. We include all top model summaries for which the delta AICc score is < 2.0 and below the null model. The 'Intercept' is included as a covariate if the null model was ranked highest.

Response	Model covariates	Estimate	SE	t value	*P* value
Scaled-mass index	(Intercept)	20.2	0.57	35.6	**<0.001**
	Number of sires per brood 2	1.8	0.66	2.7	**0.01**
	Number of sires per brood 3+	1.5	0.80	1.9	0.06
Hematocrit	(Intercept)	0.51	0.01	57.2	**<0.001**
	Number of sires per brood 2	0.02	0.01	2.1	**0.04**
	Number of sires per brood 3+	0.01	0.01	1.1	0.29

Table S7. Contrast summaries for body condition metrics in Table S6.

Condition metric	Contrast	Estimate	SE	df	t ratio	*P* value
Scaled-mass index	1 – 2	-1.8	0.66	56	-2.7	**0.03**
	1 – (3+)	-1.5	0.80	56	-1.9	0.15
	2 – (3+)	0.23	0.66	56	0.3	0.94
Hematocrit	1 – 2	-0.02	0.01	53	-2.1	0.10
	1 – (3+)	-0.01	0.01	53	-1.1	0.54
	2 – (3+)	0.01	0.01	53	0.9	0.63

Table S8. Model summaries for the reproductive success (average brood mass, hatch success, fledging success) of females based on (A) age and (B) number of sires per brood. The reference groups are 'SY', which describes first-time breeding females, and 'Number of sires per brood 1', which describes broods with one sire.

(A) Reproductive success with respect to female age

Reproductive success metric	Model covariates	Estimate	SE	t value	p value
Average brood mass	(Intercept)	9.0	1.2	7.4	**<0.001**
	Female age	0.6	0.5	1.2	0.25
	Nestling age at sampling	1.1	0.2	7.2	**<0.001**
Fledging success	(Intercept)	4.8	0.2	23	**<0.001**
	Female age	-0.5	0.3	-1.9	0.07
Reproductive success metric	**Model covariates**	**Estimate**	**SE**	**z value**	**p value**
Hatch success	(Intercept)	2.2	0.1	21	**<0.001**
	Female age	-0.6	0.1	-5.3	**<0.001**

(B) Reproductive success with respect to number of sires per brood

Reproductive success metric	Model covariates	Estimate	SE	t value	p value
Average brood mass	(Intercept)	8.7	1.3	6.5	**<0.001**
	Number of sires 2	1.3	0.7	1.7	0.09
	Number of sires 3+	1.2	0.9	1.3	0.18
	Nestling age at sampling	1.0	0.2	6.0	**<0.001**
Fledging success	(Intercept)	4.2	0.3	15	**<0.001**
	Number of sires 2	0.5	0.3	1.6	0.12
	Number of sires 3+	0.8	0.4	1.9	0.06
Reproductive success metric	**Model covariates**	**Estimate**	**SE**	**z value**	**p value**
Hatch success	(Intercept)	1.9	0.1	14	**<0.001**
	Number of sires 2	0.1	0.1	1.0	0.32
	Number of sires 3+	0.3	0.2	1.6	0.12

Table S9. AICc-based model selection parameters and output for circulating (log-transformed) 17ß-estradiol levels. 'Number of sires per brood' refers to the number of sires per brood, based on paternity analyses. 'Female age' refers to a female's age, based on plumage scores. The reference age group is 'SY', which describes first-time breeding females. Estradiol levels were natural log transformed.

g1 =lm(Number of sires per brood ~ Log estradiol levels *Female age)
g2 =lm(Number of sires per brood ~ Log estradiol levels +Female age)
g3 =lm(Number of sires per brood ~ Log estradiol levels)
g4 =lm(Number of sires per brood ~ Female age)
g5 =lm(Number of sires per brood ~ 1)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g5	2	116.75	0.00	0.38	0.38	-56.25
g3	3	117.02	0.27	0.33	0.71	-55.26
g4	3	118.72	1.97	0.14	0.85	-56.12
g2	4	119.11	2.36	0.12	0.96	-55.14
g1	5	121.44	4.69	0.04	1.00	-55.08

Table S10. Model summaries for the top ranked models from each model set in Table S9. The response variables are bolded in the first column and the independent covariates specified in the top-ranking model(s) are in the second column. The reference age group is 'SY', which describes first-time breeding females. We include all top model summaries for which the delta AICc score is < 2.0 and below the null model. The 'Intercept' is included as the only covariate if the null model was ranked highest.

Response	**Model covariates**	**Estimate**	**SE**	**t value**	***P* value**
Number of sires per brood	(Intercept)	2.0	0.1	21	**<0.001**
	(Intercept)	2.1	0.1	18	**<0.001**
	Log estradiol levels	-0.3	0.2	-1.4	0.17
	(Intercept)	2.1	0.2	13	**<0.001**
	Female age	-0.1	0.2	-0.5	0.61

Table S11. AICc-based model selection parameters and output for (A) amplicon sequence variant (ASV) richness, (B) Shannon index, and (C) Faith's phylogenetic distance.

(A) Model selection based on AICc for **ASV richness**.

g1 = lm(ΔRichness ~ Treatment +Year, +Number of days implanted)
g2 = lm(ΔRichness ~ Treatment +Year)
g3 = lm(ΔRichness ~ Treatment +Number of days implanted)
g4 = lm(ΔRichness ~ Year +Number of days implanted)
g5 = lm(ΔRichness ~ Treatment)
g6 = lm(ΔRichness ~ Year)
g7 = lm(ΔRichness ~ Number of days implanted)
g8 = lm(ΔRichness ~ 1)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g7	3	474.66	0.00	0.42	0.42	-234.03
g4	4	475.17	0.51	0.33	0.75	-233.07
g6	3	477.65	2.98	0.09	0.84	-235.52
g8	2	478.01	3.34	0.08	0.92	-236.86
g3	5	479.52	4.86	0.04	0.96	-233.97
g1	6	480.37	5.70	0.02	0.98	-233.05
g2	5	482.37	7.70	0.01	0.99	-235.39
g5	4	482.47	7.80	0.01	1.00	-236.72

(B) Model selection based on AICc for **the Shannon index**.

g1 = lm(ΔShannon ~ Treatment +Year, +Number of days implanted)
g2 = lm(ΔShannon ~ Treatment +Year)
g3 = lm(ΔShannon ~ Treatment +Number of days implanted)
g4 = lm(ΔShannon ~ Year +Number of days implanted)
g5 = lm(ΔShannon ~ Treatment)
g6 = lm(ΔShannon ~ Year)
g7 = lm(ΔShannon ~ Number of days implanted)
g8 = lm(ΔShannon ~ 1)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g8	2	188.30	0.00	0.36	0.36	-92.00
g7	3	189.11	0.81	0.24	0.61	-91.26
g6	3	190.45	2.15	0.12	0.73	-91.93
g4	4	191.19	2.89	0.09	0.82	-91.08
g5	4	191.59	3.29	0.07	0.89	-91.28
g3	5	191.67	3.37	0.07	0.95	-90.04
g1	6	193.81	5.51	0.02	0.98	-89.77
g2	5	193.93	5.63	0.02	1.00	-91.18

(C) Model selection based on AICc for **Faith's phylogenetic distance**.

g1 = lm(ΔFaith's ~ Treatment +Year, +Number of days implanted, data =data)
g2 = lm(ΔFaith's ~ Treatment +Year, data =data)
g3 = lm(ΔFaith's ~ Treatment +Number of days implanted, data =data)
g4 = lm(ΔFaith's ~ Year +Number of days implanted, data =data)
g5 = lm(ΔFaith's ~ Treatment, data =data)
g6 = lm(ΔFaith's ~ Year, data =data)
g7 = lm(ΔFaith's ~ Number of days implanted, data =data)
g8 = lm(ΔFaith's ~ 1, data =data)

Model	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
g4	4	299.70	0.00	0.52	0.52	-145.34
g6	3	301.81	2.11	0.18	0.70	-147.61
g7	3	301.85	2.15	0.18	0.88	-147.63
g1	6	304.80	5.09	0.04	0.92	-145.26
g8	2	304.99	5.28	0.04	0.96	-150.35
g2	5	306.03	6.33	0.02	0.98	-147.23
g3	5	306.67	6.96	0.02	1.00	-147.54
g5	4	309.03	9.32	0.00	1.00	-150.00

Table S12. Model summaries for the best performing model for (A) amplicon sequence variant (ASV) richness, (B) Shannon index, and (C) Faith's phylogenetic distance in Table S11. The response variables are bolded in the first column and the independent covariates specified in the top-ranking model are in the second column. We include all top model summaries for which the delta AICc score is < 2.0 and below the null model. The 'Intercept' is included as the only covariate if the null model was ranked highest. The reference sampling year is '2018'.

Response	**Model covariates**	**Estimate**	**SE**	**t value**	***P* value**
Change in ASV Richness	(Intercept)	-33.5	15.1	-2.2	**0.03**
	Days implanted	1.4	0.58	2.4	**0.02**
	(Intercept)	-11.9	21.9	-0.54	0.59
	Sampling year	-24.6	18.2	-1.4	0.18
	Days implanted	1.3	0.58	2.2	**0.03**
Response	**Model covariates**	**Estimate**	**SE**	**t value**	***P* value**
Change in Shannon	(Intercept)	-0.05	0.30	-0.16	0.88
Response	**Model covariates**	**Estimate**	**SE**	**t value**	***P* value**
Change in Faith's PD	(Intercept)	-0.13	3.0	-0.04	0.97
	Sampling year	-5.3	2.5	-2.1	**0.04**
	Days implanted	0.17	0.1	2.1	**0.04**

Table S13. PERMANOVA summaries for female cloacal bacterial community structure (based on Bray-Curtis and Jaccard,) during (A) nest building and (B) incubation.

(A) Nest building sampling period

Distance metric	Model factors	df	F model	R^2	Pr(>F)
Bray-Curtis	Treatment	2	1.0	0.05	0.39
	Residuals	41		0.95	
	Total	43		1.0	
Jaccard	Treatment	2	0.99	0.05	0.47
	Residuals	41		0.95	
	Total	43		1.0	

(B) Incubation sampling period

Distance metric	Model factors	df	F model	R^2	Pr(>F)
Bray-Curtis	Treatment	2	1.5	0.07	0.06
	Residuals	41		0.93	
	Total	43		1.0	
Jaccard	Treatment	2	1.3	0.06	0.07
	Residuals	41		0.94	
	Total	43		1.0	

Table S14. Betadisper summaries for female cloacal bacterial community structure (based on Bray-Curtis and Jaccard) during (A) nest building and (B) incubation.

(A) Nest building sampling period

Distance metric	Model factors	df	F model	Pr(>F)
Bray-Curtis	Groups	2	1.3	0.30
	Residuals	41		
Jaccard	Groups	2	1.7	0.19
	Residuals	41		

(B) Incubation sampling period

Distance metric	Model factors	df	F model	Pr(>F)
Bray-Curtis	Groups	2	2.0	0.16
	Residuals	41		
Jaccard	Groups	2	2.3	0.11
	Residuals	41		

Table S15. Model summaries for the reproductive success (average brood mass, hatch success, fledging success) of females per treatment group. The reference group is 'Treatment None', which describes control females that were not implanted.

Reproductive success metric	Model covariates	Estimate	SE	t value	p value
Average brood mass	(Intercept)	6.1	1.4	4.4	**<0.001**
	Treatment Blank	-0.1	0.7	-0.1	0.89
	Treatment Estradiol	0.3	0.7	0.4	0.70
	Nestling age at sampling	1.6	0.2	9.3	**<0.001**
Reproductive success metric	**Model covariates**	**Estimate**	**SE**	**z value**	**p value**
Hatch success	(Intercept)	0.9	0.2	5.5	**<0.001**
	Treatment Blank	-0.3	0.2	-1.6	0.11
	Treatment Estradiol	-0.8	0.2	-3.5	**<0.001**
Fledging success	(Intercept)	4.4	0.6	7.2	**<0.001**
	Treatment Blank	-0.7	0.8	-0.9	0.35
	Treatment Estradiol	-1.6	0.8	-2.1	0.05

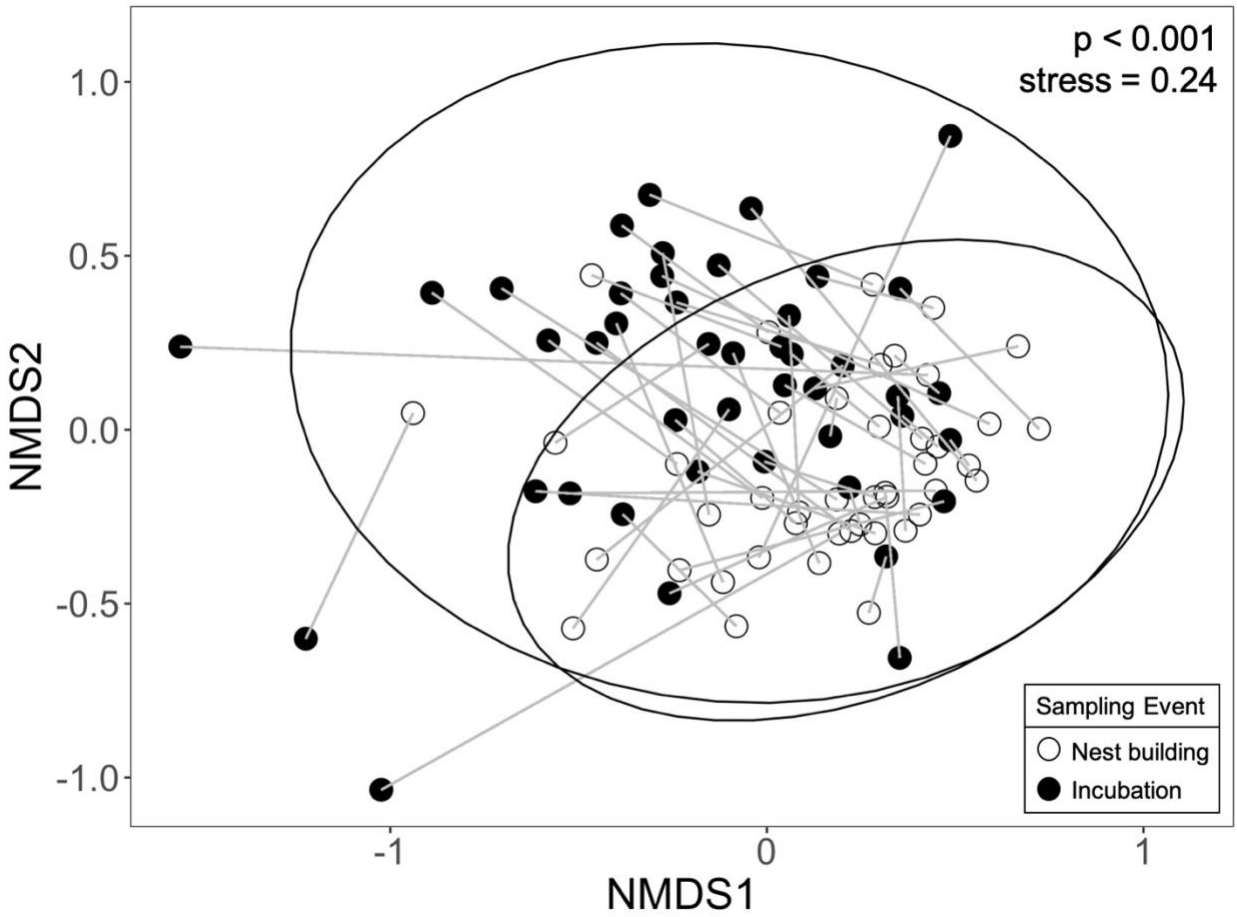

Figure S1. Cloacal bacterial beta diversity (non-metric multidimensional scaling plot, NMDS based on Bray-Curtis dissimilarity) of female tree swallows at two breeding stages (nest building, incubation). Each circle represents an individual female. Open circles = females sampled during nest building, filled circles = females sampled during incubation. Gray lines connect the two points per individual female. Circles closer together indicate individuals with more similar cloacal bacterial community composition. Sample sizes include *n* = 44 females per sampling period. NMDS based on Jaccard dissimilarity (not pictured here) is similar. P-value is based on a PERMANOVA model.