UNRAVELING THE NEST MICROBIOME: CHARACTERIZING AVIAN-ASSOCIATED BACTERIAL COMMUNITIES AND THEIR INFLUENCES ON HOST TRANSGENERATIONAL IMMUNE INVESTMENT

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UNRAVELING THE NEST MICROBIOME:

CHARACTERIZING AVIAN-ASSOCIATED BACTERIAL COMMUNITIES AND THEIR INFLUENCES ON HOST TRANSGENERATIONAL IMMUNE INVESTMENT

Anna Maria Forsman, Ph.D.

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To determine how bacterial communities that Tree Swallows encounter in the nesting environment influence maternal deposition of protective antibodies into eggs, I experimentally altered the microbiome of nests in Ithaca and collected samples of nest bacteria and yolk antibodies from across the species' North American range.

Chapter 1 describes an experiment to test whether alterations of the nest microbiome affect maternal antibody deposition and to evaluate the hypotheses that levels of transmission correlate positively with a) bacterial abundance or b) diversity. I found that maternal antibody transmission is a flexible trait that can rapidly respond to experimental changes in the antigenic environment and that yolk antibody deposition was positively related to bacterial diversity but not bacterial abundance. Immune-based maternal effects extended into the nestling period where young hatchlings from nests with decreased bacterial diversity also had lower levels of circulating antibodies and decreased bactericidal immune activity. My results provide support for the hypothesis that maternal contribution of protective antibodies increases with the likelihood of antigenic challenge and that antigen diversity contributes more strongly to this relationship than does antigen abundance.

Chapters 2 and 3 describe the comparative component of my dissertation research. To characterize the relationship between naturally occurring nest bacteria and maternal antibody deposition, sets of Tree Swallow eggs and bacterial samples were collected from nests at 15 sites across North America over three years. There was a significant and positive correlation between bacterial diversity and maternal antibody deposition into eggs across the breeding range, but no significant effects of latitude, longitude, or elevation on maternal antibodies.

The second objective of my comparative project was to characterize the bacterial communities that naturally occur in Tree Swallow nests across North America. I found no evidence that these communities exhibit a latitudinal diversity gradient or a significant distance-decay relationship. Climate variables contributed more to the variation observed in alpha and beta diversities of bacteria than did geographic variables. As predicted, both pre-breeding season temperature and precipitation influenced bacterial communities. However, I also found evidence to suggest that climate effects on the nest microbiome may be mediated through variation in maternal behavior.

BIOGRAPHICAL SKETCH

Anna Forsman was born in Sweden in 1979 to parents Lena and Roland Forsman. Shortly after her birth the newly formed family moved to the United States to pursue new adventures, which began a series of moves back and forth across the Atlantic as Anna, and later also brother Johan, grew up as Swedish Americans. Summers were almost exclusively spent in Sweden where Anna had the opportunity to independently explore nature with a freedom seldom experienced by children today. These early experiences combined with the support and encouragement of her parents kindled a deep appreciation for the natural world that set in motion Anna's path towards a career in the field of biology.

Anna completed her undergraduate work at The College of William and Mary in Williamsburg, Virginia, where she discovered her love for birds and field ecology. She also became interested in microbiology and was thus nudged in the direction of multi-disciplinary research by a Professor who had started his career through work on birds and their bacteria; coincidentally this became the very same topic that Anna pursued for her own dissertation research several years later. However, before graduate school she spent two years in the beautiful state of New Hampshire, trying out life as a non-student and working as a research associate at the Dartmouth Medical School. The forests, lakes, and mountains reminded Anna of Sweden and solidified her plans for graduate work involving a major field component. However, her experiences while helping to establish and run the Microarray Core Laboratory at Dartmouth made her eager for the opportunity to apply her newfound laboratory skills to her own research questions. This opportunity presented itself in the laboratory of Charles F. Thompson at Illinois State

University where Anna pursued a Masters project combining behavioral ecology and immunology. To the tune of house wren song and Charlie Thompson's formative influence, Anna felt a strong calling to academia and so began her doctoral studies at Cornell immediately after completing her M.S. at ISU in 2007. Although she was excited about her new adventures in Ithaca, she was equally sad to leave behind some of the best friends and memories of her life that were collected during her years in Illinois.

Anna's time at Cornell has seen both extremes of the spectrum of joy and hardship and has weathered more significant life events than originally anticipated. The unquestionable highlight was the birth of her son, Anders Wren, in December of 2012, followed by the defense and completion of her dissertation work during the summer of 2016. Through the incredible support of her family, friends, and committee, Anna was able to complete her goals despite unforeseen and very heavy hardships. Her advisor, David Winkler, provided a steady hand of support both academically and personally that allowed Anna to see her fiercely independent work to completion. She now leaves Cornell to pursue a research position at the University of Central Florida in Orlando to help build UCF's new Genomics Cluster.

For Anders Wren, who reminds me every day to love, appreciate, and live in the moment – because there is, and never will be, anything better than that.

For my parents, Lena and Roland, who have given me everything I need to make my own way in this world and who have always believed in my ability to find it.

For my brother, Johan, who is my dearest friend and protector – despite the thousands of miles that separate us, we are always near (and no bird is safe).

”Drunknat? Åh nej!

Det är lika omöjligt för mig att sjunka som för en kamel att trä på en synål”.

-Astrid Lindgren via Pippi Långstrump

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I would like to thank my advisor, David Winkler (i.e., Wink), and all of the students and researchers associated with his lab group. Life doesn't stop just because you're a graduate student, and I am so thankful for Wink's patience and support through my own personal struggles during my time at Cornell. I am so grateful that Wink believed in my ability to work through hardship and always supported and defended my decisions on how best to proceed towards my goals. Thank you also to all of my academic siblings, past and present. I am especially grateful to David Cerasale who covered for me at my Ithaca field site in 2009 while I collected samples in Alaska. I could not have completed both experimental and comparative components of my project without his help. I am also grateful to Valentina Ferretti whose input on my

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Finally, I would like to thank my amazing family: my son Anders, my parents Roland and Lena, my brother Johan, and all of my extended family across the pond that have cheered me on over the years. Anders: you may only be three years old, but with your spoonbill-finding skills and ability to cut to the heart of any matter, we make quite a team; I am so honored and inspired to have you as my co-pilot. Although the course may not be exactly as we had planned, I believe that you and I together will continue to crest the hills that seem insurmountable. You make every day an adventure, and I cannot wait to discover with you all the exciting things that lie ahead. Jag älskar dig. Mom and Dad: thank you for everything. Thank you for all the childhood experiences and opportunities that have shaped me both personally and professionally. Thank you for believing in me and for putting up with my unending ideas and, at times, completely exhausting enthusiasm about this, that, and the other. Thank you to my parents and also my brother, Johan, for providing both anchor and sails these last few years after I was thrown into completely uncharted waters. I am so grateful for your unfailing love and support of me and Anders, and I am so thankful for the previously unexpected helping of time that we now get to spend together as a family.

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Chapter 1

EXPERIMENTAL TEST OF THE ANTIGEN ABUNDANCE AND DIVERSITY HYPOTHESES OF TRANSGENERATIONAL IMMUNE INVESTMENT

INTRODUCTION

Female animals transfer a variety of immune compounds (e.g., antibodies, antimicrobials) to their embryos that impose maternal effects on offspring growth, protection, and immune trajectories (Bernardo 1996, Grindstaff, Brodie & Ketterson 2003). Henceforth, we will refer to these transgenerational effects as immune-based maternal effects. Species that undergo at least some development within the womb have a prolonged window for transmission of maternal compounds, which may be further extended through lactation (Grindstaff, Brodie & Ketterson 2003). Oviparous females, on the other hand, must deposit their entire contribution into one package, the egg. Studies of both domestic and wild birds have documented significant immune-based maternal effects on hatchling health through passive immunity (e.g., Heller *et al.* 1990, Mondal & Naqi 2001, Sahin *et al.* 2001, Rahman *et al.* 2002, Ahmed & Akhter 2003) and growth promotion (e.g., Heeb *et al.* 1998, Grindstaff 2008, Lozano & Ydenberg 2002), as well as long-term effects on life-time immune function (e.g., Gasparini *et al.* 2006, Reid *et al.* 2006). With the prospect of such transgenerational benefits, it is likely that maternal egg provisioning is subject to significant positive selection (Grindstaff *et al.* 2003). However, many immune compounds must be synthesized by females prior to egg laying and, therefore, require the maintenance or

even up-regulation of immune function, which is both energetically costly and may be accompanied by collateral damage to maternal tissues (Sheldon & Verhulst 1996, Råberg *et al.* 1998, Ilmonen, Taarna & Hasselquist 2000, Ots *et al.* 2001, Bonneaud *et al.* 2003, Martin, Scheuerlein & Wikelski 2003, Hanssen *et al.* 2004). Recent work indicates that high levels of maternal antibody transmission may also have direct negative consequences for offspring immune system development by blocking immunological memory against early encountered antigens (Siegrist 2003, Siegrist 2007) and by limiting the B-cell repertoire in offspring (Addison *et al.* 2009, Lemke *et al.* 2009). Females should be selected to invest in immune-based maternal effects when the protective benefits of doing so outweigh any maternal or transgenerational costs that reduce fitness. Because very young animals have limited capacity to produce an efficient adaptive immune response (Brambell 1970, Solomon 1971, Lawrence & Arnaud-Battandier 1981), we hypothesized that the strength of immune-based maternal effects should increase with likelihood of hatchling infection.

Maternally derived antibodies (MAb's) are arguably the most well-known molecules associated with immune-based maternal effects in vertebrates (reviewed by Grindstaff *et al.* 2003). Antibodies (i.e., immunoglobulins) are specialized proteins produced by B-lymphocytes of the adaptive immune system. Highly specific binding sites allow antibodies to recognize and attach to foreign antigens initiating the process of pathogen destruction. Antibodies produced by a donor individual confer passive immunity to a host by neutralizing and flagging antigens, thereby circumventing the need for endogenous antibody production; this is the concept underlying the use of anti-sera to neutralize some toxins and pathogenic molecules in humans (Murphy 2012).

Similarly, vertebrates confer passive immunity to their offspring by transmitting antibodies directly to the embryo through the placenta or by depositing antibodies into their eggs, milk, or colostrum (Grindstaff *et al.* 2003). Although passive immunity is temporary, there is some evidence that maternal antibodies also confer longer lasting protection by priming the developing immune systems of offspring for more efficient endogenous defenses later in life (Lemke & Lange 1999, Lemke, Hansen & Lange 2003, Lemke, Coutinho & Lange 2004, Grindstaff *et al.* 2006, Reid *et al.* 2006, Lemke et al. 2012). A primary antibody response to a novel antigen takes approximately one week in passerines (Killpack & Karasov 2012), whereas a secondary response to a previously encountered antigen is more rapid because of preformed antigen-specific memory cells (Murphy 2012). Therefore, female birds likely transfer maternal antibodies with specificities for both common and novel antigens that they encounter in the nesting environment prior to egg laying. Given both the immediate protection and vaccination-like effects conferred by maternal antibodies, there is likely strong selection for immune-based maternal effects under conditions of high antigen pressure in the offspring environment. However, this hypothesis has yet to be directly addressed, largely because of technical limitations on the identification of relevant antigens and pathogens.

The immune system is continuously faced with antigenic stimuli, most of which are innocuous but some of which are associated with harmful pathogens. This complex antigenic landscape includes any particles or microorganisms that have the capacity to stimulate an immune response, such as bacteria, fungi, viruses, and allergens. Antigens (Ag's) are diverse and ubiquitous in the environment, and there is much variation in immunological response to Ag's among host individuals. Studies investigating individual

immune function in response to antigenic challenge have focused on detecting or manipulating specific antigens. For example, vaccination studies aim to characterize individual responses to a particular antigen upon initial or repeated exposure (e.g., Mondal & Naqi 2001, Grindstaff *et al.* 2006, Forsman *et al.* 2008a, Forsman *et al.* 2008b, Hermans *et al.* 2014). This approach is powerful because investigators can select experimental antigens that address different types of host responses and that vary in their degree of novelty to the host immune system. However, the antigen-specific approach does not address how the immune system interacts with the broader antigenic landscape as a whole, which is much more complex (Horrocks, Matson & Tieleman 2011). We have known for some time that bacteria and other microorganisms are ubiquitous in the environment (Gilbert *et al.* 2011) and that they form highly diverse communities, both free-living and in association with other organisms (Ley *et al.* 2008, Gilbert, Sapp & Tauber 2012). However, it is less clear how the antigenic landscape that these microbial communities create influence immune system function in organisms encountering them. In this study we were specifically interested in testing how whole bacterial communities encountered by female birds in the nesting environment influence the transmission of maternal antibodies to eggs and subsequently to developing offspring. Although vaccination studies have indicated that maternal immune challenge influences maternal antibody transmission, little work has been done to determine how changes to the antigenic landscape as a whole affect transgenerational immune investment.

In previous microbiological studies of wild bird populations, bacterial communities have been sampled from avian plumage (e.g., Burtt & Ichida 1999, Shawkey *et al.* 2005,

Kilgas *et al.* 2012a, Alt *et al.* 2015), cloaca, (e.g., Brittingham, Temple & Duncan 1988, Mills, Lombardo & Thorpe 1999, Klomp *et al.* 2008, van Dongen *et al.* 2013, Matson *et al.* 2015), the digestive tract (Godoy-Vitorino *et al.* 2008, Roggenbuck *et al.* 2014), feces (Lu *et al.* 2008, Banks, Cary & Hogg 2009, Colles *et al.* 2009, Benskin *et al.* 2015), nests (e.g., Goodenough & Stallwood 2010, Peralta-Sanchez *et al.* 2010, Peralta-Sanchez, Moller & Soler 2011, Brandl *et al.* 2014, Grizard *et al.* 2015) and eggs (e.g., Soler *et al.* 2011b, Peralta-Sánchez *et al.* 2012, Peralta-Sánchez *et al.* 2013, Grizard *et al.* 2014, Javůrková *et al.* 2014). Microbial infection of eggs is well-documented within the poultry industry (De Reu *et al.* 2005, De Reu *et al.* 2006, Gantois *et al.* 2009), and has also been described for wild bird populations (Pinowski *et al.* 1994, Bruce & Drysdale 1994, Cook *et al.* 2003, Cook *et al.* 2005b, Beissinger, Cook & Arendt 2005). Bacterial contamination of eggshells occurs through vertical transmission from passage through the maternal cloaca and horizontal transfer from nesting materials and incubating adults (Protais *et al.* 2003, Brandl *et al.* 2014, Soler *et al.* 2015, Martínez-García *et al.* 2015). Recent studies indicate that microbial infections of eggs occur at higher rates in areas that experience wetter and more humid environmental conditions as compared to areas with hot and dry conditions (Beissinger *et al.* 2005, Cook *et al.* 2003, Cook *et al.* 2005a, Cook *et al.* 2005b, Wang, Firestone & Beissinger 2011). Similarly, bacterial load in nests and nestlings has been found to correlate positively with precipitation (Berger, Disko & Gwinner 2003). Based on such work, studies of the antigenic landscape experienced by hosts have largely used climatic or ecological proxies for infection risk, as opposed to characterizing antigen communities directly (e.g., Addison *et al.* 2009, Horrocks *et al.* 2014, Horrocks *et al.* 2015). Such proxies may

be useful when microbial samples are not available, but they are not ideal because their assumed relationships with microbial diversity have not been rigorously tested over a broad range of environments. Another approach has been to estimate bacterial abundance through culture-based methods, as a measure of antigenic pressure experienced by hosts (Cook *et al.* 2003, Cook *et al.* 2005b). Although culturing of environmental bacteria is known to exclude large portions of bacterial communities that are not readily cultured (McCaig *et al.* 2001), this technique is still useful because it samples bacterial communities directly as opposed to relying on assumptions underlying environmental proxies. Estimates of bacterial abundance using culture-based techniques have in fact been found to correlate with risk of infection of wild bird eggs exposed to ambient conditions (Cook *et al.* 2003, Cook *et al.* 2005b). However, it is likely that other aspects of bacterial community structure, such as diversity or evenness, may be equally or relatively more important in shaping host immune response and transgenerational immune investment, especially if higher diversity communities are more likely to contain opportunistic pathogens. Culture-independent approaches are critical for addressing such questions because of their ability to survey a broader proportion of bacteria present in any given sample, regardless of culturability. Methods that seek to probe and sequence the 16S ribosomal RNA gene using universal bacterial primers offer a robust alternative to traditional culture-dependent methods. Such studies that target specific genes to assess biodiversity are referred to as marker gene studies (Wu et al. 2013) or metabarcoding studies (Taberlet et al. 2012), and they produce sequence data that may be classified taxonomically and used to calculate the relative abundances of taxonomic groups, alpha-diversity within samples, and beta-

diversity between samples. Although culture-independent approaches have become the preferred mode for assessing microbial biodiversity, technical limitations cannot be completely eliminated (e.g., primer biases) and should be considered when comparing results from studies using different protocols.

The first objective of the current study was to manipulate natural bacterial communities in active Tree Swallow nests (*Tachycineta bicolor*) to test the hypothesis that female birds deposit maternally-derived antibodies into their eggs in accordance with the antigenic landscape they experience prior to egg laying. Three experimental treatments were applied to nest bacterial communities to modify bacterial abundance and diversity; therefore, we predicted that yolk antibody deposition would differ significantly among these three treatment groups. If MAb deposition is a flexible trait that can be altered in response to changes in the antigenic landscape, then female birds may be able to protect their offspring from rapid changes in bacterial communities. Flexibility in immune compound deposition would also suggest that immune strategies are not necessarily locally adapted, but instead have the capacity to change over single generations in response to antigenic conditions. Currently there are limited data indicating whether free-living birds can alter deposition of immune compounds into eggs in response to differences in ambient bacterial communities. However, studies have shown that deposition of antigen-specific antibodies into eggs can be manipulated by immunizing females with the antigen prior to egg laying (e.g., Grindstaff *et al.* 2006, Ismail *et al.* 2013, Hermans *et al.* 2014, Merrill & Grindstaff 2014). Therefore we expected that nest bacteria would impose similar antigenic stimuli upon breeding females. Female Tree Swallows at our study site spend one to two weeks building their

nests (*pers. obs.*), suggesting that there is sufficient time for both primary and secondary maternal responses to antigens encountered in the nest prior to egg laying.

The second objective of this study was to test the Antigen Abundance and Antigen Diversity Hypotheses to evaluate how different aspects of the nest microbiome affect female deposition of maternal antibodies into eggs and their resulting immune-based maternal effects on offspring. The Ag-abundance Hypothesis proposes that maternal antibody transmission is positively correlated with antigenic abundance, whereas the Ag-diversity Hypothesis proposes that maternal antibody transmission is instead positively correlated with antigenic diversity. To our knowledge, these hypotheses have not yet been tested experimentally in a wild population to explain variation in maternal antibody transmission. Based on the Ag-diversity Hypothesis, we predicted that females experiencing more diverse bacterial communities deposit relatively more MAb's into their eggs than do females experiencing lower bacterial diversity. This prediction is based on the assumption that higher antigenic diversity produces a stronger immunological stimulus and hence results in the production and transmission of more diverse antibodies by the maternal immune system. To test this prediction of the Ag-diversity Hypothesis we reduced bacterial diversity in one treatment group by adding an experimental mixture of bacteria to natural nest communities (i.e., Bac+ treatment); we predicted and confirmed that experimental additions of large amounts of bacteria representing just six isolates would overwhelm natural communities and hence reduce community evenness and diversity. Alternatively, the Ag-abundance Hypothesis predicts that females exposed to higher bacterial abundances, regardless of taxonomic distributions, should transfer more MAb's to their eggs than females

experiencing lower bacterial abundance. To address the Ag-abundance Hypothesis we experimentally reduced bacterial abundance in a second treatment group to test the prediction that females experiencing fewer bacteria in the nesting environment deposit fewer MAb's into their eggs than do control females. Although similar experiments have been conducted to look at bacterial community effects on egg albumen defenses (Bedrani *et al.* 2013, Jacob *et al.* 2015), no information is currently available regarding how bacterial communities affect yolk antibody deposition.

FIELD AND LABORATORY METHODS

This research was conducted at the Cornell University experimental ponds in Tompkins County, NY (42°30'10.40"N, 76°26'14.49"W) between the months of April and July in 2009 and 2010. This fieldsite contains approximately 120 manmade wooden nestboxes mounted approximately one meter off the ground on metal poles affixed with predator guards. Nestboxes are used primarily by the Tree Swallow (*Tachycineta bicolor*), which is a common migratory passerine species that breeds throughout the northern United States and Canada (Winkler *et al.* 2011). Tree Swallows are socially monogamous, and females are primarily responsible for nest building, which means that they spend a significant amount of time in the nesting environment prior to egg laying. Nests are constructed of grasses and are often lined with waterfowl feathers. Females lay 4-7 eggs (Winkler *et al.* 2011), and very rarely fledge more than one brood per season at this location (*pers. obs.*). All work conducted for this study were approved by the Cornell University IACUC, and egg collecting permits were issued by U.S. Fish & Wildlife Service and the New York State Department of Environmental Conservation.

At the start of the breeding season, in late April, we began monitoring nestboxes every three days for signs of nest-building activity. Nestboxes were swept clean before the start of the breeding season, and thus, active nests were identified when a visible ring of nesting material was observed on the nestbox floor. Active nestboxes were randomly assigned to one of three treatment groups: Control, reduced bacterial load (i.e., Bac-), or enhanced bacterial load (i.e., Bac+). Treatment applications for each individual nest began the day immediately following their identification as being active, and continued until the 5th egg was laid and subsequently collected (detailed treatment description below). Once egg laying commenced, nests were visited each day to mark new eggs with a pencil to allow us to differentiate freshly laid eggs from those laid earlier. We collected only the 5th laid egg from each nest and allowed all remaining eggs in the clutch to undergo normal incubation and development. Upon egg-collection, each nest was sprayed once with 70% ethanol and no further treatments were applied throughout the duration of the breeding season. Collected eggs were immediately frozen and stored at -20°C until further analyses.

Nests were monitored throughout incubation and chick rearing, until either nest failure or successful fledge was observed. In both years, nestlings were weighed at hatch (i.e., brood day 0) and 6 and 12 days of age to assess growth throughout the nestling period. We collected adult and nestling blood samples into heparinized capillary tubes from a small puncture of the brachial vein. Adult females were captured during incubation or chick rearing to collect morphological measurements and blood samples. Nestling blood samples were collected at 6 (BD6) and 12 (BD12) days of age. All blood samples were kept on ice in the field, and were centrifuged later that same day to

extract plasma. In 2010, an aliquot of fresh plasma was reserved from each sample for conducting bacterial killing assays. Plasma samples collected in both years were frozen and stored at -80°C until antibody analyses. In 2010 we also conducted an in vivo phytohaemagglutinin (PHA) assay to measure cutaneous immune activity in 12-day old nestlings (Martin *et al.* 2006), following Forsman *et al*. (2010).

Bacterial Manipulation Treatments

To experimentally increase antigenic abundance of Bac+ nests, we inoculated nests with a mixture of bacteria that we collected, isolated, and cultured from old Tree Swallow nests. Briefly, in 2009 we sampled naturally occurring nest bacteria by exposing tryptic soy agar (TSA) plates to old nests remaining in several nestboxes from the previous field season at the Cornell experimental ponds. Inoculated plates were incubated at room temperature for 48 hours to allow for bacterial colony growth. Morphologically distinct colonies were isolated on fresh TSA plates to create one-cell cultures. The resulting cultures were identified by extracting total DNA (Powersoil kit, MoBio 12888), and then amplifying and Sanger-sequencing the 16S rRNA gene at the Cornell University Biotechnology Resource Center (universal bacterial primers: 27F-AGAGTTTGATCMTGGCTCAG/1492R- TACGGYTACCTTGTTACGACTT). Nucleotide BLAST searches revealed sequence similarity to five Gram+ bacteria and one Gram- bacterium (Fig. 1.1). Glycerol stocks of these six bacterial isolates were maintained at -80°C, and were used to prepare fresh cultures for the Bac+ treatment every three days during the experiment in both years of the study. On the day prior to each Bac+ treatment application, tubes of TSB were inoculated with glycerol stocks and cultured at

room temperature in a shaker; each bacterial isolate was cultured separately. The following morning, bacterial cell density of each culture was estimated spectrophotometrically so that equal amounts of each bacterial isolate could be combined to create the Bac+ treatment mixture. In the lab, 500 uL aliquots of the bacterial mixture were prepared in microcentrifuge tubes, which were immediately transported at ambient temperature to the fieldsite for application. We used a sterile Dacron swab to apply 500 uL of bacterial mix to the top layer of material in nest cups and to the nestbox opening of Bac+ treatment nests. To slow bacterial growth for the Bac- treatment group, we sprayed Bac- nests with 70% ethanol once every day between the start of nest building and egg collection. We used a standard household spray bottle (fine mist setting) to administer 4 hand-pumps per application, covering any eggs present with a gloved hand to avoid their exposure to the ethanol. Control nests received no treatment other than continued monitoring to standardize level of disturbance among treatments. All nests were visited at least every two days up until egg collection, and no nesting material was handled prior to egg collection except with single-use sterile swabs to apply Bac+ treatments.

Bacterial Treatment Testing

Prior to egg collection, we sampled nest bacterial communities to test the efficacy of the bacterial manipulation treatments. We used a culture-based approach to test for treatment effects on the abundance of culturable bacteria (2009 and 2010) and culture-independent techniques to test for treatment effects on bacterial diversity and community composition (2010). To sample bacteria at each nest, we dampened a

sterile Dacron swab in a microcentrifuge tube containing 500 uL of UV sterilized 1X PBS, which was then rubbed across exposed surfaces of the nest cup for a total of 10 seconds. The swab tip with collected bacteria was then returned to the microcentrifuge tube with remaining PBS and transported back to the laboratory under ambient conditions for culturing later that same day. In the lab, bacterial samples were vortexed to dislodge bacteria attached to the swab head. The swab head was then removed using flame-sterilized tweezers, and 50uL of the bacterial solution was removed and diluted 100-fold for culturing. We plated 50uL of each diluted bacterial sample on duplicate TSA plates and incubated overnight to allow for colony growth. Nest bacterial abundance was estimated by quantifying the number of visible colonies 24 hours later (i.e., CFU, colony forming units). We predicted that bacterial samples collected from Bac+ nests would contain more CFUs than samples from Control nests, and that samples from Control nests would contain more CFUs than samples from Bac- nests. In 2010 we retained the bacterial solution remaining after culture preparations to characterize alpha-diversity and bacterial community composition among treatment groups with 16S rRNA gene surveys. Bacterial samples retained for sequencing were centrifuged at 16,000 x g for 10 minutes to pellet cells, which were stored at -20°C until DNA extraction (MoBio PowerSoil Kit, cat. no. 12888). Bacterial DNA samples were subsequently submitted to the Earth Microbiome Project for library preparation and Illumina sequencing.

High-throughput sequencing

PCR amplification and amplicon preparation for sequencing were performed following the protocols described in Costello et al. (2009 and 2012), and can be found on the Earth Microbiome Project (Gilbert, Jansson & Knight 2014) web page (http://www.earthmicrobiome.org/emp-standard-protocols/). Universal bacterial primers (515F: 5'-GTGCCAGCMGCCGCGGTAA-3'; 806R: 5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the bacterial and archaeal 16S rRNA variable region 4 (Walters *et al.* 2011, Caporaso *et al.* 2012). Each DNA sample was amplified in triplicate and amplicons were pooled together and quantified using a PicoGreen dsDNA assay kit (Invitrogen P7589). Negative controls included no-template controls at both DNA extraction and PCR amplification steps. All barcoded amplicons were pooled in equal concentrations for sequencing and purified using the UltraClean PCR Clean-up kit (Mo Bio Laboratories 12500). Amplicons were sequenced in two different runs on the Illumina HiSeq2000 sequencing platform at the BioFrontiers Institute Next-Generation Genomics Facility at University of Colorado (Boulder, USA).

Amplicon sequences were analyzed using QIIME (version 1.8.0; Caporaso *et al.* 2010b). Sequences were de-multiplexed and quality-filtered according to default parameters within QIIME. We assigned sequences to operational taxonomic units (OTUs) with a closed-reference protocol, which captures the full range of bacterial diversity within our data set, following the default parameters in QIIME (http://qiime.org/tutorials/open_reference_illumina_processing.html; see also (Navas-Molina *et al.* 2013). Sequences were clustered into OTUs with a sequence similarity threshold of 97% using UCLUST (Edgar 2010) and then, a representative sequence

from each OTU was matched against the Greengenes ribosomal RNA reference database (August 2013 release version; http://greengenes.secondgenome.com; (DeSantis *et al.* 2006, Mcdonald *et al.* 2012). Representative OTUs that matched the reference database received a taxonomic classification standardized in Greengenes with the RDP classifier (Wang *et al.* 2007). The representative sequences of all OTUs were then aligned to the Greengenes reference alignment using PyNAST (Caporaso *et al.* 2010a), and this alignment was used to construct a phylogenetic tree using FastTree (Price, Dehal & Arkin 2010). Chloroplasts, mitochondria, Archaea, sequences not assigned to phyla, and OTUs representing less than 0.00005% of the total reads in the data set were filtered out as recommended for Illumina-generated sequence data (Bokulich *et al.* 2013). Rarefaction for sequencing effort standardization was performed at a depth of 9700 sequences. After the described data processing, 43 samples were retained for subsequent analyses. We calculated alpha diversity for each bacterial sample as a) OTU richness, b) Shannon's Diversity Index, c) Faith's Phylogenetic Diversity index (Faith 1992), and d) community evenness. The objective of this approach was to characterize bacterial communities to a degree that would allow for comparisons of alpha-diversity and bacterial community composition between treatment groups.

Yolk and Plasma IgY

Total immunoglobulin Y (IgY) content of egg yolks and plasma IgY concentration from nestling and maternal blood samples were measured using ELISA (enzyme-linked immunosorbent assays). IgY is an avian antibody isotype that is analogous to the

mammalian isotype IgG (review Murai 2013). Frozen eggs were thawed one at a time on ice. Eggshells were carefully removed, washed with deionized water, and dried to obtain eggshell mass. Albumen thaws faster than yolk and can thus be removed by this method without disturbing yolk integrity. Each thawing yolk was rolled briefly on a clean Kimwipe tissue to remove any residual albumen, and weighed on an analytical balance to obtain yolk mass. Each yolk was then homogenized with a small stainless steel spatula and 0.05 grams of homogenized yolk was added to a microcentrifuge tube containing three glass beads and 200 uL of PBS-T wash buffer (1x PBS, 1% bovine serum albumin, 0.05% Tween-20). Diluted yolk samples were mounted on a vortex and shaken vigorously for 10 minutes, and then frozen at -20°C until ELISA.

96-well ELISA plates were coated overnight at 4°C with a polyclonal capture antibody diluted in carbonate buffer (0.15 mol l^{-1}, pH 9.6) to a concentration of 5.96 ug/mL. The capture antibody has been developed for detecting heavy and light chain IgY in dove, duck, sparrow, and chicken (Bethyl Laboratories, A140-110A), and it has been used successfully for detecting yolk IgY in passerine species (e.g., (Grindstaff, Demas & Ketterson 2005, Grindstaff 2008). Coated plates were washed three times with ELISA wash buffer to remove unbound capture antibodies and to block well surfaces not coated with antibodies. Diluted yolk samples were added to wells in triplicate at a final concentration of 62.5 ug/mL in ELISA wash buffer (i.e., 6.25 ug per well). A standard curve was run in duplicate on each ELISA plate by adding a chicken plasma sample diluted 1:80,000 and serially diluting across the ELISA plate. Samples were incubated on ELISA plates overnight at 4°C. The following day, plates were washed and a horseradish peroxidase-conjugated secondary antibody (1:1000 dilution;

Bethyl Laboratories A140-110P) was added to detect Tree Swallow IgY. ELISA plates were analyzed by spectrophotometer (405 nm) 15 minutes after the enzyme substrate was added. To correct for background noise, optical density (OD) for each sample was normalized by subtracting OD values from blanks run in triplicate on each plate. Arbitrary Chicken Units (CU) were assigned to each standard curve (1-2,048 CU) to allow for the calculation of comparable units of IgY concentration among ELISA plates, based on the fact that 6.25 ug of yolk were added to each well. Total yolk deposition of IgY was calculated by multiplying IgY concentration (CU/ug) by measured yolk mass and converted to megaCU units.

For BD-6 plasma samples 25uL of a 1:1000 plasma dilution was added to each ELISA well, and for BD-12 samples 2.5uL was added instead. Plasma sample plates were read at 10 minutes after substrate addition for BD-6 samples, and 25 minutes for BD-12 samples. Because older nestlings have significantly higher concentrations of circulating antibodies than younger nestlings, ELISA conditions had to be optimized accordingly. The dilutions and read times were selected to maximize variation among samples (Forsman unpublished). Plasma IgY concentration is reported as CU/uL of plasma.

Plasma Bactericidal Activity

Blood samples were collected from incubating females during the 2010 field season to quantify plasma concentrations of IgY (N=44) and bactericidal activity (N=48). Bactericidal activity of nestling plasma was also only measured in 2010. Assays were conducted following Forsman et al. (2008). Volume of nestling plasma and incubation

periods used for bacterial killing assays (BKA) were optimized for 6 day old and 12 day old nestlings to maximize variation among nestlings within each age group.

STATISTICAL METHODS

Bacterial Alpha-Diversity and Abundance

Illumina sequence analyses produced three measures of alpha-diversity (2010 only): species richness (i.e., OTU count), phylogenetic diversity (PD), and species diversity (Shannon Diversity Index). Species richness and diversity were used to calculate community evenness (lnS/H). We used a fixed-effects ANOVA to test for effects of treatment, date of sampling, and their interaction on each of the four indices of alpha diversity considered. No data transformations were necessary. A similar model was used to test for treatment effects on nest bacterial abundance, as measured by the numbers of bacteria cultured from nests among treatment groups (i.e., CFU count); this response variable was ln transformed to normalize the distribution of residuals produced by the model. The fixed effects model, describing nest bacterial abundance, included treatment, date of sampling, and year effect, as well as all first-order interactions.

Bacterial Community Composition

The Unifrac community distance metric (Lozupone & Knight 2005) was calculated and applied to beta diversity distance matrix analyses. We performed a Procrustes ANOVA (Anderson 2001, Collyer, Sekora & Adams 2015) using bacterial manipulation treatment as a fixed factor and the unweighted Unifrac distance matrix as an independent matrix. Principal Coordinate Analyses (PCoA) were performed to reduce dimensionality of

bacterial communities and to maximize variance of orthogonal axes. PCoA visualizations were performed with EMPeror (Vázquez-Baeza *et al.* 2013). Multivariate analyses of bacterial community composition data were performed with R statistical environment (version 3.1.3.; R_Core_Team, 2015) using the packages *geomorph* and *vegan*. *P* values below 0.05 were considered significant.

Egg Yolk IgY Deposition

Generalized linear mixed models (GLMM) were used to analyze variation in total yolk IgY deposition among female Tree Swallows. Treatment, year, lay date, and all first-order interactions were entered as fixed main effects. Lay date was initially entered as a continuous variable (i.e., Julian date). The dependent variable (megaCU IgY) was ln transformed based on residual analysis from this model. Because of a significant interaction between Julian date and Treatment, we ran a second, follow-up model with lay date instead entered as a categorical variable (i.e., early- or late-laying). Females were assigned as early-layers if clutch initiation began prior to the mean lay date for females included in the study in each year. Clutch initiation for these females ranged from Julian day 124 through 138 in 2009 and Julian day 124 through 145 in 2010. In both models, female identity was entered as a random variable to control for the fact that 13 females were sampled in both years of the study. Female identity could not be determined until the incubation stage without risking nest abandonment. Therefore, during the 2010 breeding season we were not aware whether females had been part of the experiment in the previous year until treatments had already been applied and eggs collected. We also included ELISA plate ID as a random factor to account for inter-plate

variation when quantifying yolk IgY concentration. Total yolk IgY data were ln-transformed to normalize residuals.

Egg Size

A generalized linear mixed model (GLMM) was used to analyze variation in absolute egg mass, yolk mass, and eggshell mass, which were all measured in both years of the study. Treatment, year, lay date, and clutch size were entered as fixed main effects, and female ID was entered as a random effect. First-order interaction effects were considered but subsequently dropped from the model as none were significant. No data transformations were necessary. In 2010 we also collected egg length and width measurements, allowing us to calculate egg volume and surface area. Incorporating these size variables into a second model allowed us to investigate variation in relative mass of eggs, yolk, and eggshell. Thus, this second mixed model included the fixed main effects of treatment, lay date, clutch size, and the covariate describing egg size. Hoyt's egg volume was used as the egg size covariate for analyses of egg mass and yolk mass, whereas egg surface area was used as the covariate for the analysis of eggshell mass. Non-significant interactions were dropped from the model.

Nestling Plasma IgY

Variation in blood plasma antibody concentration among nestlings was analyzed for both 6-day and 12-day-old nestlings in one GLMM using ln-transformed IgY data (CU/uL plasma). Treatment, year, age, mass nested within age group, lay date, and all first-order interactions were entered as fixed effects. We nested mass within age group

because 12-day old nestlings are significantly heavier than 6-day old nestlings, and so we wanted to capture the relationship between nestling mass and IgY within each age group separately. The continuous Julian lay date variable was entered as a squared term because we found the relationship between lay date and nestling plasma IgY to be better described as quadratic than linear. Nestling ID and nestbox were included as random effects to control for the fact that nestlings were sampled twice (BD6 and BD12) and to control for non-independence of siblings within broods. However, nestlings were not individually marked at day 6 in 2009, which means that day 6 and day 12 plasma samples could not be matched to one nestling in this year. Thus, controlling for Nestling ID in the overall model effectively only accounts for variation attributable to individuals in 2010 when nestlings were marked at 6 days of age. We feel confident that this does not affect the interpretations of the overall model because side-by-side analyses of 2010 data both with and without the random Nestling ID effect result in the same interpretation. Female ID and ELISA plate ID were also included as random effects. Tukey's honest significant difference (HSD) tests were used for follow-up comparisons of LS Means among treatment groups, while controlling for multiple tests.

Nestling Immune Activity

Bactericidal activity of nestling plasma was analyzed separately for 6-day-old and 12-day-old nestlings. The GLMM included fixed effects of treatment, nestling mass, Julian lay date, and interactions between treatment and each of the other variables; nestbox ID was included as a random variable, and no transformations were performed on the BKA data for either age group. We used the same model to analyze variation in PHA

response among nestlings, which was measured in 12-day old nestlings and in 2010 only.

Nestling Growth

Mean nestling mass was calculated for each nest at day of hatching (i.e., BD-0), BD-6, and BD-12. We first ran a repeated measures model including main effects only to assess the relative contributions of bacterial treatment, seasonal effects, sibling competition, and female quality on nestling mass; nest and female ID were included as random factors. Nestling mass data were ln-transformed to improve homogeneity of residuals produced from this model. Significant interactions resulting from this model were followed up with separate models for each age group, using raw nestling mass data, which improved homogeneity of residuals within age groups.

Maternal Immune Status

Simple linear regression analysis was used to characterize the relationship between total yolk IgY content and maternal plasma IgY concentration during incubation. Both variables were ln-transformed. To test for effects of treatment, season, and female quality on maternal plasma IgY we used a main effects model including treatment, lay date, female age, residual mass, and wing length. Residual mass is commonly used to characterize body condition of birds and is produced by regressing body mass on some structural measure of body size, such as head-plus-bill length, used here (r^2=0.0812, F=4.07, P=0.0496). A similar model design was used to analyze maternal plasma bactericidal activity.

RESULTS

Treatment Effects on Alpha-Diversity and Abundance of Nest Bacteria

Based on culture-independent methods for characterizing alpha-diversity of nest bacterial communities, we found significant overall effects of bacterial treatment on both bacterial community diversity and community evenness, which were not dependent on date of bacterial sampling (Table 1). However, there was no significant treatment effect on bacterial community richness. Nests that received experimental bacteria additions (Bac+) had lower bacterial community diversity ($LSMean_{Shannon}$=6.46, SE=0.18) and evenness ($LSMean_{Evenness}$=1.026, SE=0.020) than Control nests ($LSMean_{Shannon}$=7.23, SE=0.15, $LSMean_{Evenness}$=1.124, SE=0.017), whereas nests treated to reduce bacterial growth (Bac-) did not differ significantly in bacterial community diversity ($LSMean_{Shannon}$=6.85, SE=0.22) or evenness ($LSMean_{Evenness}$=1.093, SE=0.024) from either Control ($P_{Shannon}$=0.3324, $P_{Evenness}$=0.5709) or Bac+ nests ($P_{Shannon}$=0.3666, $P_{Evenness}$=0.0985). Richness of nest bacterial communities increased significantly with date of sampling, as did bacterial community diversity (Table1). However, bacterial community evenness did not evidence any significant seasonal effects across the pre-incubation period during which we sampled nest bacteria.

Analyses of culture-based data indicate significant effects of experimental treatment (F_2=103.68, P<0.0001) and year (F_1=20.166, P<0.0001) on nest bacterial load. We also detected a significant interaction between these two variables (F_2=10.03, P<0.0193), indicating that differences in bacterial abundance among treatment groups were stronger in 2010 than in 2009 (Fig. 1.3). The numbers of CFUs recovered from Bac- nests were significantly lower than those from Control and Bac+ nests in both

years (Fig. 1.3). Estimated abundance of culturable bacteria did not differ significantly between Control and Bac+ nests in either year (P_{2009}=1.000, P_{2010}=0.8623). Although variation between the two years of the study was dependent on treatment, the average estimated bacterial load was higher in 2009 (LS mean=177.3 CFUs) than in 2010 (49.0 CFUs). We also found that nest bacterial load tended to increase with advancing date during the pre-incubation period when nests were sampled (F_1=3.48, P=0.0655). There were no significant interactions between year and sampling date (F_1=0.01, P=0.9329) or between treatment and sampling date (F_2=0.05, P=0.9531).

Treatment Effects on Community Composition of Nest Bacteria

Bacterial communities sampled from Tree Swallow nests were dominated by the bacterial classes Alphaproteobacteria, followed by Betaproteobacteria, Cytophagia, Gammaproteobacteria, Actinobacteria, and Bacilli (Table 1.2, Fig. 1.4). The two bacterial classes represented in the experimental treatment mixture were Bacilli and Gammaproteobacteria (Fig. 1.1). As predicted, we found a significant overall treatment effect on the relative abundance of Bacilli (F_2=45.93, P<0.0001; Fig. 1.5); Bac+ nests had a significantly higher abundance of Bacilli than Bac- (P<0.0001) and Control nests (P<0.0001), whereas Control and Bac- nests did not differ from one another (P=0.9001). There were no significant treatment effects on the relative abundance of Gammaproteobacteria (F_2=1.21, P=0.3076).

Bacterial treatment did not significantly explain observed differences in bacterial community composition between samples (Procrustes ANOVA; PCo axes as dependent matrix; treatment as fixed factor, $F_{2,40}$=0.98, P=0.474). Therefore, bacterial samples do

not clearly cluster by treatment (Fig. 1.6). PCoA captured 26.86% of the total variance in beta diversity.

Total Egg Yolk IgY Deposition

From our initial model, considering lay date as a continuous variable (Table 1.3A), we found a significant overall effect of bacterial load manipulation on female deposition of antibodies into egg yolks (interaction effect, $F_{2,65.9}$=4.1073, P=0.0208). However, this treatment effect was significantly influenced by lay date (interaction, $F_{2,65.15}$=4.7581, P=0.0118). This interaction arises from the fact that unmanipulated control females deposited more IgY into egg yolks as the season progressed, whereas females exposed to experimentally modified nest bacterial communities exhibited similar levels of IgY deposition across the season (Fig. 1.7); these same trends hold when samples are separated by collection year. To better visualize the interaction between lay date and bacterial load manipulation, we assigned nests as belonging to either early or late season breeding attempts (see statistical methods). Running the model with lay date entered as a categorical variable confirmed the results obtained when using the continuous Julian date variable (Table 1.3), with no additional significant effects emerging. Tukey-Kramer follow-up analyses of least squares means indicate that yolk IgY deposition did not vary among treatment groups for early-breeding females, whereas later in the season, Bac+ deposited lower concentrations of yolk-bound antibodies (LSMean=4.29 megaCU) than control females (LSMean=6.90 megaCU; Fig. 1.8). There was no overall effect of year on yolk antibody deposition in either model,

and we observed no interannual variation in either treatment or laydate effects (Table 1.3).

Egg Morphology

No significant effects of treatment, year, lay date, or clutch size were found for absolute measures of egg mass, yolk mass, or eggshell mass for eggs collected across both years of the study (Table 1.4). However, egg mass tended to increase with advancing lay date, and yolk mass tended to decrease with increasing clutch size. To test for relative investment in different egg components, we examined variation in egg-, yolk-, and eggshell mass while controlling for egg volume or surface area, which were calculated from egg length and width measurements that were only collected in the second year of the study. From this subset of data we found a significant treatment effect on relative egg mass (Table 1.5); females exposed to the Bac+ treatment produced lighter eggs, when controlling for egg volume, than control females ($P=0.0263$). There was no significant difference in relative egg mass between Bac+ and Bac- nests ($P=0.0749$), nor between Bac- and Control nests ($P=1.0000$). No treatment effects were observed for either egg volume-adjusted yolk mass or surface area-adjusted eggshell mass (Table 1.5), suggesting that females do not modify relative investment in these egg compartments in response to environmental bacterial load. Instead we found a strong effect of clutch size on relative yolk size such that females producing larger clutches of eggs deposited lighter yolks, based on total egg volume, than females producing smaller clutches. There were no significant effects of clutch size

on relative egg mass or relative eggshell mass, and no significant effects of lay date on relative egg-, yolk-, or eggshell mass (Table 1.5).

Nestling Plasma IgY

We found a significant overall treatment effect of bacterial manipulation on circulating antibody concentration in nestlings (Table 1.6). This effect was not dependent on nestling age, lay date, or year. In accordance with the results observed for yolk antibody content, nestlings hatched and raised in Bac+ nests had lower concentrations of plasma antibodies (10,197 CU/uL) than control nestlings (13,769 CU/uL; p=0.0247), but did not differ significantly from those of nestlings in Bac- nests (12,101 CU/uL; p=0.2219). Control nestlings and Bac- nestlings did not differ in plasma antibody concentration (p=0.3996; Fig. 1.9). Unlike the results for yolk IgY, there was no significant interaction between bacterial manipulation treatment and lay date for nestling plasma IgY. Nestling mass was positively correlated with plasma antibody concentration for both 6-day old and 12-day old nestlings (Table 1.6), which is not surprising as immune measures are often found to correlate with body condition (e.g., Møller et al. 1998, Navarro et al. 2003). The significant relationship between lay date and plasma antibody concentration was quadratic in nature so that nestlings from early and late initiated broods had lower plasma IgY than nestling from broods initiated during the middle of the breeding season. As expected, we found a significant effect of age such that 12-day old nestlings had higher concentrations of plasma antibodies (26,322 CU/uL) than 6-day old nestlings (5,409 CU/uL; Table 1.6). By 12 days of age, the majority of antibodies detected in circulation are produced endogenously as maternal antibodies degrade (Grindstaff *et al.*

2003). Nestling plasma antibody concentrations were overall higher in 2009 (17,933 CU/uL) than in 2010 (7,940 CU/uL; Table 1.6). This inter-annual difference corresponds with the year effect observed for cultured bacterial abundance, which was also higher in 2009 than in 2010. A significant interaction effect between nestling age and year revealed that although 6-day old nestlings had similar levels of circulating antibodies in 2009 (6,405 CU/uL) and in 2010 (4,568 CU/uL), 12-day old nestlings in 2009 had significantly more plasma antibodies (50,208 CU/uL) than 12-day old nestlings the following year (13,800 CU/uL). One would predict that plasma antibody levels would vary less among very young nestlings as they are influenced primarily by maternal effects conferred through egg contributions prior to endogenous antibody production has begun. These results corroborate the non-significant year effect on yolk IgY.

Nestling Bactericidal Activity

There was a significant effect of bacterial load manipulation on nestling bactericidal capacity at 6 days of age but not at 12 days of age (Table 1.7). At 6 days of age, nestlings from Bac+ nests exhibited lower bactericidal capacity (33.4% of *E. coli* killed) than control nestlings (50.6 % killed; P=0.0239). Nestlings from Bac- nests (45.9% killed) did not differ from either control (P=0.8277) or Bac+ nests (P=0.2842). These results suggest that treatment-induced maternal effects influence antimicrobial defenses shortly after hatch but then dissipate as endogenous immune activity ramps up in developing nestlings. Body mass had strong positive effects on bactericidal activity in both 6-day and 12-day old nestlings, which were independent of treatment effects (Table 1.7).

Nestling PHA Response

We found a significant negative effect of body mass on PHA response ($F_{1,73.71}$=5.0580, P=0.0275), such that heavier nestlings mounted weaker PHA responses. However, there were no significant effects of bacterial manipulation ($F_{2,22.6}$=0.0078, P=0.9923) or lay date ($F_{1,27.49}$=2.0541, P=0.1631) on PHA response, and no significant interactions between treatment and either lay date ($F_{2,27.37}$=0.8129, P=0.4540) or mass ($F_{2,74.56}$=0.1145, P=0.8919).

Nestling Growth

We found significant effects of sampling year ($F_{1,69.9}$=46.8022, P<0.0001), lay date ($F_{1,71.02}$=9.8337, P=0.0025), nestling age ($F_{2,142.8}$=4559.049, P<0.001), and brood size ($F_{1,68.22}$=5.4984, P=0.0220) on nestling body mass. Because there were no significant effects of treatment ($F_{2,70.2}$=0.8678, P=0.4243) or female body condition ($F_{1,67.93}$=1.2421, P=0.2690), we dropped these factors from the model to preserve degrees of freedom. The final model used to explain variation in mean nestling mass included year, lay date, nestling age, brood size, and all first-order interactions (Table 1.8). There were no observed effects of bacterial manipulation treatment on nestling mass throughout the nestling period. Repeated measures analysis indicated that nestlings were significantly heavier in 2009 than in 2010, at all nestling ages measured (Table 1.8). As expected, nestling mass differed among age groups. Although there was no overall effect of lay date, we found a strong lay date by age interaction, suggesting that seasonal effects may be relatively more important at different points during the nestling period; lay date effects also differed between years. Similarly, we found that the

effect of brood size on nestling mass tends to vary among age groups (Table 1.8). Follow-up analyses by nestling age revealed that increasing brood size had a negative effect on nestling mass at 12 days of age ($F_{1,64.53}$=4.7476, P=0.0330), but not at earlier points during the nesting period (BD-0: $F_{1,59.74}$=0.2938, P=0.5898; BD-6: $F_{1,64.74}$=1.1632, P=0.2848). Follow-up analyses also indicate a significant lay date by year interaction effect on nestling mass at 6 days of age ($F_{1,66}$=14.7735, P=0.0003), but not at hatch or at 12 days of age (all P>0.6642). In 2009, mean nestling mass at 6 days of age increased with advancing lay date (r^2=0.1257, P=0.0367), whereas in 2010 6-day old nestling mass decreased with advancing lay date (r^2=0.5538, P<0.0001). Early in the nestling period, nestlings may be more dependent on female quality and environmental conditions since they are not yet able to thermoregulate. Competition among younger siblings may not yet be so intense, perhaps because parents can keep up with a more equal distribution of resources since demand is less than for older nestlings. If insect abundances were lower in 2010, then later-laying poor quality females would have had a harder time keeping up with the demands of brooding and provisioning than would females breeding in 2009.

Female plasma antibodies

The total amount of IgY transferred to egg yolks was significantly and positively correlated with the concentration of IgY in maternal circulation during incubation (N=34, P=0.0222, r^2=0.1529), although there was no significant treatment effect on maternal plasma IgY (see statistical methods).

There were no significant effects of treatment (F_2=0.3959, P=0.6759) or female age (F_1=0.2422, P=0.6256) on plasma IgY concentration in post-laying females; thus, these factors were dropped from the model to focus on potential effects of female quality on plasma IgY. The final model describing maternal plasma IgY included the main effects of lay date, residual mass, wing length, and all first-order interactions. Lay date had a strong positive effect on plasma IgY (F_1=28.7654, P<0.0001), indicating that females initiating clutches later in the season had higher levels of circulating antibodies than earlier laying females. However, as lay date is confounded with date of blood sampling (R^2=0.8791, P<0.0001), we cannot decipher if these date related effects are related to season or female quality. But as we found no significant effects of wing length (F_1=2.39, P=0.1305), residual mass (F_1=0.10, P=0.7523), or interactions (all, P>0.3506) on maternal IgY levels, it is likely that seasonal effects are more influential than maternal condition.

Maternal Immune Status

There were no significant effects of treatment or maternal age on plasma bactericidal activity of post-laying females (Treatment, F_2=1.45, P=0.2475; Age, F_1=0.70, P=0.4096), and these variables were thus removed from the model. The final model included lay date, maternal condition and wing length, and all first-order interactions. We found a significant positive effect of lay date on maternal bactericidal capacity (F_1=4.17, P=0.0480); plasma samples collected from females that initiated egg-laying earlier in the field season exhibited less efficient bacterial killing activity than samples collected from later-laying females. Female condition was negatively related to BKA

(F_1=5.11, P=0.0294), however, there was no significant effect of wing length on maternal BKA (F_1=0.06, P=0.8016). None of the interaction effects were significant (all, P>0.1102).

DISCUSSION

The objective of this study was to experimentally test the Antigen Diversity and Antigen Abundance hypotheses of transgenerational immune investment. Our results provide evidence that diversity and evenness of the nest microbiome enhance maternal deposition of yolk antibodies in breeding Tree Swallows, whereas no direct support was established for the Antigen Abundance hypothesis. We created three different treatment groups with the intention of manipulating abundance and diversity of bacteria inhabiting the natural nests of free-living Tree Swallows. We predicted that experimental additions of cultured nest bacteria to active nests would increase bacterial abundance and concomitantly decrease bacterial diversity of the nest microbiome as compared to that of unmanipulated and ethanol-treated nests. Because our bacterial treatment mix was composed of just six different isolates already known to be common in Tree Swallow nests at this field site (Fig. 1.1), we predicted that their experimentally increased abundance in Bac+ nests would decrease community evenness and, as a result, decrease community diversity. Our results from 16S rRNA gene sequence-based analyses confirm these predictions as Bac+ nests had both lower evenness and diversity than did control nests. Bac- nests did not differ significantly in evenness or diversity from either Bac+ or Control nests, suggesting that our ethanol treatment disrupted the nest microbiome slightly but not significantly. We also initially predicted

that bacterial additions would increase bacterial abundance in the nesting environment. However, our results indicate that overall abundance of culturable bacteria was not significantly higher in Bac+ nests than control nests. The abundance of culturable bacteria was significantly reduced in Bac- nests as compared to both Bac+ and Control nests. The lack of a difference between Bac+ and Control nests may be because some portion of experimentally added bacteria did not survive post-transfer or may have remained dormant for some time after being transferred from ideal laboratory conditions to ambient nest conditions (McDougald *et al.* 1998). But the lower abundance of bacteria in the Bac- nests indicates that the ethanol treatment did indeed depress bacterial numbers. Our culture-based measures of bacterial abundance were limited by the fact that we could only detect those bacteria capable of growing on a nutrient rich agar medium within 48 hours of collection; any slow-growing bacteria would not have been detected. However, even if not immediately active or viable, bacteria retain antigenic properties and are thus still able to stimulate the host immune system. Similarly, our ETOH treatment likely slowed the proliferation of bacteria in Bac- nests, but did not remove the bacterial antigens present prior to treatment.

The quantification of differences among treatment groups in bacterial abundance and diversity allowed us to refine our predictions for testing the Ag-abundance and Ag-diversity hypotheses. Based on the Ag-abundance Hypothesis we thus predicted that females from Bac+ and Control treatment groups (i.e., high Ag abundance) would deposit more protective maternal antibodies into their eggs than would females from the Bac- treatment group (i.e., low Ag abundance). In contrast, under the Ag-diversity Hypothesis, we predicted that females from the Control treatment group (i.e., high Ag

diversity) would deposit more MAb's than would females from the Bac+ treatment group (i.e., low Ag diversity). Because nests in the Bac- experimental group did not differ from either Control or Bac+ nests in bacterial diversity, females from the Bac- treatment group were not predicted by the Ag-diversity Hypothesis to differ from either Control or Bac+ treatment females in MAb deposition.

Our work demonstrates that female swallows exposed to experimentally reduced Ag diversity prior to egg-laying deposited lower amounts of total antibodies into their eggs than did control females. This treatment effect persisted throughout the nestling period as indicated by different levels of plasma IgY in offspring between Bac+ and Control treatment groups. These results support the hypothesis of maternal immune investment in response to differences in antigenic diversity. Additional support is lent by the observation that 6-day old nestlings from Bac+ nests also expressed lower plasma bactericidal activity than nestlings from control nests, suggesting that Ag diversity also influences other types of maternal immune contributions. Bac+ females also produced relatively lighter eggs than Control females, providing further evidence for differential maternal investment with Ag diversity. No immunological differences were observed between Bac+ and Bac- treatment groups, which supports our second prediction based on the Ag-diversity hypothesis since no differences in bacterial diversity were observed between these treatment groups. It is possible that nestling differences among treatment groups were caused by differential endogenous immune activity in direct response to differences in nest bacterial communities persisting after treatment administration had ceased (i.e., after clutch completion). But, if this were the case then we would predict that treatment effects would persist throughout the nestling period.

Although treatment effects on nestling plasma IgY levels were detectable at both 6 and 12 days after hatching, the treatment effects on plasma bactericidal activity observed at BD6 were no longer detectable at BD12. Maternal antibodies degrade after approximately 10 days in passerines, at which point endogenous antibody production has taken over (Lozano & Ydenberg 2002, Nemeth, Oesterle & Bowen 2008, King, Owen & Schwabl 2010). It is possible that other maternally derived immune compounds, such as antimicrobial proteins, also degrade or decrease in relative abundance as nestling immune function ramps up. It is likely that treatment effects observed in nestlings resulted from a combination of immune-based maternal effects and direct environmental stimulation of the nestling immune system. We suspect that the relative influence of maternal immune investment lessens as endogenous immune function develops with increasing nestling age.

We did not find any evidence that antigenic abundance *per se* significantly influences maternal transmission of immune compounds to eggs or immune-based maternal effects on offspring. Abundance of culturable bacteria was significantly lower in Bac- nests as compared to Control and Bac+ nests. However, contrary to the predictions of the Ag-abundance Hypothesis, we did not detect any significant differences in immune measures of eggs or nestlings either between Bac- and Control nests or between Bac- and Bac+. Immunological differences were only observed between Bac+ and Control treatment groups, which did not differ in abundance of culturable bacteria. It is possible that effective antigen abundance was in fact increased in Bac+ nests as compared to Control nests, but that our culture-based approach could not accurately capture this variation. Even when taking this possibility into account, the

observed differences in immune-based maternal effects between Bac+ and Control nests still do not lend support to the Ag-abundance hypothesis. In fact, our results are in direct opposition to the predictions of this hypothesis because eggs and nestlings from Bac+ nests had lower IgY and bactericidal activity than those from Control nests. Therefore, we conclude that under the experimental conditions imposed in this study, antigen diversity is more important in influencing the level of maternal antibody transmission and immune-based maternal effects on nestlings than is antigen abundance.

Our results indicate that we successfully manipulated the nest microbiome among treatment groups. Adding large numbers of bacteria from just six different isolates significantly reduced community evenness and diversity. By overwhelming natural communities with just a few bacterial species, we likely decreased the effective antigenic diversity available for the host immune system to perceive. Antigenic diversity may be a stronger predictor of immune system activation than antigenic abundance *per se*, unless the abundant antigens are particularly immunogenic. However, the bacteria we used for the Bac+ treatment are very common and abundant in natural Tree Swallow nests, and are most likely innocuous commensal species. Our results may have differed if a known pathogenic microorganism had been included in the bacterial mix. We do not dispute that antigenic abundance may contribute significantly to degree of host immune investment, as has been shown in other systems (e.g., Soler *et al.* 2011a). In fact, we found that 12-day old nestlings had much higher levels of circulating antibodies in 2009 than in 2010, which corresponds to the significantly higher abundances of bacteria detected across treatment groups in 2009. This difference between years was not

detected for IgY measured in yolk or samples from 6-day old nestlings. Endogenous antibody production and the degradation of maternal antibodies likely occurred between 6 and 12 days of age, which is why it is not surprising that the lack of year effect observed for yolk IgY was reflected in plasma IgY for 6-day old nestlings whose antibodies are still mostly of maternal origin. Plasma IgY levels of older nestlings should be more reflective of the current antigenic environment since their immune systems are more developed and capable of endogenous surveillance and response.

Neither Ag diversity nor Ag abundance appeared to influence nestling growth, as there were no significant treatment effects on nestling mass at hatch, 6 days of age, or 12 days of age. Nor did bacterial manipulation have a significant effect on cutaneous immune activity of nestlings at 12 days of age. We did not test for PHA responsiveness of 6-day old nestlings because we did not want to stimulate any endogenous immune activity prior to blood sampling at BD-12. Our primary objective was to measure plasma IgY in nestlings, and the introduction of additional immune activity associated with PHA challenge may have affected endogenous antibody production. Previous studies suggest that relative investment in different arms of the immune system vary among broods of developing nestlings (Forsman *et al.* 2008a), and so the expression of one type of immune response may not predict the expression of another. We speculate that differences in PHA response among treatment groups may have existed in younger nestlings but then dissipated, such as for bactericidal activity, by 12 days of age. Between the ages of 6 and 12 days of age, endogenous immune function is ramping up and is likely to minimize variation in immunity attributable to maternal effects contributed through egg provisioning. Both measures of nestling immune function were strongly

influenced by nestling mass, suggesting that individual quality is a better predictor of immunity, particularly in older nestlings when maternally derived immune compounds have either degraded or become outnumbered by the nestlings' own immune constituents.

We did not observe any treatment effects on absolute egg-, yolk-, or eggshell mass, nor were there any treatment effects on relative yolk- or eggshell mass when controlling for egg size. However, eggs produced by Bac+ females had lower total egg mass than did eggs from Control females when controlling for egg volume. Similar treatment effects were not observed for yolk or eggshell mass, suggesting that the variation in egg density arises from differences in albumen or membranes. Egg albumen is an important component of egg defense from microbial infection during embryonic development (Board & Fuller 1974, D'alba & Shawkey 2015). Increased albumen volume may decrease the risk of yolk infection by increasing the distance between eggshell and yolk. Three previous studies have tested for effects of abundance and diversity of environmental bacteria on the deposition of albumen defenses (Bedrani *et al.* 2013, Grizard *et al.* 2015, Jacob *et al.* 2015). The first such study found that chickens raised under germ-free conditions laid eggs with higher albumen bactericidal activity and pH than chickens exposed to higher Ag abundance and diversity during development. However, no treatment effects were detected for albumen lysozyme, ovotransferrin, or antiproteases (Bedrani *et al.* 2013). Similarly, albumen lysozyme and ovotransferrin did not correlate significantly with variation in environmental bacteria under either natural (Grizard *et al.* 2015) or manipulated conditions (Jacob *et al.* 2015) in wild bird populations. The mixed results obtained thus far for albumen defenses

suggest that further work is necessary to determine whether female swallows modify investment in the albumen component in relation to the antigenic environment.

We tested the efficacy of our treatments in altering the nest microbiome by collecting bacterial samples from study nests around the time of egg laying. This sampling design also allowed us to test for seasonal effects on bacterial abundance and diversity. We found that bacterial richness and diversity increased with advancing date, whereas community evenness did not change over the period sampled. Bacterial abundance also tended to increase with sampling date. Previous studies of bacterial abundance on egg and nest surfaces have also detected similar increases in abundance with season (Berger *et al.* 2003, Kilgas *et al.* 2012b, Soler *et al.* 2015). It is likely that the nest microbiome changes throughout the swallows' breeding season due to natural succession in response to climatic variation and also as a result of changing bird activity, which may influence the nest microclimate (e.g., egg laying vs. incubation). The seasonal effects observed for nest bacterial communities parallel the seasonal effects observed for immune measures collected for Tree Swallows. We found that levels of circulating antibodies in post-laying adult females increased with advancing date across treatment groups. Similarly, total maternal deposition of antibodies into eggs increased with season within the Control treatment group, which represents the most natural nest conditions; total egg size also tended to increase with advancing lay date. We also found a significant seasonal effect on nestling plasma antibody levels, which was best described by a quadratic relationship.

In our analyses of maternal and nestling immune function and growth, lay date is necessarily confounded with sampling date. This poses some difficulty when

interpreting date-related effects as they are likely influenced by both season and maternal quality. For example, it is possible that the interaction effect we observed between lay date and treatment on female deposition of yolk IgY may be a result of variation in quality between early- and late-laying females. In fact, previous work indicates that earlier-laying Tree Swallows are of higher quality than later-laying swallows in this study population (Hasselquist, Wasson & Winkler 2001, Ardia 2005). Hasselquist et al. (2001) found that earlier laying females mounted a more robust humoral immune response to vaccination with a novel antigen than did later laying females. Although our results indicate that both bacterial richness and swallow immune parameters increase with advancing season, we suggest that female quality may have a modifying effect on this relationship. Further study is needed to appropriately test the relative contributions of antigenic pressure and female quality on the nature and magnitude of immune-based maternal effects.

In conclusion, our work demonstrates that female birds have the capacity to respond to differences in bacterial communities in the nesting environment as evidenced by differential levels of antibody transmission to eggs among bacterial treatment groups. Specifically, our results support the Antigen Diversity Hypothesis because females exposed to higher bacterial diversity deposited more antibodies into egg yolks than did females exposed to lower bacterial diversity. We found no support for the Antigen Abundance Hypothesis. We conclude that antigenic abundance *per se* may not be as important in determining transgenerational immune investment as other aspects of the antigenic environment such as diversity, evenness, and antigen identity.

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Figure 1.1A. Species assignments for the six bacterial isolates used to increase bacterial load in Bac+ treatment nests.

Photo ID	Top BLAST match	Sequence Similarity	Phylum	Class
A	*Bacillus cereus*	877/886 (99%)	Firmicutes	Bacilli
B	*Bacillus megaterium*	875/881 (99%)	Firmicutes	Bacilli
C	*Bacillus weihenstephanensis*	827/828 (99%)	Firmicutes	Bacilli
D	*Sporosarcina aquimarina*	868/880 (99%)	Firmicutes	Bacilli
E	*Staphylococcus saprophyticus*	878/880 (99%)	Firmicutes	Bacilli
F	*Pseudomonas fulva*	800/801 (99%)	Proteobacteria	γ Proteobacteria

Figure 1.1B. Photomicrographs of the six bacterial isolates cultured from Tree Swallow nests in 2009 and subsequently used to increase bacterial load in Bac+ treatment nests in 2009 and 2010.

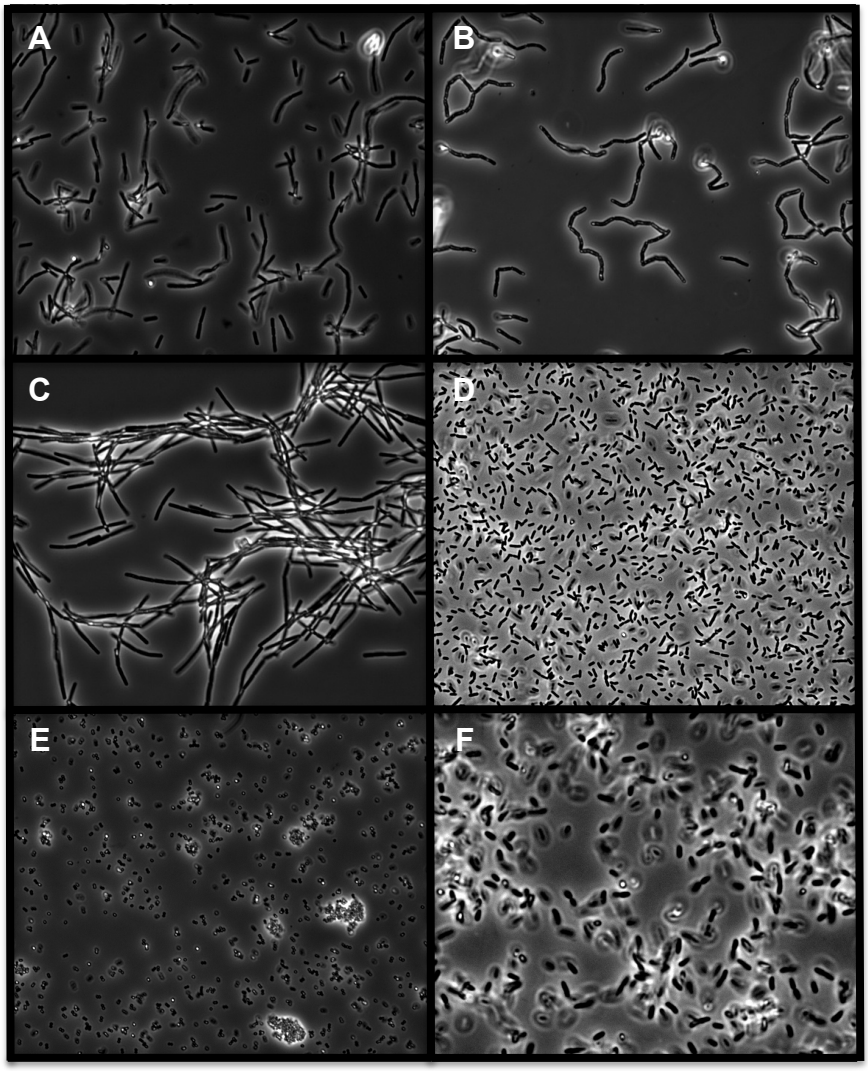

Table 1.1. Summary of results from fixed effects GLMM testing for the effects of Treatment and Sampling Date

	A	**Bacterial Richness**		B	**Bacterial Diversity (Shannon's Index)**		C	**Bacterial Evenness**	
Source	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Treatment	2	2.01	0.1489	2	5.32	**0.0093***	2	6.85	**0.0029***
Test Date	1	12.70	**0.0010***	1	5.48	**0.0248***	1	1.36	0.2514
Treatment*Date	2	2.93	0.0656	2	1.84	0.1726	2	0.70	0.5017

Figure 1.2. The experimental addition of bacterial cultures to Bac+ nests successfully decreased both a) Shannon Diversity Index, and b) Evenness of nest bacterial communities as compared to Control nests.

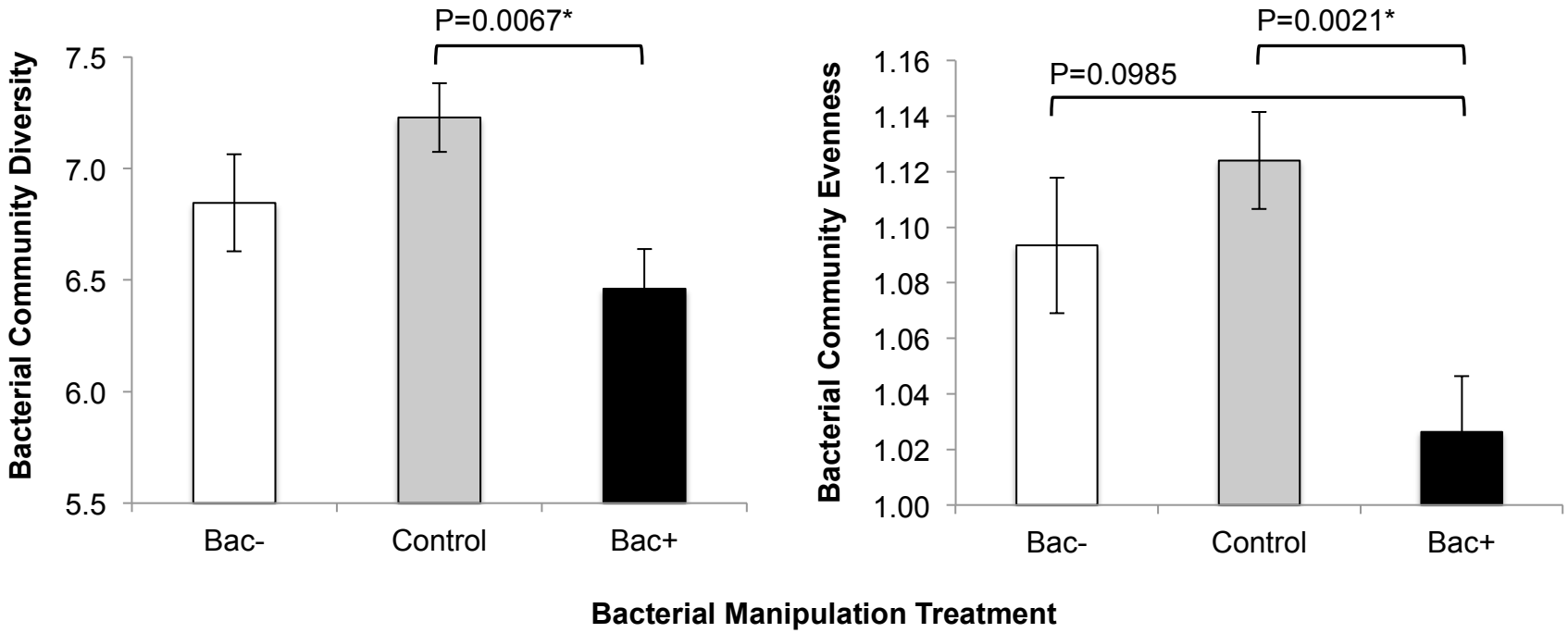

Figure 1.3. The experimental ethanol treatment that was administered to Bac- nests successfully decreased the abundance of culturable bacteria in Bac- nests as compared to in Control and Bac+ nests. The experimental addition of bacterial cultures to Bac+ nests did not increase bacterial abundance in Bac+ nests as compared to in Control nests.

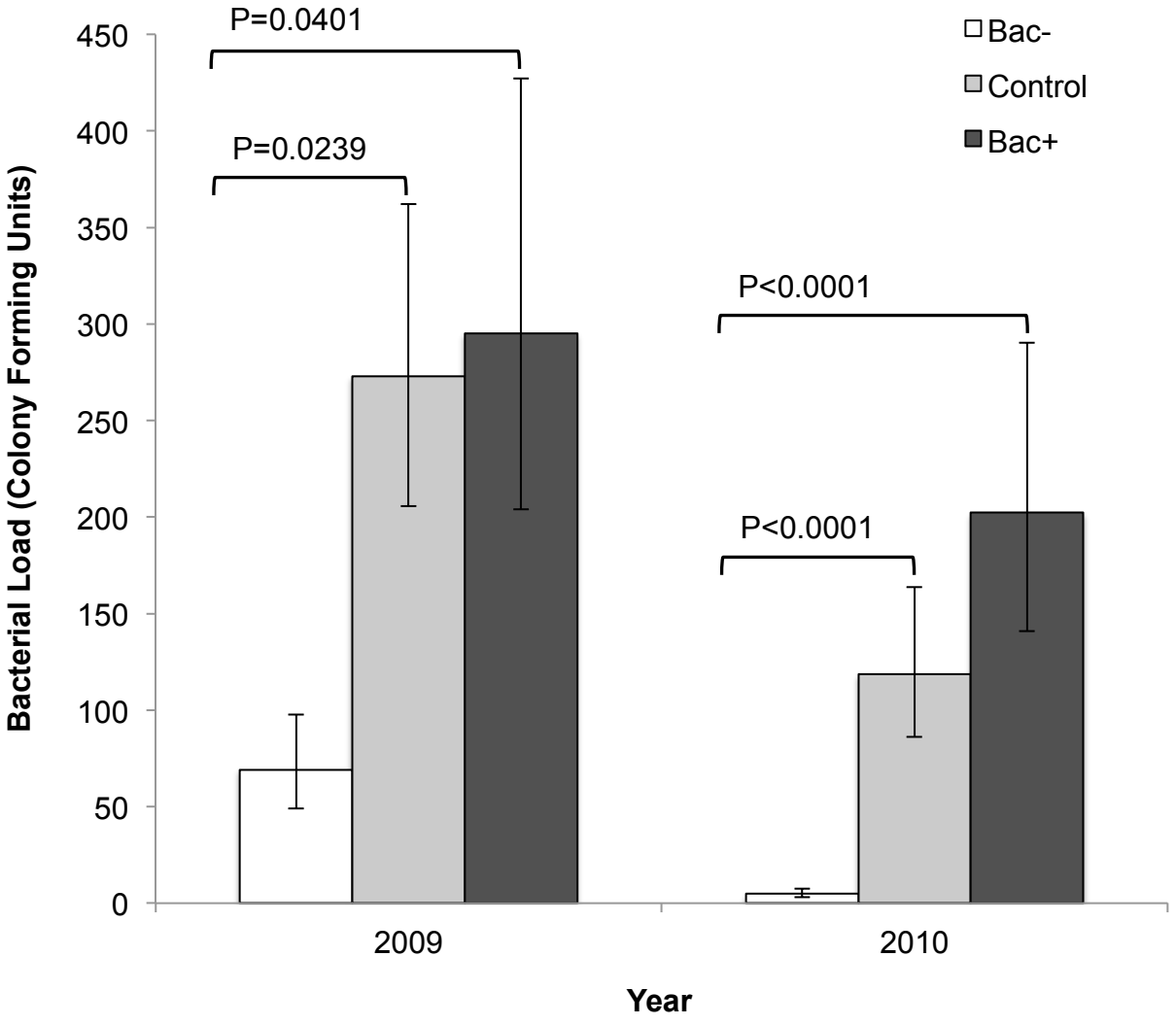

Table 1.2. Relative abundances of bacterial classes detected through 16S rRNA sequencing among all samples, and among samples within treatment groups. Asterisks denote classes represented in experimental treatment mixture.

Phylum; Class	**All**	**Bac-**	**Control**	**Bac+**
Proteobacteria; Alphaproteobacteria	43.85%	52.23%	44.53%	36.94%
Proteobacteria; Betaproteobacteria	9.85%	8.93%	10.53%	9.58%
Bacteroidetes; Cytophagia	8.56%	5.17%	11.24%	7.35%
***Proteobacteria; Gammaproteobacteria**	8.27%	**8.91%**	**7.28%**	**9.15%**
Actinobacteria; Actinobacteria	8.00%	9.84%	8.53%	5.95%
***Firmicutes; Bacilli**	7.52%	**0.78%**	**1.80%**	**20.10%**
Bacteroidetes; [Saprospirae]	3.44%	2.68%	4.27%	2.86%
Bacteroidetes; Sphingobacteriia	2.22%	1.55%	2.81%	1.91%
Proteobacteria; Deltaproteobacteria	1.28%	1.43%	1.42%	0.98%
Actinobacteria; Thermoleophilia	1.25%	1.54%	1.46%	0.76%
Acidobacteria; Acidobacteria-6	0.91%	0.75%	1.21%	0.63%
Cyanobacteria; Oscillatoriophycideae	0.80%	1.50%	0.48%	0.72%
Actinobacteria; Acidimicrobiia	0.47%	0.54%	0.54%	0.33%
Bacteroidetes; Flavobacteriia	0.42%	0.38%	0.47%	0.38%
Verrucomicrobia; [Spartobacteria]	0.41%	0.32%	0.58%	0.24%
Acidobacteria; [Chloracidobacteria]	0.35%	0.48%	0.36%	0.25%
Verrucomicrobia; Verrucomicrobiae	0.26%	0.23%	0.32%	0.20%
FBP; not identified	0.24%	0.19%	0.26%	0.24%
Cyanobacteria; Nostocophycideae	0.22%	0.31%	0.20%	0.19%
Gemmatimonadetes; Gemmatimonadetes	0.17%	0.31%	0.13%	0.11%
Planctomycetes; Phycisphaerae	0.17%	0.16%	0.20%	0.13%
Acidobacteria; Acidobacteriia	0.16%	0.14%	0.18%	0.14%
Planctomycetes; Planctomycetia	0.15%	0.19%	0.17%	0.10%
Chloroflexi; Ellin6529	0.14%	0.18%	0.13%	0.13%
Acidobacteria; Solibacteres	0.12%	0.08%	0.18%	0.06%
Cyanobacteria; Synechococcophycideae	0.11%	0.22%	0.10%	0.05%
Firmicutes; Clostridia	0.11%	0.18%	0.08%	0.10%
Bacteroidetes; [Rhodothermi]	0.06%	0.12%	0.04%	0.05%
Actinobacteria; MB-A2-108	0.05%	0.10%	0.04%	0.02%

Figure 1.4. Relative abundances of bacterial classes observed among treatment groups. For clarity, key has been abbreviated to include only to those classes found at >0.5% abundance in at least one treatment group.

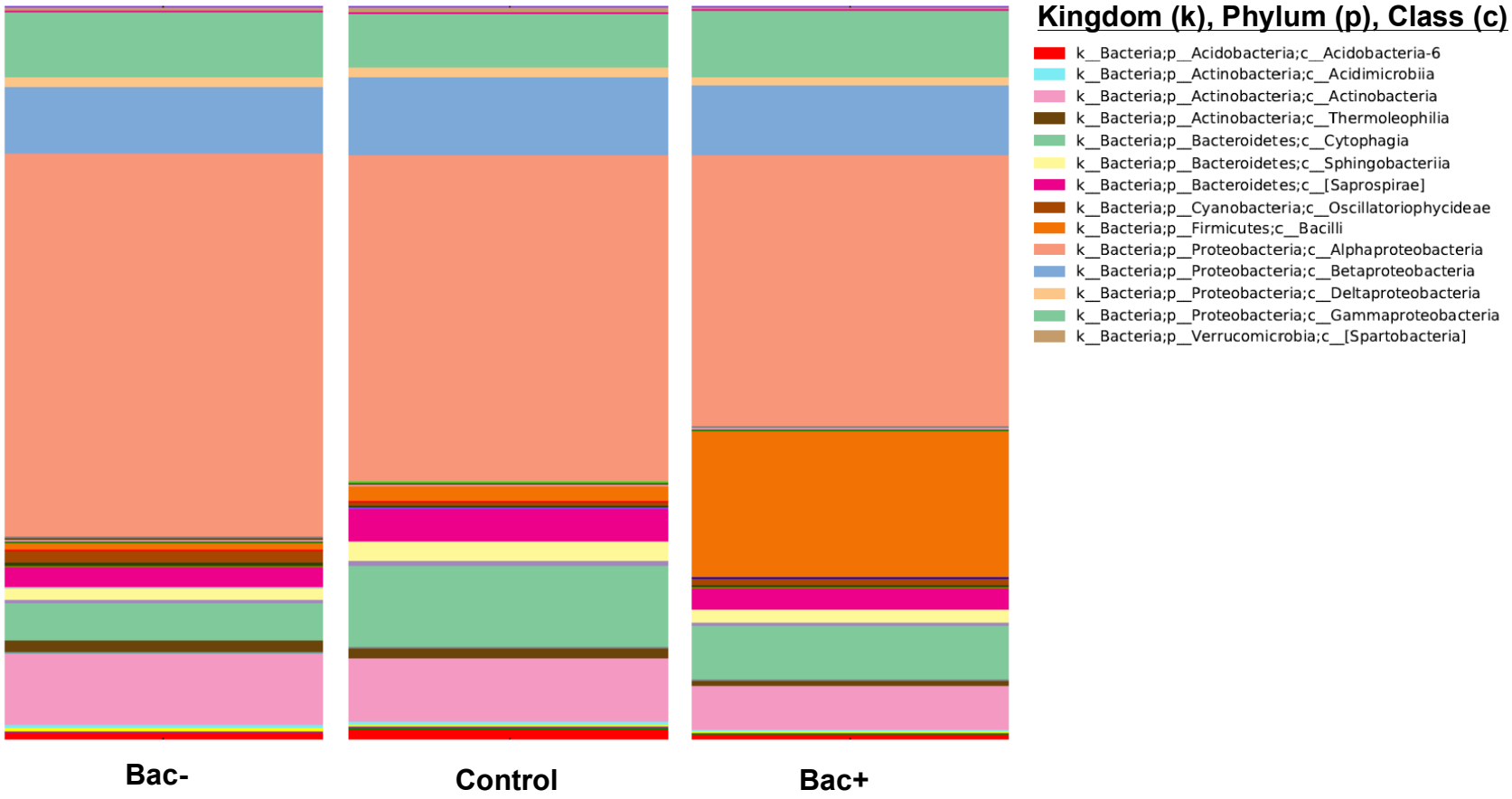

Figure 1.5. Treatment effects on the relative abundances of bacteria belonging to the Bacilli and Gammproteobacteria, which are the two bacterial classes that were represented in the experimental bacterial mix that was applied to Bac+ treatment nests. Relative abundances were calculated from bacterial samples collected from individual Tree Swallow nests in 2010. The Bac+ treatment significantly increased the relative abundance of Bacilli but not Gammaproteobacteria in Bac+ nests as compared to in Control and Bac- nests.

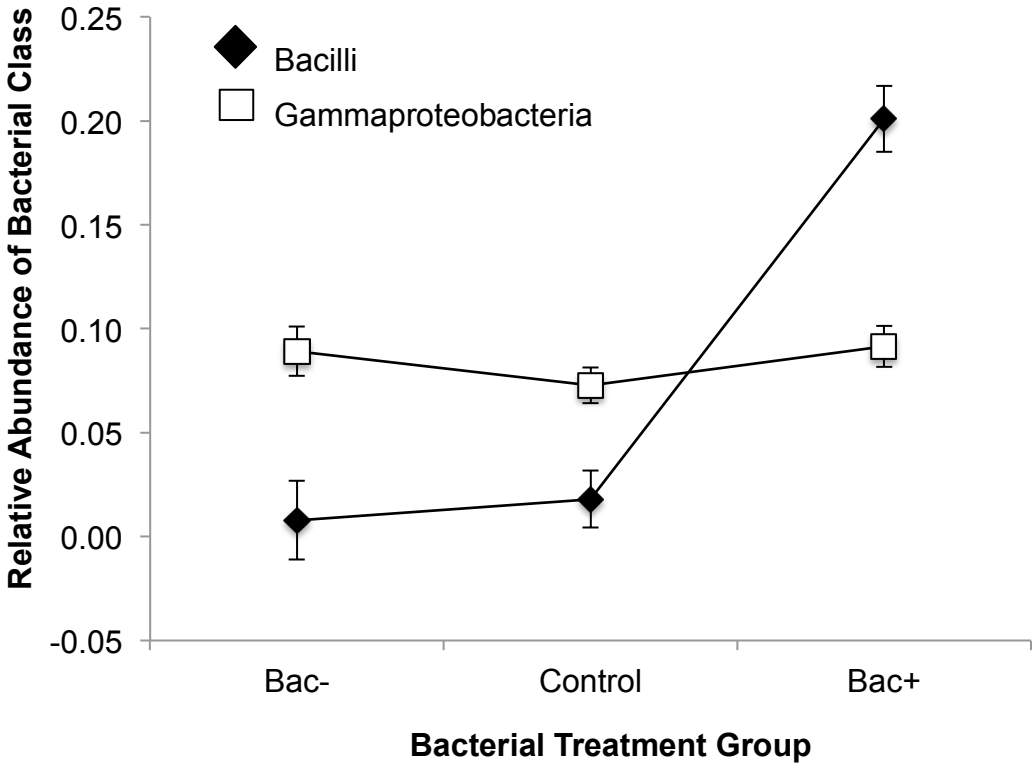

Figure 1.6. Principal Coordinate Analysis based on the unweighted Unifrac distance matrix was conducted to reduce dimensionality of bacterial community composition between samples. Each sphere represents a bacterial community sampled from a single Tree Swallow nest. Treatment groups are differentiated by color: Bac+ in red, Control in blue, and Bac- in orange. The first two principle coordinates are shown with the proportion of variation explained by each.

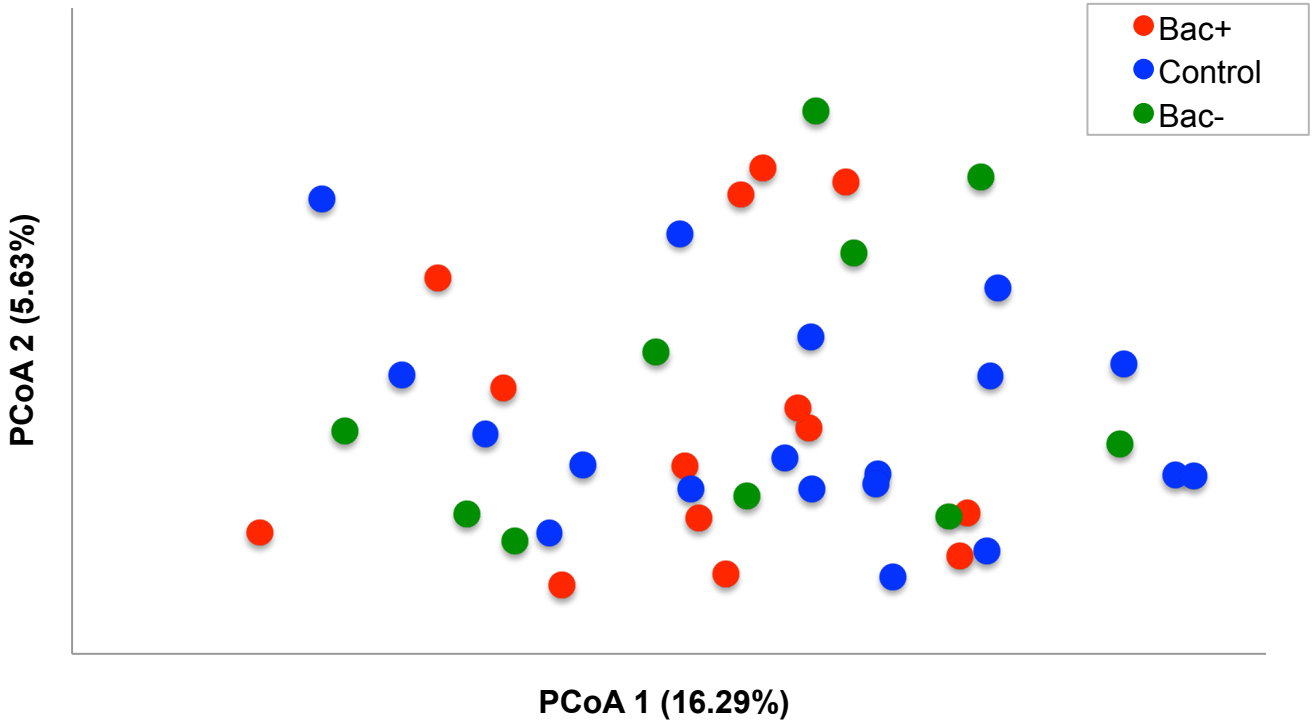

Table 1.3. Summary table describing mixed models of treatment effects on total yolk IgY deposition including either A) Julian date variable (continuous), or B) early vs. late season variable (categorical). The three-way interaction was not significant, and was thus not included in either model.

	Laydate = Julian Date			**Laydate = Season**		
	A Continuous			B Categorical (early vs. late)		
Source	***df***	***F***	***P***	***df***	***F***	***P***
Treatment	2, 65.9	4.1073	0.0208*	2, 65.85	3.6212	0.0322*
Year	1, 2.005	0.0013	0.9745	1, 1.986	0.0010	0.9782
Lay Date	1, 74.02	0.0536	0.8175	1, 74.03	0.1768	0.6754
Treatment*Year	2, 65.29	1.8138	0.1712	2, 68.89	1.2642	0.2889
Treatment*Lay Date	2, 65.15	4.7581	0.0118*	2, 62.42	3.2856	0.0440*
Lay Date*Year	1, 60.43	0.0925	0.7620	1, 53.78	0.0503	0.8235

Figure 1.7. Total yolk IgY values for all eggs included in study plotted against the Julian date that each was laid. Open rectangles represent samples collected from control nests, grey circles from nests with reduced bacterial load (Bac-), and black triangles from nests with increased bacterial load (Bac+). Regression lines were added to indicate change in yolk antibody deposition across the breeding season; red is control, blue is Bac-, and green is Bac+.

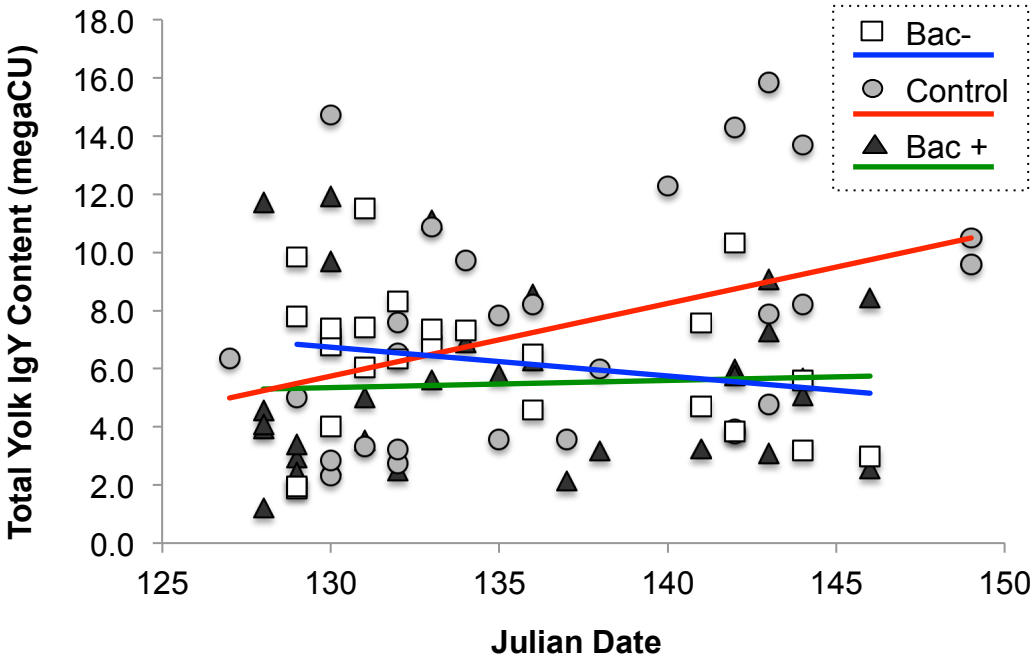

Figure 1.8. Back-transformed least squares mean values for total yolk antibody deposition illustrating the interaction effect of bacterial load manipulation and lay date. Later breeding females deposited significantly fewer antibodies to their yolks when exposed to higher bacterial as compared to control females breeding at the same time.

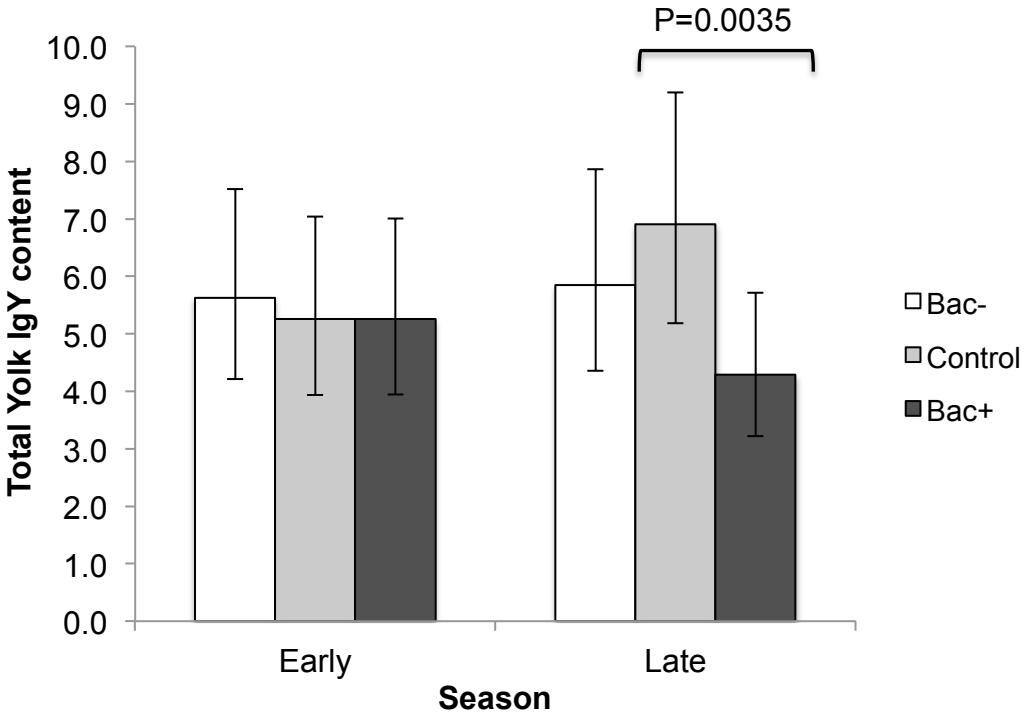

Table 1.4. Summary table from GLMMs describing the temporal and treatment effects on absolute egg and yolk mass for eggs collected in both years of the study.

	A Egg Mass			B Yolk Mass			C Eggshell Mass		
Source	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Treatment	2,62.82	1.1054	0.3374	2,77.64	1.9835	0.1445	2,74.49	0.4481	0.6406
Year	1,38.05	1.7713	0.1911	1,52.76	1.1012	0.2988	1,52.65	0.8798	0.3525
Lay Date	1,81.64	3.8796	0.0523	1,85	0.6600	0.4188	1,80.86	1.4541	0.2314
Clutch Size	1,45.97	0.1932	0.6623	1,70.95	3.2608	0.0752	1,65.47	0.0292	0.8648

Table 1.5. GLMM summary table describing the effects of bacterial treatment, lay date, and clutch size on relative investment in different egg compartments for eggs collected in 2010. Relative egg mass (a) and yolk mass (b) were investigated by controlling for egg volume, whereas eggshell mass was analyzed in terms of eggshell surface area.

	A	Egg Mass		B	Yolk Mass		C	Eggshell Mass	
Source	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Treatment	2	4.3308	0.0192*	2	0.5872	0.5602	2	2.4311	0.0999
Lay Date	1	2.9934	0.0906	1	0.2816	0.5983	1	0.0091	0.9245
Clutch Size	1	0.0796	0.7791	1	10.2483	0.0025*	1	0.8100	0.3731
Egg Volume	1	1177.2140	<.0001*	1	23.1337	<.0001			
Surface Area							1	60.2555	<.0001*

Table 1.6. Summary table from GLMM describing main effects and interaction effects on plasma concentration of IgY in 6- and 12-day old nestlings. Laydate was entered as squared term because of a quadratic relationship with nestling IgY.

Source	***df***	***F***	***P***
Treatment	2, 22.96	4.4235	0.0237*
Mass[Age]	2, 134.5	5.209	0.0066*
Year	1, 27.37	70.3647	<.0001*
Age	1, 115.5	153.5018	<.0001*
Lay Date^2	1, 21.15	5.5308	0.0284*
Treatment*Year	2, 35.44	1.9423	0.1583
Treatment*Age	2, 230.1	0.6005	0.5494
Treatment*Lay Date^2	2, 21	0.241	0.7880
Age*Year	1, 23.47	36.2813	<.0001*
Lay Date^2*Year	1, 25.13	0.3151	0.5796
Lay Date^2*Age	1, 79.81	2.3506	0.1292

Figure 1.9. Back-transformed Least Squares Means values for nestling plasma IgY concentration shown by treatment group. Nestlings produced by females from the Bac+ treatment group had significantly lower levels of circulating antibodies than nestlings produced by Control females.

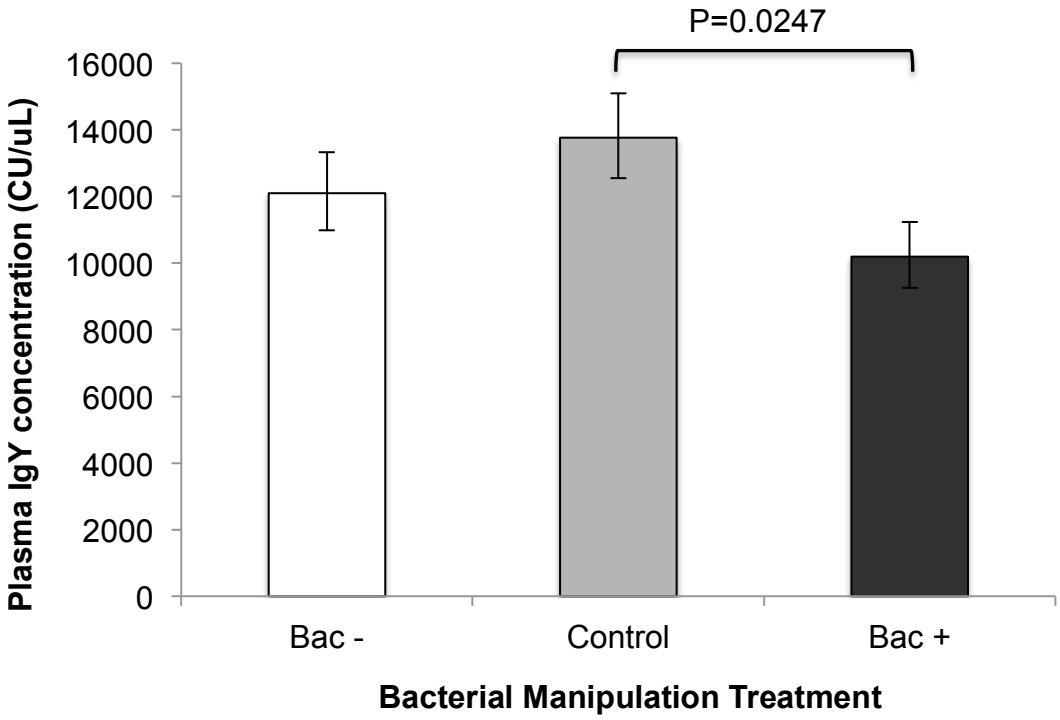

Figure 1.10. Least squares mean values for plasma bacterial killing capacity measured from 6-day old nestlings and 12-day old nestlings. There was a significant treatment effect such that 6-day old nestlings hatched and raised in nests with increased bacterial load demonstrated lower bactericidal activity than control nestlings. This treatment effect was no longer evident when nestlings reached 12 days of age.

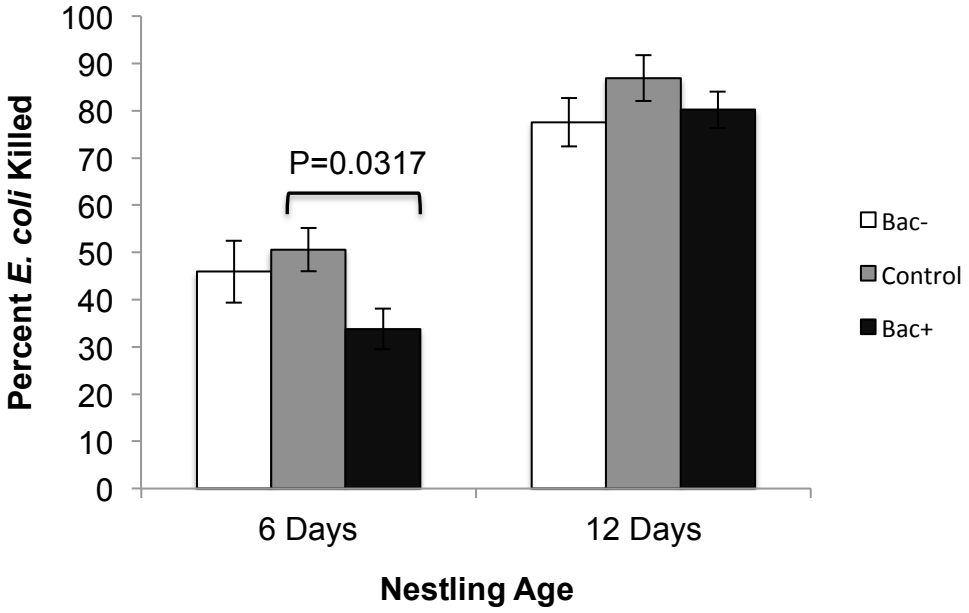

Table 1.7. Summary table of results from GLMMs describing treatment effects on plasma bactericidal capacity for A) 6-day old nestlings, and B) 12-day old nestlings. Lay date represents the Julian date on which the first egg of the clutch was laid.

	A	**6-Day Old Nestlings**		B	**12-Day Old Nestlings**	
Source	***df***	***F***	***P***	***df***	***F***	***P***
Treatment	2, 32.21	3.7212	0.0352*	2, 24.71	0.9804	0.3893
Mass	1, 107.7	13.4968	0.0004*	1, 88.18	40.4384	<.0001*
Lay Date	1, 56.75	0.9942	0.3229	1, 27.52	2.6461	0.1152
Treatment*Lay Date	2, 53.4	0.0428	0.9582	2, 27.07	0.1320	0.8769
Treatment*Mass	2, 105.7	0.1925	0.8252	2, 89.31	0.5411	0.5840

Table 1.8. Summary table for repeated measures GLMM describing main effects and treatment effects on mean nestling mass measured at 0 (i.e., hatch), 6, and 12 days of age.

Source	***df***	***F***	***P***
Year	1,69.38	52.4342	<.0001*
Lay date	1,85.63	0.0000	0.9998
Age	2,136.50	5054.6180	<.0001*
Brood size	1,71.02	1.8918	0.1733
Lay date * Year	1,87.42	5.4537	0.0218*
Age * Year	2,137.20	1.0514	0.3522
Brood size * Year	1,72.88	0.7769	0.3810
Lay date * Age	2,134.70	9.1699	0.0002*
Brood size * Age	2,135.50	2.8150	0.0634
Brood size * Lay date	1,72.01	0.0597	0.8077

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Chapter 2

TESTING THE ANTIGEN DIVERSITY AND PACE OF LIFE HYPOTHESES OF MATERNAL ANTIBODY TRANSMISSION ACROSS THE BREEDING RANGE OF THE NORTH AMERICAN TREE SWALLOW

INTRODUCTION

Female birds deposit a variety of compounds into their eggs that provide nutrition and protection for developing embryos (Mousseau & Fox 1998, Grindstaff, Brodie & Ketterson 2003), including antibodies (Grindstaff *et al.* 2003, Gasparini *et al.* 2001, Muller *et al.* 2004), lysozyme (Saino *et al.* 2002a, Horrocks *et al.* 2014), and carotenoids (Surai & Speake 1998, Biard, Surai & Moller 2005). An increasing number of studies have identified functional effects of such immune compounds on offspring immunity (Lemke, Hansen & Lange 2003, Grindstaff *et al.* 2006, Reid *et al.* 2006) and survival (Heller *et al.* 1990, Leitner *et al.* 1990, Buechler *et al.* 2002), suggesting fitness-related benefits (Mousseau & Fox 1998, Lindström 1999). Passive immune protection and growth enhancement conferred by maternally derived immune compounds are especially important for naïve hatchlings before their endogenous immune function is fully developed (Hassan & Curtiss 1996, Sahin *et al.* 2003). However, many of these maternally-derived immune compounds must be synthesized by females prior to egg-laying (Grindstaff *et al.* 2003), and therefore require the maintenance or even up-regulation of costly immune function (Kowalczyk *et al.* 1985) during an already demanding life history stage (Nilsson & Raberg 2001, Williams 2005). In addition, recent

work has suggested potential developmental costs of such immune-based maternal effects on offspring immunity (Addison *et al.* 2009, Lemke *et al.* 2009). Thus, reproductive females should be selected to enhance deposition of immune compounds into eggs when the protective benefits to offspring outweigh any detrimental effects on maternal survival or reproductive success. Two main hypotheses have been put forth to explain variation in transgenerational immune investment within and among populations: the Antigen Diversity Hypothesis (Ag-diversity Hypothesis; e.g., Forsman et al. *in review*, Bedrani *et al.* 2013, Horrocks *et al.* 2014, Jacob *et al.* 2015, Grizard *et al.* 2015) and the Pace of life Hypothesis (PoL Hypothesis; Addison *et al.* 2009). From an ecological perspective, the Ag-diversity Hypothesis proposes that investment in offspring immunity should be selectively tuned to the probability of hatchling infection or the diversity of immune-stimulating antigens in the environment. The Ag-diversity Hypothesis thus predicts that females exposed to more antigens prior to breeding will transfer more protective antibodies to their eggs than will females exposed to fewer antigens. From an evolutionary perspective, the PoL Hypothesis treats transgenerational immune investment as a life-history trait subject to trade-offs resulting from differential selection for reproduction and self-maintenance; selection for increased reproductive investment should, thus, include enhanced immune investment in offspring. The PoL Hypothesis thus predicts that females exhibiting a faster pace of life will invest more in each reproductive attempt and will hence transfer more antibodies to their eggs than will females exhibiting a slower pace of life. Although the Ag-diversity and PoL hypotheses explaining maternal immune compound deposition have received some treatment in the literature, they have rarely been evaluated simultaneously in a

single study. Therefore, the objective of the current research was to test the Ag-diversity and PoL hypotheses side-by-side through a large-scale investigation of maternal transmission of yolk antibodies in relation to environmental bacterial diversity across a gradient of life-history variation in the North American Tree Swallow (*Tachycineta bicolor*). To our knowledge, this study is the first to characterize the relationship between maternal antibody transmission and whole-community characteristics of host-associated bacteria under natural conditions and across an entire breeding range of a wild vertebrate species. Unlike the one previous study examining both Ag-diversity and PoL hypotheses of transgenerational immune investment, which relied on ecological proxies of antigen pressure (Addison *et al.* 2009), we used Next Generation Sequencing technology to directly quantify richness, diversity, and community composition of bacteria collected from active Tree Swallow nests across North America. We used these data to evaluate the relative effects of antigen diversity vs. geographic correlates of life history variation on maternal deposition of yolk antibodies into eggs collected from these same nests.

The immune system represents the most important integrative defense that an organism possesses against harmful pathogens and toxins encountered throughout a lifetime. Thus, the benefits of effective immune responsiveness (i.e., immunocompetence) to individual health and survival are clear. Extensive work on the transmission of maternally derived antibodies (MAb) to offspring have demonstrated both short-term and longer-term protective benefits through passive immunity and the enhancement of endogenous immune activity (reviewed in Grindstaff *et al.* 2003). Antibodies, also known as immunoglobulins (Ig), are specialized proteins produced by

B-lymphocytes of the adaptive immune system. Highly specific binding sites allow antibodies to recognize and attach to foreign antigens (Ag's), initiating the process of pathogen destruction (Murphy 2012). Antibodies produced by a donor individual and transferred to a recipient can confer passive immunity to the recipient by neutralizing Ag's directly (Rollier *et al.* 2000) or by flagging them for destruction by the host immune system, thereby circumventing the need for endogenous antibody production; this is the concept underlying the use of anti-sera to neutralize some toxins and pathogenic molecules in medicine. Maternally derived antibodies function similarly when transferred from mother to recipient offspring through the placenta, yolk, or milk (reviewed in Grindstaff *et al.* 2003). Birds have been found to transfer immunoglobulin Y (IgY) to developing yolks in proportion to the levels present in maternal circulation at the time of laying (Sun *et al.* 2013). Antibodies of the IgA and IgM isotypes are also transferred, but in lesser quantities to the egg albumen (Bencina *et al.* 2005). The specificities of MAb reflect historical exposure of females to antigens during a significant period of time prior to egg-laying (Reid *et al.* 2006, Staszewski *et al.* 2007), which may extend to encompass life-time exposure (Lemke *et al.* 2003). Maternal antibodies persist for a relatively brief time in hatchlings, and the duration of effects in chicks appears to depend on the level of antibodies in maternal circulation (Grindstaff 2009). In house sparrows, maternal antibodies persist for 8-9 days, during which time hatchlings commence their own production of antibodies (Nemeth, Oesterle & Bowen 2008, King, Owen & Schwabl 2010). In chickens, MAb persist in neonates for one to two weeks (reviewed in Grindstaff *et al.* 2003, Hamal *et al.* 2006), and in Tree Swallows, antigen-specific MAb were no longer detectable in offspring by ten days of age (Lozano &

Ydenberg 2002). During this early-life window, before endogenous immune activity has fully developed, maternally-derived antibodies protect hatchlings directly through passive immunity (e.g., Heller *et al.* 1990, Rollier *et al.* 2000). Increased endogenous immune activity during early life may divert resources away from other ongoing developmental processes and growth, resulting in reduced fledging success (Romano *et al.* 2013). But because passive immunity circumvents full engagement of the neonatal immune response, MAb allow hatchlings to maintain growth despite immune challenge (Grindstaff 2008, Martyka, Rutkowska & Cichon 2011). Thus, MAb confer short-term benefits to neonates through direct passive immune protection and indirectly by allowing continued growth in the face of concurrent immune challenge. Despite their ephemeral nature, MAb also confer medium- and long-term benefits through priming and imprinting of the offspring immune system (Grindstaff, Brodie & Ketterson 2003). Immunological priming occurs when the presence of maternal antibodies enhances a juvenile's own humoral response to an antigenic challenge. Because of MAb priming, an immune response to an antigen encountered for the first time may more closely resemble a secondary response than a primary response. Several studies in wild systems have demonstrated immunological priming through enhanced antibody responses of nestlings to antigenic challenges also experienced by mothers prior to egg-laying (e.g., Grindstaff *et al.* 2006, Gasparini *et al.* 2006). Immune imprinting, on the other hand, describes the phenomenon whereby maternally derived antibodies influence the juvenile B- and T-cell repertoires by selecting for particular antigen specificities (i.e., idiotypes). MAb idiotypes are recognized as antigens by the juvenile immune system and, thus, induce endogenous production of anti-idiotypic antibodies (Lemke *et al.* 2009). Because some

of the MAb are also anti-idiotypic in nature, the juvenile response produces anti-anti-idiotypic antibodies, which can bind to the original antigen (Lemke et al. 2012). Thus, maternal antibodies effectively educate the neonatal immune system without the need for exposure to the original antigens and may hence confer long-term immunological memory that may be transmitted over multiple generations (e.g., Reid *et al.* 2006). With the prospect of such far-reaching benefits, females should be selected to maximize MAb deposition to enhance growth and survival of their offspring. However, the associated costs and benefits that together shape transgenerational immune investment are likely to vary among populations depending on the risk of offspring infection and life history considerations, as predicted by the Ag-diversity and PoL Hypotheses.

The Ag-diversity Hypothesis proposes that females transmit immunoprotective compounds to offspring in accordance with the environmental prevalence of antigens capable of eliciting an immune response (e.g., Hasselquist 2007, Horrocks, Matson & Tieleman 2011). Such antigens include any pathogens, allergens, and commensal microorganisms that stimulate host immune activity. Because very young birds have limited capacity to produce an efficient adaptive immune response (Brambell 1970, Solomon 1971, Lawrence & Arnaud-Battandier 1981), immune-based maternal investment is expected to increase with increased likelihood of hatchling infection. There have been many studies indicating that maternal immune challenge, through experimental vaccination, results in increased transmission of antigen-specific antibodies (reviewed in Grindstaff *et al.* 2003). However, there is very little available evidence suggesting that maternal exposure to increased antigenic richness or diversity increases overall transmission of total antibodies. Vaccination studies are useful

because they focus on the effects of specific antigens on a study subject's immune response. However, this targeted approach does not yield information regarding how the antigenic landscape that a host encounters, as a whole, influences host immune function. This gap in our knowledge, which is likely a product of methodological difficulties associated with characterizing Ag communities, is significant because the immune system is faced with an immense diversity of antigens under natural conditions. A few studies of wild birds have focused on Ag abundance, instead of Ag diversity. For example, Gasparini et al. (2001) found that abundance of the tick-vector of *Borrelia* correlated positively with Ag-specific yolk IgY in host populations of seabirds. Similarly, a study of adult finches found evidence for positive relationships between natural variation in ectoparasite load and both circulating natural antibodies and Ag-specific antibody response (Lindstrom *et al.* 2004), but this study did not examine egg provisioning. Others have approached the Ag-diversity Hypothesis by using climatic and ecological variables as proxies for microbial diversity (e.g., MAb: Addison *et al.* 2009; egg albumen provisioning: Horrocks *et al.* 2014, Horrocks *et al.* 2015). Although it has been demonstrated that microbial growth on eggs is enhanced under humid conditions (Cook *et al.* 2005, Wang, Firestone & Beissinger 2011), there is much less evidence available describing how microbial communities vary with climatic and ecological variables over large geographic scales. Therefore, direct and quantitative measures of microbial community structure are needed to properly evaluate the Ag-diversity hypothesis. These criteria have been met in two recent studies of the Ag-diversity hypothesis to explain egg albumen contributions (Grizard *et al.* 2015, Jacob *et al.* 2015), but not yet to explain maternal antibody deposition.

Alternatively, the PoL hypothesis proposes that maternal immune investments are selected to balance the differential costs and benefits associated with life history variation. Life history theory suggests that organisms fall along a pace-of-life continuum, ranging from slow ("live slow, die old") to fast ("live fast, die young"; Promislow & Harvey 1990). Fast-paced organisms are predicted to invest relatively more in reproduction, at the expense of self-maintenance, as future reproductive opportunities may be limited (Stearns 1992). Slow-paced, longer-lived organisms, when faced with stressors that jeopardize their own survival and future reproduction are instead predicted to reduce investment in reproduction at any one reproductive event in favor of self-maintenance (Drent & Daan 1980). Life history theory also predicts that fast-paced organisms should be selected to invest relatively more in early-life growth and development to maximize the number of offspring that reach independence, whereas slow-paced organisms should be selected to invest relatively more in long-term strategies that promote offspring longevity (i.e., quantity vs. quality). The PoL Hypothesis, thus, predicts that fast-paced females should transmit more MAb to their eggs than slow-paced females to enhance offspring growth and passive immunity to maximize fledging success. Further, fast-paced females may be less constrained in their level of MAb transmission by transgenerational costs that negatively influence life-time immune efficiency (Addison *et al.* 2009). Slow-paced birds are also expected to skew investment towards self-maintenance at the expense of reproductive effort, and so may be less likely to accept the physiological or energetic costs associated with MAb transmission (e.g., Ardia 2005a). One previous study comparing maternal antibody deposition among neotropical bird species found support for these predictions of the PoL hypothesis (Addison *et al.*

2009), suggesting that life history variation influences transgenerational immune investment. Other studies, that have instead assessed individual immune function as opposed to egg provisioning, have found mixed support for the PoL Hypothesis (Lee *et al.* 2008, Horrocks *et al.* 2015). However, none of these studies incorporated measurements of antigen richness or diversity, and they were thus unable to evaluate both PoL and Ag-diversity Hypotheses simultaneously.

Through the research described here we concomitantly tested both Ag-diversity and PoL Hypotheses to explain transgenerational immune investment at a geographic scale large enough to encompass the full range of variation in antigenic environments and host life histories. We sampled bacteria and collected eggs from the nests of Tree Swallows breeding at 15 sites across North America (Fig. 2.1) to characterize bacterial community structure and maternal antibody deposition into eggs. This study is the largest and most geographically comprehensive survey of bacterial communities associated with any wild bird species to date. It is also the first to describe geographic patterns in immune-based maternal effects across an entire species range.

METHODS

Study System

Tree Swallows are insectivorous migratory birds that breed throughout much of Canada and the northern United States (Fig. 2.1); they readily nest in man-made nestboxes and can be handled throughout the incubation and nestling period with minimal disturbance. Tree Swallows are an ideal system for investigating maternal effects because of their tractability and the pre-existence of established nesting sites throughout their range.

Golondrinas de las Americas (http://golondrinas.cornell.edu) is an NSF-funded network of researchers and fieldsites throughout North America that support standardized, man-made nestboxes utilized by *Tachycineta* species, including the Tree Swallow. For this study we collected eggs and bacterial samples from 15 sites, including several *Golondrinas* sites (Fig. 2.1). This large geographic range was selected to maximize variation in pace of life and nest bacterial communities among sampling sites.

Field Methods

Paired samples of bacteria and eggs were collected from Tree Swallow nests prior to the onset of incubation between 2008 and 2010. Five to ten pairs of samples were collected from each study site per year, however, not all sites were sampled in all years of the study. Prior to each field season, we provided field collaborators with sample collection kits and protocols to maximize standardization of sample collection, storage, and shipment. At time of collection, nest bacterial communities were sampled first as to avoid any contamination that might occur when removing eggs. Briefly, we first dampened an individually wrapped sterile swab in 100uL of pre-prepared phosphate-buffered saline (1x PBS), which was then used to swab all surfaces of the nest cup material for a total of 10 seconds. Each swab head was placed in a microcentrifuge tube with 500uL of 70% ethanol. Next, one egg was collected from the bacteria-sampled nest and weighed to determine total egg mass. In 2008, samples were stored and shipped at 4°C to Cornell University for processing. In 2009 and 2010, samples were instead stored at -20°C and shipped on dry ice. All samples were stored at Cornell University at -20°C until processing.

Maternal Antibody Quantification

We quantified immunoglobulin Y (IgY) concentration of egg yolk samples using ELISA (enzyme-linked immunosorbent assay) following Grindstaff et al. (2005). Frozen eggs were removed from the freezer and measured with digital calipers to determine length and width. We then thawed eggs, one at a time, on ice. Eggshells were carefully removed, washed with deionized water, and dried to obtain eggshell mass. Because albumen thaws faster than yolk, it may be removed without disturbing yolk integrity. We briefly rolled each partially thawed yolk on a clean Kimwipe tissue to remove any residual albumen. Yolks were weighed on an analytical balance to obtain yolk mass. Each yolk was then homogenized with a small stainless steel spatula and 0.05 grams was added to a microcentrifuge tube containing three glass beads and 200 uL of ELISA wash buffer (1x PBS, 1% bovine serum albumin, 0.05% Tween-20). Microcentrifuge tubes containing diluted yolk samples (250ug/uL) were mounted on a vortex and shaken vigorously for 10 minutes. Yolk dilutions were stored at -20C until ELISA analysis.

96-well ELISA plates were coated overnight at 4C with a polyclonal capture antibody diluted in carbonate buffer (0.15 mol l^{-1}, pH 9.6) to a concentration of 5.96 ug/mL. The capture antibody has been developed for detecting heavy and light chain IgY in dove, duck, sparrow, and chicken (Bethyl Laboratories, A140-110A), and it has been used successfully for detecting yolk IgY in other passerine species (e.g., Grindstaff, Demas & Ketterson 2005, Grindstaff 2008). Coated plates were washed three times with ELISA wash buffer to remove unbound capture antibodies and to block well surfaces not coated with antibodies. Diluted yolk samples were added to wells in triplicate at a final concentration of 187.5 ug/mL in ELISA wash buffer. Samples were

incubated on ELISA plates overnight at 4°C. The following day, we washed plates and added a horseradish peroxidase-conjugated secondary antibody (1:1000 dilution; Bethyl Laboratories A140-110P) to detect Tree Swallow IgY. ELISA plates were analyzed by spectrophotometer (405 nm) 15 minutes after the enzyme substrate was added. To correct for background noise, optical density (OD) units for individual samples were normalized by subtracting OD values from blanks run in triplicate on each plate.

Next-generation Illumina Sequencing

PCR amplification and amplicon preparation for sequencing were performed following the protocols described in Costello et al. (2009; 2012), and can be found on the Earth Microbiome Project (Gilbert, Jansson & Knight 2014) web page (http://www.earthmicrobiome.org/emp-standard-protocols/). Universal bacterial primers (515F: 5'-GTGCCAGCMGCCGCGGTAA-3'; 806R: 5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the bacterial and archaeal 16S rRNA variable region 4 (V4; Wang *et al.* 2007, Caporaso *et al.* 2012). Each DNA sample was amplified in triplicate and amplicons were pooled together and quantified using PicoGreen (Invitrogen P7589). Negative controls included no-template controls for DNA extraction and PCR amplification. All barcoded amplicons were pooled in equal concentrations for sequencing and purified using the UltraClean PCR Clean-up kit (Mo Bio Laboratories 12500). Amplicons were sequenced in two different runs on the Illumina HiSeq2000 sequencing platform at the BioFrontiers Institute Next-Generation Genomics Facility at University of Colorado (Boulder, USA).

Amplicon sequences were analyzed using QIIME, an open source software package for comparison and analysis of microbial communities (version1.8.0; Caporaso *et al.* 2010b). Sequences were de-multiplexed and quality-filtered according to default parameters within QIIME. We assigned sequences to operational taxonomic units (OTUs) with a closed-reference protocol, which captures the full range of bacterial diversity within our data set, following the default parameters in QIIME (http://qiime.org/tutorials/open_reference_illumina_processing.html; see also Navas-Molina *et al.* 2013). Sequences were clustered into OTUs with a sequence similarity threshold of 97% using UCLUST (Edgar 2010), and then a representative sequence from each OTU was matched against the Greengenes ribosomal RNA reference database (August 2013 release version; http://greengenes.secondgenome.com; DeSantis *et al.* 2006, Mcdonald *et al.* 2012). Representative OTUs that matched the reference database received a taxonomic classification standardized in Greengenes with the RDP classifier (Wang *et al.* 2007). The representative sequences of all OTUs were then aligned to the Greengenes reference alignment using PyNAST (Caporaso *et al.* 2010a), and this alignment was used to construct a phylogenetic tree using FastTree (Price, Dehal & Arkin 2010). Chloroplasts, mitochondria, Archaea, sequences not assigned to bacterial phyla, and OTUs representing less than 0.00005% of the total reads in the data set were filtered out as recommended for Illumina-generated sequence data (Bokulich *et al.* 2013). Rarefaction for sequencing effort standardization was performed at a depth of 15,000 sequences. After the described data processing, 196 samples were retained for subsequent analyses.

STATISTICAL METHODS

Univariate Analyses

Moran's I test was applied for studying spatial autocorrelation of yolk IgY concentration (i.e., mean residual OD units). Because our data were collected from most of North America, geographical weights were calculated using the close neighbor algorithm, setting the number of neighbors (k) to 3. Using different k values did not change the results.

Alpha diversity for each bacterial sample was calculated as OTU richness, Shannon's Diversity Index, Faith's Phylogenetic Diversity index (Faith 1992), and community evenness. Species delineation for bacteria is fraught with controversy, and therefore it is common practice to instead assign 16S sequences to operational taxonomic units (OTU), which may or may not match with known bacterial species when compared against a reference rRNA gene database such as Greengenes. Therefore, detected OTUs may be included in calculations of alpha and beta diversity regardless if they are identified to the species level, limiting biases associated with differential annotation completeness among bacterial taxa in reference databases.

To determine the effects of geography and Ag diversity on maternal antibody deposition and egg morphology, we used a mixed model design including latitude, longitude, elevation, and bacterial alpha-diversity as fixed effects. Site and year(site) were entered as random effects to control for multiple sampling within sites and also within years nested within sites. For analyses of MAb deposition, we used residual OD values as the dependent variable describing yolk IgY concentration for each egg sampled (see ELISA methods above). Because alpha-diversity variables may influence

maternal immune status and MAb deposition differently, we ran four separate models to test for the effects of each measure of alpha-diversity relative to geographic variables. However, for analyses of egg morphology, we selected OTU richness to be entered as the fixed alpha-diversity effect. This model was used to analyze variation in total egg mass, egg volume, yolk mass, and eggshell mass. Model residuals were normally distributed and so no data transformations were performed for these morphological measures.

We used GLMM to test for correlations between the mean relative abundances of bacterial phyla and mean yolk IgY deposition across sites and years (N=31 site/year means). Models included mean phylotype abundance and year as fixed effects, site as a random effect, and mean residual OD as the response variable describing yolk antibody concentration. All GLMM analyses were performed with JMP Pro, Version 10 (SAS institute Inc, Cary, NC).

Multivariate Analyses

Prevalence of each OTU was calculated for each Site/Year sampled. Thus, all samples collected at a site within a particular year were combined to create one community representing that Site/Year. We calculated Unifrac distances between pairs of samples (Lozupone & Knight 2005), which were utilized in beta diversity distance matrix analyses. These unweighted Unifrac distance matrices were used in subsequent analyses. Samples that did not amplify in the first run where re-amplified in a second run. To validate the inclusion of samples amplified in either run, we performed Generalized Procrustes Analyses (Gower, 1975) with 22 samples that were successfully

amplified in both runs. Procrustes analyses transform principal coordinate axes to minimize the distance between pairs of samples. Both low p and M^2 values are criteria for considering pairs of samples as resembling each other. We found that pairs of bacterial samples collected from the same nest were significantly more similar between the two amplification batches than for pairs of samples collected from different nests within a single amplification batch ($p < 0.005$, $M^2 = 0.069$; Forsman et al. *in review*); therefore, we have included sequence data from both runs in our analyses.

Principal Coordinate Analyses (PCoA) were performed in order to reduce dimensionality of the bacterial community composition data and to maximize variance of orthogonal axes. We performed a Procrustes Anova (Anderson 2001, Collyer, Sekora & Adams 2015) where the PCoA axes were used as an independent matrix, latitude,longitude and elevation as covaribles and mean yolk IgY concentration as a fixed response variable. Procrustes ANOVAs use the full set of axes from Principal Coordinate Analyses (PCoA). Visualizations of PCoA were performed with EMPeror (Vázquez-Baeza *et al.* 2013).

Multivariate statistical analyses were performed with the R statistical environment 3.1.3. (R_Core_Team, 2015) using the packages *spdep, RANN, ade4, geomorph* and *vegan*. Significant *P* values were considered below 0.05.

RESULTS

Univariate Analyses

OTU richness, diversity, and phylogenetic diversity of nest bacterial communities each had significant and positive effects on yolk IgY concentration (Table 2.2, Fig. 2.2). No

significant effects of latitude, longitude, elevation, or community evenness on yolk IgY were detected (Table 2.2). We did not detect any evidence of spatial autocorrelation for mean IgY concentration across sites and years (Table 2.1).

The most common bacterial phyla detected in Tree Swallow nests across North America (Fig. 2.3) include Proteobacteria (47.7%), Actinobacteria (22.2%), Bacteroidetes (19.7%), and Firmicutes (4.1%), and Acidobacteria (1.2%). At the site/year level, mean residual yolk IgY concentration was positively correlated with the relative abundances of the bacterial phyla Acidobacteria ($F_{1,25.11}$=4.57, P=0.0423), Actinobacteria ($F_{1,25.43}$=5.48, P=0.0273), Chloroflexi ($F_{1,25.49}$=6.13, P=0.0202)), Gemmatimonadetes ($F_{1,26.38}$=9.89, P=0.0041), and negatively correlated with Proteobacteria ($F_{1,23.97}$=6.70, P=0.0161).

Bacterial richness of nest microbiota had no significant effects on any morphological measurements collected from eggs (Table 2.2; egg mass, egg volume, yolk mass, eggshell mass). Total egg mass and volume were significantly and negatively correlated with site latitude, such that females breeding on the east coast produced larger eggs than females breeding on the west coast (Fig. 2.4). However, there were no significant effects of longitude on either eggshell or yolk mass. Neither site latitude nor elevation had significant effects on any of the measures of egg size considered.

Multivariate Analyses

Principle coordinate analysis produced three principle coordinates that together explained 36.02% of variation in bacterial communities (PCo1 = 16.36%, PCo2 =

10.88%, PCo3 = 8.78%). We found that bacterial community composition explained a significant portion of the variation observed in mean yolk IgY concentration across sites and years sampled (Table 2.3). But we did not detect any significant effects of geographic variables on yolk IgY, though an elevation effect was almost significant (Table 2.3). Mean yolk IgY tended to correlate negatively with the first PCo axis explaining bacterial community composition (Spearman correlation: R = -0.34, P = 0.059), suggesting that some aspects of bacterial community composition tend to vary consistently with maternal antibody deposition across the Tree Swallow breeding range.

DISCUSSION

We found that maternal deposition of yolk antibodies was positively correlated with bacterial richness and diversity of the nest microbiota across the breeding range of the Tree Swallow, whereas no significant relationships were detected for geographic variables associated with life history variation. These results clearly lend support to the Ag-diversity Hypothesis, and suggest that ecological pressures influencing maternal immune status are relatively more important in shaping transgenerational immune investment than are sources of selection from life-history trade-offs. Through multivariate analyses, we also detected a significant effect of overall bacterial community composition on yolk IgY concentration. In accordance with the results produced from the univariate analyses, there were no significant effects of geographic variables on yolk IgY in the multivariate models. Follow-up analyses revealed significant correlations between yolk IgY and the relative abundances of five bacterial phyla suggesting that some groups of bacteria are more influential than others in shaping

maternal immune provisioning of eggs. This work represents the first study comparing both Ag-diversity and PoL Hypotheses for transgenerational immune investment utilizing direct and quantitative measures of environmental antigen pressure as opposed to climatic or ecological proxies.

Our current understanding of antibody transmission from the ovary to developing ova in both wild and domestic bird species indicates that levels of MAb transmission are proportional to antibody concentrations in maternal circulation at the time of the yolking up of the ovum prior to ovulation (e.g., Gasparini *et al.* 2002, Saino *et al.* 2002b, Grindstaff *et al.* 2005, Morales, Sanz & Moreno 2006, Sun *et al.* 2013). Based on this evidence, the Ag-diversity Hypothesis seems like a plausible explanation for the variation observed in transgenerational immune investment since one would expect maternal immune activity to increase with increased exposure to immune-eliciting stimuli. Although the movement of antibodies into yolk is a selective and receptor-mediated process (Hamal *et al.* 2006, Kowalczyk *et al.* 1985, West, Herr & Bjorkman 2004), the level of antibody transmission appears to be determined passively based on current levels of antibodies in maternal circulation and individual transmission efficiency (Kowalczyk *et al.* 1985, Abdelmoneim & Abdelgawad 2006, Coakley *et al.* 2014); there is little evidence that females can differentially allocate antibody resources between herself and her eggs or between specific eggs within a clutch (but see Saino *et al.* 2003), other than through laying-order effects (Blount *et al.* 2002, Muller *et al.* 2004, Pihlaja, Siitari & Alatalo 2006, Hargitai, Prechl & Torok 2006). Domestic hens transmit approximately 30% of circulating antibodies to developing yolk, however, there is variation in the degree of transmission among poultry lines (Abdelmoneim &

Abdelgawad 2006). Similarly, Coakley et al. (2014) found significant variation among individual female quail in the transmission of Ag-specific antibodies to yolks (9.2%-38.4% of maternal circulation). Interestingly, individual females were consistent in their relative levels of antibody transmission across time and different challenges both in poultry (Coakley *et al.* 2014) and in free-living kittiwakes (Gasparini *et al.* 2001). Variation in MAb transmission efficiency among females may arise from differential receptor density or specificity. During vitellogenesis (i.e., yolk formation), maternal IgY molecules bind to receptors on the membranes of developing oocytes and are incorporated into yolks via endocytosis (Roth, Cutting & Atlas 1976). In poultry, maximum transfer rate is approximately 45mg/day, which translates to a maternal loss of 10-20% of circulating IgY during egg-laying since multiple yolks develop simultaneously (Kowalczyk *et al.* 1985). The seemingly broad and consistent variation in MAb transmission efficiency suggests that populations may vary in their capacity for transgenerational immune investment while still responding as predicted to antigenic pressure in the environment, as documented in this study. Thus, individuals with low MAb transmission efficiency may deposit equivalent amounts of yolk IgY when faced with high antigen diversity to individuals with high MAb transmission efficiency but low environmental antigen diversity. The Ag-diversity and PoL Hypothesis are certainly not mutually exclusive, and it is likely that both ecological and evolutionary forces shape maternal immune investment in offspring. It is possible that relative MAb transmission (e.g., IgY receptor density, maternal immune responsiveness) may be shaped by evolutionary forces leading to local adaptation, whereas absolute transmission may be adjusted by ecological forces resulting in phenotypic flexibility. However, if the

ecological forces relevant to MAb deposition vary significantly in space and predictably in time then variables such as Ag pressure could also lead to local adaptation. In support of this hypothesis, Coakley et al. (2014) found that the relative level of MAb transmission to eggs was not related to the strength of the maternal immune response. Further, significant variation in antigen-specific MAb transfer but not in maternal antibody response among different color morphs of pigeons (Jacquin *et al.* 2013) illustrates this decoupling of a) antibody production and b) antibody transmission as separate mechanisms contributing to variation in MAb deposition (Boulinier & Staszewski 2008, Coakley *et al.* 2014).The current study contributes to our understanding of maternal antibody transmission by demonstrating that ecological variables are relevant in shaping transgenerational immune investment at a large geographic scale. Although differences among domestic poultry lines in yolk IgY deposition suggest that degree of transmission may be selected for (reviewed in Grindstaff *et al.* 2003), further study is needed to determine the heritability of this trait and to evaluate transfer-efficiency as a potential mechanism contributing to any heritable variation that may be detected.

To our knowledge, the Ag Diversity Hypothesis has only been tested once previously to explain maternal deposition of yolk antibodies in birds (Forsman *et al. in review*). Although vaccination studies have investigated how maternal exposure to specific antigens influences the transmission of MAb to egg yolks, there is very limited data as to how maternal exposure to the antigenic environment as a whole influences MAb deposition. A few studies considering prevalence of specific ectoparasites and vectors suggest that maternal deposition of antibodies is in fact responsive to variation

in Ag abundance (Gasparini *et al.* 2001, Buechler *et al.* 2002); Ag diversity, however, was not considered in these studies. In a direct test of the Ag-diversity Hypothesis, Forsman et al. (*in review*) manipulated abundance, diversity, and community composition of bacteria in active nests of free-living Tree Swallows and found that females at nests with experimentally reduced Ag diversity, but not abundance, produced eggs and nestlings with lower levels of IgY than females at control nests. The current study builds on these results by testing how natural variation in nest bacterial communities relates to maternal deposition of yolk IgY across multiple, and geographically disparate, breeding sites. Although the current work, and that conducted by Forsman et al. (*in review*), represent the first studies to test the Ag-diversity Hypothesis in terms of maternal antibody deposition, several other studies have looked for similar effects on maternal deposition of antimicrobial proteins into the egg albumen. The first such study investigated various aspects of albumen defenses in domestic chickens reared under germ-free (GF), specific pathogen-free (SPF), or control conditions (Bedrani *et al.* 2013). As predicted by the Ag-diversity Hypothesis, GF birds produced eggs with lower albumen pH and bactericidal activity than SPF and control females, which both experienced more environmental Ag's than did GF females. However, Bedrani et al. (2013) found no treatment effects on albumen lysozyme activity, ovotransferrin, anti-proteases, or total antibody protein. Using wild populations of red-capped larks (*Calandrella cinerea*), Grizard et al. (2015) compared alpha- and beta-diversity of naturally occurring bacteria on eggshell surfaces with pH, lysozyme, and ovotransferrin in albumen, but found no significant correlations. Similarly, Horrocks et al. (2014) studying nine different lark species (family *Alaudidae*) did not find a

relationship between ovotransferrin in albumen and climate variables used as proxies for bacterial abundance in the environment. They did, however, find a positive correlation between lysozyme and temperature, which the authors explain may have resulted from the role of lysozyme in albumen stabilization at high temperatures (Horrocks *et al.* 2014). Because bacteria were not directly sampled in that study, the Ag-diversity Hypothesis could not be tested directly. In another experimental study, similar to the one conducted by Forsman et al. (*in review*), Jacob et al. (2015) found no effects of bacterial abundance manipulations on maternal deposition of lysozyme or ovotransferrin into egg albumen; females exposed to fewer nest bacteria, however, deposited lower concentrations of yolk carotenoids. Thus, the balance of available evidence, including the current study, suggests that natural variation in albumen-bound immune compounds may be less sensitive to the maternal antigenic environment than are yolk-bound antibodies. These differences may arise because of differential mechanisms of synthesis and deposition. Whereas maternally synthesized antibodies are transported from maternal circulation to the ovum across the ovary (Kowalczyk *et al.* 1985), lysozyme is produced by uterine tissues, as well as by other tissues (Wellman-Labadie, Picman & Hincke 2007), and may thus constitute a separate pool of lysozyme than the pool produced and maintained for maternal use.

Although the PoL Hypothesis explaining MAb deposition has been tested previously (Addison *et al.* 2009), it has not been tested in direct comparison with the Ag-diversity Hypothesis. Addison et al. (2009) found support for the PoL hypothesis through a comparative study of 23 tropical bird species, which demonstrated yolk IgY concentration to be negatively correlated with developmental period. However, no

information was available regarding Ag pressure experienced by these tropical species. The authors suggested that lower levels of MAb deposition may limit maternal and transgenerational costs, and should thus be selected for in long-lived species. Although there is limited evidence that antibody production *per se* imposes significant energetic costs to females (Klasing 1998), there are other possible physiological costs associated with immune activity (e.g., oxidative stress) that may negatively affect survival and future reproduction. Specifically, Addison et al. (2009) propose that transgenerational costs to offspring immune development may impose relatively stronger constraints on MAb transmission than any direct costs to females. If present in large amounts, MAb can negatively influence immunological development trajectories of juveniles by a) blocking endogenous immune activation (Lung *et al.* 1996, Staszewski *et al.* 2007), which under normal conditions would produce antigen-specific memory cells that contribute to long-term protection upon re-exposure (Mondal & Naqi 2001, Siegrist 2003, Glezen 2003, Staszewski *et al.* 2007), and by b) reducing B-cell repertoire (i.e., antigen recognition capacity) by obscuring early-encountered antigens necessary for proper B-cell development (Carlier & Truyens 1995). Thus, the same mechanisms that confer short-term benefits through passive immunity may simultaneously result in long-term costs to endogenous immunity depending on the level and specificities of MAb present. Based on these results, we predicted that yolk IgY levels would increase with increasing pace of life, and hence latitude, if the PoL Hypothesis was supported. However, we did not detect any significant geographic patterns (i.e., latitude, longitude, or elevation) in MAb deposition across the breeding range of the Tree Swallow. Instead we found significant variation in yolk IgY concentration to be attributable to nest

bacterial diversity and composition, supporting the Ag-diversity Hypothesis. It is possible that the life-history gradient exhibited by this relatively short-distance migrant is not steep enough to readily detect intraspecific variation in maternal immune investment with pace of life, and that intraspecific comparisons for species with greater PoL variation (e.g., house sparrow, Martin *et al.* 2005) or interspecific comparisons (e.g., Lee *et al.* 2008, Addison *et al.* 2009, Horrocks *et al.* 2015) are more likely to yield significant effects. However, previous work with Tree Swallows at some of the same sites sampled in the current study indicate that faster-paced females in the north of the range do indeed maintain offspring quality but reduce their own immune function with increased reproductive effort, whereas slower-paced females further south maintain their own immune function at the cost of offspring quality (Ardia 2005b, Ardia 2005a).

Effective immune function is clearly beneficial, however, maximal immune investment is not always the optimal strategy (Viney, Riley & Buchanan 2005). Activation of the immune system is energetically costly (Lochmiller & Deerenberg 2000, Norris & Evans 2000, Martin, Scheuerlein & Wikelski 2003, Bonneaud *et al.* 2003) and is often accompanied by immunopathology, such as oxidative tissue damage (Graham, Allen & Read 2005, Bertrand *et al.* 2006). Misdirected immune responses toward innocuous molecules and autoimmunity may result in decreased fitness; therefore, selection should favor robust immune activity only when the protective benefits outweigh the associated energetic and physiological costs. Reproduction is an especially demanding life-history stage encompassing activities such as mate-choice, incubation, and parental care; female birds bear the additional costs associated with egg production. Because of energetic constraints, maternal investment in costly

reproductive activities may necessitate temporary down-regulation of self-maintenance activities, such as immune function, to maximize reproductive success (Norris & Evans 2000, Stearns 1992). There is accumulating evidence in support of trade-offs between maternal immune function and reproductive effort, indicating that individuals cannot invest maximally in both simultaneously. Studies of wild birds that have experimentally increased reproductive effort of post-laying females have documented concomitant decreases in immune responsiveness (Deerenberg *et al.* 1997, Nordling *et al.* 1998, Moreno, Sanz & Arriero 1999, Ardia, Schat & Winkler 2003, Ardia 2005a). Maternal antibody transmission presents an interesting counter-example to the typical trade-off model of reproduction vs. self-maintenance (Roff 1992, Stearns 1992, Ricklefs & Wikelski 2002) because increased investment in offspring immunity necessitates increased maternal immune activity, which under different circumstances (i.e., non-laying) would be considered an investment in self-maintenance. This convergence may create different predictions for the immune status of adult females depending on whether they are actively producing eggs or not. Reproductive females do in fact increase antibody production immediately prior to egg-laying (Klasing 1998), and circulating antibody levels may change rapidly because of their relatively short half-life; the half-life of IgY in chicken circulation is 36 hours (Patterson *et al.* 1962). Although differential immune investment with reproductive stage has not been directly tested across a life-history gradient, there is evidence that these differences may exist. For example, slower-paced neotropical bird species were found to have higher levels of circulating natural antibodies than faster-paced species (Lee *et al.* 2008), whereas

slower-paced species deposited lower levels of MAb into their eggs (Addison *et al.* 2009).

In conclusion, we found evidence suggesting that antigenic diversity in the nesting environment is relatively more important in shaping patterns of MAb deposition than are life-history correlates at a large geographic scale. OTU richness, diversity, and phylogenetic diversity were all significantly and positively correlated with maternal transmission of antibodies to egg yolks across the Tree Swallow species range. Additional support for this conclusion comes from a similar study in old world larks that documented positive correlations between adult immune activity and climate proxies for antigen exposure (Horrocks *et al.* 2015). Although we did not find any support for the Pace of Life Hypothesis when directly evaluated against the Antigen Diversity Hypothesis, other work suggests that both individual and transgenerational immune investment are related to life history, especially in comparisons across species. Further work is needed to determine how MAb transmission capacity and phenotypic flexibility contribute to the observed variation among individuals and populations.

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Figure 2.1. Breeding range (blue) map of the North American Tree Swallow; winter range is designated in orange. Field sites sampled for this study are indicated by stars. Map modified from Winkler et al. 2011.

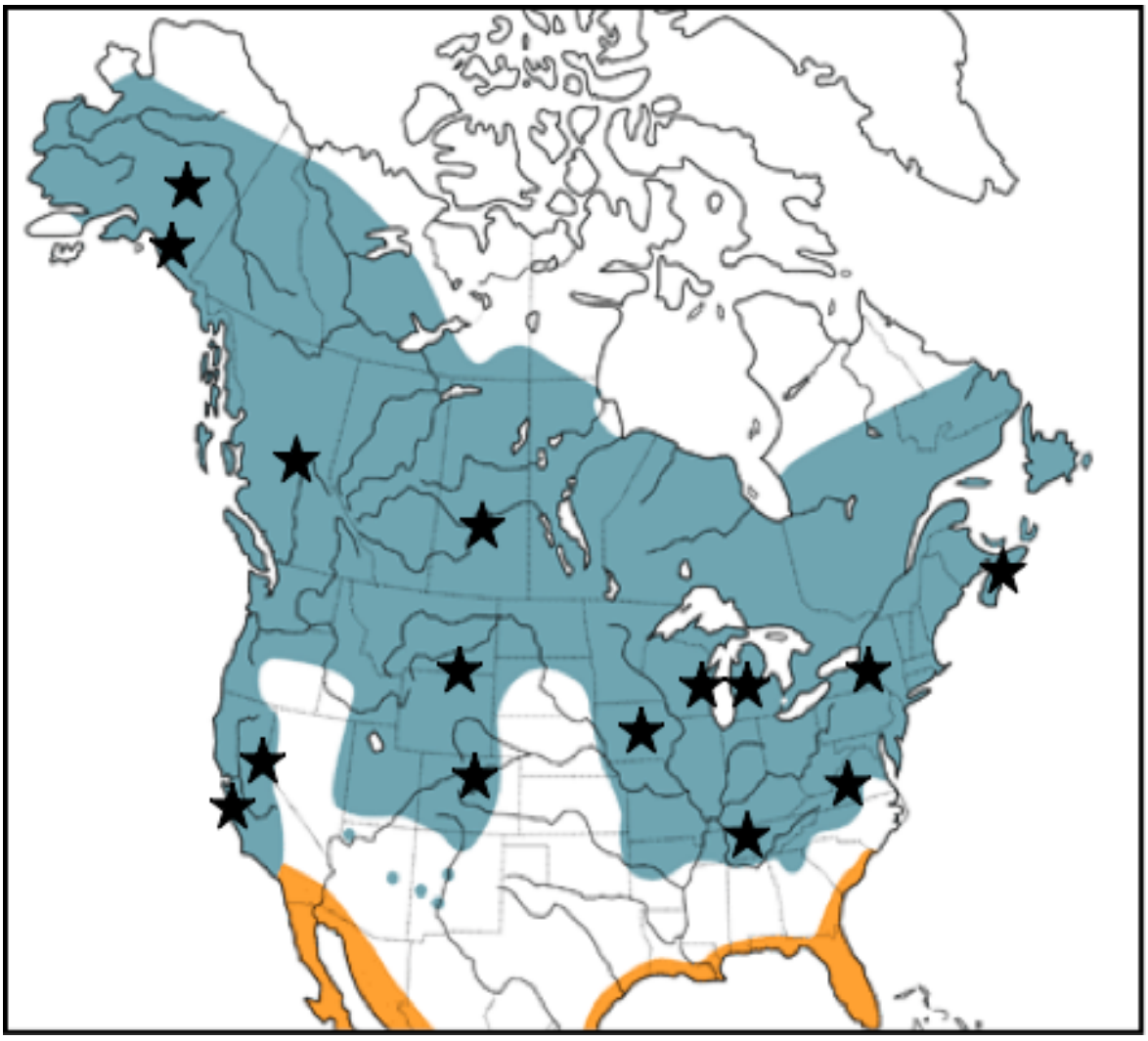

Table 2.1. Results from Moran's I testing for spatial autocorrelation of yolk IgY concentration in Tree Swallow eggs across North America.

	Moran's I	
	I	**P**
All years	**-0.09**	**0.067**
2008	-0.14	0.080
2009	-0.14	0.660
2010	-0.08	0.471

Table 2.2. Summary tables from four mixed effects models describing variation in yolk IgY concentration in Tree Swallow eggs collected from across North America in relation to alpha diversity of nest bacterial communities and geographic variables associated with life history variation.

	A **Bacterial Richness**			B **Phylogenetic Diversity**			C **Bacterial Diversity**			D **Evenness**		
Source	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Microbiome	**1,14.89**	**4.66**	**0.0477***	**1,23.48**	**4.29**	**0.0495***	**1,85.21**	**4.00**	**0.0487***	1,135.50	1.05	0.3070
Latitude	1,3.228	2.57	0.2011	1,4.148	2.24	0.2060	1,4.482	2.72	0.1666	1,7.459	1.59	0.2454
Longitude	1,4.815	1.35	0.2996	1,5.484	1.28	0.3044	1,5.59	1.35	0.2932	1,8.123	0.73	0.4173
Elevation	1,3.991	6.32	0.0659	1,4.256	6.	0.0641	1,4.214	6.63	0.0586	1,5.598	4.42	0.0837

Figure 2.2. Regression between mean OTU richness and mean residual yolk IgY concentration (r^2=0.1122, P=0.0655). Means were calculated for each year of collection within sites (i.e., Year(Site)).

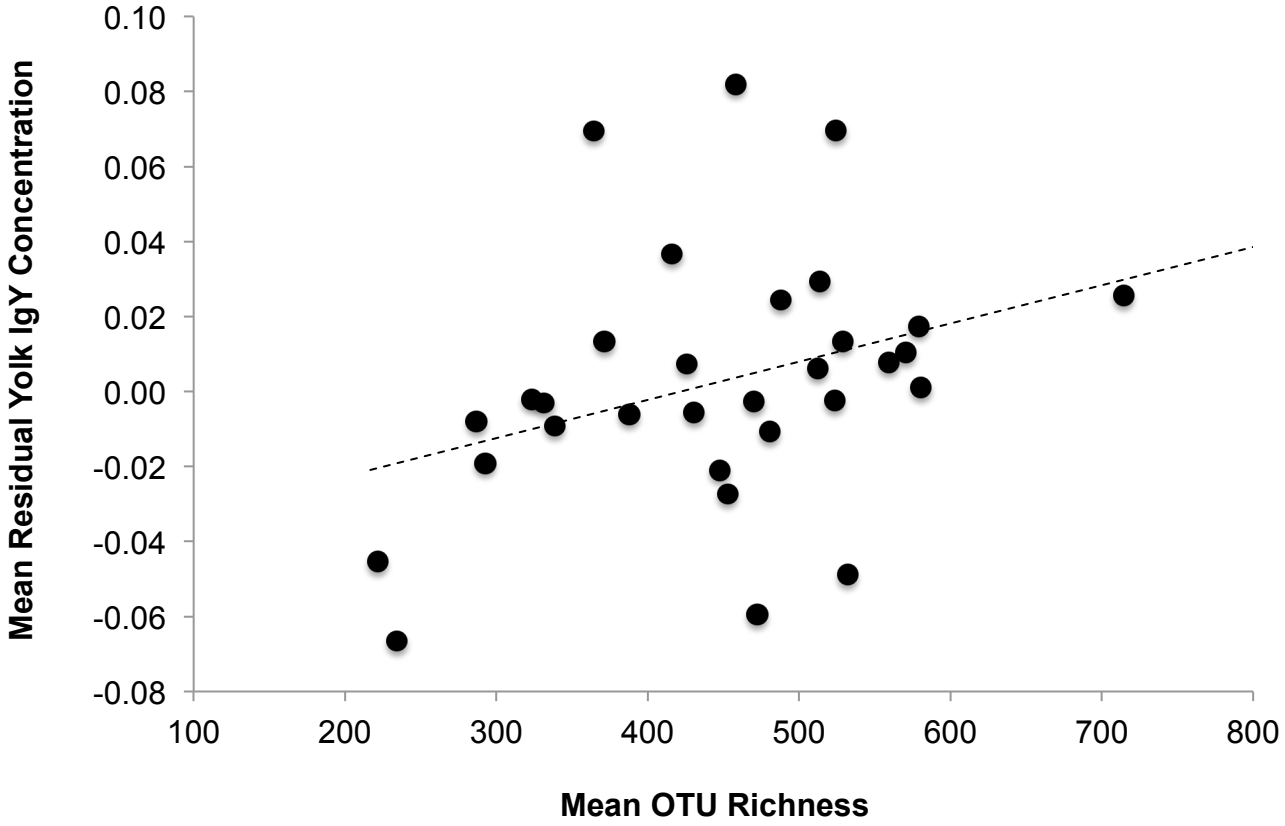

Figure 2.3. Relative abundances of bacterial phyla detected in Tree Swallow nests for each site/year.

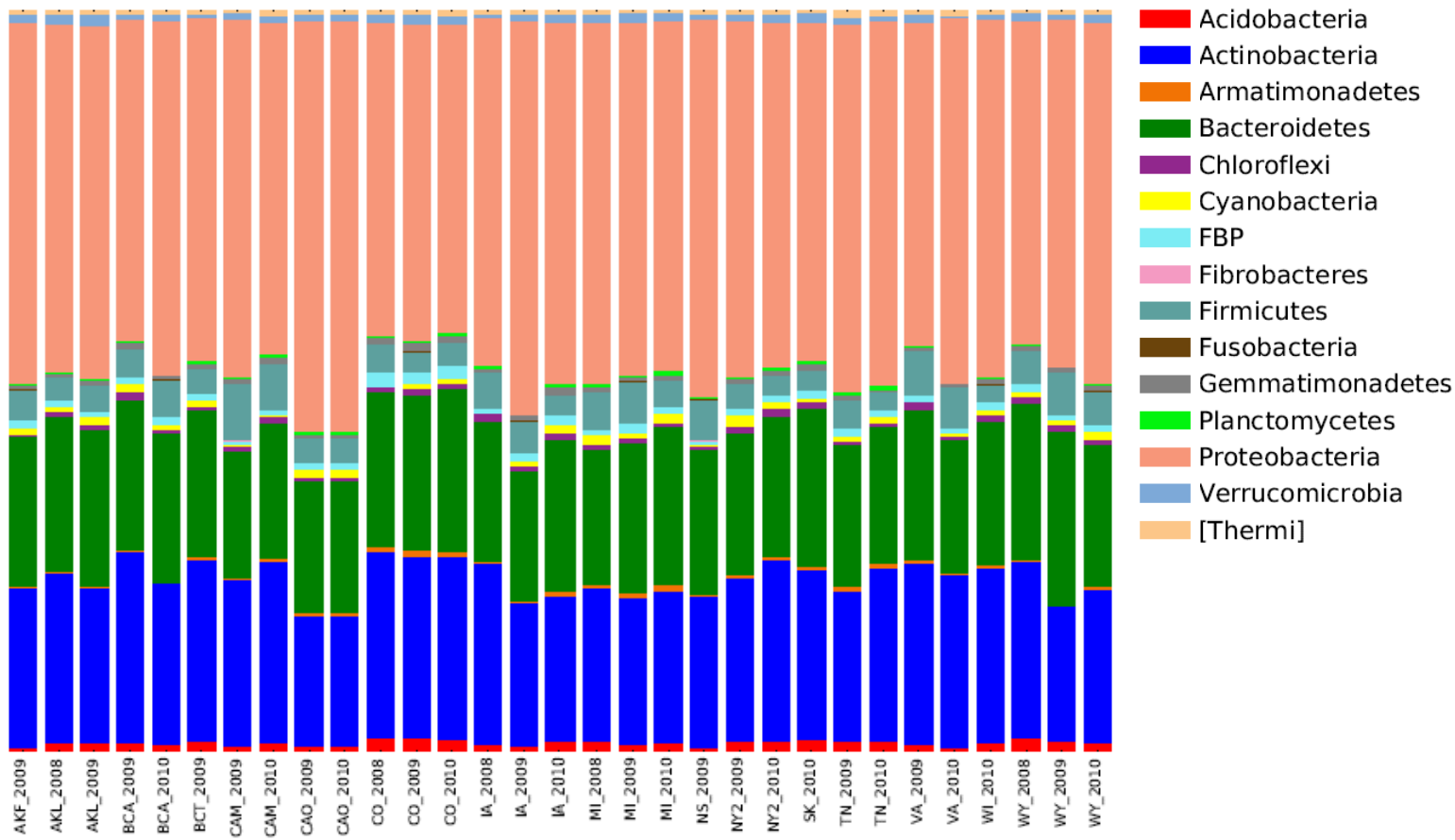

Table 2.3. Summary tables from four mixed effects models describing egg size variation across North America in relation to nest bacterial richness (i.e., number of OTUs) and geographic variables associated with life history variation. Asterisks indicate significant effects.

	A	**Egg Mass**		B	**Egg Volume**			**Eggshell Mass**		D	**Yolk Mass**	
Source	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
OTU Richness	1127.6	0.36	0.5515	1,72	0.36	0.5489	1148.9	0.07	0.7885	1,128.00	0.00	0.9546
Latitude	1,12.17	0.83	0.3807	1,10.7	1.86	0.2005	1,10.19	0.46	0.5118	1,15.86	0.55	0.4694
Longitude	**1,12.81**	**8.55**	**0.0120***	**1,10.69**	**9.09**	**0.0121***	1,10.95	1.91	0.1944	1,15.3	2.57	0.1294
Elevation	1,8.628	0.69	0.4298	1,7.027	1.13	0.3225	1,7.347	2.08	0.1901	1,9.984	0.08	0.7784

Figure 2.4. Regression between site longitude and mean egg mass, which was calculated for each year of collection within sites (i.e., Year(Site)).

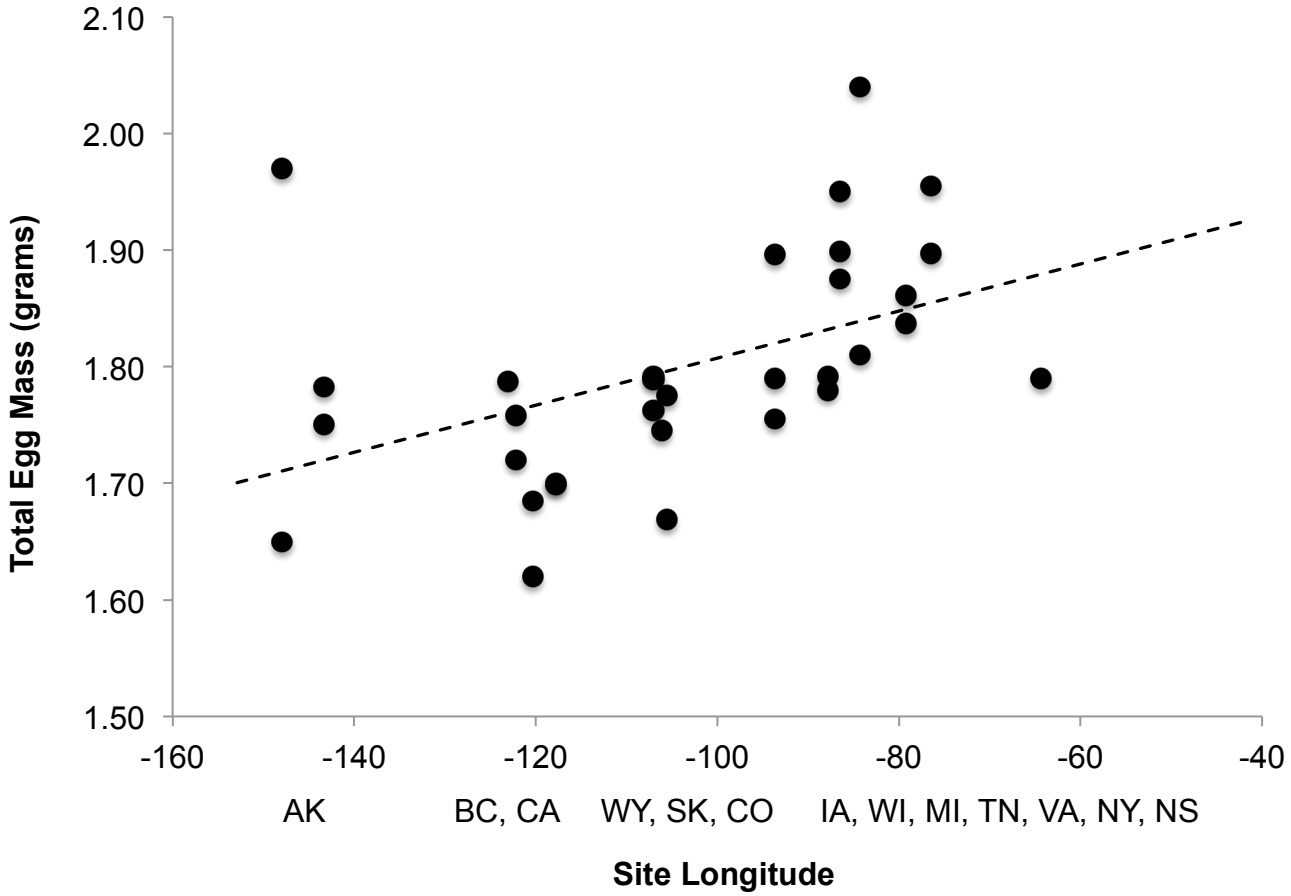

Table 2.4. Table 1. PROCRUSTES ANOVA analyzing the relationship between bacterial community (unweighted UNIFRAC distance matrix after transformation by means of Principal Coordinate Analysis) and geography on yolk Ig concentration. Asterisks indicate significant effects.

	df	F	P
Model 1			
Latitude	1,30	<0.01	0.913
Longitude	1,30	1.33	0.319
Elevation	1,30	4.56	0.060
Microbiome	13,30	2.25	**<0.001***
Model 2			
Latitude	1,30	0.01	0.913
Longitude	1,30	0.57	0.319
Elevation	1,30	2.11	0.060
Locality	12,30	1.39	0.079
Year	2,30	4.56	**0.001***
Microbiome	13,30	2.25	**0.001***
Model 3			
Latitude	1,30	0.01	0.913
Longitude	1,30	0.57	0.319
Elevation	1,30	2.11	0.060
Year	2,30	3.28	**0.006***
Locality	12,30	1.56	**0.020***
Microbiome	13,30	2.25	**0.001***

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Chapter 3

THE NEST MICROBIOME: AVIAN-ASSOCIATED BACTERIAL COMMUNITIES ACROSS THE CLIMATICALLY VARIABLE RANGE OF THE NORTH AMERICAN TREE SWALLOW

INTRODUCTION

Biogeography describes the geographic and temporal distributions of organisms across the globe. Species distributions are shaped by many factors including life history, ecology, and evolutionary history (reviewed by Hanson *et al.* 2012). Historical biogeography focuses on the dispersal and diversification of taxa over evolutionary time, whereas ecological biogeography focuses on contemporary interactions among organisms and between organisms and their environment (Lindstrom & Langenheder 2012). Biogeographic research on plant and animal taxa has a long and rich history of investigating macroecological patterns and processes. However, similar studies of historical and ecological biogeography in microbial systems have only made major strides relatively recently. The development of Next Generation DNA sequencing (NGS) technology has yielded an unprecedented view into the structure of complex microbial communities, which has led to rapid advances in our understanding of both free-living and host-associated bacteria (e.g., Consortium 2012a, Consortium 2012b, Gilbert, Jansson & Knight 2014). Yet, comprehensive studies of large-scale biogeographic patterns of microorganisms still trail behind those of macroorganisms, and results are much less consistent across studies. Microorganisms are generally assumed to be

minimally constrained by dispersal ability because of their small physical size, large population numbers, and capacity for passive dispersal via air and water (reviewed by Horner-Devine, Carney & Bohannan 2004b). Based on this assumption, the frequently cited Baas-Becking hypothesis posits that all microbes are found everywhere, but that the environment selects for the particular microbes that persist in any one location (Baas Becking 1934). This ecological view of biogeography has been predominant among microbial macroecologists for some time, and a multitude of studies have revealed that specific microbial taxa are, in fact, cosmopolitan and detectable at geographically distant sites across the globe (Glöckner, Zaichikov & Belkova 2000, Massana, DeLong & Pedrós-Alió 2000). In further accordance with the Baas-Becking hypothesis, microbial community composition can vary dramatically between different habitat types and is known to be sensitive to a variety of environmental variables that may impose differential selection among microbial taxa such as climate, substrate chemistry, and primary productivity. There also appears to be significant interactions between habitat type and community sensitivity to different types of environmental variables. For example, community composition of soil bacteria has been found to vary with pH (Fierer & Jackson 2006, Baker *et al.* 2009, Lauber *et al.* 2009, King *et al.* 2010) and plant abundance (King *et al.* 2010). Additionally, a comprehensive study of samples collected from a broad range of terrestrial and aquatic environments (including soil) found that salinity was relatively more important in shaping microbial community composition than were other environmental variables such as temperature and pH (Lozupone & Knight 2007), which suggests that structuring may be occurring at multiple levels. As the applications of NGS technology have expanded, computational methods

have followed suit to accommodate the analysis of multivariate beta diversity data in the context of complex ecosystems by allowing for comparisons between two distance matrices while controlling for a third. In this way one may test whether microbial communities that share more similar climate conditions also share more similar community composition than do communities that share less similar climate conditions, all the while controlling for geographic proximity between communities. Incorporating such corrections into biogeographic studies is critical to discernment of which of the correlated variables is most important in its effect on microbial communities.

Although there are plenty of studies that have demonstrated that ecological variables contribute to structuring of microbial communities, there is also accumulating evidence for biogeographic structure consistent with historical biogeography. The primary approach taken by such studies has been to look for evidence of dispersal limitation, which has been documented for numerous microbial communities at different spatial scales (e.g., Cho & Tiedje 2000, Whitaker, Grogan & Taylor 2003, Telford 2006, Cadillo-Quiroz *et al.* 2012, Andam *et al.* 2016). There is also evidence for local diversification of microbial communities (Pearson *et al.* 2009, Boucher *et al.* 2011, Andam *et al.* 2016), which constitutes a second generative mechanism underlying historical biogeography. Dispersal limitation may be evidenced by significant “distance-decay” relationships across sampling sites (e.g., Vos & Velicer 2008, Bissett *et al.* 2010, (Andam *et al.* 2016). This distance-decay relationship describes the negative correlation between taxonomic similarity and geographic distance among communities (Harte & Kinzig 1997, Green & Bohannan 2006), and has been detected across a broad range of both eukaryotic and prokaryotic taxa (Soininen, McDonald & Hillebrand 2007). When

communities exhibit biogeographic structure due to dispersal limitation, similarity in community composition is expected to decrease with increasing physical distance between communities. Thus, beta diversity (Anderson *et al.* 2011) is expected to increase with increasing geographic distance. Distance-decay effects have been documented for some microbial communities (Vos & Velicer 2008, Bissett *et al.* 2010, Martiny *et al.* 2011, Finkel *et al.* 2012, Andam *et al.* 2016), but not for others (Bell 2010). It has been suggested that distance-decay effects may be weaker at smaller spatial scales (Horner-Devine *et al.* 2004a, Bell 2010), and that dispersal limitation may be more easily detectible at a lower taxonomic scale (Andam *et al.* 2016). Therefore, different conclusions may be drawn regarding distance-decay effects simply because of differences among studies in sampling regime and scope. Comprehensive sampling of bacterial communities across spatial scales is thus necessary for identifying biogeographic patterns and attributing variation to historical vs. ecological mechanisms. For example, a well established biogeographic pattern for plant and animal taxa at the largest spatial scale is the latitudinal species gradient, which indicates that diversity tends to increase from polar to equatorial regions (Hillebrand 2004). Similar studies have been conducted with microorganisms but have produced much less consistent results (Soininen 2012). Whereas some have found evidence for a latitudinal diversity gradient (e.g., Fuhrman *et al.* 2008, Andam *et al.* 2016), others have not (Fierer & Jackson 2006, Corby-Harris *et al.* 2007, Lauber *et al.* 2009). Andam et al. (2016) suggest that this lack of consensus may result from differential taxonomic scope among studies, and that analyses of geographic patterns within classes of bacteria may be more likely to detect latitudinal species gradients than lower resolution metagenomic

analyses considering the entire domain *Bacteria*. Indeed, there is accumulating evidence that the detection of ecological and geographic effects on microbial communities is dependent on both the spatial and temporal scales of inquiry (spatial: Ramette & Tiedje 2007, Bissett *et al.* 2010, Martiny *et al.* 2011; temporal: Gilbert *et al.* 2012, Hatosy *et al.* 2013). This may also explain the variable results obtained from studies investigating elevation effects on alpha diversity (e.g., negative correlation: Bryant *et al.* 2008, no correlation: Fierer *et al.* 2011, positive correlation: Wang *et al.* 2011b) and beta diversity (Cordier *et al.* 2012) of microbial communities.

To date, the majority of work on microbial biogeography has focused on free-living bacteria in soil, aquatic, and marine systems, although an increasing number of studies are investigating distribution patterns of microbes across phyllosphere communities (e.g., Redford *et al.* 2010, Finkel *et al.* 2011, Meyer & Leveau 2012, Qvit-Raz *et al.* 2012, Vacher *et al.* 2012, Izhaki *et al.* 2013) and symbiont systems such as the gut microbiome (Banks, Cary & Hogg 2009, Fierer *et al.* 2012, Godoy-Vitorino *et al.* 2012). Far less is known about structuring and distribution of microbial communities living in close or consistent contact with non-human animals over large spatial scales in the wild (e.g., McKenzie *et al.* 2012, Kueneman *et al.* 2014). Field studies of avian-associated bacteria in wild bird populations are by no means new or uncommon. To date bacterial communities have been sampled from avian plumage (e.g., Burtt & Ichida 1999, Shawkey *et al.* 2005, Kilgas *et al.* 2012, Alt *et al.* 2015), cloaca, (e.g., Brittingham, Temple & Duncan 1988, Mills, Lombardo & Thorpe 1999, Klomp *et al.* 2008, van Dongen *et al.* 2013, Matson *et al.* 2015), the digestive tract (Godoy-Vitorino *et al.* 2008, Roggenbuck *et al.* 2014), feces (Lu *et al.* 2008, Banks *et al.* 2009, Colles *et al.* 2009,

Benskin *et al.* 2015), nests (e.g., Goodenough & Stallwood 2010, Peralta-Sanchez *et al.* 2010, Peralta-Sanchez, Møller & Soler 2011, Brandl *et al.* 2014, Grizard *et al.* 2015) and eggs (e.g., Soler *et al.* 2011, Peralta-Sánchez *et al.* 2012), Peralta-Sánchez *et al.* 2013, Grizard *et al.* 2014, Javŭrková *et al.* 2014). However, the majority of these studies were conducted using culture-based techniques, which we now know only capture a very small portion of community constituents. The development of sequence length-based methods (e.g., t-RFLP, DGGE) significantly improved breadth of sampling among constituents by eliminating the need for culturing. Although these methods allow for the characterization of bacterial richness, they are limited in that they do not allow for taxonomic assignment of sequences. Next generation sequencing has alleviated these limitations and now allows for culture-independent identification of bacterial taxa and the estimation of their relative abundances, as well as characterization of alpha and beta diversities.

In the current macroecological study, our objective was to characterize alpha and beta diversities of bacterial communities collected across North America from a novel and relatively consistent microhabitat: the active nests of breeding Tree Swallows (*Tachycineta bicolor*). Specifically, we tested the hypotheses that a) bacteria exhibit a latitudinal species gradient, and b) nest bacterial communities exhibit a distance-decay relationship, suggesting dispersal limitation and historical structuring. As an alternative to historical effects, we tested for environmental filtering effects by considering climate variables at each site prior to and during the breeding season. We predicted that temperature and precipitation prior to the breeding season would correlate positively with alpha diversity of nest bacteria and that these climatic variables would significantly

affect beta diversity of nest communities. We were particularly interested in evaluating the relative influences of geography and climate on the structuring of nest bacterial communities. Therefore, we tested different models including both geographic and climatic variables to address hypotheses of historical and ecological structuring of bacterial communities. In the current study we leverage our broad geographic scope of sampling to test for latitudinal and elevational gradients in diversity of bacterial communities inhabiting the relatively consistent nest microhabitat created by breeding Tree Swallows. Because there is overwhelming evidence that microbial populations do indeed exhibit biogeographic structure (Martiny *et al.* 2006, Tamames *et al.* 2010, Lindstrom & Langenheder 2012, Hanson *et al.* 2012, Soininen 2012), we did not anticipate randomly distributed microbial taxa. However, the extent to which local ecological factors and regional historical factors contribute to alpha and beta diversities of microbial communities is not well understood. Because of the difficulties associated with assessing ecological variables and the statistical complexities of dealing with multivariate data, studies have tended to focus on testing either one or the other: ecological or historical hypotheses of microbial biogeography (Lindstrom & Langenheder 2012).

FIELD AND LABORATORY METHODS

Bacterial samples were collected from Tree Swallow nests at 17 sites across North America (Table 3.1, Fig. 3.1) during the 2008-2010 breeding seasons. The sampling range for latitude was from 33°39'42.38"N to 64˚52'45.83"N, longitude: -147°54'06.17”W to -064°20'11.86”W, and elevation: 4.9 m to 2719 m. Tree Swallows

are insectivorous migratory birds that breed throughout much of Canada and the northern United States and readily nest in man-made nestboxes. Tree Swallows are an ideal system for investigating large-scale patterns of variation because of the pre-existence of established nesting sites throughout their range. *Golondrinas de las Americas* (http://golondrinas.cornell.edu) is an NSF-funded network of researchers and fieldsites throughout North America that support standardized, man-made nestboxes utilized by *Tachycineta* species, including the Tree Swallow.

Prior to each field season, we provided collaborators with sample collection kits and protocols to maximize standardization of sample collection, storage, and shipment. To collect bacterial samples, we first dampened an individually-wrapped sterile swab in 100uL of pre-prepared phosphate-buffered saline (1x PBS), which was then used to swab all surfaces of the nest cup material for 10 seconds. Each swab head was placed in a microcentrifuge tube with 500uL of 70% ethanol. In 2008, samples were stored and shipped at 4°C to Cornell University for processing. In 2009 and 2010, samples were instead stored at -20°C and shipped on dry ice. All samples were stored at -20°C until processing.

Samples were processed by first vortexing to dislodge bacteria attached to the swab head. The swab head was then removed using flame-sterilized tweezers, and collection tubes were centrifuged at full speed for 10 minutes to pellet cells. After centrifugation, supernatant was decanted off and pellets were used to extract DNA samples (MoBio PowerSoil Kit, cat. no. 12888), which were subsequently submitted to the Earth Microbiome Project for library preparation and Illumina sequencing.

Geographic coordinate data used to establish latitude, longitude, and elevation variables were provided by collaborators at each collecting site. Climate data were acquired from the National Oceanic and Atmospheric Administration's Climate Data Online database for the months of January, April, May, and June during 2008-2010.

High-throughput Illumina sequencing

PCR amplification and amplicon preparation for sequencing were performed following the protocols described in Costello et al. (2009, 2012), and can be found on the Earth Microbiome Project (Gilbert *et al.* 2014) web page (http://www.earthmicrobiome.org/emp-standard-protocols/). Universal bacterial primers (515F: 5'-GTGCCAGCMGCCGCGGTAA-3'; 806R: 5'-GGACTACHVGGGTWTCTAAT-3') were used to amplified bacterial and archaeal 16S rRNA variable region 4 (V4) (Caporaso *et al.* 2012, Walters *et al.* 2011). Each DNA sample was amplified in triplicate and amplicons were pooled together and quantified using PicoGreen (Thermo Fisher Scientific co). Negative controls included no template controls for DNA extraction and PCR amplification. All barcoded amplicons were pooled in equal concentrations for sequencing and purified using the UltraClean PCR Clean-up kit (Mo Bio laboratories inc.). Amplicons were sequenced in two different runs on the Illumina HiSeq2000 sequencing platform at the BioFrontiers Institute Next-Generation Genomics Facility at University of Colorado at Boulder (USA).

Amplicon sequences were analyzed using QIIME, an open source software package for comparison and analysis of microbial communities (version1.8.0) (Caporaso *et al.* 2010b). Sequences were demultiplexed and quality-filtered according

to default parameters within QIIME. We assigned sequences to OTUs with the closed-reference protocol that captures the full bacterial diversity within our data set, following the default parameters in QIIME (http://qiime.org/tutorials/open_reference_illumina_processing.html; see also (Navas-Molina *et al.* 2013). Sequences were clustered into operational taxonomic units (OTUs) with a sequence similarity threshold of 97% using UCLUST (Edgar 2010) and then, a representative sequence were matched against the August 2013 release of the Greengenes 97% reference data set (http://greengenes.secondgenome.com; DeSantis *et al.* 2006, Mcdonald *et al.* 2012). Representative OTUs that matched the reference database received the taxonomic classification standardized in Greengenes with the RDP classifier (Wang *et al.* 2007). The representative sequences of all OTUs were then aligned to the Greengenes reference alignment using PyNAST (Caporaso *et al.* 2010a), and this alignment was used to construct a phylogenetic tree using FastTree (Price, Dehal & Arkin 2010). Chloroplasts, mitochondria, Archaea, sequences that could not be assigned to phyla, and OTUs representing less than 0.00005% of the total reads in the data set were filtered out as recommended for Illumina-generated sequence data (Bokulich *et al.* 2013). Rarefaction for sequencing effort standardization was performed at 15733 sequences and 279 samples were retained for subsequent analyses. (Table 3.1).

STATISTICAL ANALYSES

Bacterial DNA samples that did not amplify initially were re-amplified. In order to validate the use of data produced from the two separate amplification batches, we

performed Generalized Procrustes Analyses (Gower, 1975) with 22 samples that were successfully amplified in both batches. Procrustes analysis transforms principal coordinate axes in order to minimize the distance between pairs of samples. Both low p and M2 values are required to assess the resemblance between pairs of samples. We found that pairs of bacterial samples from the same nest (i.e., technical replicates) were more similar than could reasonable by expected by chance than were pairs of bacterial samples from different nests (i.e., biological replicates, $p < 0.005$, M2 = 0.069, Figure 3.2). Therefore, we combined data from both amplification batches in this study. Sequence data produced in the second amplification batch were only used for those samples that did not amplify efficiently in the first amplification batch; no samples are represented twice.

Alpha diversity Analyses

Bacterial species richness, Faith's phylogenetic diversity index (Faith 1992), Shannon's diversity index (Shannon 1948) and evenness were calculated for each individual bacterial community sampled. Mean alpha diversities were then calculated at the site-year level to use in analyses since our independent variables (i.e., climate and geography) were measured at the site-year level.

Moran's I test was applied for studying spatial autocorrelation of site-year mean bacterial species richness, Faith's phylogenetic Diversity index, Shannon's diversity index and evenness. Because our data covered most of North America, geographical weights were calculated using the close neighbor algorithm, setting the number of neighbors (k) to 3. Different k values did not qualitatively change our results.

The effects of locality and year were analyzed by means of Generalized Linear Models (GLM), where bacterial species richness, Faith's Phylogenetic Diversity index, Shannon's diversity index and evenness per site and year (i.e., site-year means) were used as the dependent variables in each model, and site and year as fixed factors.

We used General Linear Mixed Models (GLMM) to examine the effects of a) geographic variables and b) climate variables on the four measures of alpha diversity in two separate sets of models. For the geographic models we included year, site latitude, site longitude, and site elevation as fixed main effects and site as a random effect to control for multiple sampling at sites across years. Mean site-year alpha diversity measures were used as dependent variables. For the climate models we used a backward-removal selection approach to identify best models. Full climate models included site, year, number of days in month with greater than or equal to 1.0 inch of precipitation (DP10), number days in month with minimum temperature less than or equal to 32.0 °F (DT32), total precipitation amount for the month (TPCP), monthly mean minimum temperature (MMNT), monthly mean maximum temperature (MMXT), and monthly mean temperature (MNTM). Climate variables were considered for the month of January preceding the breeding season (winter models), for the month of April immediately preceding the breeding season (pre-breeding models), and for the month in which samples were collected (breeding models). Climate data were obtained from NOAA for weather stations located in close proximity to each study site. Results from full climate models are summarized in Appendix 1.

Finally, we constructed a set of complete GLMs to simultaneously investigate the effects of geographic and climatic variables on mean site-year alpha diversity

measures. Best model selection was employed as described above including the main effects of latitude, longitude, climate variables DP10, DT32, TPCP, MMNT, MMXT, and MNTM for the months of January, April, and breeding. Results from full climate and geography models are summarized in Appendix 2.

Beta Diversity Analyses

Bacterial species prevalence per site-year and unweighted Unifrac distance were used to construct distance matrices (Lozupone & Knight 2005) that were applied to beta diversity analyses. Mantel tests were performed with R to test for correlations between variation in mean site-year bacterial communities and the geographic distances between them. Geographic distance matrices were calculate using Vincenty's formula (Vincenty 1975). Mantel analyses were each set to run for 9999 permutations.

Multivariate spatial analyses (MSA; Smouse & Peakall 1999) test for spatial autocorrelation in a multivariate dependent variable such as community composition. Because our data covered much of North America, geographical weights were calculated using the close-neighbor algorithm, setting the number of neighbors (k) to 3. Using different values of k did not qualitatively change our results. MSAs were based on Monte-Carlo simulations, for which we set the number of permutations to 9999. MSAs were performed for site-year mean communities and also for individual nest communities using centroid coordinate data for each locality. For a subset of bacterial samples we also had specific nestbox coordinates, which allowed us to test for spatial autocorrelation across samples collected within and among sites.

To test for effects of sampling site and year we performed Procrustes ANOVAs (Anderson 2001, Collyer, Sekora & Adams 2015) where the unweighted Unifrac distance matrix was included as the response variable matrix, and site and year were included as main effects. Because this particular analysis does not include positional data we were not constrained by the lack of nestbox coordinate data for some samples. Therefore, all data available were included in this analysis, thus, incorporating both within- and among-site and year variation. Principal Coordinate Analyses (PCoA) were performed to reduce dimensionality of beta diversity data and to maximize variance of orthogonal axes. Visualizations of PCoA were performed with EMPeror (Vázquez-Baeza *et al.* 2013).

Procrustes ANOVAs (Anderson 2001, Collyer *et al.* 2015) were also performed to test for effects of geographic variables on mean community composition of nest bacteria per site and year. For these analyses, we used the unweighted Unifrac distance matrix as the response matrix, and we included year, latitude, longitude, and elevation as main effects. Unifrac distances were calculated based on mean site-year bacterial communities because latitude, longitude, and elevation were derived from site centroid coordinates, as opposed to individual nestbox coordinates, we used mean site-year community composition as the response variable as opposed to individual nest community composition. Therefore, a second PCoA was performed to reduce dimensionality of beta diversity data collected at the site-year level.

Generalized Least Squares (GLS) analyses were performed to investigate the effects of climate on the bacterial communities of Tree Swallow nests. GLS allows for the inclusion of spatial correlations into the error terms of the models. Therefore, our

GLS analyses allowed us to test for climate effects while simultaneously controlling for geographic effects. Exponential correlations were applied for inclusion of any geographical autocorrelation in the error terms. The unweighted Unifrac distance matrix was used as the response term, year was entered as a fixed factor, and all of the following climate metrics were included as covariates: number of days in month with one or more inches of precipitation (DP10), number days in month with minimum temperature less than or equal to 32.0°F (DT32), total precipitation amount for the month (TPCP), monthly mean minimum temperature (MMNT), monthly mean maximum temperature (MMXT) and monthly mean temperature (MNTM). These climatic variables were obtained from NOAA for the same month when nest samples were collected (breeding model), for the month previous to breeding (pre-breeding models) and for January (winter model). The best model was chosen by backward-stepwise selection.

GLMM analyses were performed with JMP Pro 10 and all other multivariate statistical analyses with R statistical environment 3.1.3. (R_Core_Team, 2015) using packages *ape, spdep, geomorph* and *vegan*. *P* values below 0.05 were considered significant.

RESULTS

The nest microbiome

Bacterial communities sampled from Tree Swallow nests were dominated by phylum Proteobacteria (56.8%, average relative sequence abundance per sample, N=279), followed by Bacteroidetes (19.6%), Actinobacteria (16.0%) and Firmicutes (4.5%; Figure 3.3a, Table 3.2). These phyla were present in all samples. Within these groups, the

genus *Sphingomonas* dominated communities and was found at all localities and in all samples (mean: 16.3%, range: 0.2% - 46.8%; Figure 3.3b, Table 3.3). *Hymenobacter* spp. (8.1%) and an unidentified genus belonging to family *Oxalobacteraceae* (5.3%) were also found in all samples. Finally, *Pseudomonas* spp. (5.8%; 275 out of 279 samples), and other unidentified genera belonging to *Sphingomandaceae* (4.4%, 278 samples) and to *Microbacteriaceae* (2.5%; 277 samples) were relatively abundant in nest samples (Table 3.3).

Bacterial Alpha Diversity

We found no evidence of spatial autocorrelation for any of the measures of alpha diversity considered: OTU richness, Faith's phylogenetic diversity index, Shannon's diversity index, or evenness (Table 3.4). There were no significant effects of site or year on mean alpha diversities of nest bacteria. However, Shannon's diversity and community evenness were more strongly influenced by locality than were OTU richness and phylogenetic diversity (Table 3.5).

Geographic Effects on Alpha diversity

Latitude and longitude had significant effects on community evenness of nest bacteria (Table 3.6). Evenness decreased with increasing latitude (Figure 3.4a) and also decreased from west to east (Figure 3.4b). Shannon's diversity index also tended to decrease with increasing latitude. There were no significant effects of latitude or longitude on OTU richness or phylogenetic diversity, and no significant effects of site elevation on any of the alpha diversity variables considered (Table 3.6).

Climate Effects on Alpha diversity

The majority of climatic effects on nest bacterial alpha diversity were observed during the pre-breeding period (i.e., April; Table 3.7). We observed significant negative relationships between all measures of alpha diversity and the number of days in the month of April with ≥ 25.4mm of precipitation (TP10) and significant positive relationships between all measures of alpha diversity and total precipitation (mm) for the month of April (TPCP). Both OTU diversity and evenness were positively correlated with the number of days in the month of April with a minimum temperature ≤ 0°C (DT32). OTU diversity was positively correlated with mean temperature (MNTM), but negatively correlated with mean minimum temperature (MMNT) and mean maximum temperature (MMTM) for the month of April. Although we also observed a positive relationship between the number of days in the month of sample collection with a minimum temperature ≤ 0°C and phylogenetic diversity, no other climate variables were retained in any of the best breeding models explaining alpha diversity (Table 3.7). Similarly, none of the climate variables measured during the preceding winter (i.e., January) predicted any measures of alpha diversity. Locality was retained as a significant explanatory variable in all best models for Shannon diversity and evenness, but not for OTU richness or phylogenetic diversity.

Relative Effects of Geography & Climate on Alpha Diversity

We tested for the relative effects of climatic and geographic variables on alpha diversity of nest bacteria by running models including predictors of both types. Models from the

pre-breeding season retained the largest number of significant predictor variables (Table 3.8). As was the case for the models with climatic variables only, all measures of alpha diversity were positively correlated with total precipitation for the month of April (TPCP), and negatively correlated with the number of days in the month of April with ≥ 25.4mm of precipitation (TP10). These effects were relatively strong, and were the only variables retained in the pre-breeding models describing variation in OTU richness and phylogenetic diversity. The best pre-breeding models for both Shannon's diversity and evenness also included DT32, MNTM, and elevation; diversity and evenness were positively correlated with mean monthly temperature (MNTM) and the number of days with a minimum temperature ≤ 0°C (DT32) during the month of April. Interestingly, bacterial community diversity and evenness decreased with elevation across sites when controlling for pre-breeding temperature and precipitation. However, elevation effects were weaker than were climatic effects in these models.

The best breeding season models resulting from the inclusion of both geographic and climatic variables indicate that OTU richness, phylogenetic diversity, and Shannon's diversity were positively correlated with the number of days with a minimum temperature ≤ 0°C during the month of breeding (DT32) and negatively correlated with latitude (Table 3.8). No other variables were retained in the best breeding-season models, and the best breeding-season model for evenness retained no significant variables after model selection. Again, geographic effects were weaker than climatic effects for the breeding-season models.

No geographic or climatic variables were retained in the best winter-season models for OTU richness, phylogenetic diversity, or evenness. The best winter-season

model for Shannon's diversity described negative relationships with latitude, longitude, and monthly mean maximum temperature in month of January prior to sample collection (Table 3.8).

Bacterial Beta diversity

Spatial Autocorrelation of Bacterial Community Composition

Results from Multivariate Spatial Analyses (MSAs) suggest that spatial autocorrelation of bacterial community composition is influenced by both spatial and temporal scales of sampling. When testing for spatial autocorrelation of mean community composition per site and year (i.e., site-year means) we found significant autocorrelation when considering communities across all three years of the study (Table 3.9a). However, when years of sampling were considered separately, spatial autocorrelation was only detected in 2010. We ran this same MSA using bacterial community data from individual nests and found no evidence for spatial autocorrelation either across years or within years of sampling (Table 3.9b). Site centroid coordinates were used for both of these MSAs. Additionally, we had a subset of samples for which we had specific nestbox coordinates. The results from MSAs using this subset of data to look at spatial autocorrelation of individual bacterial communities in relation to distances between individual boxes (within and between sites) did not indicate significant autocorrelation across years (Table 3.9c). No spatial autocorrelation was detected within 2009 or 2010 and only marginal spatial autocorrelation was detected in 2008.

We found no evidence of a significant distance-decay effect for mean site-year bacterial communities (Figure 3.5). There was no significant correlation between Unifrac

distance and geographic distance matrices, indicating that sites located in closer proximity did not harbor more similar bacterial communities than did more distantly located sites (Mantel test: r = - 0.005, p = 0.488).

Site and Year Effects on Bacterial Community Composition

Locality and year both contributed significantly to the differences observed between bacterial communities (site as fixed factor, $F_{16,245}$= 2.17, P = 0.001; year as random factor nested within locality, $F_{17,245}$ = 1.56, P = 0.001). Principle coordinate analysis attributed 22.74% of the variation in community composition among individual nests to PC1, PC2, and PC3. Because of the relatively low proportion of variance explained by PCoAs, clustering of samples by collection site and year within sites did not yield clearly defined clusters (Figure 3.6).

Geographic Effects on Bacterial Community Composition

We found significant effects of both latitude ($F_{1,28}$= 2.35, P = 0.012) and elevation ($F_{1,28}$= 1.67, P = 0.028) on beta diversity of nest bacterial communities among site-years. No significant effects of longitude ($F_{1,28}$= 1.43, P = 0.086) or sampling year ($F_{2,28}$= 0.78, P = 0.714) on beta diversity were detected. Principle coordinate analysis was used to reduce dimensionality of bacterial composition among mean site-year communities. PC1 explained 20.57% and PC2 explained 9.30% of the variation in mean community composition among sites and years. The first two principle components were plotted against site latitude (Fig. 3.7) and site elevation (Fig. 3.8) to visualize the relationships between these geographic variables and beta diversity of nest bacteria.

Climatic Effects on Bacterial Community Composition

We used General Least Squares analyses to investigate the effects of climatic variables on beta diversity of nest bacteria, which allowed us to control for geographic effects. There were no significant effects produced by the full climate models considering either winter conditions or pre-breeding conditions. The best climate model for January preceding bacterial sampling retained only monthly mean minimum temperature (MMNT), which had a marginally significant effect on bacterial community composition (Table 3.10). The best climate model for April immediately preceding the Tree Swallow breeding season and retained only monthly mean maximum temperature (MMXT), which had a significant effect on community composition (Table 3.10).

DISCUSSION

This study provides foundational insight into the composition of bacterial communities living in the active nests of Tree Swallows across their species range in North America. We found these communities to be dominated by phylum Proteobacteria and, to a lesser extent, Bacteroidetes, Actinobacteria, and Firmicutes. A previous study using NGS to characterize bacterial communities on the eggshells in active magpie nests (*Pica pica*) found very similar relative abundances of bacterial phyla to these during early incubation (Lee *et al.* 2014). However, a second study of eggshell bacteria in wild red-capped larks (*Calandrella cinerea*) found the relative abundance of phylum Proteobacteria to be much higher (95.8%; Grizard *et al.* 2015) than what is reported for nesting material in the current study (56.8%) and for eggshells by Lee *et al*. (2014). The only other phylum represented at ≥ 1% on eggshell surfaces of lark eggs was

Actinobacteria (3.8%, Grizard *et al.* 2015), which we found to be nearly four times as abundant in nest bacterial communities (16.0%). We found 18 genera with relative abundances greater that 1% across samples, in contrast to only two genera found on eggshells in larks; Lee *et al*. (2014) detected at least 10 genera ≥ 1% on magpie eggs. Bird eggs have numerous physical and chemical defenses against environmental microbes (D'Alba & Shawkey 2015) that likely impose strong selection against bacterial colonization but may vary in strength among species and populations. Of the top ten most abundant genera found on magpie eggshells (Lee *et al.* 2014), only *Sphingomonas* and *Pseudomonas* were also in the top ten most abundant genera that we detected for nesting material communities. *Pseudomonas* was relatively more abundant in eggshell communities and *Sphingomonas* was relatively more abundant in nest communities. *Pseudomonas* species found in the nest may preferentially colonize and proliferate on eggshell surfaces. Future study is needed to characterize differences between bacterial communities from eggs and the nests in which they were collected. Preliminary information has been established by Brandl et al. (2014) who found that bacterial communities collected from nesting material harbored more OTUs (31.7 ± 2.7) than did eggshell communities (8.5 ± 0.6).

The current study, together with those conducted by Lee *et al.* (2014) and Grizard *et al.* (2015) are the first to use NGS to identify bacterial taxa and their relative abundances from communities sampled from the nesting environment of wild birds. Although microbiological surveys of wild birds and their environments have been conducted for some time, the methods employed have not been as comprehensive as NGS. For example, previous non-NGS studies of egg and nest bacteria have indicated

that these communities are dominated by phylum Firmicutes (e.g., Grizard *et al.* 2014; 16S cloning). However, our results and those of Grizard et al. (2015) show that, instead, the predominant phylum detected in nests and on eggs via NGS technology was Proteobacteria, not Firmicutes. Similarly, culture-based studies frequently report the presence of bacterial families *Enterobacteriaceae, Enterococcaceae, and Staphylococcaceae,* which are known to include egg pathogens (e.g., Potter *et al.* 2013). Yet, neither Grizard et al. (2015) nor Shawkey et al. (2009), using microarrays to identify 16S sequences, detected OTUs belonging to *Enterococcaceae* or *Staphylococcaceae*; our study detected only two genera belonging to *Enterococcaceae* and only three genera belonging to *Staphylococcaceae*. Non-NGS studies have also frequently reported high abundances of genus *Pseudomonas*, whereas we found that *Pseudomonas* spp. had an average relative abundance of 5.8% and were not detected in all of the nest bacterial samples we collected. Instead we observed the genus *Sphingomonas* (phylum Proteobacteria) to dominate nest communities (16.3%) followed by *Hymenobacter spp.* (8.0%, phylum Bacteroidetes) and species belonging to family *Oxalobacteraceae* (5.3%, Proteobacteria), all of which were present in every bacterial community sampled from Tree Swallow nests. Given the known biases associated with culture-based techniques and lack of resolution associated with sequence-length approaches, we suggest that NGS-based studies are needed to gain a deeper understanding of bacterial community assemblages.

In addition to characterizing communities of nest bacteria, we also aimed to leverage our large geographic scope of sampling to uncover patterns of bacterial alpha and beta diversity across the Tree Swallow breeding range. We did not find evidence for

a significant distance-decay relationship when comparing geographic distance and UniFrac distance matrices between mean site-year bacterial communities. These results indicate that Tree Swallow nests from sites located in closer proximity to each other do not harbor bacterial communities with more similar composition than do nests from sites located farther apart. Bacterial community composition may be either equally differentiated from one another or equally overlapping. Because we observed relatively weak clustering of bacterial communities, we conclude that there is a high degree of similarity among Tree Swallow nest communities across North America. These results suggest that breeding birds create a relatively consistent microhabitat in the nesting environment that selects for a particular suite of bacterial taxa from the broad diversity that is introduced through nest-building activity (e.g., nesting material, plumage bacteria, etc.).

In this study we evaluated the relative contributions of both geographic and climatic effects on nest bacterial community composition. We did, in fact, detect evidence for both and also identified some interesting differences between patterns of alpha and beta diversity in relation to these variables. For example, we detected significant effects of locality on bacterial community composition (i.e., beta diversity) but not on measures of alpha diversity. This suggests that local bacterial community assemblages are somewhat site-specific whereas comparable levels of richness and diversity may be found at multiple locations across a large sampling range. We need to emphasize, however, that individual bacterial communities did not cluster strongly by site in this study (Fig. 5), indicating that there is a high degree of overlap between communities. Our results from multivariate spatial analyses also suggest that there is a

high degree of variation in community composition from nest to nest within sampling sites; although we detected some degree of spatial autocorrelation when comparing average community composition among site-years, this spatial effect disappeared when we incorporated within-site variation in community composition (i.e., nest-level analyses). Because of high within-site variation, we evaluated geographic and climatic effects using site-year means for alpha and beta diversity, to focus on patterns at scales larger than nest-to-nest within sites.

One of the objectives of this study was to test for latitudinal and elevational diversity gradients, which have been observed consistently for plant and animal taxa. At this large geographic scale of sampling we detected both latitudinal and elevational effects on bacterial community composition. We found that bacterial community evenness was significantly correlated with latitude and with longitude such that evenness decreased with increasing latitude and from west to east. We also detected a similar latitudinal trend for OTU diversity. Phylogenetic diversity and OTU richness, on the other hand, were not correlated with latitude. Although these results do not provide strong evidence for a latitudinal diversity gradient, they do suggest that taxonomic diversity is sensitive to geographic variation and in the direction predicted by the latitudinal diversity gradient established for many plant and animal taxa. Our results also indicate that bacterial community composition varies to some extent with elevation. No elevation effects were detected for alpha diversity when only geographic variables were considered. However, when controlling for climatic effects we found that both OTU diversity and evenness were negatively correlated with elevation. Variation in microbial communities in relation to elevation has only recently been considered in the literature,

and there does not appear to be one consistent pattern across environments or taxa (Bryant et al. 2008, Fierer et al. 2011, Wang et al. 2011, Cordier et al. 2012). Our results suggest that elevation effects may be obscured or subsumed by the effects of other environmental variables such as temperature and precipitation, and we suggest that such covariates should be accounted for in future studies of elevational effects on bacterial communities, especially at a large geographic scale.

We found that climatic effects immediately prior to the breeding season appear to have relatively stronger effects on bacterial community composition in Tree Swallow nests than do climatic effects during the month of breeding or during the preceding winter. All four measures of alpha diversity were significantly affected by precipitation during the month of April, whereas only Shannon's diversity and evenness appeared to be sensitive to temperature effects during this same time period. Alpha diversity of nest bacterial communities increased with average rainfall during the month of April, which corroborates results from previous studies that have also demonstrated a positive relationship between precipitation and bacterial growth on bird eggs (Berger, Disko & Gwinner 2003). We also found alpha diversity to correlate negatively with the number of days in the month of April with ≥ 25.4mm of precipitation. These results suggest that although precipitation may encourage bacterial colonization and growth, heavy rainfall events may have the opposite effect, perhaps because of the reduced nest activity of birds at these times. Tree Swallows are aerial insectivores and, thus, capture insects while flying. During heavy rainfall events, numbers of flying insects are reduced, forcing swallows to stop foraging or forage in other areas. Swallows may leave the breeding area for several days during periods of inclement weather prior to egg-laying (Forsman,

pers. obs.) and may delay clutch initiation until conditions improve. The resulting reduction in nest activity associated with heavy rainfall events may, thus, reduce potential alpha diversity of bacterial communities by limiting bacterial colonization from adult swallows to nesting material or by delaying the establishment of an active nest microhabitat that may be favorable for bacterial establishment and proliferation. Additional support for this hypothesis that female activity at the nest directly influences nest bacterial communities is suggested by the counter-intuitive negative relationships we observed between minimum and maximum temperatures on alpha diversities. We initially predicted that ambient temperature would have positive effects on nest bacteria based on previous studies of microbial communities that have shown positive correlations between temperature and indices of alpha diversity (e.g., Fuhrman *et al.* 2008, Andam *et al.* 2016). Although we detected a similar positive relationship between mean monthly temperature and bacterial diversity, we also found negative correlations between diversity and both mean monthly minimum and maximum temperatures. These results are less puzzling when female nesting behavior is taken into consideration. Breeding birds spend a significant amount of time at the nest during nest-building and incubation, creating temperature and humidity conditions suitable to the physiological development of embryos. Birds breeding in localities experiencing a larger differential between ambient and developmentally optimal temperatures are required to exert more effort in closing that temperature gap than will females breeding in localities with small temperature differentials. Thus, localities with lower mean minimum and maximum temperatures may necessitate more female activity at the nest, which in turn may provide more opportunities for horizontal transfer of plumage bacteria from adult birds to

nesting material, thus increasing community diversity. Previous studies have shown that bacterial contamination of bird eggs occurs through both horizontal transfer from nesting materials and incubating adults and through vertical transmission from passage through the maternal cloaca (Protais *et al.* 2003, Brandl *et al.* 2014, Soler *et al.* 2015, Martínez-García *et al.* 2015). The hypothesized relationship between nest-activity level and bacterial community assemblage could not be tested directly with the data collected from this study, nor has it been reported elsewhere. We suggest that female modification of the nesting environment may result in interaction effects with climatic variables that may be more effectively interpreted in the context of maternal nesting behavior.

When we compared the relative effects of geographic and climatic variables on alpha diversity, we found that climate exerted stronger and more consistent effects than did geography. During the pre-breeding period, when climate appears to be the most predictive of nest bacterial alpha diversity, we found significant effects of elevation but not of latitude or longitude; when climate conditions during the month of April were controlled for, we observed negative relationships between elevation and both Shannon's diversity and evenness of bacterial communities among sampling sites. Previous studies of microbial communities have also reported negative relationships between diversity and elevation (Bryant *et al.* 2008), as well as both positive (Wang *et al.* 2011b) and non-significant relationships (Fierer *et al.* 2011), and so no consistent elevational pattern seems to be present across microbial communities. Winter models were not very effective in predicting variation in alpha diversity of nest bacteria, which suggests that these particular community assemblages may be less influenced by local

overwintering bacteria and may instead be composed of a large proportion of bacteria originating from adult birds returning from wintering grounds where they and their associated microbiota experienced different climate conditions than those present at the breeding grounds in the middle of winter. Breeding-season models considering both geographic and climatic variables indicate a significant negative effect of latitude and a positive effect of the number of days below 0°C (DT32) during the month breeding on all measures of alpha diversity except for evenness. In all cases, temperature exerted a stronger effect on alpha diversity than did latitude. Taken as a whole, our results indicate that geographic variables are relatively less influential than climatic variables in affecting bacterial communities. Thus, our results are more consistent with the ecological view of biogeography than the historical view.

Studies investigating ecological and historical biogeography of microorganisms frequently obtain conflicting results, making it difficult to identify consistent macroecological patterns like those that have been established for multicellular organisms. Several authors have pointed out that these divergent results may be due to differences among studies in taxonomic, spatial, and temporal scales of inquiry (e.g., Ramette & Tiedje 2007, Bissett *et al.* 2010, Martiny *et al.* 2011, Gilbert *et al.* 2012, Hatosy *et al.* 2013). We also suggest that different results may arise from the use of different measures of alpha diversity. Although we found negative relationships between latitude and bacterial community evenness, and to a lesser extent, diversity, we did not find similar latitudinal effects on OTU richness or phylogenetic diversity. These different measures of alpha diversity do in fact characterize different aspects of communities. Richness summarizes taxonomic diversity, whereas phylogenetic diversity takes into

account shared evolutionary histories of community constituents and can, thus, provide insight into deep phylogenetic diversification. OTU diversity (e.g., Shannon's diversity index) and community evenness, on the other hand, may be thought of as measures of ecological diversity. Our analyses revealed locality to be a significant variable explaining Shannon's diversity and evenness, but not OTU richness or phylogenetic diversity. These results suggest that although overall richness of bacterial OTU's or taxa does not vary among sites, the relative abundances of community constituents among taxa do vary significantly, thus, influencing ecological diversity. There is some evidence that community function increases with community evenness (Haig *et al.* 2015), which in turn influences diversity, suggesting that localities may vary significantly in stability and efficiency across a large geographic area. Therefore we suggest that future studies consider analyzing multiple measures of alpha diversity to better understand how different aspects of community structure and function relate to ecological and historical variation.

In conclusion, we have shown that active bird nests harbor diverse bacterial communities that are sensitive to variation in both geographic and climatic variables, but that climate exerts a relatively stronger influence than does geography. Specifically, average monthly temperature and rainfall were positively correlated with alpha diversity, and temperature immediately prior to the breeding season significantly affected bacterial community composition. We also suggest that climatic effects on nest bacterial diversity may be mediated by maternal behaviors in response to climate conditions not conducive for embryonic development. Although we detected some evidence for a latitudinal diversity gradient, it is likely that some of this variation may be attributable to

the correlation between climatic variables and latitude. In fact, we uncovered a negative relationship between alpha diversity and elevation when controlling for climate effects that was not detectable in models considering only geographic variables. These results illustrate the importance of collecting metadata and constructing comprehensive models to understand large-scale patterns of microbial distributions. Our results also corroborate previous calls for taking into account scale of sampling when interpreting results from 16s rRNA gene surveys. This work contributes important baseline data describing the relatively uncharacterized bacterial communities associated with breeding birds and paves the way for research into the relationships between these communities and the physiologies of the adult and hatchling birds with which they come into contact.

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Table 3.1. Summary of nests sampled per Site and Year to characterize bacterial communities of Tree Swallow nests across their North American breeding range.

Site/Year ID	Site	State	Country	Year	N (nests)
AKF_2008	AKF	Alaska	USA	2008	3
AKF_2009	AKF	Alaska	USA	2009	6
AKL_2008	AKL	Alaska	USA	2008	10
AKL_2009	AKL	Alaska	USA	2009	13
BCA_2009	BCA	British Columbia	Canada	2009	9
BCA_2010	BCA	British Columbia	Canada	2010	6
BCT_2009	BCT	British Columbia	Canada	2009	9
CAM_2009	CAM	California	USA	2009	11
CAM_2010	CAM	California	USA	2010	9
CAO_2009	CAO	California	USA	2009	9
CAO_2010	CAO	California	USA	2010	3
CO_2008	CO	Colorado	USA	2008	9
CO_2009	CO	Colorado	USA	2009	7
CO_2010	CO	Colorado	USA	2010	9
IA_2008	IA	Iowa	USA	2008	5
IA_2009	IA	Iowa	USA	2009	5
IA_2010	IA	Iowa	USA	2010	5
MI_2008	MI	Michigan	USA	2008	9
MI_2009	MI	Michigan	USA	2009	9
MI_2010	MI	Michigan	USA	2010	10
NS_2009	NS	Nova Scotia	Canada	2009	8
NY6_2008	NY6	New York	USA	2008	9
NY2_2009	NY2	New York	USA	2009	10
NY2_2010	NY2	New York	USA	2010	14
SK_2010	SK	Saskatchewan	Canada	2010	10
TN_2009	TN	Tennessee	USA	2009	9
TN_2010	TN	Tennessee	USA	2010	10
VA_2009	VA	Virginia	USA	2009	7
VA_2010	VA	Virginia	USA	2010	10
WI_2008	WI	Wisconsin	USA	2008	4
WI_2010	WI	Wisconsin	USA	2010	9
WY_2008	WY	Wyoming	USA	2008	9
WY_2009	WY	Wyoming	USA	2009	6
WY_2010	WY	Wyoming	USA	2010	8

Figure 3.1. Map of North America showing the Tree Swallow breeding range in blue. Winter range is indicated in orange and sample collection sites are denoted by stars. Map modified from Winkler et al. 2011.

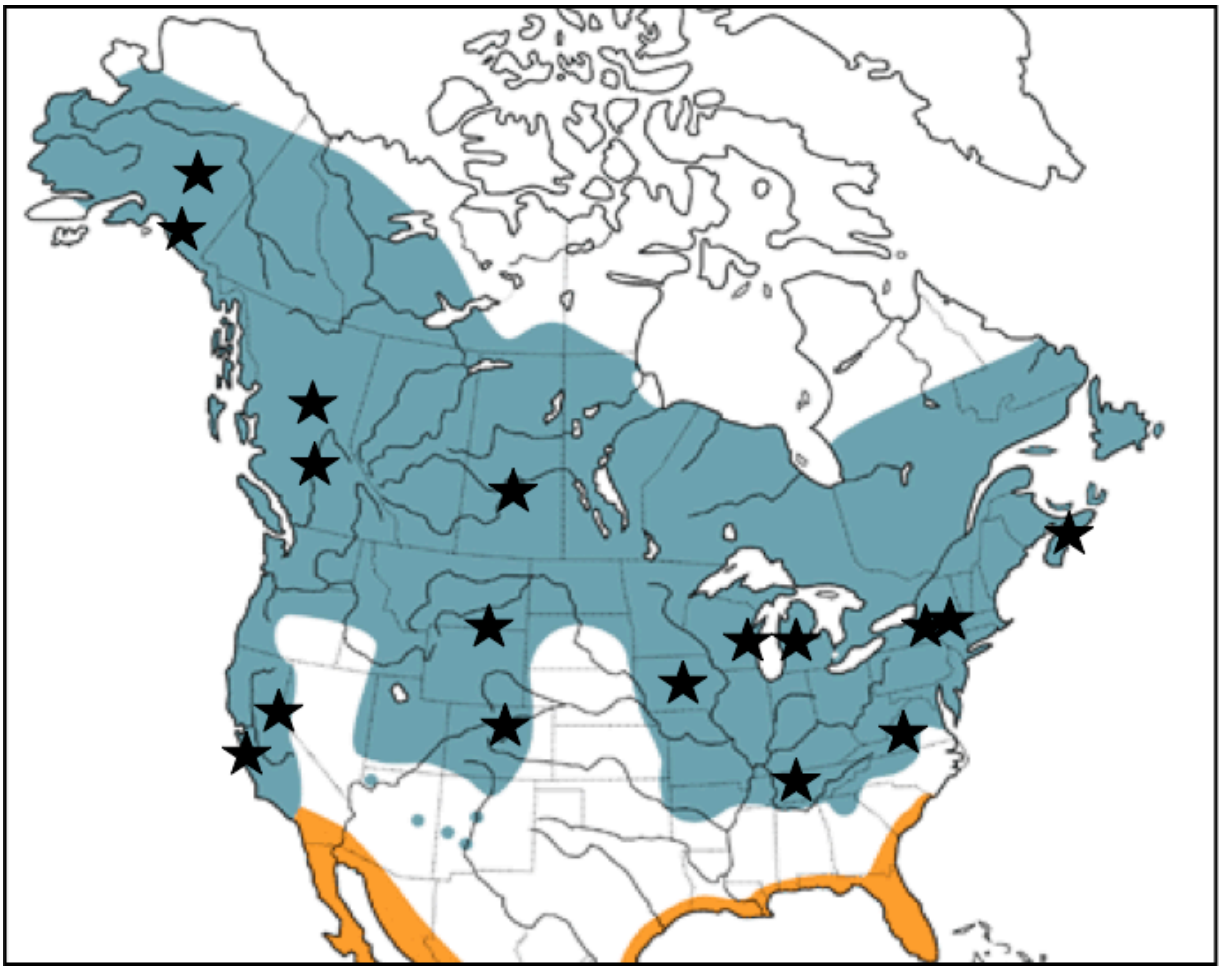

Figure 3.2. Principal coordinate analysis based on the unweighted Unifrac distance matrix of nest bacterial communities. Procrustes analysis of bacterial community composition produced short vectors joining samples that were amplified twice. Bacterial community composition was more similar between amplification batches for samples that were amplified twice than between different samples within the same amplification batch.

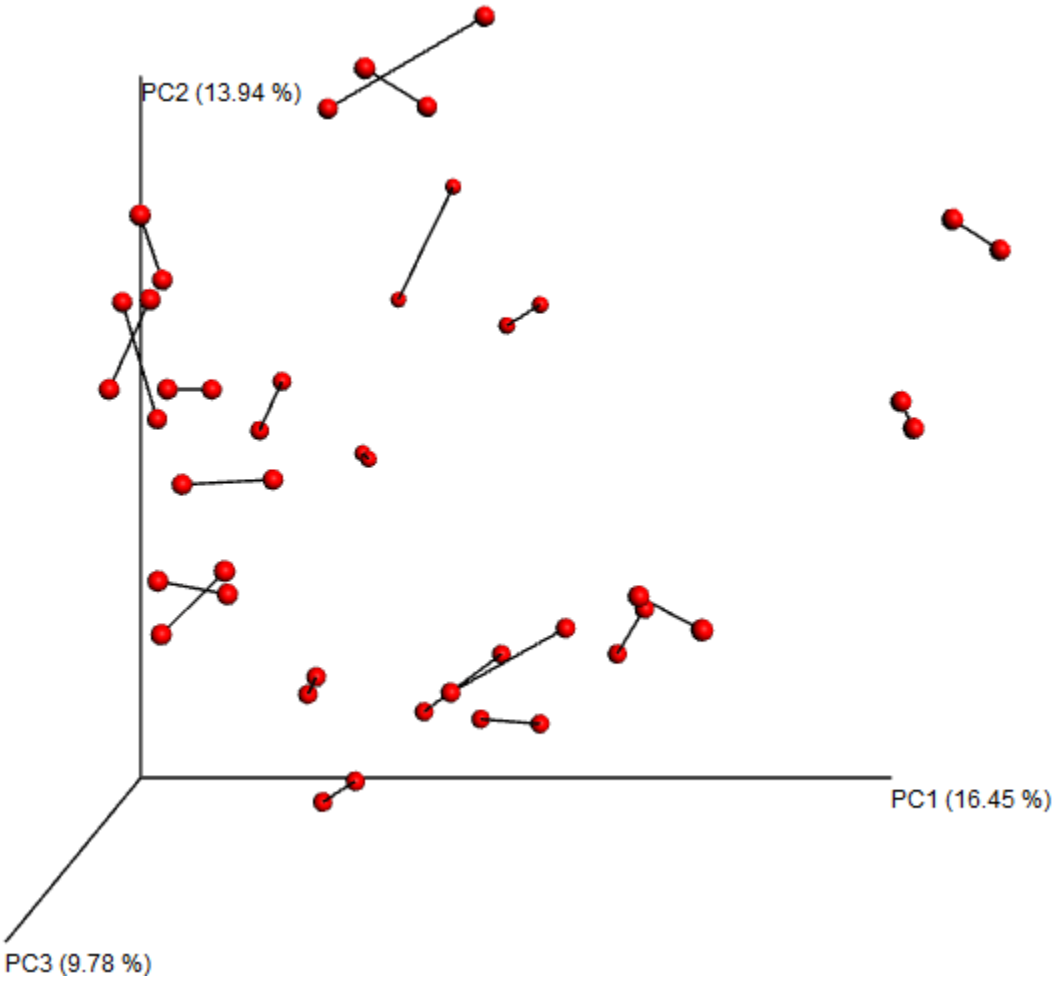

Figure 3.3a. Relative sequence abundances for each bacterial phylum from Tree Swallow nests sampled across sites and years (Table 3.1). **AKF**: Fairbanks, Alaska; **AKL**: Long Lake, Alaska; **BCA**: Alkali Lake, British Columbia; **BCT**: Telachick, British Columbia; **CAM**: Merced, California; **CAO**: Irvine, California; **CO**: Boulder, Colorado; **IA**: Ames, Iowa; **MI**: Stony Lake, Michigan; **NS**: Wolfville, Nova Scotia; **NY6**: Harford, New York; **NY2**: Ithaca, New York; **SK**: Saskatoon, Saskatchewan; **TN**: Lenoir City, Tennessee; **VA**: Swoope, Virginia; **WI**: Shorewood, Wisconsin; **WY**: Big Horn, Wyoming.

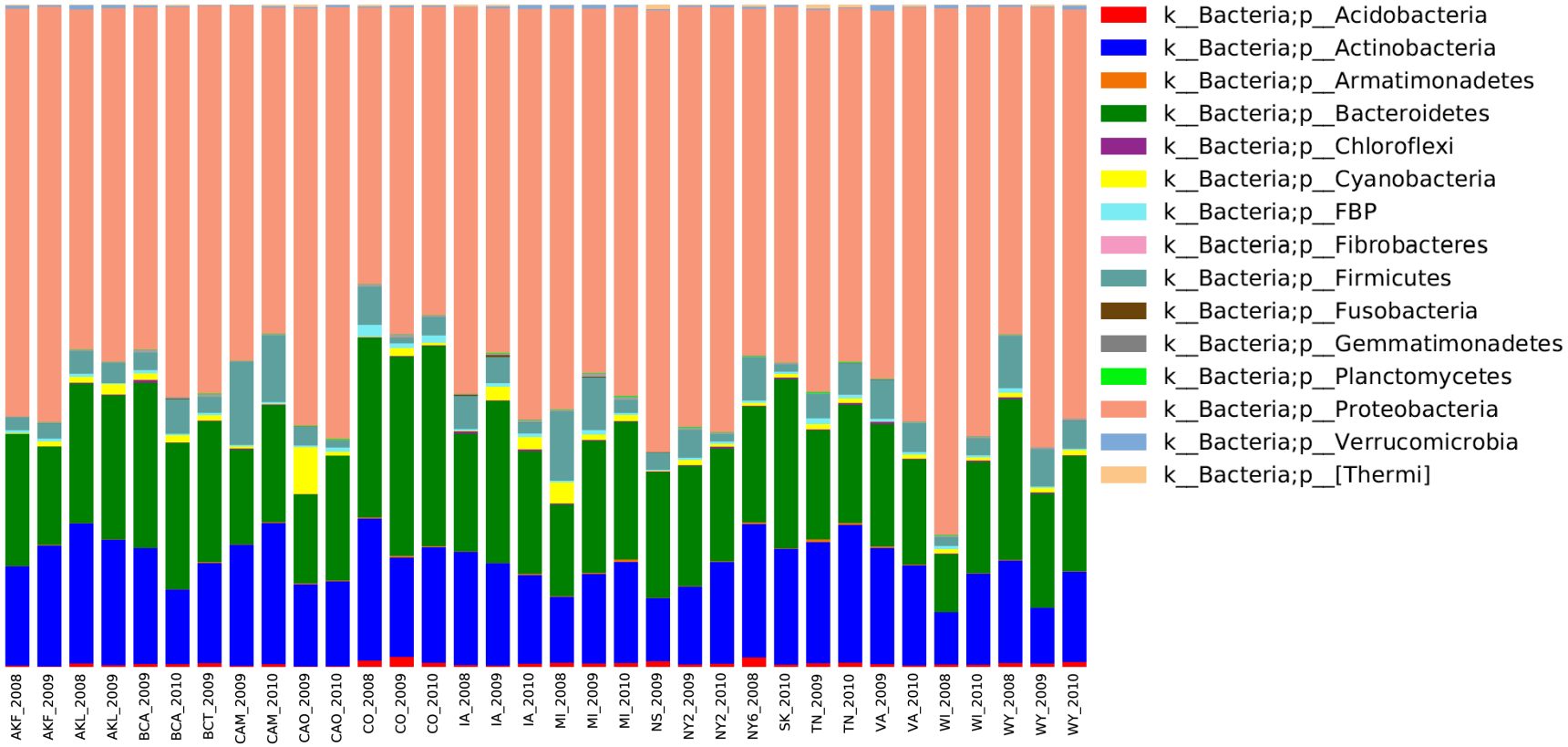

Figure 3.3b. Relative sequence abundances for bacterial genera from Tree Swallow nest sampled across sites and years. The most abundant genera and their relative abundances are listed in Table 3.2. *Sphingomonas* (16.34%) is shown in red, *Hymenobacter* (8.06%) in brown, *Pseudomonas* (5.78%) in lavender, and *Oxalobacteraceae* (5.32%) in green.

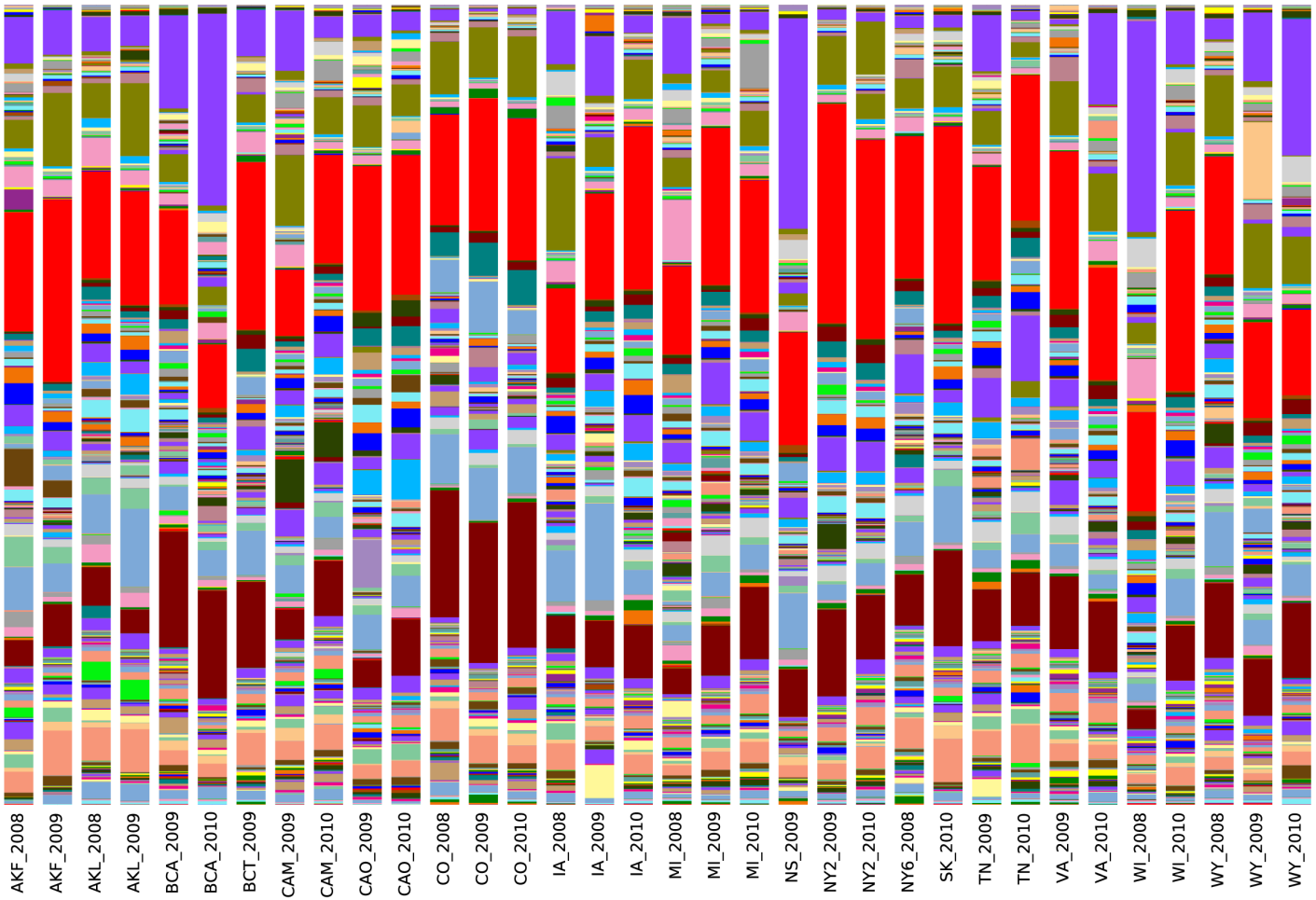

Table 3.2. Mean relative abundances of bacterial phyla detected across the 279 bacterial samples collected for this study. Relative abundances were averaged across all samples collected and were calculated as the number of sequences assigned to a particular genus divided by the total number of sequences retained after data filtering.

Phylum	Relative Abundance
Proteobacteria	56.85%
Bacteroidetes	19.62%
Actinobacteria	15.97%
Firmicutes	4.49%
Cyanobacteria	0.98%
Acidobacteria	0.54%
FBP	0.45%
Verrucomicrobia	0.31%
Gemmatimonadetes	0.23%
Thermi	0.17%
Chloroflexi	0.16%
Armatimonadetes	0.14%
Planctomycetes	0.07%
Fusobacteria	0.02%
Fibrobacteres	0.01%

Table 3.3. Mean relative abundances of bacterial genera found at a prevalence of 1% or higher across the 279 bacterial samples collected for this study. Relative abundances were averaged across all samples collected and were calculated as the number of sequences assigned to a particular genus divided by the total number of sequences detected. Genera denoted in bold text were detected in all samples. Several OTU's did not match previously annotated genera in the Greengenes database.

Phylum	Family	Genus	Relative Abundance
Proteobacteria	***Sphingomonadaceae***	***Sphingomonas***	**16.34%**
Bacteroidetes	***Cytophagaceae***	***Hymenobacter***	**8.06%**
Proteobacteria	*Pseudomonadaceae*	*Pseudomonas*	5.78%
Proteobacteria	***Oxalobacteraceae***	**-**	**5.32%**
Bacteroidetes	*Sphingobacteriaceae*	-	4.41%
Actinobacteria	*Microbacteriaceae*	-	2.84%
Proteobacteria	*Methylobacteriaceae*	*Methylobacterium*	2.70%
Proteobacteria	*Comamonadaceae*	-	1.74%
Proteobacteria	*Sphingomonadaceae*	-	1.56%
Bacteroidetes	*Chitinophagaceae*	-	1.43%
Proteobacteria	*Hyphomicrobiaceae*	*Devosia*	1.30%
Actinobacteria	*Nocardioidaceae*	-	1.31%
Proteobacteria	*Rhizobiaceae*	-	1.15%
Proteobacteria	*Acetobacteraceae*	-	1.24%
Bacteroidetes	*Sphingobacteriaceae*	*Pedobacter*	1.13%
Proteobacteria	*Caulobacteraceae*	*Mycoplana*	1.12%
Bacteroidetes	*Cytophagaceae*	*Dyadobacter*	1.05%
Firmicutes	*Bacillaceae*	*Bacillus*	1.03%

Table 3.4. Moran's I test results showing the correlation between the geographical distance matrix and different alpha diversity indexes. The geographical distance matrix was calculated using the Vincenty's formula.

	Moran's I	
	I	**P**
OTU Richness	0.01	0.659
Faith's Phylogenetic Diversity	0.03	0.589
Shannon's Diversity Index	-0.08	0.594
Community Evenness	-0.16	0.203

Table 3.5. Summary of results from General Linear Models testing for effects of sampling year and locality on site-year mean alpha diversity.

	A	**Bacterial Richness**		B	**Phylogenetic Diversity**		C	**Bacterial Diversity**		D	**Evenness**	
Source	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Year	2	0.67	0.5276	2	0.38	0.6923	2	0.13	0.8800	2	1.17	0.3385
Site	16	1.47	0.2326	16	1.54	0.2044	16	2.11	**0.0781**	16	2.35	**0.0530**

Table 3.6. Summary of results from General Linear Mixed Models testing for effects of geographic variables on site-year means for a) bacterial OTU richness, b) Faith's phylogenetic diversity index, c) Shannon's diversity index, and d) evenness. Site was included as a random factor.

	A **Bacterial Richness**			B **Phylogenetic Diversity**			C **Bacterial Diversity**			D **Evenness**		
Source	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Year	2,18.94	0.37	0.6941	2,18.76	0.17	0.8477	2,17.71	0.71	0.5036	2,16.97	2.13	0.1493
Site Latitude	1,13.2	0.31	0.5845	1,13.09	0.15	0.7012	1,11.96	3.86	**0.0731**	1,10.94	7.79	**0.0176***
Site Longitude	1,13.32	0.24	0.6319	1,13.24	0.16	0.6913	1,12.13	3.04	0.1063	1,10.98	5.68	**0.0363***
Site Elevation	1,7.652	0.00	0.9672	1,7.841	0.02	0.8834	1,7.007	0.51	0.4964	1,5.533	1.61	0.2558

Figure 3.4. Correlations between bacterial community evenness and a) latitude, and b) longitude.

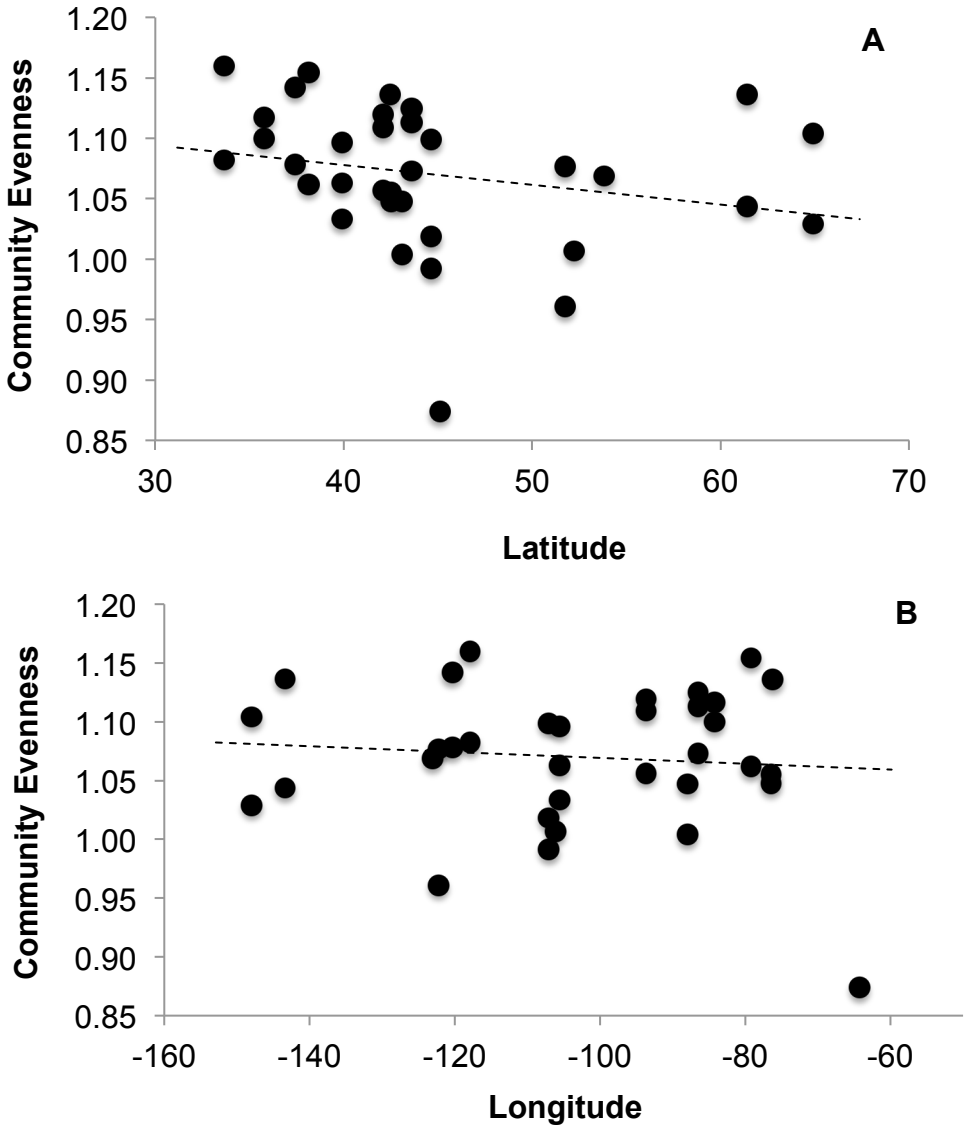

Table 3.7. Climatic General Linear Models explained variation in alpha diversity measures of bacterial community of Tree Swallow nests: a) OTU richness, b) Faith's phylogenetic diversity, c) Shannon's diversity index, and d) evenness. Number of days in month with greater than or equal to 1.0 inch of precipitation (DP10), Number days in month with minimum temperature less than or equal to 32.0 F (DT32), Total precipitation amount for the month (TPCP), Monthly mean minimum temperature (MMNT), Monthly mean maximum temperature (MMXT) and Monthly mean temperature (MNTM) were considered as continuous climatic variables as well as locality as fixed factor and year as random factor. Climatic data were considered at month of hatching (breeding models), in January (winter models) and in April (pre-breeding models). SD means standard deviation and significant values are in bold.

Table 3.7.

	Best Model	Variables	Beta	SD	d.f.	F	P
OTU Richness	Winter	-	-	-	-	-	-
	Pre-breeding	APRIL_DP10	-0.620	0.228	1	7.37	0.0107
		APRIL_TPCP	0.519	0.228	1	5.16	0.0302
	Breeding	-	-	-	-	-	-
Phylogenetic	Winter	-	-	-	-	-	-
Diversity	Pre-breeding	APRIL_DP10	-0.597	0.230	1	6.71	0.0145
		APRIL_TPCP	0.506	0.230	1	4.82	0.0357
	Breeding	DT32	0.384	0.163	1	5.52	0.0252
Shannon's	Winter	Locality	-	-	16	2.44	0.0389
Diversity	Pre-breeding	APRIL DT32	3.377	0.958	1	12.42	0.0048
		APRIL DP10	-1.265	0.245	1	26.70	0.0003
		APRIL TPCP	1.397	0.332	1	17.69	0.0015
		APRIL MMXT	-15.533	7.042	1	4.87	0.0496
		APRIL MMNT	-16.404	6.281	1	6.82	0.0242
		APRIL MNTM	34.044	13.347	1	6.51	0.0270
		Locality	-	-	16	4.56	0.0073
	Breeding	Locality	-	-	16	2.44	0.0389
Evenness	Winter	Locality	-	-	16	2.37	0.0437
	Pre-breeding	APRIL_DT32	1.540	0.604	1	6.50	0.0231
		APRIL_DP10	-0.758	0.274	1	7.67	0.0151
		APRIL_TPCP	0.774	0.332	1	5.43	0.0352
		Locality	-	-	16	2.78	0.0306
	Breeding	Locality	-	-	16	2.37	0.0437

Table 3.8. General Linear Models explained variation in bacterial species richness, Faith's Phylogenetic Diversity index, Shannon diversity index and Evenness of bacterial community of Tree Swallow nests per locality. Number of days in month with greater than or equal to 1.0 inch of precipitation (DP10), Number days in month with minimum temperature less than or equal to 32.0 F (DT32), Total precipitation amount for the month (TPCP), Monthly mean minimum temperature (MMNT), Monthly mean maximum temperature (MMXT) and Monthly mean temperature (MNTM) were considered as continuous climatic variables as well as locality as fixed factor and year as random factor. Climatic data were considered at month of hatching (breeding models), in January (winter models) and in April (pre-breeding models). SD means standard deviation and significant values are in bold.

Table 3.8.

	Best Model	Variables	Beta (SD)	SD	d.f.	F	P
OTU Richness	Winter	-	-	-	-	-	-
	Pre-breeding	APRIL_DP10	-0.620	0.228	1	7.37	0.0107
		APRIL_TPCP	0.519	0.228	1	5.16	0.0302
	Breeding	Latitude	-0.448	0.189	1	5.60	0.0244
		DT32	0.587	0.189	1	9.64	0.0040
Phylogenetic Diversity	Winter	-	-	-	-	-	-
	Pre-breeding	APRIL_DP10	-0.597	0.230	1	6.71	0.0145
		APRIL_TPCP	0.506	0.230	1	4.82	0.0357
	Breeding	Latitude	-0.421	0.187	1	5.08	0.0314
		DT32	0.622	0.187	1	11.11	0.0022
Shannon's Diversity	Winter	Latitude	-1.389	0.468	1	8.80	0.0059
		Longitude	-0.686	0.256	1	7.17	0.0119
		JANUARY_MMXT	-0.869	0.377	1	5.31	0.0283
	Pre-breeding	Elevation	-0.421	0.182	1	5.35	0.0283
		APRIL_DT32	1.796	0.627	1	8.22	0.0078
		APRIL_DP10	-1.117	0.213	1	27.48	0.0000
		APRIL_TPCP	1.099	0.254	1	18.74	0.0002
		APRIL_MNTM	1.771	0.586	1	9.15	0.0053
	Breeding	Latitude	-0.441	0.200	1	4.88	0.0347
		DT32	0.422	0.200	1	4.47	0.0426
Evenness	Winter	-	-	-	-	-	-
	Pre-breeding	Elevation	-0.425	0.185	1	5.27	0.0293
		APRIL_DT32	1.773	0.637	1	7.76	0.0095
		APRIL_DP10	-1.057	0.216	1	23.85	0.0000
		APRIL_TPCP	0.998	0.258	1	14.98	0.0006
		APRIL_MNTM	1.801	0.595	1	9.16	0.0053
	Breeding	-	-	-	-	-	-

Table 3.9. Summary of results from multivariate spatial analyses (MSA) showing correlations between the geographical distance matrices and the bacterial community distance matrices. Geographical distance matrices were calculated using the Vincenty's formula and bacterial community distance matrices were calculated using the UNIFRAC index. Significant p-values are denoted with an asterisk. MSA was performed using mean bacterial communities per site and year (a) and using individual nest communities sampled per nest (b and c). Centroid coordinates were used to calculate distance matrices in both site/year (a) and nest-based analyses (b), and nestbox coordinates were used to calculate a distance matrix for the subset of samples for which we had specific collection coordinates.

		MSA	
		statistic	**P**
a) Site/Year Means	**All years**	**0.07**	**<0.001**
	2008	-0.19	0.745
	2009	-0.06	0.298
	2010	-0.02	0.019*
b) Nests (centroid coordinates)	**All years**	**-0.0067**	**0.839**
	2008	-0.0159	0.415
	2009	-0.0052	0.206
	2010	-0.0187	0.410
c) Nests (nest coordinates)	**All years**	**-0.0018**	**0.163**
	2008	-0.009	0.045*
	2009	-0.0195	0.757
	2010	-0.0195	0.956

Figure 3.5. UniFrac distance between mean site-year bacterial communities was not significantly correlated with geographic distance between sites (Mantel test: r = - 0.005, p = 0.488), indicating that there was no evidence for a distance-decay relationship at this large spatial scale.

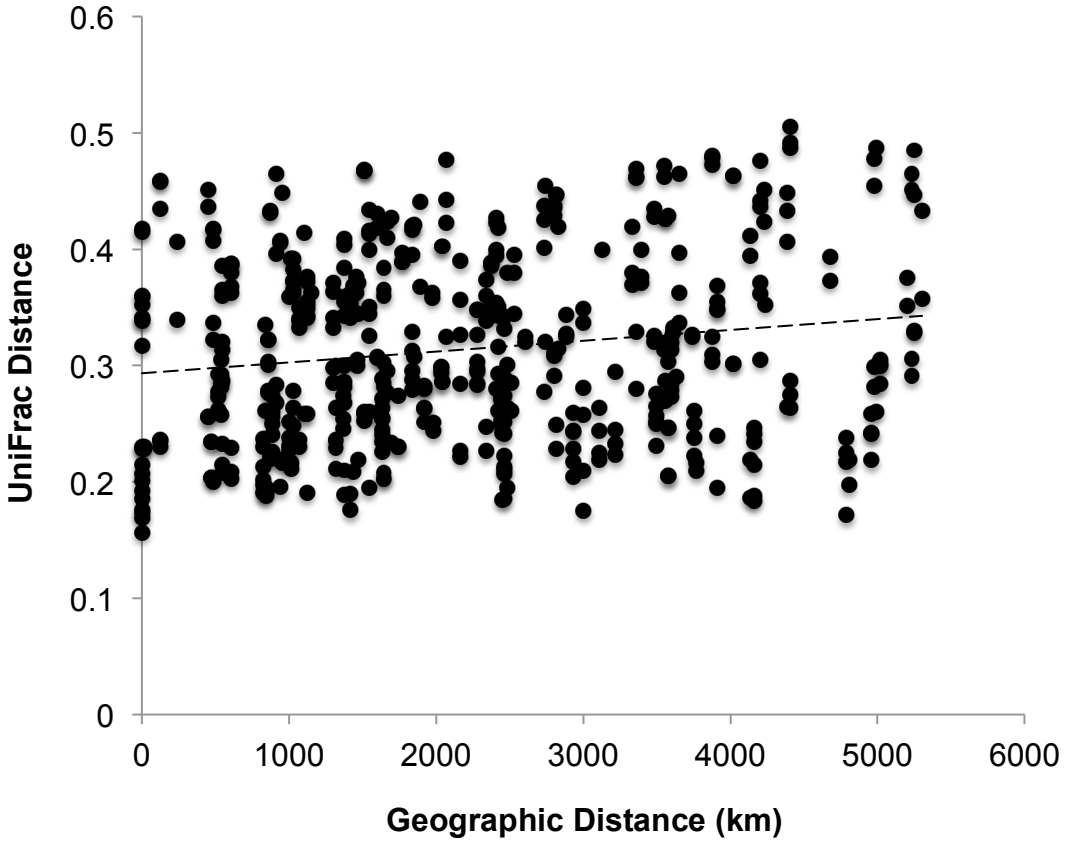

Figure 3.6. Principal Coordinate Analyses based in unweighted Unifrac distance matrix. Each sphere represents a bacterial community for a single Tree Swallow nest. Samples are colored by locality (A) or year (B). Number of samples of each group is in parentheses.

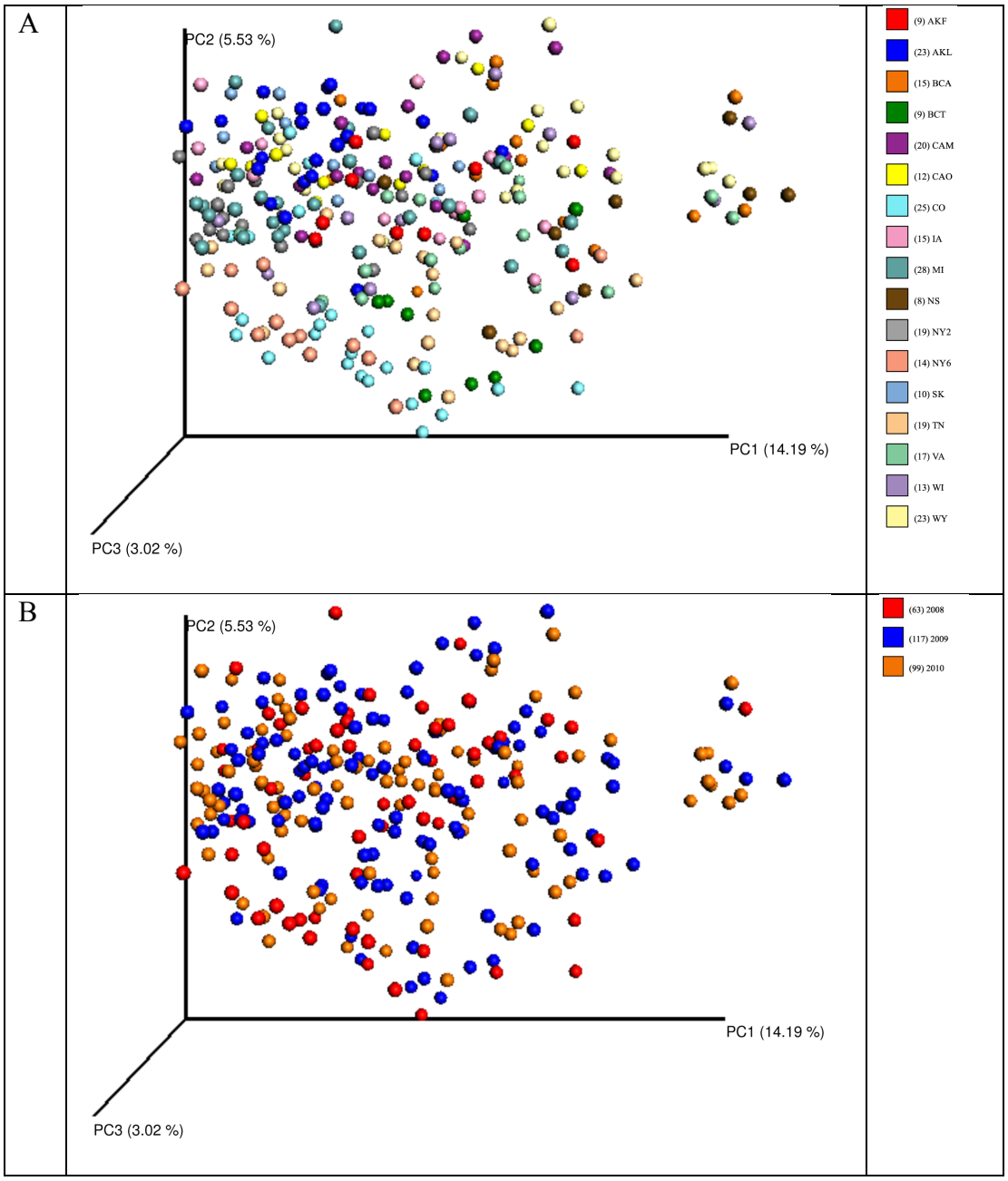

Figure 3.7. Relationships between principle coordinates for bacterial community composition and latitude of sample collection. Principal Coordinate Analysis reduced dimensionality of composition data and was based on the unweighted Unifrac distances between site-year mean bacterial communities. Each sphere represents one mean bacterial community (N=31 site-year means).

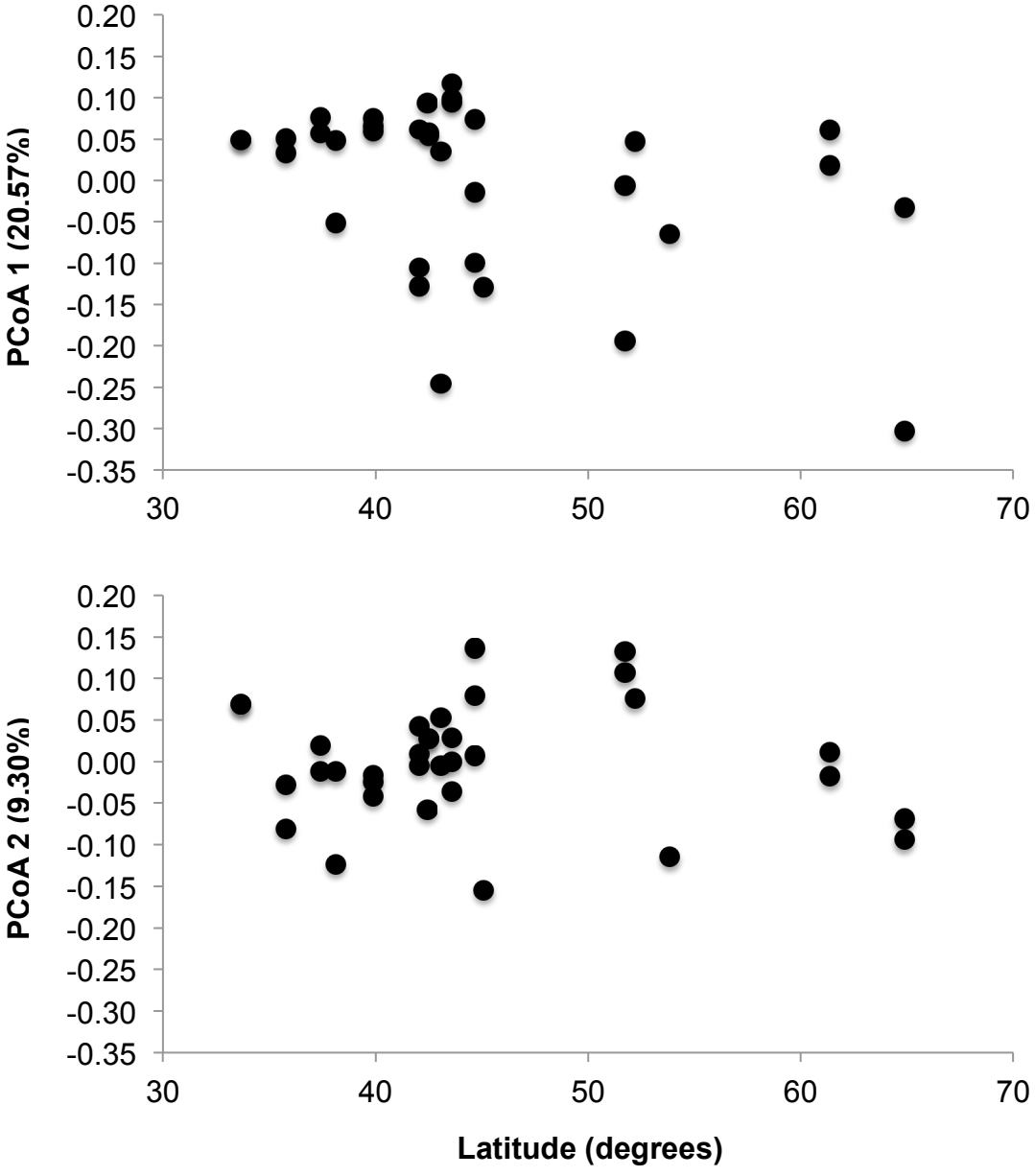

Figure 3.8. Relationships between principle coordinates for bacterial community composition and elevation of sample collection. Principal Coordinate Analysis was based on the unweighted Unifrac distances between site-year mean bacterial communities. Each sphere represents one mean bacterial community (N=31 site-year means).

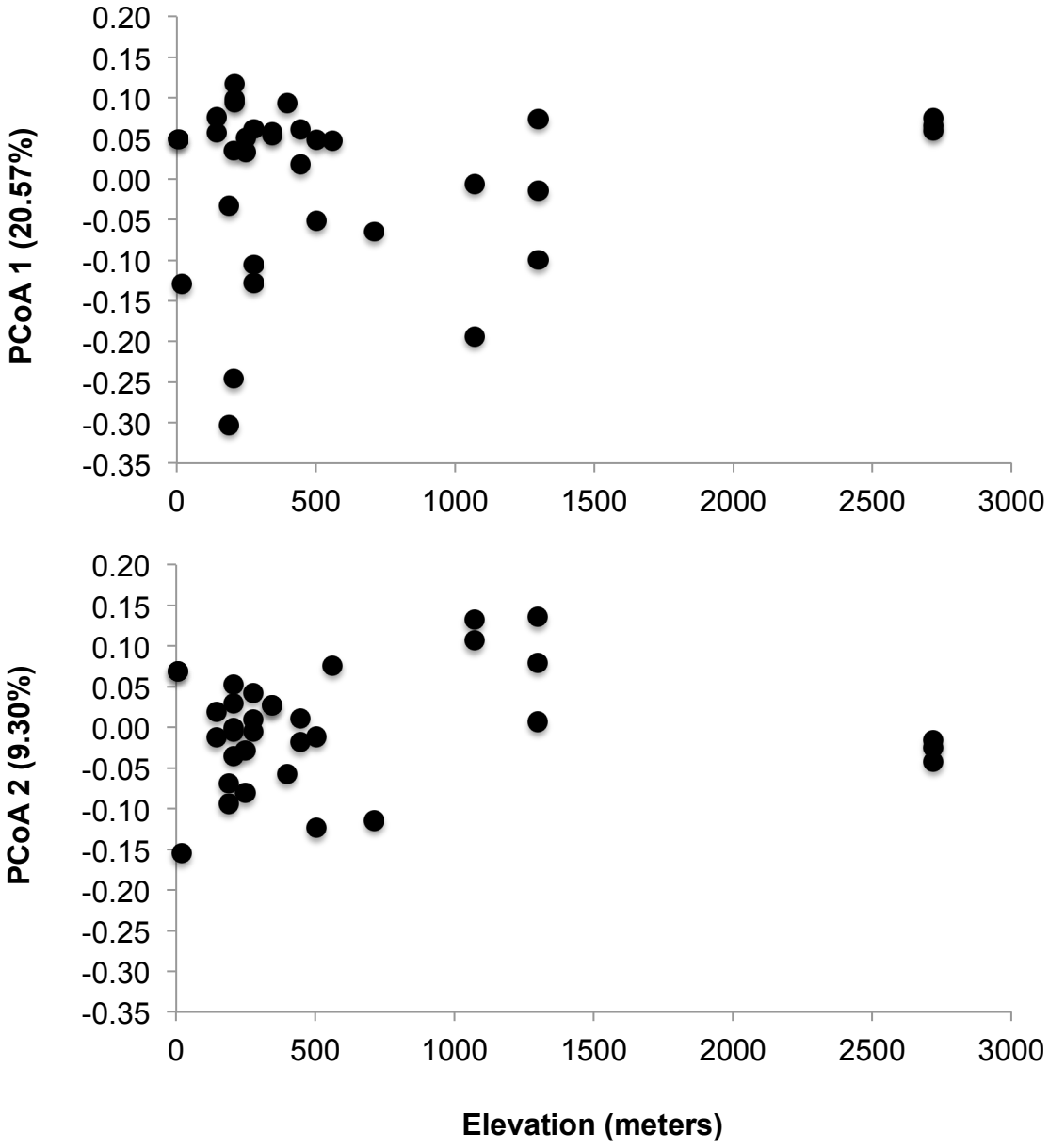

Table 3.10. Summary table from Generalized Least Squares analyses showing the relationships between bacterial communities of Tree Swallow nests and climatic variables (Number of days in month with greater than or equal to 1.0 inch of precipitation (DP10), Number days in month with minimum temperature less than or equal to 32.0 F (DT32), Total precipitation amount for the month (TPCP), Monthly mean minimum temperature (MMNT), Monthly mean maximum temperature (MMXT) and Monthly mean temperature (MNTM)). Climatic data were considered in January (winter model) and in April (pre-breeding model). Geographical distances were included in the error term. Significant p-values are denoted by an asterisk.

		Winter model		**Pre-breeding model**	
	d.f.	Pseudo-F	P	Pseudo-F	P
Full model					
DP10	1	0.40	0.533	2.26	0.145
DT32	1	<0.01	0.996	0.02	0.879
MMNT	1	4.14	0.052	0.22	0.641
MMXT	1	0.30	0.587	2.92	0.099
MNTM	1	0.06	0.803	1.13	0.298
TPCP	1	0.32	0.575	3.10	0.090
Year	1	1.64	0.211	0.38	0.544
Best model					
MMNT	1	3.89	**0.057**		
MMXT	1			4.62	**0.039***

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APPENDIX 3.1.

Climatic General Linear Models explained variation in Faith's Phylogenetic Diversity index of bacterial community of Tree Swallow nests. Number of days in month with greater than or equal to 1.0 inch of precipitation (DP10), Number days in month with minimum temperature less than or equal to 32.0 F (DT32), Total precipitation amount for the month (TPCP), Monthly mean minimum temperature (MMNT), Monthly mean maximum temperature (MMXT) and Monthly mean temperature (MNTM) were considered as continuous climatic variables as well as locality as fixed factor and year as random factor. Climatic data were considered at month of hatching (breeding models), in January (winter models) and in April (pre-breeding models). SD means standard deviation and significant values are in bold.

APPENDIX 3.1A.

	Model	Variables	Beta	SD	d.f.	F	P
OTU Richness	Full winter model	JANUARY DT32	1.363	1.735	1	0.62	0.4525
		JANUARY DP10	-0.527	1.120	1	0.22	0.6494
		JANUARY TPCP	0.226	1.211	1	0.03	0.8561
		JANUARY MMXT	6.138	48.838	1	0.02	0.9028
		JANUARY MMNT	9.984	41.697	1	0.06	0.8161
		JANUARY MNTM	-17.487	89.620	1	0.04	0.8496
		Locality	-	-	16	1.24	0.3811
		Year	-	-	2	0.09	0.9143
	Best winter model	-	-	-	-	-	-
	Full pre-breeding model	APRIL DT32	2.332	1.464	1	2.54	0.1458
		APRIL DP10	-1.318	0.393	1	11.23	0.0085
		APRIL TPCP	1.545	0.522	1	8.76	0.0159
		APRIL MMXT	-13.938	12.276	1	1.29	0.2856
		APRIL MMNT	-15.965	10.751	1	2.21	0.1717
		APRIL MNTM	32.910	23.355	1	1.99	0.1924
		Locality	-	-	16	2.40	0.0928
		Year	-	-	2	1.63	0.2486
	Best pre-breeding model	APRIL_DP10	-0.620	0.228	1	7.37	0.0107
		APRIL_TPCP	0.519	0.228	1	5.16	0.0302
	Full breeding model	DT32	0.999	0.627	1	2.54	0.1454
		DP10	-0.102	0.565	1	0.03	0.8615
		TPCP	-0.244	0.818	1	0.09	0.7726
		MMXT	-11.346	20.038	1	0.32	0.5851
		MMNT	19.029	39.012	1	0.24	0.6374
		MNTM	-8.086	21.072	1	0.15	0.7101
		Locality	-	-	16	0.97	0.5413
		Year	-	-	2	0.23	0.7969
	Best breeding model	-	-	-	-	-	-

APPENDIX 3.1B.

	Model	Variables	Beta	SD	d.f.	F	P
Phylogenetic Diversity	Full winter model	JANUARY DT32	1.482	1.706	1	0.75	0.4077
		JANUARY DP10	-0.721	1.102	1	0.43	0.5290
		JANUARY TPCP	0.372	1.191	1	0.10	0.7620
		JANUARY MMXT	13.463	48.027	1	0.08	0.7856
		JANUARY MMNT	15.535	41.004	1	0.14	0.7136
		JANUARY MNTM	-30.146	88.131	1	0.12	0.7402
		Locality	-	-	16	1.29	0.3608
		Year	-	-	2	0.07	0.9364
	Best winter model	-	-	-	-	-	-
	Full pre-breeding model	APRIL DT32	2.400	1.417	1	2.87	0.1245
		APRIL DP10	-1.274	0.381	1	11.20	0.0086
		APRIL TPCP	1.507	0.505	1	8.90	0.0154
		APRIL MMXT	-15.729	11.878	1	1.75	0.2181
		APRIL MMNT	-17.309	10.403	1	2.77	0.1305
		APRIL MNTM	36.248	22.598	1	2.57	0.1432
		Locality	-	-	16	2.70	0.0668
		Year	-	-	2	2.20	0.1668
	Best pre-breeding model	APRIL_DP10	-0.597	0.230	1	6.71	0.0145
		APRIL_TPCP	0.506	0.230	1	4.82	0.0357
	Full breeding model	DT32	1.030	0.615	1	2.81	0.1281
		DP10	-0.129	0.555	1	0.05	0.8218
		TPCP	-0.224	0.802	1	0.08	0.7864
		MMXT	-15.167	19.658	1	0.60	0.4602
		MMNT	26.675	38.271	1	0.49	0.5034
		MNTM	-12.315	20.673	1	0.35	0.5661
		Locality	-	-	16	1.02	0.5060
		Year	-	-	2	0.29	0.7538
	Best breeding model	DT32	0.384	0.163	1	5.52	0.0252

APPENDIX 3.1C.

	Model	Variables	Beta	SD	d.f.	F	P
Shannon's Diversity	Full winter model	JANUARY DT32	2.025	1.317	1	2.37	0.1584
		JANUARY DP10	-0.458	0.850	1	0.29	0.6033
		JANUARY TPCP	0.220	0.919	1	0.06	0.8161
		JANUARY MMXT	20.286	37.061	1	0.30	0.5974
		JANUARY MMNT	23.576	31.642	1	0.56	0.4752
		JANUARY MNTM	-45.205	68.008	1	0.44	0.5229
		Locality	-	-	16	2.35	0.0979
		Year		-	2	0.48	0.6357
	Best winter model	Locality	-	-	16	2.44	0.0389
	Full pre-breeding model	APRIL DT32	3.437	0.923	1	13.87	0.0047
		APRIL DP10	-1.370	0.248	1	30.52	0.0004
		APRIL TPCP	1.482	0.329	1	20.29	0.0015
		APRIL MMXT	-21.164	7.737	1	7.48	0.0230
		APRIL MMNT	-21.140	6.776	1	9.73	0.0123
		APRIL MNTM	45.110	14.720	1	9.39	0.0135
		Locality	-	-	16	4.30	0.0159
		Year	-	-	2	1.47	0.2810
	Best pre-breeding model	APRIL DT32	3.377	0.958	1	12.42	0.0048
		APRIL DP10	-1.265	0.245	1	26.70	0.0003
		APRIL TPCP	1.397	0.332	1	17.69	0.0015
		APRIL MMXT	-15.533	7.042	1	4.87	0.0496
		APRIL MMNT	-16.404	6.281	1	6.82	0.0242
		APRIL MNTM	34.044	13.347	1	6.51	0.0270
		Locality	-	-	16	4.56	0.0073
	Full breeding model	DT32	0.467	0.580	1	0.65	0.4414
		DP10	-0.057	0.523	1	0.01	0.9150
		TPCP	0.140	0.757	1	0.03	0.8577
		MMXT	2.290	18.554	1	0.02	0.9045
		MMNT	-7.046	36.122	1	0.04	0.8497
		MNTM	5.309	19.511	1	0.07	0.7917
		Locality	-	-	16	1.20	0.4038
		Year	-	-	2	0.33	0.7276
	Best breeding model	Locality	-	-	16	2.44	0.0389

APPENDIX 3.1D.

	Model	Variables	Beta	SD	d.f.	F	P
Evenness	Full winter model	JANUARY DT32	1.975	1.187	1	2.77	0.1306
		JANUARY DP10	-0.269	0.766	1	0.12	0.7334
		JANUARY TPCP	0.131	0.829	1	0.02	0.8780
		JANUARY MMXT	32.136	33.412	1	0.93	0.3613
		JANUARY MMNT	34.136	28.526	1	1.43	0.2620
		JANUARY MNTM	-67.362	61.313	1	1.21	0.3004
		Locality	-	-	16	2.23	0.1122
		Year	-	-	2	2.22	0.1650
	Best winter model	Locality	-	-	16	2.37	0.0437
	Full pre-breeding model	APRIL DT32	3.234	1.086	1	8.87	0.0155
		APRIL DP10	-1.097	0.292	1	14.14	0.0045
		APRIL TPCP	1.142	0.387	1	8.70	0.0162
		APRIL MMXT	-20.540	9.105	1	5.09	0.0505
		APRIL MMNT	-19.317	7.974	1	5.87	0.0385
		APRIL MNTM	41.652	17.323	1	5.78	0.0396
		Locality	-	-	16	2.66	0.0698
		Year	-	-	2	1.18	0.3508
	Best pre-breeding model	APRIL_DT32	1.540	0.604	1	6.50	0.0231
		APRIL_DP10	-0.758	0.274	1	7.67	0.0151
		APRIL_TPCP	0.774	0.332	1	5.43	0.0352
		Locality	-	-	16	2.78	0.0306
	Full breeding model	DT32	-0.077	0.498	1	0.02	0.8814
		DP10	-0.048	0.450	1	0.01	0.9177
		TPCP	0.423	0.651	1	0.42	0.5314
		MMXT	13.130	15.938	1	0.68	0.4313
		MMNT	-27.385	31.030	1	0.78	0.4005
		MNTM	15.486	16.761	1	0.85	0.3796
		Locality	-	-	16	1.62	0.2327
		Year	-	-	2	0.36	0.7047
	Best breeding model	Locality	-	-	16	2.37	0.0437

APPENDIX 3.2.

General Linear Models explained variation in bacterial species richness, Faith's Phylogenetic Diversity index, Shannon diversity index and Evenness of bacterial community of Tree Swallow nests per locality. Number of days in month with greater than or equal to 1.0 inch of precipitation (DP10), Number days in month with minimum temperature less than or equal to 32.0 F (DT32), Total precipitation amount for the month (TPCP), Monthly mean minimum temperature (MMNT), Monthly mean maximum temperature (MMXT) and Monthly mean temperature (MNTM) were considered as continuous climatic variables as well as locality as fixed factor and year as random factor. Climatic data were considered at month of hatching (breeding models), in January (winter models) and in April (pre-breeding models). SD means standard deviation and significant values are in bold.

APPENDIX 3.2A.

	Model	Variables	Beta (SD)	SD	d.f.	F	P
OTU Richness	Full winter model	Year	-0.036	0.240	2	0.03	0.9662
		Latitude	0.054	0.211	1	0.14	0.7165
		Longitude	0.277	0.752	1	0.00	0.9563
		Elevation	-0.017	0.303	1	0.11	0.7461
		DT32	-0.161	0.491	1	1.90	0.1825
		DP10	1.198	0.870	1	8.52	0.0079
		TPCP	-0.840	0.288	1	7.54	0.0118
		MMXT	0.983	0.358	1	2.47	0.1306
		MMNT	-19.931	12.693	1	2.67	0.1164
		MNTM	-19.720	12.065	1	2.70	0.1148
	Best winter model	-	-	-	-	-	-
	Full pre-breeding model	Year	-	-	2	2.89	0.0770
		Latitude	-1.659	0.578	1	8.23	0.0089
		Longitude	-0.943	0.471	1	4.01	0.0578
		Elevation	-0.344	0.262	1	1.73	0.2022
		DT32	0.252	0.336	1	0.57	0.4602
		DP10	0.444	0.348	1	1.63	0.2153
		TPCP	-0.196	0.342	1	0.33	0.5720
		MMXT	-0.017	29.832	1	0.00	1.0000
		MMNT	3.964	26.810	1	0.02	0.8838
		MNTM	-4.712	56.180	1	0.01	0.9339
	Best pre-breeding model	DP10	-0.620	0.228	1	7.37	0.0107
		TPCP	0.519	0.228	1	5.16	0.0302
	Full breeding model	Year	-	-	2	0.60	0.5581
		Latitude	-0.595	0.416	1	2.04	0.1668
		Longitude	-0.236	0.389	1	0.37	0.5504
		Elevation	-0.013	0.245	1	0.00	0.9571
		DT32	0.658	0.293	1	5.05	0.0351
		DP10	-0.165	0.421	1	0.15	0.6984
		TPCP	0.218	0.497	1	0.19	0.6654
		MMXT	4.967	11.641	1	0.18	0.6738
		MMNT	6.065	13.842	1	0.19	0.6655
		MNTM	-10.558	24.205	1	0.19	0.6670
	Best breeding model	Latitude	-0.448	0.189	1	5.60	0.0244
		DT32	0.587	0.189	1	9.64	0.0040

APPENDIX 3.2B.

	Model	Variables	Beta (SD)	SD	d.f.	F	P
Phylogenetic Diversity	Full winter model	Year	-	-	2	0.09	0.9181
		Latitude	-0.053	0.235	1	3.77	0.0652
		Longitude	-0.063	0.252	1	0.97	0.3353
		Elevation	-1.399	0.721	1	0.35	0.5604
		DT32	-0.579	0.588	1	0.19	0.6637
		DP10	-0.193	0.326	1	0.57	0.4574
		TPCP	-0.185	0.419	1	0.09	0.7684
		MMXT	-0.328	0.434	1	0.47	0.4993
		MMNT	0.127	0.426	1	0.41	0.5273
		MNTM	-25.547	37.189	1	0.42	0.5212
	Best winter model	-	-	-	-	-	-
	Full pre-breeding model	Year	-	-	2	0.06	0.9376
		Latitude	0.297	0.747	1	0.16	0.6952
		Longitude	-0.018	0.301	1	0.00	0.9536
		Elevation	-0.232	0.488	1	0.23	0.6395
		DT32	1.282	0.865	1	2.20	0.1523
		DP10	-0.873	0.286	1	9.32	0.0058
		TPCP	1.056	0.356	1	8.81	0.0071
		MMXT	-18.274	12.611	1	2.10	0.1614
		MMNT	-18.350	11.987	1	2.34	0.1401
		MNTM	37.492	24.457	1	2.35	0.1395
	Best pre-breeding model	DP10	-0.597	0.230	1	6.71	0.0145
		TPCP	0.506	0.230	1	4.82	0.0357
	Full breeding model	Year	-	-	2	0.58	0.5708
		Latitude	-0.636	0.416	1	2.33	0.1409
		Longitude	-0.281	0.389	1	0.52	0.4779
		Elevation	-0.074	0.245	1	0.09	0.7640
		DT32	0.638	0.293	1	4.74	0.0405
		DP10	-0.175	0.421	1	0.17	0.6825
		TPCP	0.289	0.497	1	0.34	0.5675
		MMXT	2.225	11.640	1	0.04	0.8502
		MMNT	2.625	13.840	1	0.04	0.8513
		MNTM	-4.753	24.202	1	0.04	0.8461
	Best breeding model	Latitude	-0.421	0.187	1	5.08	0.0314
		DT32	0.622	0.187	1	11.11	0.0022

APPENDIX 3.2C.

	Model	Variables	Beta (SD)	SD	d.f.	F	P
Shannon's Diversity	Full winter model	Year	-	-	2	0.78	0.4712
		Latitude	-1.790	0.655	1	7.46	0.0122
		Longitude	-0.897	0.534	1	2.82	0.1072
		Elevation	-0.276	0.296	1	0.87	0.3619
		DT32	0.035	0.380	1	0.01	0.9274
		DP10	0.179	0.394	1	0.21	0.6543
		TPCP	-0.084	0.387	1	0.05	0.8295
		MMXT	-16.792	33.797	1	0.25	0.6242
		MMNT	-11.703	30.374	1	0.15	0.7037
		MNTM	27.251	63.647	1	0.18	0.6727
	Best winter model	Latitude	-1.389	0.468	1	8.80	0.0059
		Longitude	-0.686	0.256	1	7.17	0.0119
		MMXT	-0.869	0.377	1	5.31	0.0283
	Full pre-breeding model	Year	-	-	2	1.37	0.2739
		Latitude	0.283	0.180	1	0.16	0.6895
		Longitude	-0.009	0.159	1	0.69	0.4140
		Elevation	0.229	0.565	1	0.35	0.5604
		DT32	-0.190	0.228	1	6.22	0.0206
		DP10	-0.218	0.369	1	23.85	0.0001
		TPCP	1.630	0.653	1	18.27	0.0003
		MMXT	-1.055	0.216	1	4.27	0.0508
		MMNT	1.149	0.269	1	4.44	0.0468
		MNTM	-19.684	9.527	1	4.74	0.0405
	Best pre-breeding model	Elevation	-0.421	0.182	1	5.35	0.0283
		DT32	1.796	0.627	1	8.22	0.0078
		DP10	-1.117	0.213	1	27.48	0.0000
		TPCP	1.099	0.254	1	18.74	0.0002
		MNTM	1.771	0.586	1	9.15	0.0053
	Full breeding model	Year	-	-	2	1.40	0.2684
		Latitude	-0.916	0.395	1	5.38	0.0301
		Longitude	-0.687	0.369	1	3.47	0.0759
		Elevation	-0.155	0.232	1	0.44	0.5125
		DT32	0.534	0.278	1	3.70	0.0676
		DP10	-0.237	0.400	1	0.35	0.5600
		TPCP	0.532	0.472	1	1.27	0.2721
		MMXT	5.492	11.044	1	0.25	0.6240
		MMNT	6.501	13.132	1	0.25	0.6255
		MNTM	-11.427	22.964	1	0.25	0.6237
	Best breeding model	Latitude	-0.441	0.200	1	4.88	0.0347
		DT32	0.422	0.200	1	4.47	0.0426

APPENDIX 3.2D.

	Model	Variables	Beta (SD)	SD	d.f.	F	P
Evenness	Full winter model	Year	-	-	2	2.89	0.0770
		Latitude	0.446	0.188	1	8.23	0.0089
		Longitude	-0.087	0.202	1	4.01	0.0578
		Elevation	-1.659	0.578	1	1.73	0.2022
		DT32	-0.943	0.471	1	0.57	0.4602
		DP10	-0.344	0.262	1	1.63	0.2153
		TPCP	0.252	0.336	1	0.33	0.5720
		MMXT	0.444	0.348	1	0.00	1.0000
		MMNT	-0.196	0.342	1	0.02	0.8838
		MNTM	-0.017	29.832	1	0.01	0.9339
	Best winter model	-	-	-	-	-	-
	Full pre-breeding model	Year	-	-	2	3.29	0.0561
		Latitude	0.438	0.173	1	0.05	0.8183
		Longitude	-0.082	0.152	1	1.35	0.2583
		Elevation	0.126	0.542	1	0.40	0.5313
		DT32	-0.254	0.218	1	6.62	0.0174
		DP10	-0.225	0.354	1	21.85	0.0001
		TPCP	1.613	0.627	1	14.94	0.0008
		MMXT	-0.969	0.207	1	2.37	0.1378
		MMNT	0.997	0.258	1	2.34	0.1404
		MNTM	-14.081	9.143	1	2.68	0.1155
	Best pre-breeding model	Elevation	-0.425	0.185	1	5.27	0.0293
		DT32	1.773	0.637	1	7.76	0.0095
		DP10	-1.057	0.216	1	23.85	0.0000
		TPCP	0.998	0.258	1	14.98	0.0006
		MNTM	1.801	0.595	1	9.16	0.0053
	Full breeding model	Year	-	-	2	2.92	0.0753
		Latitude	-0.951	0.368	1	6.68	0.0169
		Longitude	-0.870	0.344	1	6.41	0.0190
		Elevation	-0.242	0.217	1	1.24	0.2766
		DT32	0.297	0.259	1	1.31	0.2643
		DP10	-0.261	0.372	1	0.49	0.4898
		TPCP	0.675	0.440	1	2.36	0.1391
		MMXT	5.067	10.288	1	0.24	0.6272
		MMNT	5.888	12.232	1	0.23	0.6350
		MNTM	-10.430	21.391	1	0.24	0.6307
	Best breeding model	-	-	-	-	-	-