Reproductive physiology, avian malaria, and the cloacal microbiome in tropical Rufous-collared Sparrows (*Zonotrichia capensis*)

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ABSTRACT

Life-history strategies are adaptations in behavior, physiology, and anatomy that influence survival and reproductive success. Variation in life-history strategies is often determined by adaptations to environmental conditions and trade-offs with sexually-selected signals. One of the aspects controlling life-history trade-offs is the endocrine system. Testosterone is a hormone that mediates several key aspects of male reproduction, yet little is known about the causes and consequences of variation in testosterone. Using rufous-collared sparrows (*Zonotrichia capensis*), a Neotropical songbird with a wide distribution, I explored geographical patterns of variation in testosterone levels and infection by haemosporidians, a type of blood parasite. I found that testosterone did not vary with elevation, nor predict haemosporidian infection, but males in breeding condition were more likely to be infected (Chapter I). High levels of testosterone have been associated with an increased number of sexual contacts and can suppress the immune response, thus it may increase the risk of sexually transmitted infections. By studying the communities of bacteria that reside in the cloaca of birds, I found that they were different depending on testosterone levels, and that high-testosterone males had higher relative abundance of Chlamydiae, a class of intracellular pathogens (Chapter II). During the breeding season there is an increase in physical contacts among individuals, testosterone levels increase in males, and there are additional energetic demands, all of which can increase exposure to bacteria or facilitate infection. I compared the cloacal microbiome of the same individuals between breeding and non-breeding seasons, and found that in males, but not in females, bacterial richness and

phylogenetic diversity increased when birds were in reproductive condition. This suggested that the cloacal microbiome in birds is dynamic and responsive to breeding condition and sex of the host (Chapter III). Lastly, I synthesized the most relevant findings and suggested directions for future work (Chapter IV). I conclude that variation in testosterone is not always associated with immune suppression, and that the links among reproductive physiology, behavior, and the microbiome can provide insight into the evolution of life-history strategies.

DEDICATION

To my family.

To my mentors.

To the lush mountains and their thunder.

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ATTRIBUTION

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CHAPTER I. INTRODUCTION

Camilo Escallón

The physiology of life-history strategies in the tropics

Life histories are sets of interrelated adaptations in behavior, physiology, and anatomy that influence survival and reproductive success of organisms. Life-history traits vary across environments and as a result of trade-offs imposed by resource allocation (Stearns 1992). Environmental gradients, such as those formed by latitude and elevation, have allowed us to study the variation in life-histories from multiple perspectives. One of the patterns that has been historically studied is how reproductive strategies vary with latitude. For example, why high latitude birds produce clutches with more eggs than low latitude species (Martin *et al.* 2006), or how organisms respond to environmental cues across latitudes to time their breeding season (Miller 1965). More recently, a macro-physiological approach has been taken to understand large-scale physiological patterns across different environments (Chown, Gaston & Robinson 2004; Chown & Gaston 2008; Gaston *et al.* 2009), and associate those patterns to physiological aspects of life-history strategies (Ricklefs & Wikelski 2002). The fast-slow pace of life continuum is an interesting example to study how trade-offs along large-scale environmental gradients are regulated by various physiological factors.

Life-history strategies of birds in tropical latitudes have converged into a common set of characteristics that have placed many of them near the slow end of the slow-fast pace of life axis. Tropical latitudes are characterized by long breeding seasons, which are reflected in greater investment in self maintenance, and lower investment in each reproductive attempt (Ricklefs & Wikelski 2002). Associated with those characteristics are hormones involved in modulating trade-offs in life-history strategies, in particular testosterone, which is responsible for regulating various aspects of reproduction in males

(Hau 2007). How tropical organisms regulate testosterone levels in response to the environment likely dictates how life-histories evolve. This dissertation explores the variation in testosterone levels, associated with both abiotic and biotic changes in the environment.

Testosterone modulation by social factors

As testosterone has such a wide range of effects on the development, physiology, and behavior of all vertebrates, it is not surprising that it has been extensively investigated. Testosterone plays a major role in male reproduction and its fluctuations during the breeding season are explained in some cases by the Challenge Hypothesis (Wingfield *et al.* 1990; Wingfield & Soma 2000), which postulates that plasma testosterone concentrations are influenced by social interactions. In short, there is a rise in male testosterone baseline levels during the breeding season, and during the non-breeding season testosterone levels remain relatively low. This rise in testosterone levels is associated with periods of social instability associated with, finding and defending territories, and attracting a mate. The Challenge Hypothesis has the widest support in temperate birds where there is a marked breeding season (Goymann, Landys & Wingfield 2007; Garamszegi *et al.* 2008) and high testosterone concentrations during the breeding season (Hau, Perfito & Moore 2008). However, life history strategies in birds vary across a latitudinal gradient and the challenge hypothesis is not always applicable (Goymann *et al.* 2004). For example, the territorial defense of the rufous-collared sparrow *Zonotrichia capensis* in Ecuador does not seem to be modulated by testosterone, since the hormone levels did not increase after aggressive interactions, and experimentally implanted birds with artificially elevated testosterone were not more aggressive (Moore *et al.* 2004; Addis *et al.* 2011). In the same way, in the buff-banded rail, *Gallirallus philippensis* (Wiley & Goldizen 2003), and the wire-tailed manakin, *Pipra filicauda*, testosterone seems to be disassociated from male-male aggression (Ryder, Horton & Moore 2011). This poses a possibility that the dynamics of testosterone fluctuations in tropical birds are regulated by additional factors other than social instability (Robinson *et al.* 2010).

Testosterone modulation by environmental factors

Abiotic

Geographic variation in hormone levels has been identified for some vertebrates. For birds and amphibians, there is a positive relationship between testosterone and latitude (Goymann & Wingfield 2004; Garamszegi *et al.* 2008; Eikenaar *et al.* 2012). In a large-scale comparative analysis in birds, Garamszegi et al. (2008) tested if migration or breeding season length explained this latitudinal trend. They found that when controlling for phylogeny, the social factors explained very little of the variation in testosterone and attributed the remaining variation to environmental factors related to latitude. This variation may be explained by the hypothesis that higher latitudes result in shorter and more synchronous breeding seasons with increased male-male competition, thus favoring higher testosterone levels (Stutchbury & Morton 2001). This suggests that the variation in hormone levels seen across a latitudinal gradient can be a result of life-history responses to different environments (Ricklefs & Wikelski 2002)

Elevation is considered a major modulator of the evolution of life histories (Grant & Dunham 1990). Variation in climate, food limitation, predation rate and duration of the breeding season have been proposed as the main factors shaping phenotypic variability across elevational gradients (Badyaev 1997). In the tropics, ecosystems change, often dramatically, due to the relation of altitude and climate. Environmental heterogeneity creates different selection pressures that may lead to local adaptations (Cheviron, Whitehead & Brumfield 2008; Cheviron & Brumfield 2009). For example, populations of *Zonotrichia capensis* distributed across elevational gradients vary in physiological parameters like metabolic rate and critical temperature (Castro *et al.* 1985; Castro & Wunder 1991). Hormonal variations also exist along elevation, as birds breeding at higher elevations have higher peak testosterone concentrations than the birds from lower elevations (Goymann *et al.* 2004), suggesting that adaptations for the shorter breeding seasons favor higher testosterone levels.

Selection pressures related to elevation occur in mountains and drive a special case for the evolution of life histories. First, temperature fluctuations are stable at tropical latitudes throughout the year, but change dramatically across an elevational gradient. Lower elevations tend to be warmer and higher elevations colder, but at high elevations temperature variation within a day becomes more drastic, with daily fluctuations of over 17º C (Sarmiento 1986). This selects for physiological adaptations that can accommodate wide fluctuations in temperature within short daily intervals. Second, rainfall cycles, which are what drive most of the biological activity, are characterized by wet and dry seasons (Leigh 1975), but this seasonality is more pronounced at higher elevations (Sarmiento 1986; Buytaert *et al.* 2006). Hormonal adaptations in testosterone could be selected by the particular temperature and seasonality patterns that vary across the elevational gradient.

Biotic

The variation in testosterone in tropical birds might also be explained by parasite infection. Artificially increased levels of testosterone positively affected fitness related factors in *Junco hyemalis*, but at the same time decreased male survival rate (Ketterson *et al.* 1996). This cost could be related to a suppression of immune function by testosterone (Folstad & Karter 1992), or stimulation of behaviors that increase a male's chances of becoming infected (Mougeot *et al.* 2005), although whether testosterone is mediating such tradeoffs is still unclear (Roberts, Buchanan & Evans 2004). Nevertheless, parasite prevalence and diversity could be important drivers that affect the energy budget, obligating a differential resource allocation from reproduction to disease defense (Norris & Evans 2000).

Haemosporidia

Haemosporidians are one group of parasites that vary in intensity with the environment and can potentially vary with testosterone levels too. Hematozoan blood parasites are common avian parasites that have been linked to trade-offs between self-maintenance and

reproduction (Tomás *et al.* 2007; Martínez-de la Puente *et al.* 2010; Karell *et al.* 2011). The most common species belong to the genera *Plasmodium* and *Haemoproteus,* and while in some cases they are referred to as avian malaria (*sensu* Pérez-Tris & Bensch 2005), I prefer to use the more taxonomically accurate term of haemosporidians. These parasites are commonly found in birds, and their prevalence changes with elevation (Loiseau *et al.* 2013; Jones, Cheviron & Carling 2013).

Haemosporidians infect erythrocytes, resulting in red blood cell destruction, i.e. hemolysis, as well as cause injury to internal organs, but in some cases their effects are dependent on the infecting parasite species (Atkinson & van Riper 1991). Some of the negative effects of haemosporidians reported in wild populations are associated with a reduction in host oxygen transport capacity (Yorinks & Atkinson 2000), body condition (Merino *et al.* 2000), fitness (Asghar, Hasselquist & Bensch 2011), and survival (Martínez-de la Puente *et al.* 2010; Lachish *et al.* 2011). However, there is still some debate on whether their effects are always negative. For example, there have been reports of null or positive relationships between infection and host fitness, which defy the common negative concept of parasitism (Kilpatrick & LaPointe 2006; Podmokła *et al.* 2014; de Jong *et al.* 2014).

Sexually transmitted infections

Multi-cellular organisms carry a plethora of microbes, including many bacterial species, but until recently we were unable to identify the vast majority of bacteria, as many were unculturable in laboratory conditions. Newly developed sequencing approaches that are culture-independent have allowed us to accurately catalogue the real diversity of microbial communities from any sample (e.g. soil, skin, water). In vertebrate animals, multiple of bacterial species have been found inhabiting different body parts (Faust *et al.* 2012), and transmission of bacteria between individuals can occur through physical contact. Of particular relevance to my research, physical contact during copulation in birds allows bacterial transmission between their cloacas (Westneat & Rambo 2000; Hupton *et al.* 2003; Kulkarni & Heeb 2007).

The microbiome is the community of microorganisms and their associated genomes, which reside in and on animals. In birds, the cloaca is a joint opening for excretion and the urogenital tract. Therefore, the cloacal microbiome is composed of a mixture of bacteria coming both from the digestive tract and from contact with other individuals during copulation. Most bacteria inhabiting the cloaca are likely not pathogenic, and instead may be beneficial bacteria (Lombardo, Thorpe & Power 1999; Clay 2014). However, there are several sexually-transmitted bacteria that can be pathogenic, and may be transmitted through the population by individuals who have the most sexual partners (Smith & Dobson 1992; Sheldon 1993; Lombardo 1998; Poiani 2010; Ben Ashby & Gupta 2013). The cloacal microbiome is dynamic and may be altered by multiple factors, such as sex, age, reproductive condition, and hormone levels. Studying the ecology of the microbes that reside in the cloaca can provide us with a wider understanding of the factors that shape those bacterial communities, and importantly how the microbiome can shape the evolution of life-history trade-offs.

Current study

In this dissertation, I address questions related to variation in reproductive physiology, and how that variation relates to the communities of pathogens and microorganisms that reside on birds. I focused on the following related questions:

Do testosterone levels vary among populations across an elevational gradient?
Are testosterone levels correlated with infection of pathogenic microorganisms?
How does the reproductive physiology of the host relate to the cloacal microbiome?

I studied rufous-collared sparrows (*Zonotrichia capensis*). This is the only species in the genus *Zonotrichia* with a tropical distribution. The range encompasses southern Mexico to the southern tip of South America and in tropical areas it inhabits mostly montane areas from ~1000 to 3300 meters above sea level (Chapman 1940). The species has a sexually monomorphic plumage, is socially monogamous, and is a year-round permanent

resident at tropical latitudes (Miller & Miller 1968). It has high rates of extra pair paternity (Eikenaar *et al.* 2013). Both males and females exhibit parental care and males defend territories that can be usurped by floater males (Smith 1978). Additionally, *Z. capensis* has a seasonal breeding period in most of its range, but in tropical populations a portion of the population is frequently breeding aseasonally (Moore 2005; Class *et al.* 2011; Addis *et al.* 2011).

This species provides a good opportunity to investigate relationships between geographic variation of testosterone, parasites, and the cloacal microbiome. Previous work has established a good baseline of understanding of the role of testosterone in the reproductive and territorial behaviors of this species. By addressing how aspects of the reproductive physiology relate to variation in microorganism communities, I have been able to significantly advance our understanding of the costs of reproduction in tropical birds.

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CHAPTER II. TESTOSTERONE AND HAEMOSPORIDIAN PARASITES ALONG A TROPICAL ELEVATIONAL GRADIENT IN RUFOUS-COLLARED SPARROWS (ZONOTRICHIA CAPENSIS)

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Abstract

Elevation has been proposed as a dominant ecological variable shaping life-history traits and subsequently their underlying hormonal mechanisms. For example, in a meta-analysis of tropical birds, elevation was negatively related to breeding season length and positively related to testosterone levels. Further, parasitism by avian haemosporidians should vary with elevation as temperature and humidity affect vector abundance, and while testosterone is needed for breeding, it is hypothesized to be immunosuppressive and thus could exacerbate infection. Our objective was to examine relationships between elevation, testosterone levels, and parasitism by avian haemosporidians. We surveyed males of a single tropical bird species, the rufous-collared sparrow (*Zonotrichia capensis*) across a wide elevational range along the equator. We measured baseline testosterone levels, haemosporidian infection, and red blood cell regeneration rates in breeding males at four elevations spanning the species' natural range in the Ecuadorian Andes (600 m, 1500 m, 2100 m, 3300 m). Testosterone levels from breeding males were not related to elevation, but there was high intra-population variability. The likelihood of being infected by haemosporidian parasites was greater when in breeding condition, but testosterone levels were not related to the probability of parasitism. Infected birds had a higher degree of red blood cell regeneration, which is consistent with an adaptive physiologic response to erythrocyte destruction. In conclusion, our results suggest that parasitism by avian

haemosporidians is a cost of reproduction but the mechanism is not directly through testosterone, and testosterone levels are not related to elevation.

Introduction

In male vertebrates, testosterone is often presented as a key mediator of life-history trade-offs (Ketterson and Nolan 1992; Stearns 1992; Sinervo and Svensson 1998). A major trade-off associated with testosterone is long-term self-maintenance versus immediate reproduction. Males benefit from high testosterone as it mediates the production of secondary sexual characters, reproduction, and its associated behaviors (e.g. territorial aggression). Yet, not all breeding males have high testosterone and instead there is large inter-individual variability in testosterone levels in many species (Kempenaers et al. 2008; Williams 2008). In male birds, this variability in testosterone has been mainly attributed to factors like the timing of the breeding season, mating system, paternal care, male-male aggression, and responses to the costs of testosterone (Wingfield et al. 1990; 2001; Goymann et al. 2007). Many studies have proposed costs associated with maintaining high testosterone concentrations, such as suppressed immune function, increased parasite load, and ultimately decreased survival (Casto et al. 2001; Wingfield et al. 2001; Robinson et al. 2010; Fuxjager et al. 2011). These costs have been formalized in the immunocompetence handicap hypothesis, which states that high testosterone comes at the cost of suppressed immune function (Folstad and Karter 1992). While there is limited support for testosterone as an immune suppressor, there is some evidence for a positive relationship between testosterone and parasitism (Roberts et al. 2004; Ezenwa et al. 2011; Fuxjager et al. 2011). As such, the benefits of high testosterone levels could be constrained by the costs of increased parasitemia risk (Norris and Evans 2000; Wingfield et al. 2001).

One way to investigate the causes of testosterone variation is by surveying populations across natural gradients that also vary in life-history traits mediated by testosterone. Differences in environmental conditions should exert different selective pressures on life-history traits and ultimately on testosterone levels (Ricklefs and Wikelski 2002). At a

geographic scale, latitudinal gradients have been previously investigated to compare variation in life-histories and testosterone. For example, it has been shown that testosterone levels are positively related to absolute latitude in birds (Goymann et al. 2004; Garamszegi et al. 2008; Hau et al. 2010) and amphibians (Eikenaar et al. 2012). This latitudinal variation may be explained by the hypothesis that higher latitudes result in shorter breeding seasons with reduced opportunities to breed, thus selecting for greater investment in each reproductive event by having high testosterone levels (Stutchbury and Morton 2001). Another geographic source of environmental variation is elevation. Elevation may affect abiotic and biotic aspects of the environment including climate, predation rate, and duration of the breeding season (Badyaev 1997; Bears et al. 2009; Hille and Cooper 2015). This elevational variation may thus shape life-history traits such as fecundity, extra-pair paternity rates, and parental care (Grant and Dunham 1990; Badyaev and Ghalambor 2001; Bonier et al. 2014; Hille and Cooper 2015). One possible scenario is that long breeding seasons in the lowland tropics are associated with minimal territory establishment periods, fewer male-male interactions, and greater parasite and immunological challenges (Stutchbury and Morton 2001). This situation may have resulted in lowland species displaying a slower pace of life with lower peak testosterone concentrations (Robinson et al. 2010). A previous meta-analysis within tropical birds identified elevation as an environmental variable that significantly explained some of the variation in testosterone levels among species, with high elevation birds having higher testosterone levels (Goymann et al. 2004). While these results and possibilities are intriguing, the relationship between testosterone and elevation does not persist after controlling for phylogeny (Goymann et al. 2004). Further, it is unclear whether such a relationship exists within a single species.

Parasitism is another factor that varies with environmental conditions. Hematozoan blood parasites are common avian parasites that have been linked to trade-offs between self-maintenance and reproduction (Tomás et al. 2007; Martínez-de la Puente et al. 2010; Karell et al. 2011). While some people refer to *Plasmodium* sp. and *Haemoproteus* sp. as avian malaria (*sensu* Pérez-Tris and Bensch 2005), we will use the term haemosporidian parasites. These parasites are commonly found in birds, and their prevalence changes

with elevation (Jones et al. 2013; Loiseau et al. 2013). *Plasmodium* sp. organisms can infect erythrocytes, resulting in red blood cell (RBC) destruction, i.e. hemolysis, as well as causing injury to internal organs, while *Haemoproteus* sp. may be incidental or may cause disease in some avian species (Atkinson and van Riper 1991). The negative effects of haemosporidian parasites reported in wild populations include reduction in host oxygen transport capacity (Yorinks and Atkinson 2000), body condition (Merino et al. 2000), fitness (Asghar et al. 2011), and survival (Martínez-de la Puente et al. 2010; Lachish et al. 2011). On the other hand, a number of studies have reported null or positive relationships between infection and host reproductive success (Kilpatrick and LaPointe 2006; de Jong et al. 2014; Podmokła et al. 2014).

We investigated the relationships between elevation, testosterone, and haemosporidian infection in equatorial populations of the rufous-collared sparrow (*Zonotrichia capensis*) across an elevational gradient. This species has a sexually monomorphic plumage, is socially monogamous, and is a year-round permanent resident at tropical latitudes (Miller & Miller 1968). It has a wide elevational range in the tropics (~500 to 4000 meters above sea level) and high elevation populations have similar testosterone levels as temperate zone species of the genus *Zonotrichia* (Moore et al. 2002). Furthermore, high elevation populations breed seasonally and mid-elevation populations breed aseasonally (Moore 2005; Class et al. 2011), suggesting that there are differences in life-history traits along elevation. Populations at mid-elevation (~2000 m) have been described as having higher haemosporidian parasitism relative to low- and high-elevations (Jones et al. 2013), thus there is variation in parasitism pressure.

In the current study, we predicted that testosterone and elevation would be positively related, because higher elevation populations are subject to shorter breeding seasons and thus we presume increased male-male competition (Class et al. 2011). Further, as testosterone increases during breeding but results in parasitemia risk (Folstad and Karter 1992; Deviche and Parris 2006), we predicted that birds with high testosterone would be more likely to be infected with haemosporidian parasites. To test these predictions, we measured baseline testosterone levels, parasitemia, and RBC regeneration rates in males

from four populations along an elevational transect (600 m, 1500 m, 2100 m, 3300 m) along the equator in the Ecuadorian Andes. By avoiding the confounding factors of phylogenetic relatedness, this study is a strong test for geographic variation of testosterone levels within a single tropical species.

Methods

Study species and sites

We studied four populations of rufous-collared sparrows (*Zonotrichia capensis*) on the eastern slope of the Ecuadorean Andes (Table 2.1). The populations spanned an elevation range of ~2700 m, with a maximum linear distance between populations of 70 km. All populations have similar annual rainfall cycles, but at low elevations there are larger precipitation volumes and the rainy season is one to two months more extended (Bendix and Rafiqpoor 2001; Class et al. 2011). Each population was sampled during their respective breeding season (as established by Moore 2005; Class and Moore 2011) when testosterone levels are elevated. The majority (84%) of the captured males were in breeding condition (the height of the cloacal protuberance was greater than 5 mm which corresponds to fully grown testes (Moore 2005)) and were actively defending their territories, but we did not know the exact reproductive sub-stage (i.e. pre-breeding, feeding young in the nest, feeding fledglings) of each individual. We sampled males by setting up mist-nets in their territories and capturing them passively only during the 2 hours immediately after dawn, thereby minimizing the chances of testosterone variation due to diel cycles. Upon capture, each male was bled, weighed (using a 30 g Pesola scale), and wing and tarsus length were measured to the nearest mm. This investigation adhered to animal-care protocols by the Institutional Animal Care and Use Committee of Virginia Tech (#11-146-BIOL).

Blood samples and Hormone analysis

To measure plasma testosterone level, we took blood samples (ca. 250 µl) from the brachial vein within 10 min of capture. With the exception of a small amount used for a

blood smear (see below), blood samples were kept on ice (less than 4 h) until centrifugation and plasma was separated and frozen for later analysis. Plasma volumes ranged from 33–100 μl (mean: 75 μl). Plasma testosterone concentration was measured by direct radioimmunoassay following the procedures of Moore et al. (2002). The samples were run in duplicate in a single assay, with a mean extraction efficiency of 88% and an intra-assay variation of 12.3%. Limits of detection for the assay were ~0.08 ng/ml. The testosterone antibody used was T-3003s (Fitzgerald: Catalog # WLI-T3003s, New catalog #20R-TR018W). As we performed a direct assay without chromatography and the antibody has significant cross-reactivity with other androgens, we were measuring total androgen concentration (i.e. testosterone, 5α-dihydrotestosterone, and others). However, as testosterone is the major androgen in birds we will refer to our measured androgen levels as testosterone levels.

Blood parasites

To quantify haemosporidian infection and degree of erythroid regeneration, blood smears were created in duplicate. From blood collected at venipuncture, approximately 10 μl were smeared onto glass slides and air-dried. The slides were fixed in absolute methanol and stained with Diff-Quick (IMEB INC., San Marcos, CA, USA). The presence/absence of intraerythrocytic organisms was assessed in each blood smear by screening at least 25 fields at low magnification (400x) and then at least 25 fields at high magnification (100x) by N.M.W. If no hemoparasite was identified on the first slide examined or if smear quality was poor, the duplicate slide was evaluated in a similar fashion. The degree of erythroid regeneration, known as polychromasia on routine Romanovsky stains (Campbell and Ellis 2007), was estimated by enumerating polychromatophilic RBCs. Polychromatophilic RBCs are less mature than fully hemoglobinized erythrocytes and stain slightly bluer due to an increase in RNA and organelles (Campbell and Ellis 2007). We scored the degree of polychromasia by counting the number of polychromatophilic RBCs in 50 fields at (100x). A polychromasia score, ranging from zero to three, was assigned based on the average number of polychromatophilic RBCs/100x field: 0 (0 polychromatophilic RBCs), 2 (<7), 3 (7-15), or 4 (>15).

Statistical analysis

The distribution of plasma testosterone levels was normalized with a log transformation. We used linear regression to analyze the relationship between elevation and testosterone levels, including in the regression only males that were in breeding condition. To confirm that time of capture or handling time did not affect testosterone levels, we used linear regressions analyzing minutes since sunrise and minutes since capture against testosterone levels. To analyze if populations differed in body condition, we used a linear regression of elevation against mass and the scaled mass index as a proxy for body condition (Peig and Green 2009).

We tested if testosterone was a predictor of avian haemosporidian infection by using a logistic regression. For this analysis we included only males in breeding condition, and compared their infection status. We also tested whether the proportion of breeding and non-breeding males differed between infected and uninfected males, by employing a contingency analysis using a likelihood ratio Chi-square test. To test if being infected with haemosporidian parasites had a physiological cost in terms of an increased RBC regeneration rate, we ran an ANOVA followed by Tukey HSD *post hoc* tests comparing polychromasia scores. We also ran a linear regression to test if testosterone levels had an effect on RBC polychromasia scores. Finally, to investigate if infection by avian haemosporidian parasites had wider body condition effects, we used a t-test to see if there are differences in the scaled mass index between infected and uninfected birds.

Results

Testosterone and elevation

Across the four populations, 84% of the adult males had an enlarged CP (N = 60), 92% of the males were not molting (N = 67), and 49% of the females (N = 23) were defeathering or had a defeathered brood patch (Table 2.1). Out of all the captured birds (N = 121), only five were juveniles based on plumage. There was no relationship between elevation

and baseline testosterone levels ($t = 0.21$, $P = 0.83$, $R^2 = 0.0008$; Fig. 2.1). There was substantial variation in breeding male testosterone levels within each population. This variation in testosterone levels was not explained by time since sunrise ($t = 0.30$, $P = 0.25$, $R^2 = 0.02$), time since capture ($t = 0.20$, $P = 0.84$, $R^2 = 0.0006$), mass ($t = 1.40$, $P = 0.16$, $R^2 = 0.03$), or the scaled mass index ($t = -0.79$, $P = 0.43$, $R^2 = 0.01$).

Blood parasites

When screening for blood parasites among all four populations, we found that 40% of the males (N = 24) were infected with a haemosporidian parasite. Analyzing the elevational distribution of infection, we found that avian haemosporidian infection prevalence was the highest in the population at 1500 m, and the high elevation population at 3300 m had the lowest prevalence with only one case (Table 2.1).

Across all populations, males in breeding condition had a higher probability of being infected with haemosporidian parasites than non-breeding individuals ($\chi^2 = 3.98$, $P = 0.04$; Fig. 2.2A). Since birds at high elevation (3300 m) were less likely to be infected ($\chi^2 = 9.85$, $P = 0.002$), we subsequently excluded this population and still obtained similar results for all other populations combined ($\chi^2 = 6.19$, $P = 0.013$). While breeding males had on average higher testosterone levels than non-breeding males ($t = 6.56$, $P < 0.0001$, $R^2 = 0.37$; Fig. 2.2B), within the breeding males, testosterone was not related to the probability of being infected with avian haemosporidians ($\chi^2 = 0.002$, $P = 0.97$; Fig. 2.2C). To exclude the effects of the high-elevation population, we tested the relationships between testosterone and parasitism in each population separately. Again, there was no relationship between testosterone and the probability of being infected within each population (2100 m population: $\chi^2 = 1.91$, $P = 0.17$; 1500 m population: $\chi^2 = 1.16$, $P = 0.28$; 600 m population: $\chi^2 = 0.01$, $P = 0.97$).

Males that were both breeding and infected with haemosporidian parasites had increased levels of erythrocyte regeneration compared to uninfected individuals ($F = 3.98$, $P = 0.007$; Tukey HSD *post hoc* tests, $P < 0.02$; Fig. 2.3). Similar results were obtained when

the high elevation population was excluded due to natural low occurrence of haemosporidian parasites (F = 7.00, $P = 0.002$). The interaction between breeding condition and parasitemia could not be statistically tested because there was only one individual that was both infected and in non-breeding condition. Testosterone levels were not related to the RBC polychromasia scores (t = 1.36, $P = 0.11$, $R^2 = 0.04$). Within breeding males, there was no difference between infected and non-infected males in the scaled mass index (t = 1.58, $P = 0.12$).

Discussion

In contrast to our prediction based on previous multi-species comparisons, testosterone levels in male *Zonotrichia capensis* were not related to elevation among our populations. However, there was substantial individual variation in testosterone levels within each population, even though we sampled during each one's early to mid-breeding season (Table 2.1). While there was no direct relationship between testosterone levels and haemosporidian infection, we did find that birds in breeding condition were more likely to be infected with haemosporidian parasites.

Many life-history traits vary with elevation, including breeding season length, parental investment, breeding synchrony, and extra-pair paternity rates (Badyaev and Ghalambor 2001; Bonier et al. 2014; Hille and Cooper 2015). Variation in these traits is often also associated with testosterone (Raouf et al. 1997; Wingfield et al. 2001; McGlothlin et al. 2007). As such, we predicted that in a tropical species testosterone levels would be positively related with elevation. Indeed, Goymann et al., (2004) found a positive relationship between testosterone levels and elevation among tropical birds, but that pattern disappeared when controlling for phylogenetic relatedness of the species. The present study seems to corroborate the negative findings of Goymann et al. (2004). The fact that testosterone levels did not increase with elevation suggests the following, possibilities: 1) the life-history traits were not divergent enough to reflect differences in testosterone levels, or 2) life-history traits are indeed divergent, but evolutionary constrains my be preventing testosterone levels from evolving independently of the rest

of the hypothalamic pituitary gonadal axis. Previous studies on the genus *Zonotrichia* did not find latitudinal patterns in testosterone levels (Moore et al. 2002), thus it may be that in this group there is diminished inter-population variation in testosterone levels. Another possibility is that different results may be obtained in taxa that do not display prolonged paternal care, as *Zonotrichia capensis* does, as testosterone negatively affects this behavior (Lynn et al. 2009).

Breeding seasonality in our populations ranged from extended breeding seasons at high elevations (3300 m) (Moore 2005) to aseasonal breeding at mid-elevations (1500 m and 2100 m) (Class et al. 2011). Therefore, it could be that we just do not have any truly short breeding seasons with high synchronicity to influence testosterone levels as in higher latitude species/populations. In climatically aseasonal populations of *Zonotrichia capensis peruviensis*, testosterone levels were not different across the year (González-Gómez et al. 2013), suggesting that stable conditions could relax selective pressures on testosterone modulation. However, similar to our results, two temperate-zone populations of black redstarts (*Phoenicurus ochruros*) differing in elevation and thus in breeding season length and number of broods, had no differences in baseline testosterone levels (Apfelbeck and Goymann 2011). Relationships between seasonality and testosterone levels may therefore be hard to detect along elevational gradients as evolutionary constraints imposed by the other components of the hypothalamic pituitary gonadal axis may be restraining the plasticity of testosterone (Hau 2007).

Extra-pair paternity rates have been implicated as determinants of testosterone levels in males (Garamszegi et al. 2005). It was previously shown that two of the populations we studied (3300 m and 2100 m) have comparable, and elevated, extra-pair paternity rates (Eikenaar et al. 2013). Similarly, there is no obvious difference between the populations in the degree of paternal care provided to the young (I.T.M. unpublished data; Lynn et al. 2009). In other words, there may not be sufficient divergence in at least these life-history traits along the populations to select for differences in testosterone levels. Additionally, in *Zonotrichia capensis* testosterone seems to be decoupled from antagonistic social stimuli, as they do not increase their testosterone levels during simulated territorial intrusions

(Moore et al. 2004; Addis et al. 2010; 2011) similarly to some avian species (Goymann et al. 2007).

Our results relating breeding condition and haemosporidian infection are similar to those reported in temperate-zone birds (Ots and Hõrak 1996; Christe et al. 2012), where birds in breeding condition were more likely to be infected with haemosporidian microorganisms. Breeding effort in birds constitutes a major energetic challenge, with the potential to increase susceptibility to parasitic infections (Norris and Evans 2000). High reproductive effort can decrease immune system function (Zera and Harshman 2001) and thus directly result in increased susceptibility to haemosporidian infection (Ots and Hõrak 1996; Allander 1997). One possible mechanism to explain this is that reproductive effort presumably entails elevated levels of glucocorticoids (Romero 2002), which can also regulate immune function (Martin 2009).

We observed that infected *Zonotrichia capensis* had higher RBC regeneration rates than non-infected birds. Increase in erythropoiesis is thought to be a response to hemolysis in infected birds (Campbell and Ellis 2007). The degree of RBC polychromasia is a good indicator of erythrocyte regenerative response; with decreased oxygen tension secondary to anemia, the bone marrow produces greater numbers of erythrocytes and releases increased numbers of the earlier forms, resulting in increased polychromasia on a peripheral blood smear (Campbell and Ellis 2007). While microscopy estimates of haemosporidian infection correlate positively with molecular estimates, there is a small chance of incurring in false negatives (Valkiūnas et al. 2008). The fact that we documented higher RBC polychromasia values in infected birds reassures us that our procedure for haemosporidian blood parasite detection is accurate, and that the infection is causing a physiological response by the host. However, we cannot discern if the infection we are seeing is a resurgence of the acute phase that comes from seasonally new inoculations or a relapse from a low-level chronic infection. We observed no relationship between infection rates and body condition index, indicating that energy reserves were not impacted by infection. However, it is clear that simply looking at body condition as a response to haemosporidian infection is not sufficient to determine if there is a cost to the

infection. It is possible that infected individuals have just upregulated their feeding rates to maintain body condition in response to the cost of manufacturing new RBCs.

As expected, we found that males in breeding condition had higher circulating testosterone than non-breeding males, but contrary to our initial prediction, testosterone levels were not related to the likelihood of being infected with haemosporidian parasites. Few other studies have looked at the direct relation between naturally varying levels of testosterone and avian haemosporidian infection. Studies of blue jays (*Cyanocitta cristata*) (Garvin and Schoech 2006) and white-winged crossbills (*Loxia leucoptera*) (Deviche et al. 2010) found no correlation between plasma testosterone and *Haemoproteus* infection, while a study of red crossbills (*Loxia curvirostra*) did find a relationship (Cornelius et al. 2014). Experimental evidence for the effects of testosterone on infection by blood parasites is not conclusive either, as in dark-eyed juncos (*Junco hiemalis*), testosterone implants increased *Leucocytozoon fringillinarum* infection (Deviche and Parris 2006), but in white-plumed honeyeaters (*Lichenostomus penicillatus*) exogenous testosterone had no effect on *Haemoproteus* infection levels (Buttemer and Astheimer 2000). Our study thus does not provide support for the hypothesized immunosuppressive effects of testosterone, and we further found no relationships between testosterone and RBC polychromasia or body condition. Birds in breeding condition may be resource limited and haemosporidian infection could be a result of the trade-off between the energetics of reproduction versus self-maintenance and not the direct immunosuppressive effects of testosterone itself (Braude et al. 1999; Råberg et al. 2009).

In summary, we did not find a relationship between testosterone and elevation in our study species as predicted from previous multi-species comparisons. If the effect of elevation on testosterone levels exists for tropical birds, wider divergence in seasonality and life-history traits would probably be needed to detect an effect. However, we did find that breeding birds were more likely to be infected by haemosporidian parasites than non-breeding birds, regardless of testosterone levels. These results do not support the immunocompetence handicap hypothesis. Instead, we hypothesize that the energetic

demands of reproduction increase the likelihood of haemosporidian infection, leading to increased rates of RBC regeneration but no overall negative effects on body condition.

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Tables

Table 2.1. Characteristics of sampled populations.

Population	Elevation (m a.s.l.)	Coordinates	Sample size (males)	Proportion breeding	Proportion molting	Haemosporidia prevalence
Papallacta	3300	0° 21.83' S, 78° 8.92' W	13	100%	0%	8%
Yanayacu	2100	0° 36.43' S, 77° 53.82' W	26	85%	0%	35%
El Chaco	1500	0° 20.16' S, 77° 48.80' W	15	100%	0%	73%
Tena	600	1° 0.645' S, 77° 48.85' W	20	50%	30%	31%

Figure Legends

Figure 2.1. Testosterone levels of males in breeding condition in relation to elevation of the population. There is no relationship between elevation and mean testosterone levels of the population. Hollow circles represent individuals, filled points the mean for each population, and bars the 95% confidence intervals.

Figure 2.2. A. The proportion of males infected as a function of breeding status. The likelihood of being infected by haemosporidian blood parasites was greater for males in breeding condition. Bars show proportion of infected males, and sample sizes in each category are indicated in parentheses. B. Circulating testosterone levels in breeding and non-breeding males. The boxplots show median, quartiles, and extreme values for each breeding status. C. The relationship between circulating testosterone levels and the probability of being infected with haemosporidian parasites in males in breeding condition. Hollow circles represent individuals, filled points the global mean, and bars the 95% confidence intervals. There was no effect of testosterone on infection probability. The same result was obtained when testing each population separately.

Figure 2.3. Red blood cell polychromasia score of infected and non-infected males for non-breeding (shaded boxes) and breeding (open boxes) males. Independent of breeding condition, infected males had higher RBC regeneration rates. Post-hoc Tukey test results, depicted in letters above each box, show that infected breeding males were different from the other two groups. The boxplots show median, quartiles, and extreme values for each group of males.

Figures

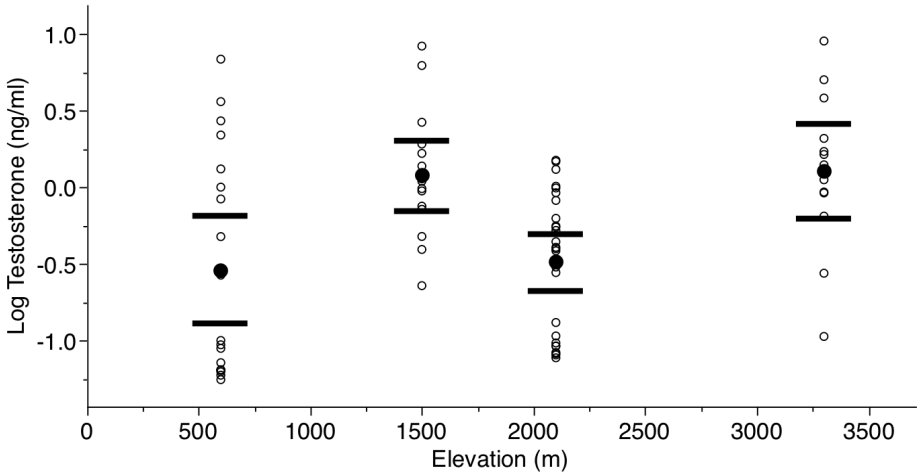

Figure 2.1. Testosterone levels of males in breeding condition in relation to elevation of the population

Figure 2.2. Breeding status, testosterone and haemosporidian infection

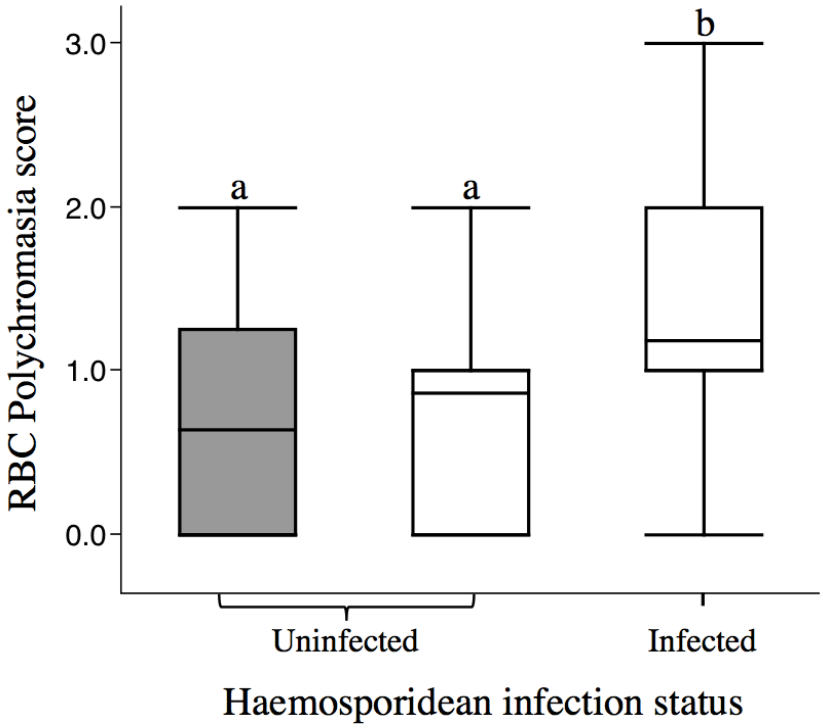

Figure 2.3. Red blood cell polychromasia score of infected and non-infected males

CHAPTER III. CLOACAL BACTERIAL PHYLOGENETIC DIVERSITY, AND RELATIVE ABUNDANCE OF CHLAMYDIAE, INCREASES WITH TESTOSTERONE LEVELS IN A FREE-LIVING TROPICAL BIRD

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Abstract

Testosterone mediates several key aspects of male reproduction, but maintaining high testosterone levels can reduce long-term survival. One of the pathways by which testosterone can influence survival is increased risk of parasite infection. High levels of testosterone have been associated with an increased number of sexual contacts. Therefore, testosterone has the potential to affect the transmission of sexually transmitted infections by promoting behaviors that increase sexual contact rates and/or by decreasing immune function. We hypothesized that if testosterone is associated with increased copulation rates, then males with high levels of testosterone would increase their chances of being infected with sexually transmitted bacteria, which would manifest as increased diversity of cloacal bacteria. To test this hypothesis, we quantified circulating testosterone levels in breeding male rufous-collared sparrows (*Zonotrichia capensis*) and collected cloacal swabs to quantify bacterial diversity using 16S rRNA gene amplicon sequencing. The most dominant bacterial phyla in the cloacal communities were Proteobacteria, Tenericutes, Firmicutes, and Actinobacteria. There was a positive correlation between testosterone levels and the phylogenetic diversity of cloacal bacteria. In addition, individuals with high and medium testosterone levels had cloacal bacterial communities that were more similar to each other than to those of low testosterone individuals. Finally, when considering bacterial taxa that are potential avian pathogens, we found that the relative abundance of Chlamydiae, a class of intracellular pathogens, was positively correlated with testosterone levels. Two nonexclusive explanations for these results are that testosterone affects behaviors that lead to increased sexual contacts

and thus the exposure and acquisition of additional phylogenetically diverse bacteria, and/or that testosterone is altering the immune system or the cloacal environment, thus making it easier for bacteria to colonize. These data suggest that increased exposure to sexually transmitted pathogens in the form of cloacal bacteria could be a cost of maintaining high testosterone levels.

Introduction

Testosterone has often been investigated as a mediator of life-history trade-offs, especially the trade-off between immediate reproduction and long-term survival (Ketterson & Nolan 1992; Hau 2007). For example, high testosterone levels can be beneficial to males, as they are associated to increased territoriality, extra-pair paternity, and the production of sexually-selected ornaments (Adkins-Regan 2005). However, even during the peak of the breeding season, significant variation in testosterone levels can be found among males (Kempenaers, Peters & Foerster 2008; Williams 2008). The immunocompetence handicap hypothesis proposes that there are survival costs associated with maintaining elevated testosterone (Folstad & Karter 1992) and that the primary cost is compromised immune function. In other words, testosterone supports short-term reproduction at the expense of increased risk of disease and consequent decreases in long-term survival. The immunocompetence handicap hypothesis has motivated extensive research, but has somewhat limited empirical support (Roberts, Buchanan & Evans 2004), perhaps because of the complexities in assessing the full array of immune responses (Martin, Weil & Nelson 2006). Further, linking immune function with disease resistance is problematic (Adamo 2004). However, while linking testosterone, immune function, and disease has been challenging, there have been multiple studies that have found links between high testosterone levels and increased parasite loads (Roberts *et al.* 2004; Deviche & Parris 2006; Cox & John-Alder 2007; Cornelius *et al.* 2014).

There are multiple ways in which parasitism could be linked to testosterone levels. Testosterone could directly alter the immune response or indirectly modify behaviors that increase exposure to parasites (Mougeot *et al.* 2005). In support of the later case, it has

been shown that elevated testosterone is associated with increased contact rates between individuals (Grear, Perkins & Hudson 2009) either through aggressive (Wingfield *et al.* 1990) or copulatory behaviors (Raouf *et al.* 1997) that can increase transmission of parasites between infected and uninfected individuals (Anderson & May 1979). As male birds with elevated testosterone levels can have increased rates of extra-pair copulations (Wingfield 1984; Raouf *et al.* 1997; Garamszegi *et al.* 2005), testosterone also has the potential to increase exposure to sexually transmitted infections (STI).

During copulation, there is cloacal contact and insemination, which allow bacterial transmission between individuals. In birds, copulation results in transmission of cloacal bacteria (Westneat & Rambo 2000; Hupton *et al.* 2003; Kulkarni & Heeb 2007). Having multiple partners has the potential to result in increased exposure to different cloacal bacteria. This can be seen in the common lizard (*Zootoca vivipara*), where females with polyandrous mating behavior had higher cloacal bacterial diversity than monogamous females (White *et al.* 2011). Therefore, the transmission rate of cloacal bacteria should also be positively correlated with the number of extra-pair copulations that are mediated by testosterone levels. As such, maintaining high levels of testosterone could have both survival and fitness costs for the male, as STIs could, in the short term, induce a costly immune response (Gustafsson *et al.* 1994; Sheldon & Verhulst 1996), and, in the long term, potentially sterilize the host (Lockhart, Thrall & Antonovics 1996). STIs have been suggested previously as a major cost of sexual reproduction (Sheldon 1993; Lockhart *et al.* 1996; Poiani & Wilks 2000; Ben Ashby & Gupta 2013); therefore, linking testosterone levels to cloacal bacterial diversity may offer additional insights into the disease-associated costs of high testosterone.

New culture-independent sequencing approaches have advanced our ability to understand complex microbial communities and how they are shaped. The cloacal microbiome in birds is composed of a mixture of bacteria coming both from the digestive tract and from contact with the exterior surface of the cloaca during copulation. Females, additionally, receive bacteria through contamination of the seminal fluid, and thus there is likely greater transmission from males to females (Westneat & Rambo 2000; Kulkarni & Heeb

2007). Most bacteria inhabiting the cloaca are likely not pathogenic, and instead may be beneficial bacteria (Lombardo, Thorpe & Power 1999; Clay 2014). However, there are several sexually-transmitted bacteria that can be pathogenic, and may be important in determining mating-behavior evolution (Sheldon 1993; Lombardo 1998; Poiani 2010). For instance, the bacterium *Chlamydia psittaci* has been implicated in avian chlamydiosis, a systemic disease linked with mortality of domestic poultry (Vanrompay, Ducatelle & Haesebrouck 1995), California gulls (*Larus californicus*), ring-billed gulls (*Larus delawarensis*) (Franson & Pearson 1995), and blue tits (*Parus caeruleu*) (Holzinger-Umlauf *et al.* 1997). *Chlamydia psittaci* can also infect mammals, and has been associated with a sterilizing urogenital infection in koalas (*Phascolarctos cinereus*) that reduces fecundity and is linked to population declines (Weigler *et al.* 1988).

The spread of STIs through the population is largely determined by individuals who have the most sexual partners (Smith & Dobson 1992; Ben Ashby & Gupta 2013). This suggests that promiscuous individuals are more likely to have a high diversity of cloacal bacteria, and their mates, consequently, are more likely to have a more diverse cloacal microbiome. For example, in female black-legged kittiwakes (*Rissa tridactyla*) with experimentally blocked insemination, the diversity of cloacal bacteria declines, suggesting that the cloacal microbiome is dynamic and altered by copulatory contact (White *et al.* 2010). In addition to copulatory contact, the establishment success of bacteria within this complex community depends on the host's immune defense, aspects of the cloacal environment, and the current bacterial community structure, which affects processes of competitive exclusion, coexistence and facilitation (Poiani 2010).

In this study, we investigated the relationship between testosterone levels and cloacal bacterial communities in free-living male rufous-collared sparrows (*Zonotrichia capensis*). We hypothesized that males with high testosterone levels would have a higher diversity of cloacal bacteria than males with low testosterone levels. This relationship could be due to increased exposure to bacteria through increased sexual contacts and/or through immunosuppressive effects of testosterone or changes in the cloacal environment. Rufous-collared sparrows offer a good opportunity to investigate these

relationships, as this species modulates its testosterone levels during the breeding cycle and has a high extra-pair copulation rate (Eikenaar *et al.* 2013). To test our hypothesis, we measured breeding-season testosterone levels, assessed cloacal bacterial diversity, and estimated the functional metagenome of the bacterial communities in breeding males from three tropical populations of rufous-collared sparrows.

Materials and Methods

Sampling of testosterone and cloacal bacteria

We studied three populations of rufous-collared sparrows on the eastern slope of the Ecuadorean Andes (Papallacta, 0°21.83' S, 78°8.92' W; Yanayacu, 0°36.43' S, 77°53.82' W; Tena, 1°0.645' S, 77°48.85' W). Each population was sampled during their respective breeding season (as established by Moore 2005; Class & Moore 2011) when testosterone levels are elevated. The majority (76%, N = 45) of the 59 captured males were in breeding condition, as they were actively defending their territories and the height of the cloacal protuberance was greater than 5 mm, which corresponds to fully grown testes (Moore 2005). We sampled males by setting up mist-nets in their territories and captured them passively only during the two hours immediately after dawn, thereby minimizing the chances of testosterone variation due to diel cycles. Upon capture, each male was bled to measure plasma testosterone and weighed, and wing and tarsus lengths were measured. To collect bacteria from the cloaca we used PurFlock® sterile micro-ultrafine nylon-tipped swabs (#3318 PN, Puritan, USA). Samples were collected by gently introducing a swab approximately 4 mm into the cloaca and rotating it once. After collection, swabs were stored in RNALater (Ambion, USA) and frozen for later DNA extraction. This investigation adhered to animal-care protocols approved by the Institutional Animal Care and Use Committee of Virginia Tech.

Hormone analysis

Testosterone was measured as part of a previous study (Escallón et al, in review) and a subset of those data was used for the current study. Briefly, to measure plasma

testosterone, we took blood samples (ca. 250µl) from the brachial vein within 10 min of capture. Blood samples were kept on ice (less than 4h) until centrifugation, when plasma was separated and frozen for later analysis. Plasma volumes ranged from 33–100µl (mean: 75µl). Total plasma testosterone concentration was measured by direct radioimmunoassay following the procedures of Moore et al. (2002). We measured total androgens (e.g. testosterone, 5α–dihydrotestosterone and others) in our direct assay, however, testosterone is the major androgen in birds, so we will refer to our measured androgen levels as testosterone levels. The samples were run in duplicate in a single assay, with a mean extraction efficiency of 88% and an intra-assay variation of 12.3%. The limit of detection for the assay was ~0.08ng/ml. The testosterone antibody used was T-3003s (Fitzgerald: Catalog #20R-TR018W).

Bacterial sample preparation and sequencing

Out of all the captured male birds (N = 59), we randomly selected 18 breeding males that spanned a range of testosterone levels to analyze their cloacal microbiota; Papallacta (N = 9), Yanayacu (N = 4), and Tena (N = 5). To remove the bacteria from the collection swab, we vortexed the tube and then centrifuged it at 12,000 RPM for five minutes to form a pellet in the bottom. The RNALater was pipetted out and the remaining pellet was used to extract bacterial DNA. We extracted DNA from each sample with the DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA, USA). The V4 region of the 16S rRNA gene was amplified by PCR using the primers 515F and 806R (Caporaso *et al.* 2011). The reverse primers contained a 12 base error-correcting Golay code (Fierer *et al.* 2008), which was used to uniquely tag PCR products of each sample. We prepared PCR reactions similar to Costello et al. (2009). Briefly, triplicate reactions of each sample contained 1µl template DNA, 12µl DNA-free PCR water (MO-BIO, Carlsbad, California), 10µl 2.5x HotMasterMix (5 PRIME, Gaithersburg, Maryland), 1µl of 20mg/ml bovine serum albumin (Fisher Scientific, Pittsburgh, Pennsylvania), and 0.5µl of each primer at 10µM concentration. We ran controls without template for each sample. We diluted extracted DNA samples that contained PCR inhibitors 1 to 10 in PCR water. The amplification conditions were as follows: an initial cycle for 3min at 94 ºC followed

by 35 cycles of 34s at 94 ºC, 60s at 50 ºC, and 90s at 72 ºC, with a final cycle for 10min at 72 ºC. Triplicate reactions of each sample were pooled, visualized on a 1% agarose gel, and quantified with a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, California). An equimolar mixture of all the samples was then sequenced at the Dana-Farber Cancer Institute of Harvard University on an Illumina MiSeq instrument with a 150 bp paired-end strategy, following methods similar to Caporaso et al. (2012). To compensate for the low base diversity of the amplicon pool, the sample was run with a 10% PhiX control. Version 1.18.42 of the MiSeq Real-Time Analysis software (Illumina) was used to perform base calling and quality scoring.

Sequence data processing

We assembled overlapping forward and reverse paired reads with Fastq-join (https://code.google.com/p/ea-utils/wiki/FastqJoin) with default parameters and processed them with the Quantitative Insights Into Microbial Ecology pipeline (QIIME v. 1.7.0; Caporaso *et al.* 2010b). Sequences were de-multiplexed and quality-filtered following methods similar to those described by Bokulich et al. (2013). Specifically, we discarded sequences if there were any ambiguous base calls, errors in the barcode, less than 75% of the read length had consecutive base calls with a phred quality score greater than 20, or there were more than 10 consecutive low-quality base calls. Sequences were extracted in Geneious and filtered to 150 bp. After quality filtering, the number of reads retained per sample ranged from 2,769 to 59,559 (Average 23,307). We then clustered the quality-filtered sequences into operational taxonomic units (OTUs) at a sequence similarity threshold of 97% with the UCLUST method (Edgar 2010) and a minimum cluster size of 0.001% of the total reads (Bokulich *et al.* 2013). Sequences were first clustered against the Greengenes database (May 2013 release; DeSantis *et al.* 2006). Sequences that did not match the database were then *de novo* clustered at a 97% sequence similarity threshold. The most abundant sequence for a given cluster was assigned as the representative sequence for that OTU. Taxonomy was assigned for each OTU with RDP classifier (Wang *et al.* 2007) at a 50% confidence threshold and the Greengenes database, as recommended by Claesson et al. (2009) for the v4 region of the 16s rRNA gene. We

aligned the representative sequences to the Greengenes database with PyNAST (Caporaso *et al.* 2010a) and constructed a phylogenetic tree with FastTree (Price, Dehal & Arkin 2009). We rarefied all samples to 10,000 sequences prior to analysis to standardize sampling effort. At this point, species curves had plateaued, indicating that we captured most of the taxonomic diversity. Rarefaction resulted in the removal of two samples that had low sequences per sample (< 10,000 reads).

Pathogenic bacteria

To determine the relative abundance of pathogenic bacteria, we compiled data from previously published sources listing bacterial pathogens isolated from cloacas of wild birds (Benskin *et al.* 2009; Poiani 2010). We compiled the list of pathogenic bacteria at a class level to include most of the OTUs that had limited taxonomic resolution. In this way, we could include possible undescribed species. Not all members of each class we examined are pathogens, but these represent groups that could contain avian pathogens. From the 61 classes of bacteria we found in the samples, we considered six for the analysis (Chlamydiae, Actinobacteria, Bacilli, Gammaproteobacteria, Epsilonproteobacteria, and Mollicutes).

Additionally, because we had numerous individuals infected with Chlamydiae, which are all intra-cellular bacterial pathogens, (1) we further checked if *Chlamydia psittaci* was present in our samples by aligning our Chlamydiales sequences to the *C. psittaci* 6BC genome (Accession number: NC_017287.1), and (2) we estimated and compared the metagenomic functional profile between the six infected birds and ten uninfected birds. The functional capacity of the cloacal microbial communities was analyzed with a bioinformatics tool that predicts gene family abundances from 16S metagenomic data by correlating the OTUs present in the samples to reference databases of microbial genomes (PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (http://picrust.github.com) (Langille *et al.* 2013)). We first picked OTUs against the Greengenes database (13_5) and then uploaded the biom file into the online Galaxy terminal (http://huttenhower.sph.harvard.edu/galaxy/), where OTUs were normalized by 16S rRNA gene relative abundance levels, and functional predictions were made based

on OTU membership. Functional predictions were categorized into Kyoto Encyclopedia of Genes And Genomes (KEGG) pathways representing gene counts of each predicted metagenome (Kanehisa & Goto 2000). We focused our analysis only on the KEGG functional category for "infectious disease", as we were interested in whether we could identify any predicted functional genome differences in the microbiota between Chlamydiae-infected and non-infected birds. Lastly, we tested if Chlamydial infection had an effect on males' mass and body condition.

Statistical analysis

All statistical analyses were carried out in R (version 3.1.0) (R Development Core Team 2014). The distribution of plasma testosterone concentrations was normalized with a log transformation. We computed measures of alpha diversity per individual (OTU richness and phylogenetic diversity) using QIIME. OTU richness is the number of OTUs present in an individual. Bacterial phylogenetic diversity is an estimate of phylogenetic breadth contained within each community. The core microbiota was defined as OTUs that were present in 90% or more of individuals across all populations, and we visualized their relative abundance on a heat map. To examine the relationship between circulating testosterone levels and both OTU richness and phylogenetic diversity, we used linear mixed models, assigning population as a random factor for each of these two analyses.

To assess whether bacterial community structure differed among populations and covaried with circulating testosterone levels, we calculated pairwise weighted and unweighted UniFrac distances for the bacterial communities. UniFrac uses branch length overlap to calculate the amount of phylogenetic distance between pairs of communities, and avoids some of the disadvantages associated with comparing communities at only a single level of taxonomic resolution (Hamady & Knight 2009). The unweighted UniFrac distance was visualized in a nonmetric multidimensional scaling plot (NMDS). To determine if there were differences in beta diversity among the three bird populations, we performed an Analysis of Similarity (ANOSIM) with 1000 permutations, which evaluates differences in community composition between pre-defined groups (i.e. populations) (Ramette 2007). However, as ANOSIM can confound mean tendency and dispersion it is

hard to determine the extent to which statistical differences are being driven by differences in variance among the groups. Therefore, we tested for differences in the variance among populations using a multivariate homogeneity of group dispersions (permdisp) test that determines whether the variances of groups of samples are significantly different. We used a Mantel test to assess the correlation between the composition of bacterial communities and testosterone levels. As testosterone levels did not significantly differ among the three populations, we grouped individuals from the three populations into a single analysis for the Mantel test.

We used gamma regressions to examine the relationships between testosterone levels and the relative abundance of the six classes of bacteria that potentially contained pathogens. We used a Kruskal-Wallis sum-rank test to assess differences in gene counts associated with "infectious disease", identified by PICRUSt, between Chlamydiae-infected and uninfected birds. To analyze if Chlamydiae-infected and uninfected birds differed in body condition, we used a Student's *t*-tests comparing infection status against mass and the scaled mass index (i.e. a proxy for body condition (Peig & Green 2009)).

Results

Dominant bacterial phyla and OTUs

The most dominant bacterial phyla (mean relative abundance > 1%) in the cloacal communities of the male rufous-collared sparrows were: Proteobacteria (43% of total relative abundance), Tenericutes (26%), Firmicutes (15%), Actinobacteria (8%), Bacteroidetes (2%), Cyanobacteria (1%), and Verrucomicrobia (1%) (Fig. 3.1). These seven phyla were present in the cloacas of all of the birds, although there was considerable variation among individuals in the diversity and the relative abundance of each phylum (Fig. 3.1). The total dataset contained 6009 OTUs, with a range of 573 to 1100 OTUs per bird. A large proportion of these OTUs were at low prevalence (proportion of individuals that have a particular OTU): 89% of them (5328/6009) had a prevalence ≤ 30%. But the dominant members of the bacterial community (OTUs with a mean relative abundance > 0.5%) also had higher prevalence, being present on more than

75% of the individuals (Fig. 3.2). There were 28 dominant OTUs present in male cloacas and these were all classified in the phyla Proteobacteria, Actinobacteria, Firmicutes, and Tenericutes (Fig. 3.2). The two OTUs with the highest overall relative abundance were *Mycoplasma* sp. (Greengenes OTU # 644706; 23%, Fig. 3.2) and *Acinetobacter lwoffii* (Greengenes OTU # 159711; 11%). The core microbiome (bacteria present on > 90% of birds) was composed of 45 OTUs belonging to six bacterial families (Pseudomonadaceae, Micrococcaceae, Staphylococcaceae, Aerococcaceae, Sphingomonadaceae, and Moraxellaceae) (Fig. 3.3). The core contained many of the dominant OTUs, but also contained many OTUs at lower abundance.

Testosterone and cloacal bacterial alpha diversity

All of the sampled males (N = 16) were in breeding condition and their testosterone levels varied from 0.11 to 9.05 ng/ml (Average: 2.5 ng/ml). There were no differences in testosterone levels among the individuals from the three populations (ANOVA, $F = 2.3$, $P = 0.13$). We found that bacterial phylogenetic diversity was positively correlated with circulating testosterone levels (LMM, $t = 2.2$, $P = 0.04$, Fig. 3.4a), such that males with higher testosterone levels had a more taxonomically diverse cloacal microbiome. However, there was no relationship between circulating testosterone levels and OTU richness (LMM, $t = 0.81$, $P = 0.43$, Fig. 3.4b).

Testosterone and cloacal bacterial community structure (beta diversity)

When comparing cloacal bacterial community structure among populations, we found a substantial amount of variation. In general, cloacal communities of birds from Tena and Yanayacu clustered together and formed a distinct group from Papallacta birds (ANOSIM, Global $R = 0.42$, $P = 0.0003$, Fig. 3.5), and dispersion analyses indicated that all populations had equal variability (Permdisp, $F = 2.01$, $P = 0.16$). Testosterone levels were not associated with cloacal bacterial community structure when the relative abundance of each OTU was taken into account (Weighted UniFrac, Mantel test, $r = -0.02$, $P = 0.55$). However, analyzing only presence/absence of OTUs resulted in

testosterone levels being associated with shifts in the composition of the bacterial communities (Unweighted UniFrac, Mantel test, r = 0.38, $P = 0.005$), with low testosterone individuals (Log Testosterone < -0.5 ng/ml) having different cloacal bacterial communities compared to other birds (Fig. 3.5). Similar results were obtained when analyzing the populations separately: Papallacta (Spearman, r = 0.61, $P = 0.014$) and Tena (Spearman, r = 0.65, $P = 0.025$). The population from Yanayacu could not be statistically analyzed with a Mantel test because of a low sample size, but Figure 5 suggests that the low testosterone male from Yanayacu also has a different community composition. Visualization of the core microbiome with a heatmap revealed that the low testosterone males were missing, or had low relative abundance of, some of the most abundant OTUs in the other birds (Fig. 3.3).

Pathogenic bacteria

The relative abundance of Chlamydiae was positively correlated with testosterone levels (Table 3.1, Fig. 3.6a), but relative abundances of the other five groups containing potential pathogenic bacteria were not correlated with the male's testosterone levels (Table 3.1, Fig. 3.6). The Chlamydiales sequences present in our samples only aligned to the *Chlamydia psittaci* 6BC genome with a similarity of 82-88%. When comparing Chlamydiae-infected and uninfected birds, we found no difference in the gene counts associated with "infectious disease" in the estimated metagenome (Kruskal-Wallis, $\chi^2 = 0.012$, $P = 0.91$). Lastly, there were no significant differences between Chlamydiae-infected and uninfected birds in their body mass (*t*-test, t = -0.35, $P = 0.7$) or body condition (*t*-test, t = 0.96, $P = 0.36$).

Discussion

We found that while overall bacterial richness did not vary based on testosterone levels, the cloacas of males with high testosterone had more phylogenetically diverse bacteria than low testosterone males. The higher phylogenetic diversity in the high testosterone males hints at two non-exclusive possibilities: (1) testosterone is immunosuppressive or

alters the cloacal environment, thus facilitating the establishment of new bacteria, and/or (2) testosterone alters male behavior, making them more promiscuous and thereby increasing exposure to bacteria. Evidence for the immunosuppressive effect of testosterone in relation to bacterial infection is scant. However, some studies have examined testosterone levels in relation to innate immune functions against bacteria. These studies have reported mixed results (Ezenwa, Stefan Ekernas & Creel 2012). In an assessment of plasma bacterial-killing ability, one study found that testosterone enhanced the immune response of food-supplemented lizards (Ruiz *et al.* 2010), while a study in red-winged blackbirds (*Agelaius phoeniceus*) found a negative relationship between plasma bacterial-killing ability and testosterone levels (Merrill *et al.* 2015). These results suggest that, at least in regards to bacteria in the bloodstream, males with high testosterone might have a higher risk of bacterial infections, but that risk could be condition dependent. Besides the plasma bacterial-killing ability, in the case of bacteria living on the cloacal skin, protection against infection can also include anatomical barriers (mucus, skin), chemical barriers (pH), or resident microbes (commensal bacteria). Testosterone could potentially down-regulate any of these defenses and make it easier for new bacteria to establish. For example, in an experimental study, testosterone implants slowed wound healing in rufous-collared sparrows (Moore & Small 2011); a slow response to tissue regeneration could make it easier for bacteria to colonize.

Testosterone mediated behavior could also lead to increased cloacal bacterial diversity as higher testosterone levels are associated with higher copulation rates, which increases exposure to diverse bacteria. It is well established that testosterone affects behaviors linked to reproduction, such as territoriality and copulation rate, and as a consequence, it influences rates of potentially infectious contact (Mougeot *et al.* 2005; Grear *et al.* 2009). In experimental studies where testosterone was artificially increased, white-crowned sparrows (*Zonotrichia leucophrys*) (Wingfield 1984) and dark-eyed juncos (*Junco hyemalis*) (Raouf *et al.* 1997) both were more likely to be polygamous. Such behaviors that result in increased cloacal contact rates between individuals could influence microbial spread through the population (Kulkarni & Heeb 2007). Experiments involving testosterone implants and contraceptive devices (Michl *et al.* 2002; White *et al.* 2010)

would be needed to explicitly test the mechanism that could explain the link we found between high testosterone and increased phylogenetic diversity of the cloacal bacterial community.

We also found that there was a correlation between testosterone levels and the composition of the cloacal microbiome, with low testosterone individuals having different cloacal bacterial communities compared to the mid- and high-testosterone birds (Fig. 3.5). We expected that if high testosterone males were mating promiscuously, then they would be accumulating more rare bacteria from their multiple mates and would have more variable bacterial communities. For example, greater variability in cloacal microbial composition in polyandrous females of superb fairywrens (*Malarus cyaneus*), white-browed scrubwren (*Sericornis frontalis*), and common lizards (*Zootoca vivipara*) was best explained as a result of the sexual transmission of different bacterial strains by multiple mates (Poiani & Gwozdz 2002; White *et al.* 2011). Indeed, sexually transmitted diseases have been postulated as a drivers of mating system evolution (Lombardo 1998; Ben Ashby & Gupta 2013). It is possible that the community similarity between the mid- and high-testosterone males could be an indication of a threshold effect, where at certain testosterone levels the amount of contacts increases resulting in a level of exposure sufficient to acquire the microbiome characteristic of mid- and high-testosterone males. Two lines of evidence suggest that the low testosterone males differ from the others because they are missing some bacteria. First, when we analyzed OTU abundance (weighted UniFrac) in relation to testosterone levels, the pattern in community structure disappeared, suggesting that presence/absence of bacteria is more important than relative abundance in driving the observed patterns. Second, the heatmaps showed that low testosterone males were missing many OTUs from their core microbiomes (Fig. 3.3a, c), indicating that their community structure is different because they do not have some OTUs that are abundant in most of the other birds. Alternatively, the low-testosterone individuals with diverging microbial communities might be facing a challenge, such as active infection, that impedes them from raising their testosterone to normal breeding levels.

The relative abundance of Chlamydiae was positively associated with testosterone levels, suggesting a possible cost of testosterone by increasing the risk of infection with a sexually transmitted pathogen. Chlamydiae are obligate intracellular bacteria that rely on their host cell for energy and nutrients (Brand 1989). They have a widespread geographic distribution, infect a broad spectrum of species (Kaleta & Taday 2003), and have a high lineage diversity as revealed by environmental and clinical samples (Corsaro & Venditti 2004). Infected birds shed Chlamydia in fecal material (Vanrompay *et al.* 1995; Andersen 1996), making it possible for these gastrointestinal pathogens to get sexually transmitted during cloacal contact. A commonly known infection in birds is produced by *Chlamydia psittaci*. The impacts of *C. psittaci* on wild populations are still poorly understood, but it is thought to typically have mild to moderate virulence, with low mortality rates in avian hosts (Brand 1989; Vanrompay *et al.* 1995). However, on some occasions large die-offs have been reported in several bird species (Vanrompay *et al.* 1995; Andersen & Franson 2007 and references therein). Based on the initial sequences, *Chlamydia psittaci* was not found in our samples, but we did find five other OTUs belonging to the Chlamydiales order (Fig. 3.6). The low match between the Chlamydiales sequences and the *C. psittaci* 6BC genome confirmed that *C. psittaci* was not infecting these birds. Furthermore, the infected individuals did not have a lower body condition index, nor did we find differences in the relative abundance of predicted infectious disease genes between Chlamydiae-infected and uninfected birds. This initial evidence suggests that the Chlamydiae infection we found does not affect body condition in these birds. In the metagenome analysis, it is possible that the sampled Chlamydiae do not have any known infectious disease genes, and/or there is still not enough information about these bacteria from free-living populations.

We found that the cloacas of rufous-collared sparrows were dominated mostly by four bacterial phyla: Proteobacteria, Tenericutes, Firmicutes and Actinobacteria. Other studies that have investigated cloacal bacterial composition in birds found the same dominant phyla, but generally in different proportions (Xenoulis *et al.* 2010; Ruiz-de-Castañeda *et al.* 2011; Santos *et al.* 2012; van Dongen *et al.* 2013). This suggests that, at a broad taxonomic resolution, there is convergence in the dominant phyla residing in the cloaca

of taxonomically diverse birds. Because a significant proportion of the cloacal microbiota is probably derived from the gastrointestinal microbiota, it is not surprising that the dominant phyla we detected are similar to the dominant phyla found in the crop, ileum, cecum, and feces of other avian taxa (see references in Kohl 2012; Hird *et al.* 2014; Mirón *et al.* 2014; Sergeant *et al.* 2014; Waite & Taylor 2014; Mohd Shaufi *et al.* 2015). However, different communities are found in mammals. Mammalian guts are often dominated by Firmicutes and Bacteroidetes (Ley *et al.* 2008), and the vaginal microbiome of baboons (*Papio anubis*) was dominated by Bacteroidetes, Fusobacteria, and Firmicutes (Uchihashi *et al.* 2015). Overall, while there is some variability in the bacterial community composition among individuals of a single species, the dominant taxa and their abundance appears to be host species specific (Kohl 2012; but see Hird *et al.* 2014; Waite & Taylor 2014).

At a finer taxonomic scale, the most abundant OTUs in our samples were *Mycoplasma* sp. (23%) and *Acinetobacter lwoffii* (11%). The genus *Mycoplasma* contains over 100 species, most of which are likely commensals or facultative parasites. *Mycoplasma* have been reported in a variety of organisms, including humans (Ling *et al.* 2010), birds (Ley, Berkhoff & McLaren 1996), and tortoises (Wendland *et al.* 2010). The genus *Acinetobacter* is ubiquitous, living in soil and animals, but has also been associated with clinical infections in humans (Doughari *et al.* 2011), specifically with gastric illness from *Acinetobacter lwoffii* (Rathinavelu, Zavros & Merchant 2003). *Acinetobacter lwoffii* was also detected in high prevalence on eggshells of pied flycatchers (*Ficedula hypoleuca*) (Ruiz-de-Castañeda *et al.* 2011). Other studies on birds reported *Corynebacterium* sp. and *Lactobacillus* sp. as dominant bacteria in cloacas of black-legged kittiwakes (*Rissa tridactyla*) (van Dongen *et al.* 2013), *Staphylococcus saprophyticus* in cloacas of wild parrots (mealy Parrots, *Amazona farinosa*, blue-and-yellow Macaws, *Ara ararauna*, and red-and-green Macaws, *Ara chloropterus*) (Xenoulis *et al.* 2010), and *Lactobacillus* sp. in the gut of house sparrows (*Passer domesticus*). All of these genera were dominant members of the microbiome in rufous-collared sparrows, except for *Lactobacillus*, which was present, but at low abundance. Similarly, Mohd et al. (2015) also found a low abundance of *Lactobacillus* in the ilea and ceca of chickens. Our results showed that the

OTUs present in the core microbiome had markedly different relative abundance among birds (Fig. 3.3), which suggests that environmental factors such as diet, age, and physical location could also be playing an important role in structuring the cloacal microbiome, making it unique for each individual. Indeed, we found that 24% of the OTUs were unique to each individual, similar to the 28.1% reported for the cloacal microbiome of spotted towhees (*Pipilo maculatus*) (Klomp et al. 2008) and 25% in kittiwakes (*Rissa tridactyla*) (White et al. 2010).

To conclude, the benefits of having high levels of testosterone have been demonstrated both in experimental and observational studies, but the costs are not nearly as well-established. Our study links cloacal bacterial phylogenetic diversity, and at least one group of potential pathogens, with testosterone levels. This latter result provides some support for the immunocompetence handicap hypothesis. While previous studies have proposed STIs as a potential selective force for the evolution of animal mating systems (Lombardo 1998; Ben Ashby & Gupta 2013), we propose that STIs could also be a substantial cost of maintaining high testosterone levels in male vertebrates.

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Tables

Table 3.1. Relationship between the relative abundance of the classes containing potential pathogens and testosterone levels. Reported are parameter estimates and *P*-values from the generalized linear models using a gamma distribution. Parameter estimates are on the inverse scale.

Bacterial Class	Prevalence (%)	Relative abundance; Mean (min – max)	Estimate	SE	*P*-value
Chlamydiae	38	0.006 (0 - 0.042)	-353.7	124.7	0.013
Actinobacteria	100	7.7 (0.4 - 22.3)	-0.062	0.064	0.3
Bacilli	100	14.7 (0.1 - 86.7)	-0.021	0.047	0.7
Gammaproteobacteria	100	30.2 (0.6 - 85.6)	0.015	0.014	0.3
Epsilonproteobacteria	88	5.6 (0 – 86.8)	-2.049	2.057	0.3
Mollicutes	100	25.9 (0.003 - 78.0)	-0.021	0.028	0.5

Figure legends

Figure 3.1. Average relative abundance of bacterial phyla in the cloaca of rufous-collared sparrows (n = 16). Four dominant phyla represented 93% of the cloacal microbiome (Proteobacteria, Tenericutes, Firmicutes, and Actinobacteria). Birds are organized in order of increasing testosterone levels.

Figure 3.2. Mean relative abundance (bars) and prevalence (proportion of individuals that have a particular OTU; closed circles) of dominant bacteria present in cloacas of male rufous-collared sparrows. Only operational taxonomic units (OTUs) with a mean relative abundance greater than 0.5% are shown. The lowest taxonomic resolution that could be defined for OTU identification is shown on the y-axis. Bacterial phylum for each OTU is listed in parentheses (Pro = Proteobacteria, Act = Actinobacteria, Fir = Firmicutes, Ten = Tenericutes). Error bars represent standard error.

Figure 3.3. Heat map of the relative abundances (panels A and B) and presence/absence (panels C and D) of the core microbiome (bacteria present on > 90% of birds, N = 45). Rows indicate unique OTUs and columns indicate individual birds. Taxon list in panels A and C is organized by the average relative abundance of core OTUs in all birds. Taxon list in panels B and D is organized by the average relative abundance of OTUs in the three low testosterone birds (outlined by black box). The lowest taxonomic resolution that could be defined for OTU identification is shown. Bacterial phylum for each core OTU is listed in parentheses (Pro = Proteobacteria, Act = Actinobacteria, Fir = Firmicutes, Ten = Tenericutes, Bac = Bacteroidetes, Cya = Cyanobacteria, The = Thermi).

Figure 3.4. Relationship between circulating testosterone levels with A. cloacal bacterial phylogenetic diversity and B. Bacterial OTU richness. All males in this analysis were in breeding condition.

Figure 3.5. Ordination plot of nonmetric multidimensional scaling (NMDS) using unweighted UniFrac pairwise distances. Symbol shapes represent populations and fill color represents circulating testosterone levels. Increasing distance between symbols indicates increasing dissimilarity in phylogenetic composition (i.e. points that are closer to each other share more bacterial OTUs).

Figure 3.6. The relationship between the relative abundance (in proportion of sequences) of avian pathogenic bacterial phyla and male testosterone levels. Gamma regressions were used to test the relationship between the pathogens' relative abundance and circulating testosterone levels. Panels (A)-(F) depict the relationship between testosterone and specific bacterial taxa as defined in each panel.

Figures

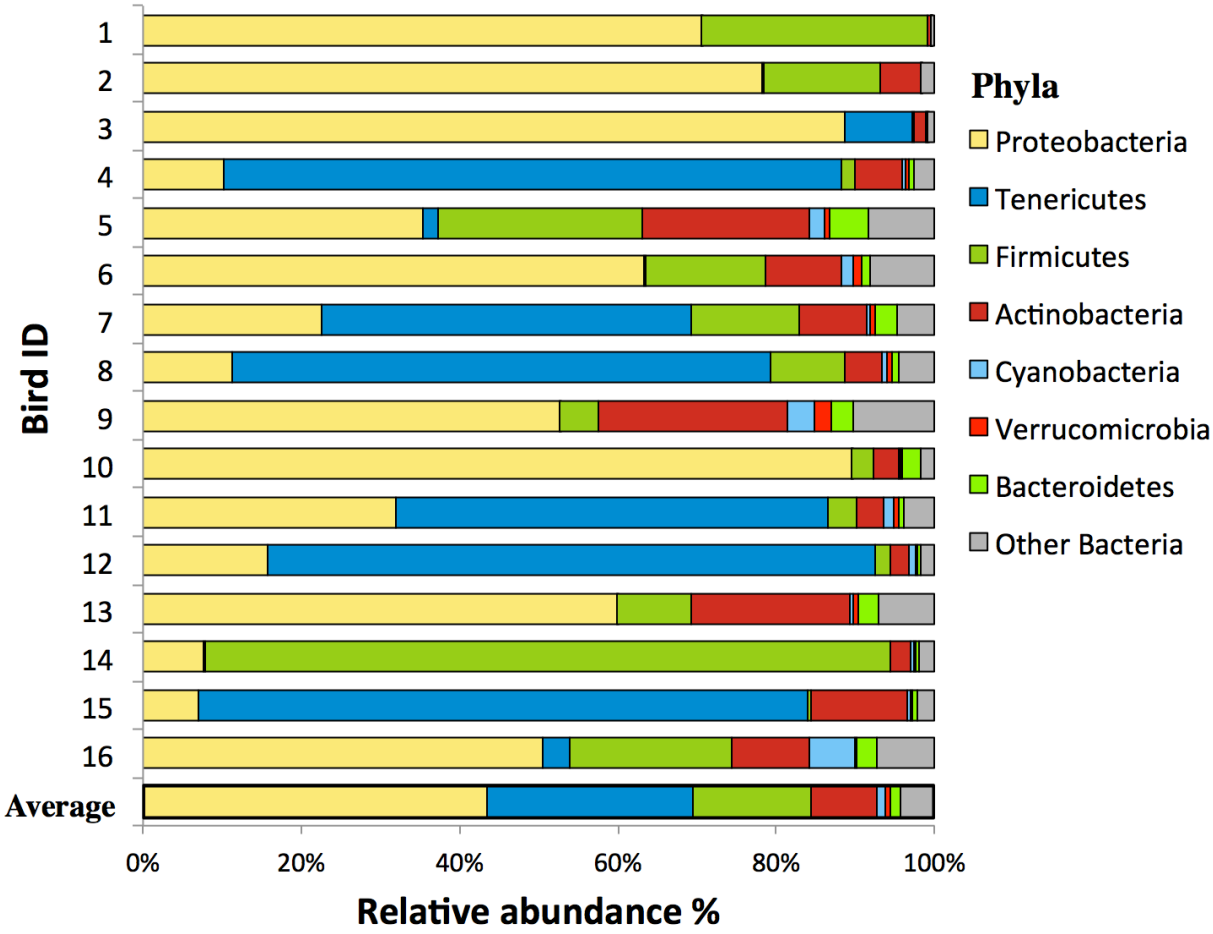

Figure 3.1. Average relative abundance of bacterial phyla in the cloaca of rufous-collared sparrows

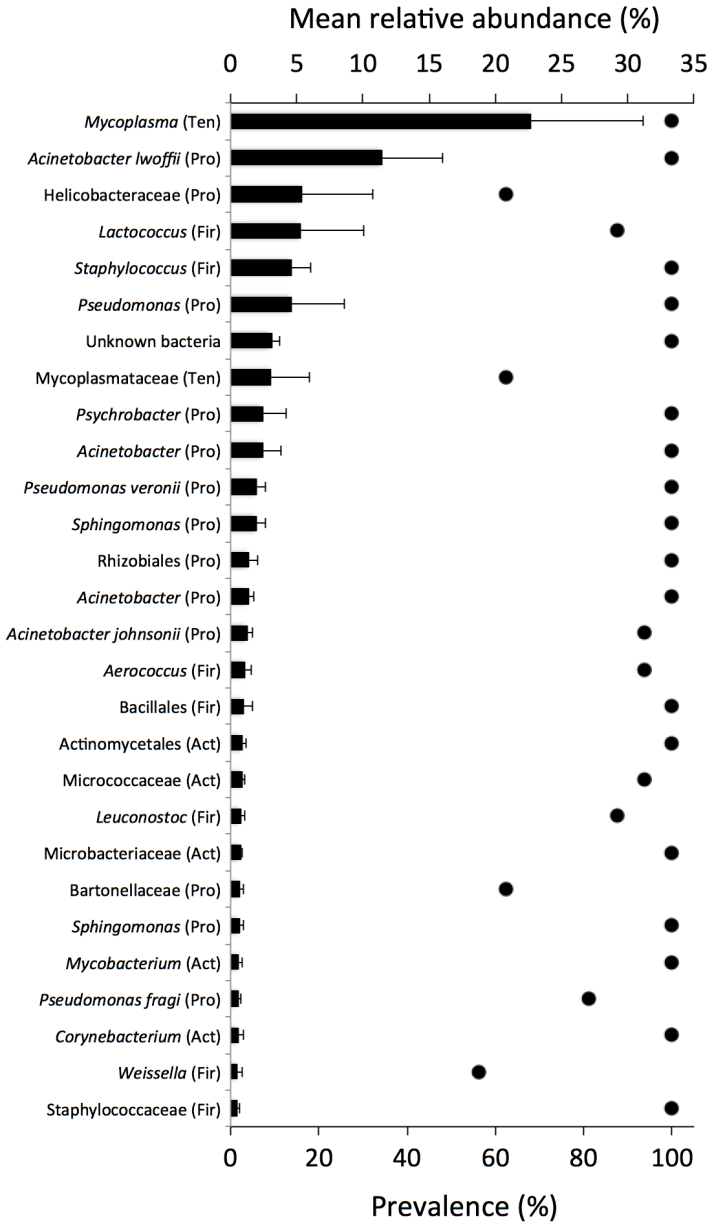

Figure 3.2. Mean relative abundance and prevalence of dominant bacteria present in cloacas of male rufous-collared sparrows

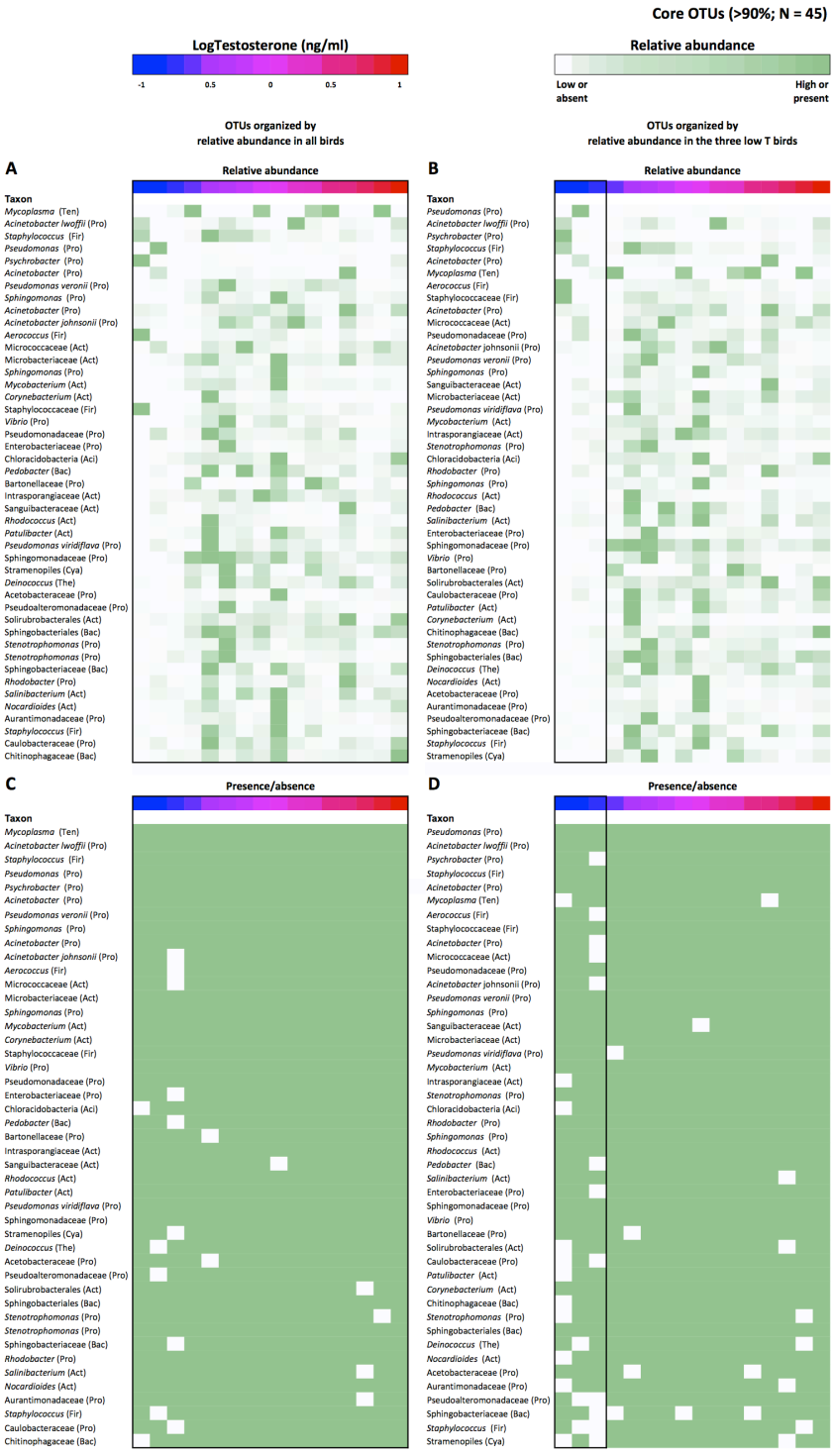

Figure 3.3. Heat map of the relative abundances and presence/absence of the core microbiome

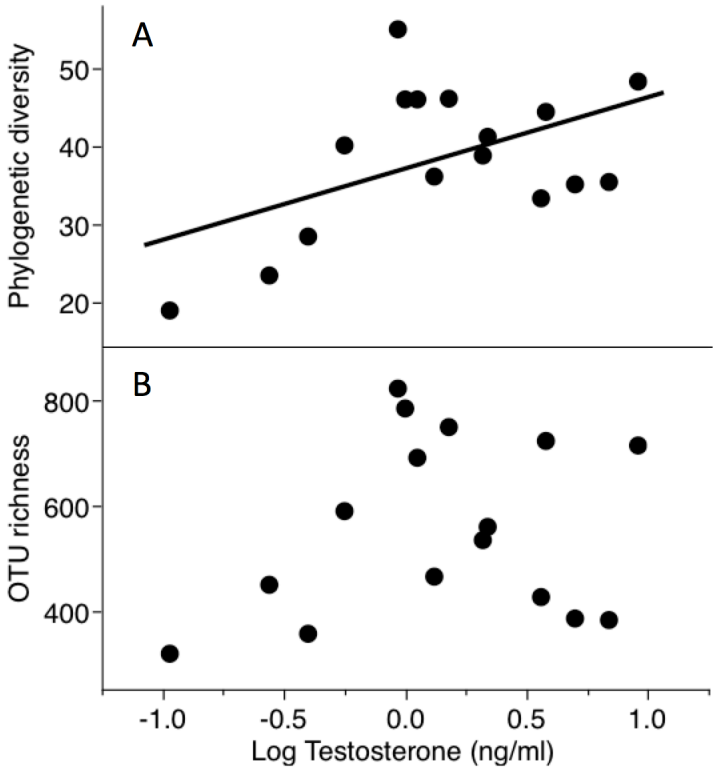

Figure 3.4. Relationship between circulating testosterone levels with A. cloacal bacterial phylogenetic diversity and B. Bacterial OTU richness.

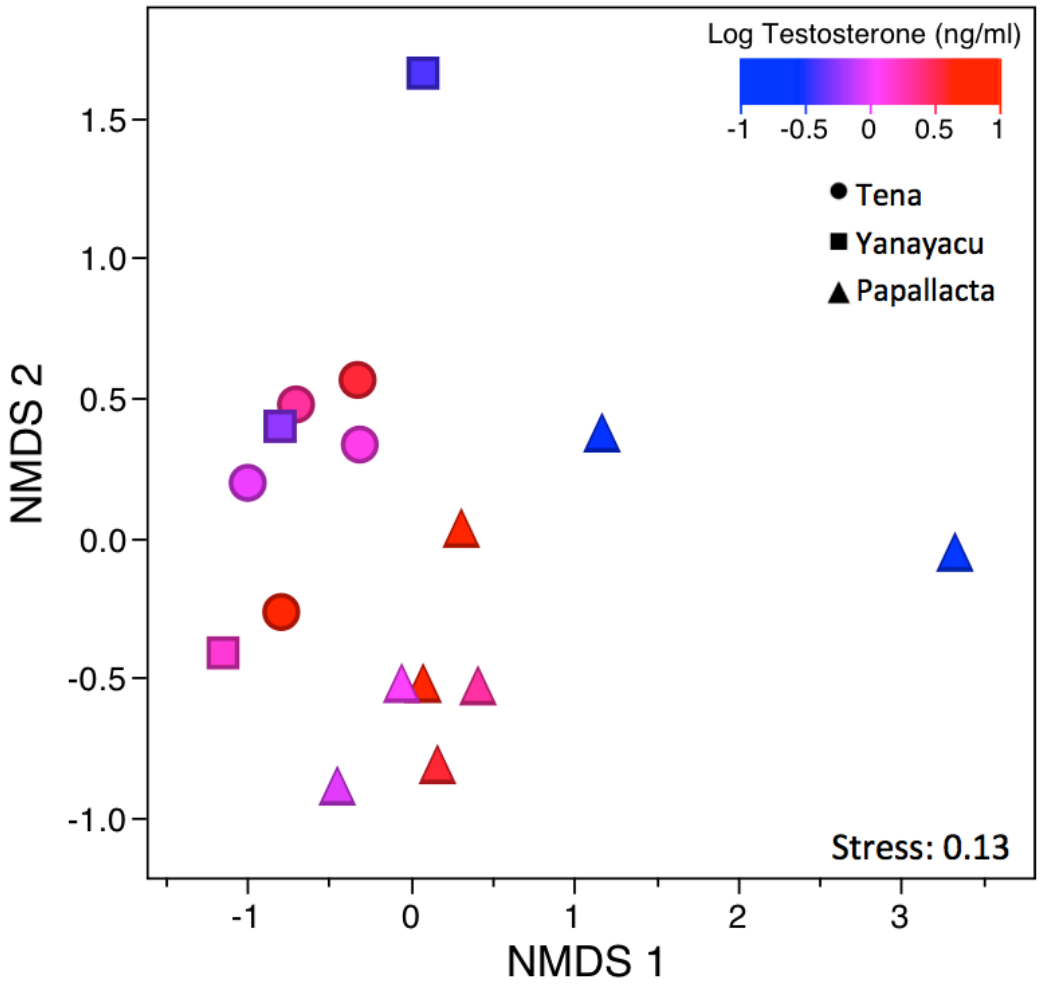

Figure 3.5. Ordination plot of nonmetric multidimensional scaling (NMDS) using unweighted UniFrac pairwise distances

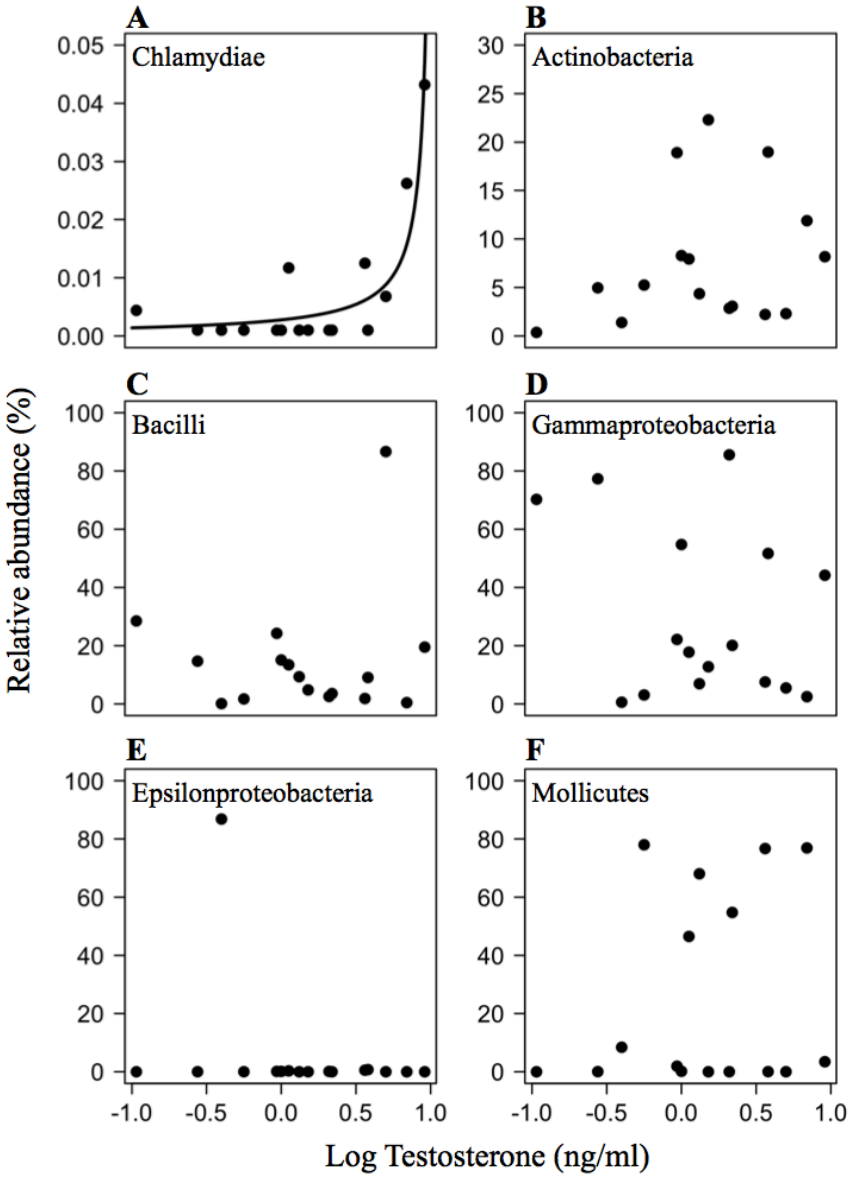

Figure 3.6. The relationship between the relative abundance (in proportion of sequences) of avian pathogenic bacterial phyla and male testosterone levels

CHAPTER IV. VARIATION IN THE CLOACAL MICROBIOME IN A WILD BIRD: THE ROLE OF REPRODUCTION AND SEX

Camilo Escallón, Lisa K. Belden, Ignacio T. Moore

Abstract

The microbial communities that reside on animals are dynamic and can be affected by the behavior and physiology of the host. These microbial communities provide critical functions to their host, but can also represent health costs if constituents are pathogenic. In birds, bacteria residing in the cloaca form a complex community including both transient gut bacteria and sexually transmitted bacteria. During the breeding season there is an increase in physical contacts among individuals and physiological tradeoffs can draw resources from self-maintenance, all of which can increase exposure to bacteria or facilitate infection. Additionally, males and females differ in many aspects of their physiology and behavior, which can make the risk of infection sex-dependent. As such, we hypothesized that (1) cloacal bacterial communities would be more diverse during the breeding season than in the non-breeding season, (2) males would have more diverse cloacal bacterial communities than females, and (3) individuals would accumulate bacterial species across breeding seasons. We surveyed the cloacal microbial communities in free-living male and female rufous-collared sparrows (*Zonotrichia capensis*) through sequential breeding and non-breeding seasons in the Andes of Colombia. We found that the cloacal microbiome was different between the sexes when they were in breeding condition. Further, in males, but not in females, the bacterial community became more diverse with the onset of reproduction by increasing its phylogenetic diversity and OTU richness and then decreased its diversity as they transitioned to non-breeding condition. Individuals sampled across sequential breeding seasons did not accumulate more bacterial species but changed their community composition compared to their previous season. Among males, those with higher

testosterone levels during the breeding season had a more phylogenetically diverse cloacal microbiome. Our results show that the cloacal microbiome in birds is dynamic and responsive to breeding condition and sex of the host.

Introduction

The microbes, and their associated genomes, that reside in and on animals are termed the microbiome. Each individual's microbiome is dynamic and can change through time (Turnbaugh *et al.* 2007; McFall-Ngai & Hadfield 2013). The relationship of animals with their microbiome likely has major implications for the evolution, behavior, and physiology of their hosts, by contributing critical functions to the host, and by being a selective force in the form of pathogenic microbes (Archie & Theis 2011; Ezenwa *et al.* 2012; Clay 2014). Microbes are most abundant in the vertebrate gut, but they also line most other mucosal surfaces, including the nasal passages, lungs and urogenital tract. In the vertebrate gut, most microbes colonize early in life from a mixture of environmental and, for some species, maternal sources (van Dongen *et al.* 2013; Smith & Mueller 2015). However, on other mucosal surfaces, microbial symbionts are picked up almost entirely from direct contact with other individuals. For example, the urogenital tract is an interesting case because pathogenic microbes can invade the normal, healthy community of microbes through sexual contact and can thus represent a potential cost of sexual reproduction (Sheldon 1993; Lockhart, Thrall & Antonovics 1996). The basic natural history of the microbes that reside in the urogenital tract is not well understood. For example, males and females vary in physiology and reproductive behavior, and yet it is not clear how these differences relate to the urogenital microbiome composition. Further, as reproductive activity often varies seasonally, the urogenital microbiome might vary seasonally as well. The widespread presence of sexually transmitted infections (STI) in wild animals underlines the importance of understanding the factors that contribute to variation in the urogenital microbiome.

The cloaca of birds, reptiles, and amphibians is a combined opening for the digestive, urinary, and reproductive systems. Thus, the cloacal microbiome is a mixture of bacteria

coming primarily from the digestive tract and from contacts during copulation, making it a complex bacterial community. Establishment success of new members of the bacterial community depends on the host's immune system, the physical environment of the cloaca, and the current bacterial community structure, which can affect the ecological niche of immigrant bacteria (Poiani 2010). Most bacteria inhabiting the cloaca are likely not pathogenic, and instead may be beneficial to the host, so we will refer to them as STIs, and not as sexually transmitted pathogens (Lombardo, Thorpe & Power 1999; Smith & Mueller 2015). Beneficial bacteria can provide crucial physiological functions to their host, for example by helping to extract energy and nutrients from food resulting in increased survival (Ley *et al.* 2008). They can also have longer-term benefits, including potentially mate choice, as individuals with beneficial microbes could be preferred as mates (Lombardo *et al.* 1999; Smith & Mueller 2015). However, there are several sexually transmitted bacteria that are pathogens and may ultimately impose a selective pressure on the evolution of polygamous mating systems (Sheldon 1993; Lombardo 1998; Poiani 2010). From an epidemiological perspective, the spread of STIs through the population is largely influenced by individuals who have a large number of sexual partners (Smith & Dobson 1992; Ben Ashby & Gupta 2013). For instance, promiscuity has an impact on the overall community composition, increasing bacterial diversity in the cloacas of polygamous lizards (White *et al.* 2011), and in the vaginal microbiome of mice (MacManes 2011) and primates (Yildirim *et al.* 2014). Thus, multiple matings may lead to a greater sampling of the bacterial metacommunity within the host population, making the evolution of promiscuity dependent on the costs and benefits of each type of STI.

In vertebrate animals, life-cycle events are partitioned into discrete stages such as growth (i.e. self maintenance), reproduction, and offspring rearing. Coupled with those distinct stages are specific behaviors, hormone levels, and immune conditions, which can influence bacterial communities. In particular, reproduction has three features that can affect bacterial communities living on individual animals. First, there is an increase in physical contacts and consequently more exposure to infectious microorganisms (Anderson & May 1979). For example, in female kittiwakes (*Rissa tridactyla*) the cloacal microbiome started to change after cloacal contacts were experimentally blocked by a

contraceptive device, suggesting that the cloacal microbiome is dynamic and can be affected by copulatory activity (White *et al.* 2010). Furthermore, it has been hypothesized that there could be a sex bias in the sexual transmission of bacteria by contamination of the seminal fluid. Kulkarni and Heeb (2007) experimentally infected zebra finches (*Taeniopygia guttata*) with *Bacillus licheniformis* and demonstrated that this bacterium was transmitted through sexual contact at a higher rate from males to females than vice versa. Second, male testosterone levels are usually elevated during reproduction, and the hormone has often been investigated as a mediator of life-history trade-offs between immediate reproduction and survival (Hau 2007). Testosterone could influence bacterial communities by promoting behaviors that increase contact rates, such as extra pair copulations (Wingfield 1984; Raouf *et al.* 1997; Grear, Perkins & Hudson 2009), or by suppressing immune function (Folstad & Karter 1992). Immune suppression by testosterone was proposed as a hypothesis to explain the costs of testosterone-dependent sexual signals, but this hypothesis has only mixed support (Roberts, Buchanan & Evans 2004; Roberts & Peters 2009). We previously found a positive relationship between testosterone and cloacal bacterial phylogenetic diversity in wild male rufous-collared sparrows (*Zonotrichia capensis*) sampled during the breeding season (Escallón et al., *In prep*). Third, reproduction is an energetically challenging life-history stage (Stearns 1992; Sheldon & Verhulst 1996), which can result in changes in the cloacal environment in preparation for breeding (i.e. changes in pH, mucus secretion, and tissue bactericidal capacity). This could facilitate colonization by opportunistic bacteria or by bacterial species dependent on the bird's breeding cycles, such as STIs (Martinez-Bakker & Helm 2015).

In this study, we investigated whether reproductive condition is associated with changes in the cloacal microbial communities in free-living rufous-collared sparrows by comparing the individual cloacal microbiome of males and females through sequential breeding and non-breeding seasons. This bird is socially monogamous, but has a high level of extra pair paternity, with an average of 48% of the nests containing nestlings that do not belong to the social father (Eikenaar *et al.* 2013). Thus, cross infection of cloacal bacteria among individuals is likely. Rufous-collared sparrows are year-round residents

in tropical regions, where there is constant food availability, and in some populations they are able to breed several times a year (Miller 1962). We tested two hypotheses that could explain temporal variation in bacterial cloacal communities in rufous-collared sparrows. In the bacterial clearance hypothesis, we predicted that bacteria are cyclically added and lost in each breeding season, such that cloacal bacterial diversity of adults would be higher during the breeding season than in the non-breeding season. In the bacterial accumulation hypothesis, we predicted that cloacal bacterial diversity would increase through sequential breeding seasons, as each season presents additional opportunities for microbial transmission through copulations and accumulation of diverse bacteria over time. As a comparison, we also sampled the cloacas of juveniles, who should have low bacterial diversity levels, because they have not yet copulated and should only be infected with bacteria mainly associated with the digestive tract.

Methods

Sampling of cloacal bacteria and testosterone

We studied a wild breeding population of rufous-collared sparrows (*Zonotrichia* capensis) in the rural town of Guatavita in the Cundinamarca Department of the Eastern Colombian Andes (4.978158°; -73.791961°; 2940 m a.s.l.). At this site, there is high rainfall variability throughout the year, but there is a small average decrease from December to February, and an increase from May to September. In addition, temperature is nearly constant (~13º C) throughout the year (Instituto de Hidrología, Meteorología y Estudios Ambientales, Colombia). Tropical rufous-collared sparrows have an extended breeding season, and there can be some asynchrony in the timing of the breeding season among individuals (Moore 2005; Class *et al.* 2011). In this population, the largest proportion of individuals were in breeding condition between May and September (Males, 77%, N = 181/235; Females, 61%, N = 89/142), whereas from December to January the proportion of breeding individuals was lower (Males, 18.4%, N = 14/76; Females, 17.6%, N = 9/51).

We sampled rufous-collared sparrows by setting up mist-nets and captured them passively only during the two hours immediately after dawn. Upon capture, we performed a cloacal swab on every bird, and males were bled to measure plasma testosterone. To collect bacteria from the cloaca we used PurFlock® sterile micro-ultrafine nylon-tipped swabs (Puritan, USA). Samples were collected by gently introducing a swab approximately 4 mm into the cloaca and rotating it once. After collection, swabs were stored in RNALater (Ambion, USA) and frozen for later DNA extraction. For males, we determined breeding condition based on the height of the cloacal protuberance (Breeding = greater than 5 mm, which corresponds to fully grown testes) (Moore 2005). For females, we defined breeding condition as the presence of a brood patch. Juveniles were identified based on plumage coloration. This investigation adhered to animal-care protocols approved by the Institutional Animal Care and Use Committee of Virginia Tech.

The sampling for cloacal bacteria in this population was done approximately every five months for three consecutive years (2011-13). All captured birds were banded with a numbered metal ring, which allowed individual identification on subsequent recaptures. To analyze the changes in cloacal microbiome diversity between breeding and non-breeding seasons, we selected the birds that had been sampled in either breeding or non-breeding condition and then resampled five to six months later in a different breeding condition from the previous one. Similarly, to analyze changes in bacterial diversity between breeding seasons, we selected individuals that had been sampled in breeding condition and then sampled them again ten to twelve months later in breeding condition. This left us with paired samples across breeding stages with a total of 122 cloacal swabs collected from 27 males, 22 females, and 9 juveniles during that time.

Bacterial sample preparation and sequencing

To remove the bacteria from the collection swab, we vortexed the tube and then centrifuged it at 12,000 RPM for five minutes to form a pellet in the bottom of the tube. The RNALater was pipetted out and the remaining pellet was used to extract bacterial

DNA. We extracted DNA from each sample with the DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA, USA). The V4 region of the 16S rRNA gene was amplified by PCR using the primers 515F and 806R (Caporaso *et al.* 2011). The reverse primer contained a 12 base error-correcting Golay code (Fierer *et al.* 2008), which was used to uniquely tag PCR products of each sample. We prepared PCR reactions similar to Costello et al. (2009). Briefly, triplicate reactions of each sample contained 1µl template DNA, 12µl DNA-free PCR water (MO-BIO, Carlsbad, California), 10µl 2.5x HotMasterMix (5 PRIME, Gaithersburg, Maryland), 1µl of 20mg/ml bovine serum albumin (Fisher Scientific, Pittsburgh, Pennsylvania), and 0.5µl of each primer at 10µM concentration. We ran controls without template for each sample. We diluted extracted DNA samples that contained PCR inhibitors 1 to 10 in PCR water. The amplification conditions were as follows: an initial cycle for 3min at 94 ºC followed by 35 cycles of 34s at 94 ºC, 60s at 50 ºC, and 90s at 72 ºC, with a final cycle for 10min at 72 ºC. Triplicate reactions of each sample were pooled, visualized on a 1% agarose gel, and quantified with a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, California). An equimolar mixture of all the samples was then sequenced at the Dana-Farber Cancer Institute of Harvard University on an Illumina MiSeq instrument with a 250 bp paired-end strategy, following methods similar to Caporaso et al. (2012). To compensate for the low base diversity of the amplicon pool, the sample was run with a 10% PhiX control. Version 1.18.54 of the MiSeq Real-Time Analysis software (Illumina) was used to perform base calling and quality scoring.

Sequence data processing

The reverse reads were of low quality, therefore we only used the forward reads in our analysis. Using the Quantitative Insights Into Microbial Ecology pipeline (QIIME v. 1.9.1; Caporaso *et al.* 2010b), sequences were de-multiplexed and quality-filtered following methods similar to those described by Bokulich et al. (2013). Sequences were quality filtered, by discarding sequences if there were any ambiguous base calls, errors in the barcode, less than 50% of the read length had consecutive base calls with a phred quality score greater than 20, or there were more than 10 consecutive low-quality base

calls. After quality filtering, the number of reads retained per sample ranged from 2,408 to 177,819 (Average 36,116). We imported the data into Geneious and filtered an additional 830,258 reads that that were assembled to PhiX and were not at 250 bp, leaving a final count of 5,145,224 reads. We then clustered the quality-filtered sequences into operational taxonomic units (OTUs, the bacterial equivalent of species) at a sequence similarity threshold of 97% with the UCLUST method (Edgar 2010) and a minimum cluster size of 0.001% of the total reads (Bokulich *et al.* 2013). Sequences were first clustered against the Greengenes database (May 2013 release; DeSantis *et al.* 2006). Sequences that did not match the database were then *de novo* clustered at a 97% sequence similarity threshold. The most abundant sequence for a given cluster was assigned as the representative sequence for that OTU. Taxonomy was assigned for each OTU with the RDP classifier (Wang *et al.* 2007) at a 50% confidence threshold and the Greengenes database, as recommended by Claesson et al. (2009) for the v4 region of the 16s rRNA gene. We filtered out mitochondria and chloroplast sequences from the OTU table. We aligned the representative sequences to the Greengenes database with PyNAST (Caporaso *et al.* 2010a) and constructed a phylogenetic tree with FastTree (Price, Dehal & Arkin 2009). We rarefied all samples to 2,400 sequences prior to analysis to standardize sampling effort. At this point, species accumulation curves had plateaued, indicating that we captured most of the taxonomic diversity. Rarefaction resulted in the removal of two samples that had low sequences per sample (< 2,400 reads), so the final dataset consisted of 120 samples and 7101 unique OTUs.

Hormone analysis

To measure plasma testosterone levels, we took blood samples (ca. 250 µl) from the brachial vein within 10 min of capture and kept them on ice (less than 4 h) until centrifugation, when plasma was separated and frozen for later analysis. Plasma volumes ranged from 16–100µl (mean: 48µl). Total plasma testosterone concentration was measured by direct radioimmunoassay following the procedures of Moore et al. (2002). While we did measure total androgens (e.g. testosterone, 5α–dihydrotestosterone and others) in our direct assay, testosterone is the major androgen in birds, so we will refer to

our measured androgen levels as testosterone levels. The samples were run in a single assay, with an intra-assay variation of 18.8% and a limit of detection of ~0.16ng/ml. The testosterone antibody used was T-3003s (Fitzgerald: Catalog #20R-TR018W).

Statistical analysis

To analyze the differences in phylogenetic diversity and number of observed OTUs across breeding conditions and between breeding seasons, we used paired t-tests. We also calculated, for each individual's paired sample, the net change in diversity, by subtracting the initial diversity value from the final value, and compared the breeding transitions with a t-test. Analyzing the net change in diversity gave us information on the magnitude of the change as well as whether there are differences between groups. We computed measures of alpha diversity per individual (OTU richness and phylogenetic diversity) using QIIME. OTU richness is the number of OTUs present in an individual. Bacterial phylogenetic diversity is an estimate of phylogenetic breadth contained within each community.

To assess whether the bacterial community composition differed between sex, age, and breeding condition, we calculated pairwise weighted (OTU relative abundance) and unweighted (presence/absence of OTUs) UniFrac distances with the phyloseq package (McMurdie & Holmes 2013). UniFrac uses branch length overlap to calculate the amount of phylogenetic distance between pairs of communities, and avoids some of the disadvantages associated with comparing communities at only a single level of taxonomic resolution (Hamady & Knight 2009). We used principal coordinate analysis (PCoA) of weighted and unweighted UniFrac distances to visualize bacterial community composition. Differences between groups were evaluated for weighted and unweighted UniFrac distances with Adonis from the vegan package using 1000 permutations (Oksanen *et al.*). To test for different variance among groups, we used analysis of multivariate homogeneity of group dispersions (betadisper) that determines whether the variances of groups of samples are significantly different. To test for shifts in the community composition between breeding conditions, we performed t-tests with the first

axis of the PCoA. To visualize the differences in the core bacterial community, we calculated the core microbiota, defined as OTUs that were present in at least 90% of the birds on the rarefied data set, and plotted their relative abundance on a heat map. To detect shared OTUs between groups, we calculated the core microbiome individually for males, females, and juveniles.

To examine the relationship between male circulating testosterone levels and both phylogenetic diversity and number of observed OTUs, we used linear mixed models, assigning individual as a random factor to account for repeated samples per individual. We also ran an additional linear mixed model incorporating the breeding condition of each male. The distribution of plasma testosterone levels was normalized with a log transformation. All statistical analyses were carried out in the R environment (version 3.2.1) (R Development Core Team 2014).

Results

The cloacal microbiome between breeding and non-breeding seasons

Male birds transitioning from non-breeding to breeding condition increased their cloacal phylogenetic diversity by 46% (t = -4.3, $P = 0.001$, Fig. 4.1a) and the number of observed OTUs by 81% (t = -4.1, $P = 0.001$, Fig. 4.2a). When males transitioned from breeding to non-breeding, there was a strong trend towards a decrease of 21% for phylogenetic diversity (t = 2.3, $P = 0.058$, Fig. 4.1b) and no change in the number of observed OTUs (t = 1.6, $P = 0.15$, Fig. 4.2b). For females, the phylogenetic diversity and number of observed OTUs did not change during either reproductive transition. From non-breeding to breeding condition, phylogenetic diversity (t = -1.2, $P = 0.28$, Fig. 4.1c) and the number of observed OTUs (t = -0.4, $P = 0.67$, Fig 2c) did not change, and similarly there was no change from breeding to non-breeding in phylogenetic diversity (t = 1.7, $P = 0.17$, Fig. 4.1d) and in the number of observed OTUs (t = 1.6, $P = 0.19$, Fig. 4.2d). Analysis of the net change within individuals revealed a significant difference between reproductive states in phylogenetic diversity ($P = 0.0003$, Fig. 4.3a) and number of OTUs ($P = 0.001$,

Fig. 4.3b) in males but not in females (phylogenetic diversity, $P = 0.07$, Fig. 4.3a; number of OTUs, $P = 0.2$, Fig. 4.3b).

The cloacal community composition of breeding males was different from breeding females both when considering only the presence/absence of OTUs (Unweighted UniFrac, Adonis, psuedoF = 2.1, $P = 0.001$, $R^2 = 0.03$, Fig. 4.4a) and when considering OTU relative abundance (Weighted UniFrac, Adonis, psuedoF = 3.5, $P = 0.001$, $R^2 = 0.05$, Fig. 4.4b), but the cloacal community composition was not different between non-breeding males and non-breeding females (Unweighted UniFrac, Adonis, psuedoF = 1.0, $P = 0.4$, $R^2 = 0.03$, Fig. 4.4a; Weighted UniFrac, Adonis, psuedoF = 1.2, $P = 0.2$, $R^2 = 0.03$, Fig. 4.4b). Juveniles had a cloacal community composition most similar to adult males (Unweighted UniFrac, Adonis, psuedoF = 1.3, $P = 0.07$, $R^2 = 0.02$, Fig. 4.4a; Weighted UniFrac, Adonis, psuedoF = 1.3, $P = 0.19$, $R^2 = 0.02$, Fig. 4.4b), differing significantly from that of females in the unweighted, but not in the weighted distance, despite some overlap in the PCoA ordination (Unweighted UniFrac, Adonis, psuedoF = 1.3, $P = 0.03$, $R^2 = 0.02$, Fig. 4.4a; Weighted UniFrac, Adonis, psuedoF = 0.8, $P = 0.57$, $R^2 = 0.01$, Fig. 4.4b**)**. Significant multivariate dispersion among groups (Unweighted UniFrac, Beta dispersion, F = 3.5, $P = 0.03$) could be driving the significant Adonis results, as significant differences between females and juveniles may be caused by different within-group variation rather than different means across groups.

In males, the community composition between breeding and non-breeding birds was different when analyzing the presence/absence of OTUs (Unweighted UniFrac, Adonis, psuedoF = 2.8, $P = 0.001$, $R^2 = 0.04$, Fig. 4.5a) and OTU relative abundance (Weighted UniFrac, Adonis, psuedoF = 2.9, $P = 0.003$, $R^2 = 0.04$, Fig. 4.5b). In fact, with only one exception, the community composition has a consistent direction of change on the first multivariate axis of the unweighted analysis (Non-breeding to breeding, t = -4.5, $P = 0.001$, Fig. 4.6a; Breeding to non-breeding, t = 2.7, $P = 0.03$, Fig. 4.6b), and a similar, strong trend in the weighted analysis (Non-breeding to breeding, t = -2.1, $P = 0.058$, Fig. 4.7a; Breeding to non-breeding, t = 0.1, $P = 0.91$, Fig. 4.7b). In contrast, females' cloacal community composition was not different between breeding and non-breeding stages

(Unweighted UniFrac, Adonis, psuedoF = 1.1, $P = 0.19$, $R^2 = 0.02$, Fig. 4.5c; Weighted UniFrac, Adonis, psuedoF = 1.3, $P = 0.2$, $R^2 = 0.03$, Fig. 4.5d). However, there was a consistent direction of change, similar to that observed in males, for the first multivariate axis of the unweighted analysis (Non-breeding to breeding, t = -3.1, $P = 0.03$, Fig. 4.6c; Breeding to non-breeding, t = 3.1, $P = 0.04$, Fig. 4.6d). For the weighted analysis there was no consistent change from non-breeding to breeding (t = -1.8, $P = 0.14$, Fig. 4.7c) but a strong trend toward a consistent direction from breeding to non-breeding (t = 2.6, $P = 0.06$, Fig. 4.7d).

The core microbiome (bacteria present on > 90% of birds) was composed of eight OTUs belonging to the phyla Proteobacteria and Actinobacteria. When comparing the relative abundance of core OTUs between breeding stages, there were no significant differences for the males (t = -1.4, $P = 0.22$, Table 4.1) or for the females (t = 0.8, $P = 0.44$, Table 4.1). Interestingly, juveniles had many of the core OTUs of breeding males in high abundance. When comparing the number of shared OTUs in the whole microbiome, juveniles shared 35% of the OTUs (1193 out of 3368) with males and 42% of the OTUs (1005 out of 2380) with females. Juveniles had a larger core microbiome than adults with 25 OTUs (Table 4.2), and shared 40% of the OTUs (10 out of 25) with the core microbiome of males and 32% of the OTUs (8 out of 25) with the core microbiome of females.

The cloacal microbiome across breeding seasons

The cloacal bacterial phylogenetic diversity and number of observed OTUs in males and females did not increase over sequential breeding seasons separated by 10 to 12 months (Table 4.3, Fig. 4.8). However, for males and females, there were strong trends in community composition change through the sequential breeding seasons, as revealed by the first axis of the PCoA (Table 4.3, Fig. 4.9).

Testosterone and cloacal bacterial alpha diversity

Testosterone levels in the non-breeding males varied from 0.05 to 0.49 ng/ml (average: 0.13 ng/ml) and in the breeding males varied from 0.06 to 9.96 ng/ml (average: 1.08 ng/ml) and were significantly different between reproductive states ($P < 0.001$). There was a positive relationship between testosterone levels and phylogenetic diversity ($R^2 = 0.23$, $P = 0.0004$) and testosterone levels and number of OTUs ($R^2 = 0.16$, $P = 0.001$). When accounting for breeding condition, the positive relationship between testosterone levels and phylogenetic diversity was only significant for males in breeding condition (Breeding, $R^2 = 0.28$, $P = 0.02$; Non-breeding, $P = 0.6$, Fig. 4.10a) and there was a marginally significant relationships to the number of observed OTUs in breeding, but not non-breeding males (Breeding, $P = 0.06$; Non-breeding, $P = 0.4$, Fig. 4.10b).

Discussion

We studied the cloacal microbiome of a wild tropical songbird and describe variation in bacterial community composition dependent on the reproductive status of the bird. In particular, the cloacal microbiome of males changed more drastically than that of females, increasing in phylogenetic diversity and OTU richness during the breeding season and then decreasing in diversity with the transition to a non-breeding condition. Similarly, the bacterial community composition had a consistent direction of change when transitioning between breeding conditions. However, the relative abundance of the eight core OTUs did not change between breeding conditions. When analyzing the cloacal microbiome across breeding seasons, the bacterial diversity did not change, indicating that birds do not accumulate more bacteria with additional reproductive seasons, but there were strong trends in community composition change. The results presented here provide support for the bacterial clearance hypothesis, because the core microbiome stayed stable, but the diversity, richness, and composition of the microbiome changed with the reproductive condition of the birds. Thus, it is likely that birds are cyclically acquiring new bacteria during reproduction and losing them during the non-breeding season.

Reproduction is a physiologically demanding activity wherein organisms temporarily change their behavior and self-maintenance priorities to maximize their fitness (Stearns 1992). The breeding condition is known to result in increases in metabolic rate (Harshman & Zera 2007), oxidative stress (Monaghan, Metcalfe & Torres 2009), and ultimately it has been associated with increased susceptibility to infection by parasites (Oppliger & Christe 1996; Norris & Evans 2000). This increased susceptibility to infection is consistent with what we report here, as the richness and diversity of cloacal bacteria increased, and community composition changed, when birds were in breeding condition. Interestingly, these patterns only occurred in male birds.

Four other, non-exclusive processes can influence these changes in the male microbiome. First, during reproduction there is an increase in physical contacts that could facilitate the sexual transmission of bacteria between individuals (Kulkarni & Heeb 2007). For example, when cloacal contacts during copulations were experimentally blocked in kittiwakes (*Rissa tridactyla*), the bacterial diversity in the female's cloaca started to decrease, presumably because it stopped receiving bacteria from the males' ejaculates (White *et al.* 2010). It would be interesting to follow a mating pair through different reproductive conditions to see changes in the microbiome similarity within the pair and whether extra-pair copulations can indirectly affect the pair's cloacal microbiome.

Second, when birds are in reproductive condition the internal environment of the cloaca may change. Such alterations can be directly related to the presence or absence of certain key species that secrete acidic fermentation products and create an acidic environment that restricts the growth of most bacteria (Witkin, Linhares & Giraldo 2007), or changes in the level of mucous secretion that affects the capacity of bacteria to colonize. Third, at the same time these changes in the cloacal environment can affect competition and cooperation between the hundreds of microorganims living there and create additional niches for additional bacteria (Faust *et al.* 2012). Fourth, seasonal shifts in environmental bacterial communities (i.e. in soil, water, or food) could be reflected in the bids' cloacal microbiome. However, if this were true one would expect to see the same changes in the cloacal microbiome in both sexes, and it was only in the males that we saw the changes.

The bacterial diversity within an individual bird did not change from one breeding season to the next (i.e. when sampling twelve months apart). We expected that independent of the bird's age, additional opportunities for copulation would result in an accumulation of different bacterial species in their cloacas. Instead, we found that in both sexes the levels of diversity and richness remained unchanged from one breeding season to the next. This could occur because birds are essentially resetting their cloacal microbiome during the non-breeding season and losing many of the new bacterial strains that they accumulated during the previous breeding season. White et al. (2011) found that older female lizards (*Zootoca vivipara*) had lower levels of cloacal bacterial diversity and hypothesized that older individuals could have communities that are more resistant to colonization by foreign bacteria. In the present study, we were not able to determine the birds' age, but did find strong trends for changes in the bacterial community composition. Environmental changes across years could be driving these shifts in community composition. Long term studies following the microbiome of individual birds would be necessary to disentangle the effect of age and multiple reproductive opportunities.

There were differences in the cloacal community composition between sexes, but only when in breeding condition, which suggests that sex-specific physiological factors, like testosterone levels in males, could be playing an important role. Additionally, we only documented cloacal microbiome changes for males, whereas the females' cloacal microbiome did not change consistently between breeding and non-breeding condition. These findings were contrary to our initial expectations, because we predicted that females would exhibit greater changes in their cloacal microbiome, as they receive a higher proportion of bacteria during copulations and normally experience higher physiological costs of reproduction compared to males (Cox & Calsbeek 2010; Cox *et al.* 2010)(Kulkarni & Heeb 2007). We determined females' breeding status based on the presence of a brood patch, which starts before egg laying and continues during incubation. Use of this definition might have masked changes in the cloacal microbiome over the breeding cycle. Further research would be needed to understand if the female's cloacal microbiome varies at all in the short time span of pre-breeding, egg laying, and

incubating. On the other hand, the changes in the cloacal microbiome of the males could be related to an intrinsic characteristic of that sex. We found that bacterial phylogenetic diversity increased with testosterone levels in males, agreeing with previous findings by Escallón et al. (*In prep*). Testosterone could be directly affecting immune defenses and thus facilitating the invasion of new bacterial strains, or it could, indirectly, be affecting males' reproductive behavior by making them more promiscuous and thus having more chances of receiving sexually transmitted infections. One way to differentiate between those two possibilities would be to experimentally manipulate testosterone levels and copulatory contacts, such that one could artificially create a highly promiscuous male that is unable to copulate.

Unlike most studies that analyze the microbiome composition at a single time-point, in this study we incorporated important information about the temporal and inter-individual variability of the microbiome, showing that the cloacal microbiome in birds is dynamic and responsive to breeding condition and sex of the host. Birds and their cloacal microbiome are involved in complex reciprocal interactions, with potential fitness costs to the host (Jacob *et al.* 2015) and repercussions in the population (Lockhart *et al.* 1996), thus likely driving the evolution of mating strategies in various ways (Thrall, Antonovics & Dobson 2000; Kokko *et al.* 2002; Smith & Mueller 2015).

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Tables

Table 4.1. Heat map of the core OTUs for males and females showing the change in OTU relative abundance between breeding conditions, and the OTU relative abundance in Juveniles

Consensus Lineage	Green genes OTU #	Males		Females		Juveniles
		Non-breeding	Breeding	Non-breeding	Breeding	
(Pro) *Sphingomonas*	4449609	3.09	5.92	2.41	3.36	4.65
(Act) *Frigoribacterium*	589376	1.30	1.61	2.76	2.64	4.32
(Act) *Curtobacterium*	842941	1.56	0.67	7.41	1.02	1.33
(Pro) *Methylobacterium*	4303249	1.22	2.13	1.40	1.45	1.85
(Pro) *Pseudomonas*	4386920	2.17	2.42	1.28	0.44	0.30
(Pro) *Methylobacterium*	979344	0.89	1.15	0.75	0.80	1.04
(Act) *Rhodococcus fascians*	4460853	0.51	0.77	0.70	1.04	1.20
(Pro) *Methylobacterium*	4396717	0.57	0.89	0.51	0.72	0.94

The number on each cell and color scale represent the average percentage relative abundance of the core OTUs found on all birds from the population. Blue represents higher abundance and white lower abundance. The lowest taxonomic resolution that could be defined for OTU identification is listed; bacterial phylum for each core OTU is listed in parentheses (Pro = Proteobacteria, Act = Actinobacteria). Core OTUs are sorted in descending order of the sum of mean relative abundances across all groups.

Table 4.2. List of core OTUs (>90% prevalence in each age/sex group) from cloacas of rufous-collared sparrows, and the mean relative abundances of those OTUs.

		Males	Females	Juveniles
N		27	22	9
No. of OTUs		3368	2380	1353
No. of core OTUs		10	8	25
Consensus Lineage	Greengenes OTU #	Mean relative abundances (%) of the core OTUs		
(Pro) *Sphingomonas*	4449609	5.1	3.0	4.7
(Act) *Frigoribacterium*	589376	1.5	2.8	4.3
(Act) *Curtobacterium*	842941	0.9	4.0	1.3
(Pro) *Methylobacterium*	4303249	1.9	1.4	1.8
(Pro) *Pseudomonas*	4386920	2.3	0.8	0.3
(Pro) *Methylobacterium*	979344	1.1	0.8	1.0
(Act) *Rhodococcus fascians*	4460853	0.7	0.8	1.2
(Pro) *Methylobacterium*	4396717	0.8	0.6	0.9
Unassigned	denovo13229	0.9		0.2
(Act) *Rhodococcus*	73846	0.4		0.5
(Pro) *Agrobacterium*	4333206			3.1
(Pro) *Agrobacterium*	220269			1.8
(Act) *Rathayibacter caricis*	1821516			0.8
(Pro) Rhizobiaceae	5364			0.7
(Pro) Aurantimonadaceae	662915			0.7
(Pro) *Erwinia*	1123414			0.7
(Pro) *Methylobacterium adhaesivum*	591699			0.7
(Act) *Aeromicrobium*	140401			0.7
(Pro) Methylobacteriaceae	denovo16236			0.6
(Pro) Aurantimonadaceae	982912			0.5
(Pro) *Sphingomonas echinoides*	4453466			0.3

(Pro) Enterobacteriaceae	274754	0.3
(Pro) *Methylobacterium*	1109067	0.2
(Act) Microbacteriaceae	808021	0.1
(Act) *Rhodococcus*	denovo26817	0.1

The lowest taxonomic resolution that could be defined for OTU identification is listed. Bacterial phylum for each core OTU is listed in parentheses (Pro = Proteobacteria, Act = Actinobacteria). Core OTUs are sorted in descending order of the sum of mean relative abundances across all groups.

Table 4.3. Results for the linear mixed model between sequential breeding seasons, diversity metrics, and community composition

		Breeding season						
		1	2	3	Mean			
Sex	Metric	Mean (min - max)			change	SE	t	*P*-value
Males	Phylogenetic diversity	23.5 (8.9 - 31.0)	26.0 (21.4 - 36.4)	27.9 (17.9 - 33.9)	1.87	1.76	1.2	0.24
	Observed OTUs	295 (59 - 487)	312 (241 - 473)	381 (210 - 520)	40.33	31.56	1.4	0.17
	PCoA1 (Unweighted UniFrac)						2.1	0.06
	PCoA1 (Weighted UniFrac)						1.8	0.09
Females	Phylogenetic diversity	17.8 (9.2 - 22.8)	21.6 (10.0 - 28.5)	22.5 (17.1 - 28.2)	1.48	1.75	0.8	0.41
	Observed OTUs	184 (73 - 266)	256 (90 - 393)	280 (188 - 393)	20.54	24.50	0.8	0.42
	PCoA1 (Unweighted UniFrac)						1.6	0.14
	PCoA1 (Weighted UniFrac)						2.1	0.06

Figure legends

Figure 4.1. Individual shifts in phylogenetic diversity from non-breeding to breeding condition (A & C) and breeding to non-breeding condition (B & D). Time between sampling was five to six months. Lines connect data from the same individual across breeding conditions. Males are the top row (A & B) and females are the bottom row (C & D).

Figure 4.2. Individual shifts in the number of observed OTUs from non-breeding to breeding condition (A & C) and breeding to non-breeding condition (B & D). Time between sampling was five to six months. Lines connect data from the same individual across breeding conditions. Males are the top row (A & B) and females are the bottom row (C & D).

Figure 4.3. Net change between breeding conditions within individuals in phylogenetic diversity (A) and the number of observed OTUs (B) for males (black bars) and females (white bars).

Figure 4.4. Principal coordinates illustrating dissimilarities between the cloacal microbiome of males, females, and juveniles. PCoA with Unweighted UniFrac pairwise distances (A), and PCoA with Weighted UniFrac pairwise distances (B). Each point represents a cloacal sample from an individual. Blue points represent males, red points females, and green points juveniles. Breeding status is represented by different shapes. Increasing distance between points indicates increasing dissimilarity in cloacal community composition.

Figure 4.5. Principal coordinates illustrating dissimilarities between the cloacal microbiome of adult birds in breeding (black points) and non-breeding condition (grey points). PCoA with Unweighted UniFrac pairwise distances (A & C), and PCoA with Weighted UniFrac pairwise distances (B & D). Males are the top row (A & B) and

females are the bottom row (C & D). Each point represents a cloacal sample from an individual.

Figure 4.6. Individual shifts in bacterial community composition represented by the first principal coordinate of Unweighted UniFrac distances, from non-breeding to breeding condition (A & C) and breeding to non-breeding condition (B & D). Time between sampling was five to six months. Lines connect data from the same individual across breeding conditions. Males are the top row (A & B) and females are the bottom row (C & D)

Figure 4.7. Individual shifts in bacterial community composition represented by the first principal coordinate of Weighted UniFrac distances, from non-breeding to breeding condition (A & C) and breeding to non-breeding condition (B & D). Time between sampling was five to six months. Lines connect data from the same individual across breeding conditions. Males are the top row (A & B) and females are the bottom row (C & D)

Figure 4.8 Individual shifts in phylogenetic diversity (A & C) and number of observed OTUs (B & D) across sequential breeding seasons. Time between sampling was ten to twelve months. Lines connect data from the same individual across breeding seasons. Only one male was sampled across the three seasons, all other birds were sampled in just two seasons. Males are the top row (A & B) and females are the bottom row (C & D).

Figure 4.9. Individual shifts in bacterial community composition represented by the first principal coordinate of Unweighted UniFrac distances (A & C) and Weighted UniFrac distances (B & D) across sequential breeding seasons. Time between sampling was ten to twelve months. Lines connect data from the same individual across breeding seasons. Only one male was sampled across the three seasons, all other birds were sampled in just two seasons. Males are the top row (A & B) and females are the bottom row (C & D).

Figure 4.10. Relationship between circulating testosterone levels with (A) cloacal bacterial phylogenetic diversity and (B) number of observed OTUs. Each point represents a cloacal sample from a male in either breeding (black points) or non-breeding condition (grey points). The trend line represents the positive relationship between testosterone levels and phylogenetic diversity in breeding males.

Figures

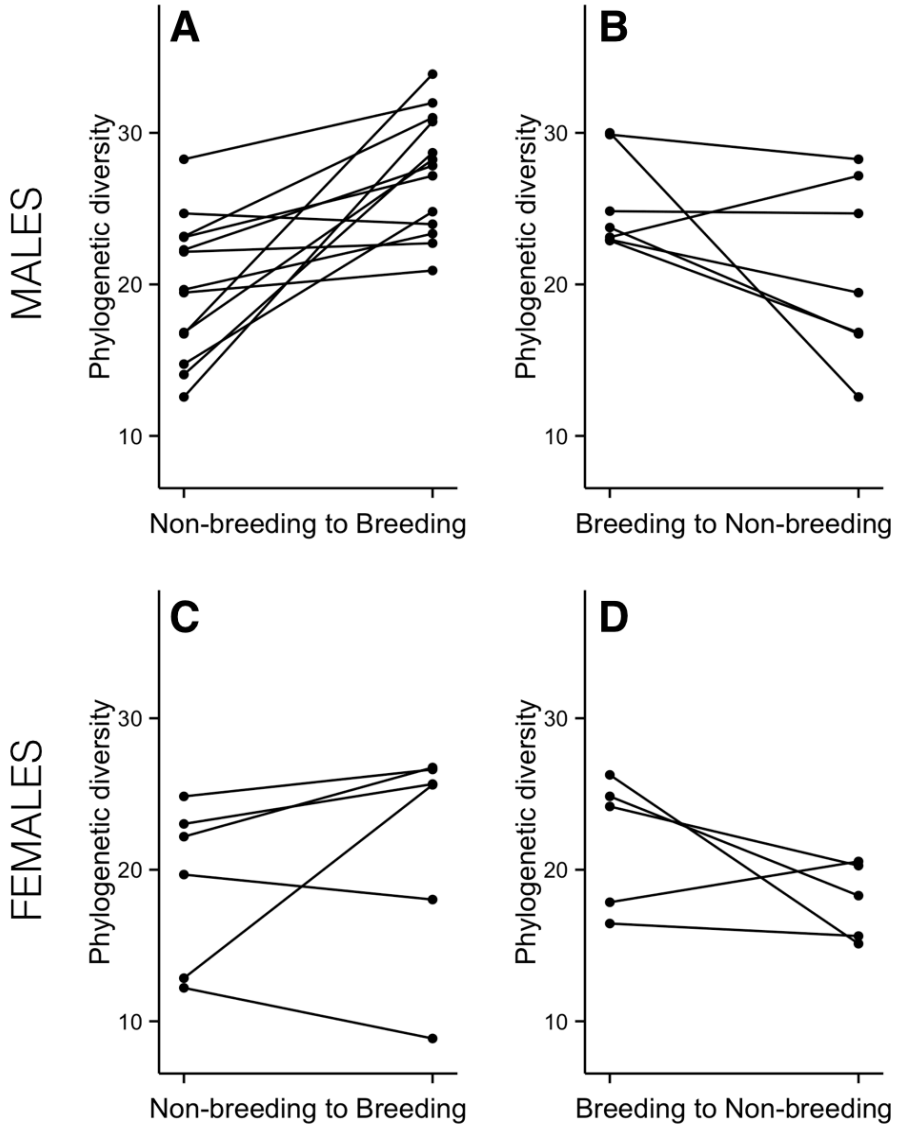


Figure 4.1. Individual shifts in phylogenetic diversity from non-breeding to breeding condition and breeding to non-breeding condition.

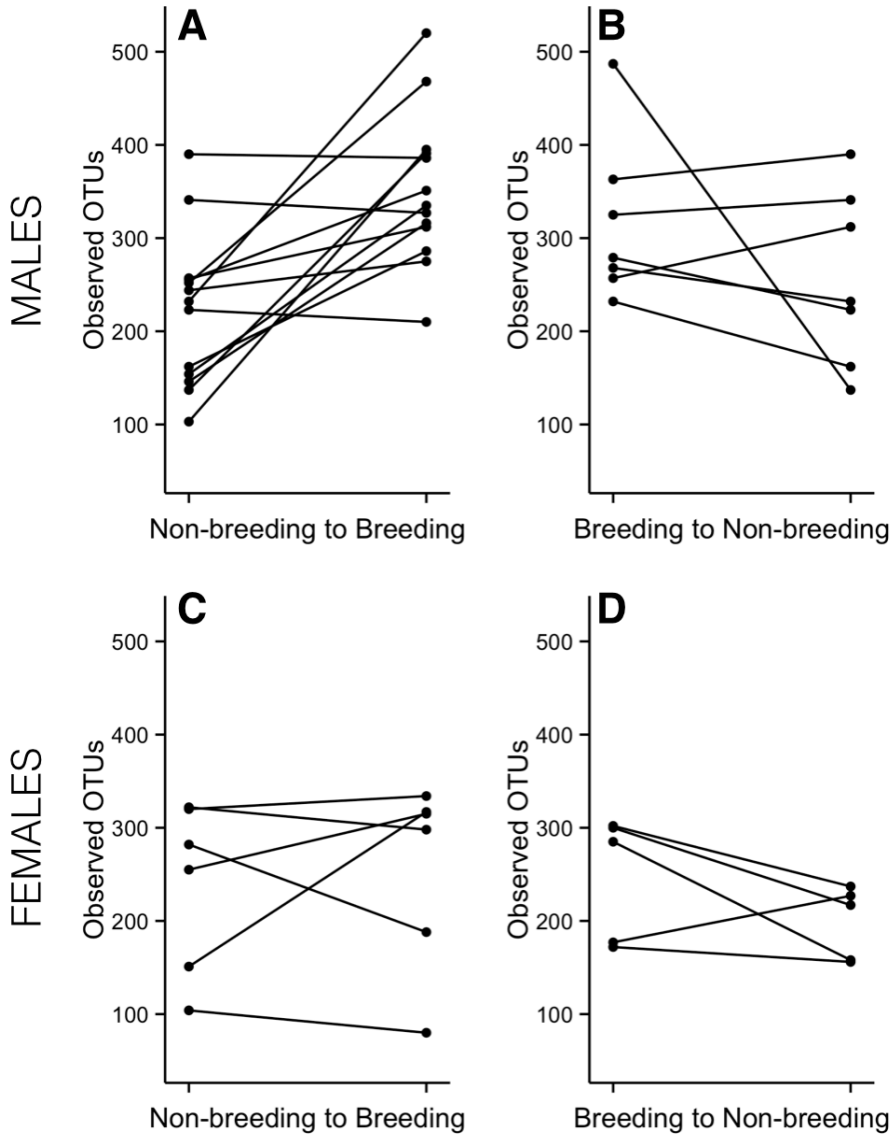


Figure 4.2. Individual shifts in the number of observed OTUs from non-breeding to breeding condition and breeding to non-breeding condition.

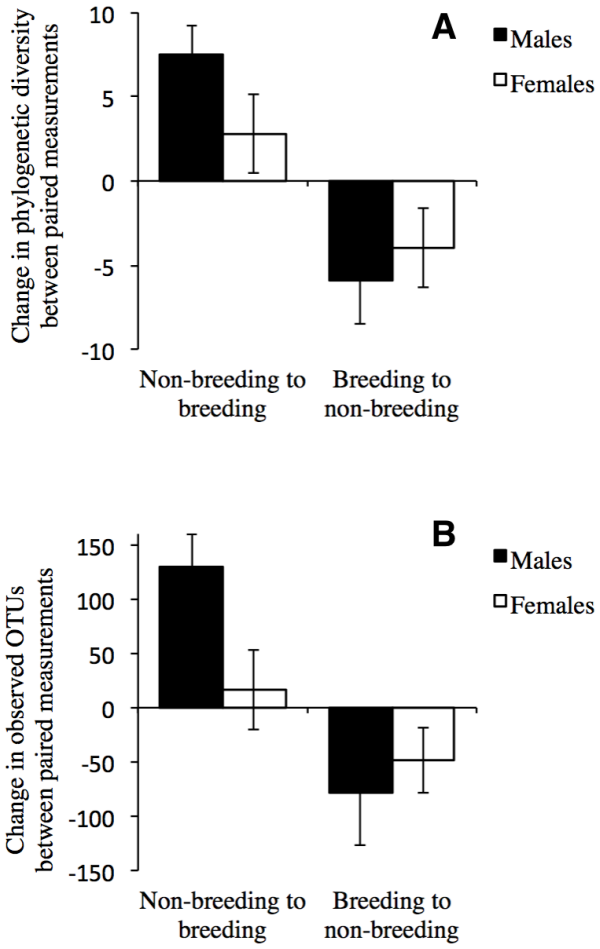


Figure 4.3. Change in individual alpha diversity between breeding conditions in phylogenetic diversity and the number of observed OTUs.

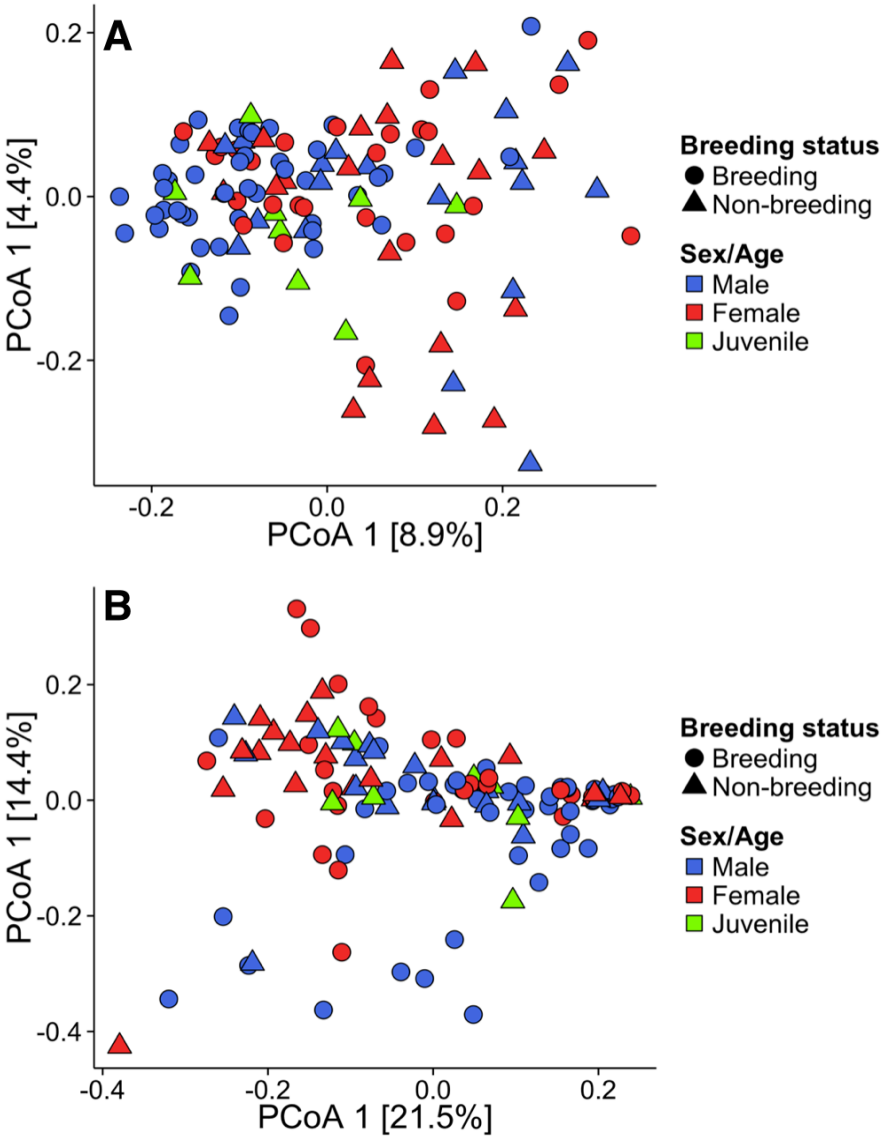


Figure 4.4. Principal coordinates illustrating dissimilarities between the cloacal microbiome of males, females, and juveniles

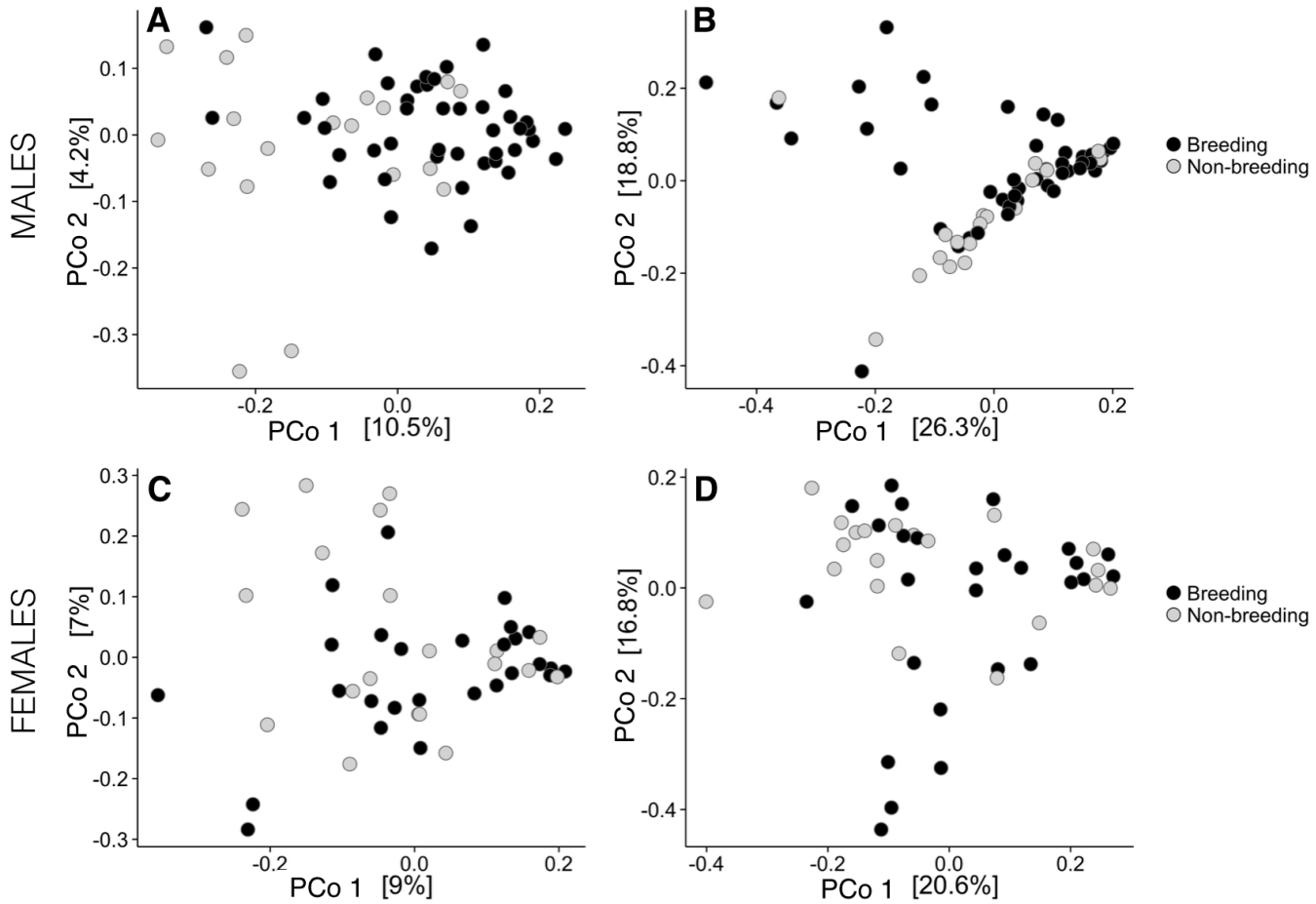


Figure 4.5. Principal coordinates illustrating dissimilarities between the cloacal microbiome of breeding and non-breeding adult birds.

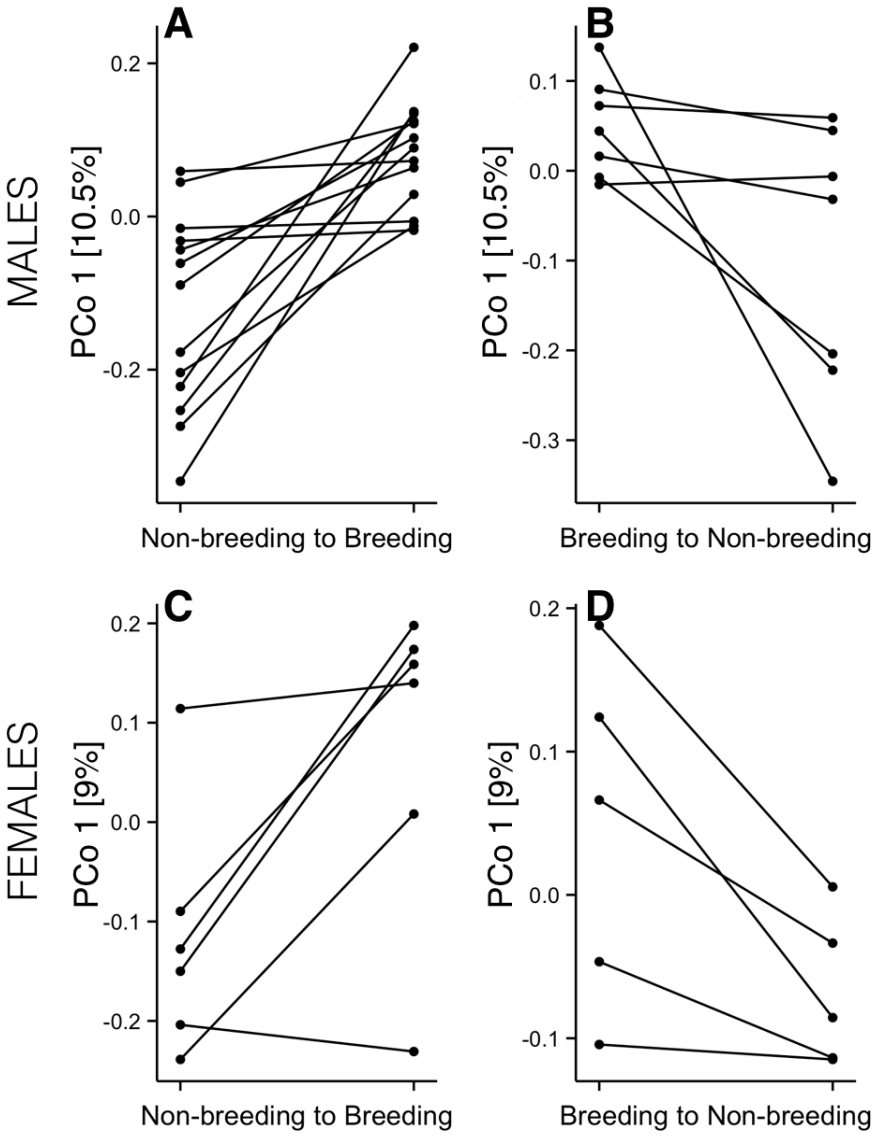


Figure 4.6. Individual shifts in bacterial community composition represented by the first principal coordinate of Unweighted UniFrac distances (presence/absence)

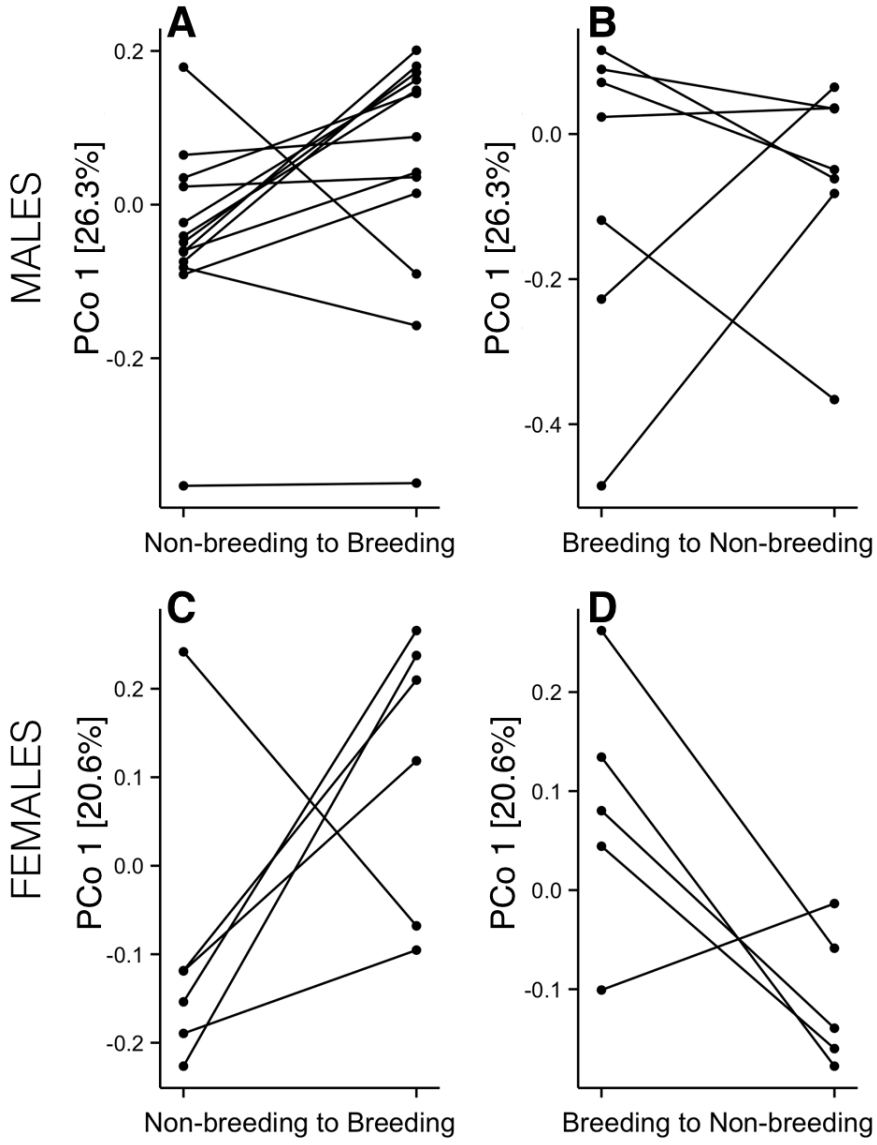


Figure 4.7. Individual shifts in bacterial community composition represented by the first principal coordinate of Weighted UniFrac distances (relative abundance)

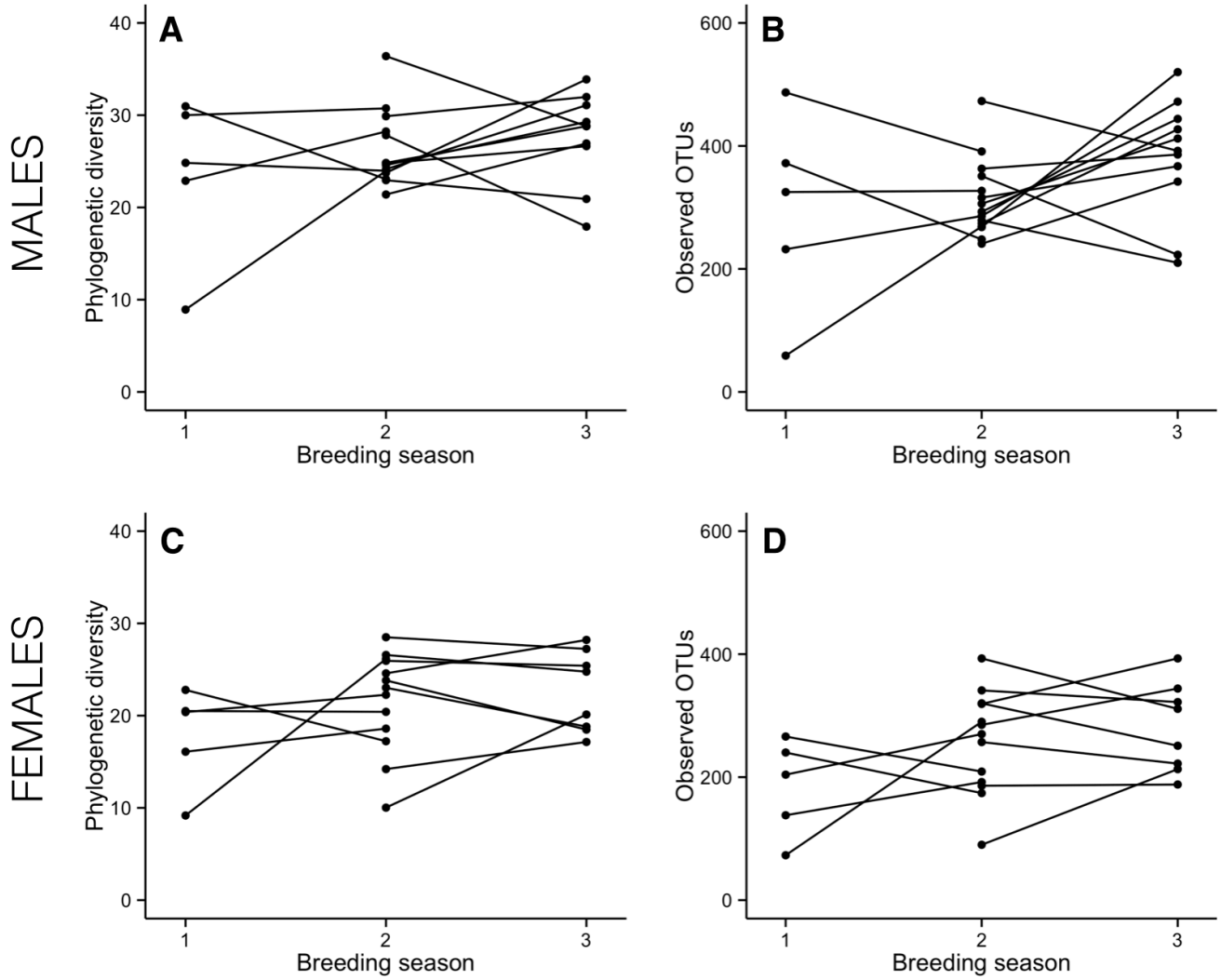


Figure 4.8. Individual shifts in phylogenetic diversity and number of observed OTUs across sequential breeding seasons

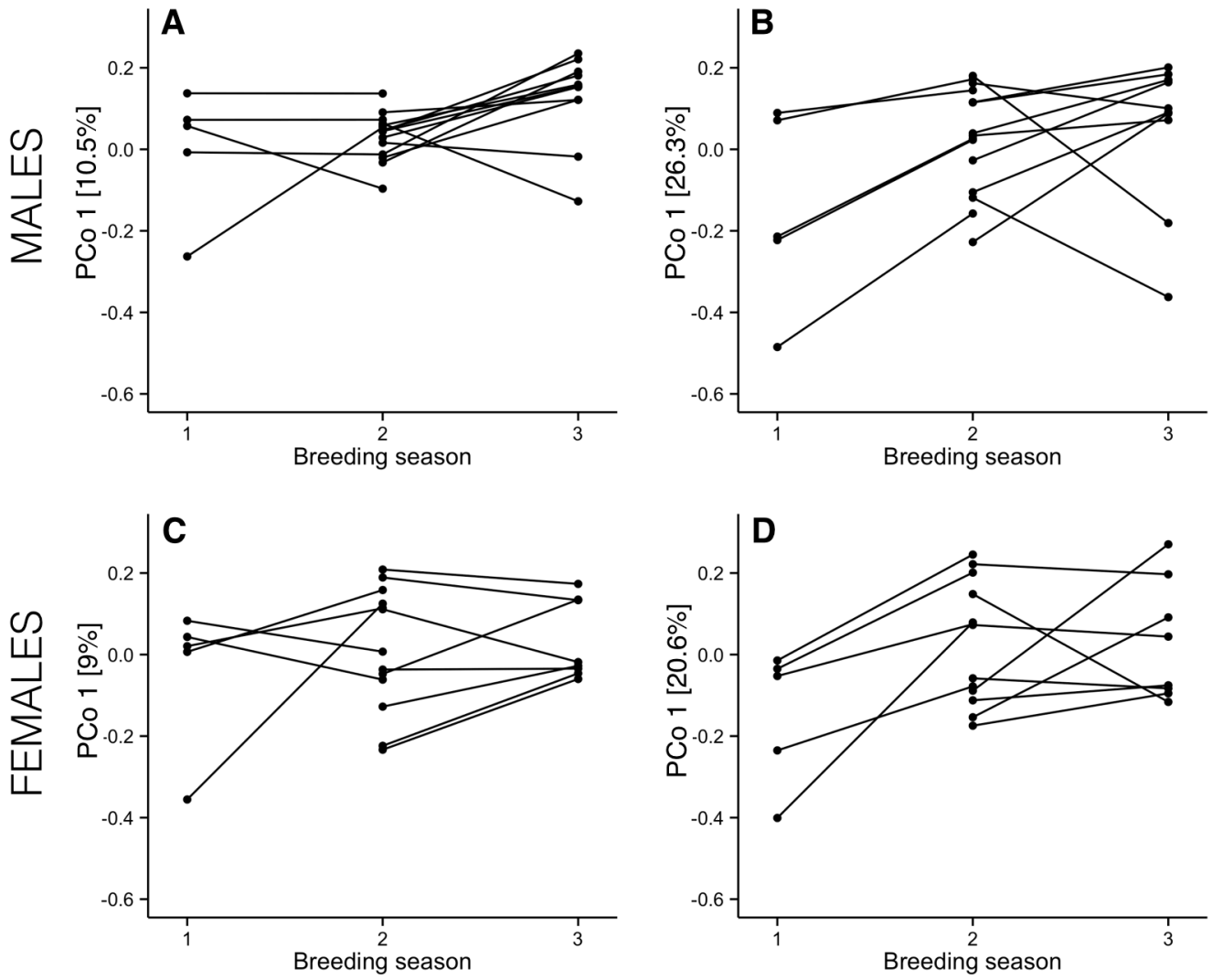


Figure 4.9. Individual shifts in bacterial community composition represented by the first principal coordinate of Unweighted UniFrac distances and Weighted UniFrac distances across sequential breeding seasons

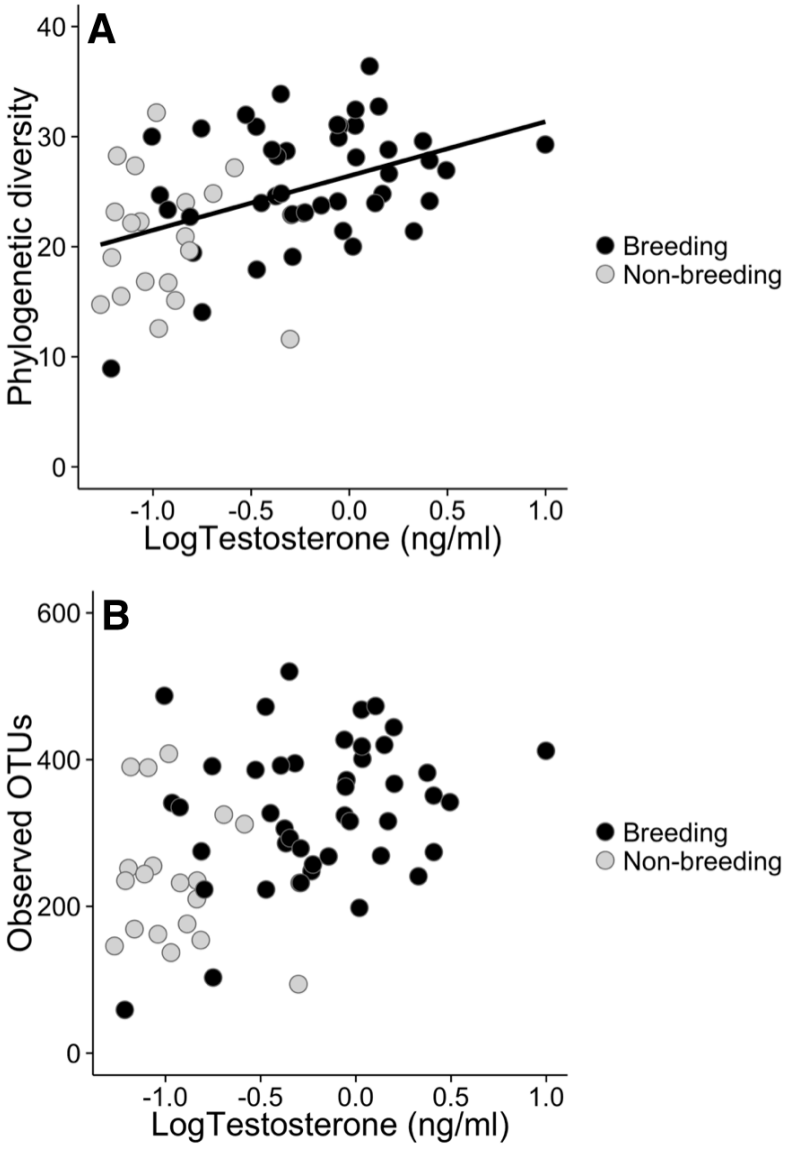


Figure 4.10. Relationship between circulating testosterone levels with cloacal bacterial phylogenetic diversity and number of observed OTUs.

CHAPTER V. CONCLUSIONS

Camilo Escallón

Geographic variation in testosterone levels

My dissertation touches on various aspects of the reproductive physiology of tropical birds. I initially explored macro-geographical patterns of testosterone variation under the premise that variation in hormone levels would match variation in life-history strategies in populations that differ in breeding synchronicity. I sampled along one of the largest altitudinal transect that can be found for rufous-collared sparrows (*Zonotrichia* capensis) in their tropical distribution. I predicted that high elevation birds would have high testosterone levels because of short breeding seasons at high altitude (Fig. 5.1). The shorter breeding seasons would presumably entail increased male-male competition, which favors high testosterone levels that modulate secondary sexual traits and let male birds take advantage of the reduced breeding opportunities (Wingfield *et al.* 1990), and conversely that long breeding season would select for low testosterone levels to avoid its physiological costs (Wingfield, Lynn & Soma 2001). However, I did not find a relationship of testosterone with elevation, but instead found high levels of intra-population testosterone variation. Maybe there was significant variation among individuals in their life-histories, with some males more focused on extra-pair copulations and others more focused on parental behavior, or maybe the pulsatile release of testosterone makes it vary greatly over the reproductive cycle. This result made me realize that I could have overestimated the degree of seasonality along tropical elevations, even though we knew that rufous-collared sparrows at mid elevations breed aseasonally through the year (Class & Moore 2011) and that at high elevations there is more seasonality but still an extended breeding season (Moore 2005). Sampling along a wider elevational range could make it possible to find greater seasonal divergence, however, it is unlikely that climatic seasonality would diverge much more than in our 2,700 m transect.

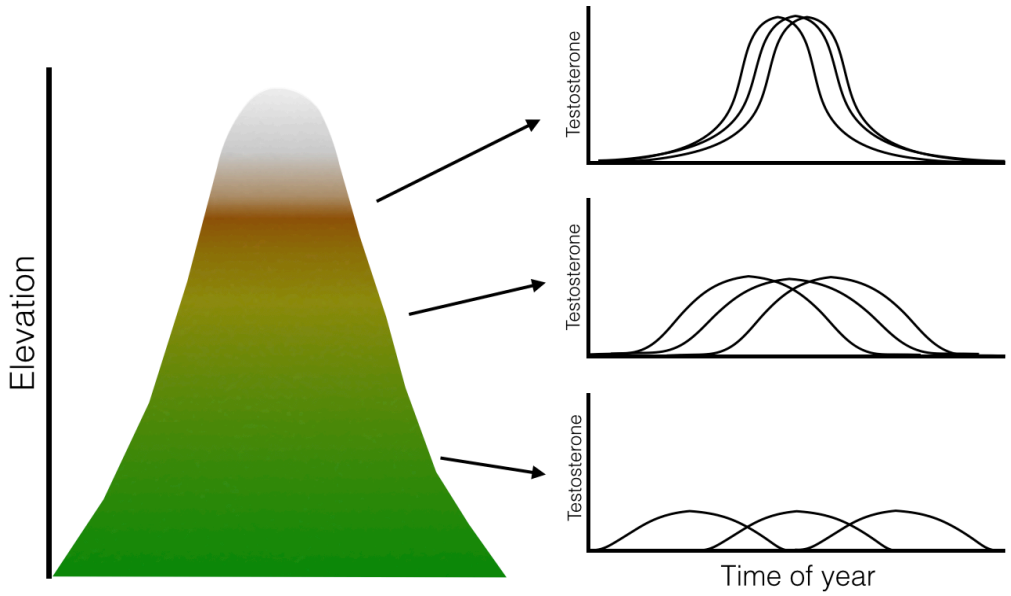


Figure 5.1. Predicted variation in testosterone levels and breeding synchrony along a tropical elevational gradient. Each curve represents the testosterone profile of a single individual.

Future directions

I sampled testosterone levels to measure the variation during the period of elevated testosterone (i.e. breeding season) (Kempenaers *et al.* 2008). Another possibility to find evidence for physiological adaptations to different life-histories across elevations could be to sample individual males at different times of the year to determine the duration of elevated testosterone (*Fig. 2c in* Kempenaers *et al.* 2008). Maintaining elevated levels of testosterone for long periods of time could be a better indicator of adaptations to low seasonality than the actual testosterone levels during the breeding season. This sort of study would certainly require a great amount of effort, but with rufous-collared sparrows being year-round residents, one could easily follow individual males uninterruptedly and be able to take repeated samples of the same individual (Miller & Miller 1968).

Rufous-collared sparrows have reduced mitochondrial gene flow across populations from different elevations (Cheviron & Brumfield 2009), which suggests that local adaptations

to selective pressures at each elevation could be maintained and not get diluted by immigrants. A further experimental test to determine differences in testosterone levels between populations could involve translocating individuals from the elevational extremes to an elevational midpoint to determine if their testosterone profiles change in response to the new environment. Such an experiment would give us information on whether the testosterone response to its environment is plastic or fixed. A similar experiment was done for a temperate bird, *Junco hyemalis,* which showed that testosterone profiles represented a plastic response to the new environment (Atwell *et al.* 2014). It remains to be verified if a relationship between seasonality and testosterone levels in tropical birds can be detected along elevational gradients, or if evolutionary constraints may be restraining the plasticity of testosterone over large geographic gradients (Hau 2007).

Reproductive physiology of rufous-collared sparrows and their associated microorganisms

A second major aspect of my dissertation was testing the immunocompetence handicap hypothesis in relation to how the reproductive physiology of the host affected parasitic and commensal microorganisms. I studied haemosporidians, common blood parasites of birds that affect red blood cells and can have deleterious effects on the reproductive success of the host. I also studied the relationship between reproductive physiology of the host and its cloacal microbiome, in particular the community of bacteria that reside in the bird's cloaca. In both cases, I hypothesized that birds with high testosterone would be more likely to be infected with parasites because testosterone is associated to the production of secondary sexual characters, but it may also increase parasitemia risk (Folstad & Karter 1992; Deviche & Parris 2006). In the case of parasitism by haemosporidians, I found no relationship between infection and testosterone levels, but the males in breeding condition were more likely to be infected with the parasite, suggesting that there is an energetic tradeoff between self-maintenance and reproduction, but that it is not mediated by testosterone. For the cloacal microbiome, I found a positive relationship between testosterone and phylogenetic diversity of cloacal bacteria.

Testosterone was also positively correlated with the presence of Chlamidiae, a type of sexually transmitted pathogen. When analyzing the transition between non-breeding and breeding condition, males, but not females, increased their levels of cloacal bacterial richness and phylogenetic diversity. This showed that the cloacal microbiome in birds is dynamic and can be affected by factors associated with their reproductive physiology.

Can the immunocompetence handicap hypothesis be improved?

Sexual signals should be honest indicators of male health or genetic quality, so displaying them should be costly, and only high quality males should be able to withstand those costs (Zahavi 1975). The immunocompetence handicap was proposed as a mechanistic explanation of the costs of testosterone-dependent sexual signals, by hypothesizing that testosterone is immunosuppressive (Folstad & Karter 1992). Roberts et al. (2004), in a meta-analysis, reviewed the evidence for the effects of testosterone on immune suppression. Although there is some debate on the evolutionary significance of some immune measurements (Martin, Weil & Nelson 2006), they found weak evidence for immune suppression by testosterone. On the other hand, they found that testosterone increased the risk of parasitemia (Roberts *et al.* 2004). Thus, the empirical support for the hypothesis is limited.

Three aspects should be addressed in future studies to further evaluate the immunocompetence handicap hypothesis. (1) Measuring parasitism levels is better than measuring immune responses, because by measuring parasitemia one is directly measuring the selective agents. If the immune response is going to be measured, it is crucial to understand what the response means in terms of reproductive success. In other words, one must make it clear if the level of an immune response is a sign of tolerance or resistance (Råberg *et al.* 2009). (2) Suppression of the immune system by high testosterone levels may be condition-dependent (Roberts & Peters 2009). In nature, there is always variation in energetic reserves among individuals, and this variation can be attributed to different factors, such as breeding condition, migration, or environmental factors. Thus, when analyzing immune response, one should control for the amount of

resources that the individual is able to allocate to self-maintenance. (3) The immunocompetence handicap hypothesis states that testosterone levels suppress the immune system, but immune activation by parasite infection can also suppress testosterone levels (Boonekamp *et al.* 2008). Thus, I hypothesize that the trade-off between immunocompetence and testosterone-dependent sexual signals is constantly shifting direction and that endocrine and immune systems are mutually influencing each other (Fig. 5.2). Further research should be done to determine the inflexion point where parasite loads become more important and have a larger effect on testosterone, and vice versa.

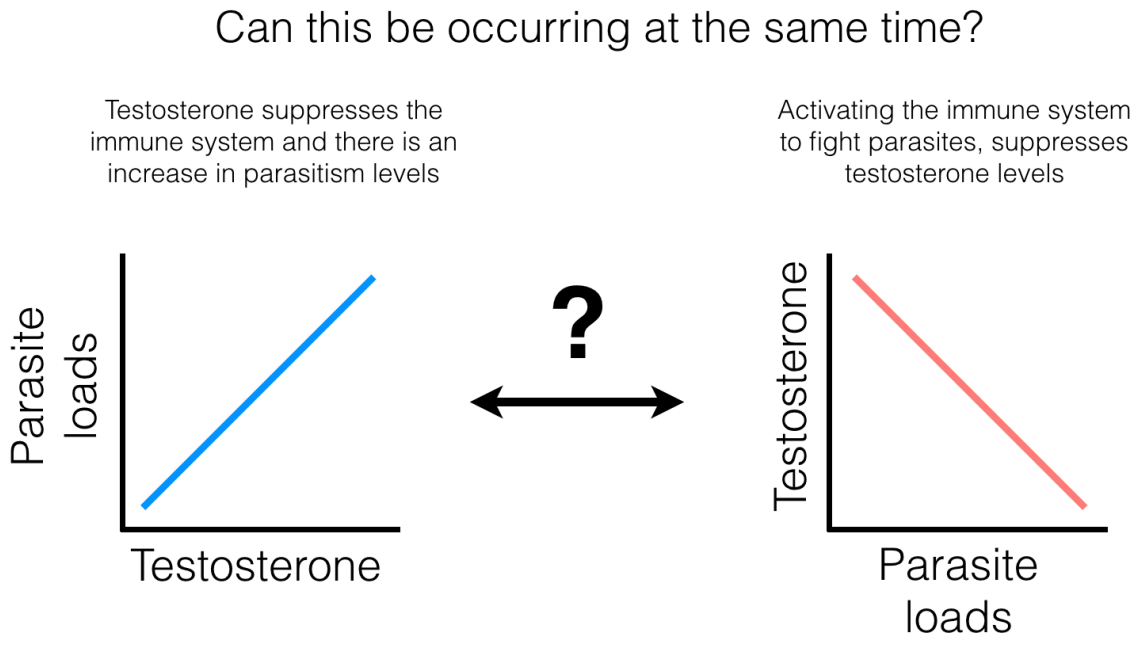


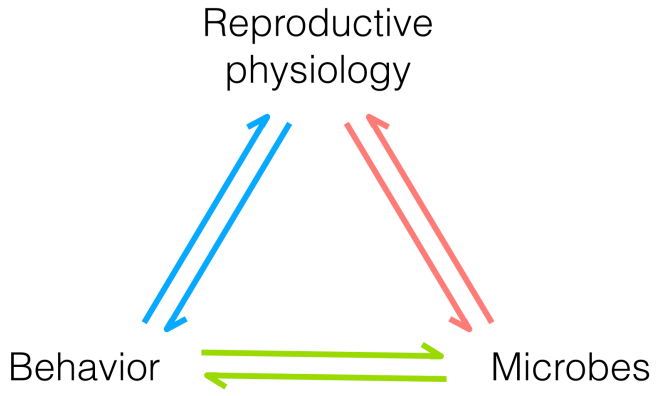
Figure 5.2. Hypothetical relationship between testosterone and parasite levels, based on the trade-offs predicted by the immunocompetence handicap hypothesis.

The link between reproductive physiology, behavior, and microbes

Reproductive condition is a cyclical and temporary state where specific behaviors and physiological systems are activated. Microbes, in turn, should adjust their life cycles to the reproduction timing of the host (Martinez-Bakker & Helm 2015). As such,

communities of cloacal bacteria represent an interesting case, because besides harboring gut microbes, there are sexually transmitted infections. In my studies, I found that the cloacal microbiome is dynamic and can be influenced by the reproductive state of the host. Interestingly, I found that in two separate populations, one in Ecuador and the other in Colombia, the phylogenetic diversity of bacteria positively correlated with testosterone levels of the males. The lack of experimental evidence limits my ability to make specific conclusions about causes and consequences of the relationships I found. Therefore, I propose a series of experiments based on the three-way interaction between host physiology, behavior, and microbes (Fig. 5.3).

gure 5.3. Relationships between reproductive physiology (e.g. testosterone levels, productive status, immune system) behavior (e.g. copulation rates, promiscuity, gression), and microbes (e.g. cloacal microbiome, bacteria on feathers, pathogens).



It has been established that testosterone levels affect reproductive behavior and that behavior affects testosterone levels (blue lines in Figure 5.3) (Raouf *et al.* 1997; Safran *et al.* 2008). Additionally, testosterone may affect the cloacal microbiome (red line in Figure 5.3), which is what I showed in this dissertation. However, the link between the microbiome and behavior has been less well studied (green line in Figure 5.3). The cloacal microbiome may be changing because testosterone is suppressing the immune system or because behaviorally increased copulations increase transmission rates. One experimental way to test whether testosterone is affecting the microbiome would be to

use contraceptive devices that limit transmission of bacteria, and simultaneously modify testosterone with implants. This would allow us to limit sexually transmitted bacteria and increase or suppress testosterone levels. However, to understand if multiple copulations affect the cloacal microbiome, one should know the promiscuity levels of the individuals. Extra pair paternity rates could be a proxy for extra pair copulations, but they are a limited measure, because not all copulations result in successful fertilization. A solution to measuring promiscuity levels would be to mark birds with, for example, radiolabeled isotopes or proximity data loggers, and then measure close contacts with other birds. Alternatively, one could infect birds with, for example, labeled *E. coli,* and determine how many other birds get infected. Lastly, determining the effects of the cloacal microbiome on behavior would be crucial to fully understand the interaction between the microbes and their hosts (Archie & Theis 2011; Ezenwa *et al.* 2012; Adamo 2014; Funk *et al.* 2015; Archie & Tung 2015).

Conclusion

Identifying the causes and consequences of variation in reproductive traits has been a major topic in evolutionary biology. Studying the immunocompetence handicap hypothesis was important in this context because I could test how immune suppression by testosterone could affect haemosporidian infection in populations with selective pressures for divergent life-histories. My dissertation research will serve as a basis for future studies on the immunocompetence handicap hypothesis in tropical latitudes. In addition, studying the cloacal microbiome in light of sexual reproduction was relevant because sexually transmitted diseases have been hypothesized as important factors in the evolution of mating systems, but the mechanisms (i.e. testosterone levels, reproductive condition, and sex) had not been considered in empirical studies. Finding that the cloacal microbiome was dynamic and affected by the reproductive physiology of the host created novel lines of research that can inform fields such as theoretical evolutionary biology, disease ecology, and applied epidemiological models. Many questions remain unanswered, but hopefully this dissertation provides a useful foundation for future studies.

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