EVOLUTION OF FEMALE ORNAMENTATION IN THE WHITE-SHOULDERED FAIRYWREN (*MALURUS ALBOSCAPULATUS*)

AN ABSTRACT

SUBMITTED ON THE TWENTY THIRD DAY OF MARCH 2018

TO THE DEPARTMENT OF ECOLOGY AND EVOLUTIONARY BIOLOGY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE SCHOOL OF

SCIENCE AND ENGINEERING OF TULANE UNIVERSITY

FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

BY

Erik David Enbody

APPROVED:

Jordan O. Karubian, Ph.D., Director

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ABSTRACT

A comprehensive understanding of sexual dichromatism and sexual selection depends on understanding selective pressures on females, which may differ from those experienced by males. Conventional theory suggests that ornamentation in females evolves as the byproduct of selection pressures on males, and is non-adaptive. My dissertation challenges this assumption through a series of linked studies related to female ornamentation in a species of tropical passerine bird, the White-shouldered Fairywren (*Malurus alboscapulatus*), of New Guinea. The White-shouldered Fairywren is ideally suited to evaluate the evolution of female ornamentation, because populations are characterized by divergence in female plumage coloration from brown (unornamented) to black-and-white (ornamented), with no variation in males, which are uniformly black-and-white. My thesis research employed field-based observation and experimentation with contemporary genomic, endocrine, and microscopy techniques to identify proximate mechanisms, current adaptive function, and evolutionary history of female ornamentation in this system.

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FORWARD

A comprehensive understanding of sexual dichromatism and sexual selection depends on understanding selective pressures on females, which may differ from those experienced by males. Conventional theory suggests that ornamentation in females evolves as the byproduct of selection pressures on males, and is non-adaptive. My dissertation challenges this assumption through a series of linked studies related to female ornamentation in a species of tropical passerine bird, the White-shouldered Fairywren (*Malurus alboscapulatus*), of New Guinea. The White-shouldered Fairywren is ideally suited to evaluate the evolution of female ornamentation, because populations are characterized by divergence in female plumage coloration from brown (unornamented) to black-and-white (ornamented), with no variation in males, which are uniformly black-and-white. My thesis research employed field-based observation and experimentation with contemporary genomic, endocrine, and microscopy techniques to identify proximate mechanisms, current adaptive function, and evolutionary history of female ornamentation in this system.

My first chapter assessed the degree to which mechanisms underlying ornament production are conserved between the sexes and across populations. Empirical research suggests that ornamented traits and reproductive behaviors in males are often testosterone-regulated, but less is known about the association between testosterone, ornaments, and behaviors among females. A null hypothesis consistent with the non-adaptive scenario is that the same combinations of traits is regulated via the same mechanism in female White-shouldered Fairywrens as in males, namely via variation in

circulating testosterone levels. Instead, I found that elevated levels of testosterone in ornamented females corresponds to a greater aggressive response to experimental trials, but male testosterone levels did not differ between populations, despite higher male aggression in the population with ornamented females. These finding suggest that elevated androgens are not simply a byproduct of selection on males. Furthermore, my results indicate that female ornaments may function in territorial defense, which is consistent with a role for social selection and suggest a potential adaptive function for the observed patterns of plumage variation.

My second chapter characterized the nanoscale mechanisms of melanin based plumage production in males and females of both the White-shouldered Fairywren and its sister species, the Red-backed Fairywren (*Malurus melanocephalus*). Between these two species, both ornamented and unornamented phenotypes in each sex are present, providing an opportunity to test the null hypothesis that female traits evolve in direct correlation with male traits. Consistent with this, I expected that across all ornamented sexes and lineages, plumage is produced through similarities in barbule density and fine scale arrangement of melanin in barbules. I showed that the ornamented female phenotype in White-shouldered Fairywren is distinctive from that in males and differs in lacking complex and structured feather barbules of males. In contrast, unornamented plumage in all populations and both sexes is produced by similar structural and pigment distributions. Together, these findings contradict the idea that female ornamentation in White-shouldered Fairywrens was achieved via a simple switch to produce an equivalent ornament to that expressed in males. This work provides an additional line of evidence

that the mechanisms for producing an ornamented phenotype in females differ from those of males, consistent with selection on female ornamentation on this system.

My third chapter used high-throughput sequencing to identify regions of the genome that may be under selection using whole genome resequencing and to test for differences in gene expression between female phenotypes. Although a number of studies have successfully identified targets of selection in male genomes that putatively mediate transitions in the ornamented phenotype, no comparable data currently exists for females, providing a powerful opportunity to assess the role of selection on the evolution of population variation in female ornamentation. I compared four populations of White-shouldered Fairywren that differ in degree of female ornamentation, with no changes in male coloration. I first demonstrate that all populations possessing a black and white phenotype are likely descended from an unornamented ancestor, which is inconsistent with female ornaments only evolving in response to sexual selection pressures on males. Using a comparative approach, I next identified signatures of selection on regions of the genome that contain key melanogenesis and steroid genes between multiple transitions in color in female fairywrens. I associated these potential regulatory elements to differences in gene expression between female phenotypes in a relevant tissue, molting feathers, and differences between populations in gene expression are inconsistent with a neutral model of evolution. I additionally demonstrated how testosterone influences transcriptional regulation of a putative signal, the white shoulder patch, and find that experimental elevation of testosterone results in activating similar genes that are expressed in populations naturally exhibiting white shoulder patches. These findings provide a third line of evidence for molecular targets of selection that likely mediate female plumage

transitions and refine my understanding of the genomic, transcriptional, and endocrine control of evolutionary transitions in female phenotypes.

Taken together, my dissertation provides evidence from multiple sources for social selection acting on female ornamentation in the White-shouldered Fairywren: females integrate ornamentation and hormones in ways that differ from males to achieve a more aggressive territorial phenotype; plumage nanostructure of ornamented females differs from that of males; female ornamentation is derived within this group; and regions of genomic differentiation among populations are consistent with directional selection on female ornamentation. In doing so, this body of work provides the strongest evidence to date for selection on female ornamentation and provides novel insights into the mechanistic and evolutionary processes generating biodiversity.

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CHAPTER 1 [1]

FEMALE ORNAMENTATION IS ASSOCIATED WITH ELEVATED AGGRESSION AND TESTOSTERONE IN A TROPICAL SONGBIRD

[1]Co-authors: Boersma, J.B., Schwabl, H.S., Karubian, J.

ABSTRACT

In males, testosterone plays a key role in ornament production and linking ornamentation with reproductive behaviors and other traits to produce an integrated phenotype. Less is known about whether females couple testosterone, ornamentation, and aggressive behaviors or whether alternative mechanisms achieve female-specific combinations of traits. Assessing these alternatives is necessary to understand the degree to which selection acts on female traits. The White-shouldered Fairywren (*Malurus alboscapulatus*) provides a useful context to address these questions because populations vary in degree of female ornamentation, a derived trait, whereas male ornamentation is constant across both populations. I found that ornamented females have higher levels of circulating testosterone and respond more aggressively to experimental territorial intrusions than do unornamented females. These findings are consistent with the idea that, among female White-shouldered Fairywrens, testosterone mechanistically links plumage and behavioral traits to produce an integrated competitive phenotype, as has been reported for males of closely related species. In contrast, circulating testosterone in males did not differ significantly between populations. More broadly, my findings are

consistent with ongoing selection on the mechanisms underlying female ornaments, likely via social selection.

Introduction

Sex-based differences in visual signals are widespread in nature, and have long captured the attention of naturalists and evolutionary ecologists. A comprehensive understanding of sex-based differences depends upon understanding the underlying mechanisms of production and patterns of expression in both sexes. Yet, until recently, most studies of ornamentation have focused on males, despite females of many taxa exhibiting ornamentation (Amundsen 2000; Heinsohn 2005; Clutton-Brock and Huchard 2013). Some have proposed that female ornamentation is a non-adaptive byproduct of selection favoring ornaments in males (Darwin 1871; Lande 1980) and, consistent with this perspective, female ornaments often closely resemble those of conspecific males (e.g., Amundsen and Parn 2006; Dale et al. 2015). However, female ornaments are sometimes distinct from those of males (e.g. Douglas J. Emlen et al. 2005; Simmons and Emlen 2008; Weiss et al. 2009), and comparative phylogenetic studies have revealed frequent evolutionary transitions between ornamented and unornamented states in females (e.g., Burns 1998; Hofmann et al. 2008; Johnson et al. 2013; Shultz and Burns 2017). These patterns are inconsistent with ornaments in females being selectively neutral and potential byproducts of their selection in males (Dale et al. 2015). Accordingly, an understanding of how and why ornament expression differs between the sexes is needed to better understand sexual dimorphism (Clutton-Brock 2007).

A detailed assessment of the mechanisms underlying phenotypic variation is important for understanding the evolution of ornamentation. Understanding the role of selection is particularly important, as it is a mechanism that can shape phenotype evolution (Ketterson et al. 2009; Adkins-Regan 2012). Sex steroids also can play a key mechanistic role (Hau 2001), for females as well as males (Staub and De Beer 1997; Kimball and Ligon 1999; Muck and Goymann 2011). Among males, androgen testosterone is often associated with both individual (Roberts et al. 2009) and interspecific variation in plumage ornaments (Dijkstra et al. 2012; Rosvall et al. 2016) and is the subject of tradeoffs between survival and reproductive investment. Similarly, in females, testosterone can be associated with singing (Kriner and Schwabl 1991), aggression (Sandell 2007), non-reproductive physiological processes (Zysling et al. 2006), and fitness (Veiga and Polo 2008; Cain and Ketterson 2012). However, testosterone may have different regulatory roles in females than in males (Wolfgang Goymann and Wingfield 2014) and, in some cases, testosterone levels can also be correlated across the sexes without having a measurable function in females (e.g., Ketterson et al. 2005; Møller et al. 2005; Wolfgang Goymann and Wingfield 2014). A better understanding of the degree to which testosterone mechanistically mediates transitions between ornamentation states in females will therefore advance my understanding of the evolution of sex-based differences.

Importantly, testosterone has the potential to link ornament expression with other morphological or behavioral traits to produce an integrated phenotype (Ketterson and Nolan 1992; Cox et al. 2017), in which suites of functionally and mechanistically related traits are coupled in an adaptive manner (Pigliucci 2003). For example, it is well

established that changes in circulating testosterone levels regulate expression of both aggressive and reproductive behavior (Wingfield et al. 1990) and male ornaments are often testosterone regulated (e.g. fish: Fernald 1976, reptiles: Cox et al. 2008, and birds: Lank et al. 1999; Peters et al. 2000; Mougeot et al. 2004; Lindsay et al. 2011). When present in females, ornaments are predicted to function in intrasexual competition over ecological or social resources (West-Eberhard 1979). Indeed, empirical work has corroborated the idea that aggression in females is often associated with the development of female ornamented traits (e.g. Pryke 2007; Rubenstein and Lovette 2009; Crowhurst et al. 2012) and female birds possess androgen receptors in brain regions associated with aggression (Rosvall et al. 2012). Aggression may also be associated with circulating sex steroids in female birds (Kriner and Schwabl 1991; Pärn et al. 2008; Cain and Ketterson 2012) and interact with testosterone to affect fitness (Veiga and Polo 2008). Testosterone may become elevated as a response to aggressive encounters (Wingfield et al. 1990; Langmore et al. 2002), but in other species testosterone is not necessarily required to elevate aggression (Jawor et al. 2006).

The extent to which testosterone acts as a phenotypic integrator underlying differences between the sexes and populations remains poorly understood, despite major implications for my understanding of evolutionary processes (Williams 2008). For example, testosterone may stimulate ornament expression in males of some species (e.g. van Oordt and Junge 1934; Fernald 1976; Mougeot et al. 2004), but not others (Owens and Short 1995), suggesting species-specific effects of testosterone on ornamentation. Similarly, between the sexes, females may decouple associations between testosterone and traits to produce female-specific phenotypes and behaviors (Rosvall 2013), though

the degree to which females are able to do so is unclear. Sexes could use different mechanisms (e.g., hormone target sensitivity, circulation, and conversion to other hormones, or non-endocrine mechanisms to achieve ornamentation (Rosvall et al. 2012), such that ornamentation in females is effectively de-coupled from associated behavioral traits observed in males. The evolution of androgen-mediated sexual dimorphism can also be achieved by coupling trait expression to testosterone in males without decoupling these traits from testosterone in females (Cox et al. 2015). Alternatively, the integrated phenotype may remain highly conserved across the sexes so that the same mechanisms involved in expression of male ornaments and associated behaviors exist in females.

Resolving between these alternatives requires information about the associations between testosterone, ornamentation, and behavioral traits in females, appropriate comparison points with males, and phylogenetic history. In the current study, I assessed associations between testosterone and territorial aggression between two subspecies of the White-shouldered Fairywren (*Malurus alboscapulatus*) that differ in degree of female, but not male, ornamentation (Rowley and Russell 1997; Karubian 2013; Enbody et al. 2017). The White-shouldered Fairywren is sister to the Red-backed Fairywren (*Malurus melanocephalus*), and although the current study is focused exclusively on the White-shouldered Fairywren, some basic information on Red-backed Fairywren provides an informative context for the hypotheses I present and test. Among Red-backed Fairywrens, male plumage ornamentation (Karubian 2002) is associated with increased aggression (Karubian et al. 2008) and both naturally and experimentally elevated circulating testosterone titres (Lindsay et al. 2009; Lindsay et al. 2011). This relationship has led to the expectation that testosterone acts on a mechanistic level to integrate these

traits in male Red-backed Fairywrens (Webster et al. 2010). However, recent work has shown that this relationship may vary by age, body condition, and population (Barron et al. 2015; Dowling and Webster 2017; Lantz et al. 2017), making additional research on the degree to which testosterone acts as an integrator a priority for future work in this system. The naturally unornamented female Red-backed Fairywrens have lower circulating testosterone than do Red-backed Fairywren males (Schwabl et al. 2015), but testosterone-implanted females develop a partial, male-like phenotype (red, but no black coloration; Lindsay et al. 2016). This implies that sex differences in ornamentation of Red-backed Fairywrens are in-part mediated by sex differences in circulating levels of testosterone, but the role of testosterone in integration of female ornamentation with behavioral traits is not known.

Prior research on the Red-backed Fairywren findings provide a useful context in which to study the mechanistic basis and degree of integration between White-shouldered Fairywren female ornaments and behavior. More specifically, they provide the opportunity to assess the degree to which the role of testosterone in microevolutionary processes is conserved in the progression from individual flexibility (male Red-backed Fairywrens) to fixed differences between populations (i.e. between female White-shouldered Fairywrens). Accordingly, the null hypothesis (conservation of mechanisms) predicts that the same combinations of traits (morphological and behavioral) will be regulated via the same mechanism in male and female White-shouldered Fairywrens as in male Red-backed Fairywrens, namely via variation in circulating testosterone levels. Alternatively, the integrated phenotype and its underlying mechanisms may be labile, with components added or subtracted from the hormone-dependent module in response to

selective pressures that differ across species, populations, or sexes. Here I test this null hypothesis, by assessing the corresponding predictions that populations of White-shouldered Fairywren with ornamented female plumage show: (1) higher circulating testosterone levels in females, (2) coordinated increases in aggressive behavior in females, and (3) constant levels of testosterone and aggression in males.

Methods

Study System & General Field Methods

Malurus fairywrens are predominantly socially monogamous, though sexually promiscuous passerine birds (Buchanan and Cockburn 2013). The White-shouldered Fairywren (*Malurus alboscapulatus*) of New Guinea forms a monophyletic clade with the sexually dichromatic Red-backed Fairywren and White-winged Fairywren (*Malurus leucopterus,* Driskell et al. 2011) of Australia. Female ornamentation is likely a derived trait in White-shouldered Fairywren populations with female ornamentation, as females are unornamented in both sister taxa (Driskell et al. 2011; Lee et al. 2012; Karubian 2013).

My study was designed to compare females from two populations: one with ornamented females (black-and-white, *M. a. moretoni*) and the other with unornamented females (cryptic brown, *M. a. lorentzi*). Ornamented *M. a. moretoni* females are qualitatively similar to ornamented males, but differ in lacking a dense barbule structure and satin sheen (Enbody et al. 2017). I worked at two sites in Papua New Guinea with color-banded study populations: 1) *M. a. moretoni* (‘ornamented’ population hereafter) in Milne Bay Province (150^{o}30’E,10^{o}15’S, 0-20m ASL, Figure 1.1) and 2) *M. a. lorentzi*

('unornamented' population hereafter) in Western Province (141° 19'E, 7° 35'S, 10-20m ASL, Figure 1.1). Both sites are centered in rural villages surrounded by tropical lowland savannah. The study site in Milne Bay province includes less contiguous grassland patches and slightly higher (2,682 mm/year) and less variable (23% precipitation seasonality, WorldClim) rainfall than the site in Western Province (2,339 mm/year, 49% precipitation seasonality, WorldClim).

I monitored populations at the Milne Bay Province site from 2013–2016 and the Western Province site during visits in 2014-2016. In each of these periods I captured most or all adults in an area of approximately 200 ha and monitored their behavior and breeding. As in many other tropical passerines (Stutchbury and Morton 2001), White-shouldered Fairywrens in both populations breed year-round and males in both populations possess enlarged cloacal protuberances year-round (E.D. Enbody, J. Boersma, unpublished data). Individuals were captured in mist nets by flushing or using playback and banded with a unique combination of plastic color bands and single aluminum band with a unique number provided by the Australian Bird and Bat Banding Scheme. At the time of capture, I collected blood samples (ca. 60-100 µl, mean net to bleeding time = 6min, range = 1-57min, but samples taken at >20min were excluded from analyses), which were spun in a centrifuge for 5 min for separation. Plasma was stored in 100% ethanol (Goymann et al. 2007) for analyses.

Androgen radioimmunoassay methods

I used a radioimmunoassay, closely following previously published methods (Lindsay et al. 2009; Lantz et al. 2017), for measuring levels of circulating androgens to

approximate testosterone levels for 139 females (105 ornamented, 34 unornamented) and 143 males. The protocol for the present study differed in that plasma samples were stored in ethanol and were only assayed for androgens. Samples ranging from 16.8–83.5 μl (mean: 39.49 μl; median: 41.65 μl) were vortexed and centrifuged, and the supernatant was transferred to extrelut columns (MilliporeSigma, Billerica, MA) for extraction of steroids. After extracting with diethyl ether, each sample was redissolved with 125 μl of phosphate-buffered saline with gelatin before being assayed for total androgens. Samples were assayed in single 100 μl aliquots using tritium-labeled testosterone (Perkin Elmer Life Science NET-553, Waltham, MA, USA) and a testosterone antibody (Wien Laboratories, T-3003, Flanders, NJ, USA) that has 100 % reactivity with testosterone, 60% with 5α- dihydrotestosterone, 5% with aldosterone, and <15% reactivity with other steroids. Note that I refer to testosterone although my radioimmunoassay cross-reacts with other androgens such as 5α- dihydrotestosterone. I ran samples in singlets rather than duplicates to maximize hormone detection in small sample volumes, following the validated protocol of Lindsay et al. (2009). Samples from both populations and sexes were randomly distributed across 8 separate assays, with a between assay coefficient of variation of 11.46% and a mean within assay coefficient of variation of 7.34% (range = 4.07–11.33%; coefficients of variation calculated according to Chard 1995). Androgen recovery rates were determined for each sample using tritiated testosterone, with a mean across assays of 64.12%. The minimum detectable androgen concentration was 228.09 pg/ml based on a 16 μl plasma sample cutoff and average recovery rate. Detectable samples had androgen titres ranging from 84.78– 6,025.95 pg/ml. I back-calculated plasma androgen titres from undetectable samples using my assay's minimal detectable

levels of 1.95 pg/tube, which yielded a range of 58.46–389.17 pg/ml, depending on plasma volume.

Presentation Experiment

I designed a presentation experiment to test the response of both sexes to the ornamented and unornamented phenotype in each subspecies between January and March, 2016. During this time in both populations, a small number of individuals were nesting or nested after playback experiments were completed. In each trial, a pair that was known to be territorial was presented with artificial mounts created from cardstock and accompanied by playback consisting of a previously recorded pair duet from their own population. Twelve cardstock mounts were adapted from designs for bird models by an artist (http://www.johanscherft.com). Each mount was painted using a combination of spray paint and gouache paint to resemble the two female phenotypes (n=4 ornamented and unornamented each) and male phenotype (n=4) and tail feathers from the respective phenotype were attached to the mount. The use of artificially colored mounts in this study (and others, e.g. Greig et al. 2015) could influence the degree to which aggression measurements reflect interactions between live birds. However, I are interested in the relative difference of aggression between receivers with different phenotypes and interpret my findings in light of comparative aggression differences. I quantified responses to artificial mounts using a male mount with an ornamented female mount or a male mount with an unornamented female mount. I used both a male and a female mount to simulate a territorial intrusion by a White-shouldered Fairywren pair and to distinguish between responses to an ornamented female or a male. Preliminary trials suggested that

when presented with a lone female combined with female song, males would often respond alone in an apparent attempt to incite courtship (E.D. Enbody, unpublished data), so presenting a pair with duet song was used to present a biologically meaningful intrusion in an agonistic context.

Five duets from each population were recorded from pairs using a Marantz PMD 661 Mk II (96kHz sampling rate, 24-bit depth; D&M Professional, Itasca, IL) with a Sennheiser ME66 shotgun microphone and K6 power module (Sennheiser Electronic Corporation, Old Lyme, CT). Each duet stimulus for playback consisted of the same duet repeated separated by 10s, filtered for noise below 500 Hz, and amplitude standardized using Audacity. Playbacks were broadcasted using an iPod touch (Apple Inc, Cupertino, CA) and an Altec Lansing iM227 Orbit MP3 speaker (Altec Lansing LLC, New York, NY).

Prior to running a trial, I located a territorial pair and observed their behavior for 3-5min prior to placing mounts on a location known to be within their territory. I only tested pairs where both individuals were in adult plumage. I randomly selected a stimulus mount and a local population stimulus song using the following protocol prior to each trial: 1) Randomly selected stimulus phenotype (ornamented or unornamented) 2) Randomly selected female mount (1-4; of the selected phenotype), 3) Randomly selected male mount (1-4), and 4) Randomly selected song stimulus (1-5; recorded locally). Each duet stimulus was used an average of 8 times (range: 3-15), each female mount an average of 9 times (range: 6-12), and each male mount an average of 19 times (range: 15-21). I only used songs recorded from pairs not adjacent to the focal territory to avoid the possibility that pairs were familiar with the song stimulus. Thus each focal pair was

presented with either of two combinations: ornamented female mount and male mount (with local song) or unornamented female mount and male mount (with local song). This experimental design was chosen to test the response to different visual stimuli, but not to different vocal stimuli.

Male and female mounts were placed on two similar 1.5m tall sticks that I used for all experiments. Each stick included three stems 25cm from the top where one stem held the mount and the two other were available for the responding pair to perch on. Male and female mounts were separated by 1m and a speaker, controlled remotely by observers, was placed in vegetation between and below the mounts. Next, I set up a small blind 20m away from the mount location for two observers. At the start of the trial, one observer began recording using the Marantz PMD 661 MK II sound recorder and another using a Sony DCRSX40/L camcorder. Each trial began with 1 min of acclimation time, followed by playback that continued until the focal female came within 1m of the mount or 5 min had passed, whichever was first. In the former scenario (female approaches within 1m), three more songs were played before ceasing playback, and for the latter scenario playback ceased at the end of 5 min. If the focal female never approached within 10m after 5 min then the trial was scored as "no response," ceased, and repeated another day. This usually occurred if the pair had traveled out of hearing distance or was engaged in another territorial dispute. A successful trial ended 10 min after the cessation of playback. Depending on the latency to response to 1m, trials varied in length between 10 and 15 min (13.34 min +/- 1.57 min), so continuous time variables are analyzed as rates (duration or # of behavior / trial length). Trials where focal pairs interacted with neighboring territorial holders were discarded (n=9). Following Greig et al. (2015), this

protocol allowed me to assess the response to the mounts after a constant number of songs had been played with the pair in close proximity. One observer focused on the female and the other the male and narrated behaviors into the microphone or video camera, respectively. I only include trials in which the female responded, excluding male-only responses. For both sexes, I recorded the duration of time at 0-5m from the mounts, the number of duets, flybys (when an individual flew within 0.5m of the mount), and leapfrogs (when one responding individual hopped over the other responding member of the pair), and latency to each behavior (see Table 1). These behaviors were selected based on their use in previous experimental playback trials for recording aggressive behaviors (Pearson and Rohwer 2000; Uy et al. 2009).

Though I conducted 75 total presentations (44 in the ornamented population, 31 in the unornamented population), females only responded in 55 trials (73% of trials), and I only analyzed trials that included a female response (33 in the ornamented population, 22 in the unornamented population). Males responded together with the female in 51 of the 55 trials (93% of responsive trials). Raw data on male response in n = 15 trials was irretrievably lost in the field due to failure of a data storage device (corresponding data on female response from these trials was not lost). I analyzed my data with and without these trials and obtained qualitatively similar results; for this reason I decided to include the 15 trials in which only data on female response was available in the analyses presented below.

Statistical analysis

Circulating testosterone

Although all individuals were potentially in breeding condition (see above) I excluded individuals known to be nest building (n=8), egg laying (n=4), incubating (n=11), or at nestling provisioning (n=11) stages due to well-documented short term impacts on circulating androgen levels associated with these reproductive activities in this and other species (Lindsay et al. 2009; Schwabl et al. 2014). The remaining dataset included individuals (n=235) in a reproductive stage I consider comparable to temperate species' "pre-breeding" levels, allowing me to avoid potentially confounding breeding stage effects on testosterone titres in males and females (Lindsay et al. 2009; Schwabl et al. 2014). Age may also influence testosterone levels (Lantz et al. 2017), but my sample sizes for known age birds in the unornamented population were insufficient to include as a predictor in the analysis. Delay between capture and blood sampling has a significant negative effect on circulating testosterone levels in other species (Lindsay et al. 2009), so as noted, I included only samples obtained within twenty minutes of capture and include time delay as a fixed effect (Lindsay et al. 2009; Lantz et al. 2017). Delay up to twenty minutes had, however, no significant effect on testosterone titres. Furthermore, the use of playback did not appear to have an effect on circulating testosterone titres (students t-test; $t=1.0459, df=127.59, p=0.298$). I included individual as a random effect in all models because some individuals were re-captured within and/or between years. After these filtering steps, I analyzed circulating testosterone from a total of 30 adult females and 26 adult males in the population with unornamented females and 84 adult females and 95 adult males in the population with ornamented females.

I first built a generalized linear model with a binomial distribution to test if subspecies differed in the probability that the androgen assay detected testosterone using

androgen detection as a binary response variable. I then used linear mixed models to test whether testosterone levels were related to subspecies or sex and other life history and extrinsic factors. I built models with known predictors of testosterone levels including subspecies, sex, year, time delay (until bled), and Julian date and two interaction terms (see Table S1). Following initial model comparisons, I corrected for heteroscedasticity in the subspecies and sex interaction term using a correction for variance structure. I were interested in the effect of subspecies and sex on circulating testosterone, so I performed stepwise model selection using AIC to remove nonsignificant effects. I present measures of support for my top models based on AIC. The linear mixed model for testing predictors of testosterone were conducted in R v3.3.2 (R Core Development Team 2016) using *nlme* (Pinheiro et al. 2017).

Response to simulated territorial intrusion

I first tested the effect of song stimulus (i.e. 1-5 at each site) and subspecies on the probability that an individual would respond using the expanded dataset that included both responding individuals and non-responders. I report the results of a generalized linear model using a binomial distribution on a binary response variable (i.e. "yes" or "no"). I then used principal components analysis (PCA) to quantify responses of free-flying birds from two subspecies of White-shouldered Fairywren in my field-based mount presentation experiments. All response variables were log transformed to improve normality, scaled and centered prior to running the PCA (following Filardi and Smith 2008; Uy, Moyle, and Filardi 2009). I assessed the effect of subspecies, sex, and stimulus type (and all interactions) on the top three principal components using linear-mixed

models in the package *lme4* in R (links to R scripts are available from the journal office), because *lme4* handles multiple random effects (Bates et al. 2015). I reduced the effect of pseudoreplication in my results (Kroodsma et al. 2001) by including male mount stimulus (1-4), female mount stimulus (1-4, nested in female phenotype), and song stimulus (1-5, nested in subspecies) as random effects in the model. I removed trials with individuals known to be nest building, incubating, or with nestlings (unornamented n= 7, ornamented n=5), because breeding stage can influence aggression in other species (e.g. Hunt et al. 1995). Residuals of the full model were normally distributed and did not violate homoscedastic assumptions. I tested for significant predictors of each model (i.e. the top three PCs) using a Wald chi-square test with alpha set at 0.05 using the Anova (Type II) command in the R package "car" (Fox and Weisberg 2011).

Results

Circulating testosterone

A greater proportion of ornamented female samples had detectable levels of testosterone (n=42, 50%) than did unornamented female samples (n=9, 30%), but this effect was not significant (glm: z= 1.865, df=112, p=0.062). The opposite, non-significant trend, was found in males, as a lower proportion of males from the population with ornamented females had of detectable testosterone (n=76, 80%) than males from the unornamented population (n=25, 96%): 0.96; glm: z= -1.743, df=119, p=0.081).

The top model for the effect of different predictors on circulating testosterone levels included the interaction between subspecies and sex, the interaction between subspecies and year, and Julian date (Table 1.1; I report AIC values for stepwise model

comparisons in Table S1.1). However, circulating testosterone was only significantly predicted by the interaction between subspecies and sex and also year; other variables had nonsignificant effects on testosterone (Table 1.1). Between sex and between population comparisons (following a Tukey's adjustment for multiple comparisons) of circulating testosterone levels indicate that ornamented females had higher levels of testosterone than did unornamented females and females of both populations were lower in circulating testosterone than males (Figure 1.2, Table 1.2). In contrast, males did not differ between populations in circulating testosterone (Figure 1.2, Table 1.2).

Response to simulated territorial intrusion

Stimulus song type (i.e., different song types from the same population that was being presented to; glm females: z=0.135, p=0.893; glm males: z=1.719, p=0.086) did not predict the likelihood that an individual would respond to the simulated territorial intrusion. Neither subspecies was more likely to respond to a trial (glm females: z=0.156, p=0.876; glm males: z=-0.101, p=0.919).

Among the 55 trials I included in my analyses, the first 3 PCs cumulatively explained 78.7% of variation in behavioral responses in both sexes to simulated territorial intrusion (Eigenvectors and variable loadings shown in Table 1.3). I interpret higher values of PC1 as an index of increased aggression, characterized by faster response time and more time spent close to the mount, as well as greater rates of pair coordination behavior (duets and leapfrogs). Interpretation is less clear for PC2, but the component seems to correspond to responses that were rapid, but of short duration, or characterize the overall motivation of an individual to respond. PC3 was also associated with

increased aggressive response, in particular a high number of flybys, and by less pair coordination behavior (duets and leapfrogs). Individual response variables are shown in Figure S1.

Subspecies was a significant predictor of PC1, PC2, and PC3 (Table 1.4, Figure 1.3), indicating that birds in the population with ornamented females exhibited a more aggressive response to simulated territorial intrusion than did birds in the population with unornamented females. Responses were tightly correlated between the sexes, consistent with the observation that pairs generally responded together with similar intensity. However, sex was a significant predictor of PC2, indicating that, between the two populations, females may have been overall less quick to approach and remain close to the mount, while still interacting to a high degree in pair coordination behavior and song. No other effects, including stimulus type or any interactions, were significant predictors of PC1, PC2, or PC3 (Table 1.4).

Discussion

This study examined the association between circulating testosterone, ornamentation, and aggression in females and males of two recently diverged populations of passerine bird, White-shouldered Fairywrens (*Malurus alboscapulatus*), that differ in female ornamentation. In doing so, my goal was to explore the extent to which the integrated ornamented phenotype is conserved between the sexes and across populations with variable ornamentation. I found that females with an ornamented plumage phenotype have higher levels of circulating testosterone than do females with an unornamented phenotype. Females from the population with elevated levels of

testosterone and ornamentation also exhibited greater aggressive response to experimental territorial intrusions. My results indicate that a hormonal mechanism mediates integration of plumage ornamentation and aggression in female White-shouldered Fairywrens, following the same associations between these traits previously documented in male Red-backed Fairywrens (Lindsay et al. 2009; Webster et al. 2010; Lindsay et al. 2011, but see Barron et al. 2015). Notably, I recovered no corresponding relationship between testosterone and aggression among male White-shouldered Fairywrens suggesting that selection acts on female traits independent of selection on males. More broadly, these findings point to the ways in which regulation of trait expression and degree of coupling among those traits can vary among different levels of biological organization, and suggest directions for future research, as outlined below.

My finding that two populations with variation in female ornamentation differ in circulating levels of testosterone suggests a role for testosterone in mediating the female ornamented phenotype. It has been demonstrated that testosterone induces ornamented trait expression in younger male Red-backed Fairywrens (Lindsay et al. 2009; Lindsay et al. 2011), but the relationship between testosterone and behavior in different plumage types is less clear among older males (Barron et al. 2015) and across populations (Lantz et al. 2017). Experimental doses of testosterone also activate the development of a partial male-like plumage and bill color in the naturally unornamented female Red-backed Fairywrens (Lindsay et al. 2016). Age is associated with increased testosterone in female Red-backed Fairywrens, which may result in the production of a few red feathers and a darker bill in some older birds (Lindsay et al. 2016). I lack the long-term data necessary to explore the effect of age in White-shouldered Fairywrens, but all females molt into

subspecific adult plumage in their second year. In other sexually dimorphic species, testosterone-implanted females produce only a portion of the male's morphological phenotype (*Malurus cyaneus*; Peters 2007) or song type (*Sturnis vulgaris*; Ridder et al. 2002). Together these results are consistent with circulating testosterone being partly responsible for regulating sexual dimorphism, suggesting that other circulating hormones (e.g. oestrogen; Owens and Short 1995) or transcriptional regulation (Rosvall et al. 2012) also mediate ornament expression differences between the sexes. Yet in White-shouldered Fairywrens, the association between elevated plasma testosterone, the production of an ornamented plumage and increased aggression implies similarity in the relationship between testosterone and ornament production between the sexes. This finding has broader implications, because differential sex steroid production and secretion in adulthood is a potential mechanism through which sexual dimorphism can be maintained, despite males and females sharing most of their genetic architecture (Adkins-Regan 2005). Despite my robust findings, the pattern I observe is limited to two allopatric populations and is therefore limited by overall sample size. Thus, while the differences detected in my statistical analysis appear sufficient for documenting the general patterns between the different female phenotypes, the direct functional role of testosterone in ornament production is still unknown and will be best addressed using experimental testosterone-implant studies.

Intriguingly, the observation that testosterone is elevated in ornamented females, but not males of that population, suggests that testosterone is not elevated in females simply as a byproduct of selection for higher androgen levels in males (Ketterson et al. 2005; Møller et al. 2005). Accordingly, adaptive explanations for the appearance of

ornamentation and higher aggression should be considered for the evolution of ornamentation in females. Female aggression can have fitness consequences in both vertebrates (Dloniak et al. 2006; Stockley and Bro-Jørgensen 2011; Cain and Langmore 2016) and invertebrates (Elias et al. 2010; Bath et al. 2017). Female Red-backed Fairywrens also respond aggressively to female intruders (Karubian et al. 2008). Therefore, it is parsimonious to suggest that female ornaments would function in similar contexts where they are present in White-shouldered Fairywrens. Aggression is important for establishing dominance relationships and mediating intrasexual conflict, a process that is associated with sexual selection and speciation.

My study demonstrates that aggressive behavior differs between females of two populations that vary in female, but not male ornamentation. I suggest that female ornaments in this system function in territory defense and may mediate access to limiting resources (ecological or social) by gaining access to higher quality territories. In addition, heavily weighted principal components for duets and leapfrogs suggests that ornamented females may be coordinating to a greater degree with their mate to improve territory defense. If this hypothesis is true, social selection may play a role in shaping female phenotypic traits by improving access to ecological or social resources (West-Eberhard 1979). It is possible that habitat or environmental differences drive this pattern, as the site with unornamented females shows greater variability in seasonality of rainfall (see Methods, above). For example, resources may be more evenly distributed in the less seasonal environment where the ornamented population occurs, and species that experience little seasonality may have low territory turnover between years (Stutchbury

and Morton 2001), which could lead to long-term investment in territory maintenance and elevate the importance of territory defense behaviors.

My findings provide compelling evidence that males and female in a population with ornamented females respond with a greater degree of territorial defense than individuals in a population with unornamented females. Differential aggression towards heterospecific stimuli can influence species interactions by mediating aggressive interactions (e.g. Jankowski et al. 2010; Tobias et al. 2013; Freeman 2016), but I find that presentation of different stimulus types (ornamented or unornamented female) does not elicit differential aggression in either population. The artificial mounts used in this project may elicit a different amplitude of response compared to live birds, but the lack of differential response to different stimuli at least suggests that broader patterns in aggression represents a true difference in baseline aggression in the two sub-species, rather than a differential response to the stimulus type I presented. These results also suggest that unornamented females do not have a preexisting bias to respond with greater aggression towards a perceived aggressive phenotype (e.g. through sensory drive: Endler 1992). However, future research investigating responses to song types vs. plumage phenotypes from the two populations would provide clarify whether there are pre-existing biases for signal types in White-shouldered Fairywrens.

Male testosterone levels were not elevated in the White-shouldered Fairywren population that showed higher male and female territorial responses, and in which females had higher testosterone levels. In Red-backed Fairywrens, intraspecific differences in plumage color between males are associated with suites of behavioral and other traits, including aggression (Karubian et al. 2008) and reproductive behaviors

(Karubian 2002; Barron et al. 2015; Dowling and Webster 2017). These differences in behaviors also covary with circulating testosterone during all reproductive phases (Lindsay et al. 2009). Although circulating testosterone is regulated, at least in part, by the social environment (Karubian et al. 2011a) in Red-backed Fairywrens, little is known about how testosterone influences behavior directly (Barron et al. 2015). My findings suggest that in White-shouldered Fairywrens, as in Red-backed Fairywrens, testosterone levels are elevated in males relative to females, but that relative sub-specific differences in testosterone for males do not mirror differences in aggression. Similar research in songbirds with differing aggression between populations have suggested that testosterone sensitivity may have a more direct effect than differences in circulating levels (Bergeon Burns et al. 2013; Bergeon Burns et al. 2014), which may apply to male White-shouldered Fairywrens as well. It remains to be shown whether sex differences in White-shouldered Fairywrens suggest decoupling of male territorial behavior from testosterone levels or result from differences in habitat structure, breeding density, and seasonality that could affect male testosterone levels and territorial behavior. I hesitate to speculate extensively on this pattern in males, as differences may be attributable to a small number of individuals in the population with unornamented females exhibiting extremely high levels of testosterone. Consistent with this observation, males in the population with unornamented females appear to spend more time interacting with members of other groups than in the population with ornamented females (E. D. Enbody, J. Boersma, unpublished data), which may elevate testosterone levels (e.g., Challenge Hypothesis; Wingfield et al. 1990).

Conclusions

The effects of testosterone couple different components of an individual's physiology, morphology, and behavior and therefore have the potential to play a critical role in maintaining integrated phenotypes (Ketterson et al. 2009). Findings in this study complement prior work on males suggesting that testosterone, ornamentation, and behavior are interrelated in the production of competitive reproductive phenotypes (Wingfield et al. 1990; Hau 2001; Archer 2006). Overall, my findings are consistent with the idea that similar morphological and behavioral traits are integrated into phenotypes across sexes and populations by the same hormone, in this case testosterone. The gain or loss of ornamentation in females has been shown to influence macroevolutionary patterns of sexual dichromatism (Irwin 1994; Burns 1998; Figuerola and Green 2000; Friedman et al. 2009; Price and Whalen 2009; Johnson et al. 2013; Shultz and Burns 2017) and my study suggests that it similarly influences differentiation among populations. Evidence of associations between population-specific female ornamentation, testosterone levels, and aggressive behavior suggest that females hormonally integrate morphological and competitive traits using a similar process as males in other species (namely testosterone, Cain and Ketterson 2012). This lays out promising avenues for further study of female ornament evolution focusing on mechanisms and underlying components of integrated male and female phenotypes.

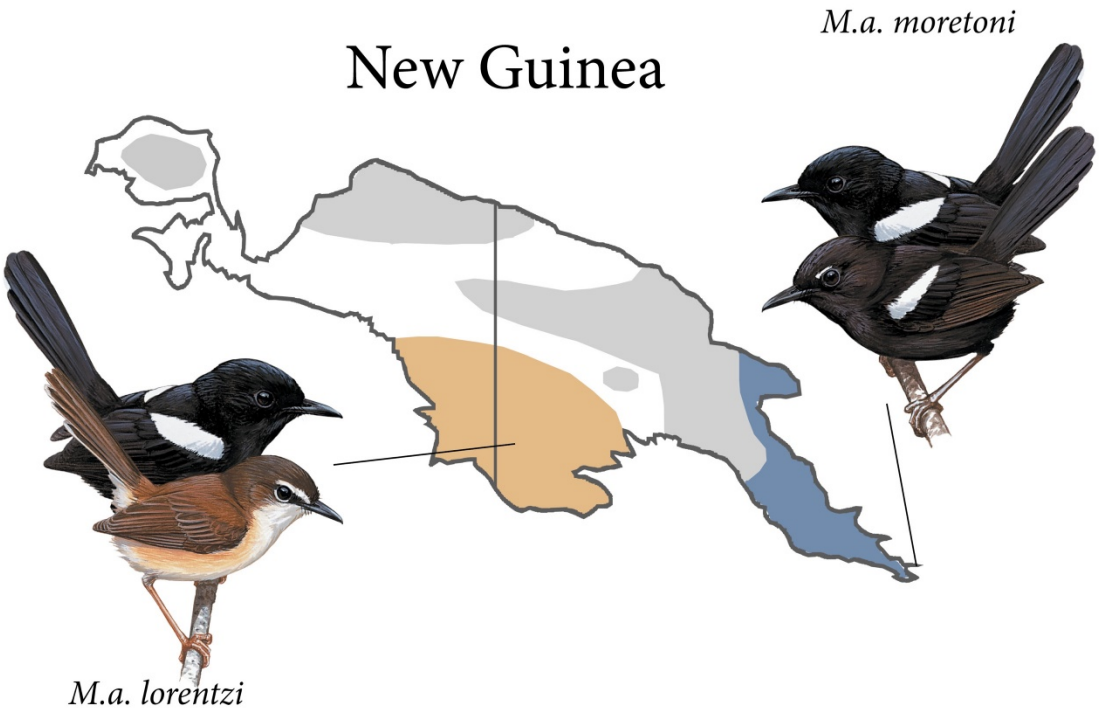

Figure 1.1: Illustrations of the two taxa included in this study (del Hoyo et al. 2017, reproduced with permission). Females are either lacking in melanized ornamentation ("unornamented") and the white shoulder patch (left: *M.a. lorentzi*) or possess a melanized black and white plumage ("ornamented", right: *M. a. moretoni*). Males, illustrated behind females, have similar plumage features in both populations. Gray regions on the map of the island of New Guinea (with the political border drawn in the middle) refer to other populations of White-shouldered Fairywren not included in this study and ranges are based on Birdlife International and NatureServe (2013).

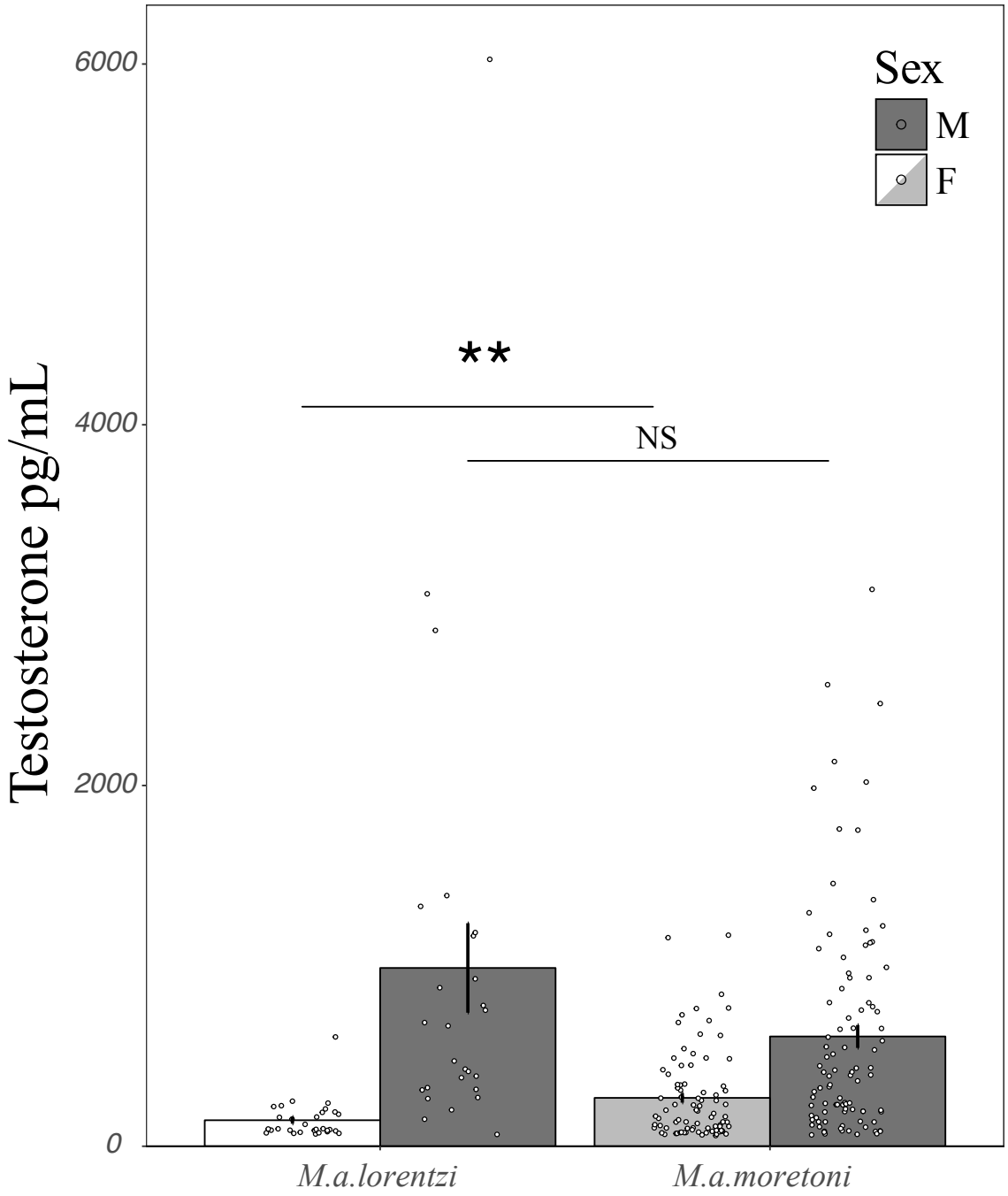

Figure 1.2: The relationship between circulating testosterone titres (untransformed) and subspecies in male (grey) and female (white or light grey) White-shouldered Fairywrens. Error bars denote standard error and significant comparisons are denoted by an asterisk (**=p<0.01).

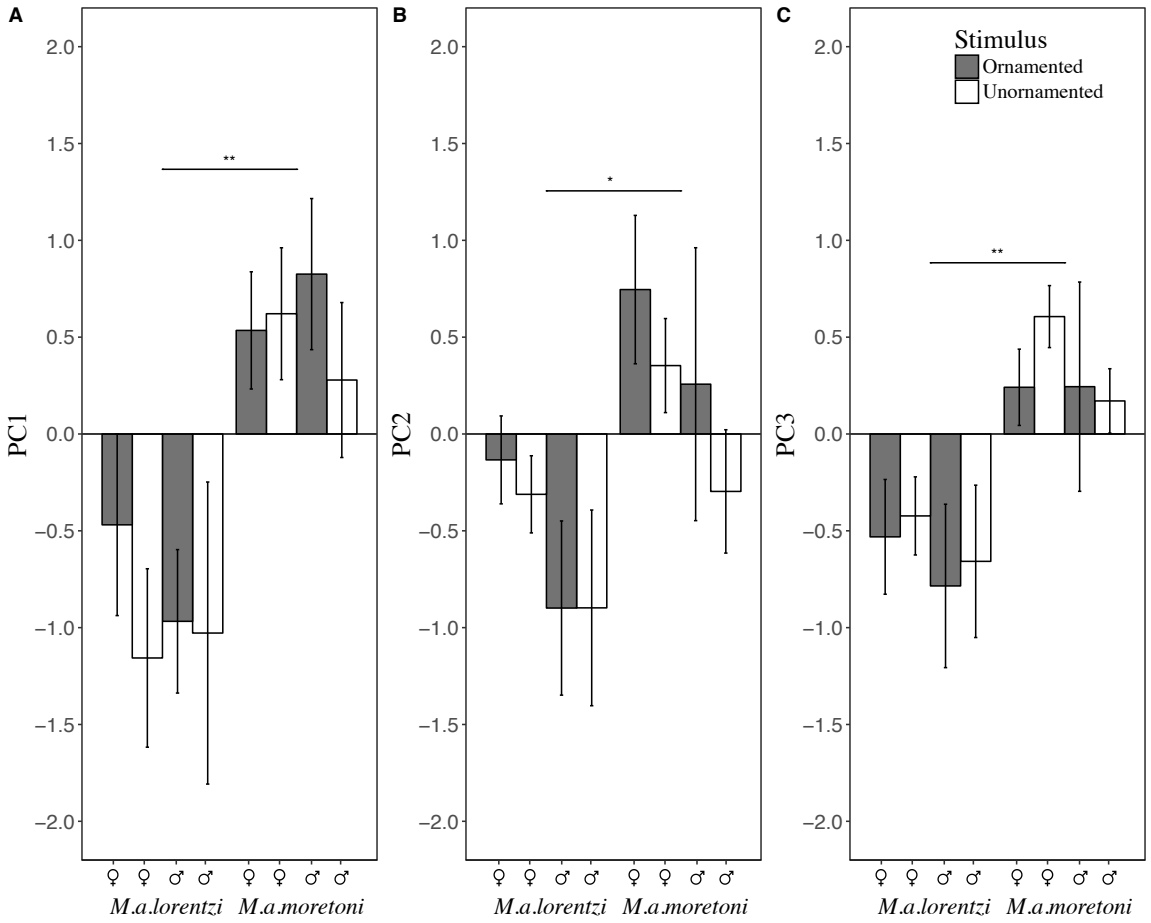

Figure 1.3: Response of White-shouldered Fairywrens to simulated territorial intrusions. Shown are responses by free-flying females and males of two populations that differ in degree of female ornamentation. In *M. a. lorentzi*, females are unornamented and in *M. a. moreotni*, females are ornamented. Color of the bar represents the type of stimulus presented: white denotes trials where an unornamented stimulus was presented and grey denotes trials where an ornamented stimulus was presented. Both sexes are plotted on the x-axis. Individuals of both sexes in the population with ornamented females responded with greater values in PC1 (A) and PC3 (B). Females trended to responded with greater overall values of PC2 than males. Error bars show standard error and significance is shown for comparisons between sexes (NS=not significant) and between populations (**=p<0.01, *=p<0.05).

Table 1.1: The effect of the fixed predictor variables on circulating testosterone using a linear mixed model, with individual as a random effect.

	Value	Std Error	Df	t-value	*p*
(Intercept)	4.052	0.386	160	10.509	<0.001*
Subspecies	0.79	0.229	160	3.449	<0.01*
Sex	1.628	0.188	160	8.639	<0.001*
Year (2015)	0.101	0.203	66	0.495	0.622
Year (2016)	1.144	0.309	66	3.698	<0.001*
Julian Date	0.003	0.002	66	1.405	0.165
Subspecies*Sex	-0.98	0.233	160	-4.208	<0.001*
Subspecies*Year(2015)	-0.434	0.287	66	-1.515	0.135
Subspecies*Year(2016)	-0.545	0.332	66	-1.642	0.105

Table 1.2: Contrasts for linear mixed model comparing circulating testosterone between subspecies and sex following a Tukey's adjustment for multiple comparisons.

Sex	Subspecies	estimate	SE	Df	t.ratio	*p*
F - F	*M. a. lorentzi* - *M. a. moretoni*	-0.464	0.136	66	-3.4	<0.01*
M - F	*M. a. lorentzi* - *M. a. lorentzi*	-1.628	0.188	160	-8.639	<0.0001*
	M. a. lorentzi - *M. a. moretoni*	-1.111	0.148	66	-7.49	<0.0001*
	M. a. moretoni - *M. a. lorentzi*	-1.164	0.210	66	-5.542	<0.0001*
	M. a. moretoni - *M. a. moretoni*	-0.648	0.137	160	-4.731	<0.0001*
M - M	*M. a. lorentzi* - *M. a. moretoni*	0.516	0.218	66	2.368	0.0935

M. a. moretoni have ornamented females and *M. a. lorentzi* have unornamented females.

Table 1.3:PCA eigenvectors (standard deviation), variance explained, and loadings for the top three PCs.

	PC1	PC2	PC3
Standard deviation	1.462	1.283	0.968
Proportion of Variance	0.356	0.275	0.156
Leapfrogs	0.377	0.363	-0.458
Duets	0.47	0.456	0.271
Time < 5m	0.383	-0.441	-0.442
Latency to 5m	-0.367	0.524	0.149
Latency to first duet	-0.566	-0.154	-0.257
Flybys	0.188	-0.41	0.659

Leapfrogs is the rate of leapfrog behavior in each trial, duets are the rate of coordinated songs per trial, time <5m is the proportion of time spent at a distance of <5m from the mounts, and flybys are flights within 0.5m of the mounts.

Table 1.4: Model estimates, standard error, and t-value for the effect of subspecies (focal *M. a. moretoni* or focal *M. a. lorentzi*), sex, mount stimulus (ornamented or unornamented), and all interactions on the top three principal components.

	Predictor	Estimate	Std Error	t-value	Chisq	Df	*p*
PC1	Subspecies	1.004	0.575	1.745	27.002	1	*<0.01**
	Sex	-0.498	0.664	-0.75	0.129	1	*0.72*
	Stimulus	-0.688	0.601	-1.144	0.886	1	*0.346*
	Subspecies*Sex	0.789	0.85	0.928	0.058	1	*0.81*
	Subspecies*Stimulus	0.774	0.754	1.027	0.209	1	*0.648*
	Sex*Stimulus	0.627	0.944	0.664	0.087	1	*0.768*
	Subspecies*Sex*Stimulus	-1.26	1.187	-1.061	1.127	1	*0.289*
PC2	Subspecies	0.635	0.566	1.12	4.846	1	*<0.05**
	Sex	-0.765	0.564	-1.356	6.445	1	*<0.05**
	Stimulus	-0.281	0.591	-0.476	0.364	1	*0.546*
	Subspecies*Sex	0.341	0.726	0.47	0.06	1	*0.806*
	Subspecies*Stimulus	0.175	0.677	0.258	0	1	*0.988*
	Sex*Stimulus	0.145	0.811	0.179	0.065	1	*0.799*
	Subspecies*Sex*Stimulus	-0.426	1.021	-0.417	0.174	1	*0.676*
PC3	Subspecies	0.872	0.379	2.298	19.151	1	*<0.01**
	Sex	-0.253	0.388	-0.653	2.365	1	*0.124*
	Stimulus	0.139	0.445	0.313	0.173	1	*0.678*
	Subspecies*Sex	0.27	0.499	0.541	0.044	1	*0.834*
	Subspecies*Stimulus	0.185	0.465	0.397	0.007	1	*0.931*
	Sex*Stimulus	-0.078	0.558	-0.14	0.921	1	*0.337*
	Subspecies*Sex*Stimulus	-0.389	0.703	-0.553	0.306	1	*0.58*

Significance tested using a Wald's chi-square test and I report chi-square values, degrees of freedom, and p-value with significant effects marked with an asterisk. Subspecies is a significant predictor of both PC1 and PC3.

Supplementary figures

Table S1.1: White-shouldered Fairywren models built to predict the effect of various fixed effects on circulating testosterone. The best model (according to AIC) included only the interaction between subspecies and sex and Julian date.

Model effects	AIC	df	ΔAIC	weight
Subspecies* Sex + Subspecies* Year+ Julian Date + Time Delay + T after sunrise	613.1	13	2	0.1461
Subspecies* Sex + Subspecies* Year + Julian Date + Time Delay	613.1	12	1.9	0.1501
Subspecies* Sex + Subspecies* Year + Julian Date	611.2	11	0	0.388
Subspecies* Sex + Subspecies* Year	612.9	10	1.7	0.1679
Subspecies* Sex + Year	613.2	8	2	0.1432
Subspecies* Sex	620	6	8.8	0.0047

All models compared using a stepwise model selection are presented and all models include individual as a random effect. AIC is the Akaike information criteria, ΔAIC is the difference between each model and the top model, and weight is the model weight.

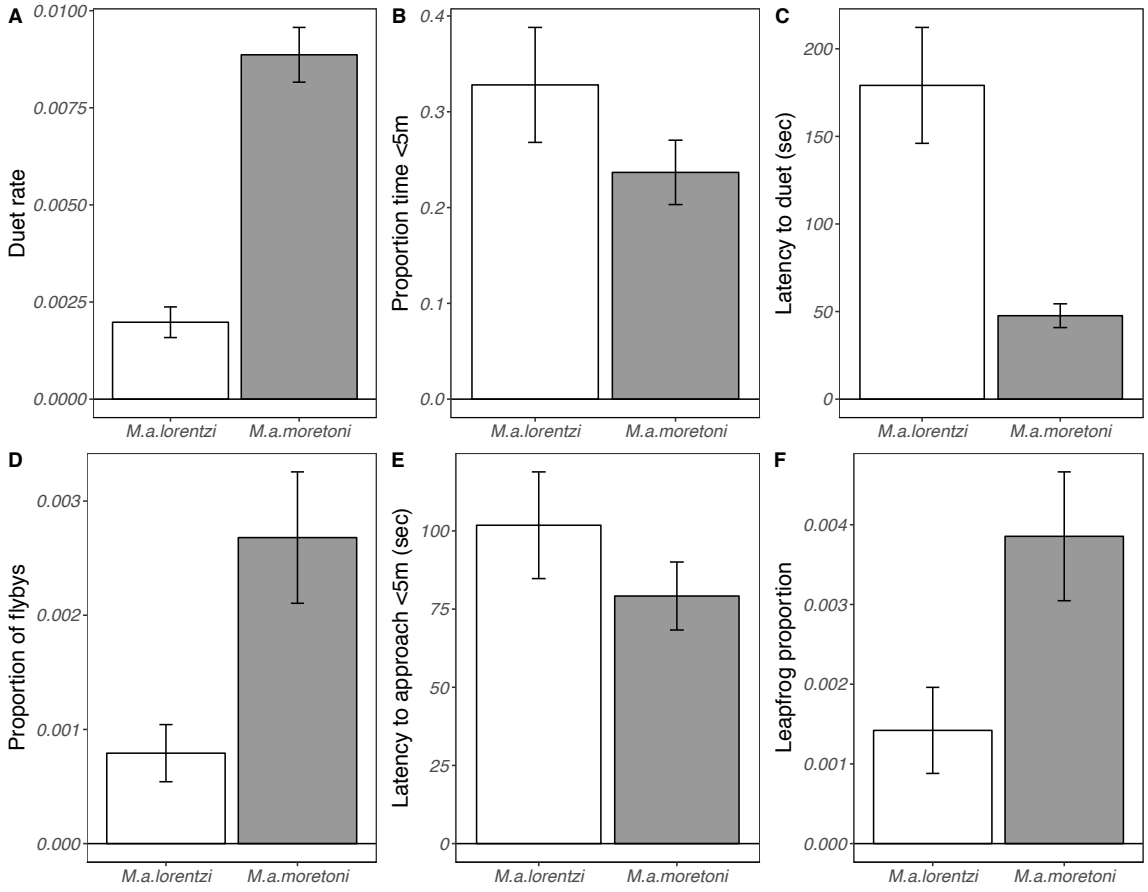

Figure S1.2: Each response variable used in the principal components analysis of the main text. Duet rate (A) is given as number of songs per trial length and proportions (B,D,F) are number of behaviors per trial lengths. Dark grey bars denote the population with ornamented females, white represents the population with unornamented females. Error bars show standard error.

CHAPTER 2

PRODUCTION OF PLUMAGE ORNAMENTS AMONG MALES AND FEMALES OF TWO CLOSELY RELATED TROPICAL PASSERINE BIRD SPECIES

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ABSTRACT

The evolution of elaborate secondary sexual traits (i.e., ornaments) is well-studied in males but less so in females. Similarity in the appearance of ornaments between males and females supports the view that female ornaments arise as a neutral by-product of selection on male traits due to genetic correlation between sexes, but recent research suggests an adaptive function of female ornaments in at least some contexts. Information on the degree to which production of ornaments differs between the sexes can shed light on these alternative perspectives. I therefore characterized the structural underpinnings of melanin-based plumage production in males and females of two closely related passerine bird species (genus *Malurus*). Importantly, both ornamented and unornamented phenotypes in each sex are present between these two species, providing an opportunity to test the null expectation of equivalent modes of production in male and female ornamented phenotypes. In *Malurus alboscapulatus*, ornamented females are qualitatively similar to males, but I describe a distinctive ornamented female phenotype that differs from that of males in lacking a blue sheen and in lower feather barbuledensity. In *M. melanocephalus*, unornamented males and females are also similar

in appearance, and I describe a similarity between unornamented phenotypes of males and females in both color and underlying feather barbule structure and pigment composition. Unornamented male *M. melanocephalus* can flexibly transition to the ornamented phenotype in weeks, and I found extreme differences in color and feather structure between these two alternative male phenotypes. These results contradict the idea that female ornaments have evolved in this system following a simple switch to male-like plumage by demonstrating greater complexity in the production of the ornamented phenotype in males than in females.

Introduction

There is growing awareness that a comprehensive understanding of sexual selection depends upon better understanding the female perspective as well as that of males, in part because the selective pressures experienced by females may differ from those experienced by males (Amundsen 2000; Clutton-Brock 2007; Rosvall 2011; Tobias et al. 2012). This has spurred renewed interest in the evolutionary history and current adaptive function of secondary sexual traits, or ornaments, and expression between the sexes (Kraaijeveld et al. 2007; Price and Whalen 2009; Nordeide et al. 2013). Ornaments may convey information about an individual's health and quality if production of the ornament is physiologically costly (Zahavi 1975). An understanding of the proximate mechanism for producing ornaments in each sex is an important component of understanding the form and function of ornaments in both sexes. For example, in the lizard *Sceloperus virgatus*, females produce an honest, sex-specific throat ornament (Weiss et al. 2009) which uses a pigment, lacking in males, that is thought to

limit tradeoffs with egg development (Weiss et al. 2012). Female *Onthophagus sagittarius* (dung beetles) have horn weaponry that is similar to males, but produced in a different location (Douglas J. Emlen et al. 2005; Simmons and Emlen 2008), which is associated with competition for ecological resources (rather than competition for mates, as in males; Watson and Simmons 2010). However, variation in sex specific ornaments within and across systems means that a comprehensive explanation for the production and adaptive function of ornaments in both sexes remains incomplete.

In birds, when ornamentation is present in both sexes, the appearances of males and females are often similar (Amundsen and Parn 2006). The observation that females possess identical or rudimentary forms of male ornaments first motivated the idea that ornaments evolve in females only as a neutral byproduct of selection on males (Darwin 1871). The genetic correlation model proposed by Lande (1980) suggests that selection on one sex can be strong enough to produce a correlated inheritance of those traits in the other sex in the absence of selection pressures. However, recent research has identified numerous examples of adaptive benefits to female ornamentation (reviewed in Kraaijeveld et al. 2007). An understanding of the mechanisms underlying female ornament production and expression provides an opportunity to assess the degree to which ornaments in females are rudimentary or analogous to those found in males. For example, careful examination has revealed differences between the sexes in subtle features of color (e.g. in colors in the ultraviolet range; Hunt et al. 1998) and structural components (Shawkey et al. 2005) that may imply sex-specific selection pressures (Heinsohn et al. 2005). However, such studies remain relatively rare, and a better understanding of the proximate sources of color variation can provide important insights

into how selection acts on male and female ornaments (Maia, Rubenstein, et al. 2013; Gluckman 2014).

The underlying architecture for color production is largely conserved among birds (Prum 2006; Shawkey et al. 2006), and as a result, there is reason to expect that similarly ornamented phenotypes in each sex follow similar mechanistic pathways (Shawkey et al. 2005). Coloration in bird feathers is produced by pigments, or through the fine scale arrangements of feather materials into nanostructures that selectively scatter light, or both (Hill and McGraw 2006; Eliason et al. 2015). Melanin is an endogenously produced pigment that is present across all bird taxa (Stoddard and Prum 2011) and is the basis for black, brown, or gray coloration in feathers (Fox and Vevers 1960). In contrast, feather structure properties are responsible for white, matte, and iridescent colors (Shawkey et al. 2006). There is evidence that ornament production by each of these mechanisms has associated physiological costs (Hill and McGraw 2006) and that these costs may vary across mechanisms (e.g. carotenoid pigments, reviewed in Svensson and Wong 2011; melanin pigments, reviewed in Guindre-Parker and Love 2014; structural properties, Keyser and Hill 1999). In addition, melanin-based color production can have pleiotropic effects on physiology and behavior (Roulin and Ducrest 2013). Therefore, plumage of any variety can potentially serve as an honest signal and face associated selective pressures, making plumage a suitable trait for studying ornament evolution in males and females. I ask how mechanisms of production differ between variable phenotypes of both male and female birds. I reason that patterns of similarity between the sexes for ornament production would provide evidence for a conserved underlying mechanism across sexes,

whereas exceptions may suggest alternative selection pressures driving ornamentation in males and females.

The Australasian *Malurus* fairywrens provide a useful system for studying male and female traits, due to extensive existing research into the behavior, life-history, and ecology of the group (Buchanan and Cockburn 2013) and the considerable intra- and interspecific variation in plumage coloration within the group (Johnson et al. 2013; Karubian 2013). In the current study, I compared the anatomical basis for variation in the melanin-based color in the crown, a putative plumage ornament (Rowley and Russell 1997), in three sister lineages in the 'bi-colored' clade of *Malurus* fairywrens (family Maluridae). I examine two subspecies of *M. alboscapulatus* (White-shouldered Fairywren: WSFW*;* Meyer 1874) and in *M. melanocephalus* (Red-backed Fairywren: RBFW*;* Latham 1801) that exhibit considerable variation in both male and female ornamentation (Figure 2.1). Phylogenetic evidence suggests that these lineages are descendent from a monochromatic ornamented ancestor within Maluridae (Driskell et al. 2011; Lee et al. 2012; Johnson et al. 2013; Karubian 2013) and that female ornamentation was lost in the genus *Malurus* relatively recently (Friedman and Remeš 2015). For my purposes in the current study, however, the relevant female ancestral state is of an unornamented ancestor at the level of the bi-colored clade and female ornamentation can be considered a derived character that occurs only in some populations of WSFW following a recent, rapid color change in females (Johnson et al. 2013). Ornamented WSFW populations have been treated as sexually monomorphic in comparative studies (Johnson *et al.*, 2013; Karubian, 2013, but see Friedman & Remeš, 2015), although it has been noted that sexes differ in a “satin sheen” possessed by males and not females

(Schodde 1982). Thus, one open question concerns the degree to which ornamented female WSFW resemble males and, if they differ, what the underlying structural causes of this variation may be. Male RBFW within a population express one of two plumage phenotypes, ornamented or unornamented, and females are unornamented (Rowley and Russell 1997; Karubian 2002). Males can flexibly transition from an unornamented to ornamented phenotype within a few weeks (Lindsay et al. 2009; Karubian et al. 2011b). While the ornamented male RBFW is unmistakable, unornamented male and female RBFW are generally indistinguishable in plumage to the human eye; however, experimental evidence suggests that both males and females can distinguish between the two (Karubian et al. 2008). Therefore, a second area of inquiry concerns the degree of similarity between dull male and female RBFW, and the structural change in feathers required for males to transition from an unornamented to ornamented state.

I assessed how feather morphology mediates differences in visual signal expression within and among sexes in these two closely related species using photospectrometry and electron microscopy. My over-arching null hypothesis was that, across all ornamented sexes and lineages, plumage is produced through similarities in barbule density and fine scale arrangement of melanin in barbules. I find that ornamented plumage in WSFW females is distinct from that of ornamented male WSFW and RBFW. This difference between the ornamented females and males corresponds to differences in feather barbule structure. I also find notable differences in feather morphology (barbule density and melanin content) between ornamented and unornamented male RBFW. In contrast, I found similarity between RBFW unornamented males and unornamented females in color and feather structure. Taken together, these findings demonstrate that the

mechanisms of ornament production are similar among males of distinct species, but differ among males and females of a single species. These findings also suggest that that the flexible transition when molting from unornamented to ornamented male phenotypes is associated with substantial structural changes.

Materials and methods

Study species and sample collection

Both RBFW and WSFW are socially breeding, tropical, insectivorous passerines that live in grassland environments in Australasia (Rowley and Russell 1997). The WSFW is endemic to New Guinea, where it is widespread, and the RBFW is endemic to northern and eastern Australia (Rowley and Russell 1997). Male WSFW are black with a bluish sheen and white shoulder patches and females of the focal populations can either look similar (although perhaps visually duller; Schodde 1982), or are overall brown in color (Figure 2.1). Ornamentation in some populations of female WSFW is extensive compared to other *Malurus* species (Karubian 2013) and this intraspecific variation in female ornamentation is rare amongst birds (but see other examples in Bleiweiss, 1992; Andersen *et al.*, 2014; Kearns *et al.*, 2015). Male RBFW are black (with a colorless sheen) with red backs but also have a brown plumage and females are always brown (Figure 2.1). Male RBFW within a single population exhibit a high degree of flexibility in male plumage development, in contrast to the static interpopulation differences discussed in WSFW above. Most male RBFW molt (pre-alternate molt) into the black and red ornamented plumage before breeding. First-year male RBFW can breed as unornamented brown birds (qualitatively similar to females), but are socially subordinate

to ornamented individuals (Karubian 2002; Karubian et al. 2008) and most or all males are unornamented in the nonbreeding season (following the pre-basic molt). Female RBFW always molt an unornamented brown plumage (Rowley and Russell 1997), although a small number of older females (<5%) produce a few red, but not black, feathers (Lindsay et al. 2016). Both species possess a violet-sensitive single cone ('SWS1'), meaning they are sensitive to some ultraviolet wavelengths (Ödeen et al. 2009; Ödeen et al. 2012).

I collected adult crown feathers from 67 ornamented male, 33 ornamented female, and 27 unornamented female WSFW and 7 ornamented male, 13 unornamented male, and 8 unornamented female RBFW in May-August, 2014. I collected samples from WSFW for ornamented females in Milne Bay Province, Papua New Guinea ($150^{o}30'E,10^{o}15'S$, 0 -20 m ASL, Figure 2.1) and for unornamented females from Western Province, Papua New Guinea (141° 19'E, 7° 35'S, 10-20m ASL, Figure 2.1). I collected samples from RBFW in Northern Territory, Australia (13°02' S, 131°02' E, 50m ASL, Figure 2.1). I took a small blood sample from each individual and stored red blood cells in lysis buffer for subsequent genetic determination of sex.

Laboratory sexing

To assign sex to unknown individuals, I extracted DNA from blood samples using a DNeasy blood and tissue kit (Qiagen) and amplified a sex-specific intron within the CHD gene using primers 2550F/2718R (Fridolfsson and Ellegren 1999). I ran CHD intron fragments through electrophoresis using a 2% agarose minigel and stained with

SYBR Safe DNA gel stain (Life Technologies). Bands were scored visually following Kahn et al. (1998), using positive controls to confirm accuracy.

Color Spectrometry

I used photospectrometry to measure spectral reflectance of all crown feathers. I mounted all sampled crown feathers on black illustration board (Dick Blick Art Materials, Ultra-black Mounting Board) in an overlapping pattern. I recorded reflectance using an Ocean Optics USB-2000+ spectrometer (R400-7-UV-VIS probe, RPH-1 probe holder) with a PX-2 pulsed xenon light source under laboratory conditions. I recorded % reflectance relative to a WS-1 white standard (Ocean Optics) for each feather with the probe 7mm from, and perpendicular to, the surface. Although other angles were investigated, I chose a perpendicular orientation, as I were able to achieve repeatable measurements and observe the saturation of blue sheen of male WSFW feathers (following Shawkey et al. 2006). I used SpectraSuite (Ocean Optics) software to record reflectance curves at 20 scans per sample with an integration time of 100. I averaged three reflectance measurements taken by completely removing the probe and placing it back down. I re-calibrated against the white standard and two color standards at regular intervals to ensure consistency of measurements throughout data collection.

I generated color variables for analysis using the pavo package version 0.5-5 (Maia, Eliason, et al. 2013) in R version 3.3.0 (R Core Team 2016). To describe achromatic plumage variation and the strength of color signal properties, I calculated brightness as mean reflectance over the entire avian visual spectral range (300-700nm; Montgomerie 2006). Low values of brightness represent dark colors and high values

represent light colors. I found that hue was not a useful metric to describe chromatic variation in either species (as used in some studies of sexual selection in carotenoid-based plumage ornaments in birds, e.g., Baldassarre and Webster 2013), because in the avian tetra color space model, hue is a measure of the horizontal and vertical deviance from the achromatic origin, and my measurements of black/brown feathers were largely clustered around the achromatic origin (Stoddard and Prum 2008). Instead I examined chroma, which describes the distance a color is from the achromatic origin (Stoddard and Prum 2008) and is a measure of the relative strength of the plumage color (Endler and Mielke 2005). Chroma is a commonly used metric to describe phenotypic variation and quality of sexual signals (e.g. Shawkey et al. 2003; Doucet 2004; Cornuault et al. 2015) and captures variation from blue to black in this species. Chroma was analyzed using the average VS cone-type retina (Ödeen et al. 2012) and idealized illumination in avian tetrahedral color space following Stoddard and Prum (2008).

To compare relative overlap in color between the sexes and phenotypes, I also plotted colors of each sex in tetrahedral color space to represent total color variation of that phenotype (Stoddard and Prum 2008; Stoddard and Stevens 2011). I then calculated volume of color space occupied by each sex and present the overlap (relative to the small volume) on a scale of 0-1 to illustrate the overall similarity or difference between sexes following Stoddard and Stevens (2011).

Scanning Electron Microscopy

I visualized barbule structure using scanning electron microscopy (SEM), which provides a valuable tool for describing the structural component of color production in

feathers (Shawkey et al. 2003). I used a subset of feathers from 14 ornamented male, 7 ornamented female, and 7 unornamented female WSFW and 7 ornamented male, 6 unornamented male, and 8 unornamented female RBFW. I mounted individual crown feathers with carbon tape and viewed them using a scanning electron microscope (SEM; Hitachi S4800). I visualized images using ImageJ software (U.S. National Institutes of Health; http://rsb.info.nih.gov/ij/). Differences between the lineages were visible in barbule structure and I measured barbule structure in two ways. First, following D'Alba et al. (2014), I counted the number of barbules along a 500-μm transect on the second and third barbs from the distal tip of the feather. Additionally, I measured the density of barbules in a 1 mm^2 box located at the tip of each crown feather using Image J. Specifically, I used the Threshold tool to isolate the feather barbules from the dark background of the image, and then measured feather area in a 1 mm^2 box using the Analyze Particles tool to give a summary of the percent area of the box that was covered by feather barbules. These two measurements differ in that the first would detect the total number of barbules per barb and the second would detect differences in barbule shape and size.

Transmission electron microscopy

Two crown feathers from each phenotype (Table S2.1) were embedded for transmission electron microscopy (TEM) following Shawkey et al. (2003). Because of their small size, I prepared and embedded the entire crown feather. I cut barbs using a Leica Reichert Ultracut S microtome, and placed sections on 200 mesh copper grids (Ted Pella, Redding CA, USA) with Formvar support, post-stained with uranyl acetate, and

viewed on a FEI G2 F30 Tecnai TEM (FEI Inc, Hillsboro, OR, USA). Using ImageJ, I compared relevant metrics to melanin and structural colors (Doucet et al. 2006; Shawkey et al. 2006) including the number of melanin-containing melanosomes per barbule ('melanosome density'), thickness of the keratin cortex (distance from the outermost melanin granule to the edge of the barbule), and the thickness of the outer layer of melanosomes (distance from the outermost melanin granule to the innermost contiguous melanin granule). Both thickness of the keratin cortex and thickness of the outer layer of melanosomes were averaged across six different points following Maia et al. (2011).

Statistical analyses

For analysis, males and females were each characterized as possessing either an ornamented (e.g. ornamented male) or unornamented phenotype (e.g. ornamented female). I used a nested analysis of variance (ANOVA) to compare differences in color variables, barbule density and barbule number (number of barbs per 500μm) between phenotypes nested within lineage. I compared differences in means between each group using a Tukey Honest Significant Difference test, which corrects for multiple comparisons. Sample sizes for TEM are prohibitively small for statistical analysis, so I present them as tabulated values and qualitative visuals. Brightness measurements were log transformed to achieve homoscedasticity for the above analysis; the other variables had equal variances. Individual linear regressions were performed to test associations between structural properties and color. All analyses were performed in R (R version 3.3.0, R Core Team, 2016), and alpha was set to 0.05.

Results

Sexes and lineage differed in both brightness and chroma (Table 2.1, see details below). The number of feather barbules and density of barbules differed between sexes and lineages (Table 2.1). However, in pairwise comparisons below, only the density of barbules differed suggesting that the shape and structure of barbules, but not overall number of barbules, differed between groups.

Feather ornamentation in White-shouldered Fairywren (WSFW)

Ornamented female WSFW were brighter and lower in chroma than unornamented female WSFW, and the same was true when comparing ornamented males to unornamented females (Table 2.1, Table S2.1, Figure 2.2). I also observed lower chroma in ornamented female than ornamented male WSFW, but no difference in brightness (Table 2.1, Table S2.1, Figure 2.2). Ornamented male WSFW did not differ in either measure between the two populations (Figure 2.2). Using a measure of color space overlap, I found slight overlap between ornamented male and ornamented female WSFW, but no overlap between ornamented male WSFW and unornamented female WSFW (Figure 2.3).

These differences in color between lineages were associated with differences in barbule structure in WSFW. In terms of barbule density (via SEM), ornamented males had a greater density of barbules than did ornamented and unornamented females, and there was no difference in number of barbules (Table 2.1, Figure 2.2). Overall, ornamented females were intermediate between ornamented males and unornamented females in barbule density (Figure 2.2,2.4). In cross sections (via TEM), barbules of

ornamented male and ornamented females were qualitatively similar in numbers of melanosomes, thickness of the outer melanosome layer, and thickness of the keratin cortex (Table S2.2, Figure 2.4). When all ornamented individuals were pooled, chroma was moderately correlated with the thickness of the outer melanosome layer, although this relationship was not significant (r^2=0.30, p=0.160). Ornamented females also differed from unornamented females in barbule cross sections by a higher density of melanosomes in each barbule, with a corresponding thick outer layer of melanin and thinner outer keratin layer (Figure 2.4). With all individuals included, the thickness of the outer keratin layer was positively correlated with brightness (r^2=0.82, p<0.0001).

Feather ornamentation in Red-backed Fairywrens (RBFW)

Ornamented males were brighter and lower in chroma than were unornamented male and female RBFW, which were similar to each other (Table 2.1, Table S2.1, Figure 2.2). Ornamented males and unornamented RBFW (of both sexes) overlapped little in color, but unornamented male and female RBFW overlapped to a high degree (Table 2.1, Table S2.1, Figure 2.3).

In terms of barbule density (via SEM), ornamented males had a greater density of barbules than both unornamented males and females, but did not differ in number of barbules (Figure 2.2,2.4). In cross section (via TEM), barbules in ornamented males had a higher density of melanosomes, a thick outer melanosome layer, and thinner keratin layer compared to both unornamented males and females (ornamented males were similar to that found in male WSFW; Table S2.2). Unornamented male RBFW were similar to

unornamented female RBFW in low number of melanosomes, lacking in a distinct melanosome layer, and a thick keratin layer (Table S2.2, Figure 2.4).

Discussion

A better understanding of the proximal mechanisms underlying ornament production, in combination with related information on phylogeny, behavior and ecology, has the potential to provide insights into signal evolution and adaptive significance. In the current study on *Malurus* fairywren feather coloration and microstructure, my findings point to an ornamented female *Malurus alboscapulatus* (White-shouldered Fairywren: WSFW) phenotype that differs from that of males. More specifically, the male ornamented phenotype involves more components (i.e. greater barbule density) than does the female ornamented phenotype. Ornamented plumage in females is recently derived in WSFW (Johnson *et al.*, 2013; above), and these results contradict the idea that female ornamentation was achieved via a simple switch to produce an equivalent ornament to that expressed in males. Moreover, I found that feathers in ornamented male RBFW differ from the unornamented RBFW male plumage both in having a high density of structured melanosomes within barbules and in high barbule density. It is therefore striking that males of this species are able to molt between these alternative plumage states in relatively short time windows. In contrast, I found overall similarity in the color and underlying structure of unornamented males and females of both species.

Feather ornamentation in Malurus alboscapulatus (White-shouldered Fairywren)

Specialized barbule morphology is a widespread mechanism for iridescent plumage in birds (Prum 2006) and barbule size, shape and the organization of melanosomes within barbules have also been associated with iridescent color production (Doucet et al. 2006; Shawkey et al. 2006; Maia et al. 2011). Compared to the saturated, blue plumage of male WSFW, the matte black feathers of ornamented female WSFW lack a high density of barbules. The high density of barbules in male WSFW appears to be caused by enlarged and flattened barbules, but not an increase in the number of barbules. This suggests that the production of the blue iridescent sheen in male WSFW is associated with an increased exposure of the nanostructural characteristics found within barbules (as in *Ptilonorhynchus violaceus*, Doucet et al. 2006). In addition, there is a correlative relationship between the thickness of the melanin layer and chroma, and the width of the melanin layer may be involved in how the keratin cortex selectively reflects blue wavelengths (Doucet et al. 2006). In *Volatinia jacarina* (Blue-black Grassquit) a thin keratin layer over a layer of melanin granules was sufficient to produce a blue sheen (Maia et al. 2009), and a similar anatomical arrangement may be involved in male WSFW color production. In the absence of these barbule properties, a matte black coloration in ornamented females is produced by the dense melanosome composition of the barbules.

Darwin's (1871) suggestion that ornaments are correlated in their production between the sexes has received both theoretical (Lande 1980) and empirical support (Price and Pavelka 1996; Potti and Canal 2011; Schielzeth et al. 2012). Due to the similarity in overall patterning between ornamented WSFW of both sexes in the Milne Bay Population, a genetic correlation for ornamentation seems likely for plumage

expression in WSFW. However, if female ornaments appear only as a neutral byproduct to selection on males, I should expect they will be identical in form. My finding that female ornaments in WSFW did not evolve following a simple and immediate switch to male ornamentation indicates that some additional factor is likely involved in the evolution of female ornaments in this species. These findings are consistent with recent research quantifying colors across the family Maluridae that suggests females evolve elaborate colors at different rates and in response to different selective regimes than males (Friedman and Remeš 2015). However, additional work exploring the selective advantages of female ornamentation will be needed to discern the function of matte black color in this system. Some possibilities include male preference for female ornaments (Amundsen et al. 1997), a competitive advantage to female ornaments in reproductive (Rubenstein and Lovette 2009) or ecological contexts, or selection related to survival or nest success (Martin and Badyaev 1996; Nordeide et al. 2013). Alternatively, matte black color could be selectively neutral, and if producing a bluish sheen incurs costs, natural selection could prevent the evolution of blue in females. Lastly, females may lack the developmental capacity to produce the barbule structure of males, limiting the production of a fully male-like ornament. Taken together, my results do not rule out a genetic correlation model for explaining the evolution of female ornaments, but they are consistent with sex specific selection pressures acting on female ornaments.

Future research might also explore the link between testosterone, feather structure, and the deposition of melanin (Peters et al. 2000; Peters 2002; Lindsay et al. 2009; Karubian et al. 2011b). Testosterone appears to drive acquisition of ornamented plumage in male *Malurus* fairywrens (Peters et al. 2000; Lindsay et al. 2011) and

experimental testosterone implants in female *M. cyaneus* produces some male-like characteristics (without changing color), which could imply a structural change following a rise in testosterone (Peters 2007). Female RBFW produced carotenoid based, but not melanin based, coloration under experimentally elevated testosterone levels (Lindsay et al. 2016). Similarly, preliminary data suggests that unornamented female WSFW produce white feathers, but not melanin-based black feathers, when testosterone is experimentally elevated (Boersma personal communication). Future work investigating genes that associated with melanin deposition and keratin structure could be informative for describing the underlying mechanism for dichromatism and monomorphism in these groups (Uy, Moyle, Filardi, et al. 2009; San-Jose et al. 2015).

Feather ornamentation in Malurus melanocephalus (Red-backed Fairywrens)

In contrast to the WSFW, plumage coloration is similar between unornamented male and female RBFW, as are the underlying mechanisms of sparse feather barbules and randomly distributed melanosomes within barbules. Based on these findings, one might reason that unornamented male RBFW, which during the breeding season are younger individuals (Webster et al. 2008), may be mimicking females, a common explanation for delayed plumage maturation (DPM) in birds (Hawkins et al. 2012). However, experimental aviary trials using live birds demonstrate that adult female and male RBFW can distinguish between unornamented male and female RBFW (Karubian et al. 2008). I suggest that conspecifics may be discriminating between young males and females based on behavior or vocalizations, or another body patch such as bill color (Karubian 2008), or that they are able to perceive differences that do not come up as significant in my

analyses. Given the overall similarity in both color and feather structure among unornamented male RBFW and unornamented female RBFW, I propose that genetic correlation between male and female traits likely plays an important role in determining these characteristics.

Although my spectroscopy results suggest similarity in color between ornamented female WSFW and male RBFW, male RBFW have a colorless sheen to their feathers that is visible to the eye (personal observation, Figure 2.1). The high barbule density is likely involved in the production of this sheen (Doucet et al. 2006; Prum 2006), as it is the key difference between ornamented males and ornamented females in this study. Future work could focus on how male RBFW and male WSFW produce different colored plumage sheens, which may be the result of different light absorbance in the cortex of the barb rami (Doucet et al. 2006).

Male RBFW transition between unornamented and ornamented plumage between the nonbreeding and breeding seasons, indicating a high degree of flexibility in visual signal development Karubian 2002; Webster et al. 2008; Karubian et al. 2011b; Lantz and Karubian 2016. My work suggests that this transition is achieved by molting in feathers with both higher density of barbules and changes to melanosome deposition. Given that this transition can take place over just a few weeks, it is notable to find overall more structurally complex changes to feathers within male RBFW than between recognized sub-species of female WSFW. The magnitude of this change in structure over such short time periods speaks to the strength of social or sexual selection on male fairywrens.

Conclusion

By characterizing the structural differences underlying variation in ornamentation among males and females of three closely related lineages of *Malurus* fairywren, I provide insights into the underlying processes driving the evolution of ornament production and sexual dichromatism in this group of birds. I describe an evolutionary transition to ornamentation in WSFW female coloration that is inconsistent with the idea that only genetic correlation between the sexes is responsible for the evolution of female ornaments. Instead, this work implies that female-specific selection pressures may have driven production of a unique female ornament. These results underscore the importance of explicitly considering the female perspective in evolutionary biology, including work on the mechanistic underpinnings of ornament production. In contrast, I show that the rapid transition (i.e. weeks) from unornamented to ornamented state among male RBFW in response to changes in breeding status involves the greatest degree of structural change I observe in the system, highlighting the relative strength of sexual selection in this highly promiscuous species.

Tables

Table 2.1: Nested ANOVA results comparing phenotype (e.g. ornamented male, ornamented female, etc) nested within lineage.

	df	Sum Sq	Mean Sq	F	*p*
log Brightness: Lineage:Phenotype	4	23.908	5.977	97.210	<0.001
Chroma: Lineage:Phenotype	4	0.894	0.223	29.472	<0.001
Density: Lineage:Phenotype	4	5186.638	1296.660	41.442	<0.001
Num. barbules: Lineage:Phenotype	4	0.000	0.000	2.669	0.045

Figures

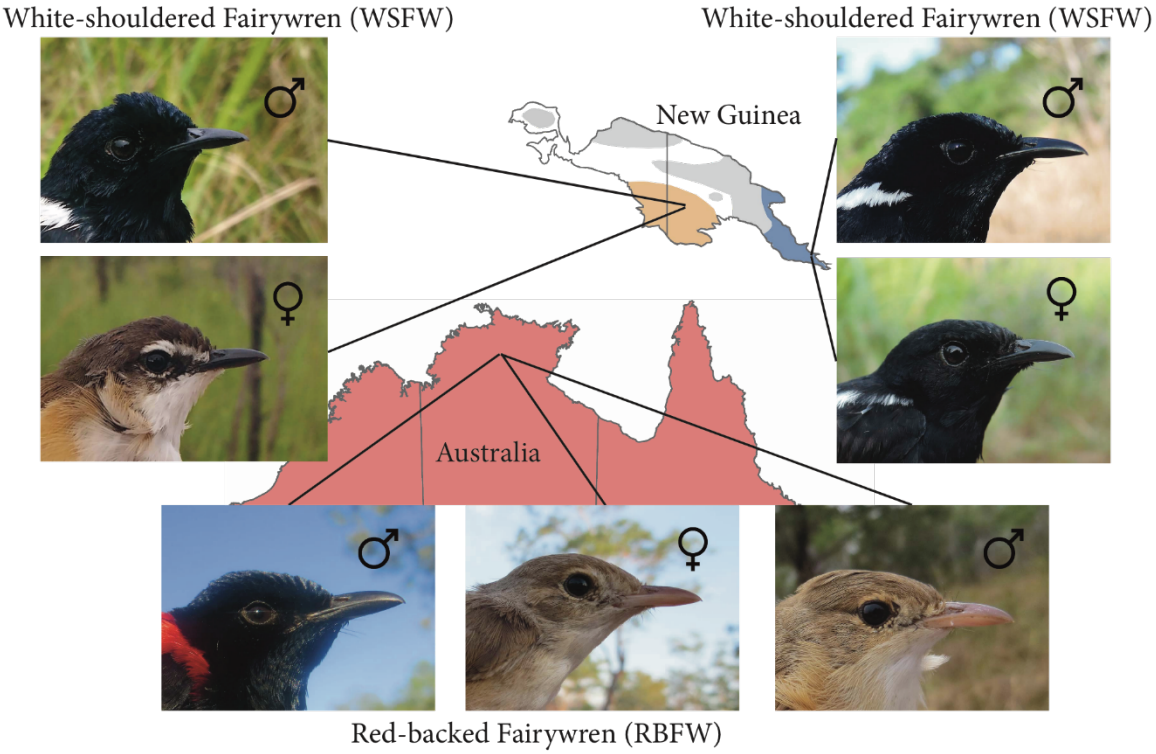

Figure 2.1: Photographs of the three taxa included in this study and their distributions in New Guinea and Australia. Within the White-shouldered Fairywren, female crown color is either brown or black in different subspecies, while males remain similarly ornamented black in all subspecies. In Red-backed Fairywrens, male crown feathers are black in nuptial plumage, but females and males in the nonbreeding season have brown crown feathers. See text for more details. Gray regions of the map refer to other populations of WSFW not included in this study (Rowley and Russell 1997); ranges based on BirdLife International and Natureserve (2013).

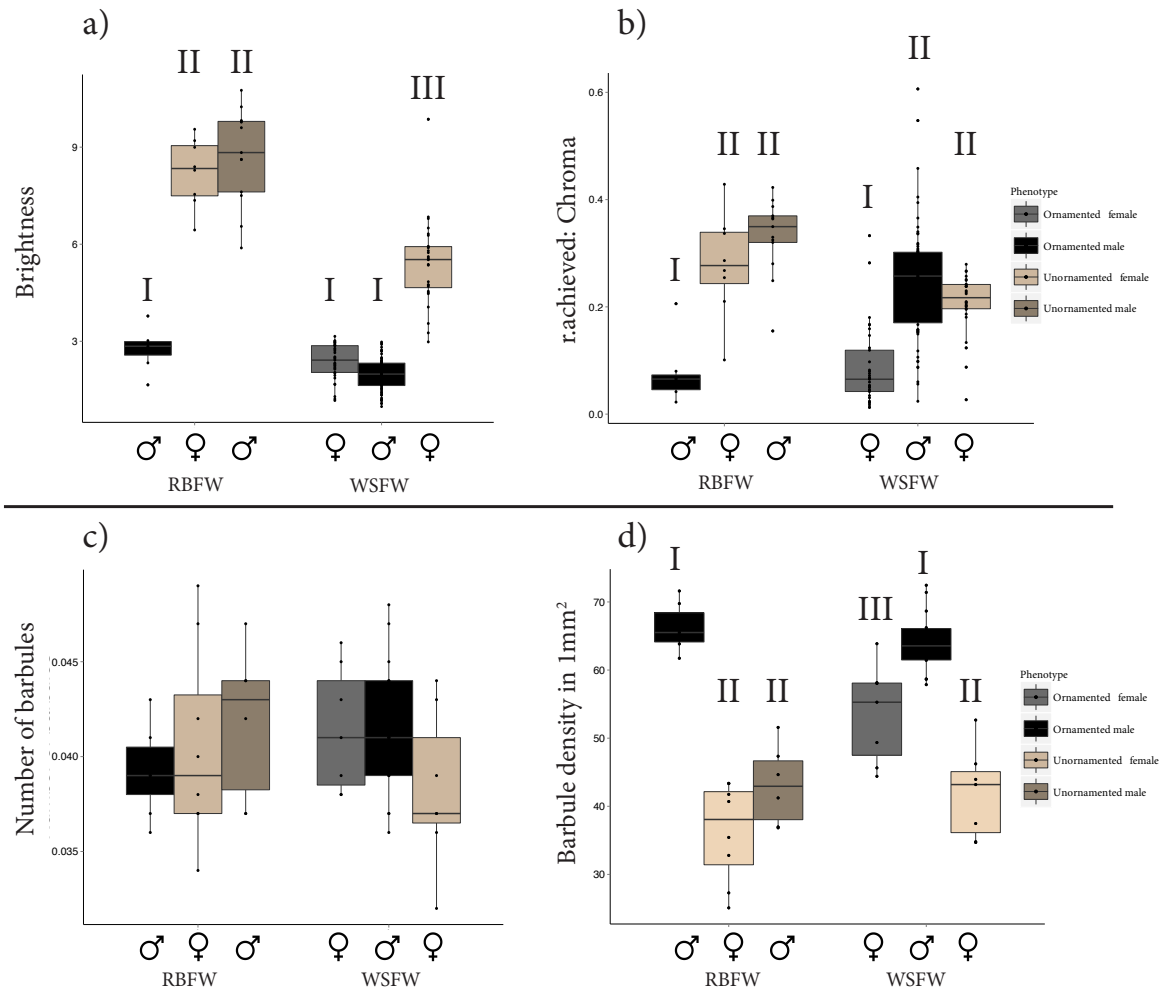

Figure 2.2: Boxplots for differences in (a) brightness, (b) chroma, (c) number of barbules and (d) barbule density between lineages and phenotypes. Numerals above each box indicate groups that differ significantly from each other; the same numeral indicates no significant difference. Ornamented female White-shouldered Fairywrens differ from ornamented male White-shouldered Fairywren in chroma and barbule density. Unornamented female White-shouldered Fairywren are less bright than other unornamented phenotypes, but otherwise all unornamented phenotypes are similar.

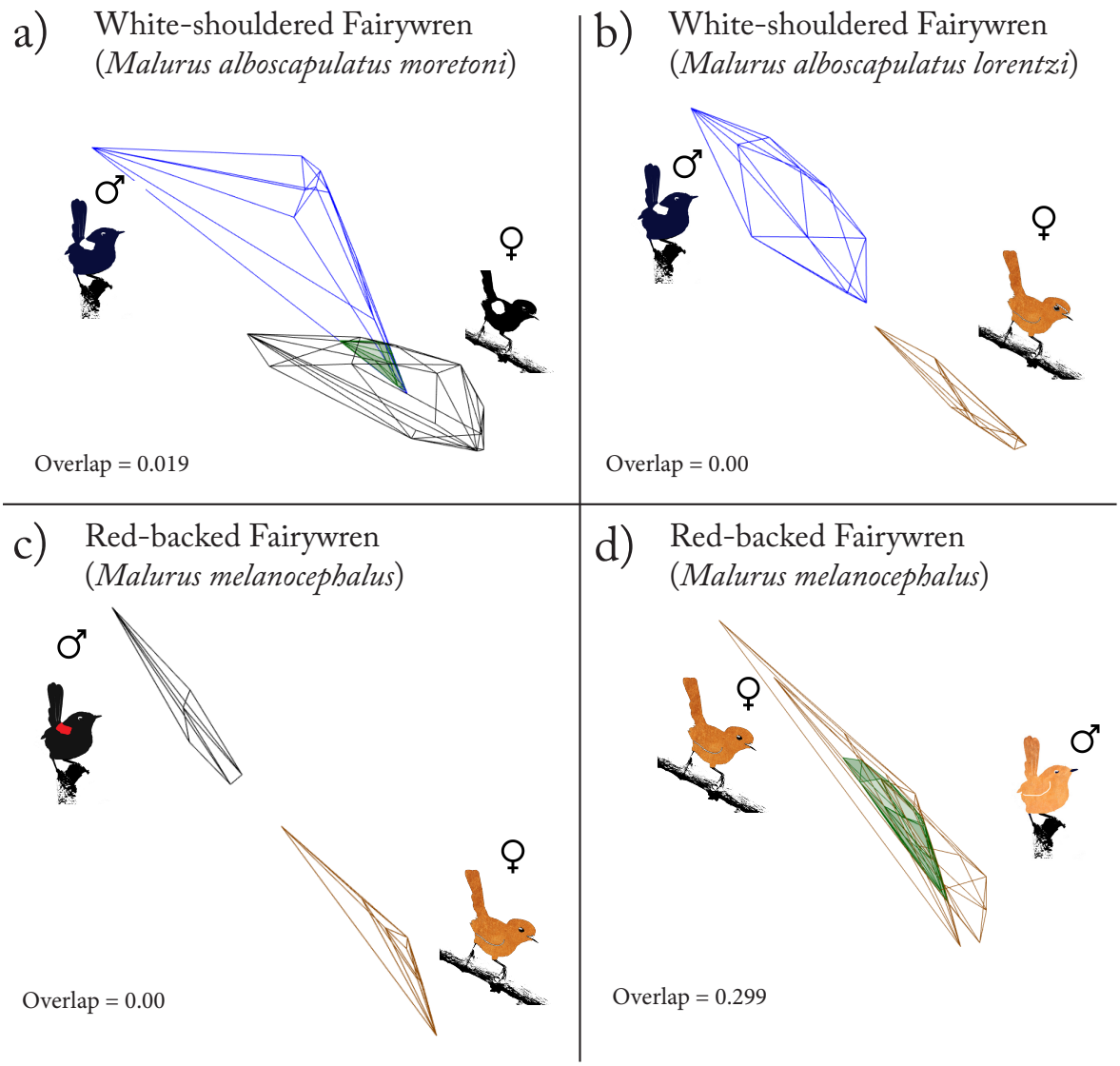

Figure 2.3: Volume overlap representing plumage color mapped in Cartesian color space to illustrate dichromatism between sexes and phenotypes. Sex and phenotype are illustrated adjacent to their respective polygon and green represents overlap. Images illustrate the separation of color volume occupied between ornamented males and unornamented females, slight overlap in color between the sexes in the population of White-shouldered Fairywrens with ornamented females, and high overlap between unornamented male and female Red-backed Fairywrens. Overlap between volumes is listed on a scale of 0 to 1.

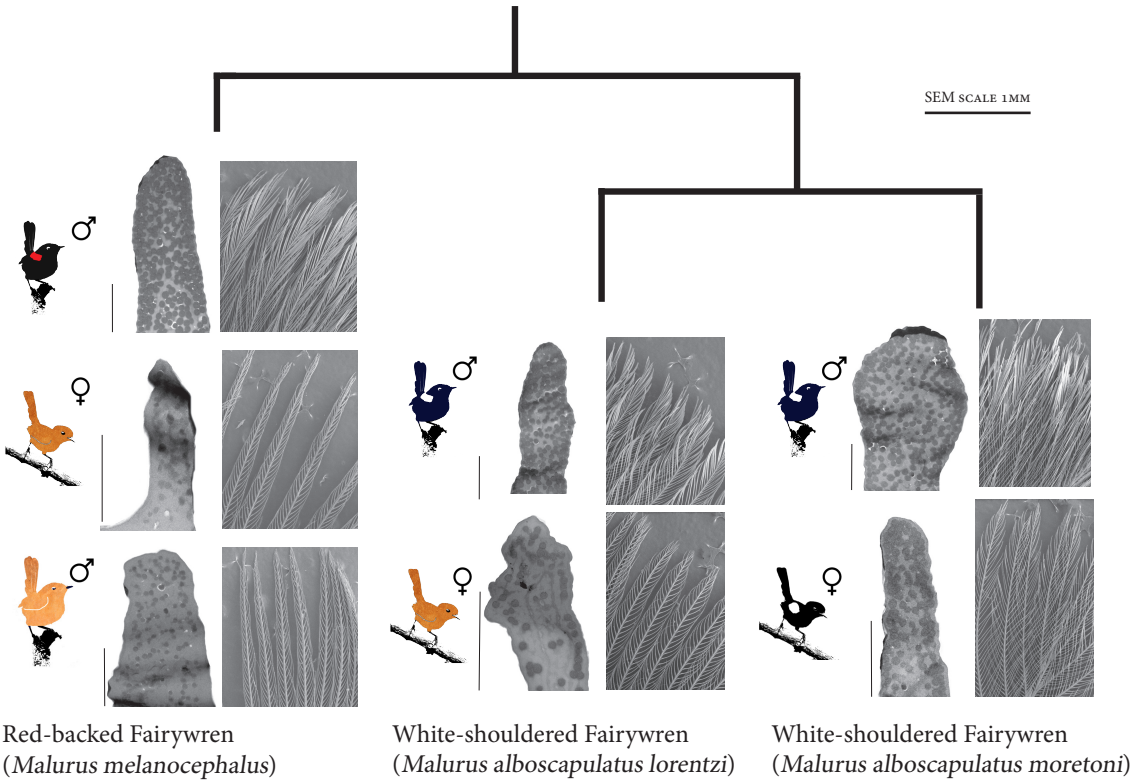

Figure 2.4: Inferred phylogeny of included lineages based on Driskell et al. (2011) with illustrated phenotypes, SEM, and TEM images of representative crown barbules. Scale bar for all SEM images are shown above, scale bars for each TEM image is the vertical bar adjacent to each image. Left, note the overall similarity in cross section of melanosome (dark spots) distribution and density between all ornamented phenotypes compared to unornamented phenotypes. Right, note similarity in barbule density between ornamented female White-shouldered Fairywrens and all unornamented phenotypes, in contrast to the highly dense and clustered ornamented male barbules.

Supplementary material

Table S2.1: Tukey's post hoc significance values (p) following a Nested ANOVA.

Comparison	Log Brightness	Chroma	Density	Num. barbules
M.a.lorentzi:OM-M.a.moretoni:OF	0.001	0.000	0.753	0.945
M.a.moretoni:OM-M.a.moretoni:OF	0.740	0.000	0.002	0.999
M.melanocephalus:OM-M.a.moretoni:OF	0.911	1.000	0.006	0.994
M.a.lorentzi:UF-M.a.moretoni:OF	0.000	0.000	0.015	0.907
M.melanocephalus:UF-M.a.moretoni:OF	0.000	0.000	0.000	1.000
M.melanocephalus:UM-M.a.moretoni:OF	0.000	0.000	0.065	1.000
M.a.moretoni:OM-M.a.lorentzi:OM	0.175	0.519	0.585	0.478
M.melanocephalus:OM-M.a.lorentzi:OM	0.002	0.008	0.697	0.405
M.a.lorentzi:UF-M.a.lorentzi:OM	0.000	1.000	0.000	0.179
M.melanocephalus:UF-M.a.lorentzi:OM	0.000	0.859	0.000	0.733
M.melanocephalus:UM-M.a.lorentzi:OM	0.000	0.009	0.001	0.983
M.melanocephalus:OM-M.a.moretoni:OM	0.227	0.000	1.000	1.000
M.a.lorentzi:UF-M.a.moretoni:OM	0.000	0.192	0.000	1.000
M.melanocephalus:UF-M.a.moretoni:OM	0.000	1.000	0.000	1.000
M.melanocephalus:UM-M.a.moretoni:OM	0.000	0.470	0.000	0.995
M.a.lorentzi:UF-M.melanocephalus:OM	0.000	0.026	0.000	1.000
M.melanocephalus:UF-M.melanocephalus:OM	0.000	0.001	0.000	1.000
M.melanocephalus:UM-M.melanocephalus:OM	0.000	0.000	0.000	0.983
M.melanocephalus:UF-M.a.lorentzi:UF	0.001	0.648	0.724	0.990
M.melanocephalus:UM-M.a.lorentzi:UF	0.000	0.002	1.000	0.852
M.melanocephalus:UM-M.melanocephalus:UF	1.000	0.969	0.506	1.000

Table S2.2: Tabulated values of measurements of one barbule cross section under TEM. Num_mel = number of melanosomes; mel.area=number of melanosomes per μm^2; ker.lay = average width in µm of keratin layer; mel.lay = average width in µm of outer layer of melanosomes.

Species	Phenotype	num_mel	mel.area	ker.lay	mel.lay
Red-backed Fairywren (RBFW)	Ornamented male	527	14.466	0.0818	0.549
Red-backed Fairywren (RBFW)	Ornamented male	413	16.928	0.11	0.452
Red-backed Fairywren (RBFW)	Unornamented female	133	5.093	0.211	0.173
Red-backed Fairywren (RBFW)	Unornamented female	90	4.074	0.269	0.156
Red-backed Fairywren (RBFW)	Unornamented male	168	16.867	0.135	0.198
Red-backed Fairywren (RBFW)	Unornamented male	211	10.038	0.287	0.159
White-shouldered Fairywren (WSFW)	Ornamented female	268	14.295	0.12	0.475
White-shouldered Fairywren (WSFW)	Ornamented female	205	8.812	0.108	0.528
White-shouldered Fairywren (WSFW)	Ornamented male	382	10.75	0.117	0.642
White-shouldered Fairywren (WSFW)	Ornamented male	352	8.61	0.116	0.64
White-shouldered Fairywren (WSFW)	Ornamented male	719	7.938	0.0858	0.681
White-shouldered Fairywren (WSFW)	Ornamented male	371	12.013	0.121	0.568
White-shouldered Fairywren (WSFW)	Unornamented female	249	14.102	0.159	0.162
White-shouldered Fairywren (WSFW)	Unornamented female	193	17.545	0.157	0.182

CHAPTER 3 [1]

GENOMIC AND TRANSCRIPTIONAL EVIDENCE FOR SELECTION ON FEMALE ORNAMENTATION

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ABSTRACT

Ornamentation, such as showy plumage, is widespread among female vertebrates, but whether ornamentation in females evolves as a neutral byproduct of selection on males, or is the subject of sexual or social selection, is not well resolved. To assess the extent to which selection has acted on female ornaments and refine understanding of the mechanisms underlying color evolution and diversification, I resequenced genomes of a tropical passerine bird in which females, but not males, vary between populations in occurrence of ornamented black and white plumage. Using a comparative approach, I show that ornamented female plumage is a derived state and demonstrate that selection has acted on regions of the genome containing genes relating to melanogenesis that are associated with evolutionary transitions in the degree of female ornamentation. I associate these divergent regions to variation in the feather transcriptome, providing a putative link between genomic targets of selection with functional control of female plumage color. I also find evidence for selection on regions of the genome related to steroid production, and leverage transcriptional variation in the context of both naturally occurring and experimentally induced differences in female shoulder patch coloration to show that variation in this trait is shaped by circulating testosterone. Finally, I

demonstrate that interpopulation variation in the transcriptome is inconsistent with a neutral model. These findings provide multiple lines of evidence that directional selection has acted on ornamentation, a classic sexually selected trait, in females of this species.

Introduction

Interest in the evolution of elaborate ornaments motivated the theory of sexual selection (Darwin 1871; Wallace 1889), and subsequent research has established the contribution of sexual selection to the origins of species (i.e., speciation, Andersson 1994; Edwards et al. 2005; Price 2008). The vast majority of this work has focused on ornamentation in males, despite the fact that females of many species are ornamented (e.g. Amundsen and Parn 2006), that female ornaments may be the subject of male choice and mediate same-sex conflicts (Amundsen et al. 1997; Douglas J Emlen et al. 2005), and that female ornamentation may rapidly evolve (Burns 1998; Hofmann et al. 2008; Johnson et al. 2013). Although attention to female ornaments has increased in recent years (e.g. Amundsen 2000; Clutton-Brock 2007; Rubenstein and Lovette 2009), few studies have been able to rule out a neutral model of evolution of female ornamentation (Kraaijeveld et al. 2007), which posits that ornamental traits in females are the result of correlated inheritance from males, and not directly subject to selection (Darwin 1871). Though this perspective has received theoretical support- as males and females share most of the genome and sexual selection is expected to be stronger in males (Lande 1980)- empirical evaluation of it and alternatives (i.e., selection on female ornamentation) requires a suitable study system characterized by variable female ornamentation independent of males, a well-resolved phylogeny, and detailed molecular analyses. To

date these conditions have only been met for males (e.g., Toews et al. 2016; Campagna et al. 2017). In addition, sex-specific expression can overcome sexual conflict in the genome through endocrine control, so an understanding of the contribution of hormones to female ornament expression also represents an important, but poorly resolved, component of female trait evolution (McGlothlin et al. 2007; Rosvall 2013). As a consequence, the degree to which evolutionary transitions in female ornamentation arise due to selection versus neutral processes and the mechanisms by which these transitions are achieved remains poorly resolved.

Evolutionary transitions in female ornamentation likely involve some combination of genetic architecture, transcriptional regulation, and endocrine signaling that cumulatively act to overcome constraints imposed by a shared autosomal genome. In terms of genetic architecture, key color genes controlling pigment morphogenesis (Mundy 2005) or pigment metabolism (Benkman 2016) may control animal phenotypes, including species-specific coloration (e.g. Uy et al. 2016), via simple loss / gain of function mutations. This also could be achieved by directional selection that shifts allele frequencies. More often, however, complex coloring and patterning is mediated by polygenic interactions (Imsland et al. 2015; Mallarino et al. 2016) and regulatory elements (Poelstra et al. 2015). As a consequence, transcriptional differences may underlie phenotypic variation, particularly in sexual dichromatism. From an endocrine perspective, testosterone is a candidate for mediating female-specific transitions in ornamentation because it is an important activator for ornamentation in males (Hau 2007), is also produced by females (W. Goymann and Wingfield 2014) and stimulates the expression of some male traits in females (Ketterson et al. 2005). Yet, differential effects

of testosterone on tissues types and body regions complicate a generalized understanding of testosterone on complex phenotypes (Staub and De Beer 1997; Ketterson et al. 2005).

To maximize insights into female ornament evolution, use of a system characterized by variation in relevant traits among females, but not males, would effectively mirror approaches that have previously been taken to infer selection on ornaments in species where males vary but females do not (e.g., Poelstra et al. 2014; Toews et al. 2016; Campagna et al. 2017). Females of populations of the New Guinea White-shouldered Fairywren (*Malurus alboscapulatus*; Figure 3.1a) possess one of three phenotypes – pied, black, or brown – and because males are uniform blue/black across populations, degree of sexual dichromatism also varies across populations (Enbody et al 2017, Karubian 2013). The occurrence of sister species with unornamented females suggests that ornamented female White-shouldered Fairywrens evolved from an unornamented ancestor (Karubian 2013), but this hypothesis lacks rigorous phylogenetic evidence and the ancestral female White-shouldered Fairywren phenotype is unclear. This is a critical piece of information, because a genetic correlation model assumes a monochromatic elaborate ancestor that has evolved as a consequence of selection on males generating ornamentation in both sexes (Kimball and Ligon 1999; Friedman and Remeš 2015). It has been suggested that variable levels of dichromatism in this species arose recently through the rise of simple genetic mutations among allopatric populations isolated by the mountainous terrain of New Guinea (Schodde 1982), which would be supported by high relatedness and low genetic structure between populations. Yet degree of genetic similarity between populations remains poorly resolved, and the mechanisms that putatively underlie this rapid diversification remain unknown. Females with the more

ornamented phenotype are more aggressive and have higher circulating testosterone (Enbody in review) and differ from males in the nanostructure of ornamented feathers (Enbody et al 2017), consistent with an adaptive function for female ornamentation, but genetic and endocrine processes responsible for transitions and the contribution of selection to genomic divergence remain unknown.

To address the contribution of social or sexual selection pressures to female ornament evolution, I first evaluate the phylogenetic relationships among White-shouldered Fairywren populations to infer the ancestral female plumage state. I next assess whether genomic regions of divergence are associated with transitions in ornamentation between populations, with the prediction that regions of high divergence relative to background variation contain genes relevant to observed patterns of female phenotypic differentiation. I subsequently assess whether gene expression differences in the feather transcriptome are consistent with selection on transitions in female coloration, by determining whether a small number of genes, predominantly responsible for different coloration, are differentially expressed, relative to population divergence. I predict that differences in expression of female ornamentation will be associated with genes linked to melanogenesis, feather morphogenesis, and testosterone sensitivity. For the latter, I additionally include an experimental test of the role of circulating levels of testosterone on the expression of the ornamental shoulder patch.

Methods:

Population sampling:

I sampled blood from 37 female White-shouldered Fairywrens from four populations: *moretoni* (n=10) between 2013 and 2016, *lorentzi* (n=10) in 2015 and 2016, *naimii* (n=10) in 2016, and *aida* (n=7) in 2017. Individuals were sexed in the field using known plumage and reproductive traits. Sex for 27 samples was confirmed using laboratory techniques (for details on methods, see Enbody et al. 2017).

I collected two molting feathers (from females) that had recently erupted from the sheath (~10% emerged from the sheath) from the shoulder and chest of *moretoni* and *lorentzi* and from the chest and shoulder of *naimii* and *lorentzi*. One *moretoni* chest feather was discarded due to its later stage of molt (i.e. >10% emerged). Three individual *lorentzi* implanted with testosterone and molting feathers were sampled before and 10-11 days after implantation (following the protocol in Lindsay et al. 2011). Thus, for these individuals, I obtained three feathers pre testosterone implantation and another three feathers post testosterone implantation from the same individual. Each feather was stored in 0.5mL of RNAlater storage buffer (ThermoFisher Scientific), incubated overnight, and transferred to a freezer 1-3 days later.

Data generation and processing:

Reference genome annotation

I annotated a previously developed reference genome for a single male White-shouldered Fairywren (Sin unpublished data). To annotate the reference genome, I first created a custom repeat library using Repeat Modeler 1.0.10 (http://www.repeatmasker.org/ RepeatModeler/). I used protein databases from other

vertebrates (n=16), RNAseq results from the current study, EST data from the zebra finch assembly (Warren et al. 2010), and gene predictions from *Gallus gallus* for the first round of MAKER (3.01.02). I subsequently ran a second iteration of MAKER using the gene models predicted in the first MAKER iteration. The second MAKER run identified a total of 17,999 gene models. I subsequently used NCBI BLAST+ to identify putative gene function using blastp on the list of proteins identified by MAKER with an evaluation threshold of 1e-6 and identified 16,629 putative orthologs.

Whole genome re-sequencing library preparation

Genomic DNA was extracted from red blood cells using a Qiagen DNeasy Kit. I first sheared genomic DNA using a Covaris S220 with a target fragment size of 300bp. I prepared a paired-end library for each individual with a Wafergen PrepX ILM 32i DNA Library Kit on an Apollo 324 as per manufacturer's instructions. I confirmed fragment sizes on an Agilent Tapestation and library concentration using real-time qPCR on a BioRad CFX96. All libraries were multiplexed and sequenced across three lanes of an Illumina HiSeq 2500. Sequencing depth ranged from 3.76x to 6.05x (mean=4.52x) per individual. Preliminary quality assessment was performed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc).

Preprocessing whole-genome resequencing

Preprocessing of whole genome resequencing reads closely followed GATK best practices recommendations (broadinstitute.org/GATK). I first converted FastQ files to SAM format, while simultaneously retaining read group information and marking

Illumina adaptors using Picard Tools (2.14.0). I converted SAM files back to FastQ format and aligned reads to the reference using BWA (0.7.15) –mem option and –M. Aligned BAM files were then merged, PCR duplicates marked, sorted and validated using Picard Tools. I realigned reads around indels using GATK version 3.8.

Separating Z chromosome

I used SatsumaSynteny (with default parameters, Grabherr et al. 2010) to align all assembled scaffolds in the reference individual against the Zebra Finch Z chromosome (Warren et al. 2010). I analyzed scaffolds that aligned to the Z chromosome for all subsequent analyses seperately to avoid bias due to differential sequencing depth on sex chromosomes.

Identity of related individuals

Population genetic analysis may be sensitive to closely related individuals in the dataset. I first used ngsRelate (Albrechtsen et al. 2009) to identify putative relatives (siblings or offspring) in my dataset by running each population separately using default parameters. I subsequently removed out one individual from *moretoni* (n=9 remaining), three individuals from *naimii* (n=7 remaining), and one individual from *aida* (n=6 remaining).

RNAseq library preperation

Feathers removed from RNAlater were bead-milled in a Qiagen TissueLyser I and messenger RNA was extracted from feathers using a Qiagen RNeasy mini-kit, following

manufacturer's instructions. RNA integrity was confirmed on an Agilent Bioanalyzer. For RNAseq library preparation, I first isolated mRNA using a Wafergen PrepX PolyA mRNA Isolateion Kit, then prepared paired-end stranded mRNA libraries using Waftergen PrepX RNA-Seq Kit on an Apollo 324. I confirmed library integrity on an Agilent Bioanalyzer, quantified fragment sizes on an Agilent Tapestation, and calculated library concentration using real-time qPCR on a BioRad CFX96. Prepared cDNA libraries were multiplexed and spread across two lanes of Illumina NextSeq 500. Preliminary quality assessment was performed using FastQC.

Preprocessing RNAseq

I first used Rcorrector (Song and Florea 2015) to correct for sequencing errors in Illumina RNAseq reads and removed kmers with errors that were unfixable using a custom python script (https://github.com/harvardinformatics/TranscriptomeAssemblyTools/blob/master/FilterUncorrectabledPEfastq.py). I subsequently trimmed adaptors and removed low quality reads (-q 5) using TrimGalore 0.4.4, which is a wrapper script around Cutadapt (Martin 2011). I next downloaded ribosomal RNA databases from Silva (Quast et al. 2013) for Small Subunit (Nr99) and Large Subunit (128) rRNA. I aligned reads to the concatenated rRNA database using BowTie2 2.3.3 (Langmead and Salzberg 2012), with the –very-sensitive-local option, and retained only those reads that did not map to the database.

Transcript alignment

I aligned transcripts to the White-shouldered Fairywren reference genome using STAR (Dobin and Gingeras 2015) with annotations generated using MAKER. I ran STAR using the BjSJout function to remove spurious splice junctions, removed noncannonical reads, and using the default twopassMode. Lastly, I used STAR to count the number of reads per gene using the –quantMode GeneCounts function, which I used as input for differential testing below.

Data analysis

Genotype likelihoods and F_{st} calculation

I estimated genotype likelihoods using ANGSD (Korneliussen et al. 2014) by first calculating the site frequency spectrum for each population. I filtered out low quality base pair reads and reads with low quality mapping and removing sites with more than 50% missing data within the population. Specifically, I used the following ANGSD settings: -uniqueOnly 1, -remove_bads 1, -only_proper_pairs 0, -trim 0, -minMapQ 20, -minQ 20, -minInd (*lorentzi*=5,*naimii*=4,*aida*=3,*moretoni*=5), -doCounts 1, -doMaf 1, -doMajorMinor 1,-GL 1, -doSaf 1. I used the output of this analysis to obtain joint frequency spectrums for each between-population comparison (using realSFS), which were subsequently used as priors for allele frequencies at each site to estimate F_{st}. I averaged F_{st} in overlapping, sliding 50kb (10kb steps) windows across the genome, following window sizes used in studies with similar coverage (e.g. Vijay et al. 2016; Campagna et al. 2017). I excluded scaffolds with <2 windows and windows with <10 variable sites. For comparing the genomic landscape between different population pair

comparisons, I used a Z transformation to standardize per window F_{st} in each pair (following Han et al. 2017). Windows with ZF_{st} above the 99% percentile were examined for gene enrichment and considered putative targets of selection. I are not able to rule out if these genes are in linkage disequilibrium with other genes under selection and the degree to which they alone are driving divergence between White-shouldered Fairywren populations, but I include additional functional tests using gene expression and hormone manipulation (below) to evaluate the potential for genes located in regions of divergence to affect downstream transcriptional regulation.

Resequencing PCA and phylogenetic network

I used the –doGeno function in ANGSD to generate a file of genotype probabilities of all populations for input for building a covariance matrix using ngsCovar script (Fumagalli et al. 2013) in ngsTools (Fumagalli et al. 2014). Principal components were plotted in R using ggplot2 (http://ggplot2.tidyverse.org).

I used ANGSD (-doFasta 2, doCounts 1, minQ 20, -setminDepth 10) to obtain consensus fasta sequences for all populations with high coverage and for the sister taxa, Red-backed Fairywren (*Malurus melanocephalus*), which was aligned to the White-shouldered Fairywren reference as described above. I next used IQ-TREE (Nguyen et al. 2015) to create a distance matrix based on all alignments and used this as input to construct a phylogenetic tree using UPGMA clustering in PHYLUCE (http://evolution.genetics.washington.edu/phylip/ doc/neighbor.html).

Differential Expression analysis

I used the DESeq2 R package from Bioconductor (Love et al. 2014) to determine if the counts of genes differed between focal comparisons. DESeq2 uses negative binomial generalized linear models to determine if a given gene is expressed differently between treatments. I were interested in the transcriptional architecture of population differences in coloration and patterning and developed comparisons accordingly. I first grouped all samples by population and body region and examined patterns of similarity using a PCA. Following these initial comparisons, I focused on two focal comparisons: 1) shared coloration genes between natural transitions in brown and white shoulder patches and pre-post testosterone treatment and 2) shared genes for repeated transitions between black and white chest patches. Differentially expressed genes were tested for significance using a Wald test and adjusted using a Benjamini-Hochberg adjustment for multiple comparisons (as implemented by DESeq2, Love et al. 2014). Alpha was set to $p<0.1$ for all differential expression comparisons.

Tests for neutrality of differentially expressed genes

To differentiate between drift and directional selection in gene expression profiles, I developed a null model of gene expression. I applied an index of M_{st} (similar to Qst used for quantitative traits), which is the proportion of total variance in gene expression explained by variance between populations (Whitehead and Crawford 2006, Hughes et al. 2015). As M_{st} values are derived from between-population variance, it is possible to compare M_{st} values directly to F_{st}. Measures of M_{st} that exceed F_{st} are putative signatures of directional selection, while values equal to F_{st} indicate neutral evolution. Following the protocol from Feiner et al. (2017) using the associated R scripts, I

simulated one million neutral M_{st} values by sampling the distribution of F_{st} values (following Lewontin and Krakauer 1983, Lind et al. 2011, Feiner 2018) and evaluated observed M_{st} values that exceeded the 97.5% confidence interval. For F_{st} calculations, I implemented a global F_{st} calculation utilizing a strict filtering protocol in ANGSD (as above, with -SNP_pval 1e-3 -skipTriallelic). I calculated the neutral expectation of the number of genes with M_{st} higher than the 97.5% confidence interval using a permutation test. I interpreted comparisons with more outliers than expected as containing genes that have been subject to directional selection.

Gene enrichment analysis

I searched differentially expressed genes for enriched pathways using the GOseq R (R Core Team 2016) package (Young et al. 2010) and the ensemble *Homo sapiens* database for GO identification and gene length data. I additionally searched UNIPROT-KB for functional annotations of particular genes of interest. Lastly, I searched a previously published list of melanogenesis related genes (Poelstra et al. 2015) in divergent regions of the genome and curated list of genes involved in feather morphogenesis (Ng et al. 2015).

Results and Discussion

Phylogenetic history

I resequenced whole genomes to a mean sequencing depth of 4.5x (range: 3.8x to 6.05) for 37 (list of samples and localities Table S1) females from four populations of White-shouldered Fairywrens: two with a black female phenotype (*moretoni* and *aida*),

one with pied (*naimii*), and one with a brown phenotype (*lorentzi*) (Figure 3.1a,b). Resequenced genomes were aligned to a recently developed draft genome of one male White-shouldered Fairywren (Sin unpublished data). Analysis of genotype likelihoods shows that the major axis of variation (Figure 3.1c) separates the three "ornamented" populations from the "unornamented" population. Moreover, the two populations on the North coast of New Guinea cluster closely together, which is suggestive of either recent divergence or ongoing gene flow. These evolutionary relationships are consistent with phylogenetic clustering from a distance matrix of autosomal loci (UPGMA clustering method, Figure 3.1d). Together, these results corroborate an ancestral unornamented state for females among White-shouldered Fairywren populations and a single evolutionary origin of black-and-white female ornamentation. This is inconsistent with a genetic correlation model where selection on males leads to elaboration in both sexes, because the ancestral phenotype would likely have been sexually dichromatic.

Regions of divergence

To search for molecular targets of selection in the genome, I estimated relative genomic divergence by calculating F_{st} in 50kb overlapping windows between all possible pairwise comparisons (n=6). Regions experiencing selective sweeps should have decreased genetic variation within populations and increased population differentiation resulting in higher measures of F_{st} relative to background variation (Wright 1950; Lewontin and Krakauer 1973; Oleksyk et al. 2010). Elevated divergence relative to the background was set at windows in the 99th percentile of Z transformed F_{st} (to facilitate comparisons between populations with different demographic histories; Lamichhaney,

Berglund, et al. 2015; Han et al. 2017). To explore enrichment in divergent regions, I identified 16,999 protein coding genes in the White-shouldered Fairywren reference genome on the basis of mRNA sequencing data and protein evidence from other species using MAKER (Cantarel et al. 2008). While the identification of extreme outliers is an established method for testing for selection on genomic regions (Oleksyk et al. 2010), the importance of islands of divergence to the speciation process (e.g. Pennisi 2014) and the timing of selection relative to population divergence have been the subject of considerable debate (Cruickshank and Hahn 2014).

I examined elevated windows (>99% percentile) for gene content with a particular focus on genes likely to be involved in generating the transition between diagnostic plumage patches: the dorsal surface, which is brown in *lorentzi* females but black with white in the other three subspecies and the chest, which is white in both *lorentzi* and *naimii* females but black in other two subspecies (Figure 3.1a). I focused on these body regions by searching for uniquely shared divergent windows between comparisons of shared transitions (see Table S3). For windows to meet this criteria, they must be 1) shared between all pairwise comparisons of the phenotypic comparison being made (i.e., shared), and 2) not be elevated in comparisons between pairwise comparisons of populations with the same phenotype (i.e., unique).

In my survey of genome-wide patterns of divergence, mean differentiation across all windows was considerably lower between *aida* and *naimii* (mean=0.116, SD=0.045, Figure 3.2a) than between other comparisons (mean=0.274, SD=0.095, Figure 3.2a). Between *aida* (black females) and *naimii* (pied females), the comparison with the lowest overall divergence, I identify nine regions containing melanogenesis genes and one

containing a putative feather morphogenesis gene (*HAND2*). Moreover, one of the windows (*KITLG*, Figure 3.2b, Figure S3.1) is common to all comparisons between populations possessing black and white chests, which implicates *KITLG* as a strong candidate for a genetic mediator of the black vs. white chest transition. I also compared all populations to *lorentzi* (the only population with a brown dorsal surface) to identify putative shared regions associated with a transition in dorsal surface coloration. Out of 310 shared windows and 165 shared genes (Table S3), only one peak contains a melanogenesis gene (*ARCN1*) and another contains a member of the SDR family (Figure 3.2c), *DHSR12*, which is involved in steroid production (Bray et al. 2009). Mutations in *ARCN1* in *Mus musculus* result in changes to coat coloration by altering melanocyte trafficking (Xu et al. 2010) and may affect MC1R signaling (Dorshorst et al. 2015). Another member of the SDR family may be involved in male polymorphisms in the Ruff (*Philomachus pugnax,* Lamichhaney, Fan, et al. 2015). Together, these data suggest that *ARCN1* may be associated with the transition from brown plumage to black patches on the dorsal surface, whereas *KITLG* may contribute to divergence in black vs. white chest coloration. Given that *moretoni* (black females) have elevated levels of circulating testosterone relative to *lorentzi* females (Enbody in review), *DHSR12* may have a role in mediating between population differences in steroid production.

Differential expression of plumage patch genes

I further explore the role of the melanogenesis and steroid pathways in mediating transitions between phenotypes by comparing the feather transcriptome between populations and plumage patches. Consistent with low genomic divergence between

populations, I observed high overall similarity in mRNA expression from RNAseq of 26 molting feather samples (from twelve individuals, Table S2) from two different body locations. Among 16,780 expressed genes in feather follicles, gene expression was strongly linked to between-population differences in color in specific body regions. Between *moretoni* (white) and *lorentzi* (brown) shoulder patch feathers, 1272 genes were differentially expressed. Up regulated genes in white feathers included a number of avian keratin genes, which may be associated with differences in the lengths of feather barbules between these two feather types (white=longer, Figure S3.2) and keratin structure is associated with the intensity of white plumage production (Igic et al. 2018). Down regulated genes included genes that were enriched for categories associated with response to peptide hormones and other chemical stimuli, which may indicate a relationship with hormonal steroids (Table S4).

To further explore the relationship between testosterone and shoulder patch color expression, I experimentally elevated levels of circulating testosterone via implants (e.g., as in Lindsay et al. 2011) in three free-flying *lorentzi* females that were naturally undergoing molt. I had reason to believe testosterone might be important in plumage expression in female White-shouldered Fairywrens, because in the sister species, the Red-backed Fairywren (*M. melanocephalus*), experimentally elevated testosterone induces male ornament production (Lindsay et al. 2009; Lindsay et al. 2011) and stimulates a male-like phenotype in females (Lindsay et al. 2016). After ten days of elevated testosterone, females molted testosterone-induced white feathers instead of naturally occurring brown feathers on the shoulder patch, with no other noticeable changes to their plumage phenotype (Figure S3.3). I examined expression differences

between molting brown feathers (pre-treatment) and molting white feathers (post-treatment) within the same individual for each of these three implanted females. Of 187 differentially expressed genes (144 down, 43 up) following treatment, 93 (73 down, 20 up) were also differentially expressed between the *moretoni* and *lorentzi* shoulder patch comparison (7% of all between population differentially expressed genes, Figure 3.3). I found 69 gene ontology categories, including categories relating to responses to various stimuli (e.g. peptide hormones, chemical), that were shared between the testosterone treatment group and between population differences in the shoulder patch (Table S4). Similar categories of response to stimuli were identified in brains of other songbird species receiving testosterone treatment (Dittrich et al. 2014). Together, these results suggest that natural variation in gene expression between the *moretoni* and *lorentzi* populations are directly linked to testosterone regulation. In addition, I found that 10 out of the 20 upregulated genes in white feathers included avian keratin groups. PNHBA (of the WNT/β-catenin pathway) was also significantly upregulated in white feathers and is involved in feather morphogenesis (Ng et al. 2014). The only melanogenesis-related gene differentially expressed in was *KITL,* which was overexpressed in white feathers.

I additionally assessed whether genes that were suppressed by testosterone treatment were underexpressed in all populations that naturally develop white shoulder patches. As previous research suggests that *moretoni* females (black phenotype) have elevated levels of circulating testosterone (Enbody in review) relative to *lorentzi* (brown females), I predicted that all melanized populations (i.e. *aida, naimii,* and *moretoni*) would show similar low expression of testosterone suppressed genes. In this subset of 144 genes, testosterone treated *lorentzi* females cluster closer with samples taken from

another population, not their own (Figure 3.4). These testosterone-suppressed genes are most highly expressed in the shoulder patch of *lorentzi*, and, although also expressed in the shoulders of some other populations, suggest that testosterone may not be mediating other color transitions (e.g. between white and black coloration Figure 3.4). This is further supported by the lack of melanin-based feathers molting in post testosterone treated individuals.

For melanized patches, I examined multiple occurrences of white (*naimii* and *lorentzi*) and black chest feathers (*aida* and *moretoni*) among females, I found 35 genes differentially expressed in both comparisons and of these, 28 were significantly overexpressed in black feathers. Only 4 were significantly under-expressed in white feathers (Figure 3.5). Of genes overexpressed in black chest feathers, six are known to be involved in melanogenesis (*HPGDS*, *FRZB*, *MLANA*, *PMEL*, *SLC24A4*, *TYR*). Although no gene ontology terms were significant for shared genes, the top gene enrichment categories include melanin biosynthesis (Table S5). Genes overexpressed in white chest feathers shared between these populations include *KRF2*, one of the avian keratin groups. In addition, *KITLG* (identified in comparisons between black and white chested populations in the window based analysis) is located upstream of *MITF*, which activates the expression of *HPGDS*, *MLANA*, and *TYR* (Poelstra et al. 2015).

To differentiate between directional selection and drift on differentially expressed genes, I calculated the proportion of variance between populations (M_{st}) and compared to values of simulated neutrality based on F_{st} (Feiner et al. 2017). I found more differentially expressed genes than expected by chance with M_{st} above the 97.5% confidence interval (following a permutation test) in chest feathers between *aida* (black)

and *naimii* (white). Nearly 50% of the differentially expressed genes in this comparison fall outside the 97.5% confidence interval of the $M_{st\_neutral}$ distribution (Table 1), which includes five of the six shared melanogenesis genes in the comparison above (*HPGDS*, *MLANA*, *PMEL*, *SLC24A4*, *TYR*) and the single shared avian keratin gene (*KRF2*). This indicates that these differentially expressed top candidate genes related to feather coloration are under directional selection. In contrast, comparisons between *moretoni* and *lorentzi* (in both chest feathers and shoulder patch feathers) resulted in a smaller proportion of genes outside of the 97.5% confidence interval than expected by chance (Figure S3.4). Thus, differentially expressed genes do not show a strong signal of directional selection in chest or shoulder patches between *moretoni* and *lorentzi*. Alternatively, divergence between *moretoni* and *lorentzi* may have occurred sufficiently long ago to mask a signature of selection on gene expression.

Conclusion

Resolving the degree to which selection contributes to female ornament evolution is central to understanding many of the processes generating biodiversity, including speciation and sexual dimorphism. Using a comparative approach, I found that transitions to ornamented female phenotypes from an unornamented ancestor are associated with genomic signatures of directional selection. I identified divergence peaks common to multiple transitions in female ornamentation that contain putatively functional genes, with the melanogenesis pathway gene *KITLG* located in divergence peaks in black versus white chest feathers and the melanocyte trafficking protein *ARCN1* located in divergence peaks between brown and all melanized populations, consistent with directional selection

acting on elements within regions containing these melanogenesis genes. These genes may in turn influence the regulation of other genes that were differentially expressed between white and black chest feathers and that also exhibit signatures of directional selection. For example, differential regulation of *KITLG* would influence the downstream expression of the central melanogenesis transcription factor *MITF*.

Intriguingly, my testosterone implant experiment demonstrates that expression of the white shoulder patch, a putative signal, is directly regulated by testosterone and is associated with the suppression of a set of genes that are not highly expressed in melanized populations of White-shouldered Fairywren. This result, combined with previous findings of elevated testosterone in *moretoni* relative to *lorentzi* (Enbody in review), suggests that elevated levels of testosterone in melanized populations may regulate the expression of genes that mediate development of the white shoulder patch. Furthermore, divergence in the region of an SDR family gene (*DHSR12*), which is involved in steroid production, appears to have a functional role in determining differences in testosterone production between brown and all melanized populations. I found no evidence that testosterone influences the other plumage regions I investigated, and suggest these may be maintained by differential regulation of a small number of melanogenesis genes, independent of circulating steroids. Differences in the regulation of *ARCN1*, known to regulate coat coloration in mice (Xu et al. 2010) and possibly cattle (Dorshorst et al. 2015), is a strong candidate for mediating transitions from a brown dorsal surface to a melanized white and black surface.

Other studies have convincingly demonstrated the role of sexual selection in shaping elaboration of male traits, and I consider it likely that social selection drives the

divergence in female plumage I have documented among White-shouldered Fairywren females (West-Eberhard 1979; Rubenstein and Lovette 2009). As differences between populations in territorial aggressive behavior (Enbody in review) suggests that ornaments function in key social contexts, future work should aim to clarify the adaptive function of female plumage ornamentation in White-shouldered Fairywren populations.

Figures & Tables

Table 3.1: Observed M_{st}values compared with neutral expectation of M_{st}, which were derived from Fst values.

Data	Mean Fst	Mean Mst neutral	Mean Mst observed	Mean Mst of DEG	Expected number of genes outside 97.5% CI	Observed number of genes outside 97.5% CI	Fold enrich.	Observed number of DEG outside 97.5% CI [percent of all DEG]
Chest: *aida* vs. *naimii* (black vs. white)	0.064	0.153	0.034	0.679	411-491	535	1.2	87[49%]
Chest: *moretoni* vs. *lorentzi* (black vs. white)	0.204	0.306	0.044	0.217	409-491	154	0.34	6[2%]
SP: *moretoni* vs. *lorentzi* (black vs. brown)	0.204	0.291	0.112	0.671	410-491	207	0.46	163[13%]

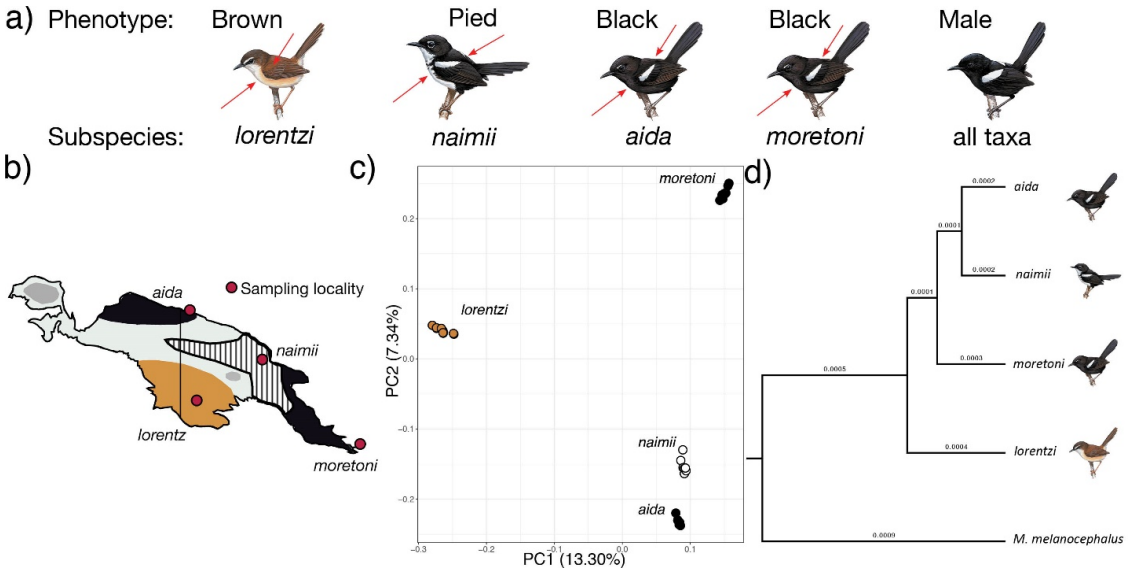

Figure 3.1: a) Descriptions of all female phenotypes by subspecies (males are similar in all populations) with illustrations reproduced from del Hoyo et al. (2017), with arrows pointing to the plumage patches that are the focus of this study. Red arrows refer to the chest and shoulder patch regions discussed in this study. b) Map of New Guinea with sampling locations marked in red and approximate ranges for each of the sampled populations (from: Birdlife International and NatureServe 2013). Unsampled populations (n=2) are marked in grey. c) PCA of the covariance matrix generated from genotype likelihoods of all unrelated samples. d) Phylogenetic relationships among all four populations using the UPGMA cluster method from the pairwise distance matrix of autosomal loci, with the Red-backed Fairywren (*Malurus melanocephalus*) as the outgroup. Branch lengths are substitutions per base.

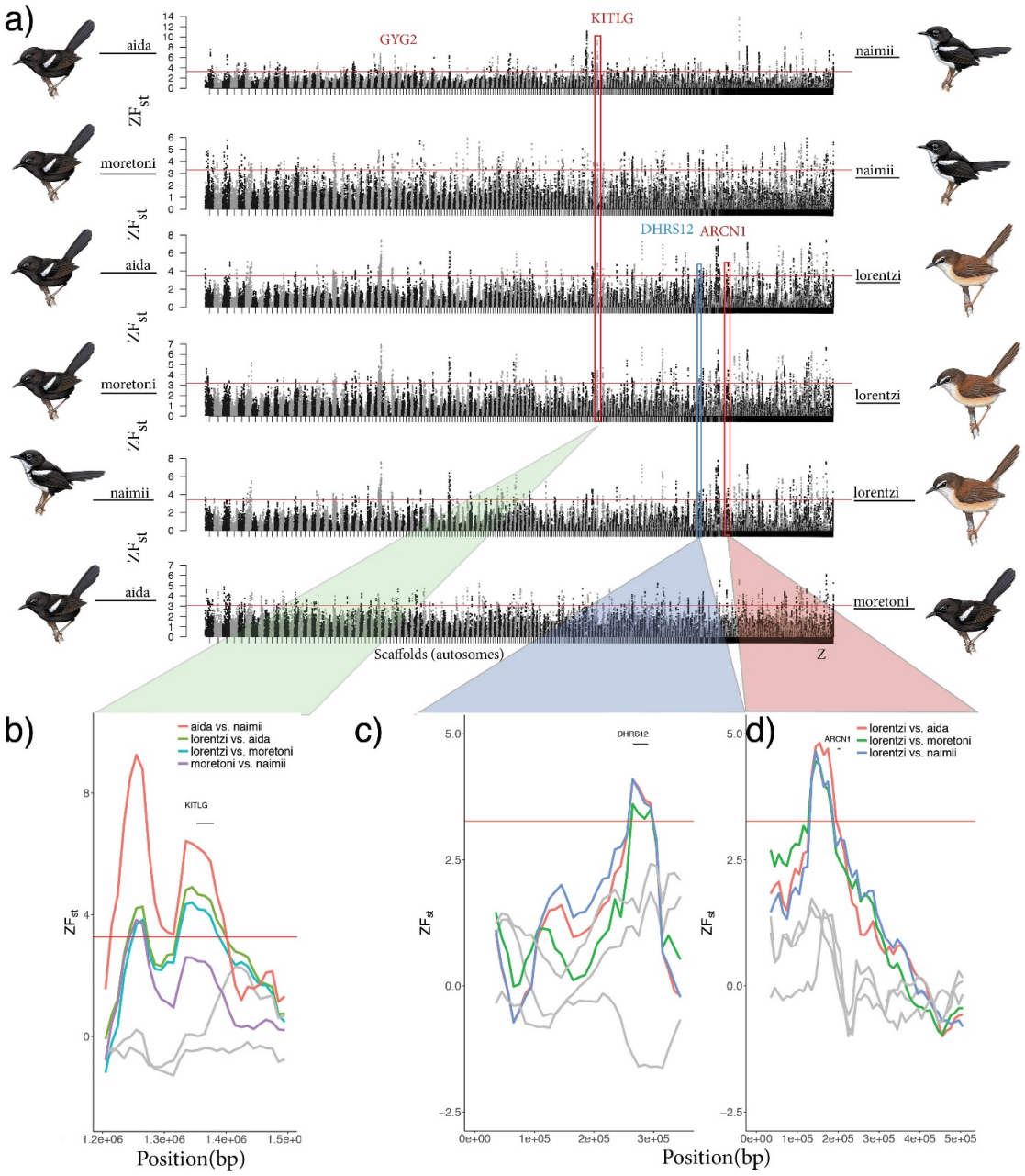

Figure 3.2: a) Manhattan plots for all pairwise comparisons between White-shouldered Fairywren populations. Points show overlapping sliding window F_{st} values in 50kb windows and points above the red line are above the 99th quantile. Scaffolds are ordered by size and differentiated by color and the Z chromosome is separated on the far right. Shared divergent peaks are outlined and labeled (red for melanogenesis gene, blue for steroid related gene). Note different y-axis scales. b) Enlargement of shared regions of divergence identified in populations that differ in black or white chests. Red horizontal line refers to the mean ZFst > 99th percentile in all comparisons. Colored lines refer to comparisons that differ in the color of the chest (black: *aida* and *moretoni* or white: *naimii* and *lorentzi*). Grey lines refer to population comparisons with no difference in chest coloration. A melanogenesis gene, *KITLG*, is labeled and its length marked. c)

Enlargement of shared regions of divergence identified between populations that differ in dorsal surface coloration. Colored lines in this figure refer to pairwise comparisons between populations that differ in color of the dorsal surface (melanized black with white shoulder: *aida* and *moretoni* or brown: *lorentzi*). Grey lines refer to comparisons that did not differ in dorsal surface coloration. *DHRS12,* an SDR gene involved in androgenesis, and *ARCN1*, a melanogenesis gene is labeled and their length marked above each corresponding peak.

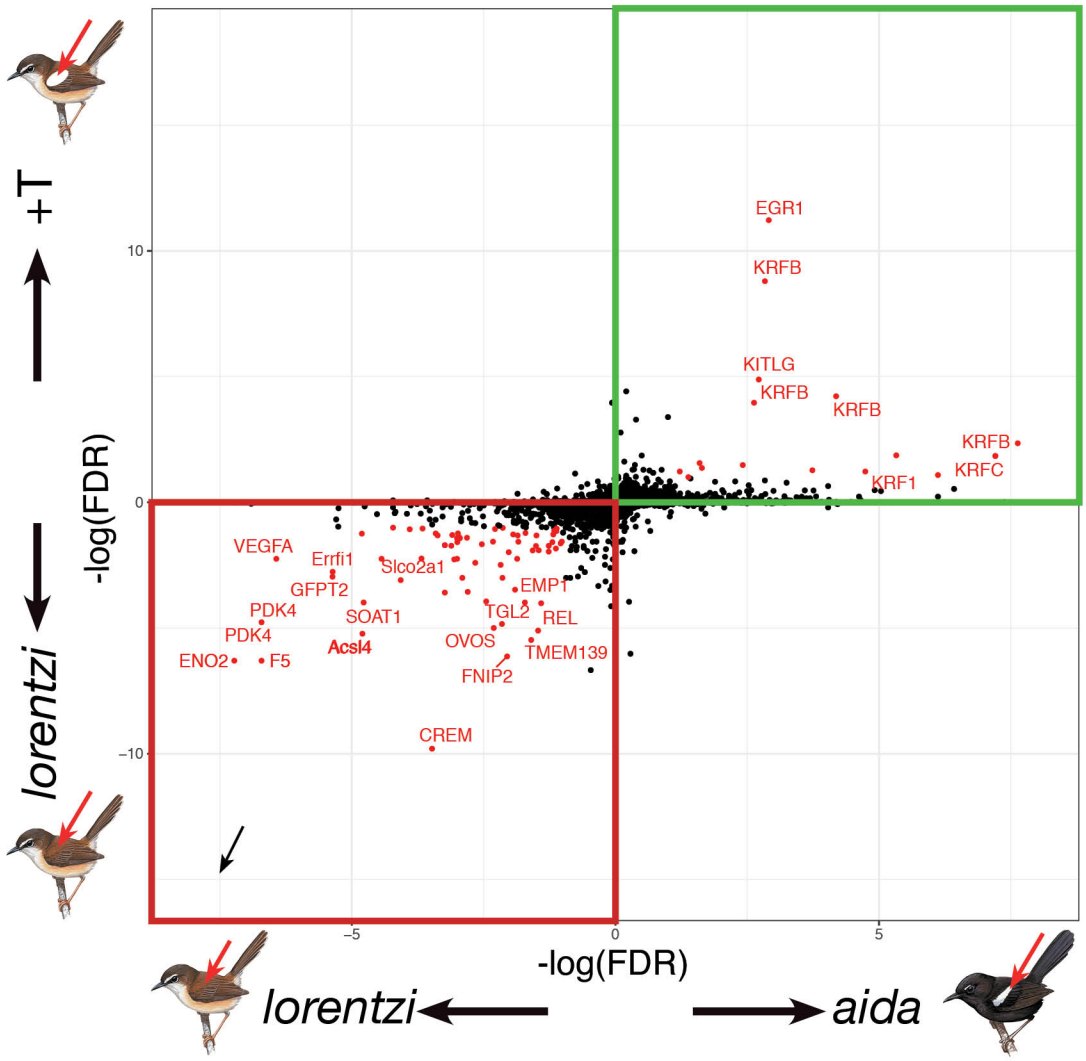

Figure 3.3: Differential expression of shared color genes between t-treatment groups and between *lorentzi* and *moretoni* shoulder patches. Genes that are significantly overexpressed in white shoulder feathers are located in the quadrant bounded by green and genes that are significantly overexpressed in brown shoulder patch feathers are bounded by red. Significantly differentially expressed genes in both plots are colored in red and outlier genes of interest are labeled. The scale on each axis is –log10[FDR], an adjusted measure of significance for direction of expression.

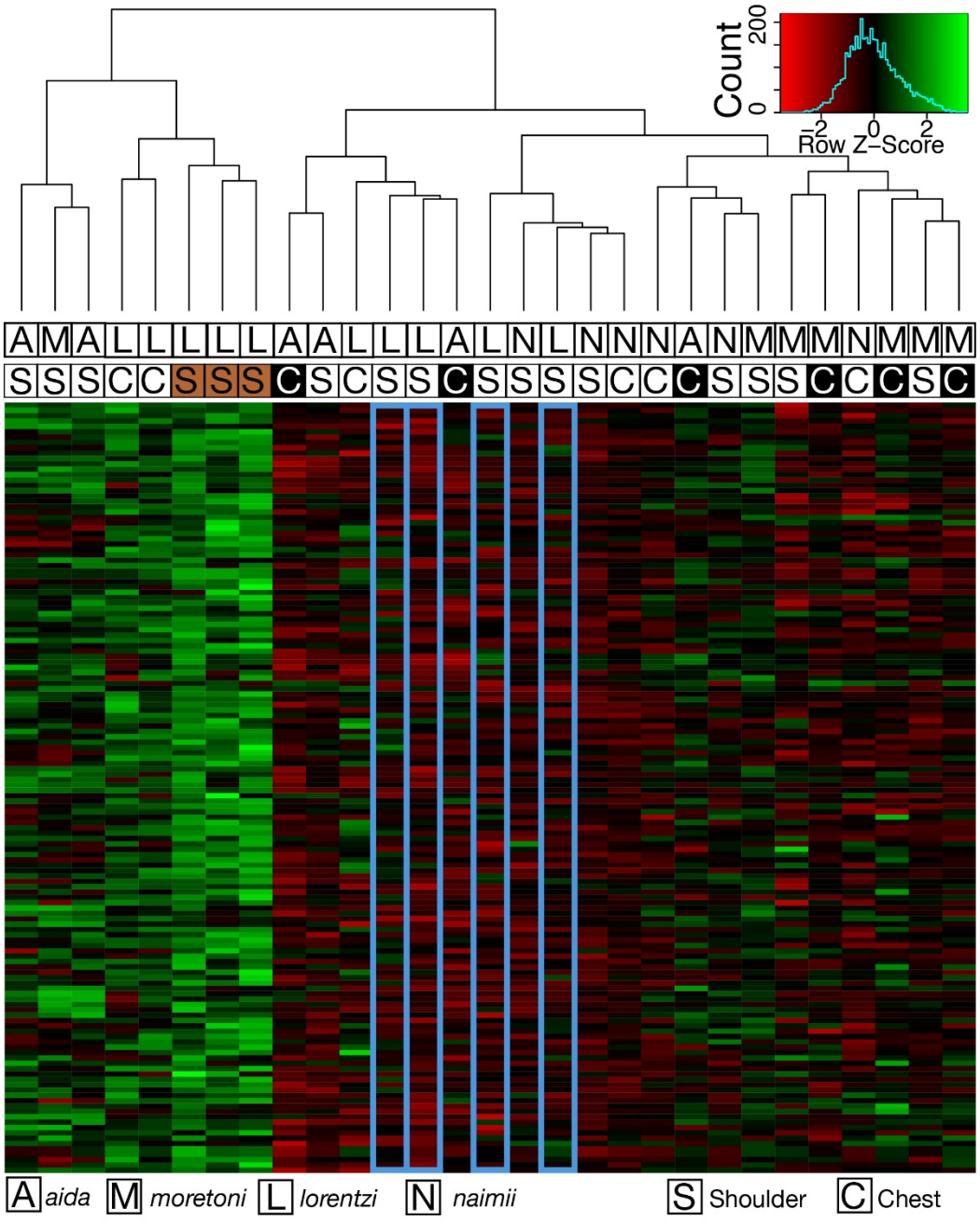

Figure 3.4: Heatmap of all tissue sampled in this study showing normalized counts of 144 genes that are down regulated following testosterone treatment in the shoulder patch of *lorentzi* females. The heatmap shows the expression of these testosterone suppressed genes in the tissue of all other populations. Dendrogram above the columns clusters samples by similarity in normalized gene counts (Z-score). Each column corresponds to one sample, whose population and body part is labeled (see key at bottom). The color of the square for each body part indicates the color of plumage for that part. Post treatment *lorentzi* are highlighted by a blue box and note that samples cluster more closely with

samples from populations *aida, naimii*, and *moretoni*, than to untreated (brown) shoulder patch samples of *lorentzi.*

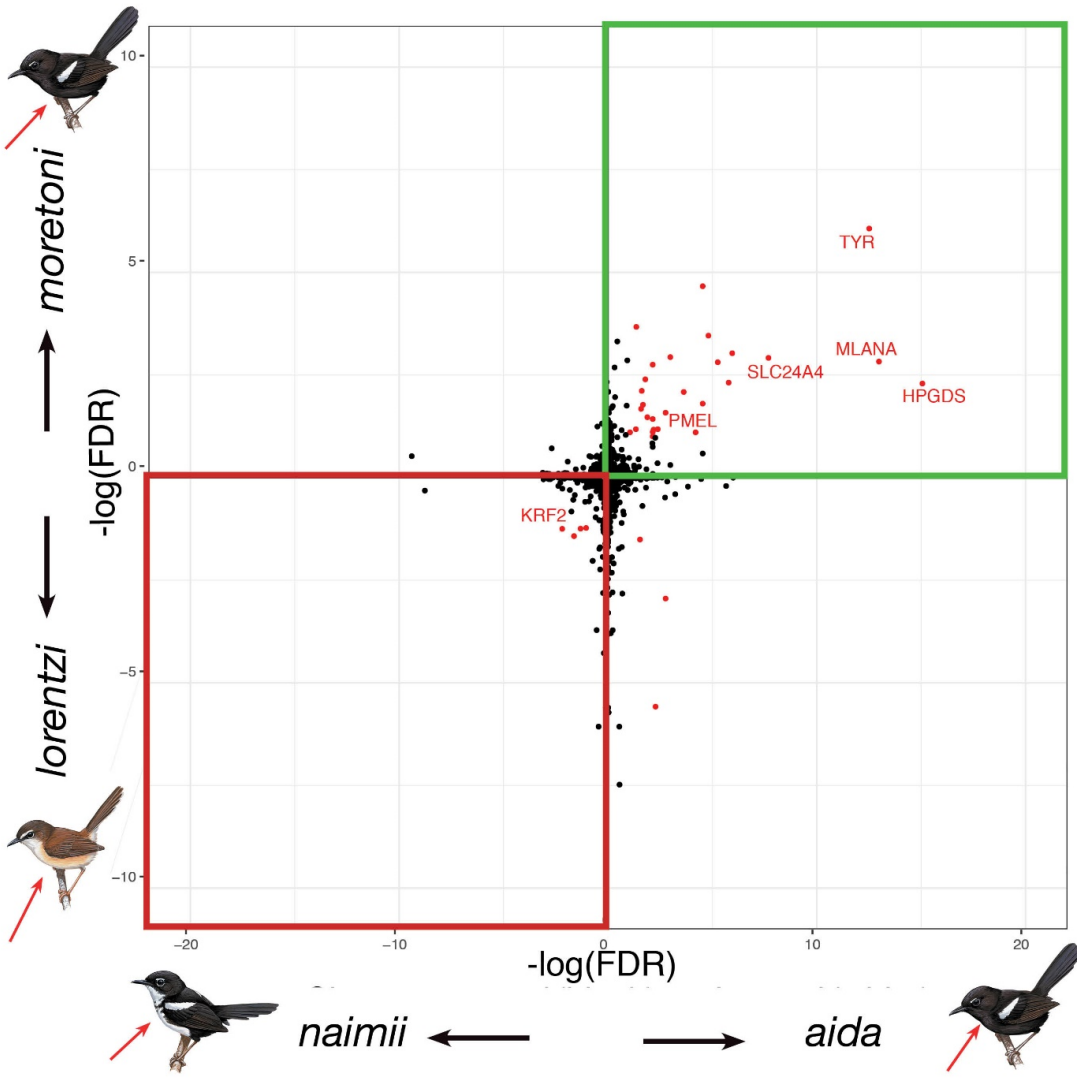

Figure 3.5: Differential expression of shared color genes in between-population comparisons. Genes that are significantly overexpressed in black feathers vs. white feathers are located in the quadrant bounded by green and genes that are significantly overexpressed in white chest feathers are bounded by red. The scale on each axis is –log10[FDR], an adjusted measure of significance for direction of expression.

Supplemental:

Table S3.1: List of all samples and sampling location used for whole genome resequencing. DNA was extracted from red blood cells, archived at Tulane University.

Identifier	Tissue Location	Subspecies	Sex	Site	Latitude	Longitude
33248	Tulane University	*aida*	F	Vanimo	-2.69226	141.29795
33249	Tulane University	*aida*	F	Vanimo	-2.69226	141.29795
33252	Tulane University	*aida*	F	Vanimo	-2.69226	141.29795
33253	Tulane University	*aida*	F	Vanimo	-2.69226	141.29795
33254	Tulane University	*aida*	F	Vanimo	-2.69226	141.29795
33256	Tulane University	*aida*	F	Vanimo	-2.69226	141.29795
33257	Tulane University	*aida*	F	Vanimo	-2.69226	141.29795
33297	Tulane University	*lorentzi*	F	Obo	-7.6017	141.30902
47617	Tulane University	*lorentzi*	F	Obo	-7.6017	141.30902
47623	Tulane University	*lorentzi*	F	Obo	-7.6017	141.30902
47631	Tulane University	*lorentzi*	F	Obo	-7.6017	141.30902
47653	Tulane University	*lorentzi*	F	Obo	-7.6017	141.30902
47657	Tulane University	*lorentzi*	F	Obo	-7.6017	141.30902
47672	Tulane University	*lorentzi*	F	Obo	-7.6017	141.30902
47683	Tulane University	*lorentzi*	F	Obo	-7.6017	141.30902
97513	Tulane University	*lorentzi*	F	Obo	-7.6017	141.30902
97528	Tulane University	*lorentzi*	F	Obo	-7.6017	141.30902
47720	Tulane University	*moretoni*	F	Garuahi	-10.2216	150.4805
36148	Tulane University	*moretoni*	F	Garuahi	-10.2216	150.4805
36149	Tulane University	*moretoni*	F	Garuahi	-10.2216	150.4805
47707	Tulane University	*moretoni*	F	Garuahi	-10.2216	150.4805
47717	Tulane University	*moretoni*	F	Garuahi	-10.2216	150.4805
36126	Tulane University	*moretoni*	F	Porotona	-10.2654	150.5795

36182	Tulane University	*moretoni*	F	Porotona	-10.2654	150.5795
36188	Tulane University	*moretoni*	F	Porotona	-10.2654	150.5795
47745	Tulane University	*moretoni*	F	Porotona	-10.2654	150.5795
47815	Tulane University	*moretoni*	F	Porotona	-10.2654	150.5795
33221	Tulane University	*naimii*	F	Gewal	-5.29228	145.628743
33223	Tulane University	*naimii*	F	Gewal	-5.29228	145.628743
33225	Tulane University	*naimii*	F	Gewal	-5.29228	145.628743
33228	Tulane University	*naimii*	F	Gewal	-5.29228	145.628743
33230	Tulane University	*naimii*	F	Gewal	-5.29228	145.628743
33232	Tulane University	*naimii*	F	Gewal	-5.29228	145.628743
33233	Tulane University	*naimii*	F	Gewal	-5.29228	145.628743
33234	Tulane University	*naimii*	F	Gewal	-5.29228	145.628743
33235	Tulane University	*naimii*	F	Gewal	-5.29228	145.628743
33240	Tulane University	*naimii*	F	Dumpu	-5.83122	145.661629

Table S3.2: List of all samples and sampling location used for RNAseq. RNAseq was extracted from molting feather tissue and "Part" refers to the region on the bird where the tissue was sampled from (see Figure 1). "T treatment" refers to if the sample was part of the experimental testosterone treatment group.

Identifier	Part	T Treatment?	Subspecies	Sex	Site	Latitude	Longitude
33253	Chest		*aida*	F	Vanimo	-2.69226	141.29795
33248	Chest		*aida*	F	Vanimo	-2.69226	141.29795
33254	Chest		*aida*	F	Vanimo	-2.69226	141.29795
33253	Shoulder		*aida*	F	Vanimo	-2.69226	141.29795
33248	Shoulder		*aida*	F	Vanimo	-2.69226	141.29795
33254	Shoulder		*aida*	F	Vanimo	-2.69226	141.29795
47631	Chest		*lorentzi*	F	Obo	-7.6017	141.30902
33297	Chest		*lorentzi*	F	Obo	-7.6017	141.30902
97528	Chest		*lorentzi*	F	Obo	-7.6017	141.30902
33287	Shoulder	After	*lorentzi*	F	Obo	-7.6017	141.30902
33297	Shoulder	After	*lorentzi*	F	Obo	-7.6017	141.30902
97513	Shoulder	After	*lorentzi*	F	Obo	-7.6017	141.30902
97528	Shoulder	After	*lorentzi*	F	Obo	-7.6017	141.30902
33297	Shoulder	Before	*lorentzi*	F	Obo	-7.6017	141.30902
97513	Shoulder	Before	*lorentzi*	F	Obo	-7.6017	141.30902
97528	Shoulder	Before	*lorentzi*	F	Obo	-7.6017	141.30902
36126	Chest		*moretoni*	F	Porotona	-10.2654	150.5795
36182	Chest		*moretoni*	F	Porotona	-10.2654	150.5795
47745	Chest		*moretoni*	F	Porotona	-10.2654	150.5795
47717	Shoulder		*moretoni*	F	Garuahi	-10.2216	150.4805
36126	Shoulder		*moretoni*	F	Porotona	-10.2654	150.5795
47815	Shoulder		*moretoni*	F	Porotona	-10.2654	150.5795
47745	Shoulder		*moretoni*	F	Porotona	-10.2654	150.5795
33230	Chest		*naimii*	F	Gewal	-5.29228	145.628743
33221	Chest		*naimii*	F	Gewal	-5.29228	145.628743
33225	Chest		*naimii*	F	Gewal	-5.29228	145.628743
33230	Shoulder		*naimii*	F	Gewal	-5.29228	145.628743
33221	Shoulder		*naimii*	F	Gewal	-5.29228	145.628743
33225	Shoulder		*naimii*	F	Gewal	-5.29228	145.628743

Table S3.3: Summary of shared windows between different comparisons. Phenotype refers to comparisons that are between black, pied, or brown birds. Specific body part comparisons are shown for all black vs. white comparisons and for all black/white dorsal vs. brown comparisons. The number of windows are the total number of windows above the 99th percentile in each comparison that are shared between datasets, the number of genes in windows refer to all genes located in divergent shared windows, and the number of comparisons refer to how many pairwise comparisons are represented. (unique) refers to filtering out windows that were also divergent between comparisons with no phenotypic change (e.g. aida vs. moreotni or for chest, naimii vs lorentzi). KITLG* is located in a local peak (i.e. not >99th quantile) between moretoni and naimii (see Figure 3).

Comparison	Number of windows	Number of genes in windows	Number of melanogenesis genes in windows	Number of comparisons	Melanogenesis genes
Phenotype: Black vs. Brown	367	196	3	2	FREM2, KITLG, ARCN1
Phenotype: Black vs. Brown (unique)	24	23	1	2	FREM2
Phenotype: Black vs. Pied	40	21	0	2	
Phenotype: Black vs. Pied (unique)	8	9	0	2	
Phenotype: Brown vs. pied	615	343	5	2	EED , CAV2, CAV1, ARCN1,HPGD
Phenotype: Brown vs. Pied (unique)	262	229	4	1	EED , CAV2, CAV1, HPGD

Chest: Black vs. White	2	2	0(1)	4	KITLG*
Dorsal: Black vs. White (dorsal) & Brown vs. White (shoulder)	310	165	1	4	ARCN1 GYG2 , NOTCH2,KITLG,RGS20,RAB32,RB1,HPGD, WNT2B,
Population: aida vs. naimii	420	244	9	1	ASIP

Table S3.4: Gene ontology categories that were significantly enriched following a BH adjustment for multiple comparisons when comparing shoulder feathers in *moretoni* vs. *lorentzi*, and in shoulder patches of *lorentzi* following testosterone treatment.

GOTerm	ontology	Num DE In Cat	Num In Cat	term
GO:0010033	BP	36	1809	response to organic substance
GO:0071310	BP	31	1473	cellular response to organic substance
GO:0042035	BP	7	48	regulation of cytokine biosynthetic process
GO:0009605	BP	26	1101	response to external stimulus

GO:0042089	BP	7	54	cytokine biosynthetic process
GO:0042107	BP	7	55	cytokine metabolic process
GO:0042221	BP	38	2293	response to chemical
GO:0032612	BP	5	27	interleukin-1 production
GO:0042127	BP	22	922	regulation of cell proliferation
GO:0034097	BP	16	542	response to cytokine
GO:0048511	BP	10	186	rhythmic process
GO:0070887	BP	31	1761	cellular response to chemical stimulus
GO:0042036	BP	4	13	negative regulation of cytokine biosynthetic process
GO:1901652	BP	12	299	response to peptide
GO:0001817	BP	12	315	regulation of cytokine production
GO:0000122	BP	15	491	negative regulation of transcription from RNA polymerase II promoter
GO:0008283	BP	24	1191	cell proliferation
GO:0071345	BP	14	484	cellular response to cytokine stimulus

GO:0001816	BP	12	346	cytokine production
GO:0031324	BP	28	1580	negative regulation of cellular metabolic process
GO:0032652	BP	4	24	regulation of interleukin-1 production
GO:0032611	BP	4	23	interleukin-1 beta production
GO:0030595	BP	6	84	leukocyte chemotaxis
GO:0043436	BP	17	768	oxoacid metabolic process
GO:0019752	BP	16	700	carboxylic acid metabolic process
GO:0042222	BP	2	2	interleukin-1 biosynthetic process
GO:0045360	BP	2	2	regulation of interleukin-1 biosynthetic process
GO:0045362	BP	2	2	positive regulation of interleukin-1 biosynthetic process
GO:0001818	BP	7	115	negative regulation of cytokine production
GO:0010558	BP	20	918	negative regulation of macromolecule biosynthetic process
GO:0006082	BP	17	774	organic acid metabolic process
GO:0008219	BP	24	1305	cell death

GO:0002687	BP	5	55	positive regulation of leukocyte migration
GO:0043434	BP	10	266	response to peptide hormone
GO:0031327	BP	20	952	negative regulation of cellular biosynthetic process
GO:0070555	BP	5	53	response to interleukin-1
GO:0010469	BP	8	193	regulation of receptor activity
GO:1901700	BP	19	903	response to oxygen-containing compound
GO:0009890	BP	20	967	negative regulation of biosynthetic process
GO:0042327	BP	15	585	positive regulation of phosphorylation
GO:0001227	MF	7	121	transcriptional repressor activity, RNA polymerase II transcription regulatory region sequence-specific binding
GO:0048519	BP	41	3096	negative regulation of biological process
GO:0008083	MF	5	62	growth factor activity
GO:0005996	BP	8	182	monosaccharide metabolic process
GO:0019221	BP	10	320	cytokine-mediated signaling pathway

GO:0045892	BP	16	672	negative regulation of transcription, DNA-templated
GO:1901564	BP	50	4334	organonitrogen compound metabolic process
GO:1901653	BP	9	226	cellular response to peptide
GO:0009719	BP	19	919	response to endogenous stimulus
GO:0000981	MF	12	393	RNA polymerase II transcription factor activity, sequence-specific DNA binding
GO:0045087	BP	11	370	innate immune response
GO:0001541	BP	4	34	ovarian follicle development
GO:0071417	BP	11	338	cellular response to organonitrogen compound
GO:0005126	MF	6	103	cytokine receptor binding
GO:0009892	BP	28	1797	negative regulation of metabolic process
GO:0042698	BP	5	62	ovulation cycle
GO:0051172	BP	25	1475	negative regulation of nitrogen compound metabolic process
GO:0050927	BP	3	14	positive regulation of positive chemotaxis
GO:1903507	BP	16	693	negative regulation of nucleic acid-templated transcription

GO:1902				
679	BP	16	694	negative regulation of RNA biosynthetic process
GO:0001				
934	BP	14	555	positive regulation of protein phosphorylation
GO:0042				
325	BP	19	944	regulation of phosphorylation
GO:0010				positive regulation of phosphorus metabolic
562	BP	15	633	process
GO:0045				positive regulation of phosphate metabolic
937	BP	15	633	process
GO:0050				
896	BP	54	4753	response to stimulus
GO:0048				
523	BP	38	2798	negative regulation of cellular process
GO:0042				
030	MF	2	3	ATPase inhibitor activity
GO:0050				
926	BP	3	15	regulation of positive chemotaxis
GO:0010				
243	BP	14	578	response to organonitrogen compound

Table S3.5: Gene ontology categories that were significantly enriched in both comparisons of black chest feathers and white chest feathers. GOTerms bolded were significant following a correction for multiple comparisons in *moretoni-lorentzi* comparison, but no terms were significantly enriched following a multiple test comparison when looking at overlapping genes in both comparisons.

GO Category	Ontology	Description	*p*	Num DE In Cat	Num In Cat
GO:1901685	BP	glutathione derivative metabolic process	5.16E-06	3	11
GO:1901687	BP	glutathione derivative biosynthetic process	5.16E-06	3	11
GO:0004364	MF	glutathione transferase activity	8.92E-06	3	13
GO:0019748	BP	secondary metabolic process	7.72E-05	3	29
GO:0030658	CC	transport vesicle membrane	0.00014 9596	4	99
GO:0030672	CC	synaptic vesicle membrane	0.00018 4997	3	40
GO:0099501	CC	exocytic vesicle membrane	0.00018 4997	3	40
GO:0016765	MF	transferase activity, transferring alkyl or aryl (other than methyl) groups	0.00018 8414	3	37
GO:0042438	BP	melanin biosynthetic process	0.00040 1863	2	11
GO:0006582	BP	melanin metabolic process	0.00048 0926	2	12
GO:0044550	BP	secondary metabolite biosynthetic process	0.00056 5461	2	13

GO:0006			0.00059		
071	BP	glycerol metabolic process	461	2	13
GO:0019			0.00082		
400	BP	alditol metabolic process	4482	2	15
GO:0016			0.00107		
486	BP	peptide hormone processing	8227	2	17
GO:0044			0.00109		
425	CC	membrane part	7816	18	3349
GO:0016			0.00146		
021	CC	integral component of membrane	2689	15	2512
GO:0030			0.00147		
659	CC	cytoplasmic vesicle membrane	899	6	461
GO:0012			0.00171		
506	CC	vesicle membrane	496	6	475
GO:0031			0.00182		
224	CC	intrinsic component of membrane	0853	15	2564
GO:0008			0.00208		
021	CC	synaptic vesicle	657	3	91

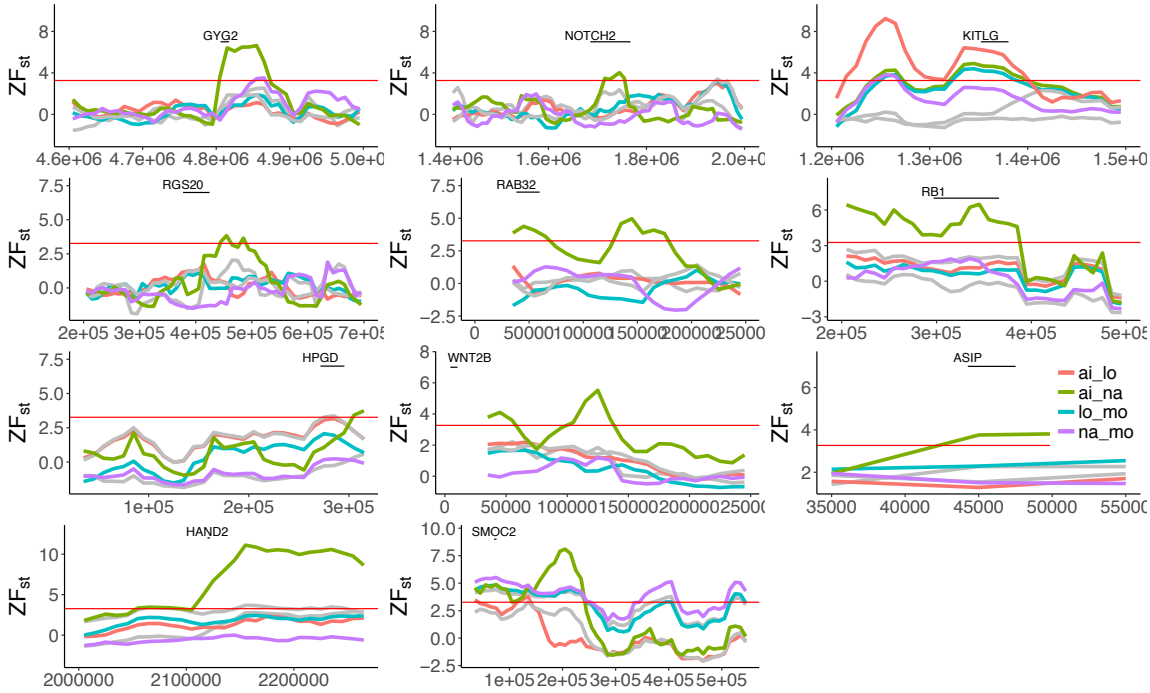

Figure S3.1: Overlap in ZF_{st} between all pairwise comparisons for 9 melanogenesis genes, *HAND2*, and *SMOC2* identified in outlier windows identified between *aida* (black females) and *naimii* (pied females). Colored lines refer to populations that differ in the color of the chest, being black (*aida* and *moretoni*) or white *(naimii* and *lorentzi*). Grey lines refer to population comparisons with no difference in chest coloration.

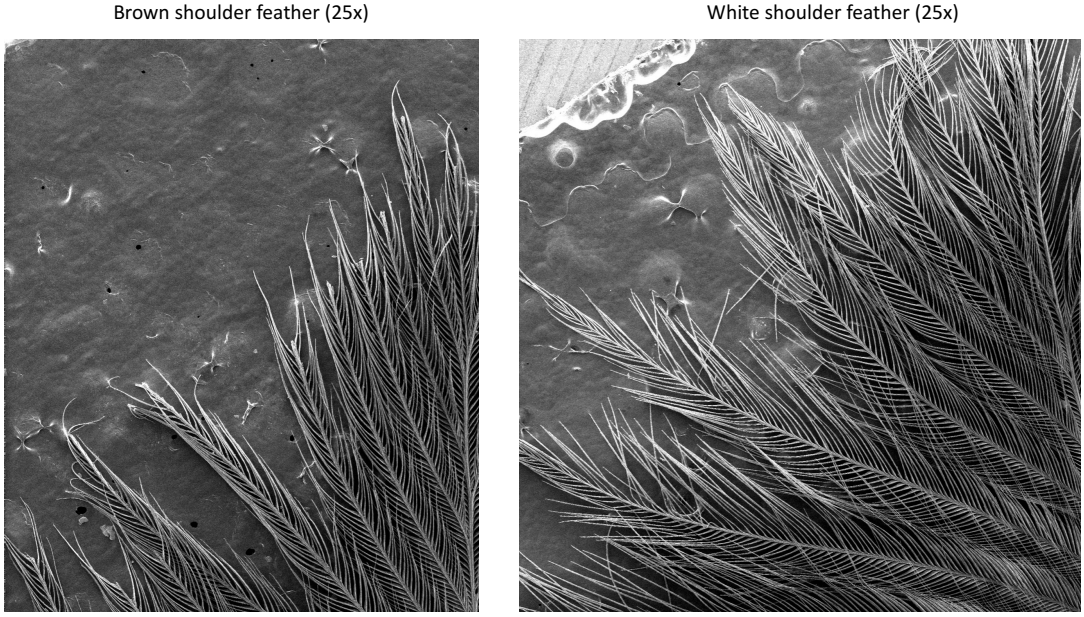

Figure S3.2: Scanning electron images of brown shoulder patch feathers from wild caught *lorentzi* (left) and white shoulder patch feathers from wild caught *moretoni*. Note that feather barbules are longer in white shoulder feathers than brown.

Figure S3.3: Photographs of female White-shouldered Fairywren of the *lorentzi* subspecies before and after testosterone treatment. Note that untreated free flying *lorentzi* females exhibit brown shoulder patches (left) and post testosterone treatment females develop a white shoulder patch (right), but the remainder of the dorsal surface remains brown.

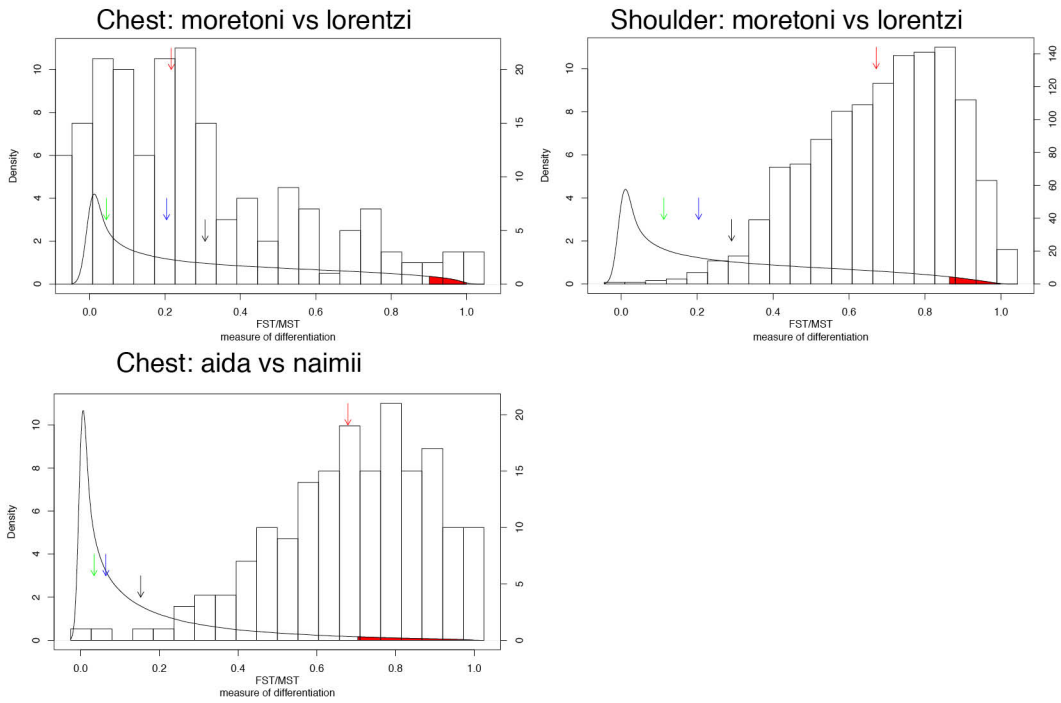

Figure S3.4: Testing the neutral expectation of differentiation in gene expression profiles. The black curve shows the simulated distribution of M_{st} values under neutral evolution, with the 2.5% tail highlighted in red. The histogram shows the distribution of M_{st} values of significantly differentially expressed genes in each comparison. The light green arrow denotes average M_{st} of all expressed genes, the red arrow shows the mean M_{st} of differentially expressed genes, the black arrow is the mean simulated (neutral) M_{st} value, and the black arrow is the global F_{st} value. Values of M_{st} exceeding the 97.5% confidence interval (outside of the red highlighted section of the tail) are expected to be under directional selection.

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BIOGRAPHY

Erik David Enbody was born in 1989 in East Lansing, Michigan where he was raised. From a very young age Erik developed a passion for wildlife and for birds in particular. He started his first research position while in High School working as a technician in a bioacoustics laboratory at Michigan State University and has since worked on ornithological research projects on five continents. He attended Colorado College where he completed his B.A. with distinction in Biology and completed a thesis with Dr. Brian Linkhart on the predation of Flammulated Owl nests by Red Squirrels. In 2013, he joined Dr. Jordan Karubian's lab at Tulane University and conducted his Ph.D. research in Papua New Guinea. Erik will join Dr. Leif Andersson's research group at Uppsala University in Sweden as a Post-Doctoral Researcher in the Department of Medical Biochemistry and Microbiology in 2018.