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Original Article

Testosterone induces plumage ornamentation followed by enhanced territoriality in a female songbird

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We know little of the proximate mechanisms underlying the expression of signaling traits in female vertebrates. Across males, the expression of sexual and competitive traits, including ornamentation and aggressive behavior, is often mediated by testosterone. In the white-shouldered fairywren (*Malurus alboscapulatus*) of New Guinea, females of different subspecies differ in the presence or absence of white shoulder patches and melanic plumage, whereas males are uniformly ornamented. Previous work has shown that ornamented females circulate more testosterone and exhibit more territorial aggression than do unornamented females. We investigated the degree to which testosterone regulates the expression of ornamental plumage and territorial behavior by implanting free-living unornamented females with testosterone. Every testosterone-treated female produced a male-like cloacal protuberance, and 15 of 20 replaced experimentally plucked brown with white shoulder patch feathers but did not typically produce melanic plumage characteristic of ornamented females. Testosterone treatment did not elevate territorial behavior prior to the production of the plumage ornament or during the active life of the implant. However, females with experimentally induced ornamentation, but exhausted implants, increased the vocal components of territory defense relative to the pretreatment period and also to testosterone-implanted females that did not produce ornamentation. Our results suggest that testosterone induces partial acquisition of the ornamental female plumage phenotype and that ornament expression, rather than testosterone alone, results in elevations of some territorial behaviors.

Key words: **female ornamentation, territorial behavior, testosterone.**

INTRODUCTION

The expression of elaborate secondary sexual traits in females, such as ornaments, was long thought to be a byproduct of sexual selection on males (Darwin 1871). However, evolutionary transitions in elaborate coloration, or ornamentation, occur more frequently in females than in males in many taxa, suggesting that selection can act on female ornament evolution independently of selection on males (Irwin 1994; Omland 1997; Burns 1998; Johnson et al. 2013; Price and Eaton 2014). Moreover, recent empirical studies provide support for adaptive functions of ornamentation in females, sometimes in the context of sexual selection (e.g., Fitzpatrick and Servedio 2017) or in the context of competing for nonmating resources via social selection (West-Eberhard 1979, 1983; reviewed in Tobias et al. 2012). One useful route to evaluating the function of a putative signal is to understand the proximate mechanisms regulating its expression (Hau 2007; Rosvall et al. 2016), yet very little is known about mechanisms of ornament production in females.

Differential secretion of sex steroids is a common mechanism underlying sex-specific trait expression from a shared autosomal genome. For instance, in males of some avian taxa, increased circulation of androgens induces molt into colorful male plumage, whereas, in other taxa, enhanced estrogen circulation in females induces molt into cryptic female plumage (reviewed in Kimball and Ligon 1999). Sex steroids like testosterone mediate development and expression of a suite of morphological and behavioral traits (reviewed in Hau 2007), thus causing some evolutionary

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endocrinologists to label testosterone as a phenotypic integrator (reviewed in Lipshutz et al. 2019). There is some empirical support for testosterone acting as a phenotypic integrator in males (Ketterson and Nolan Jr 1999; Wingfield et al. 2001; Hau 2007; Hau and Wingfield 2011), though it can be difficult to determine how traits are linked mechanistically due to the dynamic feedback between hormones and the traits they potentially regulate (Rubenstein and Hauber 2008; Safran et al. 2008; Vitousek et al. 2014).

The role of testosterone in female ornamentation is currently equivocal. However, testosterone is correlated with female ornamentation in some bird species (Muck and Goymann 2011; Moreno et al. 2014; Cantarero et al. 2017), and exogenous testosterone has been found to induce ornament production in females of several avian and nonavian species (Lank et al. 1999; Eens et al. 2000; Lahaye et al. 2012; Cox et al. 2015; Lindsay et al. 2016). Testosterone supplementation can also stimulate competitive behaviors in females, including singing (Kriner and Schwabl 1991; De Ridder et al. 2000) and territorial aggression (Zysling et al. 2006; Rosvall 2013; Cantarero et al. 2015). However, most testosterone manipulation experiments have been conducted in species lacking discrete variation in female phenotypes, so these studies, while useful for determining mechanisms of sexual dimorphism and capacity for phenotypic plasticity in these systems, do not inform our understanding of the proximate basis of naturally varying female phenotypes.

In the current study, we supplemented testosterone to female white-shouldered fairywrens (*Malurus alboscapulatus*, Maluridae), a passerine bird endemic to New Guinea. In this species, females show pronounced variation in plumage ornamentation by subspecies while males are similarly ornamented across subspecies. Previously, we found that females belonging to the subspecies (*M. a. moretoni*) with contrasting black-and-white female ornamental plumage have higher mean plasma testosterone levels and also show greater territorial defense behavior than females belonging to the subspecies lacking this ornamentation (*M. a. lorentzi*; Enbody et al. 2018), suggesting that testosterone might play a role in female trait variation. Males from a closely related species, the red-backed fairywren (*Malurus melanocephalus*), provide a useful comparison to our system, as they vary from cryptic female-typical brown plumage to red-and-black ornamental plumage. Ornamented males circulate higher testosterone and are more aggressively territorial than unornamented males (Barron et al. 2015), and exogenous testosterone stimulates the acquisition of ornamental plumage in males (Lindsay et al. 2011). Females in this species are naturally unornamented but, nonetheless, developed a partial, male-like phenotype when implanted with testosterone (Lindsay et al. 2016). Collectively, these results in red-backed fairywrens inform the prediction that the ornamented female white-shouldered fairywren phenotype is produced via enhanced testosterone circulation.

Here, we experimentally assess whether testosterone regulates the expression of ornamentation and the associated behavioral phenotype in a natural population of a female vertebrate. Second, we investigate how testosterone and associated ornament production affect territorial defense behavior. Third, we assess whether the expression of ornamentation generates a social cost in the form of increased territorial aggression from conspecifics. Specifically, we address three hypothesized pathways to expression of the integrated ornamented female phenotype: 1) exogenous testosterone supplementation in unornamented females of *M. a. lorentzi* induces molt into the ornamented plumage phenotype observed in *M. a. moretoni*; 2) testosterone also promotes territory defense behaviors, independently of plumage ornamentation; and 3) expression of ornamental plumage promotes territory defense independent of elevated testosterone.

METHODS

Study system and general field methods

We studied white-shouldered fairywrens (Figure 1a; *M. a. lorentzi*) in Obo village, Western Province, Papua New Guinea (Figure 1b; 141°19' E, 7°35′ S, 10–20 m a.s.l.). Females of this subspecies lack the black plumage with white shoulder patches that is expressed in males of this species, as well as females of other subspecies (e.g., *M. a. moretoni*; Figure 1c; Enbody et al. 2018). Nesting and territory defense occur year-round in both subspecies studied to date

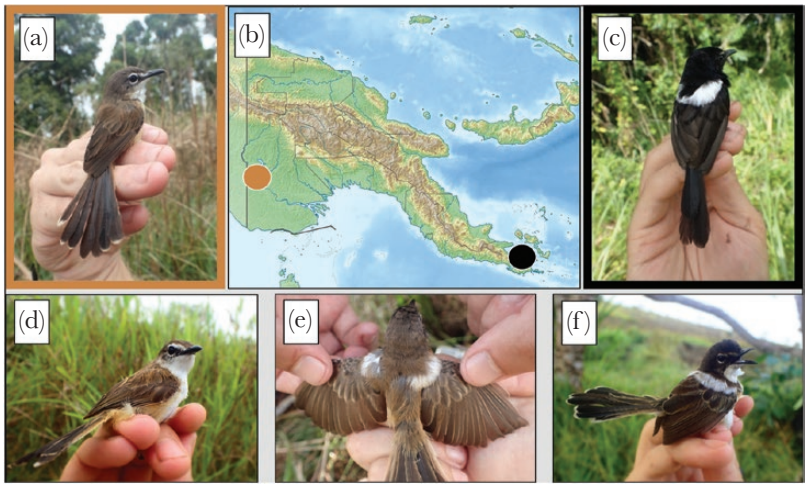

Figure 1
(a) An unmanipulated female from the unornamented white-shouldered fairywren subspecies (*M. a. lorentzi*). (b) Map of Papua New Guinea depicting the location of our two field sites (brown dot: field site of *M. a. lorentzi*, where our study was conducted; black dot: field site of *M. a. moretoni*). (c) An unmanipulated ornamented female from the *M. a. moretoni* subspecies. (d–f) Testosterone-implanted females of *M. a. lorentzi* displaying a gradient of ornamentation: (d) no ornamentation ($n = 5$), (e) partial shoulder patches ($n = 11$), and (f) prominent shoulder patches with darker brown body plumage ($n = 4$).

(Enbody et al. 2018, 2019). Sex ratios and group composition do not seem to differ between unornamented and ornamented female populations, and limited cooperative behavior has been observed in both populations (Enbody et al. 2019). The formative (first year) and adult plumage aspect are qualitatively similar in *M. lorentzi* females. In ornamented populations (e.g., *M. a. moretoni*) females differ phenotypically in all ages from *M. lorentzi* but transition to a darker phenotype in their first breeding season (Enbody et al. 2019).

In the present study, unornamented *lorentzi* females were captured predominantly by flushing into mist nets and, in rarer cases, using duet playback to attract birds to nets. Individuals who were previously unbanded were given a unique Australian Bird and Bat Banding Scheme metal band in addition to a unique combination of three plastic color bands. In order to mitigate the confounding effects of breeding stage on behavior, we excluded females who were actively nesting from the experiment.

Experimental design

Testosterone implants

Each implant consisted of 14.63 mg of beeswax (Beesworks®), 4.87 mg of peanut oil (ACROS Organics™), and 0.5 mg of crystalline testosterone (Sigma T1500), initially dissolved in 2.5 µL of 100% ethanol (Fisher Bioreagants™). Control implants were identical in composition to the testosterone implants except that they lacked testosterone. Testosterone and control implants were both roughly 2 × 3.2 mm (diameter × length) and weighed between 19.8 and 20.7 mg. We scaled implants to produce maximal levels for white-shouldered fairywren females based on our long-term sampling of this species (Enbody et al. 2018). Captive work with these implants in zebra finches (*Taenopygia guttata*) indicated that they elevate testosterone to maximal male levels without any pharmacological side effects to health or behavior (Schwabl H, Goymann W, unpublished data). A study using the same batch of implants employed in our study found that circulating androgens were elevated to 2198–4065 pg/mL (mean = 3027 pg/mL; Khalil et al., in review) in males, which is near the highest levels measured in ornamented females of our study species (highest-circulating androgens = 2816 pg/mL; Enbody et al. 2018). Each implant was inserted subcutaneously in the abdominal region after plucking feathers from the area and sterilizing the incision site with rubbing alcohol, then the incision was sealed with VetBond™ (3 M). We implanted 4 females with testosterone implants and 4 with control implants during a pilot study in September 2016 and 16 females with testosterone and 8 with control implants in March 2017, reflecting the number of readily accessible females at our study site. All assessments of behavior were conducted in 2017.

Plumage, molt, and cloacal protuberance assessments

We plucked approximately 10 feathers at initial capture (when implants were set) across four body regions: crown, shoulder patch, rump, and chest, in addition to 2 tail feathers to induce feather replacement. Ornamental female plumage is characterized by black feathers on the crown, rump, chest, and tail and white shoulder patch feathers (Enbody et al. 2017). Plucking feathers from each region does not visibly alter behavior or affect flight in this species, and females were observed consistently throughout the study period to ensure there were no adverse effects of our protocol. During the 2016 pilot study, we captured implanted females approximately 1, 2, 3, and 4 weeks postimplanting to assess the effect of testosterone on feather replacement, ornamentation, and morphology. In 2017, we captured a subset of females around 10 days after implanting when plucking-induced pin feathers were beginning to emerge from their sheaths (n = 11 testosterone females; 6 control females) and again 28+ days later (n = 11 testosterone females; 5 control females) to align our morphological measurements to the behavioral experiments described below. We assessed cloacal protuberance (CP) size by measuring the length, width, and height with digital calipers. CP volume was calculated as volume = π (D/2)W/2))L (Tuttle et al. 1996). At each capture, molt and plumage were assessed across the following body regions: head, back, chest, belly, wing, and tail. Plumage coloration was qualitatively assessed by noting the presence or absence of black or white feathers, and molt was quantified by determining the approximate proportion of actively molting feathers in each region on a scale of 0–3 (0 = no molt, 1 = 0–32%, 2 = 33–66%, 3 = 67–100% feathers molting).

Simulated territorial intrusions

We used a simulated territorial intrusion (STI) protocol for our study system (detailed methods in Enbody et al. 2018). Briefly, we placed cardstock mounts painted to resemble pairs from *M. a. lorentzi* on a focal pair's territory and lured in the pair using a duet recorded from our study population. We randomly selected one male and one female mount (n = 4 mounts for each sex) and a duet (n = 10) for each trial; in the rare case that the chosen duets were from the focal pair or their territory neighbors, we reselected a different duet. Each duet was played through a small speaker (UE Roll 2; California) and consisted of a single duet separated by 10 s of silence before repeating. Mounts were placed immediately next to each other above the speaker on a 1.5-m tall stick containing three small branches: one containing the mount pair, and the others for responding pairs to perch on. Though our main goal was to quantify female response, we used mount pairs and duets for several reasons relating to white-shouldered fairywren social behavior. First, we have never observed females entering territories without their mates. Second, territories are consistently defended in a coordinated fashion by pairs, especially through duetting (Enbody et al. 2018). Finally, the use of only a female mount and song may lead to multiple males approaching to perform sexual displays to the mount in this population (Enbody and Boersma, personal observations).

We started each trial with 1 min of acclimation time followed by 5 min of duet playback or until the focal female approached within 1 m of the mount, whichever occurred first. In the former case, we played three more duets before ceasing playback, and, in both cases, we continued to record behavior until 5 min after the playback was stopped. The goal of this approach was to standardize the number of duets each responding pair was exposed to while in close proximity to the speaker. If the pair or the focal female failed to approach within 10 m of the mount after 5 min, the trial was terminated, we searched the territory for the pair and started a new trial close to their current location. We recorded data for two female responses where the male did not approach within 10 m. The behavior of territory-holding males and females (pairs) was quantified separately by one observer ~20 m away from the mounts. For each, we quantified the rate of the following behaviors: duets, solo songs, flybys (where an individual flew within 1 m of the mount), and the proportion of time within 5 m of the mount. Additionally, we recorded latency to first vocalization (duet or solo song) and to approach within 5 m of mounts.

We repeated STI trials a maximum of three times for each pair included in the female implant experiment: 1) prior to implanting;

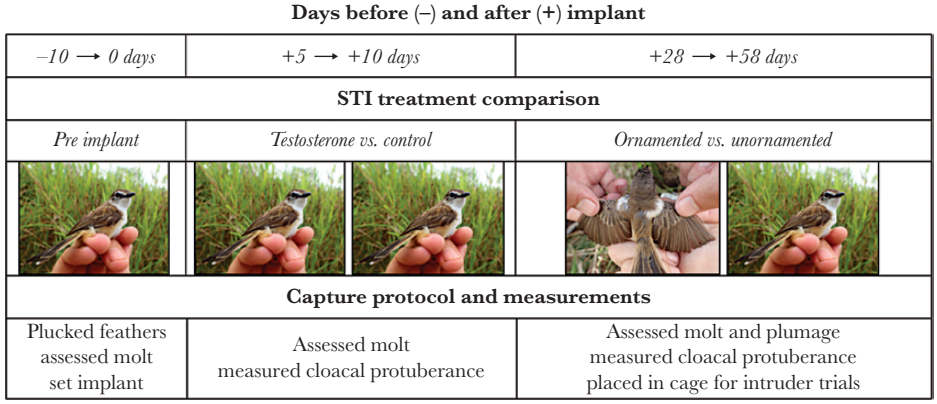

Figure 2
Experimental timeline for STI and morphological sampling. Photographs for phenotype simply reflect general physical appearance for each treatment type rather than unique photographs from each treatment type during that sampling period. Females only differed in plumage during the final sampling period (28–58 days postimplant).

2) 5–10 days after implanting with testosterone or control; and 3) 28+ days after implanting (Figure 2). At time point 2, females implanted with testosterone were expected to have levels elevated well beyond their baseline (time point 1) but have yet to develop plumage ornamentation; at time point 3, testosterone from the implant should have been exhausted (Schwabl H, Goymann W, unpublished data) and ornamental feathers have fully emerged from their sheaths and readily visible. This approach allowed us to test how both testosterone and ornament presence influenced territorial aggression independently.

Caged female intrusions

We designed a second behavioral assay to assess how the presence/absence of ornamentation influences the territorial defense behaviors of conspecifics. Following the general methods of Karubian et al. (2008), we used live females belonging to either the experimentally induced ornamented phenotype or natural unornamented phenotype as stimuli and assessed the response of free-living pairs. We built a 63.5-cm^3 cage using a wood frame and wire mesh with two small bamboo sticks for perching within the cage (Supplementary Figure S3). At the bottom of the cage was a cloth bird bag that opened into the cage so we could release the female, and the bag was cinched closed during the trial to ensure that the caged individual could not escape. The cage was placed toward the center of a known white-shouldered fairywren territory using two large sticks that held the cage ~1.5 m above the ground (Supplementary Figure S3). We chose a microhabitat that would allow for the cage to sit near the top of the grass in order to mimic where this species is often found in its habitat and also to allow for the bird to be easily visible to conspecifics. In order to minimize the time females spent in cages, we did not include any acclimation time before proceeding with behavioral trials. These trials were conducted 43–56 days after setting testosterone or control implants.

We used an amended version of our STI protocol for cage trials. As per the STIs, we played duet songs to lure the territory-holding pair to the area but, once they were within 5 m, we immediately ceased playback and then recorded their response for 10 min. During preliminary trials, we determined that pairs apparently noticed the caged individual once they were within 5 m of the cage and then directed their territory defense at the caged individual. Because we were exclusively interested in assessing response to the caged female's state of ornamentation, we stopped the vocal stimulus of playback once pairs were close enough to perceive their intruder. If pairs failed to approach the cage within 5 m after 5 min of playback, the trial was terminated, and we moved to a neighboring territory. We quantified the same behavior rates as for the STIs but analyzed time within 0.5 m of the cage instead of 5 m and only assessed the response after playback had ceased. We did not observe any males displaying to the caged females, and both members of the pair usually responded to the intruding female with changes in behavior once within 5 m. Pairs containing a testosterone-implanted female who produced any level of ornamented feathers were excluded from selection. Pairs who failed to approach within 5 m of the cage were excluded (n = 3), resulting in 12 total trials.

A camera was used to record the behavior and activity of the caged stimulus female to determine if her behavior might affect pair response in addition to her plumage phenotype. We quantified caged female activity on a four-point scale: a score of 0 indicated no movement at all, 1 corresponded to movement while the territorial pair was absent, still, when they were present, 2 denoted movement during most of the trial, and 3 represented a constant movement for the duration of the trial. We focused exclusively on the level of movement as females varied substantially in how much they explored the cage, and none exhibited singing or directional movements toward the responding pair. We did not observe any abnormal behavior of the caged female; each individual spent the trial searching for an exit from the cage.

Statistical analysis

CP volume

We analyzed CP volume from repeated measures taken from females belonging to the testosterone-treatment group (untreated and control females did not produce a CP). The purpose of these analyses was to assess whether implants were physiologically effective and whether there was any evidence for differential testosterone effectiveness among females producing ornaments versus those that did not. We used t-tests in R version 3.5.1 (R Core Team 2018) to compare CP volume between females who produced some ornamentation to those that did not produce any ornamentation.

Response to STIs

We scaled and centered all STI response variables, then used principal components analysis (PCA) with an oblique promax

rotation to quantify male and female responses individually. Sexes were run in separate PCAs due to the possibility of female treatment affecting response variably between sexes. We used the R package *psych* (v1.8.12; Revelle 2020) for both PCAs, and PCA scores were analyzed using linear mixed models in the R package *lme4* (v1.1–21; Bates et al. 2015). Scree plots were used to determine which principal components (PCs) to analyze (eigenvalues >1.0; Supplementary Figure S2). We assessed the normality of PC scores with a Shapiro–Wilk test prior to building models. Following the detection of a significant effect of treatment on PC scores, we used post hoc Tukey comparisons to determine which groups differed. We included individual ID (for both members of pair), mount stimulus (1–4 for both sexes), and duet stimulus (1–10) as random effects in the models. Terms that were estimated as zero were dropped to improve model fit. To assess whether females adjusted their response behavior to that of their mates, we included male PC score as a covariate in each model. Additionally, paired *t*-tests were used to determine if females who would later produce ornamentation differed from testosterone-implanted females who would remain unornamented during phase 2. The purpose of these tests was to determine if females who would produce ornaments were more territorial independent of ornament expression. Pairs who were later determined to have an active nest during the trial were excluded from analyses (n = 2), as were females who lost their implant (n = 1) or had an equivocal state of ornamentation (e.g., only a few ornamented feathers; n = 2). In total, we analyzed trials from 8 testosterone-implanted females and 4 controls during the pre-implant period, 10 testosterone-implanted and 5 controls 7–11 days postimplant, and 4 partially ornamented and 4 fully unornamented females 28+ days after implanting with testosterone.

Response to caged female trials

Caged female trials were analyzed similarly to STIs. First, we scaled and centered response variables, then ran each sex individually in a PCA with a promax rotation. We used scree plots to select which components to analyze (Supplementary Figure S4). We then analyzed PCA scores using a linear mixed model with individual ID and caged female activity scores as random effects. We analyzed trials from four females with prominent shoulder patches and eight lacking any ornamentation.

Ethical statement

Our testosterone implant procedure, blood sampling regimen, and STI and caged female intrusion protocols were all approved by the Institutional Animal Care and Use Committee (IACUC protocol #0395 and ASAF#04573).

RESULTS

Morphology

Plumage and molt

Fifteen of 20 testosterone-implanted *M. a. lorentzi* females produced white shoulder patch feathers, which are not naturally produced in this subspecies (Figure 1). Some of the white scapular feathers were brown at the base, while others were white throughout. Among the 15 females with white scapular feathers, 8 molted a mix of white and brown feathers in this region and, thus, did not produce a full shoulder patch. None of the implanted females produced a full complement of black feathers but four females across both study years replaced light brown feathers with darker brown plumage and a few individual black feathers (Figure 1f). None of the control-implanted females molted in white scapular feathers or any darker brown or black plumage.

Cloacal protuberances

All testosterone-implanted females developed CPs in both study years within 7–11 days of treatment (Supplementary Table S1). These CPs resembled those in males of the species in most cases; however, four individuals produced only the tip of a CP absent of the remaining dimensions measured for calculating volume (length, width, and height of bulbous swelling) and, thus, received a volume of 0 after measurement. Females captured 4 weeks postimplant (range 28–58 days) had greatly diminished CP volumes, consistent with circulating testosterone having returned to baseline levels (Supplementary Figure S1B). We did not observe measurable CPs in any females sampled 30+ days after implantation (n = 9 females), suggesting that testosterone from implants was, as expected from pilot work (Schwabl H, Goymann W, unpublished data), fully exhausted at this point. CP volumes of females developing ornaments did not differ from those of females that did not at 7–11 ($t_{2.91} = -0.047$, $P = 0.97$) and 28+ days postimplant ($t_{6.04} = -1.18$, $P = 0.28$). None of the control-implanted females produced a CP.

STI response

Female response

We analyzed 43 total trials for female response. The first two PCs (eigenvalues >1) cumulatively explained 58% of the behavioral variation (29% for both PC 1 and PC 2; Table 2). We interpret high scores for PC 1 as indicative of the motivation to quickly approach the mounts and sustain close proximity; high scores for PC 2 reflect quicker and greater singing (solo songs and duets) in response to the stimulus (Table 1).

Testosterone treatment did not have a significant effect on PC 1 or PC 2 prior to molt of shoulder patches (+5 to +10 days after implanting; PC 1: $F_{3,17.07} = 1.61$, $P = 0.22$; PC 2: $F_{3,20.91} = 0.426$, $P = 0.74$). Additionally, we found no difference in either PC between females who would later produce some ornamentation versus those that would not in the 5–10 day postimplant period (PC 1: $t_{3.64} = -0.74$, $P = 0.50$; PC 2: $t_{4.36} = -0.14$, $P = 0.89$). PC 1 scores did not differ among testosterone-treated females differing in plumage during the final testing phase (28+ days after implanting; $F_{2,\ 7.20} = 1.76$, $P = 0.24$). However, there was a significant effect of the presence of ornamentation on PC 2 during the final testing phase ($F_{2,10.61} = 11.05$, $P = 0.003$), when T from implants was exhausted as indicated by absent CPs. A Tukey comparison revealed that partially ornamented females scored significantly higher relative to both the preimplant period ($P < 0.001$) and compared to testosterone-implanted females lacking any ornamentation ($P = 0.014$). Male PC score was a significant factor in both the

Table 1
Variable loadings for female response to STIs

Female STI response	PC 1: close to mount	PC 2: vocalizations
Standard deviation	1.47	1.44
Proportion of variance	0.29	0.29
Latency to sing or duet	0.12	−0.97
Latency to 5 m	−0.77	−0.03
Song and duet rate	0.15	0.72
Time within 5 m	0.93	−0.06
Flyby rate	0.02	0.05

testosterone to control (PC 1: $F_{1,\ 16.21} = 21.38$, $P < 0.001$; PC 2: $F_{1,\ 21.82} = 35.31$, $P < 0.001$) and partial ornamentation to no ornamentation comparisons (PC 1: $F_{1,\ 11.76} = 36.19$, $P < 0.001$; PC 2: $F_{1,8.92} = 5.91$, $P = 0.038$).

Male response

We analyzed 44 total trials for male response. Behavior variables loaded differently for males (Supplementary Table S2) compared to females (Table 1), with PC 1 explaining 48% of the variation and indicating a quick and sustained close approach to the mount together with a rapid and persistent vocal response. The eigenvalue for PC 2 was substantially lower than the value for PC 1 (1.12 and 2.40, respectively) and the main variable that loaded on that component, flyby rate, was a behavior that was not typically exhibited by males during these trials, so we excluded PC 2 from the analysis. Males did not differ in their response according to their female's initial hormone treatment ($F_{3,22.40} = 1.01$, $P = 0.41$) or later state of ornamentation ($F_{2,10.62} = 1.92$, $P = 0.63$).

Territorial response of resident pair to ornamented versus unornamented female intruder

PC loadings for responses of resident males and females were similar, with PC 1 explaining 59% of the variation in response for females and 71% of the variation for males (Table 2). Male and female PC 1 scores were positively correlated (Pearson's $r_{10} = 0.83$, $P = 0.0009$). We interpret higher PC 1 scores as greater overall aggression (faster and longer approach, faster to sing, and greater song rate) toward the caged female intruder. We did not find any effect of the female intruder's state of ornamentation (ornamented vs. unornamented; Figure 4) on the response of the resident territorial female ($F_{1,9.54} = 0.62$, $P = 0.45$) or male ($F_{1,6.99} = 0.0034$, $P = 0.96$). Furthermore, caged female intruder behavior (activity score) did not influence male or female territorial response ($F_{1,2.26} = 2.59$, $P = 0.14$) and did not differ according to the caged female's plumage ($t_{7.72} = 1.11$, $P = 0.30$)

DISCUSSION

We assessed whether elevated circulating testosterone stimulates ornamentation and the associated behavioral phenotype in female white-shouldered fairywrens (*Malurus alboscapulatus*), a species with discrete female plumage phenotypes that differ in ornamentation and territorial defense behavior across subspecies (Enbody et al. 2018, 2019). We show experimentally that exogenous testosterone caused unornamented females to produce some plumage ornamentation (predominantly shoulder patches; Figure 1e,f) and find that females with shoulder patches enhance territorial defense behavior (Figure 3). Overall, our results support our initial prediction that testosterone facilitates ornamentation and increased territoriality previously observed in the naturally ornamented subspecies (*M. a. moretoni*; Enbody et al. 2018). However, it appears that adult testosterone activates only one component of ornamentation (shoulder patches) and that expression of this putative signal, in turn, may act to enhance some components of territorial behavior (singing) independently of the hormone. Furthermore, we found no evidence that the acquisition of this putative signal comes with a social cost in the form of greater territorial aggression received by the signal bearer. Additionally, the mates of experimental females did not appear to modulate their response to a simulated intruder according to the female's initial testosterone treatment or resulting plumage phenotype.

This study is among the first to experimentally assess how testosterone mediates the production of elaborate female plumage within a species with naturally occurring alternate female phenotypes. The activational effects of testosterone on female plumage were mostly limited to the acquisition of white components (shoulder patches) of ornamental plumage (Figure 1e) and, in rare

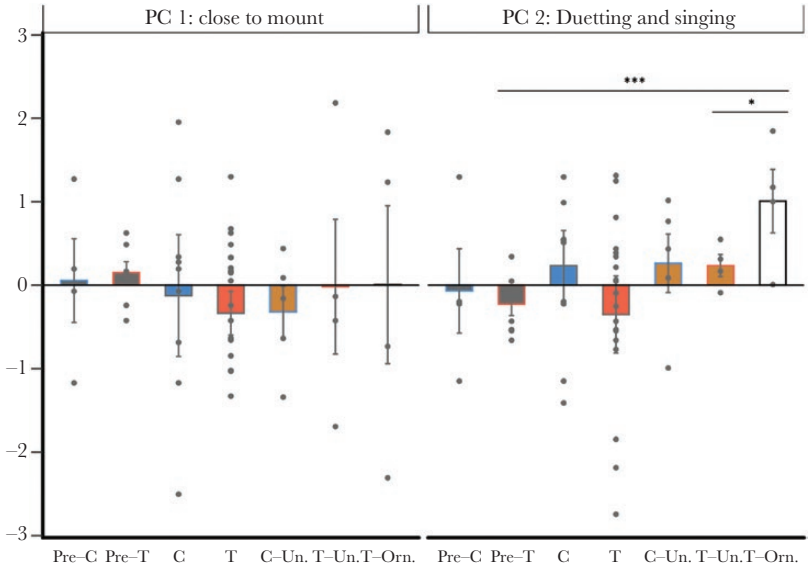

Figure 3

Female response to STIs by implant treatment, plumage phenotype, and progress of treatment. Testosterone treatment itself (7–11 days posttreatment) had no effect on either PC 1 or 2. Females who later (28–58 days posttreatment) had produced some ornamentation (T-ornamented) had significantly greater PC 2 scores (vocal territoriality) compared to females within the testosterone-treated group who failed to produce ornamentation (T-unornamented) and relative to the pretreatment period. Bars denote means with plus/minus standard error lines overlaid; significant differences between treatment groups are indicated with asterisks (***$P < 0.001$, *$P < 0.05$). Individual points represent scores from each trial by treatment and plumage category.

cases, darker brown feathers and a few black feathers in areas that are typically light brown (Figure 1f). Our findings are consistent with results from a sister species in which females lack natural female ornamentation, the red-backed fairywren (*M. melanocephalus*), where testosterone-implanted females generated male-like carotenoid-based red feathers but generally did not produce male-like melanic black feathers (Lindsay et al. 2016). Moreover, brown male red-backed fairywrens with elevated circulating testosterone (in response to an experimental shift in breeding status) responded to feather plucking by developing red feathers but not black feathers (Karubian et al. 2011). Our study differs in that white feathers are structurally produced, unlike the male-like carotenoid pigment-based red plumage induced in unornamented female red-backed fairywrens, indicating that testosterone facilitates ornament production via a separate pathway in white-shouldered fairywrens. In another member of the family Maluridae, superb fairywren (*Malurus cyaneus)*, testosterone-treated females underwent a male-like nuptial molt and feathers resembled males morphologically; however, females did not produce any of the elaborate coloration of the male ornamental plumage (Peters 2007). In two non-Malurid bird species, exogenous testosterone led to enhanced bare part coloration in females: one with naturally occurring female ornamentation (Eens et al. 2000) and one without (Lahaye et al. 2014). Finally, in reptiles, drab females typically respond to exogenous testosterone by producing male-typical skin ornamentation matching and, in some cases, exceeding male skin coloration (reviewed in Cox et al. 2015). These studies and ours highlight that females often possess the mechanisms to respond to high circulating levels of testosterone but naturally do not express male-like ornamentation in part due to low circulating testosterone.

We found that elevated testosterone, before the production and expression of ornamentation, did not enhance territorial defense behavior (close approach and singing) during STIs relative to controls (Figure 3). Exogenous testosterone increased the aggressive behavior of females in many species studied to date (e.g., Zysling et al. 2006; Sandell 2007; reviewed in Rosvall 2013). However, in some species, testosterone treatment did not enhance female aggressive behavior (DeVries et al. 2015), although it induced other androgen-regulated behavior, such as singing in female European robins (*Erithacus rubecula*; Kriner and Schwabl 1991). Our results are consistent with an indirect relationship between circulating testosterone and territorial behavior in white-shouldered fairywrens, where testosterone promotes the production of a morphological phenotype associated with enhanced territory defense, but defense behavior is not elevated in the absence of that phenotype.

We found that females that had developed partial ornamentation in response to testosterone treatment showed greater singing and duetting during territory defense compared to females that did not produce ornamentation (Figure 3). Previously, a positive correlation between female ornamentation and territorial aggression has been described in Gouldian finches (*Erythura gouldiae*; Pryke 2007), European pied flycatchers (*Ficedula hypoleuca*; Morales et al. 2014), and lovely fairywrens (*Malurus amabilis*; Leitão et al. 2019). Our results showing that females with testosterone-induced partial ornamentation exhibit a greater response during STIs (PC2: territorial singing and duetting) are consistent with previous studies of the effects of ornamentation on territorial behavior in male birds. Experimental enhancements of ornaments often result in males achieving greater ranking within the social hierarchy via increased dominance in contests (reviewed in Vitousek et al. 2014). Our finding that females who acquired some ornamentation (shoulder patches) exhibited greater territoriality highlights the potential for shoulder patches acting as a status signal in intraspecific competition. A future study that manipulates shoulder patches independently of other treatments could confirm the relationship between this putative signal and territoriality.

We believe that territorial behavior is associated with plumage but not testosterone for these reasons: 1) testosterone-implanted females lacking ornamentation did not differ in territory defense from controls when testosterone was elevated (Figure 3), 2) testosterone-treated females that would remain unornamented did not differ behaviorally from testosterone-treated females that would later produce ornamentation when assessed prior to ornament production, 3) testosterone-treated females displaying partial ornamentation differed in territorial behavior from other testosterone-implanted females lacking ornaments, and 4) size of androgen-dependent CPs did not differ among partially ornamented and unornamented females receiving the same testosterone treatment (Supplementary Table S1B and Supplementary Figure S1B), indicating that testosterone levels were elevated both in females who did and did not produce ornamentation. Furthermore, CPs were diminished by the time we were assessing the effect of plumage on territorial behavior, indicating that testosterone was exhausted at this point, as expected. However, we do note that it is possible that variation in metabolism of the testosterone from implants or in tissue sensitivity produced the pronounced variation we observed in the production

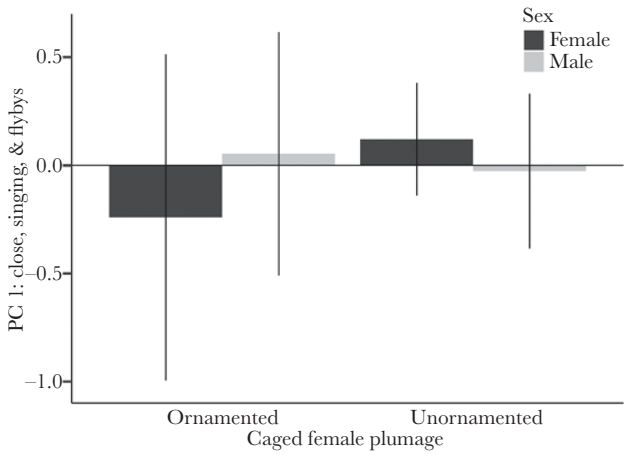

Figure 4
Response of resident females and males (pairs) to ornamented (prominent shoulder patches present) and unornamented caged female intruder. Pairs did not respond differently to female intruders showing some or no ornamentation. Bars denote means with plus/minus standard error lines overlaid.

Table 2
Variable loadings for females and males for caged female intrusions

	Females PC 1	Males PC 1
Standard deviation	2.93	3.54
Proportion of variance	0.59	0.71
Latency to sing or duet	−0.88	−0.72
Latency to 0.5 m	−0.92	−0.83
Song and duet rate	0.77	0.92
Time within 0.5 m	0.78	0.90
Flyby rate	0.30	0.30

of ornamental plumage and territorial behavior; this represents an additional opportunity for future research.

We did not find any evidence for altered behavior of conspecifics or for a social cost in the context of territorial intrusions to females who acquired shoulder patches. Previous studies in females have found mixed support for social costs to female ornament expression. In lovely fairywrens, artificial enhancement of female plumage led to increased aggression of the signal bearer toward its own mirror image, indicating a social cost to dishonest signaling (Leitão et al. 2019). Conversely, in European pied flycatchers, female decoys with a plumage signal on their head (white forehead patch) received fewer attacks than decoys lacking the signal, indicating no social costs to expression, and suggesting that the plumage patch signals fighting ability as it does in males (Morales et al. 2014). Our finding that females who acquired shoulder patches did not elicit a different territorial response from conspecific pairs is also interesting considering that altered conspecific response to manipulated signals is the proposed mechanism underlying observed transitions in the behavior of the signal bearer (reviewed in Vitousek et al. 2014; Webster et al. 2018). It is important to note that our trials with caged females did not mimic a natural situation in that females of this species have not been observed invading neighboring territories on their own. Additionally, caged females may have been perceived as on a foray, as females of other fairywren species move into other territories to seek extraterritorial copulations by Double and Cockburn (2000); we did not, however, observe any sexual displays by territory-holding males in response to these females, and the pair responding together is consistent with territory defense (e.g., duetting and close approaches) rather than sexual functions of the response. We did not detect an effect of caged female overall activity level on the response of the territorial pair, though we may have failed to quantify subtle but meaningful behavior of the caged female that affected the response.

Lastly, the acquisition of shoulder patches by females did not affect the territorial response of mates. Male responses to STIs did not differ according to either their mate's initial treatment (testosterone vs. control implant) or resulting plumage phenotype (ornamented vs. unornamented; Supplementary Figure S5). Therefore, we do not find evidence for the greater vocal territoriality in partially ornamented females being the product of altered territorial behavior by their mates or via compensating for lower territorial aggression by their mates.

CONCLUSIONS

We find support for testosterone facilitating the acquisition of a major component of a female ornament in a species with discrete variation in female ornamentation. Acquisition of ornamentation was followed by enhanced territoriality, though only vocal components of territory defense. In contrast to expectations, testosterone did not enhance territoriality independently of ornamentation, as greater responses to STI occurred only after ornamentation was developed and not before (Figure 3). Our results are inconsistent with testosterone being an overall integrator of the ornamented female phenotype in white-shouldered fairywrens. Rather, elevated testosterone appears to initiate a sequence of processes starting with the production of ornamental plumage signals, and it is these plumage signals rather than elevated testosterone per se, that are associated with enhanced territorial behavior. Our findings contribute to a growing body of research on the function of competitive female traits (Cain and Ketterson 2012; Tobias et al. 2012; Karubian 2013; Garamszegi 2014; Goymann and Wingfield 2014; Moreno et al. 2014; Cantarero and Cantarero 2015; Enbody et al. 2018; Leitão et al. 2019) and highlight the need for further experimental work testing the extent to which sexes share proximate mechanisms of phenotype expression.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Data availability: Analyses reported in this article can be reproduced using the data provided by Boersma et al. (2020).

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