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Male White-shouldered Fairywrens (*Malurus alboscapulatus*) elevate androgens greater when courting females than during territorial challenges

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ABSTRACT

Androgens like testosterone mediate suites of physical and behavioral traits across vertebrates, and circulation varies considerably across and within taxa. However, an understanding of the causal factors of variation in circulating testosterone has proven difficult despite decades of research. According to the challenge hypothesis, agonistic interactions between males immediately prior to the breeding season produce the highest levels of testosterone measured during this period. While many studies have provided support for this hypothesis, most species do not respond to male-male competition by elevating testosterone. As a result, a recent revision of the hypothesis ('challenge hypothesis 2.0') places male-female interactions as the primary cause of rapid elevations in testosterone circulation in male vertebrates. Here, we offer a test of both iterations of the challenge hypothesis in a tropical bird species. We first illustrate that male White-shouldered Fairywrens (*Malurus alboscapulatus*) differ by subspecies in plasma androgen concentrations. Then we use a social network approach to find that males of the subspecies with higher androgens are characterized by greater social interaction scores, including more time aggregating to perform sexual displays. Next, we use a controlled experiment to test whether males respond to simulated territorial intrusion and/or courtship competition contexts by elevating androgens. We found that males elevated androgens during territorial intrusions relative to flushed controls, however, males sampled during courtship competitions had greater plasma androgens both relative to controls and males sampled while defending territories. Ultimately, our results are consistent with challenge hypothesis 2.0, as sexual interactions with extra-pair females were associated with greater elevation of androgens than territorial disputes.

1. Introduction

Hormones serve a fundamental role in translating genotype to phenotype, and thus influence a population's response to selection (Cox et al., 2016). Androgens like testosterone often mediate expression of alternate male phenotypes (Ketterson and Nolan, 1999; Lindsay et al., 2011; McGlothlin and Ketterson, 2008; Williams, 2012). Specifically, testosterone is responsible for many of the most conspicuous physical and behavioral traits in nature, from ornaments and armaments (Johns, 1964; Karubian et al., 2011; Peters et al., 2000; van Oordt and Junge, 1934) to territorial aggression (reviewed in Soma, 2006) and sexual display behaviors (Day et al., 2006, 2007; Fusani, 2008; Fusani et al., 2007). For these reasons, testosterone has been referred to as a phenotypic integrator that regulates a network of traits (reviewed in Lipshutz et al., 2019). While consequences of elevated testosterone circulation are reasonably well understood thanks to testosterone-implant studies (Boersma et al., 2020; Gerlach and Ketterson, 2013; Lindsay et al., 2011; McGlothlin et al., 2004; Peters, 2007; Peters et al., 2000; Peterson et al., 2013; Veiga et al., 2004; Zysling et al., 2006), the causes of elevated endogenous levels in natural systems are more difficult to disentangle.

The social environment is a major source of variation in testosterone circulation across male vertebrates (reviewed in Oliveira, 2004;

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Goymann, 2009) and seasonal changes in the nature and intensity of social interactions are often attributed to temporal patterns, such as seasonal variation, of testosterone circulation across vertebrates (see Goymann et al., 2007; Goymann, 2009). Wingfield et al. (1990) originally proposed the challenge hypothesis to explain the seasonal elevation of testosterone levels from a low, non-breeding baseline to the highest levels during periods of social instability at the onset of breeding. In this first iteration of the challenge hypothesis, elevated testosterone was attributed to the aggressive male-male interactions that occur during this period as males compete for breeding territories (Goymann, 2009; Wingfield et al., 1990). Whereas there is abundant empirical support for testosterone mediating aggression in male vertebrates (reviewed in Soma, 2006), most species do not respond to territorial challenges by increasing circulating testosterone (Goymann et al., 2007). In contrast, interactions with fertile females are often associated with elevated testosterone in males, which provides the foundation for the revised 'challenge hypothesis 2.0' (Goymann et al., 2019). According to this hypothesis, interactions with receptive females are more important than intrasexual aggressive interactions for seasonal elevation of circulating testosterone in males. Empirical tests of both iterations of the challenge hypothesis within a single taxon are lacking and can help resolve the social contexts underlying rapid elevations of testosterone in males.

Moreover, our understanding of the causal factors underlying variation in testosterone is currently limited by a historical bias toward temperate systems. Tropical species tend to exhibit lower peak testosterone circulation than temperate counterparts (reviewed in Goymann et al., 2004), though there is considerable variation in testosterone in tropical birds (Moore et al., 2019). Among tropical birds, greater peak testosterone is associated with higher altitudes, seasonal territory defense and breeding, as well as monogamous mating systems (Goymann et al., 2004; Hau et al., 2008a, b). How social challenges affect circulating testosterone in tropical species is poorly understood and resolving this is necessary to improve the generalizability of hypotheses about causes of male testosterone variation across taxa.

Here we use a combination of long-term correlative studies and a controlled experiment to test both the territorial defense focused and female courtship focused iterations of the challenge hypothesis in males of a tropical species. White-shouldered Fairywrens (*Malurus alboscapulatus*) are split into six subspecies on the basis of female ornamentation, including *Malurus alboscapulatus moretoni* (hereafter: *moretoni*) which has ornamented females resembling males and *Malurus alboscapulatus lorentzi* (hereafter: *lorentzi*) which has unornamented females (Enbody et al., 2019a, b). Unlike females, males of these subspecies are similarly ornamented across their range with no discernable variation in plumage coloration (Enbody et al., 2019a, b). Previous studies have demonstrated that plasma androgens are higher in *moretoni* females than *lorentzi* females and that both sexes of *moretoni* are more territorial while jointly defending their territories during simulated territorial intrusions (STIs) than *lorentzi* pairs (Enbody et al., 2018). Mean androgen concentrations in males were previously found to be greater in males than females in both subspecies, with greater variation, but not mean levels in male *lorentzi* (Enbody et al., 2018). Both subspecies of White-shouldered Fairywrens defend territories and breed year-round, and are socially monogamous with biparental care of offspring, but differ in density and group size, suggesting differences in sociality (Enbody et al., 2019a, b). Here we expand on initial comparative work to compare males of the *moretoni* and *lorentzi* subspecies in circulating androgens and several metrics of social interaction. Then we use an experiment to test which social interaction contexts, specifically agonistic territorial intrusions or courtship opportunities, cause elevated androgens in males.

2. Methods

2.1. Study system

White-shouldered Fairywrens (Maluridae) are savanna and second-growth specialists endemic to New Guinea (Schodde, 1982). White-shouldered Fairywrens defend territories and breed year-round, with females building nests and incubating eggs and males assisting with offspring provisioning (Enbody et al., 2019a, b). We studied three populations of the *moretoni* subspecies, where females are ornamented like males (monochromatic plumage), and two populations of the *lorentzi* subspecies, where females are unornamented (dichromatic plumage). Our work with *moretoni* was conducted around Garuahi (150°29′ E, 10°13′ S, 0–10 m a.s.l.), Podagha (149°90′ E, 9°69′ S, 50–60 m) and Porotona, Milne Bay Province (150° 35′ E, 10°15′ S, 10–20 m a.s.l.). We conducted work with *lorentzi* near Obo village, Western Province: one population was along the western edge of the Fly River drainage (141°19′ E, 7°35′ S, 10–20 m a.s.l.), and the other was on a chain of islands in the middle of the Fly River (141°16′ E, 7°34′ S, 10–20 m a.s.l.). We used mistnets to catch Fairywrens and used a unique Australian Bird and Bat Banding Scheme metal band in addition to a unique combination of three plastic color bands to differentiate individuals for behavioral observations. All individuals employed for this study were released on their territories without injury. Capture and behavioral work were approved by the Institutional Animal Care and Use Committee (IACUC) at Washington State University (protocol #: ASAF-04573).

2.2. Long-term androgen and sociality study

2.2.1. Androgen sampling

We regularly caught male White-shouldered Fairywrens and took blood samples from the jugular vein within 10 min of capture to measure plasma androgen concentrations. At the end of each field day, we centrifuged blood samples to separate plasma from red blood cells and used a Hamilton™ syringe to transfer plasma into 0.4 ml of 200 proof Ethanol (Fisher Bioreagants™) in individual Eppendorf™ tubes. Samples were stored at room temperature in the field, then transferred to a refrigerator in our lab at Washington State University at the end of the field season. Long-term work spanned field seasons from 2014 to 2019 across most months of the year and between the hours of 5 am-noon and 3 pm to 7 pm when Fairywren activity was highest. Individuals that were exposed to playback prior to capture or any other experimental work were excluded from analysis. In addition, we excluded any males who were lacking cloacal protuberances (indicative of a non-breeding state) from our dataset so that we could compare males in breeding condition across subspecies. A small proportion of males remain on natal territories as auxiliary helpers while the remaining males maintain cloacal protuberances throughout the year (Enbody et al., 2018). We searched for nests opportunistically during sampling periods and include a binary variable (active nest versus nesting status unknown) in analyses due to the opportunistic nature of breeding in this system.

2.2.2. Social network observations

We conducted social network observations by following a focal group for a minimum of 10 and maximum of 25 min during field seasons in 2016 and 2017. Observations began when we first located the focal group, and focal groups were selected each day based on the duration of time since the last observation. Focal groups were generally mated pairs, and in some cases included adult auxiliaries or fledged young. The number of stable groups varied across field seasons and populations between 10 and 30 groups. Once we found the focal group, we recorded spatial location data (via GPS) and the color bands for each individual in the group, repeating every 5 min. Individuals within 5 m of each other were counted as associating, as we have noted in a previous experiment that conspecifics respond to a caged intruder at this proximity (Boersma et al., 2020). We took note of whether males aggregated and performed

sexual displays to females. We identified three distinct types of male sexual displays in this species: 'petal-carrying' where a male carries an orange or red flower petal, leaf, or berry; 'puff-shoulder' where males erect shoulder patches and hop between perches near a female; and 'display flights' where males erect shoulder patches and back feathers and perform a bobbing flight away from the target female's perch. If these behaviors were observed among multiple males with a female present, each male in the group was counted as attending a display flock. These display aggregations resemble a lek, though there is no designated display area as in traditional lek breeding systems (Boersma, pers. obs.).

2.3. Challenge hypothesis 1.0 and 2.0 experiment

2.3.1. Experimental design

We tested challenge hypothesis 1.0 by randomly assigning *moretoni* and *lorentzi* males to be sampled for androgens following capture by flushing into a mistnet absent a social stimulus or while actively defending territories during a simulated territorial intrusion (STI). Challenge hypothesis 2.0 was assessed separately in *lorentzi* males by comparing androgens sampled during a separate courtship competition stimulus relative to flushed controls and those sampled during STI. All males included in this experiment had a blood sample taken within 10 min of capture (mean = 4.62 min, range = 2–10 min). In total we captured 17 *moretoni* males and 8 *lorentzi* males by flushing, 14 *moretoni* males and 13 *lorentzi* males following an STI, and 14 *lorentzi* males during courtship competition. Full details on each sampling context are reported below.

2.3.2. Flush capture

Flush captures were used to assess baseline (presumably not influenced by our activities) androgen levels. During flush captures no playback was used and we avoided rapid movements toward Fairywrens that may elicit a stress response. We ensured that no conspecifics were on a focal pair's territory prior to capture; any instances of intrusions by conspecifics led to samples being excluded from analysis.

2.3.3. Simulated territorial intrusion (STI) capture

We used an established simulated territorial intrusion protocol for our species (for full details see Enbody et al., 2018) to sample androgens during an agonistic challenge. Briefly, we randomly selected a cardstock White-shouldered Fairywren mount pair ($n = 4$ exemplars of each sex) that matched the local phenotypes and played recorded duets ($n = 8$ exemplars) from the local subspecies via a Bluetooth speaker (Ultimate Ears Roll 2). Duet playbacks were previously recorded using a protocol outlined in Enbody et al. (2018). We used duets and mount pairs rather than a solo male song and mount as this is a better simulation of territorial disputes in this system (Boersma et al., personal observation). In addition, previous studies in this system have found that pairs jointly defend territories and duets are a key behavior elicited in response to an intruder and when marking territory boundaries (Boersma et al., 2020; Enbody et al., 2018). After 10 min of playback we opened a furled mistnet that was placed approx. 5 m from the mounts to capture males. We recorded the time between playback start and capture to assess the effect of playback time on androgen levels (mean playback time: 20.9 min.; range: 11–77 min.).

2.3.4. Courtship competition capture

A female mount (N = 4 separate mounts randomly assigned) painted with the local phenotype (i.e. unornamented female) was placed on a known territory and a randomly selected female solo song recorded from the *lorentzi* subspecies (N = 4 individual recordings) played by a Bluetooth speaker (Ultimate Ears Roll 2) was used to lure males from neighboring territories into forming a display flock (described in 'Social network observations' section). We only sampled *lorentzi* males in the courtship competition context due to our inability to induce display flocks absent territory defense in *moretoni* using female-only playback (Jones et al., in review). Mistnets were setup near the mount, and we confirmed that males were either actively displaying or were bringing food (generally small invertebrates) to either the mount or the territorial female immediately prior to capture. Display flock size varied from 2 to 5 males and we caught between 1 and 3 males at each trapping location. We did not observe any overt aggression between members of the flock, which is typical when males court extra-pair females (Boersma, personal observation). Territory-holding males were excluded from analysis to eliminate the possibility of measuring androgens during mate-guarding rather than courtship. We were unable to measure time between initiating playback and capturing males due to our focus on confirming that males were actively displaying and off their territories while simultaneously keeping track of net-to-bleed time. However, playback time generally ranged between 5 and 10 min prior to capture of courting males. Territory-holding females were not observed responding to the mount or soliciting males; however, we cannot preclude the possibility that variation in female behavior during these trials produced variation in male response to these trials.

2.4. Androgen radioimmunoassay methods

Prior to assay we sorted samples randomly across assays to account for inter-assay variation. We used an established radioimmunoassay protocol that measures total androgen titres instead of testosterone specifically (full details in Boersma et al., 2021; Lindsay et al., 2011) because the antibody we use (Wien Laboratories T-3003, Flanders, NJ, USA) cross-reacts strongly with 5α-dihydrotestosterone (DHT). Therefore we refer to our measurement as androgen titres. Long-term androgen dataset samples were split among 15 separate assays with an inter-assay coefficient of variation of 17.29% (calculated following methods in Chard, 1995). The experimental sampling context samples were split among 5 separate assays with an inter-assay of 13.57%. Plasma volumes ranged from 19.94–66.23 μl (mean = 39.51 μl) in our long-term sample pool, and 12.14–28.37 μl (mean = 22.43 μl) in our experimental sample pool. The minimum detectable androgen concentration calculated based on a 12 μl (lowest) plasma volume and mean recovery rate of 65.77% across assays was 308.86 pg/ml. We used our assay's minimal detectable level of 1.95 pg/tube to assign a total androgen concentration among undetectable samples (N = 23 in long-term dataset, N = 7 in experimental dataset).

2.5. Statistical methods

All analyses were conducted in R (www.r-project.org) version 3.6.1 using linear regressions in base R and mixed models in package lme4 (Bates et al., 2015). We used backward stepwise selection following Wang et al. (2008) throughout: the full set of candidate predictors was initially included in each model and any variable with $p \geq 0.4$ was removed. Post-hoc Tukey tests were used following detection of a significant effect when there were more than two treatment levels using package multcomp (Hothorn et al., 2008). We used a permutation-based approach for both long-term androgen and social network analyses following Farine (2017). In short, we compared the observed model coefficients for the subspecies variable to the distribution of coefficients from 1000 randomized datasets where subspecies identity had been randomly reassigned. When randomizing subspecies identity, we required that individuals sampled multiple times were assigned the same subspecies identity in each of the randomized datasets.

2.5.1. Plasma androgens

To improve normality for statistical analysis, we natural log (ln) transformed circulating androgen concentrations (pg/ml plasma) and inspected residuals with normal Q-Q plots. We used a linear mixed model with ln androgens as the response variable and subspecies, year, time of day bled, net-capture-to-bleed time, and nesting status (confirmed active nest or unknown) and date as fixed effects in the

initial model. To account for repeated measures, we included individual ID as a random effect.

2.5.2. Social network analysis

We used R package asnipe (Farine, 2013) to build social networks using the simple ratio index (SRI). We used the igraph package (Csardi and Nepusz, 2006) to detect and plot social network structure and the program Gephi (Bastian et al., 2009) to depict where individuals were observed using GPS data associated with each observation. We compiled data across both sampling years to generate the social network plot, but split data for each male by sampling year in our models. There were 1005 separate observations made in *lorentzi* (681 in 2016 and 324 in 2017) and 795 observations in moretoni (465 in 2016 and 330 in 2017). Prior to running models we removed individuals from the dataset that were observed less than 5 times in a sampling year. This resulted in 47 total *lorentzi* males and 43 *moretoni* males observed across years, with 28 *lorentzi* males and 23 *moretoni* males observed in 2016 and 19 *lorentzi* males and 20 *moretoni* males observed in 2017.

To determine how subspecies differ in sociality, we calculated five social metrics for each male: (1) unweighted male degree, or the number of conspecific males an individual interacted with; (2) unweighted female degree, or the number of conspecific females an individual interacted with; (3) weighted male degree, or the number of male conspecifics interacted with weighted by the total number of interactions; (4) weighted female degree, or the number of female conspecifics interacted with weighted by the total number of interactions; and (5) the number of display flocks each individual male took part in during the sampling period (full details in "Social network observations" section). We chose to analyze both unweighted and weighted degree scores because they capture different aspects of social behavior: unweighted degree indicates how many social partners each male has, while weighted degree reflects the strength of those partnerships. Each metric was analyzed in separate linear models with subspecies, year, and sight frequency (the # of times an individual was observed) as fixed effects. Individual ID was included as a random effect to account for repeated measures.

2.5.3. Challenge hypothesis 1.0 experiment

As above, we used a natural log (ln) transformation for plasma androgens and confirmed normality of residuals. We used a linear mixed models with ln androgens as the response variable. Sampling context (flush vs. STI), subspecies, sampling context * subspecies time of day bled, net-to-bleed time, and nesting status (nesting, not nesting, or unknown based on state of female's brood patch) as fixed effects with individual ID as a random effect.

2.5.4. Challenge hypothesis 2.0 experiment

We used a linear mixed model to assess whether natural log transformed androgens differed among *lorentzi* males sampled during the courtship competition context relative to *lorentzi* males sampled while defending territories or flushed into the net absent a social stimulus. We used the same model as outlined in the above "*Challenge hypothesis 1.0 experiment*" absent subspecies and subspecies * sampling context.

3. Results

3.1. Long-term study

3.1.1. Plasma androgens

We sampled plasma androgens in 42 individual *lorentzi* and 58 *moretoni* males for a total of 68 and 92 samples, respectively. Natural log transformed androgens significantly differed according to subspecies, breeding status, and bleed time (Fig. 1; full model terms: Table S1). The *lorentzi* subspecies had higher plasma androgens than the *moretoni* subspecies ($p = 0.008$, *lorentzi* mean: 1118.75 pg/ml; *moretoni* mean: 660.19 pg/ml). Males known to have an active nest ($N = 19$) had higher androgens than males without a known nest ($p = 0.028$; active nest mean: 1320.73 pg/ml; unknown nest status mean: 792.33 pg/ml). There was a positive relationship between circulating androgens and time of day bled ($p = 0.024$). Net-to-bleed time and year had no effect ($p > 0.4$) and were removed from the model.

3.1.2. Sociality

Social networks for each sampling site (subspecies) are depicted in Fig. 2. Males differed significantly by subspecies in each social metric except female weighted degree (Fig. 3; full model terms: Table S2). Males of the *lorentzi* subspecies were characterized by higher female degree (perm $p < 0.001$; *lorentzi* mean: 2.26, *moretoni* mean: 1.02) and

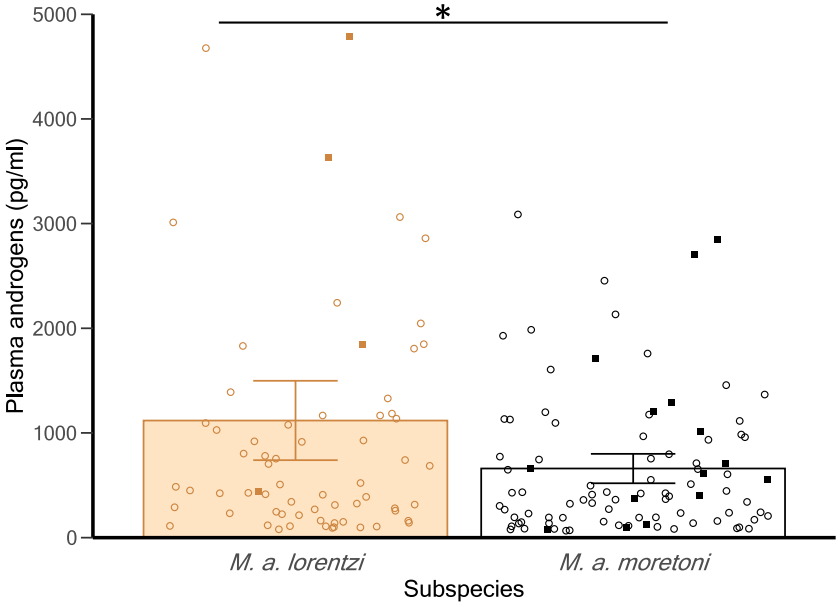

Fig. 1. Plasma androgens (pg/ml) by subspecies. Bars represent means with 95% confidence intervals overlaid, and asterisks indicate significant difference between subspecies (*$p < 0.05$). All males were in breeding condition and those known to have an active nest had higher androgens ($p = 0.03$) and are depicted with filled squares. Two potential outliers are not shown due to constraints on plot area (both *M. a. lorentzi* without known nests with 9138.28 and 6025.95 pg/ml, respectively).

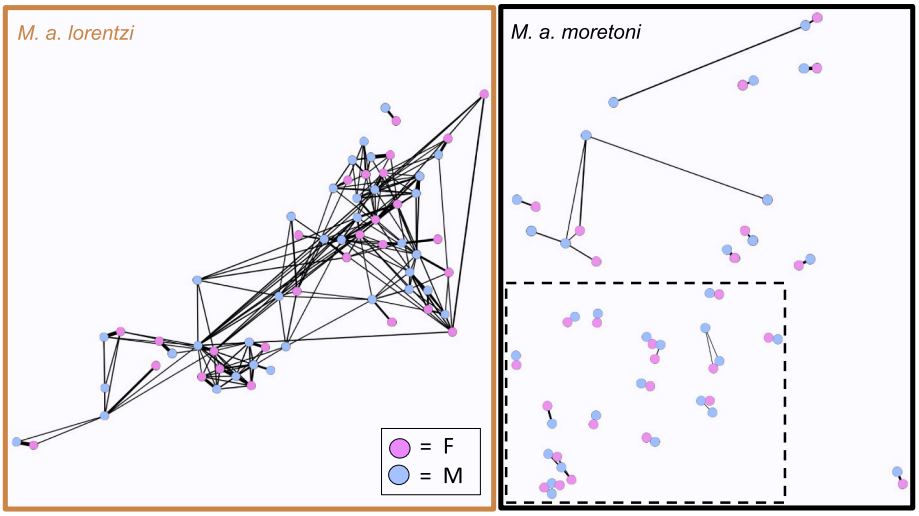

Fig. 2. Social networks by subspecies. *M. a. lorentzi* (unornamented females) are shown in left panel and *M. a. moretoni* (ornamented females) are in right panel with a dashed inset separating two populations sampled during the 2016–17 sampling period. Each node is an adult male (blue circle) or female (pink) observed 10 or more times over the study period (*lorentzi*: 26 males and 21 females; *moretoni*: 25 males and 21 females). Lines between nodes represent individuals seen within 5 m proximity.

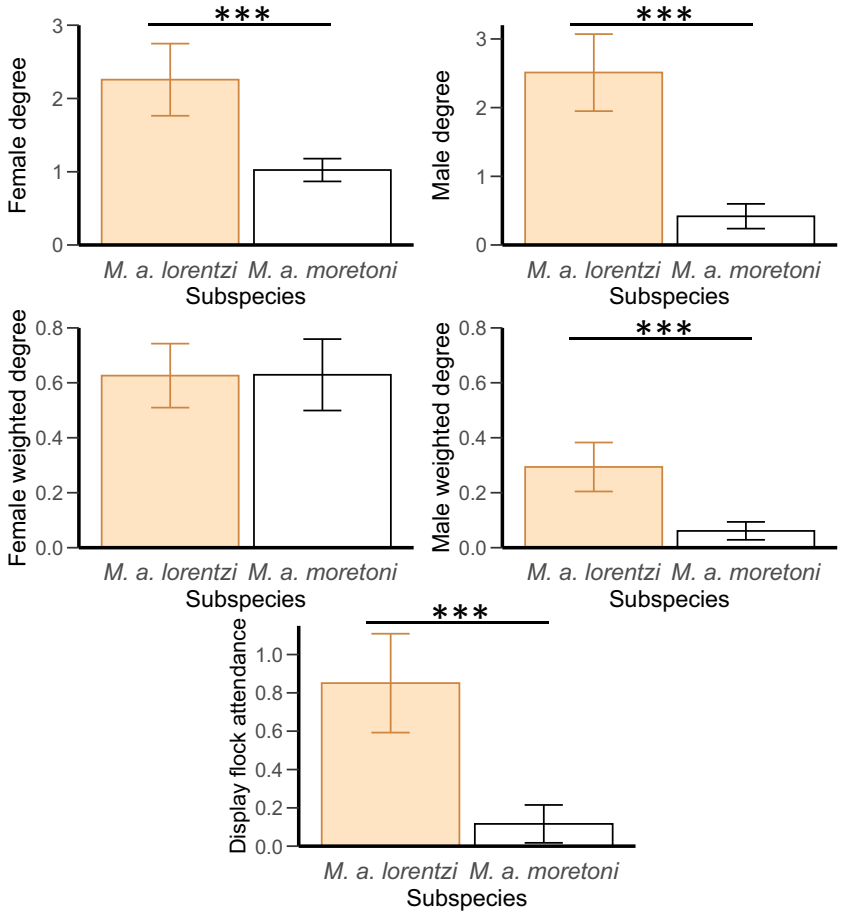

Fig. 3. Social interaction scores by subspecies. Degree scores measure how many individual females and males an individual interacted with each sample year. Weighted degree scores measure individual interactions with a female or male by frequency of interaction. Display flock attendance reflects the number of times each individual male was observed aggregating with other males displaying to females during each year. Bars represent means with confidence intervals overlaid, and asterisks indicate significant comparisons (***$p < 0.001$).

male degree scores (perm $p < 0.001$; *lorentzi* mean: 2.51, *moretoni* mean: 0.42), higher male weighted degree scores (perm $p < 0.001$; *lorentzi* mean: 0.29, *moretoni* mean: 0.06), and greater display flock attendance (perm $p < 0.001$; *lorentzi* mean: 0.85, *moretoni* mean: 0.12) than the *moretoni* subspecies, but no difference in female weighted degree (perm $p = 0.53$; *lorentzi* mean: 0.63, *moretoni* mean: 0.63). Sight frequency was not a significant predictor in any model.

3.2. Challenge hypothesis 1.0

We analyzed whether plasma androgens differed between males sampled while actively defending territories vs. those that were flushed into the net absent a social stimulus. The final model included both sampling context and subspecies, with sampling context as the only significant predictor ($p = 0.011$ for sampling context, $p = 0.272$ for subspecies); full model terms: Table S3a). Males sampled during STI had greater plasma androgens than flushed males (Fig. 3). Duration of

exposure to playback was not predictive of androgens during an STI ($p = 0.83$; Table S4).

3.3. Challenge hypothesis 2.0

We used a separate analysis in lorentzi males with the addition of the courtship competition sampling context. Sampling context was the only predictor of androgens ($p < 0.001$; full model terms: Table S3b). A post-hoc Tukey test revealed that *lorentzi* males sampled in the courtship context had higher androgen levels than *lorentzi* males who were flushed into the net (Fig. 4; $p < 0.001$) and those sampled during a simulated territorial intrusion ($p = 0.006$). Males sampled during an STI did not differ significantly from males who were flushed into the net without playback ($p = 0.102$).

4. Discussion

We used a combination of a long-term correlative study and controlled experiments to test which factors are associated with elevated circulating androgens in male White-shouldered Fairywrens of different subspecies. First, we found that males of the *lorentzi* subspecies (males ornamented, females un-ornamented) have higher mean circulating androgens and are also more social than *moretoni* males (males and females ornamented) in four out of five metrics of sociality. We then tested how different social interaction contexts (simulated territorial intrusion (STI) and courtship competition) affect androgens relative to flushed controls. We first found that males sampled while actively defending their territories from simulated intruders had higher androgens than flushed controls irrespective of subspecies. Then among *lorentzi* males we compared males sampled during STI and flushed to those sampled during an additional courtship competition context. We found that males sampled while actively courting females had significantly greater circulating androgens than flushed control males or those sampled during an STI. While we find some support for both the initial agonistic interaction focused challenge hypothesis ('1.0', Wingfield et al., 1990) our collective results are more consistent with the second iteration of the challenge hypothesis ('2.0', Goymann et al., 2019) which places sexual interactions with females as the primary drivers of elevated testosterone to the physiological maximum.

Decades of research have established a prominent role for the social environment in causing variation in male testosterone circulation. However, most of these studies have focused on gross temporal/seasonal patterns of testosterone circulation in temperate organisms. Our correlative androgen and sociality study adds to a growing body of literature illustrating a link between both social organization and finer-grained social interactions and androgens (Gleason et al., 2009; Goymann et al., 2004, 2019; Maguire et al., 2021; Oliveira, 2004; Ryder et al., 2011). Males of the *lorentzi* subspecies exhibited greater mean androgen circulation than *moretoni* males across six study years (Fig. 1). Although the previous study of male androgens in this species did not find differences by subspecies (Enbody et al., 2018), our sample size is much larger for *lorentzi* (N = 26 in previous study vs. N = 68 in current study), and here we exclude the potential confound of playback use prior to capture. Interestingly, female White-shouldered Fairywrens showed the opposite pattern by subspecies: *lorentzi* females had lower mean plasma androgens than *moretoni* females in a previous study (Enbody et al., 2018). However, this has been attributed to variation in female ornamentation, as *moretoni* females exhibit male-like ornamentation, while *lorentzi* do not, although, they do produce some ornamentation when given exogenous testosterone (Boersma et al., 2020). In the current study *lorentzi* males were found to have higher circulating androgen levels and greater social interaction scores, including interaction with more individual males and females and aggregating in display flocks (Figs. 2 and 3), which is consistent with social interactions driving subspecific male androgen levels. Due to the transient nature of androgen elevation and that our social data are aggregated across days without androgen sampling, we do not present a within-population correlation between androgens and sociality here.

We used an experiment to test which social interaction contexts affect androgen circulation. We found that males elevate androgens while engaging in territory defense relative to flushed controls, but the greatest androgen elevations were measured during courtship of extra-pair females in *lorentzi* (Fig. 4). Our study is the first direct test to our knowledge of challenge hypothesis 1.0 vs. 2.0 (Goymann et al., 2019; Wingfield et al., 1990), and our results are more consistent with the more recent iteration. Previous studies have illustrated that androgens are elevated in males during courtship, thus informing the amended, female interaction focused, 2.0 version of the challenge hypothesis

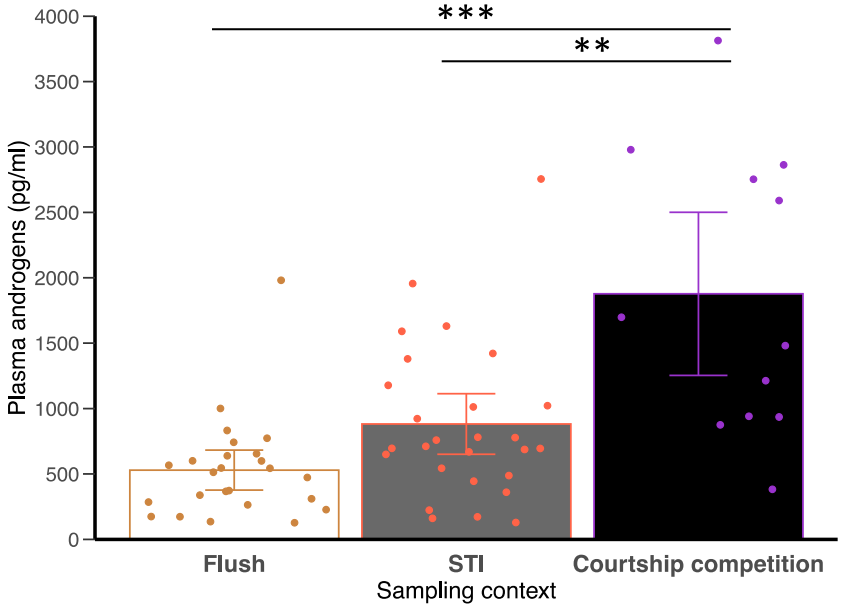

Fig. 4. Plasma androgens (pg/ml) across experimental sampling contexts. Bars represent means with confidence intervals overlaid, and asterisks indicate significant comparisons (** $p < 0.01$, *** $p < 0.001$). Flush and STI captures are pooled among male of the *moretoni* and *lorentzi* subspecies, while courtship competition captures were limited to *lorentzi*.

(Goymann et al., 2019). While we found elevated androgens in males sampled during territorial intrusions relative to flushed controls in our initial challenge hypothesis 1.0 test, the STI group did not differ from controls in our challenge hypothesis 1.0 vs. 2.0 analysis in *lorentzi* only. These nuanced results illustrate the importance of assessing multiple potential causes of androgen elevation in response to social stimulation.

Nesting status was not predictive of a male's androgen response across experimental sampling context, suggesting that the social context a male is sampled in is more important than whether he is actively providing paternal care or defending a mate. However, without finer-scale nesting stage information we cannot exclude the possibility that parental versus sexual phases of nesting contributed to variation in androgens in our dataset. It is also possible that we failed to elicit a maximal androgen response to STI because intrusions were not of long enough duration, as males of some tropical species may elevate testosterone only after 2 h (Wikelski et al., 1999). The longest intrusion test in our study lasted 77 min, with 4 males sampled after at least 30 min of playback. However, there was no relationship between playback time and androgens following an agonistic challenge in our dataset. It is important to note we measured androgens in plasma collected from the jugular vein, and thus our results might reflect the causes of variation in neural androgens specifically (Saldanha and Schlinger, 1997; Soma et al., 2008). Given that acute stress can induce season-specific effects on jugular versus brachial steroids (Newman et al., 2008), it would be useful to determine whether our experimental androgen results are generalizable to systemic circulation.

The drivers of variation in androgen levels in tropical species is poorly understood (Goymann et al., 2004; Hau et al., 2008b; Moore et al., 2019). Comparative analyses of male tropical species suggest that testosterone is typically low unless species have short breeding seasons and/or experience great social instability (Goymann et al., 2004; Hau et al., 2008b; Wikelski et al., 1999). Cooperatively breeding species, which are more common in the tropics (Feeney et al., 2013), can exhibit relatively high testosterone levels due to competition among unrelated breeders and helpers for limited mating opportunities (Khan et al., 2001; Peters et al., 2001; Poiani and Fletcher, 1994; Vleck and Brown, 1999; Wingfield et al., 1991). Likewise, tropical lek breeding species, where males court females throughout much of the year, seem to have testosterone levels comparable to temperate species (Fusani et al., 2007; Moore et al., 2019; Ryder et al., 2011), and androgens can be associated with and stimulate courtship display behavior (Chiver and Schlinger, 2017; Day et al., 2006, 2007; Wiley and Goldizen, 2003). Assuming that our measurement of androgens mostly represent testosterone, our results indicate that the testosterone levels of male White-Shouldered Fairywrens vary across a range similar to that of temperate zone passerines.

Our study species exhibits life history characteristics consistent with both high and low testosterone tropical species. They defend territories and maintain breeding readiness year-round, with long-term pair bonds and biparental care, with some level of cooperative breeding and aggregation at mobile lek display sites (Enbody et al., 2019a, b). Given the *moretoni* subspecies with ornamented females shows more aggressive territorial defense (Enbody et al., 2018), our finding that *lorentzi* males are characterized by more social interaction, including greater rates of sexual displays to females, indicate potentially stark differences in social environments across subspecies. The highest androgen values in our long-term dataset come from *lorentzi* males, and our experimental work showed that *lorentzi* males sampled while actively courting females had higher androgens than those sampled during an agonistic intrusion or absent a social stimulus. Collectively, our results indicate that courtship interactions are responsible for the highest elevations of androgens in males of this tropical species, supporting challenge hypothesis 2.0.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2022.105158.

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