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Early-moulting Red-backed Fairywren males acquire ornamented plumage in the absence of elevated androgens

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ABSTRACT

Sexual ornaments, including plumage ornamentation, are often studied during breeding periods even though signal development can take place months earlier. This temporal disconnect potentially obscures the proximate mechanisms that underlie signal expression and development. We studied the correlation between androgen levels and expression of ornamented plumage in adult Red-backed Fairywrens (*Malurus melanocephalus*) 4–6 months before breeding, when signal production occurs in some, usually older, males. We found that, during this period, ornamented males, unornamented males and females all had low plasma androgen levels that did not differ from each other. Variation in androgen levels was unrelated to phenotype or moult. These findings differ from previous research conducted immediately prior to breeding in a different population of this species, which used correlative and experimental work to demonstrate that testosterone induces prenuptial moult and acquisition of ornamented plumage in younger males. Our study demonstrates that mechanisms contributing to signal production may vary within and among populations in relation to temporal, age-dependent, or geographic parameters. These results highlight the complexity of hormonal pathways to signal production, and the importance of studying signal acquisition throughout the entire period when signals are produced, as studies conducted at different time points may have quantitatively different results.

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Introduction

Sexual ornaments are often considered indicators of male quality, among which elaborate plumage ornaments in birds have received considerable attention (reviewed in Andersson 1994). The handicap principle states that ornaments may honestly transmit information about the physiological condition or quality of the bearer (i.e. signaller) to the receiver (Zahavi 1975). High costs of signal production, as well as the social costs of having high-quality signals, are traditionally thought to maintain signal honesty (Searcy and Nowicki 2005). However, the proximate mechanisms that underlie signal honesty are contentious (Cotton *et al.* 2004), and evidence suggests that in at least some cases physiologically inexpensive signals may accurately reflect condition (e.g. Matsumasa *et al.* 2013) and/or status (e.g. Rohwer 1975).

Androgens, a family of steroid hormones (e.g. testosterone) that regulate development of male traits, may mediate signal honesty by linking sexually selected traits to male condition or immune system quality (Salvador *et al.* 1996; Duckworth *et al.* 2001; Blas *et al.* 2006; Pérez-Rodríguez *et al.* 2006). Although a number of sexual traits are largely independent of circulating androgens (e.g. Hagelin 2001; York *et al.* 2016), androgen levels have been linked to avian sexual signals including courtship (Fusani 2008), vocal signals (Galeotti *et al.* 1997), ornaments (Zuk *et al.* 1995; Eens *et al.* 2000), and plumage (reviewed in Kimball 2006). The link between androgens and plumage ornamentation is particularly well established in the order Charadriiformes: in male Ruff (*Philomachus pugnax*), testosterone is necessary for development of the ornamental ruff feathers (van Oordt and Junge 1934), and in females of sex-role-reversed phalaropes (*Lobipes lobatus* and *Phalarops tricolor*), testosterone is necessary for females to produce ornamented plumage (Johns 1964). However, other proximate mechanisms can also underlie plumage colour in birds: in addition to testosterone, luteinising hormone, oestrogen, and non-hormonal factors have been identified as mechanisms in avian species (reviewed in Kimball and Ligon 1999; Kimball 2006).

Malurus fairywrens represent a useful system to further explore the relationship between androgens

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and ornamented plumage. In the majority of fairywren species, males undergo two moults per year in which they alternate between brown female-like unornamented non-breeding plumage and colourful ornamented breeding plumage. The timing of the moult into ornamented breeding plumage in male fairywrens is extremely variable (Mulder and Magrath 1994), such that during non-breeding periods there are both unornamented males (i.e. in brown, female-like non-breeding plumage) and ornamented males (i.e. in nuptial breeding plumage). Unlike most passerine birds, for which luteinising hormone appears to be the dominant endocrine mechanism for signal production (reviewed in Kimball and Ligon 1999; Kimball 2006), acquisition of ornamented plumage in Superb Fairywrens (*M. cyaneus*) and Red-backed Fairywrens (*M. melanocephalus*) is thought to be controlled by testosterone (Peters *et al.* 2000; Lindsay *et al.* 2009, 2011). Lindsay *et al.* (2009) found significantly higher plasma androgen levels in ornamented vs. unornamented breeding males, including those undergoing the prenuptial moult, and was able to stimulate moult into ornamented plumage in second-year males with testosterone implants prior to breeding (Lindsay *et al.* 2011). However, there are two points worth noting about Lindsay *et al*'s. earlier work on this system. First, both studies were conducted in the month prior to the breeding season and second, they focused largely on second-year males, thereby excluding a substantial proportion of individuals, including older and putatively higher quality males that moulted weeks or even months previously. The strongest evidence that circulating levels of androgens control phenotype, including the implant experiments, is based on evidence from second-year males (Lindsay *et al.* 2011); indeed, among males for which age was known, there was no significant difference in circulating levels of androgens among younger vs. older males (Lindsay *et al.* 2009). As such, the relationships between androgen levels with moult and ornamentation in older, early-moulting males that develop ornamented plumage during the non-breeding period remain unclear and are the focus of this study. Additionally, studying these relationships during the non-breeding period is beneficial because it avoids potential confounding effects that may be correlated with testosterone levels (e.g. territory acquisition).

We studied the acquisition of ornamented plumage during the non-breeding season (4–6 months before the start of the breeding season) in a population of Red-backed Fairywrens (*M. melanocephalus cruentatus*) near Darwin, Northern Territory, Australia. We quantified natural variation in plasma androgen levels in ornamented and unornamented males and unornamented females, including moulting individuals, and induced feather growth through experimental plucking to determine colour production in the absence of a regular moult. In accordance with previous research on the relationship between *Malurus* fairywren ornamentation and androgens immediately prior to the breeding season (Peters *et al.* 2001; Lindsay *et al.* 2009, 2011), we predicted that males undergoing moult into ornamented plumage or expressing nuptial plumage during the non-breeding season would exhibit higher androgen levels during these periods, relative to unornamented males and females, which were expected to exhibit low androgen levels. In contrast to these expectations, we instead found that ornamented male Red-backed Fairywrens had similar androgen levels to unornamented males and females during the non-breeding season, and that there was no relationship between androgens and moult within or across sexes or phenotypes. As a consequence, early-moulting males were able to develop ornamented plumage, and to express it for a long period, in the absence of elevated androgen levels. This study highlights the importance of studying signal expression at the time when signals are produced and the complex relationship that may exist between age, seasonality, androgens and plumage ornamentation in this and other bird species.

Materials and methods

Study system and species

The Red-backed Fairywren is a small Australian passerine with two subspecies: the crimson-backed subspecies (*M. m cruentatus*), which is the focus of this study, is found in northern Australia, and the orange-backed subspecies (*M. m. melanocephalus*), which has been studied more extensively (e.g. Karubian 2002; Lindsay *et al.* 2009; Webster *et al.* 2010; Baldassarre and Webster 2013), is found in eastern Australia. Subspecies differ in back hue and other morphometric traits (e.g. body size, tail length), and have distinct breeding seasons (Rowley and Russell 1997). *M. m. cruentatus* have redder backs, are smaller with shorter tails (Rowley and Russell 1997), and breeding is in the wet season (peak in January–February; Nakamura *et al.* 2010) as opposed to austral spring and early summer (peak in November–December) as in *M. m. melanocephalus* (Webster *et al.* 2010). Both subspecies are territorial during the breeding season, but territory boundaries dissolve and birds form large flocks of 15 or more individuals during the non-breeding season (Rowley and Russell 1997; Webster *et al.* 2010). Adult Red-backed Fairywrens are sexually and seasonally

dichromatic and complete two annual moults: males acquire ornamented red/black (hereafter ornamented) plumage during a partial prenuptial moult, and brown female-like plumage (hereafter unornamented) during a complete postnuptial moult, while females produce dull brown unornamented plumage in both moults. However, Red-backed Fairywren second-year males (i.e. males in their first potential breeding season) can breed in either ornamented or unornamented plumage (i.e. delayed plumage maturation; Karubian 2002), with breeding male phenotypes also varying in volume of the cloacal protuberance (CP) for sperm storage (Karubian 2002; Lindsay *et al.* 2009).

The onset of the prenuptial moult in *Malurus* fairywrens can occur over an extended and asynchronous period; in Superb Fairywrens, onset of prenuptial moult varies among males by up to 6 months (Mulder and Magrath 1994). In Superb Fairywrens, the prenuptial moult is typically complete within 25 days (Mulder and Magrath 1994), which is similar for Red-backed Fairywrens (S. Lantz and J. Karubian, unpub. data). Variation in timing of moult into ornamented plumage is an important predictor of reproductive success in congeners, with early-moulting males having higher levels of extra-pair paternity (Mulder and Magrath 1994; Dunn and Cockburn 1999; Brouwer *et al.* 2011). Males begin displaying to females as soon as they develop nuptial plumage (Mulder and Magrath 1994). Additionally, ornamented Red-backed Fairywren males are socially dominant over unornamented males (Karubian *et al.* 2008) and are also preferred by females (Karubian 2002), which leads to higher reproductive success through extra-pair fertilisations (Webster *et al.* 2008).

We studied a colour-banded population of Red-backed Fairywrens (*M. m. cruentatus*) on privately owned Coomalie Farm (13° 02′ S, 131° 02′ E), approximately 87 km south of Darwin, Northern Territory, Australia. This area is typical of seasonal tropics in northern Australia, and is characterised by alternating wet and dry seasons, with periods of high rain and flooding from October to April, and little rain and many bush fires from May to September. Breeding in this population is concentrated in January–February after the onset of monsoon rains (Nakamura *et al.* 2010). We conducted our study in June–August of 2013 and 2014, which coincides with the dry season in the Northern Territory and with the non-breeding season for these birds, as well as the time when some males are undergoing the prenuptial moult but others have not yet begun. Because this study was conducted during the non-breeding season, we did not attempt to assign social status to birds (i.e. breeding males vs. auxiliary helpers; see Karubian 2002).

Field methods

We captured and re-captured individuals through targeted mist-netting, where birds were herded into the net, as in other hormonal studies on this same species (Lindsay *et al.* 2009, 2011; Barron *et al.* 2013, 2015). The majority of individuals in the population were colour-banded prior to the data collection period for the current publication. Because we established this study population in 2012 and there was limited breeding during the study period, we have limited data on age structure in our population. However, based on previous capture records, most birds in this study were known after-second-year adults. We include data from four first-year birds (based on skull ossification; all females), and excluding these individuals does not quantitatively change our results. We do not include male age in models because of this lack of known structure in the population (the majority of individuals had an age of 2+ or 3+).

Upon capture we immediately took a small blood sample (<70 μl) from the jugular veins of all males, which was put into capillary tubes and kept on ice until processing. Because breeding Red-backed Fairywrens can respond to increased handling time with decreased androgen levels, we only included individuals with delay since capture of less than 20 min (bleeding delay mean ± SD = 7.7 ± 2.4 min) in our analyses, as in Lindsay *et al.* (2009), measured as the amount of time from hitting the mist net until a blood sample was taken. Within the group of individuals included in our analyses, there was no relationship between bleeding delay and androgen levels (Spearman rank correlation: $r_S = 0.11$, $p = 0.30$, $n = 89$). Additionally, we scored adult plumage coloration as in Karubian (2002) by dividing each bird's body surface into five regions (head, back, belly, chest, tail) and scoring each on a scale of 0–10 for the proportion of ornamentation, with 0 being brown and 10 being red or black. We summed scores across all body regions and multiplied by 2 to get a total ornamentation score that ranged from 0 (brown) to 100 (red/black).

We visually scored moult by estimating the number of growing pins of six body regions (head, back, belly, chest, tail, and wing) on a four-point scale, with 0 being none and each subsequent point on the scale constituting up to a third of the feathers in that region in moult. Thus, a bird with between 1 and 33% of feathers in moult in each of the six regions (i.e. a score 1 for each) would have a total moult score of 6, and there was a combined possible maximum moult score of 18 if a bird were moulting heavily (score 3, corresponding to >66% of feathers in moult) in each of the six regions.

Males that were moulting into ornamented plumage and had a combined moult score of 2 or more were considered to be actively moulting in the prenuptial moult (as in Lindsay *et al.* 2009). Unornamented males with low levels of moult into unornamented plumage (i.e. brown birds moulting in brown feathers), but that were in ornamented plumage during the breeding season, were considered to be exhibiting adventitious moult rather than the prenuptial moult (see Lantz and Karubian 2016), and were not scored as moulting because this does not constitute a regular moult. When we arrived at the study site in June, we noted a few individuals already in ornamented plumage that most likely had already undergone the prenuptial moult prior to our arrival.

In order to determine plumage ornamentation of feathers re-grown outside of the moult cycle, we plucked two tail feathers from all captured males to induce standardised feather re-growth. When we recaptured these individuals, we took note of the colour of feathers that replaced these plucked feathers. Re-grown feathers were classified subjectively as 'brown', 'intermediate', or 'black' rather than the classification above of 0–10, with intermediate feathers being darker than the typical brown plumage but not fully black. We chose this classification because a score of '5' using the proportion of ornamentation scale would be a feather that was half black and half brown, whereas these intermediate feathers were uniformly intermediate in colour.

We used genetic-based sexing techniques to determine the sex of all unornamented birds using 2550 F and 2718 R primers (Fridolfsson and Ellegren 1999).

Radioimmunoassay

Upon return to the field station, we centrifuged blood samples to separate plasma from red blood cells. We stored blood plasma in liquid nitrogen until transfer to Washington State University, where samples were kept in a −20° freezer. We assayed 16–42 µl plasma samples using previously published protocols that allow simultaneous measurements of total androgen and corticosterone concentrations (Lindsay *et al.* 2011; Barron *et al.* 2013). We extracted steroids with diethyl ether and re-dissolved them in 150 µl phosphate-buffered saline with gelatin, pH 7.1 (PBSg). We conducted radioimmunoassays in 100 µl aliquots using tritium-labelled testosterone (Perkin Elmer Life Science NET-553, Waltham, MA, USA) and a testosterone antibody (Wien Laboratories T-3003, Flanders, NJ, USA) that cross-reacts with closely related steroids. For this reason we refer to total androgen concentration rather than testosterone (testosterone antibody has 60% reactivity with 5α-dihydrotestosterone (DHT) according to manufacturer specifications). To increase sensitivity, samples were run as singlet assay tubes (as per Lindsay *et al.* 2009) and samples were distributed randomly across the assay.

In 2013, the intra-assay coefficient of variation was 6.4% (calculated according to Chard 1995). Androgen recoveries were determined for each sample (mean recovery = 74%) using calculated corticosterone recoveries from a combined assay. Our robust dataset indicates that mean recovery rates from these two steroids do not differ. We detected androgen concentrations (n = 20) between 163 and 4831 pg/ml (mean = 762, median = 538). Concentrations of undetectable levels (n = 33) were back calculated from minimal detectable levels (1.95 pg/tube), which gave us androgen measurements of 87–4831 (mean = 365, median = 152) in 2013. The lower detection limit for a 16 µl plasma sample was 246 pg/ml.

In 2014 the intra-assay coefficient of variation was 7.4% (calculated according to Chard 1995). Mean recovery (71%) was calculated from four plasma samples unrelated to the present experiment. We detected androgen concentrations (n = 22) between 141 and 1731 pg/ml (mean = 436, median = 274). Concentrations of undetectable levels (n = 14) were back calculated from minimal detectable levels (androgens = 1.95 pg/tube); this gave us androgen measurements of 95–1731 (mean = 323, median = 206) in 2014. Across both years, we calculated the probability of detection for ornamented males, unornamented males, and females as the proportion of individuals in these groups that had detectable androgen levels.

Statistical analyses

To test whether androgen levels were related to individual sex, plumage coloration, and moult status, we used linear mixed models and an information theoretic approach (Burnham *et al.* 2011, Hegyi and Garamszegi 2011). We built models of androgen levels as a function of individual characteristics, and then selected the best models based on how well they fit our data using AICc (Akaike information criterion (Akaike 1973) corrected for small sample size). We conducted this analysis at two levels. First, we built a set of models to predict the probability of androgen detection, using a binomial response variable for androgen detection. To test the hypothesis that moult into ornamented plumage is driven by androgens, we built models including individual sex, plumage (ornamented or unornamented), and moult status (whether the individual had a combined total moult score of 2 or greater).

Specifically, to test whether phenotype predicted androgen levels, we built models with sex and plumage. We also built models with moult as a factor to test whether moulting individuals had higher androgen levels, which would enable us to decouple potential effects of moult and sex. Additionally, we created a full model with year, Julian date, time captured, and time delay from capture to bleeding in addition to sex, plumage, and moult, as possible predictors of androgen detection. We included individual as a random factor in all models because re-captures (potential pseudoreplicates) accounted for 21/89 samples. Of these potential pseudoreplicates, four individuals were sampled within the same season, nine were sampled across both years of the study, and four individuals were sampled both within the same season and across seasons. Models with categorical plumage (i.e. ornamented vs. unornamented) were equivalent to models using ornamentation score (from 0 to 100, where 0 is brown and 100 is red/black) to describe plumage variation.

We then subset the data to include only those individuals that had detectable androgen levels (i.e. excluding those with non-detectable androgen levels), and normalised androgen levels within this group using the inverse of the natural log transformation. We compared androgen levels within this subset of individuals using the same set of candidate models, but using normalised androgen levels instead of a binomial response.

We present measures of support for our top models based on AICc, including the model weights (w_i). All analyses were conducted in R v. 3.0.2 (R Core Team 2013). Model selection was run using the packages AICcmodavg (Mazerolle 2016), nlme (Pinheiro *et al.* 2016), and lme4 (Bates *et al.* 2015).

Additionally, we compared androgen levels between those males in which we induced moult by plucking tail feathers. We compared normalised androgen levels between males with black, dark brown, and brown regrown tail feathers using an analysis of variance.

Results

During the 2013 non-breeding season, 27% of all known adult males in the population (i.e. regardless of whether they were sampled for androgens) were in ornamented plumage; in 2014, the equivalent value was 20%. None of the males we captured had cloacal swellings. Across both years, we analysed 89 total androgen samples collected from 68 adult males and 21 samples collected from females (53 unique males and 19 unique females after accounting for repeated samples). Among male samples we analysed, 25 were collected from ornamented males (19 actively acquiring ornamented plumage through moult, and 6 already completed moult) and 43 were collected from unornamented males (not moulting).

Individuals had similar plasma androgen levels regardless of phenotype (ornamented vs. unornamented), sex (male vs. female), and moult status (actively moulting or not; Figure 1; Table 1). Consequently, the top-ranked model for probability of androgen detection was the null model (Table 2). Similarly, among individuals with detectable plasma androgen levels, the null model was also the top model (Table 2).

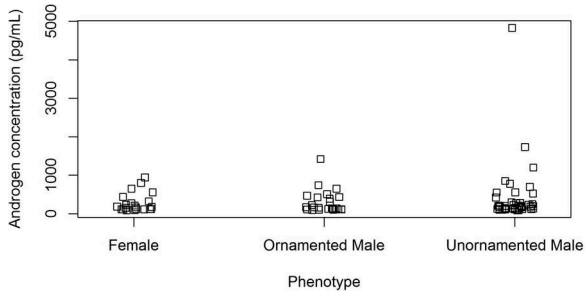

Figure 1. Plasma androgen levels of Red-backed Fairywrens during the non-breeding season did not differ between the sexes or male plumage types. All phenotypes had similar mean androgen levels, although both ornamented red/black and unornamented brown males had some individuals with elevated androgen levels.

Table 1. Non-breeding season Red-backed Fairywren plasma androgen levels. Plasma androgen levels of Red-backed Fairywren males and females during the non-breeding season, including individuals with and without detectable plasma androgen levels, and males with and without moult. Ornamented males are in red/black nuptial plumage, while unornamented males are in brown eclipse plumage. We calculated probability of detection for ornamented males, unornamented males, and females as the proportion of individuals in these groups that had detectable androgen levels

		Plasma androgen pg/ml				
Phenotype	*n*	Mean	SD	Range		Probability of detection
Ornamented males	25	296	300	95	1418	0.44
Unornamented males	43	411	762	87	4831	0.49
Females	21	283	249	89	942	0.48
Males moulting into ornamented plumage	19	297	331	95	1418	0.42
Unornamented males not in moult	43	411	762	87	4831	0.49
Ornamented males without moult	6	290	194	108	504	0.50

Table 2. Red-backed Fairywren probability of androgen detection and normalised plasma androgen levels were not related to moult, sex, or plumage. The null models were the best candidate models to predict both (a) probability of androgen detection and (b) normalised androgen levels in ornamented male and unornamented male and female Red-backed Fairywrens, including moulting individuals. All candidate generalised linear mixed models to predict detectability of androgens are presented, with model weights. The full model includes year, Julian date, time captured, and time delay from capture to bleeding in addition to sex, ornamentation score, and moult status as fixed effects. All models include individual as a random effect. K is the number of parameters in the model, AICc is the Akaike information criterion with a correction for finite sample sizes, ΔAIC is the difference between each model's AICc compared to the best model, and w_i is the model weight

Model name	K	AICc	ΔAICc	w_i
(a)				
Null	2	127.17	0.00	0.55
Sex	3	129.32	2.14	0.19
Sex + Moult	4	130.07	2.90	0.13
Sex + Plumage	4	131.31	4.13	0.07
Sex + Plumage + Moult	5	132.00	4.83	0.05
Full	9	135.94	8.77	0.01
(b)				
Null	3	−207.07	0.00	0.67
Sex	4	−204.66	2.41	0.20
Sex + Plumage	5	−202.12	4.95	0.06
Sex + Moult	5	−202.10	4.97	0.06
Sex + Plumage + Moult	6	−199.39	7.69	0.01
Full	10	−197.11	9.96	0.00

Qualitative results did not change following removal of the unornamented male outlier with the highest androgen levels in our study population.

We experimentally initiated moult by plucking tail feathers in a subset of 15 unornamented males. Among these males, seven re-grew black ornamented tail feathers, three re-grew tail feathers that were intermediate in coloration, and five re-grew brown unornamented tail feathers. There was no difference in androgen levels of males between these three categories (F_2 = 0.39, p = 0.69). There were individuals with and without detectable androgen levels among the males in each of these three categories (growing ornamented, intermediate, or unornamented tail feathers), corroborating the idea that plumage colour was not strongly linked to androgen levels.

Discussion

Red-backed Fairywrens in Australia's Northern Territory exhibited similar low plasma androgen levels during the non-breeding season, regardless of sex, phenotype (i.e. ornamented or unornamented), or moult status. Males were able to develop ornamented plumage months prior to breeding, apparently in the absence of elevated androgen levels. Also, males that grew ornamented tail feathers after plucking had similar androgen levels to males that grew unornamented feathers. Based on these results, we conclude that elevated androgens are not necessary for the production of ornamented plumage in this study population.

These findings run counter to previous correlative and experimental work demonstrating an association between androgen levels and acquisition of nuptial plumage in a different population of Red-backed Fairywrens located on the Atherton Tablelands, Queensland (Lindsay *et al.* 2009, 2011), and also in Superb Fairywrens (Peters *et al.* 2000). In Superb Fairywrens, the annual testosterone profile shows a peak in testosterone during breeding, with low testosterone levels immediately following breeding (Peters *et al.* 2001). In this species, nuptial plumage is associated with elevated testosterone, while unornamented males never had detectable androgen levels (Peters *et al.* 2000). However, plumage ornamentation was also not related to testosterone levels in Purple-crowned Fairywrens (*M. coronatus*; Peters *et al.* 2013).

In the current study, the androgen concentrations we measured in all classes of bird, including ornamented males, were much lower than those recorded for ornamented Red-backed Fairywrens in Queensland. Instead, our values were similar to levels found in the Queensland population from unornamented auxiliary helpers across pre-breeding through fledgling periods of the breeding season, and from unornamented breeding males during the pre-breeding season and prenuptial moult (Lindsay *et al.* 2009). Importantly, analyses for these two studies were conducted in the same laboratory (H.S.) and using the same techniques. Lindsay *et al.* (2011) were also able to induce moult into ornamented plumage with testosterone implants in Red-backed Fairywrens, and concluded that testosterone was necessary and sufficient for acquisition of ornamented males in this species. We propose several potential explanations for the differences in the relationship between androgens and plumage coloration between the current study and previous experimental and correlational work on this species (Lindsay *et al.* 2009, 2011).

First, we consider it likely that differences in androgen levels obtained by Lindsay *et al.* (2009) and the current study may be due to the fact that the two studies sampled birds at different points during the annual cycle. More specifically, Lindsay and colleagues sampled birds immediately prior to breeding, whereas we collected blood samples for the current study 4–6 months prior to the breeding season. Based on research from other avian systems, androgen levels

are typically highest in the weeks prior to breeding, whereas androgen levels are typically low several months prior to the onset of the breeding season (Wingfield *et al.* 1990; Goymann *et al.* 2007; Goymann and Landys 2011). Indeed, many studies measuring testosterone in avian systems focus on the breeding season, when high testosterone is often linked to territoriality (Goymann and Landys 2011), aggression (Wingfield *et al.* 1990), mate guarding and parental care (Ketterson and Nolan 1994), and courtship displays (Fusani 2008). Measuring androgen levels outside of the breeding season can avoid these potentially confounding factors. During our sample collection phase, which occurred during the non-breeding period, ornamented males failed to exhibit, or exhibited at very low levels, behavioural traits (e.g. courtship displays) associated with breeding season behaviour and high androgens in fairywrens (Karubian 2002; Lindsay *et al.* 2009) and other avian taxa (Wingfield *et al.* 1990), and did not have measurable cloacal protuberances for sperm storage. Although we are not currently aware of studies where proximate mechanisms for ornamentation change based on the annual cycle (but see Soma *et al.* 2000, where territoriality is regulated by oestrogen instead of testosterone during the non-breeding season), our results suggest either an alternative proximate mechanism to ornamentation outside of breeding in this system or that the previously documented relationship between androgens and ornamentation is more complex than simply androgens alone.

A second potential explanation for the differing patterns reported by Lindsay *et al.* (2009, 2011) and the current study may be age. More specifically, we propose that plumage ornamentation may be flexible and controlled by testosterone among 1-year-old males, but may act as a 'fixed system' (Moore 1991) among after-second-year-old male Red-backed Fairywrens. In this scenario (which our data suggest but would need further verification), older males all produce ornamented plumage in the pre-alternate moult, as appears to be the case in many North American passerines (Rohwer *et al.* 1980), and high levels of circulating androgens are not required for development of ornamented plumage. In contrast, acquisition of ornamented plumage may be more flexible among second-year males, requiring relatively high levels of circulating androgens in order to occur. Age-dependent plumage as a fixed system is common in some other male birds with delayed plumage maturation and strong sexual dichromatism (e.g. *Chiroxiphia* manakins, Foster 1987; McDonald 1989; DuVal 2005), and there are several pieces of information that are consistent with the idea that this explanation may apply to Red-backed Fairywrens. First, second-year Red-backed Fairywren males do in fact exhibit flexible strategies during the breeding season to either develop ornamented plumage or retain unornamented plumage (i.e. exhibit delayed plumage maturation; Karubian 2002), and this variation is associated with androgens among second-year males (Lindsay *et al.* 2009, 2011). Second, in both Queensland and the Northern Territory, all of the >1000 males we have monitored produced ornamented plumage in all subsequent breeding seasons after they had obtained ornamentation for the first time (Karubian 2002; pers. obs.). Third, the fact that several unornamented males from both Queensland (Karubian *et al.* 2011) and the current study were able to re-grow nuptial feathers in the absence of a regular moult and/or detectable androgen levels demonstrates that adult males are physiologically capable of producing ornamented feathers independent of the annual moult cycle. Queensland experimental work focused on second-year males (Lindsay *et al.* 2011), which breed in either ornamented or unornamented plumage (i.e. delayed plumage maturation in many, but not all males; Karubian 2002). In contrast, all or nearly all the birds we investigated in the current study were after-second-year or older. This situation, which was due to consecutive breeding seasons with little or no successful reproduction on our Northern Territory study site, raises the possibility that elevated testosterone may be necessary to achieve ornamentation for younger males (Schwarzová *et al.* 2010; Lindsay *et al.* 2011), but not for older males (Moore 1991; Gross 1996). We were not able to test for the specific effects of age in our study for reasons explained above (Methods), and a clear priority for future work is to assess relationships between age, androgens, and plumage acquisition. There is also the possibility of an age-dependent relationship between testosterone and plumage colour (see Peters *et al.* 2006), which could potentially maintain signal honesty. This could be tested by comparing spectrophotometric analysis of plumage hue with testosterone levels across age classes.

A third possibility is that these two different populations may differ in the association between androgens and plumage colour regulation. There is some evidence from the literature that spatially distinct populations may differ in androgen levels, as tropical bird species often have lower plasma testosterone levels than do temperate species (reviewed in Goymann *et al.* 2004), and amphibians show a positive correlation between latitude and testosterone levels (Eikenaar *et al.* 2012). However, these relationships occur between rather than within species, and we are not aware of any studies showing differences

in androgen levels between populations of the same species. Nevertheless, the Northern Territory and Queensland populations represent distinct subspecies separated by approximately 1600 km, with low levels of gene-flow between them (Lee and Edwards 2008; Baldassarre *et al.* 2014). Differences in the environment between these regions, or breeding ecology between the two subspecies (Rowley and Russell 1997), could theoretically have driven differences in the association between androgens and ornamentation.

Regardless of which combination of these or other factors underlie the differences between our Northern Territory population and the Queensland population, our findings raise the intriguing possibility that ornamented males may gain benefits of early and extended signal expression while avoiding the costs (e.g. immunological inhibition) associated with high levels of circulating testosterone. Potential benefits could occur if plumage ornamentation functions as a 'status signal' (Rohwer 1975) in Red-backed Fairywrens, allowing ornamented males enhanced access to resources, including food, territories, and/or mates. This is supported by experimental evidence of social dominance in ornamented vs. unornamented male Red-backed Fairywrens during the breeding season (Karubian *et al.* 2008). However, there could also be other costs associated with moult (King 1980), and/or social or survival costs of expressing ornamented plumage (Rubenstein and Hauber 2008).

In summary, this study presents correlational evidence that androgens and ornamentation are not tightly linked during the non-breeding season in older male Red-backed Fairywrens: males can acquire ornamented red/black plumage in the absence of high androgen levels, and unornamented males may exhibit elevated androgens. Our current understanding of this system suggests that part of the explanation for this may be that plumage ornamentation is a fixed system, in that second-year males may depend on androgens for signal development whereas older males do not, but this needs additional confirmation and other explanations are certainly possible. These findings suggest that a focus on androgens alone may not be adequate to explain the mechanisms behind plumage ornamentation, which in turn has important consequences for our understanding of signal honesty. Emerging research that relates behavioural and phenotypic variation to sensitivity of target tissues to steroids (e.g. abundance of androgen receptors; Ketterson *et al.* 2009, Rosvall *et al.* 2012) or other hormonally-regulated gene expression (Peterson *et al.* 2013) rather than plasma hormone levels per se, offers a promising avenue for improving our understanding of the mechanisms behind ornamentation in sexually and seasonally dichromatic species.

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Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Research was approved by the Animal Ethics Committee at Charles Darwin University, Darwin, Australia, and the Institutional Animal Care and Use Committee at Tulane University.

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