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Plumage colour acquisition and behaviour are associated with androgens in a phenotypically plastic tropical bird

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Androgens regulate male reproductive behaviour and may be a mechanistic link between sexual signals and physiological condition. However, the role of these hormones in regulating prenuptial moult and male plumage signals of passerine birds is unclear. In the red-backed fairy-wren, *Malurus melanocephalus*, plumage colour is a sexually selected trait and males express three reproductive phenotypes: males can breed in bright red and black plumage or in dull brown plumage, or assume dull plumage and act as nonbreeding auxiliaries; each phenotype differs in parental and reproductive behaviour. We found that plasma androgen concentrations differed significantly between male phenotypes, with red/black breeding males having the highest levels and auxiliaries having the lowest levels across all nesting stages. These hormonal differences were also present during the prebreeding moult when nuptial plumage is acquired. Males also differed significantly in body condition during moult based on the phenotype acquired in the subsequent breeding season, with red/black breeding males being in the best condition and auxiliaries being in the poorest condition. Moreover, androgen concentrations were positively correlated with body condition during prenuptial moult. Thus, in this species, androgens may determine plumage coloration and provide a link between the expression of sexual signals and body condition.

Male sexual signals are generally thought to be condition-dependent traits that advertise individual quality to conspecifics (Zahavi 1975; Hill 1991; Andersson 1994; Cotton et al. 2004). Theoretical models suggest that signal honesty in sexually selected traits is enforced by the costs of trait production and maintenance, such that high levels of trait expression are relatively more expensive for low-quality individuals than for high-quality individuals (Grafen 1990). The proximate mechanisms underlying these traits are central to their proposed costly nature, yet physiological control of variable trait expression is not well understood (Rhen & Crews 2002; Knapp 2004).

Androgens have been proposed as a likely proximate mediator affecting the relationship between sexually selected traits and condition (Blas et al. 2006; Perez-Rodriguez et al. 2006; Peters 2007). Androgens regulate variation in male-typical reproductive behaviour and morphology (Wingfield et al. 2001), but are also known to suppress immune function (Folstad & Karter 1992; Roberts et al. 2004), increase metabolic rate (Wikelski et al. 1999b; Buchanan et al. 2001) and interfere with parental behaviour (Hegner & Wingfield 1987; Ketterson et al. 1992; De Ridder et al. 2000; Peters 2002). Moreover, androgen levels are often correlated with body condition (Duckworth et al. 2001; Chastel et al. 2005; Perez-Rodriguez et al. 2006), social status (Schoech et al. 1991; Wingfield et al. 1991; Poiani & Fletcher 1994) and reproductive success (Raouf et al. 1997; Garamszegi et al. 2005), suggesting that they may be key to maintaining signal honesty.

The showy breeding plumage of male birds has emerged as a model trait for examining mechanisms underlying intrasexual variation in the elaboration of sexual signals (Hill & McGraw 2006a, b), and is condition dependent in several species (Hill & Montgomerie 1994; Veiga & Puerta 1996; Keyser & Hill 2000; Doucet 2002). However, most of the work examining the effects of androgens on male sexual signals in birds has focused on non-plumage traits (Zuk et al. 1995; Eens et al. 2000; McGraw et al. 2006; Siitari et al. 2007), and the role of androgens in regulating plumage signals of male quality remains unclear (Owens & Short 1995; Kimball & Ligon 1999; Wingfield & Silverin 2002; Peters et al. 2006). Some studies have shown that males with more elaborate plumage traits have higher androgen levels (Saino & Møller 1994; Gonzalez et al. 2001; Duckworth et al. 2004; Peters et al. 2006), but

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in general these studies have examined hormone levels during breeding and not during the prenuptial (prealternate) moult when breeding plumage is acquired.

Most studies of androgens and bright plumage acquisition in passerines have been conducted during the postnuptial moult, when androgen levels are typically low (Nolan et al. 1992; Goymann et al. 2006). In these studies, supplemental androgens appeared to suppress or delay moult (Hahn et al. 1992; Kimball & Ligon 1999; Stoehr & Hill 2001) and even led to the production of drab plumage in some species (Stoehr & Hill 2001), although exceptions exist (Evans et al. 2000). Less is known about the role of androgens during the prenuptial moult when sexual plumage signals are typically acquired in many species. In particular, it is unclear to what degree results from studies conducted during a postnuptial moult can be extrapolated to indicate mechanisms acting prior to breeding. Bright plumage acquisition during the prenuptial moult is androgen induced in the Charadriiformes (Witschi 1961; Stokkan 1979; Kimball & Ligon 1999; Kimball 2006), but such androgen regulation may be uncommon in the Passeriformes (but see Collis & Borgia 1992; Peters et al. 2000). Nevertheless, some recent studies suggest that androgens may be involved in mediating intrasexual variation in plumage signals of passerine birds (Gonzalez et al. 2001), particularly for melanin-based plumage badges of male quality (Bókony et al. 2008).

The red-backed fairy-wren, *Malurus melanocephalus*, is an Australian passerine that shows discrete yet flexible variation in adult male phenotype: a male undergoing his first prenuptial moult can acquire bright red and black plumage and become a breeder (red/black breeder), or acquire dull brown, female-like plumage and either become a breeder (brown breeder) or remain as a nonbreeding auxiliary on the natal territory (i.e. a helper at the nest). In addition to variation in plumage colour, the three male types differ in bill coloration (Karubian 2008) and the volume of the cloacal protruberance (CP) or sperm storage organ (Karubian 2002; see also Results), with bright breeders showing the most intense level of trait expression. After their second year, most males moult into bright plumage and assume the red/black breeding phenotype (Webster et al. 2008). All three male types (red/black breeders, brown breeders, auxiliaries) are reproductively competent and capable of siring offspring (Webster et al. 2008), but compared to brown breeders, red/black males are socially dominant (Karubian et al. 2008), preferred by females (Karubian 2002) and have higher reproductive success because of increased rates of extrapair fertilizations (Webster et al. 2008).

Because red/black breeding males appear to invest heavily in mating effort whereas brown breeding males appear to invest more in parental effort (Karubian 2002), we hypothesized that variation in red-back fairy-wren male behavioural phenotype is associated with differences in circulating levels of androgens during reproduction. Moreover, since male red-backed fairy-wren behavioural phenotypes are coupled to differences in nuptial plumage that are acquired during a complete prenuptial moult (Rowley & Russell 1997), we predicted that differences in androgen levels between male types would first appear during the prebreeding moult and would be related to male condition at that time.

METHODS

Field Methods

We studied two populations of colour-banded red-backed fairy-wrens near Herberton, Queensland, Australia (145°23′E, 17°23′S) over the course of four breeding seasons (2003–2006); breeding occurs from early October through March. Our study sites were located in open sclerophyl forest with tall eucalypt overstory and grass understory. Red-backed fairy-wrens moult twice per year, with males acquiring breeding plumage during a prenuptial moult that occurs between August and December (i.e. overlapping somewhat with breeding at the population level; see Results, Fig. 1). During the prenuptial moult, some first-year males and all older males acquire black plumage on the head, tail, belly, chest, outer wing coverts and innermost secondaries, and acquire red (carotenoid based; K. McGraw & M. Webster, unpublished) plumage on the back and scapulars, with outermost secondaries and all primaries moulting in brown. Females and most first-year males acquire brown plumage on all body parts with the occasional exception of the alula, which can be black on some first-year brown males. This nuptial plumage is maintained throughout the breeding season and is shed during the postnuptial moult to be replaced by the standard full-body brown coloration in most birds (some older males overwinter in a red/black plumage similar to the breeding plumage; personal observation).

We collected blood samples for hormonal analyses from males of known age or minimum age based on skull ossification (age range 1–5 years, 87%, 169 of 195, were a minimum age of 1 year). Capture and sampling occurred between the 0500 and 1100 hours or between 1500 and 1800 hours and between September and February of all years. We trapped males by herding focal groups into mist nets without song playback. From each captured bird, we collected a maximum of 80 μl of whole blood from the jugular vein, which was transferred to heparinized microcapillary tubes. After centrifuging, we measured haematocrit and removed plasma for storage in liquid nitrogen until transportation to Washington State University, where it was kept in a −20 °C freezer. At the time of capture, we banded all unbanded birds with a numbered Australian Bird and Bat Banding Scheme (ABBBS) aluminium leg band and three coloured plastic leg bands for individual identification. We monitored banded birds multiple times per week and were able to unambiguously determine breeding status (i.e. breeder versus auxiliary) and nesting stage.

At capture, we measured tarsus length and body mass, and collected three measures of the cloacal protruberance (CP): the length (L), width (W) and depth (D); we calculated CP volume using the formula $\pi \times D/2 \times W/2 \times L$ (Mulder & Cockburn 1993; Tuttle et al. 1996; Karubian 2002). To assess variation in bill colour, we scored the colour of four sections of the bill (top and bottom, anterior and posterior), with each section being scored visually on a scale from 1 (horn-coloured) to 10 (black); total bill colour score was the sum score for these four sections (maximum score of 40). To reduce error in our measurement of bill colour due to subjectivity, the scoring system was periodically calibrated across observers with multiple individuals scoring the same bill.

We scored the percentage of the body in bright red and black plumage versus dull brown plumage following Karubian (2002). The distribution of the percentage of red/black scores during breeding across the population was bimodal, so we were able to classify males into two discrete categories of plumage types: a male was considered 'brown' if one-third or less ($\leq$33%) of his body was covered in red and black plumage, and red/black if two-thirds or more ($\geq$67%) of his body was in red and black plumage (see Webster et al. 2008). Of 1-year-old red-backed fairy-wren males captured 2004–2006, 15% became red/black breeders, 55.6% became brown breeders and 19.5% became auxiliary helpers (Webster et al. 2008; M. S. Webster, unpublished data). A small subset of first-year birds moulted into intermediate plumage (9.8%; $N = 14$ in our final data set) and these were excluded from analyses.

We scored intensity of the prenuptial moult visually by estimating the number of growing pin feathers on six body regions (head, back, wing, belly, chest, tail) as none (0), light (1), medium

(2), or heavy (3); a bird was considered to be moulting if it had a combined moult score of 2 or more across all six body parts.

Radioimmunoassay

Plasma sample volumes ranged between 10 and 50 μl; because of the small size of these samples, we ran assays for total androgen concentration (testosterone and 5α-dihydrotestosterone (DHT); see below for antibody cross-reactivity). Androgens were extracted from plasma with diethyl-ether and were not further purified; radioimmunoassays were conducted using tritium-labelled testosterone (PerkinElmer Life Sciences NET-553, Waltham, MA, U.S.A.) and a testosterone antibody (Wien Laboratories T-3003, Flanders, NJ, U.S.A.) that cross-reacts with closely related steroids (100% reactivity with testosterone, 60% with DHT, 5% with aldosterone, less than 15% with other androgenic steroids, and less than 0.5% with oestradiol and all other steroids: values provided by the manufacturer). Samples of 30 μl or less were run as singlet assay tubes, samples of 35 μl or greater were run as duplicate tubes. Two recovery samples containing 2000 cpm tritium-labelled testosterone were run per assay using pooled plasma samples (mean recovery of 75%). Singlet samples were redissolved after extraction in 110 μl phosphate-buffered saline with gelatine, pH 7.1 (PBSg) and duplicate samples received 210 μl PBSg. Radioimmunoassays were conducted in 100 μl aliquots according to standard techniques (Schwabl 1993). The average intra-assay coefficient of variation across the six assays was 13.05% and the interassay variation was 22.14% (calculated according to Chard 1995). Samples from male types and nesting stages were distributed randomly across the six assays.

Statistical Analyses

Androgen concentrations were natural log-transformed to meet normality assumptions of standard least-squares models. Linear least-squares models were built including all potentially influential variables (male phenotype, nesting stage, moult score, capture date, year, body mass), and for each analysis, nonsignificant terms and interactions were removed. Analyses of non-normally distributed variables (bill colour, percentage of red and black plumage) were conducted using Spearman rank correlations. A principal components analysis was conducted to examine the relationship between androgens concentrations and the highly correlated, seasonally regulated nonplumage male secondary sexual traits, bill colour and CP volume. All analyses were conducted using JMP 5.1.2 (SAS Institute Inc., Cary, NC, U.S.A.) and Minitab 14 (Minitab, Inc., State College, PA, U.S.A.) and post hoc pairwise comparisons were performed using the Tukey adjustment for multiple comparisons.

Body condition was calculated from the residuals of a regression of mass on tarsus length (ANOVA: $F_{1,661} = 31.43$, $P < 0.0001$, $R^2 = 0.045$). For all analyses of condition, use of mass alone or haematocrit (percentage of red blood cells to whole blood volume) provided similar results. Additionally, our measure of condition was positively correlated with the amount of fat in the furcular cavity (measured on a scale of 0–3, where 0 = no fat, 1 = narrow band of fat, 2 = layer of fat in entire furcular cavity, 3 = fat bulging from furcular cavity; Spearman rank correlation: $r_S = 0.28$, $N = 189$, $P < 0.0001$), indicating that these mass/length residuals reflect the size of fat energy stores, a concern raised in some analyses of avian body condition (Green 2001).

Delay between capture and blood sampling had a significant effect on androgen levels (ANOVA: $F_{1,236} = 0.59$, $P < 0.0001$, $R^2 = 0.125$, controlling for variation due to male type; delay log-transformed for normality): both breeding male types responded to handling with decreased androgen levels (ANOVA: red/black breeders: $F_{1,115} = 32.02$, $P < 0.0001$, $r = 0.47$; brown breeders: $F_{1,72} = 12.41$, $P = 0.0007$, $r = 0.38$), whereas auxiliary males did not respond to handling (ANOVA: $F_{1,47} = 2.11$, $P = 0.15$, $r = 0.21$), probably because they had low androgen levels to begin with (see below). Therefore, we only used samples from breeding males collected within 20 min of capture, but included all samples from auxiliary males (delay between capture and blood sampling for auxiliary males averaged 17 min, range 3–60 min); in the resulting data set there was no effect of capture/sampling delay on androgen levels (ANOVA: $F_{1,176} = 2.109$, $P = 0.148$, $R^2 = 0.096$, controlling for variation due to male type). Androgen levels did not differ between singlet and duplicate assays (ANOVA: $F_{1,190} = 0.15$, $P = 0.698$, $R^2 = 0.004$), across the 4 years of the study (ANOVA: $F_{1,190} = 0.59$, $P = 0.444$, $R^2 = 0.047$), between the sampled populations (ANOVA: $F_{1,190} = 1.254$, $P = 0.264$, $R^2 = 0.077$), or with time of day (ANOVA: $F_{1,190} = 1.57$, $P = 0.212$, $R^2 = 0.006$), so all data were combined for analyses.

To address the possible confounding effects of male age, we examined variation in androgen concentrations between known-age bright males (using the preexclusion data set and controlling for the effects of delay between capture and blood sampling). We found that within red/black males, androgen levels were not significantly correlated with male age (ANOVA: $F_{3,24} = 2.03$, $P = 0.136$; mean ± SE: age 1 = 6.27 ± 0.26 log pg androgens/ml plasma, $N = 7$; age 2 = 6.51 ± 0.22, $N = 10$; age 3 = 6.59 ± 0.27, $N = 8$; age 4+ = 7.35 ± 0.31, $N = 5$). Because of the small sample size of 1-year-old bright males ($N = 1$), we were unable to control for male age in our final, postexclusion data set.

Pseudoreplicates accounted for 24 of 197 samples (12.2%): of these, 11 were males sampled during separate breeding seasons (5.6%) and 13 were sampled during the same season at different stages in the nesting cycle (6.6%). Including the replicate samples did not influence our major results, but increased sample sizes in underrepresented nesting stages, and therefore, we chose to incorporate them in all analyses.

Ethical Note

All animals were handled and released in a safe and humane manner, and blood samples did not exceed the maximum amount safely allowable given individual body size (according to the guidelines of the Ornithological Council). All procedures were approved by Institutional Animal Care and Use Committee (protocol no. 3067) of Washington State University, the James Cook University Animal Ethics Review Committee (approval no. A1004) and the Queensland Government Environmental Protection Agency. Export of samples from Australia was approved by the Australian Government Department of Environment and Heritage.

RESULTS

Timing of Breeding and Moult

Red-backed fairy-wrens in our population start a prenuptial moult in August, with most birds moulting heavily in September, October and November. Breeding can start in September, with most birds nesting in December and January (Fig. 1). The mean capture date for males considered to be moulting (moult ≥ 2) was 19 November, during the peak of moult for the population (see Fig. 1). Nonmoulting males were trapped significantly later in the season (ANOVA: $F_{1,145} = 19.96$, $P < 0.0001$, $R^2 = 0.121$), with mean capture date 15 December.

There was no difference between the three male types in capture date (ANOVA: $F_{2,257} = 0.30$, $P = 0.741$, $R^2 = 0.011$) or timing of CP growth (i.e. the probability of trapping a male with

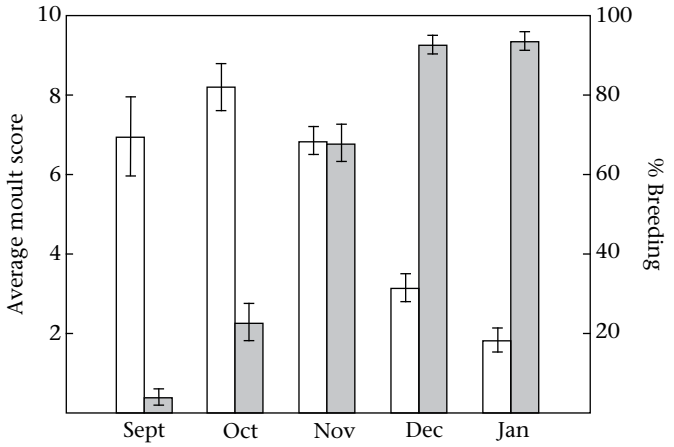

Figure 1. Mean ± SE overlap between prenuptial moult and breeding at the population level. □: mean moult score by month; ▩: percentage of the population breeding each month. Both sets of values were calculated across all 4 years of the study (2003–2006).

a measurable CP on a particular date; ANOVA: $F_{2,571} = 0.36$, $P = 0.701$). Nor was there a difference in the date that moulting males of the three types were captured (ANOVA, male type on capture date restricted to moulting males: $F_{2,375} = 0.15$, $P = 0.864$, $R^2 = 0.002$). However, the three male types differed significantly in the moult stage (as measured by total moult score) at which they were sampled (ANOVA, capture date and male type with response moult score: $F_{1,256} = 7.57$, $P < 0.001$, $R^2 = 0.258$), with auxiliary males and dull breeding males having a significantly higher total moult score at the time of capture than bright breeding males (mean ± SE: auxiliary = 7.93 ± 0.60, $N = 50$; brown breeder = 7.0 ± 0.44, $N = 51$; red/black breeder = 5.36 ± 0.32, $N = 82$). Although we were unable to record moult initiation dates, we included moult score as a controlling factor in all analyses to account for the potentially confounding effects of moult stage at the time of blood sampling. Moult score was also significantly correlated with body condition (ANOVA: $F_{1,258} = 9.79$, $P = 0.002$, $R^2 = 0.037$), and thus, further analyses examining variation among male types in body condition during moult included moult score.

Androgens and Breeding Phenotype

Plasma androgen levels were strongly associated with male phenotype (ANOVA: $F_{2,172} = 34.37$, $P < 0.0001$, $R^2 = 0.286$; Fig. 2). Post hoc tests revealed that each male type differed significantly from the others in a stepwise pattern that matched our a priori expectations: red/black breeders > brown breeders > auxiliaries (Tukey adjustment for multiple comparisons: all $P < 0.001$). As expected, male androgen levels were also associated with nesting stage (ANOVA: $F_{6,172} = 2.50$, $P = 0.024$, $R^2 = 0.081$; Fig. 2), and the interaction between male phenotype and nesting stage was marginally nonsignificant (ANOVA: $F_{12,160} = 1.77$, $P = 0.058$).

Both CP volume and bill colour differed significantly between the three male types (ANOVA: CP volume: $F_{2,542} = 48.45$, $P < 0.0001$; bill colour: $F_{2,578} = 55.986$, $P < 0.0001$, $R^2 = 0.162$), with red/black males having the most exaggerated level of trait expression. There was no significant association between either of these traits and androgen level (ANOVA, controlling for male type: CP: $F_{1,134} = 2.90$, $P = 0.091$, $R^2 = 0.103$; bill colour: $F_{1,175} = 3.42$, $P = 0.066$, $R^2 = 0.263$), although in each case, more elevated trait values tended to be positively correlated with androgen level. Indeed, androgen levels were significantly higher in males with measurable CPs than in males without measurable CPs (ANOVA, controlling for male type: $F_{1,177} = 10.29$, $P = 0.002$, $R^2 = 0.134$).

Because bill colour and CP volume were highly correlated (Spearman rank correlation: $r_S = 0.46$, $N = 158$, $P < 0.0001$) and both traits are acquired early in the season during prenuptial moult and the onset of breeding, we ran a principal components analysis for these two variables. Both traits loaded positively and strongly on the first principal component (PC1), which accounted for 75% of the total variation (eigenvector: 0.707). Androgens were positively correlated with this index of male nonplumage sexual traits (ANOVA: $F_{1,143} = 4.86$, $P = 0.0292$, $R^2 = 0.186$), as were male phenotype (ANOVA: $F_{3,143} = 65.91$, $P < 0.0001$, $R^2 = 0.569$) and date (ANOVA: $F_{1,143} = 7.95$, $P = 0.006$, $R^2 = 0.038$).

Androgens and Moult: Trait Acquisition

Androgen levels were as high during moult as during breeding and did not differ from those seen after the prenuptial moult was completed (ANOVA, pre- versus postmoult, controlling for male type and nesting stage: $F_{1,128} = 0.48$, $P = 0.492$, $R^2 = 0.006$). Males moulting into red and black plumage ($N = 26$) had higher androgen levels than males moulting into brown plumage ($N = 40$) (ANOVA: $F_{1,57} = 43.22$, $P < 0.0001$, $R^2 = 0.392$), and androgen levels were positively correlated with the percentage of red and black nuptial body plumage (Spearman rank correlation: $r_S = 0.46$, $P < 0.0001$). Androgen levels also differed significantly between moulting males according to the phenotype that each male assumed during the subsequent breeding season (ANOVA, controlling for nesting stage: $F_{2,68} = 30.26$, $P < 0.0001$, $R^2 = 0.562$; Fig. 3), mirroring differences seen during the breeding season (Tukey adjustment for multiple comparisons: bright breeder versus dull breeder: $P = 0.003$; bright breeder versus auxiliary: $P < 0.0001$; dull breeder versus auxiliary: $P = 0.002$).

There was a marginally nonsignificant correlation between androgens and the index of nonplumage sexual traits (PC1) in moulting birds (ANOVA, controlling for date: $F_{1,43} = 3.92$, $P = 0.054$, $R^2 = 0.441$; Fig. 4a), but no such correlation for nonmoulting birds (ANOVA, controlling for male type in both analyses: $F_{1,54} = 0.003$, $P = 0.95$, $R^2 = 0.109$; Fig. 4b).

Androgens and Body Condition

Condition during prenuptial moult was associated with both male plumage colour and the breeding phenotype assumed in the subsequent season. Males moulting into red and black plumage were in significantly better condition than males moulting into brown plumage (ANOVA: $F_{1,285} = 8.758$, $P = 0.003$, $R^2 = 0.03$; Fig. 5a). Condition during moult was also correlated with subsequent breeding phenotype (ANOVA: $F_{2,256} = 6.179$, $P = 0.002$, $R^2 = 0.056$; Fig. 5b): males that subsequently became breeders were in significantly better condition than males that subsequently became auxiliaries (Tukey adjustment for multiple comparisons: red/black breeder versus Auxiliary: $P < 0.001$; brown breeder versus auxiliary: $P = 0.025$); there was no significant difference in condition during moult between red/black breeders and brown breeders (red/black breeder versus brown breeder: $P = 0.294$). In contrast, condition was not associated with either plumage colour (ANOVA: $F_{1,232} = 2.578$, $P = 0.11$, $R^2 = 0.011$) or male phenotype (ANOVA: $F_{2,214} = 0.515$, $P = 0.599$, $R^2 = 0.005$) after moult was completed.

Androgen levels were positively associated with body condition for moulting males (ANOVA, controlling for male type: $F_{1,72} = 7.691$, $P = 0.007$, $R^2 = 0.52$; Fig. 6). In contrast, androgen levels of postmoulting males were not associated with condition (ANOVA: $F_{1,56} = 0.792$, $P = 0.377$, $R^2 = 0.033$).

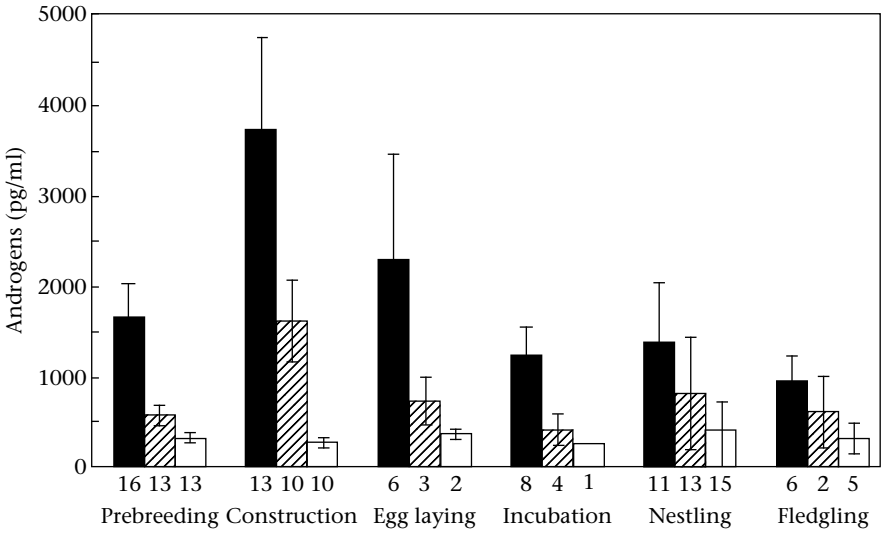

Figure 2. Mean ± SE plasma androgen levels of red/black breeders (■), brown breeders (▨) and auxiliaries (□) across all stages of the nesting cycle. Sample sizes are indicated below each bar.

DISCUSSION

Androgens and Breeding Phenotype

Species that show discrete, yet flexible adult breeding phenotypes offer uniquely powerful models for studying the evolution of adaptive plasticity in behaviour and morphology (Moore et al. 1998; Knapp 2004). Our results for the red-back fairy-wren support the hypothesis that phenotypic differences between breeding male types are mediated seasonally by activational actions of androgens (see Moore 1991). The association between elevated androgens and

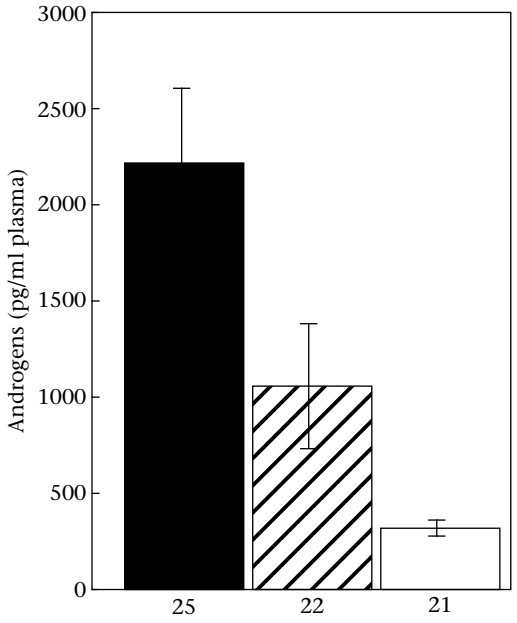

Figure 3. Mean ± SE androgen levels of red/black breeders (■), brown breeders (▨) and auxiliaries (□) during prenuptial moult, where male type represents the behavioural phenotype that was expressed during the subsequent breeding season. Of these males, 48% were observed to be members of breeding groups with active nests, 46% were prebreeding, and for 5%, the nesting stage was unknown. Sample sizes are indicated below each bar.

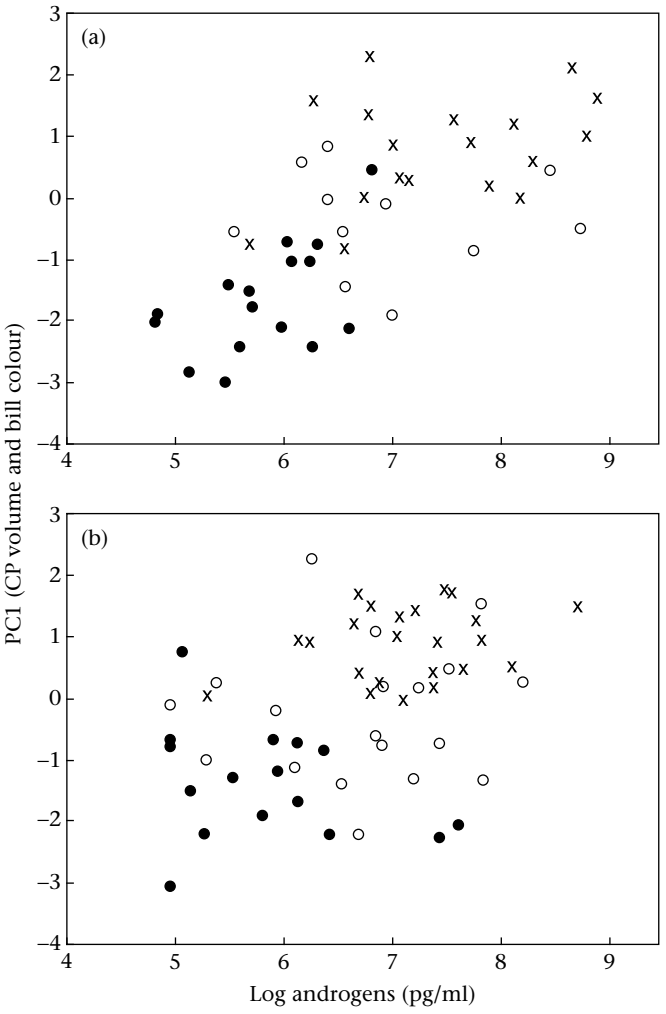

Figure 4. Regression of principal component 1 (bill colour and CP volume) on plasma androgen (log pg/ml) concentrations (a) during and (b) after completion of prenuptial moult; X: red/black breeding males; ○: brown breeding males; ●: auxiliary males.

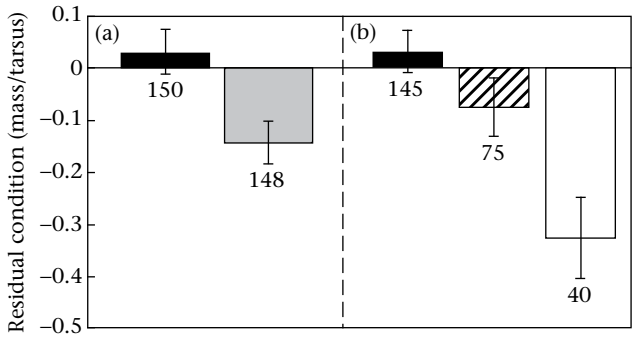

Figure 5. Mean ± SE body condition (residuals of the regression of body mass on tarsus length) of moulting males by (a) plumage colour acquired and (b) male behavioural phenotype assumed during the subsequent breeding season. ■: red/black plumage colour and red/black breeder males; ▨: brown plumage colour; ▨: brown breeder males; □: auxiliary males. Sample sizes are indicated below each bar.

red/black plumage production during the prenuptial moult, in combination with the observed correlation between androgens and exaggeration of the nonplumage sexual trait component (PC1), both support this hypothesis. In addition, the stronger association between androgens and PC1 during moult than during breeding indicates that androgens may be important in activating these morphological differences before the onset of breeding. However, experimental manipulations are needed to demonstrate whether the effects of androgens on phenotype are reversible (see also Peters et al. 2000). Continued androgen differences between the three male types during reproduction may thus represent maintenance of behavioural phenotypes, as discussed below.

Relative to brown-coloured males, red/black plumed red-backed fairy-wren males are socially dominant, engage in more off-territory forays and sire more extrapair young (Karubian 2002; Karubian et al. 2008; Webster et al. 2008), behaviours associated with high levels of androgens in other avian species (Wingfield et al. 1987, 2001; Raouf et al. 1997), and in red/black breeding males in the present study. In contrast, brown breeding males invest more in parental care (Karubian 2002) and correspondingly had lower androgen levels than red/black breeders in our study. Thus, our results are consistent with studies showing that androgens mediate investment in mating effort and parental effort (Ketterson & Nolan 1994; De Ridder et al. 2000; Van Roo et al. 2003; Hau 2007).

Subordinate auxiliary male red-backed fairy-wrens are reproductively competent and sire some offspring (Webster et al. 2008), but generally they are 'helpers at the nest' that put little effort into reproduction relative to breeding males. Accordingly, these males had the lowest levels of androgens in this study, which is consistent with results from other cooperatively breeding species (Schoech et al. 1991; Wingfield et al. 1991; Poiani & Fletcher 1994). Moreover, moulting males that eventually became auxiliaries were in significantly poorer body condition than those that became breeding males, and androgen levels were tightly associated with male body condition. These results suggest that androgen production is reduced in males that are in relatively poor body condition, leading to suppression of sexual behaviour (Schoech et al. 1991; Wingfield et al. 1991). Such a mechanism for physiological constraint may benefit auxiliary males, because low androgen levels may restrict males' ability to engage in behaviours that elicit aggression from dominant breeders or expulsion from their natal group (Schoech et al. 1991).

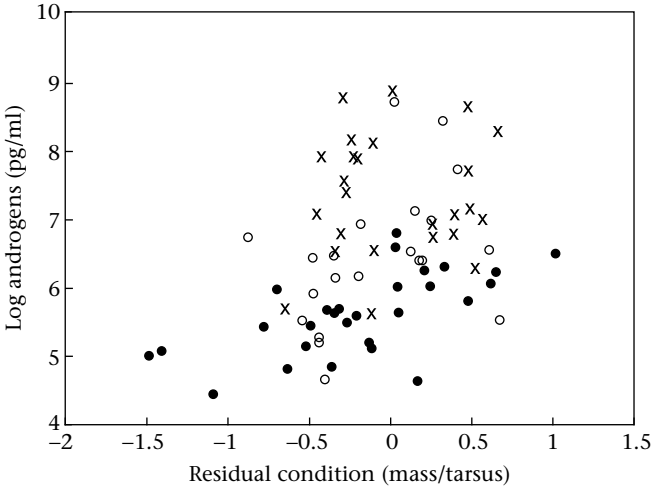

Figure 6. Regression of plasma androgen levels (log pg/ml) of males on body condition (residuals from regression of mass on tarsus length) during prenuptial moult; X: males that subsequently expressed the red/black reproductive phenotype; ○: males that subsequently expressed the brown reproductive phenotype; ●: auxiliary males.

Androgens, Plumage Colour and Condition

Androgens may be important in linking sexual signals to condition, because increased levels of androgens can lead to a more exaggerated level of trait expression (Wingfield et al. 2001) but may simultaneously have antagonistic pleiotropic costs, for example, depression of immune functions (Folstad & Karter 1992; Roberts et al. 2004) or other physiological costs (Buchanan et al. 2001; Alonso-Alvarez et al. 2007) that only males in superior condition can withstand. In support of this hypothesis, both experimental and observational studies have shown that androgens act as the proximate link between body condition and the production of sexually selected nonplumage traits (Ligon et al. 1990; Perez-Rodriguez et al. 2006).

Although plumage is an obvious and important sexual signal in birds (Andersson 1994; Hill & McGraw 2006a, b), few studies have focused on the role of androgens in acquisition of nuptial plumage signals (Peters et al. 2006). In passerine birds, acquisition of bright male nuptial plumage has been suggested to be regulated by luteinizing hormone (LH) rather than by androgens (Kimball & Ligon 1999; Kimball 2006), although androgens may affect the size of some melanin-based plumage badges (Evans et al. 2000; Gonzalez et al. 2001; Bókony et al. 2008). Moreover, some experimental implant studies of passerines have shown that testosterone does not increase plumage brightness (Day et al. 2006) and can even suppress moult and lead to drab plumage (Stoehr & Hill 2001). However, many of these studies were conducted on species with a single annual postnuptial moult occurring at a time when gonads are regressed and androgen levels low. For species that lack a prenuptial moult, androgen-independent (e.g. LH dependent) mechanisms of plumage colour acquisition have probably evolved.

Our results with red-backed fairy-wrens contrast strongly with these previous studies by showing that male androgen levels during the prenuptial moult are as high as during reproduction, and that males acquiring the bright red/black plumage have the highest androgen levels. To our knowledge, bright male nuptial plumage acquisition is directly associated with androgen levels in only one other passerine bird, the superb fairy-wren, *Malurus cyaneus*, a congener of the red-backed fairy-wren that also undergoes a prenuptial moult directly before breeding (Peters et al. 2000). In superb fairy-wrens, experimentally elevated male testosterone levels resulted in premature moult into nuptial plumage, and removal of implants caused a cessation of moult (Peters et al. 2000).

Moreover, males that moulted into dull plumage after breeding showed a decline in testosterone, whereas males that retained nuptial plumage during the nonbreeding season maintained high levels of testosterone (Peters et al. 2000). However, in superb fairy-wrens, all adult males assume bright coloration for breeding; thus, it is unclear whether variation among males in brightness is related to differences in androgen levels during moult. In red-backed fairy-wrens, breeding males are dichromatic, and we found that males that moulted into red and black plumage had much higher androgen levels than males that moulted into brown plumage. These combined results support the hypothesis that the bright nuptial plumage of *Malurus* fairy-wrens is an androgen-regulated sexual trait.

Our results suggest body condition as a proximate factor determining differences in androgen levels (see also Duckworth et al. 2001; Chastel et al. 2005), and therefore, male plumage coloration, in red-backed fairy-wrens. Male types differed significantly in body condition during moult, and condition was strongly correlated with androgen levels even after controlling for male type. Although differences in androgen levels between male types persisted across the breeding season, differences in body condition and the positive correlation between condition and androgen levels were confined to the moult. Therefore, androgen secretion itself may be condition dependent during this critical period, and thereby serve as a proximate link between condition, plumage colour and breeding phenotype. Although plumage coloration is associated with male condition in several species (e.g. Hill & Montgomerie 1994; Veiga & Puerta 1996; Doucet 2002), plumage is generally thought to be an androgen-independent trait in passerines (Kimball & Ligon 1999). Thus, our study is the first to suggest a causal link between body condition, androgens and plumage colour in this group of passerine birds. The mechanism by which body condition could affect androgen production and plumage colour is unclear, but may involve corticosterone, a metabolic stress hormone (Duckworth et al. 2001; Perez-Rodriguez et al. 2006), and possibly social interactions prior to the prenuptial moult (Wingfield et al. 1990; Wikelski et al. 1999a; McGraw et al. 2003). However, without experimental manipulations, we cannot reject the alternative hypothesis that both condition and androgens are regulated separately by some additional factor such as male age.

It is unlikely that behavioural, androgenic and conditional differences between plumage classes (red/black and brown) and reproductive phenotypes of red-backed fairy-wrens in our study were merely related to age for three reasons. First, our previous studies have indicated that first-year red/black breeders do not differ significantly from after-second-year red/black breeders in within-pair or extrapair reproductive success or other components of behaviour (Karubian 2002; Webster et al. 2008). Second, in the present study, we found that androgen levels did not differ significantly across known-age red/black males (ages 1–5 years). Thus, although age may contribute to variation in androgen levels among males, age alone cannot fully explain the plumage class and phenotype-specific variation in androgens identified in this study. Finally, first-year brown breeders did not differ in body condition from red/black breeders (of any age), suggesting that condition is not an age-related character. Experimental studies are now needed to test for the proposed causal link between condition, androgen levels and acquisition of plumage colour and reproductive phenotype.

Overall, this study presents strong correlational evidence for condition-dependent acquisition of male breeding phenotype that is mediated by androgens. This androgen dependence may help maintain the honesty of plumage signals, as well as bill colour and CP volume, which are both androgen sensitive (Keck 1933; Witschi & Miller 1938; Witschi 1961; Haase 1975). Thus, in the red-backed fairy-wren, there seems to be a short window, just prior to breeding, during which male condition and androgen levels appear to be linked to determine male breeding phenotype and, eventually, components of reproductive fitness (Webster et al. 2008).

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