HORMONAL REGULATION OF MALE REPRODUCTIVE PHENOTYPE IN A COOPERATIVELY BREEDING TROPICAL BIRD

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of WILLOW ROSELLA LINDSAY find it satisfactory and recommend that it be accepted.

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HORMONAL REGULATION OF MALE REPRODUCTIVE PHENOTYPE IN A COOPERATIVELY BREEDING TROPICAL BIRD

Abstract

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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 pre-nuptial 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 exhibit three reproductive phenotypes. Males can breed in red and black plumage, breed in brown plumage, or assume brown plumage and act as non-breeding auxiliaries: each phenotype differs in parental and reproductive behaviour. We found that both plasma androgen concentrations and body condition differed significantly among male phenotypes, with red/black breeding males having the highest androgen levels and best condition and auxiliaries the lowest levels and poorest condition. These differences were present during the pre-nuptial moult when nuptial plumage is acquired and androgen concentrations were positively correlated with body condition at that time.

While androgens appear to be involved in regulating these alternative reproductive phenotypes, their function may be mediated by the avian glucocorticoid stress hormone,

corticosterone (CORT). We tested the hypothesis that CORT regulates breeding status by suppressing breeding in auxiliary males and mediating the relationship between body condition, testosterone, and production of plumage signals. Contrary to our predictions, subordinate auxiliary males and dominant red/black breeding males had similar levels of baseline CORT. Additionally, we found no correlation between CORT and body condition and a positive rather than negative association between molting male CORT and androgens. Thus, honesty of male red-backed fairy-wren plumage signals may be enforced by the relationship between body condition and androgens without the mediating influence of CORT.

Finally, we experimentally tested the hypothesis that red/black plumage color is testosterone (T: androgen) dependent using T, control, and anti-T implants. We found that, relative to controls, both T and anti-T treatment stimulated early onset of the pre-nuptial molt, lead to the production of bright plumage, influenced feather length, and differentially effected body morphology. Across all combined characters, anti-T males produced an intermediate phenotype to T and control males. Thus, bright plumage production is T dependent but T may not be the only hormonal mechanism functioning.

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Dedication

This dissertation is dedicated to my ally in all things,

my best friend and life-coach,

my husband,

Jesse Haag.

GENERAL INTRODUCTION

The diverse and often elaborate signals produced by mating males result from the strong forces of sexual selection by which males compete for choosy females (Andersson 1994). Elaboration of these traits is not uniform across a population; instead, they vary with aspects of a male's quality including age, social status, and physiological and nutritional condition. Males that produce more elaborate breeding signals generally receive more mating opportunities and have higher fitness. One assumption of these "honest signals" is that the mechanisms involved in their production should be costly, thus deterring cheaters (Zahavi 1975). While research concerning the fitness payoffs for both males and females in their production and choice of elaborate sexual signals is well advanced, less is known about the mechanisms involved in enforcing signal honesty.

In many species, sexual signals and displays are seasonally variable, produced and expressed during the brief period of time prior to and during the annual reproductive phase. Production of such temporally flexible signals is often hormone dependent, and in particular, male sexual signals are often dependent on changes in plasma concentrations of the androgenic sex steroid testosterone (Wingfield et al. 2001). The role of testosterone in mediating production of sex signals is closely tied to the seasonality of breeding for most species. Reproductive readiness and gonadal activity are orchestrated by changing environmental conditions such as temperature and photoperiod, and thus, increases in circulating testosterone coincide with the pre-breeding phase. However, testosterone carries concomitant costs to the immune system, metabolism, and can interfere with parental behavior (Wingfield et al. 2001). As a consequence, not all males may be able to afford the costs of testosterone concentrations necessary for the

production of elaborate sexual signals. Indeed, males in relatively good condition are less affected by these costs than are males who have other drains on their health such as poor nutritional condition or heightened levels of glucocorticoid stress hormones (Folstad and Karter 1992). Although testosterone is well suited for insuring trait honesty, it is by no means clear that it functions in all systems and trait production is frequently regulated by other hormonal and genetic factors.

Avian plumage coloration is a conspicuous and highly variable signal that is known to function in sexual selection (Andersson 1994). Intrasexual variability in male plumage color often correlates with age or body condition and thus can honestly signal male quality to both mates and rivals. While the mechanisms that stimulate seasonal differences between the sexes in plumage color have been investigated (Kimball 2006), taxonomic patterns are based on relatively few species and most studies have been conducted on temperate avian species while tropical species which account for the majority of avian diversity are little studied. In addition, very little is known about the mechanisms involved in controlling intrasexual variation in plumage patterns. In particular, the role of testosterone in mediating plumage signals is contentious, as it appears to be important in some species but not in others (Owens and Short 1995).

In my dissertation I examine the hormonal mechanisms involved in controlling intrasexual variability in the production of male plumage color and breeding phenotype of the red-backed fairy-wren (*Malurus melanocephalus*). Red-backed fairy-wrens are a cooperatively breeding passerine bird native to northeastern Queensland, Australia. Plumage color is seasonally variable, with all individuals assuming a similar drab plumage type during the non-breeding season, and males molting into nuptial plumage during a pre-nuptial molt just prior to breeding (Rowley and Russell 1997). Males in this species exhibit a discrete polymorphism in

breeding plumage that is related to known differences in reproductive success and behavior (Karubian 2002; Webster et al. 2008). First-year males can either obtain mates and breed or can forgo breeding to remain as non-breeding auxiliaries on the natal territory. Auxiliary males always molt into a brown, female-like plumage and have reduced gonadal development and limited reproductive activity (Rowe et al. 2010). Breeding males can either molt into brown female-like plumage or can molt into the bright red and black nuptial plumage characteristic of this species. Red/black breeding males are socially dominant to brown breeding males and they experience greater reproductive success, primarily due to extra-pair mating opportunities (Karubian et al. 2008; Webster et al. 2008).

Thus, the red/black plumage type is a sexually selected character and male breeding phenotype (auxiliary, brown breeder, red/black breeder) has fitness consequences. In the three chapters of this dissertation I: 1) examined the condition-dependence or honesty of breeding phenotype, 2) examined the function of testosterone in regulating plumage color during the pre-nuptial molt, and 3) assessed any potential contributions of glucocorticoid stress hormones in regulating breeding strategy and the relationship between body condition and testosterone. In each chapter I tested a separate hypothesis: 1) red-backed fairy-wren breeding phenotype is condition-dependent and testosterone-mediated signal of male quality (chapter 1; assessed using a correlational approach), 2) corticosterone stress hormones regulate breeding status by suppressing breeding in auxiliary males and by mediating the relationship between body condition, testosterone, and production of plumage signals (chapter 2; assessed using a correlational approach), and 3) plumage color is testosterone dependent (chapter 3; assessed using a hormone manipulation experiment).

The research questions addressed in this dissertation, field and laboratory work, data analyses, and interpretations are my own. However, my dissertation was greatly enhanced by the input and advice of my advisors Hubert Schwabl and Michael Webster. Chapter 1 is published in the journal *Animal Behaviour* (Lindsay et al. 2009) and is coauthored by myself, my collaborator Dr. Claire Varian-Ramos, Dr. Webster, and Dr. Schwabl. Dr. John Wingfield of University of Californian, Davis analyzed plasma luteinizing hormone concentrations which are presented in chapter 3. In all chapters, I utilized a long term dataset (2003-2009) of red-backed fairy-wren behavioral and morphological measurements, two years of which were collected by Dr. Varian-Ramos.

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CHAPTER 1

PLUMAGE COLOUR ACQUISITION AND BEHAVIOUR ARE ASSOCIATED WITH ANDROGENS IN A PHENOTYPICALLY PLASTIC TROPICAL BIRD

INTRODUCTION

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 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 (Wingfield 1987; Ketterson et al. 1992; De Ridder et al. 2000; Hegner & 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, 2006b), 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 demonstrated that males with more elaborate plumage traits have higher androgen levels (Saino & Moller 1994; Gonzalez et al. 2001; Duckworth et al. 2004; Peters et al. 2006), but in general these studies have examined hormone levels during breeding and not during the pre-nuptial (pre-alternate) moult when breeding plumage is acquired.

Most studies of androgens and bright plumage acquisition in passerines have been conducted during the post-nuptial 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 pre-nuptial 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 post-nuptial moult can be extrapolated to indicate mechanisms acting prior to breeding. Bright plumage acquisition during the pre-nuptial 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). Nonetheless, 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 (Bokony et al. 2008).

The Red-backed fairy-wren (*Malurus melanocephalus*) is an Australian passerine that exhibits discrete yet flexible variation in adult male phenotype: a male undergoing his first pre-nuptial 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 non-breeding 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 exhibiting 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 due to increased rates of extra-pair 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 pre-nuptial moult (Rowley & Russell 1997) we predicted that differences in androgen levels among male types would first appear during the pre-breeding 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 4 breeding seasons (2003-2006); breeding occurs from early October through March. Our study sites were located in open sclerophyl forest with tall eucalypt over-story and grass under-story. Red-backed fairy-wrens moult twice per year, with males acquiring breeding plumage during a pre-nuptial moult that occurs between August and December (i.e., overlapping somewhat with breeding at the population level; see 'Results' Fig. 1). During the pre-nuptial 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 red (carotenoid-based; K. McGraw & M. Webster, unpublished) plumage on the back and scapulars with outer-most 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 post-nuptial

moult to be replaced by the standard full-body brown colouration in most birds (some older males overwinter in a red/black plumage similar to the breeding plumage; pers. obs.).

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 out of 195) were a minimum age of 1). 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 80µl whole blood from the jugular vein, which was transferred to heparinized microcapillary tubes. After centrifuging, we measured hematocrit 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 (max 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 percent of the body in bright red and black plumage versus dull brown plumage following Karubian (2002). Due to the bimodal distribution of percent red/black scores during breeding across the population, we were able to classify males into two discrete categories of plumage types: a male was considered "brown" if one-third or less (≤ 33%) of his body was covered in red and black plumage, and red/black if two-thirds or more (≥ 67%) of his body was in red and black plumage (see Webster et al. 2008). Of one-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; unpublished data). A small subset of first year birds moulted into intermediate plumage (9.8%; $N = 14$ in our final dataset) and these were excluded from analyses.

We scored intensity of the pre-nuptial 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 10-50µl; due to 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, Massachusetts USA) and a

testosterone antibody (Wien Laboratories T-3003, Flanders, New Jersey USA) that cross reacts with closely related steroids (100% reactivity with testosterone, 60% with DHT, 5% with aldosterone, < 15% with other androgenic steroids, and < 0.5% with estradiol and all other steroids: values provided by the manufacturer). Samples of 30µl or less were run as singlet assay tubes, samples of 35µl or greater were run as duplicate tubes. Two recovery samples containing 2000cpm tritium labelled testosterone were run per assay using pooled plasma samples (mean recovery of 75%). Singlet samples were re-dissolved after extraction in 110µl phosphate-buffered saline with gelatine, pH 7.1 (PBSg) and duplicate samples received 210µl PBSg. Radioimmunoassays were conducted in 100µl aliquots according to standard techniques (Schwabl 1993). The average intra-assay coefficient of variation across the six assays was 13.05% and the inter-assay 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, and body mass) and for each analysis non-significant terms and interactions were removed. Analyses of non-normally distributed variables (bill colour, % 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 non-plumage male secondary sexual traits, bill colour and CP volume. All analyses

were conducted using JMP 5.1.2 and MINITAB 14 and post-hoc pair-wise comparisons were performed using the Tukey adjustment for multiple comparisons.

Body condition was calculated as 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 provided similar results as did the use of haematocrit (percent red blood cells to whole blood volume). Additionally, our measure of condition was positively correlated with the amount of fat in the furcular cavity (measured on a score of 0-3; Spearman rank correlation: $r_S = 0.28$, $P < 0.0001$, $N = 189$) 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 (red/black breeders ANOVA: $F_{1,115} = 32.02$, $P < 0.0001$, $r = 0.47$; brown breeders ANOVA: $F_{1,72} = 12.41$, $P = 0.0007$, r = 0.38), whereas auxiliary males did not respond to handling (ANOVA: $F_{1,47} = 2.11$, $P = 0.15$, $r = 0.21$), likely because they had low androgen levels to begin with (see below). Therefore, we only used samples from breeding males collected within 20 minutes of capture, but included all samples from auxiliary males (delay between capture and blood sampling for auxiliary males averaged 17 minutes, 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 four 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 pre-exclusion dataset 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 and mean ± SE: $F_{3,24} = 2.03$, $P = 0.136$; 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, $N = 5$). Due to the small sample size of one-year-old bright males ($N = 1$), we were unable to control for male age in our final, post-exclusion dataset.

Pseudo-replicates accounted for 24 out 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 IACUC (protocol # 3067) at Washington State University, the James Cook University Animal Ethics Review Committee (approval #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 pre-nuptial 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 November 19th, during the peak of moult for the population (see Fig. 1). Non-moulting 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 December 15th.

There was no difference 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 a measurable CP on a particular date; ANOVA: $F_{2,571} = 0.36$, $P = 0.701$) between the three male types. Nor was there a difference in the date that moulting males of the three types were captured (ANOVA: $F_{2,375} = 0.15$, $P = 0.864$, $R^2 = 0.002$; ANOVA of male type on capture date restricted to moulting males). However, the three male types differed significantly in the moult stage (as measured by total moult score) at which they were sampled (ANOVA: $F_{1,256} = 7.57$, $P < 0.001$, $R^2 = 0.258$; factors capture date and male type and response moult score) 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, the above suggests that male types may differ in the timing of pre-nuptial moult (see also Mulder & Magrath 1994; Dunn & Cockburn 1999: high-quality congeneric superb fairy-wren (*Malurus cyanus*) males moult earlier and have higher reproductive success). As a consequence, moult score was included as a controlling factor in all analyses in order 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 which 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 non-significant (ANOVA: $F_{12,160} = 1.77$, $P = 0.058$).

Both CP volume and bill colour differed significantly among the three male types (ANOVA: CP volume $F_{2,542} = 48.45$, $P < 0.0001$, bill colour $F_{2,578} = 55.986$, $P < 0.0001$, $R^2 = 0.162$– see also Karubian 2008) with red/black males having the most exaggerated level of trait expression, but there was no significant association between either of these traits and androgens (ANOVA: CP $F_{1,134} = 2.90$, $P = 0.091$, $R^2 = 0.103$; bill colour $F_{1,175} = 3.42$, $P = 0.066$, $R^2 = 0.263$; controlling for male type) although for both there was a trend for a positive relationship of

androgens with the more elevated trait values. Indeed, males with measurable CPs had significantly higher levels of androgens than males without (ANOVA: $F_{1,177} = 10.29$, $P = 0.002$, $R^2 = 0.134$; controlling for male type).

Because bill colour and CP volume are highly correlated (Spearman rank correlation: $r_S = 0.46$, $N = 158$, $P < 0.0001$) and are both traits that are acquired early in the season during pre-nuptial 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 principle component (PC1), which accounted for 75% of the total variation (Eigenvector: 0.707). Androgens were positively correlated with this index of male non-plumage 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 pre-nuptial moult was completed (ANOVA: $F_{1,128} = 0.48$, $P = 0.492$, $R^2 = 0.006$; factors pre- vs. post-moult, controlling for male type and nesting stage). Males moulting into red and black plumage ($N = 26$) had higher androgen levels than males moulting into brown plumage (ANOVA: $F_{1,57} = 43.22$, $P < 0.0001$, $R^2 = 0.392$, $N = 40$), and androgen levels were positively correlated with the percent of the body in red and black nuptial plumage (Spearman rank correlation: $r_S = 0.46$, $P < 0.0001$). Androgen levels also differed significantly between moulting males according to the male phenotype assumed during the subsequent breeding season (ANOVA: $F_{2,68} = 30.26$, $P < 0.0001$, $R^2 = 0.562$; controlling for nesting stage; Fig.3), mirroring differences seen during the breeding season (Tukey adjustment for multiple comparisons: bright

breeder vs. dull breeder $P = 0.003$, bright breeder vs. auxiliary $P < 0.0001$, dull breeder vs. auxiliary $P = 0.002$).

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

Androgens and body condition

Condition during pre-nuptial 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) such that males eventually becoming breeders were in significantly better condition than those becoming auxiliaries (Tukey adjustment for multiple comparisons: red/black breeder vs. auxiliary $P < 0.001$, brown breeder vs. auxiliary $P = 0.025$); there was no significant difference in condition during moult between red/black breeders and brown breeders (red/black breeder vs. 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: $F_{1,72} = 7.691$, $P = 0.007$, $R^2 = 0.52$; controlling for male type, Fig. 6). In contrast,

post-moulting male androgen levels were not associated with condition (ANOVA: $F_{1,56} = 0.792$, $P = 0.377$, $R^2 = 0.033$).

DISCUSSION

Androgens and breeding phenotype

Species that exhibit 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 among breeding male types are mediated seasonally by activational actions of androgens (see Moore 1991). The association between elevated androgens and red/black plumage production during the pre-nuptial moult, in combination with the observed correlation between androgens and exaggeration of the non-plumage sexual trait component (PC1), both support this hypothesis. Additionally, the stronger association between androgens and PC1 during moult than during breeding indicates that androgens may play an important role in activating these morphological differences prior to onset of breeding. However, experimental manipulations are needed to demonstrate if the effects of androgens on phenotype are reversible (see also Peters 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 extra-pair young (Karubian 2002; Karubian et al. 2008; Webster et al. 2008): all of these behaviours have been shown to be associated with high levels of androgens in other avian species (Wingfield et al. 1987; Raouf et

al. 1997; Wingfield et al. 2001), and indeed red/black breeding males had the highest levels of androgens in this study. In contrast, brown breeding males invest more in parental care (Karubian 2002) and correspondingly had lower androgens than red/black breeders. Thus, our results are consistent with studies showing a role for androgens in mediating investment in mating effort vs. 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 exhibited 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 who became breeding males, and androgen levels were tightly associated with male body condition. These results suggest that androgen production is reduced in males who 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, as low androgen levels will restrict their ability to engage in behaviours that might elicit aggression from the dominant breeder or expulsion from their natal group (Schoech et al. 1991).

Androgens, plumage colour, and condition

Androgens may play an important role in linking sexual signals to condition, as 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 non-plumage traits (Ligon et al. 1990; Perez-Rodriguez et al. 2006).

Although plumage is an obvious and important sexual signal in birds (Anderson 1994; Hill & McGraw 2006a; 2006b) 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; Bokony et al. 2008). Moreover, some experimental implant studies of passerines have shown that testosterone does not increase plumage brightness (Day et al. 2006) and even can suppress moult and lead to drab plumage (Stoehr & Hill 2001). However, it is important to note that many of these studies were conducted on species with a single annual post-nuptial moult occurring at a time when gonads are regressed and androgen levels low. For these species that lack a pre-nuptial moult, it is likely that androgen-independent mechanisms of plumage color acquisition have evolved (such as LH-dependence).

Our results with red-backed fairy-wrens contrast strongly with these previous studies by showing that male androgen levels during the pre-nuptial 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 has been shown to be 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 undergo a pre-nuptial moult directly prior to breeding (Peters et al. 2000). In this species, 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 who moulted into dull plumage after breeding showed a decline in testosterone, whereas males who retained nuptial plumage during the non-breeding 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 moulting into red and black plumage had much higher androgen levels than did males moulting 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 among male types persist 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 time period, and thereby serve as a proximate link between condition, plumage colour, and breeding phenotype. Although plumage coloration has been found to be 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, ours is the first study 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 and stress hormone (Duckworth et al. 2001; Perez-Rodriquez et al. 2006), and possibly social interactions prior to the pre-nuptial 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.

For the following three reasons it is unlikely that behavioural, androgenic and conditional differences between the two plumage classes (red/black and brown) and three behavioural reproductive phenotypes are merely related to age. 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 extra-pair reproductive success or other components of behaviour (Karubian 2002, Webster et al. 2008). Second, in this study we found that androgen levels did not differ significantly across known-age red/black males (ages 1-5). 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 do not differ in body condition from red/black breeders (of all ages), 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, both of which are long known to be 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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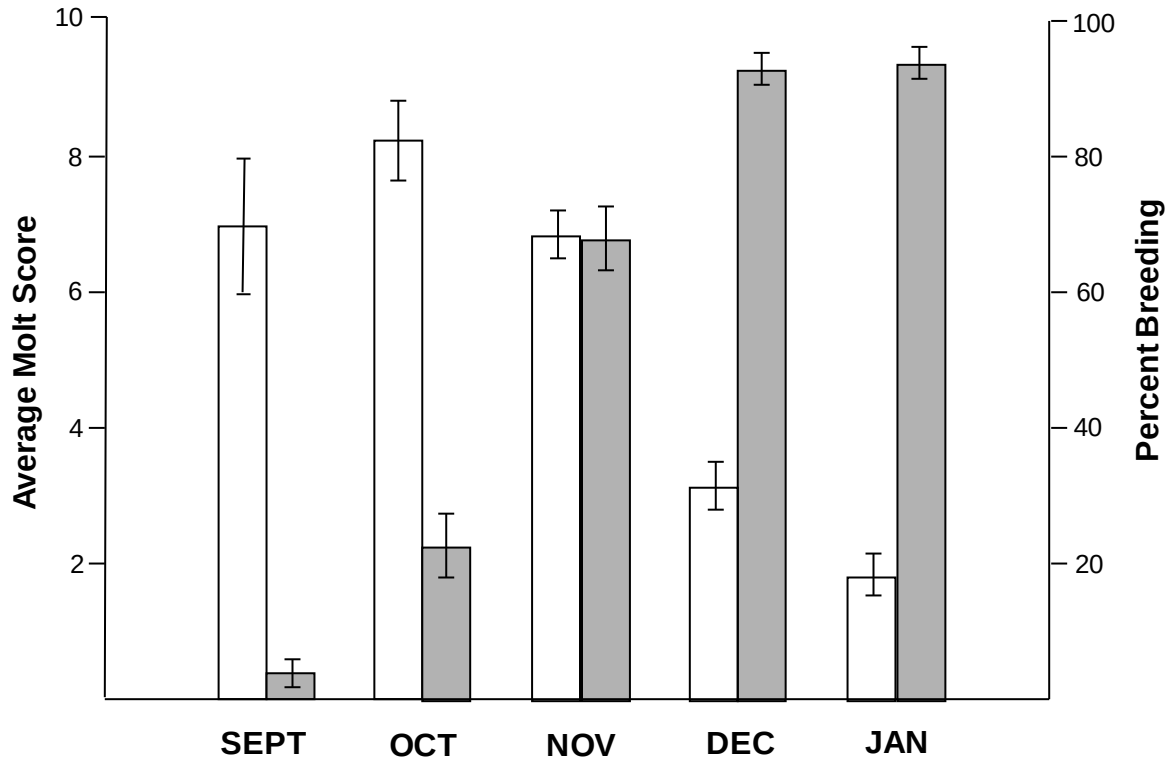

Figure 1: Overlap between pre-nuptial moult and breeding at the population level; white bars are the mean moult score by month, gray bars are the percent of the population breeding each month; error bars show ± one standard error. Both sets of values were calculated across all four years of the study (2003-2006).

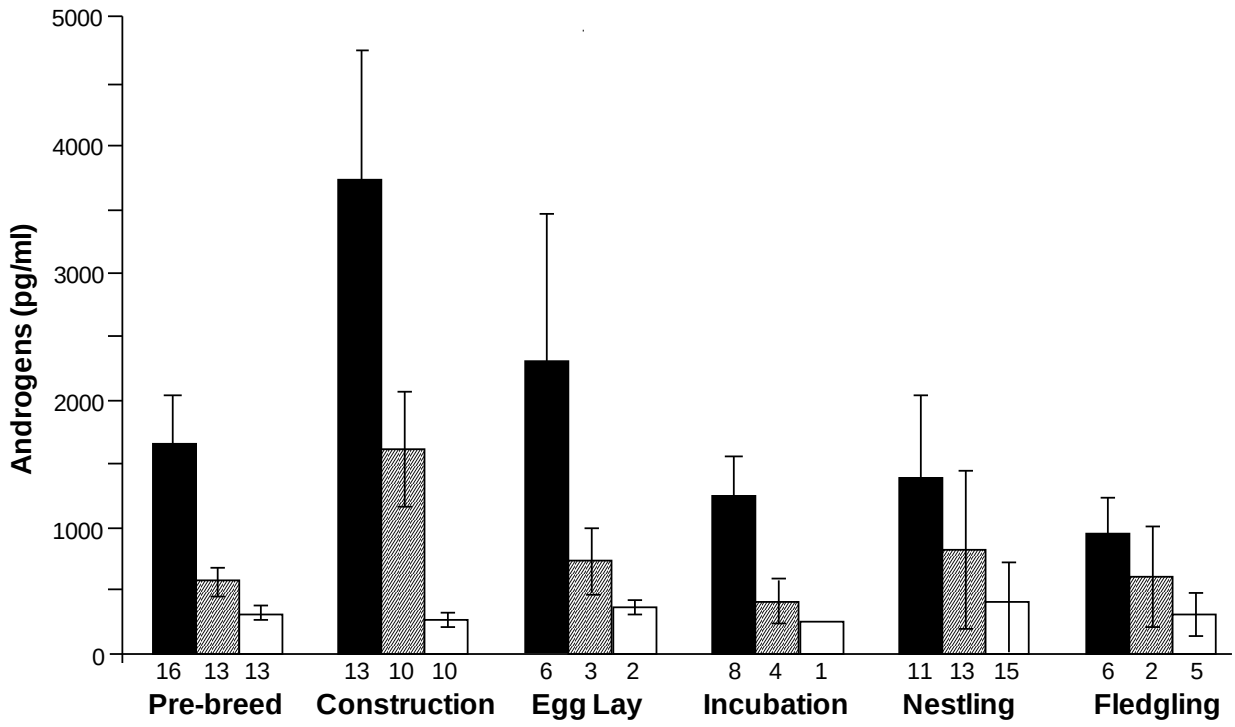

Figure 2: Plasma androgen levels of red/black breeders (solid columns), brown breeders (cross-hatched columns), and auxiliaries (white columns) across all stages of the nesting cycle. Columns are means ± one standard error and sample sizes are indicated below each column.

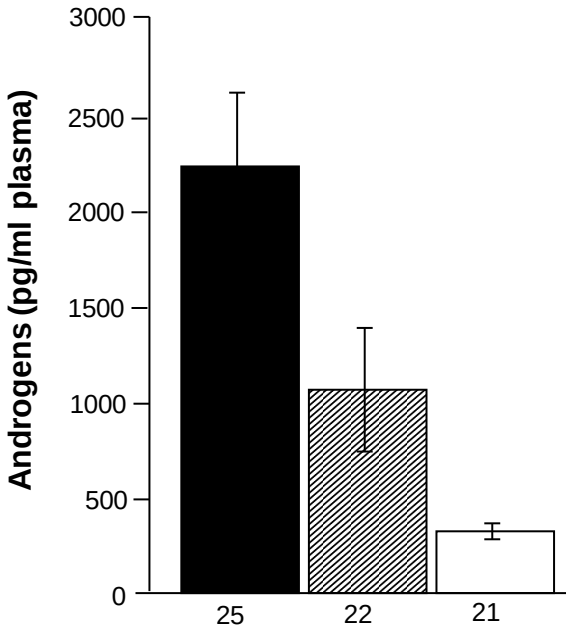

Figure 3: Androgen levels of red/black bright breeders (solid column), brown breeders (cross-hatched column), and auxiliaries (white column) during pre-nuptial moult, where male type represents the behavioural phenotype that is expressed during the subsequent breeding season. Of these males, 48% were observed to be members of breeding groups with active nests, 46% were pre-breeding, and for 5% the nesting stage was unknown. Columns are means ± one standard error and sample sizes are indicated below each column.

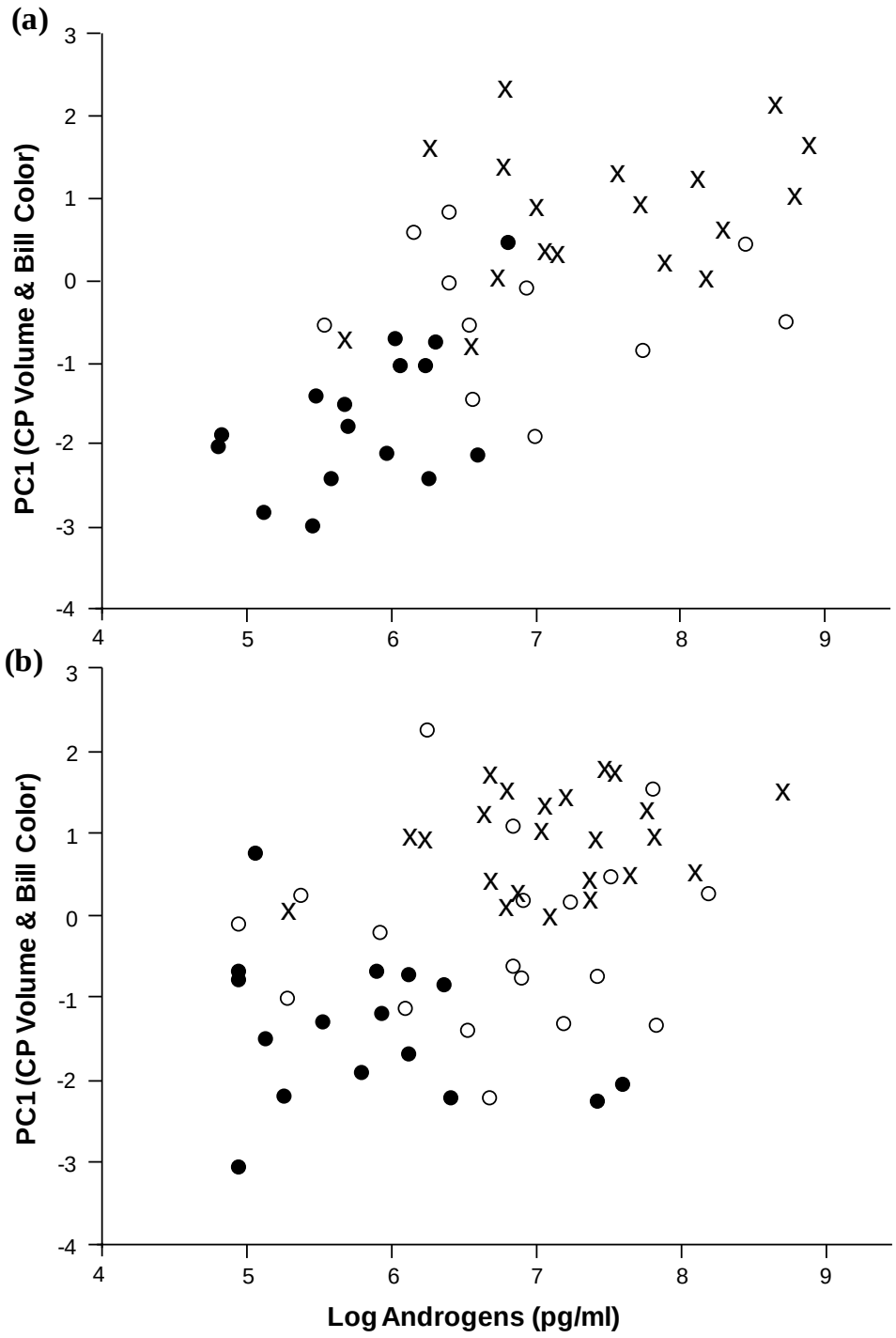

Figure 4: Regression of Principal Component 1 (bill colour and CP volume) on plasma androgen (log pg/ml) concentrations during (a) and after completion (b) of pre-nuptial moult; X's indicate red/black breeding males, open circles brown breeding males and filled circles auxiliary males.

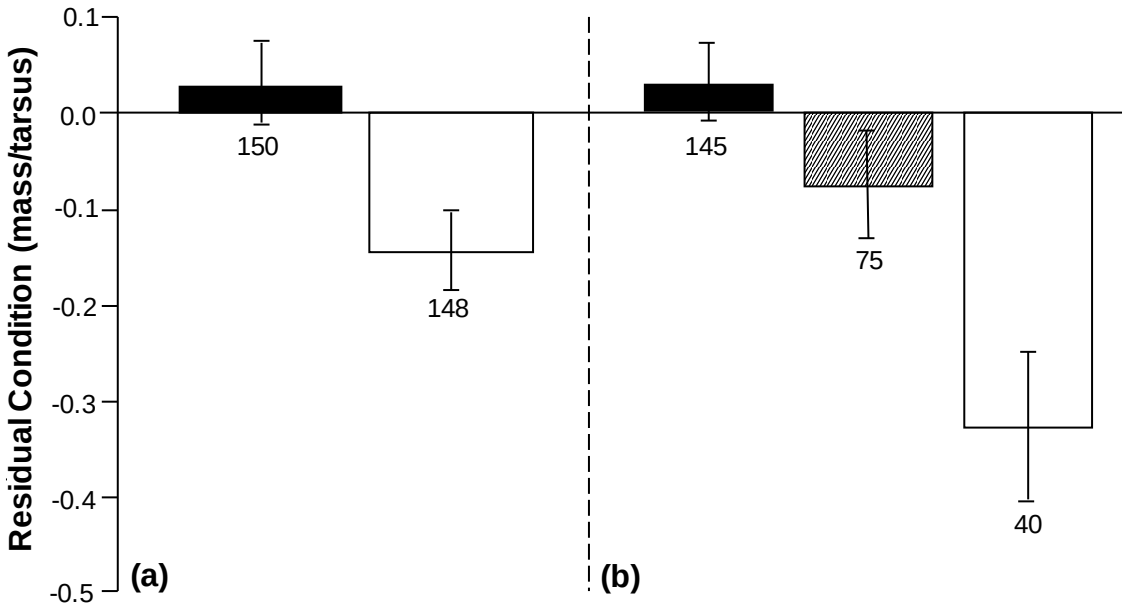

Figure 5: Condition (residuals of the regression of body mass on tarsus length) of moulting males by (a) plumage colour acquired (red and black plumage in solid column, brown plumage in white column) and (b) male behavioural phenotype assumed during the subsequent breeding season (red/black breeders in solid column, brown breeders in cross-hatched column, and auxiliaries in white column). Columns are mean ± standard error of body condition and sample sizes are indicated below each column.

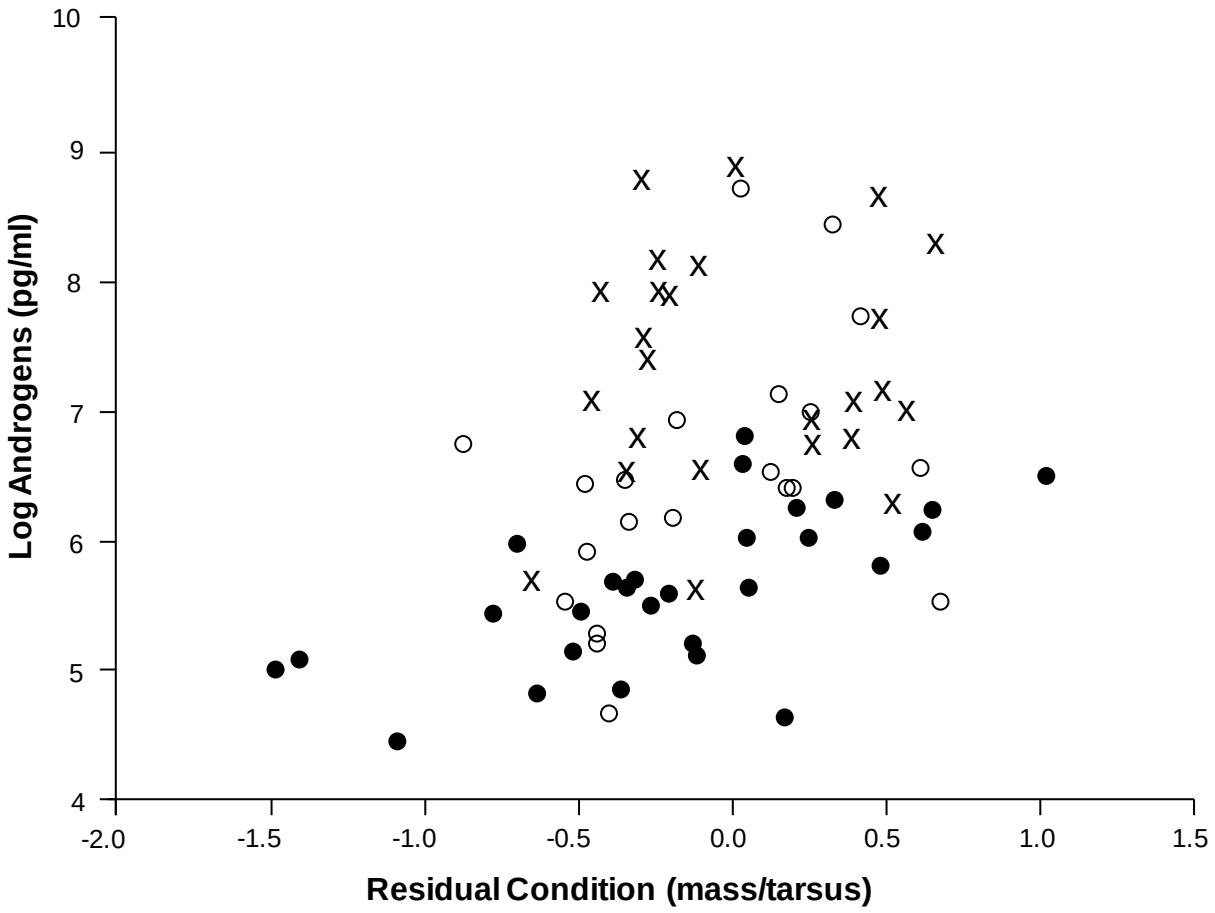

Figure 6: Regression of plasma androgen levels (log pg/ml) of males on body condition (residuals from regression of mass on tarsus length) during pre-nuptial moult; X's indicate males later expressing the red/black reproductive phenotype, open circles indicate males later expressing the brown reproductive phenotype, and filled circles indicate auxiliary males.

CHAPTER 2

THE ROLE OF CORTICOSTERONE STRESS HORMONE IN MEDIATING BREEDING STATUS AND SEXUALLY SELECTED PLUMAGE COLOR OF A COOPERATIVELY BREEDING PASSERINE

INTRODUCTION

Cooperatively breeding species are often characterized by the presence of breeding and non-breeding group members (Brown 1987). The ecological and social factors underlying cooperative breeding and the adaptive trade-offs of reduced reproduction in subordinate group members are becoming clear (Brown 1987; Stacey and Ligon 1991; Koenig et al. 1992; Arnold and Owens 1998; Hatchwell and Komdeur 2000). Less is known about the proximate mechanisms facilitating stable maintenance of cooperative breeding in the absence of continual reproductive conflict over breeding roles (Reyer et al. 1986; Schoech et al, 1991; Vleck and Brown 1999; Creel 2001). Delayed reproduction in subordinate group members is often correlated with reduced production of gonadal steroids (androgens) (Reyer et al. 1986; Schoech et al, 1991; Wingfield et al. 1991; Vleck and Brown 1999; Oliveira et al 2003; Brouwer et al. 2009; but see de la Cruz et al. 2003; Khan et al. 2001), and can be accompanied by differences in body condition (Moore and Jessop 2003; Rubenstein 2007). Glucocorticoid stress hormones, known to inhibit reproduction, may explain the simultaneous suppression of androgen production

and reproductive (sexual) behavior in non-breeding group members (Creel 2001; Young et al. 2006).

Adrenal glucocorticoids are metabolically active hormones that are also secreted in response to behavioral and environmental stressors (Sapolsky et al. 2000). Acute or short term increases in glucocorticoids above basal levels can be adaptive, transiently redirecting physiology and behavior towards survival (Wingfield et al. 1998) while long term or chronically enhanced glucocorticoid levels can result in immune suppression, disease, and reproductive failure (Sapolsky 1992; Sapolsky 2002; Wingfield and Sapolsky 2003). This adrenocortical stress response is highly variable in its magnitude, differing both within and among species, populations, and sexes (Moore and Jessop 2003) and varying with environmental conditions (Romero 2002; Rubenstein 2007) as well as individual quality such as age, body condition, and reproductive life history stage (Schwabl 1995; Wingfield and Sapolsky 2003).

Glucocorticoids are usually studied in the context of ecology (Romero 2002; Romero 2004; Wingfield and Sapolsky 2003) and very little is known about their role in sexual selection (Romero and Wikelski 2001; Husak and Moore 2008). However, the function of these metabolic and stress hormones in mediating sexual suppression of subordinate members of cooperatively breeding groups (Creel 2001; Goymann and Wingfield 2004) and the influence of stress on the expression of condition-dependent sexual traits (Buchanan 2000; Rubenstein and Hauber 2008) are areas of much current research interest. It has been proposed that glucocorticoids may function in both intra- and inter-sexual selection; first by mediating the expression of reproductive roles and dominance rank, and second by acting as an intermediary between body condition and androgen production, thus insuring honesty of androgen mediated sexual traits (Husak and Moore 2008).

Here we investigate the relationship between corticosterone (CORT; the primary avian glucocorticoid), reproductive status, and honest signal production in the cooperatively breeding red-backed fairy-wren (*Malurus melanocephalus*). Red-backed fairy-wrens are an Australian passerine in which males exhibit a discrete polymorphism in breeding strategy during their first reproductive season. One-year old males can either breed in red and black nuptial plumage (red/black breeders) or breed in brown, female-like nuptial plumage (brown breeders). Alternatively, they can forgo reproduction to remain as non-breeding brown-plumed auxiliaries on their natal territories (helpers at the nest). By their second year post-hatch, all males acquire breeding positions and molt into red/black nuptial plumage. These three phenotypes are differentiated morphologically, as well as by reproductive behavior, dominance rank, and fitness (Karubian 2002; Karubian et al. 2008; Webster et al. 2008). Plumage color production and reproductive phenotype are androgen dependent (Lindsay et al. 2009; Chapter 3) and correlated with body condition during molt. Molting red/black males are in the best body condition, have the highest concentrations of androgens, and during the subsequent breeding season, have the highest annual reproductive output. Although auxiliary males are reproductively competent (Rowe et al. 2010), they rarely sire offspring (Webster et al. 2008).

We predicted that CORT would vary with male phenotype and breeding status such that auxiliary males would have the highest CORT and red/black breeding males the lowest levels, explaining the suppression of reproductive behavior of auxiliary males. Additionally, we hypothesized that CORT would be negatively correlated with both body condition and T, explaining the observed positive correlation between androgens, body condition, and development of the sexually selected red/black plumage type.

METHODS

Field methods

We conducted our study on two adjacent color banded breeding populations of red-backed fairy-wrens outside of Herberton, Queensland in northeastern Australia (145˚23'E, 17˚23'S). All wrens were fitted with a combination of three colored plastic leg bands and an aluminum Australian Bird and Bat Banding Scheme (ABBBS) band for individual identification. Thus we were able to unambiguously determine breeding status, male phenotype, and nesting stage for sampled individuals. Sampling occurred between the months of August and January and over the course of four breeding seasons (2006-2009; breeding occurs between October and March). Birds were either trapped between the hours of 0500-1100 or 1500-1800.

We collected a series of morphological measurements from all captured individuals including (but not limited to) body weight, tarsus length, and plumage color (measured as the percent of the body in bright nuptial plumage and summed across 5 body regions; head, back, tail, belly, chest). Males were considered "brown" with brightness scores of ≤ 33% and "red/black" if their brightness scores were ≥67% (see also Webster et al. 2008). In our final dataset we excluded samples for intermediate plumed males (N = 12) as well as for males of unknown phenotype (N = 13). We visually estimated the degree of molt across six body regions (head, back, wing, belly, chest, tail) as none (0), light (1), medium (2), or heavy (3) and summed these values for a maximum molt score of 18. We assigned individuals to categories as either molting or non-molting with those individuals considered to be molting having a combined molt score of 2 or more. We drew a small blood sample (max 80µl) from the jugular vein for steroid analysis and after red/blood cells were separated from plasma, the plasma was stored in liquid

nitrogen until transport to Washington State University where it was kept at -20ºC. We recorded the time in minutes from capture to bleeding for each individual.

Radioimmunoassay

Radioimmunoassay for total plasma androgen concentrations followed previously published protocols (Schwabl 1993; Lindsay et al. 2009) with some modification in order to conduct simultaneous direct measurements of CORT concentrations (without column chromatography). Steroids were extracted from plasma with two consecutive 4ml washes of diethyl-ether and were not further purified. We added 200µl phosphate-buffered saline with gelatine, pH 7.1 (PBSg) to dried extracts; 100µl of re-dissolved extract was placed directly into singlet androgen assay vials and 20µl into individual CORT recoveries (containing recovered fraction of initial 2000cpm tritium labelled CORT; mean CORT recovery of 79.17%). We added an additional 200µl PBSg to the remaining 80ul of re-dissolved steroid extract and prepared duplicate 100µl CORT assay tubes for all samples. Four androgen recovery samples containing 2000cpm tritium-labelled testosterone were run per assay with no simultaneous measurement of CORT (mean recovery of 91.28%). Radioimmunoassays were conducted using tritium-labelled testosterone (PerkinElmer Life Sciences NET-553, Waltham, Massachusetts USA) and CORT (PerkinElmer Life Sciences NET 399) with testosterone and CORT antibodies (testosterone antibody; Wien Laboratories T-3003, Flanders, New Jersey USA; CORT antibody; Esoterix Endocrinology B3-163). Radioimmunoassays were conducted according to standard techniques (Schwabl 1993). Total androgen concentrations are reported as pg/ml and CORT as ng/ml. The average intra-assay coefficient of variation across the three assays was 3.47% for androgens and 13.69% for CORT and the inter-assay variation was 14.95% for androgens and 14.93% for

CORT (calculated according to Chard 1995; androgen intra-assay variation calculated using 5 duplicate known samples per assay). Samples from male types and nesting stages were distributed randomly across the six assays.

Body condition

We calculated body condition as the residuals from a regression of mass on tarsus; an informative index of condition for our species as it has been shown to positively correlate with the amount of fat in the furcular cavity and provide similar results in major analyses to the use of mass alone or to the use of blood hematocrit (Lindsay et al. 2009).

Statistical analysis

Plasma CORT and delay between capture and obtaining a blood sample (time delay in min) were square root transformed for normality. Androgen concentrations were marginally non-normal after log transformation and thus all analyses of androgens were conducted using either Wilcoxon / Kruskal-Wallis tests rank sums or Spearman rank correlations. However, relevant androgen figures are presented using log transformed data for greater clarity and separation between data points. We conducted all analyses using linear least squares models which we built including all potentially influential variables. All factors and interaction terms greater than P = 0.10 were removed from the final analyses.

RESULTS

Delay between capture and bleeding

Plasma CORT titers were significantly associated with time delay (in minutes) between capture and bleeding (Fig1: $F_{1,138} = 21.97$, $P < 0.0001$, $r^2 = 0.241$; mean delay = 13.58min, range = 1-45min). Although there was no significant interaction between delay and male type on CORT levels (Fig 1: $F_{2,139} = 1.28$, $P = 0.2815$ (type by delay); $F_{2,139} = 2.70$, $P = 0.0705$ (male type)), the plotted regression suggests that the increase of plasma CORT with time between capture and bleeding was blunted in auxiliary males compared to the two breeding male types. Indeed, while both breeding male phenotypes responded significantly to delay ($F_{1,64} = 17.78$, $P < 0.0001$, $r^2 = 0.22$ (brown); $F_{1,47} = 14.04$, $P = 0.0005$, $r^2 = 0.23$ (red/black)), auxiliary did not respond ($F_{1,28} = 1.504$, $P = 0.1689$, $r^2 = 0.07$).

To establish a time profile of CORT secretion in response to capture and handling stress (i.e. time delay) using individual rather than re-sampled males, we subdivided time delay into categories (1 = 1-5min, $N = 30$; 2 = 6-10min, $N = 36$; 3 = 11-15min, $N = 30$; 4 = 16-20min, $N = 27$; 5 = 21-25min, $N = 10$; 6 = 26-30min, $N = 5$; 7 = 31-45min, $N = 10$) and examined the relationship between plasma CORT and delay category. Across all males, response to delay was curvilinear (Fig 2a: $F_{6,138} = 6.70$, $P < 0.0001$, $R^2 = 0.2257$) with similar levels of CORT for the first 10 minutes (categories 1-2) (Tukey adjustment for multiple comparisons, $\alpha = 0.05$) and from 11 to 45 min (categories 3-7): categories 1-2 were both significantly lower than categories 3-7 (Fig 2a). As such we designated these categories as "baseline" CORT (1-10min, mean = 6.38min, $N = 69$) and "stress-induced" CORT (11-45min, mean = 19.56min, $N = 82$). Within the baseline category, CORT was no longer significantly correlated with delay ($F_{1,61} = 0.83$, $P = 0.3661$, $R^2 = 0.01$) and thus delay was not corrected for in further analyses of basal CORT levels. In the stress induced category, delay was still significantly associated with CORT levels ($F_{1,80} = 5.26$, $P = 0.0245$, $R^2 = 0.06$) and hence was corrected for in further analyses. As with our

analyses of time delay (as a continuum), there was a difference in response to delay category between the three male types (Fig 2b). The stress response profiles for the two breeding male types were similar to one another and closely mirrored the among male response (Fig. 2a); the stress response profile of auxiliary males was muted, with delay category 4 (16-20 min) being the only category with significantly higher CORT levels compared to all other categories (Tukey adjustment for multiple comparisons, $\alpha = 0.05$).

CORT , breeding status, and male phenotype

Baseline CORT levels (defined as up to 10 min delay) did not differ between the three male phenotypes (Fig 3a: $F_{2,60} = 1.87$, $P = 0.1632$, $R^2 = 0.06$) nor did they vary with breeding status (Fig 3b: auxiliary vs. breeder; $F_{1,61} = 0.43$, $P = 0.5141$, $R^2 = 0.01$). In contrast, stress induced CORT levels differed significantly with breeding status such that breeding males had higher plasma CORT than did auxiliaries (Fig 3b: ANOVA of status, delay, and the interaction of status by delay; $F_{1,78} = 4.52$, $P = 0.0368$, $R^2 = 0.15$ (status); $F_{1,78} = 0.40$, $P = 0.5275$ (delay); $F_{1,78} = 3.45$, $P = 0.0670$ (status by delay)). As with baseline samples, there was no difference in stress induced CORT between the three male phenotypes, although there was a trend (Fig 3a: $F_{1,78} = 2.37$, $P = 0.0998$, $R^2 = 0.12$ (type); $F_{1,78} = 4.85$, $P = 0.0306$ (delay); type by delay not significant).

CORT, body condition, and androgens

Across all male types, neither baseline nor stress-induced CORT levels were associated with body condition (Table 1, Fig 4a). However, there was a significant negative association between condition and baseline CORT in red/ black males. This association was confined to the

molting period (Table 1). Stress-induced CORT levels were unrelated to body condition among all males, within each male type, and regardless of presence or absence of molt (Table 1).

Surprisingly, androgen levels were positively associated with baseline CORT levels. This relationship was most prevalent during molt (Table 1, Fig 4b) and contrasted with our predictions. The positive association between CORT and androgens was strongest in the analysis of the relationship among all males and disappeared when examined within each male phenotype separately (Table 1). There was no association between CORT and androgen concentration for stress induced samples or in non-molting males. Consistent with our previously published work (Lindsay et al. 2009), the three male phenotypes differed significantly in plasma androgen levels (Wilcoxon / Kruskal-Wallis tests rank sums; $\chi^2 = 25.34$, $P < 0.0001$) and body condition was only correlated with androgens during the molting period (Fig 4c: Spearman rank correlations: molting males $P = 0.0442$, $r_s = 0.22$; non-molting males $P = 0.2552$, $r_s = 0.02$).

DISCUSSION

Our results contradict our proposed hypotheses that 1) chronically elevated CORT suppresses reproduction in auxiliary males, and 2) CORT enforces honesty of sexually selected plumage signals by mediating the relationship between body condition and plasma androgen concentrations. While stress induced plasma CORT differed significantly with breeding status (auxiliary vs. breeder), breeding and not auxiliary males had the highest CORT concentrations. Baseline plasma CORT did not differ with either breeding status (auxiliary vs. breeder) nor did it differ with male phenotype (auxiliary, brown breeder, red/black breeder). While there is a clear correlation between body condition and androgen production during molt and the development of breeding plumage color (Lindsay et al. 2009), we found no association between CORT and

body condition and a positive rather than negative association between molting male CORT and androgen titer. As such, it is highly unlikely that the observed relationship between body condition and androgen production is mediated by CORT levels, although CORT may be related to androgens in a different manner (see below).

CORT and breeding status

Although CORT appears to function in maintaining dominance rank for non-cooperative breeders (Creel 2001) and such a relationship has been documented for cooperative breeders (Young et al. 2006), our results do not indicate a role for CORT in cooperatively breeding red-backed fairy-wrens. These results are in line with results of other studies of cooperatively breeding birds in which elevated CORT did not predict helping behavior (Florida scrub jay, *Aphelocoma c. coerulescens*: Schoech et al. 1991; Schoech et al. 1997; Harris hawk, *Parabuteo unicinctus*: Mays et al. 1991; white-browed sparrow weaver, *Plocepasser mahali*: Wingfield et al. 1991; superb starling, *Lamprotornis superbus*: Rubenstein 2007; red-cockaded woodpecker, *Picoides borealis*: Malueg et al. 2009). Two crucial questions emerge from ours and other studies; why would dominate breeders have heightened CORT, and what suppresses androgens and reproduction in auxiliary males?

The relationship between CORT and social rank is highly variable and CORT can be elevated in subordinates or dominants under different circumstances (Creel 2001). Influential factors include the stability of the social hierarchy (Sapolsky 1992), frequency of antagonistic encounters (Creel 1996), relatedness of the members in a social group (Goymann and Wingfield 2004), and the physiological and metabolic demands of social rank (Goymann and Wingfield 2004; Rubenstein and Shen 2009). Therefore, the relative costs of acquiring and maintaining a

certain social position, rather than the social rank itself predicts variation in CORT (Creel 2001; Goymann and Wingfield 2004; Rubenstein and Shen 2009). If the costs of different social ranks (or breeding status) are equal, no differences in CORT levels are to be expected. This may be the case for red-backed fairy-wrens as baseline CORT did not vary with either breeding status or male phenotype.

CORT can also vary with familiarity and the degree of control an individual has over a particular stressor (Romero 2004). For example, subordinates living in permanent social groups are expected to have low levels of CORT as social interactions are mediated by the predictable behavioral roles of different group members and thus should rarely lead to fighting (Creel 2001). In support of this hypothesis, a recent study of cichlid fish showed that helpers exhibiting elevated levels of submissive behavior towards breeders had lower levels of cortisol, indicating that helper males may be able to reduce stress by appeasing breeder males through submission (Bender et al. 2006). Fighting within red-backed fairy-wren breeding groups is rarely observed and it may be the case that auxiliary males appease dominant breeders through submissive behavior. Because red-backed fairy-wren breeding groups consist of a breeding pair and their un-dispersed auxiliary (male) offspring from the previous season, reproductive conflict is predicted to be low for two reasons. First, auxiliaries receive indirect fitness benefits from aiding in the care of their siblings (Stacey and Koenig 1990), and second, there should be little incentive to compete for within group reproduction as a consequence of inbreeding avoidance (Varian-Ramos 2008).

It is apparent in Fig 1 and 2b that the stress response profiles for the two breeding male types differ from that of the auxiliary males; a result which explains the difference between auxiliary and breeding male types in stress-induced CORT concentration (Fig 3b). Differences in

CORT secretion in response to acute stress have been documented for other species with alternative reproductive strategies such as the white-throated sparrow, *Zonotrichia albicollis* (during non-reproductive phase, Schwabl 1995) and the tree lizard, *Urosaurus ornatus* (Knapp and Moore 1997; Moore et al. 1991). While we are beginning to understand, at a mechanistic level, how these differences might occur (eg. steroid binding globulins; Breuner and Orchinik 2000; Jenning et al. 2000; Moore and Jessop 2003), it is still unclear how they might evolve and what these differences might mean for sexual selection. A recent hypothesis gaining increasing attention posits that phenotypic differences in acute secretion of CORT during stress may be under direct selection by female choice (Husack and Moore 2008). While female choice of males with low CORT equates with selection against the negative health consequences of elevated CORT (reviewed by Breuner et al. 2008 and Bonier et al. 2009), choice of males with a rapid stress response (as seen in breeding male types) may equate with preference for males that are better prepared to cope with challenges in their environment (Wingfield et al. 1998; Husak and Moore 2008).

It seems likely that the rapid increases in circulating CORT documented in breeding male red-backed fairy-wrens may be of low cost to these male types for a number of reasons. First, while CORT is known to suppress immune function in temperate species (Sapolsky et al. 2000), experimental CORT manipulation had no effect on immunity in tropical birds (Martin et al. 2005; reviewed by Martin and Rubenstein 2008), and elevated CORT may not be a health detriment to breeding males. Second, although red-backed fairy-wren male phenotypes differ in annual reproductive output (Webster et al. 2008), they do not differ in survival (Karubian et al. 2008) and thus, there may be no fitness costs to social dominance. As such, the elevated stress response of breeding males may indicate a more flexible ability to deal with environmental,

competitive, and reproductive challenges rather than a cost to fitness, and indeed, this flexibility itself may be of selective advantage.

Our results support the hypothesis that auxiliary non-breeder behavior is a consequence of reproductive inactivation rather than complete reproductive suppression by dominate group members (Reyer et al. 1986). First, red-backed fairy-wren auxiliary males are not infertile (a hallmark of systems with "socially induced infertility"; Reyer et al. 1986). Although they do have smaller gonads (Rowe et al. 2010) and lower androgens (Lindsay et al. 2009), they are capable of siring offspring (Webster et al. 2008; Varian-Ramos unpublished) and produce viable sperm (Rowe et al. 2010). Second, given the opportunity to breed, auxiliary males rapidly assume the role of breeder, a transition accompanied by increases in androgens and enhanced expression of secondary sexual characters such as the size of the sperm storage organ and bill coloration (Karubian et al. in prep). It is therefore not surprising that chronically elevated CORT levels do not explain reproductive inactivity as this may lead to adverse physiological conditions (Chrousos and Gold 1992; Creel 2001) that would not allow for a rapid change in breeding role as observed in the red-backed fairy-wren.

The mechanism underlying low levels of androgens and reduced gonadal function of auxiliary males remains unclear. While these characteristics may result from bidirectional feedback between behavior and hormone production (Rubenstein and Hauber 2008), they could also stem from inactivation or insensitivity of the hypothalamic-pituitary-gonadal (HPG) axis upstream from the gonads (Adkins-Regan 2005). We have evidence to suggest that molting young males (assuming brown plumage) have lower levels of luteinizing hormone (LH) than molting older males (assuming red/black plumage), indicating reduced activity of gonadotropin secretion by the pituitary gland (Lindsay, unpublished data). For species in which non-breeding

cooperative group members can rapidly assume a breeding role (as is the case for red-backed fairy-wrens), reproductive inactivity is probably mediated by behavior and related to peptide production (e,g. gonadotropin-releasing hormone (GnRH)) rather than a complete shutdown of the HPG axis (Adkins-Regan 2005). Our results clearly support this hypothesis, although future studies have to be conducted to test this idea by examining pituitary LH secretion in response to GnRH challenge (see Spinney et al. 2006).

CORT, body condition, and androgens; consequences for production of honest breeding signals

Theoretically there are many reasons to predict a role for CORT in mediating acquisition of condition-dependent sexually selected signals (Buchanan 2000; Husack and Moore 2008) and there is some evidence to support such a function (Perez-Rodriguez 2006; Roberts et al. 2007), including evidence for CORT related effects on plumage signals (Roulin et al. 2008; Bortolotti et al. 2009; Mougeot et al. 2010). Our data do not support this hypothesis as we found no evidence for a correlation between CORT, body condition, and androgens to explain the honesty of sexual signal production in male red-backed fairy-wrens.

The influence of body condition on androgen secretion may be proximately mediated by the effects of CORT on some components of the HPG axis (Perez-Rodriquez 2006), and CORT is generally found to be related to condition (Moore and Jessop 2003; Rubenstein 2007, but see below). However, CORT is not a necessary intermediary in such a relationship as androgen production may be directly influenced by body condition (Wilson et al. 1979; Duckworth et al. 2001; but see Weatherhead et al. 1993; Foerster et al. 2002). Because both body condition (Lochmiller and Deerenberg 2000; Alonso-Alvarez and Tella 2001) and androgens (Roberts et

al. 2004) are related to immune function, it is possible that individuals in poor body condition are unable to produce high levels of androgens without risking cumulative negative consequences for immune health. Additionally, a two-way interaction may exist between body condition and androgens where increased sexual behavior (Vehrencamp et al. 1989; Marler and Ryan 1996) and basal metabolic rate (Buchanan et al. 2001) resulting from heightened androgens can negatively impact body condition. Thus, honesty of male red-backed fairy-wren plumage signals may be enforced by the relationship between body condition and androgens without the mediating influence of CORT.

CORT and body condition

While CORT is often negatively correlated with body condition (Moore and Jessop 2003; Rubenstein 2007), the dynamics of this association can differ taxonomically, with social rank, and with other variables that impact physiology such as parasite load and developmental experience (Sapolsky et al. 2000; Wingfield and Sapolsky 2003). In some species, CORT decreases linearly with increasing body condition, while in others, a threshold needs to be reached before a correlated increase in CORT is induced (Moore and Jessop 2003). Thus, it is possible that a relationship between CORT and body condition exists in red-backed fairy-wrens, but due to our sampling, we were unable to detect the condition threshold.

Interestingly, we did find a negative correlation between CORT and body condition for molting red/black males, a finding even more unique in that red/black breeders are in the best body condition of the three phenotypes during molt (Lindsay et al. 2009). Although the process of molt is energetically expensive (Lindström et al. 1993; references in Kurvers et al. 2008), this expenditure is presumably shared equally by all male types. However, there may be an additional

expenditure suffered by molting red/black males due to the production, mobilization, and deposition of carotenoid and melanin pigments into developing feathers (Jawor and Breitwisch 2003; Peters 2007). Thus, the heightened body condition of molting red/black males may buffer against simultaneous costs of molt, pigment deposition, high testosterone necessary for production of bright nuptial plumage, and maintenance of dominance rank. These costs would leave very little energetic resources to spare, potentially resulting in the observed negative correlation between body condition and CORT. In support of this hypothesis, CORT is not correlated with body condition in non-molting red/black males, and non-molting male phenotypes no longer differ in body condition (Lindsay et al. 2009).

CORT and androgens

The positive correlation between male androgen and CORT levels during molt is surprising although it is not unheard of for studies to report positive associations between stress hormones and reproduction (Moore and Jessop 2003). This could occur because at moderately elevated levels, CORT mobilizes energy stores and may facilitate energetically costly reproductive behavior (Moore and Jessop 2003). Indeed, many species show a seasonal increase in baseline CORT with the onset of reproduction, an effect particularly prevalent in studies of anuran amphibians where plasma CORT increases alongside of plasma androgens in response to reproductive behaviors such as amplexus and calling (Orchinik et al. 1988; Emerson 2001). Red-backed fairy-wrens demonstrate extreme levels of reproductive promiscuity (Karubian 2002; Webster et al. 2008) and sperm competition (Rowe et al. 2010), and plumage color is a sexually selected trait that varies both between as well as within phenotype (Karubian 2002; Webster et al. 2008). Additionally, reproductive display behavior is elaborate and can include puffbacks,

petal carrying, and chases (Karubian and Alvarado 2003; Karubian et al. 2008) while maintenance of pair bonds requires extensive allopreening (pers. obs.). As such, male reproductive behavior is likely to carry enough energetic costs to explain the observed positive correlation between CORT and androgens, particularly early in the season (during the pre-nuptial molt) when males face the simultaneous challenges of plumage color acquisition, territory establishment, and assumption of breeding role.

Plasma androgens can also increase at initial stages of the acute stress response (Rivier and Rivest 2003; Wingfield and Sapolsky 2003; Chichinadze and Chickinadze 2008), particularly if the stressor is an antagonistic encounter (Chichinadze and Chickinadze 2008). This phenomenon may be more common for dominant individuals who have a greater probability of winning in a given competitive interaction (reviewed in Chichinadze and Chickinadze 2008). While red/black breeding males are dominant over brown males (Karubian et al. 2008) and breeding red-backed male fairy-wrens have the highest stress induced CORT and highest androgens, no within phenotype correlations between androgens and CORT were apparent.

Conclusion

In summary, our correlational data offers little evidence to support a major role for glucocortoids in mediating condition-dependent expression of sexually selected male traits or suppression of reproductive function in auxiliary male red-backed fairy-wrens. Experimental studies of responses of the HPG axis to GnRH challenges and effects of CORT on reproductive function and male breeding phenotype are warranted to understand the mechanisms that determine male phenotype, signal honesty, and ultimately fitness.

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Table 1: Results from separate ANOVA's examining the relationship between body condition and CORT and non-parametric Spearman rank correlations between CORT and androgens for baseline and stress-induced samples. We report results from analyses conducted on all baseline and stress induced males, the three male phenotypes, and subsets of the data divided into molting and non-molting males. For molting males, we also report relationships between CORT, body condition, and androgens for males molting into brown vs. red/ black plumage.

		Body Condition		**Total Androgens**		
	N	F	P	R^2	P	r_s
BASELINE	64	$F_{1,60} = 0.754$	0.3888	0.012	**0.0347**	0.2665
Auxiliary	10	$F_{1,7} = 0.003$	0.9580	0.000	0.6628	-0.1581
Brown	32	$F_{1,30} = 0.148$	0.7032	0.005	0.4243	0.1463
Red/Black	22	$F_{1,19} = 4.443$	**0.0486**	-0.189	0.9777	-0.0065
Molting males	31	$F_{1,27} = 0.000$	0.9922	0.000	**0.0501**	0.3609
Brown Plumage	19	$F_{1,16} = 0.312$	0.5839	0.019	0.2716	-0.3636
Red/Black Plumage	12	$F_{1,9} = 5.307$	**0.0467**	-0.371	0.1458	0.3468
Non-molting males	33	$F_{1,31} = 1.197$	0.2824	0.037	0.2681	0.1985
STRESS INDUCED	82	$F_{1,76} = 1.20$	0.2981	0.000	0.8525	-0.0209
Auxiliary	20	$F_{1,18} = 0.544$	0.4704	0.029	0.8256	0.0526
Brown	34	$F_{1,29} = 1.109$	0.3009	0.037	0.1904	-0.2301
Red/Black	28	$F_{1,25} = 1.163$	0.2911	0.026	0.8704	-0.0323
Molting males	55	$F_{1,51} = 0.630$	0.4309	0.006	0.9596	0.0070
Brown Plumage	35	$F_{1,32} = 0.008$	0.9268	0.000	0.4074	0.1445
Red/Black Plumage	20	$F_{1,18} = 0.508$	0.4850	0.027	0.2430	-0.2737
Non-molting males	25	$F_{1,22} = 0.764$	0.3917	0.005	0.8637	-0.0362

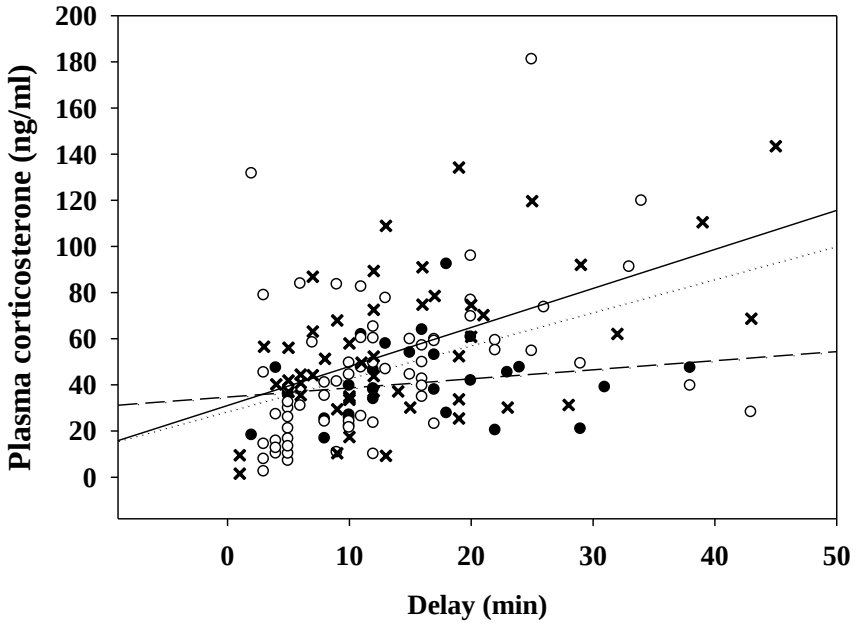

Figure 1: Independent regressions of plasma CORT (ng/ml) on time delay (min) between capture and bleeding for auxiliary males (closed circles, dashed regression line), brown breeding males (open circles, dotted regression line), and red/black breeding males (cross hatches, solid regression line).

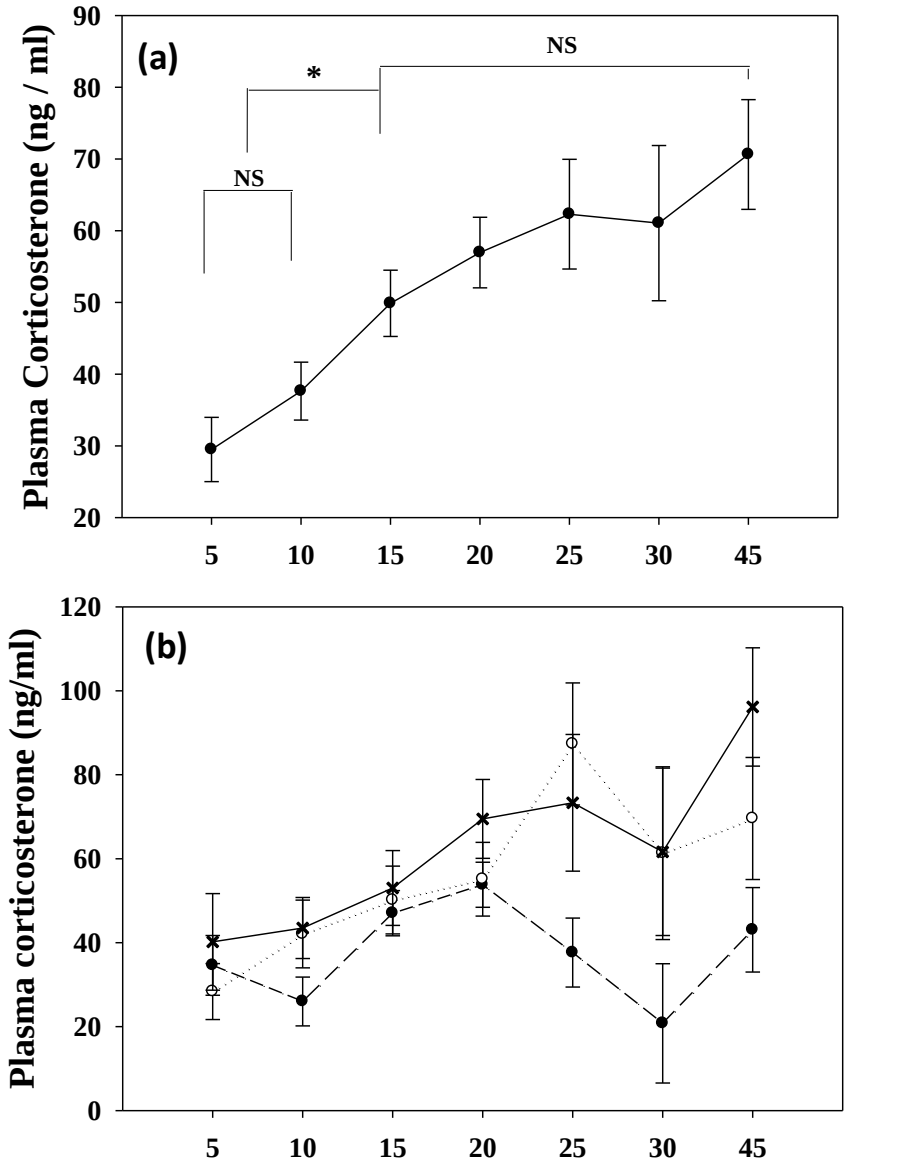

Figure 2: Stress response curves (mean per time category ± SE) averaged across all males (a) and within each male type (b; auxiliary males: closed circles, dashed line; brown breeding males: open circles, dotted line; red/black breeding males: cross hatches, solid line).

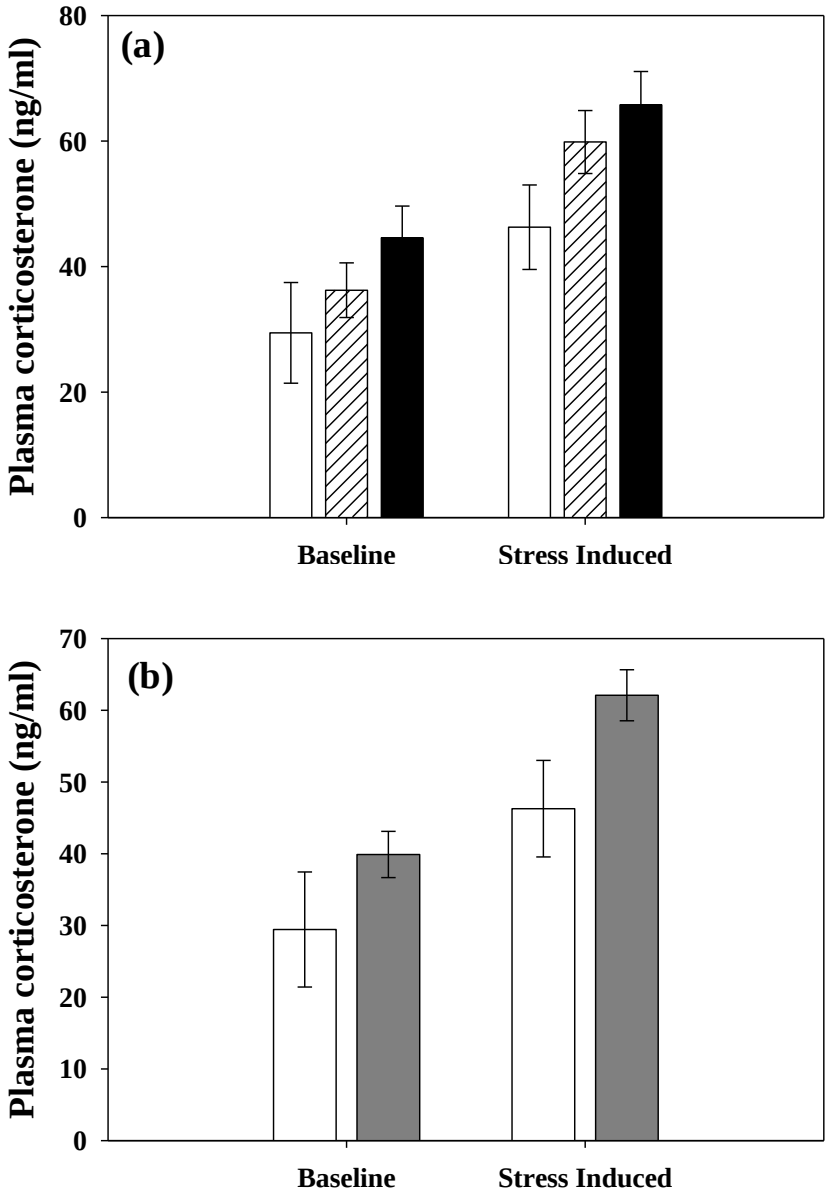

Figure 3: Differences in plasma CORT levels (ng/ml: mean ± SE) between male phenotypes (a) and between breeding status (b: auxiliary vs. breeder (gray columns)) in baseline and stress induced samples (auxiliaries are white columns, brown breeding males are cross hatched columns, red/black breeding males are black columns).

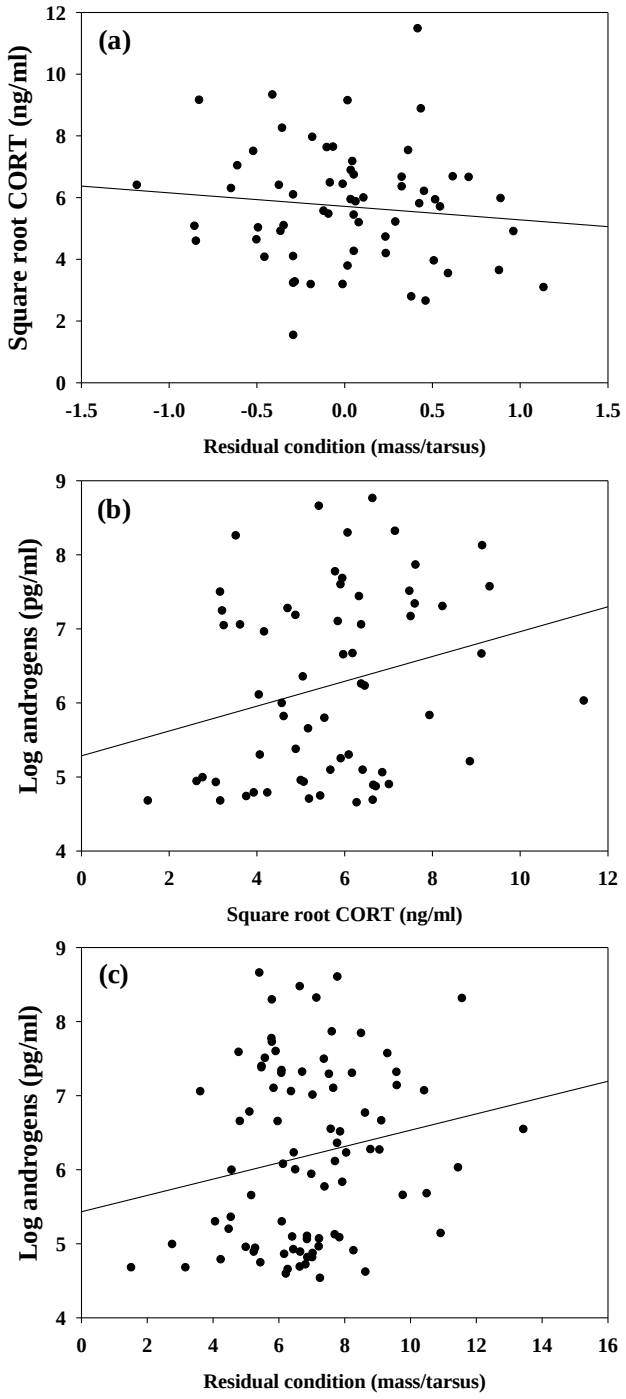

Figure 4: Regressions of baseline CORT (square root transformed ng/ml) with body condition (residuals of mass on tarsus; a) and total plasma androgens (log transformed pg/ml; b) as well as between androgens and body condition (c: molting males from both baseline and stress induced samples).

CHAPTER 3

SEXUALLY SELECTED MALE NUPTIAL PLUMAGE IS TESTOSTERONE DEPENDENT IN A TROPICAL PASSERINE, THE RED-BACKED FAIRY-WREN

INTRODUCTION

Intrasexual variation in male plumage color can act as a signal in both competitive and sexual interactions (Andersson 1994; Hill 2006; Senar 2006), and can reflect aspects of an individual's quality including age (Lyon and Montgomerie 1986), social rank (Senar 2006), and nutritional and physiological condition (Hill and Montgomerie 1994). The condition-dependence of these signals requires the presence of a costly mechanism in order to maintain honesty (Zahavi 1975; Cotton et al. 2004). Knowledge of these mechanisms informs hypotheses concerning the function and maintenance of plasticity in male reproductive plumage variation. Androgens, male gonadal steroids known to carry immunological (Folstad and Karter 1992; Roberts et al. 2004), energetic (Buchanan et al. 2001), and behavioral costs (Wingfield et al. 2001), are likely mediators of variation in many male sexual signals (Wingfield et al. 2001), but their role in plumage color variation is unclear (Owens and Short 1995).

Testosterone (T) is the primary androgenic steroid in most vertebrates, and may be involved in the control of plumage coloration in two ways. First, sexual dichromatism (i.e. plumage dimorphism during the reproductive phase of the annual cycle) can be T dependent (as is the case in Charadriiformes) whereby the presence of T induces molt into bright nuptial plumage and its absence into drab plumage (Owens and Short 1995; Kimball 2006). In Passerine

birds, an avian order for which male plumage color is repeatedly shown to honestly reflect body condition and lead to predictable variation in reproductive output (Hill and McGraw 2006), acquisition of bright male plumage appears to be T-independent and instead is regulated by luteinizing hormone (LH) (Kimball 2006). The lack of a role for T in Passerines is somewhat unclear, however, studies which established LH dependent sexual dichromatism were conducted on only a limited number of taxa, and most were performed on species with seasonally invariant plumage color that is acquired during a single annual post-nuptial molt (Witshi 1961; Kimball 2006). This latter point is particularly important because T levels are naturally low during the post-nuptial molt (Wingfield et al. 1990; Nolan et al. 1992), which occurs at a time when gonads are regressed in most species (Runfeldt and Wingfield 1985; Nolan et al. 1992). Thus, the role of T in determining production of bright nuptial plumage may hinge on hormonally active testes at the time of molt, such as for birds that undergo a pre- alternate (pre-nuptial) molt just prior to the breeding season when circulating T levels might be high.

Alternatively, T may regulate male intrasexual variation in the brightness or elaboration of color patterns; a role receiving increasing empirical support and which may even occur in species for which dichromatism is not T dependent (reviewed by Kimball 2006). The effects of T on intrasexual plumage variation may be direct (Peters et al. 2000), or may be indirect, for example through stimulating changes in other parameters that influence color patterns (e.g., preening behavior; Gonzalez et al. 2001; Roberts et al. 2009, or developmental processes; Collis and Borgia 1992; Strasser and Schwabl 2004). Moreover, in some Passerine species, T has been shown to delay onset of postnuptial molt (Schleussner et al. 1985), lead to the production of drab plumage (Stoehr and Hill 2001), or have no effect on plumage brightness (Day et al 2006). Discrepancies between passerine species in their response to T might also be explained by the

timing of molt relative to breeding (i.e. the type of molt: pre- versus post-nuptial).Thus, T might directly regulate plumage color and maintain the honesty in plumage signal production in only a subset of passerine species.

Australian fairy-wrens (*Malurus*, Maluridae) are a group of Australian Passerines that show pronounced sexual dichromatism during the breeding season, but not during the non-breeding season. In contrast to most passerines studied to date, nuptial plumage is acquired during a pre-nuptial (pre-alternate) molt (Rowley and Russell 1997) that can overlap with reproduction and thus occurs at a time when the gonads are active (Peters et al. 2000; Lindsay et al. 2009). Previous work with *Malurus* wrens suggests that they also differ from other studied passerine birds with respect to the key role of androgens in plumage color production. Indeed, the Superb Fairy-wren (*Malurus cyaneus*) is the only passerine species for which experimental evidence clearly indicates that sexual dichromatism is T dependent (Peters et al. 2000; Peters 2007a).

Red-backed Fairy-wrens (*Malurus melanocephalus*) are an intrasexually polymorphic congener of the Superb Fairy-wren. First-year males in this species adopt one of three breeding phenotypes that differ in behavior, morphology including plumage color, and reproductive success (Karubian 2002; Webster et al. 2008; Karubian et al. 2009). Specifically, males of this species can breed in red/black nuptial plumage, breed in brown female-like plumage, or remain as brown non-breeding auxiliaries on the natal territory. Correlational evidence suggests that male red-backed fairy-wren plumage color is T-sensitive as both plumage color and T are correlated with body condition during the pre-nuptial molt (Lindsay et al. 2009). Thus, red-backed fairy-wrens offer an ideal model system for assessing both the function of T in the

control of sexual plumage dichromatism as well as in regulation of condition-dependent intrasexual variation in male plumage signals.

We experimentally tested the hypothesis that T levels during molt mediate the acquisition of red/black vs. brown nuptial plumage in male red-backed fairy-wrens. To test our hypothesis we subjected first-year males to one of three hormone implant treatments: T, Anti-T (a combination of an aromatase inhibitor and an anti-androgen, see methods), and control implants. We predicted that T supplementation would lead to the production of the red/black plumage type and that both Anti-T and control treated males would acquire brown plumage (with the possibility for some control males to molt red/black as 15% of first year males naturally obtain red/black plumage; Webster et al. 2008; unpublished data). The alternative hypothesis that red/black plumage is T independent (as is appears to be case for many other passerines) predicts that all treatments should yield a similar plumage type. A second alternative, that male red/black plumage is LH dependent (as in most other passerines), would predict brown plumage production in T and control implanted males (with the same caveat for control males as stated above) and red/black plumage in Anti-T males. This latter would be due to elevated levels of LH resulting from antiandrogen treatment reducing negative feedback inhibition of T on LH secretion by the pituitary gland.

METHODS

Field Methods

Red-backed fairy-wrens are common cooperatively breeding insectivorous passerines with a widespread distribution across northern and north-eastern Australia. We conducted our

experiment on members of a partially color banded population of red-backed fairy-wrens near Herberton, Queensland (145˚23'E, 17˚23'S) during the austral spring of 2007 (August – October). This site is located in the inland open forest of the Atherton tablelands with sparse Eucalyptus overstory and ground cover dominated by tall grasses, in which the wrens build their dome shaped nests, and shrubs frequently used for forage, perching, and retreat.

Red-backed fairy-wrens molt twice per year, with most birds assuming brown plumage during a pre-basic molt (post-nuptial) that occurs after termination of breeding and all individuals acquiring breeding plumage during a pre-alternate molt (pre-nuptial) that occurs between August and December (overlapping somewhat with breeding; Lindsay et al. 2009). A small percentage of old males (ages 2+) assume a red/black nuptial-like plumage during pre-basic molt (pers. obs.). Indeed, of 40 older males trapped between Aug 22 and Sept 22 of 2007, only 4 (10%) had red/black plumage, two of which were molting to replace existing red/black plumage with new red/black feathers, indicating that the bright plumage type was maintained throughout the non-breeding season (as may be the case for some older or high quality males in other species of fairy-wren; Dunn and Cockburn 1999). Brown after-second-year males in basic (non-breeding) plumage can be differentiated from females by the presence of a black alula and darkened bill (pers. obs.), whereas second-year (age 1) males prior to the assumption of breeding morphology cannot be visually distinguished from females which have brown plumage during both the reproductive and non-reproductive phase.

The red/black nuptial plumage type acquired by many males for the breeding season consists of red carotenoid based feathers (K. McGraw and M. Webster, unpublished) on the back and scapulars, and black (presumably melanin based) feathers on the head, tail, belly, chest, and outer wing coverts. Primary and secondary wing feathers are brown on all birds, and wing

feathers appear to molt progressively through the season (ie. not confined to the pre-nuptial molting period; pers. obs.). The brown plumage type consists of brown feathers on all body regions except for the chest and the upper belly which is white. We calculated the percent of the body covered in red/black nuptial plumage (% brightness; Karubian 2002) as twice the cumulative brightness score (0-10; brown-red/black) summed across five independently scored body regions; head, back, tail, belly, and chest. While all males aged 2+ assume the red/black nuptial plumage type, young males (age 1) molt into either the red/black or the brown plumage type. As such, the distribution of brightness scores across the population is highly bimodal with few males of intermediate coloration. We designated males into two discrete plumage classes; either red/black if their brightness scores were ≥67% or brown if their brightness scores were ≤33% (only a small number of males fall in the range between 33% and 67%; Webster et al. 2008).

Bill color is a signal of breeding status in this species (Karubian 2008), ranging from a pale cream to ebony black. We visually scored bill color on a scale of 1-10 and calculated bill color as the sum of scores taken from four sections of the bill (top and bottom, anterior and posterior; cumulative bill color ranges from 1-40). To reduce subjectivity in bill color score, we designed a red-backed fairy-wren "bill color ruler". In brief, we constructed this "bill color ruler" in Adobe Photoshop using standardized digital photos (against a color checker embedded in each photo) of a range of red-backed fairy-wren bills covering the spectrum from light to dark. After sampling colors from all photographed bills, we calculated brightness (in grayscale) and arranged these sampled color patches into a continuous ruler that was divided into ten sections corresponding with our scale from 1-10. We slid the bill of each captured bird along the ruler until we settled on a matching color score.

In addition to acquiring nuptial plumage (red/black, brown, or intermediate), the onset of the reproductive phase of males is marked by the darkening of the bill (Karubian 2008) and the growth of the cloacal protuberance (CP) (Karubian 2002). As with other fairy-wrens (Rowley and Russell 1997) the posterior portion of the red-backed fairy-wren male's cloaca is swollen and enlarged and the anterior edge is elongated into a narrow tip. We took three measurements of the posterior protuberance: length (L), width (W) and depth (D) and measured the length of the anterior tip. From this we calculated the CP volume using the formula $\pi \times D/2 \times W/2 \times L$ (Mulder and Cockburn 1993; Tuttle et al. 1996; Karubian 2002).

All captured birds in our study population were either of known age and parentage or were assigned a minimum age based on the degree of skull ossification (ossification scale ranging from 0 to 6; 0-3 = age 1, 4-6 = age 1+; modified from Pyle et al. 1987 based on ossification patterns of known aged individuals). Trapping occurred either between the hours of 0500-1100 or 1500-1800. Upon capture, birds were weighed to the nearest 0.1 grams using a pesola spring balance and a series of standard morphological measurements were taken including: plumage brightness, body molt score, CP size, bill color, tarsus and wing length, the quantity of fat stored in the furcular hollow (range of 0-3), and the size of the keel muscle (range of 0-3). We scored molt visually on six body regions (head, back, wing, tail, belly, and chest) as none (0), light (1), medium (2), or heavy (3) based on the proportion of feathers in pin. A bird was considered to be molting with a cumulative molt score of 2 or more. In no case were the pins resulting from feather plucking at previous captures (see experimental methods) considered in the molt score. Birds were placed in custom designed holding bags with plastic inserts for sterile collection of fecal samples (Lindsay et al. in prep). From each captured bird we collected a maximum of 80µl whole blood from the jugular vein using a 29 gauge insulin needle, and

transferred the blood to heparinized microcapillary tubes. Within 6 hours of collection, we centrifuged blood using a capillary rotor for 5 minutes at 2000 rpm, measured haematocrit, and stored both plasma and feces in liquid nitrogen until transport to Washington State University where it was kept at -20ºC awaiting further analysis.

Experimental methods

Captured first-year birds (known age of 1) were randomly assigned to one of three treatments; a testosterone ("T males"; crystalline testosterone – Sigma T1500) implant group (N=15), a paired Anti-Testosterone treatment group ("Anti-T males"; N=13), and a control group of birds that received an empty implant ("control males"; N = 12). The effects of T on behavior and morphology can result from actions of androgens via the androgen receptor pathway or can stem from enzymatic conversion of testosterone to estrogen by aromatase and subsequent actions via the estrogen receptor pathway. Therefore, the Anti-T treatment consisted of a combination of an aromatase inhibitor (blocking T conversion into 17β-estradiol) and an anti-androgen (an androgen receptor antagonist), thus blocking both possible pathways by which T might affect plumage. Specifically, we used 1-4-6 androstatrien-3,17dione (ATD: Sigma A7710), which is an effective, irreversible aromatase inhibitor (Foidart et al. 1995) and trifluoro-2-methyl-4'-nitro-m-propionotoluidid (flutamide or FLUT; Sigma F9397), which is a reversible androgen antagonist that blocks androgen receptors. Previous studies of songbirds have combined ATD and FLUT to effectively block actions of testosterone (Schwabl and Kriner 1991; Soma et al. 1999; Canoine and Gwinner 2002; Moore et al. 2005; Van Duyse et al. 2005).

Implant length was scaled according to other studies of small passerines with an effective length of 4-5mm, inner diameter of 1.47mm and outer diameter of 1.96mm (as in Peters et al.

2000). The ends of each implant were sealed with Silicone Adhesive (MED-1037, NuSil Silicone Technology). Silastic implants (Dow Corning) were set sub-cutaneously under the skin of the back adjacent to the back feather tract (Anti-T treated birds received one implant each on both sides of the back feather tract). Implants were inserted into a small (1-2mm) skin incision which was sealed with veterinary skin adhesive and a small piece of OpSite flexible wound dressing. The skin at the site of implant was disinfected with ethanol and implants were rinsed in saline prior to insertion in order to clean the exterior of excess hormone and begin the process of diffusion. Wrens were held immobile during the process of implantation by fastening outspread wings and paired legs with smooth alligator clips to a small piece of plywood and were kept calm by covering their heads with a piece of opaque fabric. Implantation occurred at the first sign of molt in the population (the first pin feather seen on a captured adult red-backed fairy-wren) and implants were removed after the completion of molt (~1month from implantation; see below).

We determined the sex of all previously unbanded experimental birds assigned an age of 1 year based on skull ossification after experimental treatment using standard molecular genetic techniques (below). As a consequence, we manipulated both males and females with a final sample sizes of N=8 T males, N=7 Anti-T males, and N=7 control males (results of female treatment will be reported elsewhere).

Implanted birds were trapped up to three times post-implant (implants were given between Aug. 21-Sept. 6) in order to assess changes in molt, plumage color, and body morphology. A "mid" treatment recapture was attempted between 15 and 24 days post-implant (mean=17.8 days; T N=6, Anti-T N=4, control N=6; Sept. 2 – Sept. 21). A "final" recapture was attempted between 35 and 56 days post-implant (mean = 45.2 days; T N=6, Anti-T N=5, control

N=6; Oct 3-Oct. 19) and implants were removed at that time. Finally, we trapped some treated birds for a "post-treatment" recapture (Dec 26 – Jan 4) between 115 and 130 days post-implant (mean=122.1 days; T N=3, Anti-T N= 4, control N=3).

In addition to standard measurements as described above, a series of additional measurements were taken at each recapture. At the time of implant, feathers were plucked from six body regions (head, back, wing (inner wing coverts), belly, chest and tail). In this way we standardized feather growth rates between individuals from different treatments and subsequently measured the growth and color of emerging pins in these regions. Feather color was scored as described above (0-10 where 0 = brown/white and 10 = red/black with intermediate values for dark browns and pale orange colors); no score was given to those regions lacking pin feathers. The length of pin feathers to the nearest 0.5mm (mid-treatment recapture) and final full feather length (final-treatment) were measured for all regions where feathers were plucked. Feathers were re-plucked from all the same regions at the final recapture and the color and length of the resulting new feather growth was scored ~3 months later at the post-treatment recapture.

Genetic sexing methods

We determined sex using standard methods (Varian-Ramos et al. 2010) by amplifying an intron within the CHD gene with the primers 1237L and 1272H (Kahn et al. 1998). Our PCR reactions contained 0.15 mM dNTPs (each), 0.50 µM primers (each), 2.0 mM MgCl2, 2.5 units *Taq* polymerase, and 1 µl DNA suspended in sterile water (approximately 50 ng genomic DNA) and were run on an Applied Biosystems GeneAmp PCR System 9700 (3 min denaturation at 94° C, 30 cycles of 94° C for 60 sec, 57° C for 60 sec, and 72° C for 45 sec). We visualized PCR

product with electrophoresis using a 3% ethidium bromide stained agarose minigel. Two known sex adult birds were run per gel and those individuals with one band were determined to be male whereas those with two bands were scored as female.

Plasma and fecal steroid radioimmunoassay

Plasma androgen and corticosterone assays followed standard Schwabl lab techniques (Schwabl 1993; Lindsay et al. 2009; Lindsay and Schwabl in prep) as did fecal androgen, corticosterone, and estradiol assays (Lindsay and Schwabl in prep). Intra- and inter-assay coefficients of variation were within those of previous studies (Chapter 2; Lindsay et al. 2009; Lindsay and Schwabl in prep).

Luteinizing hormone assay

We analyzed plasma from the mid-treatment recapture for LH concentration including 3 Anti-T males, 6 T males, and 6 control males. In addition, we analyzed the LH concentrations of 5 unmanipulated older males (ages 1+) molting into red/black plumage and 3 young males (ages 1) molting into brown plumage (both old and young molting male samples taken from the same population during the same season as experimental birds). We measured LH by a postprecipitation, double-antibody radioimmunoassay (Follett et al., 1972, 1975; Sharp et al., 1987). This assay utilizes purified chicken LH for standard curves and for radio-iodination and goat anti-rabbit gamma globulin precipitating serum as second antibody. This assay has been used extensively for measurements of circulating concentrations of LH in a variety of avian species. Further details of the LH assay are described by Wingfield et al. (1991), and intra- and inter-assay variabilities were similar to those of previous studies.

Statistics

None of the response variables of interest (molt score, bill color, feather color, percent brightness, feather length, hormone concentration, body condition) met the assumptions of normality necessary to fit standard least-squares models, nor did they respond to transformations to normalize residuals. Therefore we assessed the influence of treatment, time trapped (implant, mid, final, or post-treatment) and the interaction between treatment and time using the Sheirer-Ray-Hare test, a nonparametric version of a two-way ANOVA modified from the Kruskal Wallace test (Sokal and Rohlf 1995). Although we conducted our experiment using a repeated measures design, we had considerable missing data with individual's trapped one or more but rarely all four times across the course of the season. In consequence, we chose to accept the error associated with violating the assumption of independent sampling between time periods in our analysis. Measures of morphology for unmanipulated aged 1+ red/black males (N=48) are plotted alongside those of implant, mid, final, and post-treatment experimental re-trap periods by averaging values across the dates surrounding these four trapping periods (Aug 21-Sept 6, Sept 7- Sept 21, Sept 22-Oct 19, and Dec 26-Jan 4). We used Kruskal Wallace tests to examine differences between treatment and unmanipulated male morphological values. We log transformed LH for normality and analyzed LH using standard least squares models. We calculated body condition as the residuals of a regression of mass on tarsus (Lindsay et al. 2009). All analyses were conducted using JMP 7 and Excel 2007 (Microsoft Office). For all figures we present means ± standard errors.

Animal care and ethics

We treated all captured and implanted birds in a safe and human manner. Silastic implants were set sub-cutaneously under the skin of the back, a location shown to reduce interference with connective tissue and chances of skin rupture (Ornithological council guidelines to use of wild birds in research). As Red-backed Fairy-wrens show high sight fidelity with defined territories, we were able to recapture almost all birds to remove empty implants. However, we failed to re-trap 4 birds that most likely dispersed from the study site. Previous research on other passerines has shown that long term maintenance of empty implants (implants from which all hormone has diffused) have no effect on survival (Wingfield 1984). All procedures were approved by the 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. The Australian Government Department of Environment and Heritage approved export of samples from Australia.

RESULTS

Androgens

T treatment effectively increased circulating and excreted levels of T: plasma androgen levels of T-implanted males were higher than for unmanipulated males sampled in the population during the same season (T implant mean=1,215pg/ml ± 405 (SE), range 123-5,127; median =473; population mean = 758±127, range 101-3,356, median = 520, N=28). These levels were physiological as they did not exceed maximum values documented in male red/backed fairy-

wrens (highest value recording for a breeding red/black male = 9,302; pop mean across 7 years of sampling [2003-2009] = 981±60; range = 64-9,302, median=512, N=473). Both plasma and fecal androgen concentrations differed with treatment (Table 1; Fig. 1a,b) such that T males had the highest concentrations and control males the lowest. This difference disappeared by the post-treatment re-trap (Table 1). Anti-T treated birds had fecal androgen levels at the mid-treatment re-trap that did not differ from either T implanted males (χ^2=1.07, df=1, p=0.302) or control males (χ^2=0.5, df=1, p=0.479). Fecal androgen concentrations changed across the course of the experiment in T males (χ^2=11.01, df=3, p=0.012) with a dramatic increase following implantation and a decline after the implant was removed (post-treatment recapture; see Fig. 1a). However, there was no effect of time in the whole model including the other two treatment types (Table 1), nor was there an interaction between time and treatment.

Molt and plumage coloration

Testosterone treatment stimulated an earlier onset of molt relative to other treatment types (Table 1; Fig. 2). Brown feathers plucked at the time of implant were replaced by red/black nuptial feathers in both T and Anti-T males but not in control males (Table 1; Fig. 3a). However, Anti-T males produced feathers with a smattering of brown coloration and/or paler shades of orange to dark brown as compared to the normal bright red and black feathers produced by T males. Feathers plucked when implants were removed (final recapture) grew back brown on T and Anti-T birds, red/black on control birds that gained breeding positions, and brown on control birds that remained as helpers (Table1; Fig. 3a). As a result, the color of regrown feathers did not differ among the three treatment types at the time of the post-treatment re-trap (Table 1).

Full body coloration (% plumage brightness) differed significantly among the three treatments across the course of the experiment (mid, final, and post-treatment recaptures). T males molted into a red/black plumage type similar to that of older unmanipulated red/black males and control males assumed a typical brown first-year plumage type (Table 1; Fig. 3b). Anti-T treated birds initially showed an increase in full body plumage brightness, but this increase in brightness was truncated at the mid-treatment re-trap (Fig. 3b). This was likely due to implant loss, as the Anti-T implanted birds rejected the FLUT and ATD implants, with the skin around the implant reddened, swollen, and sometimes pussy and the implant partially or fully expelled (the other treatment types had near-perfect implant retention, with only 1 control implant lost). As a result, only one of the 4 Anti-T males sampled at the mid-treatment re-trap had both implants intact, one had lost both implants, and the other two only had the FLUT implant remaining. By the final re-trap, no Anti-T implants remained (N = 4 recaptured birds). All three treatment types changed in plumage brightness between the final and post-treatment recaptures (brightness of both T and Anti-T decreasing and control increasing; Fig. 1c). This was not a consequence of resumed molt but due to alterations in full body coloration resulting from feather plucking at the time when the implants were removed (see corresponding reduction in plucked feather color score at the post-treatment recapture in Fig. 3a and % brightness in Fig. 3b).

Feather length

The normal red/black and brown male plumage types (of unmanipulated birds) are characterized by differences in feather length as well as feather coloration. Feathers are shorter on the head, back, and tail in red/black males than in brown males (see Fig. 4a,b; head χ^2=5.36,

df=1, p=0.021, red/black N=5, brown N=6; back χ^2=4.05, df=1, p=0.044, red/black N=5, brown N=7; tail χ^2=5.46, df=1, p=0.019, red/black N=4, brown N=4; see also Swaddle et al. 2000; see Karubian et al. 2009 for differences in tail length). In line with these differences, T treatment significantly affected feather length. Feather regrowth from plucked regions did not vary in length with treatment at the time of the mid-treatment re-trap (Fig. 4a,b; head χ^2=0.152, df=2, p=0.927; back χ^2=2.56, df=2, p=0.278; tail χ^2=4.68, df=2, p=0.096) indicating that growth rates did not differ between the treatment types. But, by the final re-trap, both back and tail but not head feather lengths differed between treatments (back χ^2=6.68, df=2, p=0.035; tail χ^2=12.26, df=2, p=0.002; head χ^2=5.56, df=2, p=0.062;). Control males had feathers of similar length to standard brown plumage on unmanipulated brown males; both T and Anti-T males had feathers of similar length to unmanipulated red/black males (Fig. 2a). Feather length at the post-treatment re-trap did not differ between treatments (head χ^2=2.79, df=2, p=0.248; back χ^2=0.87, df=2, p=0.648; tail χ^2=0.127, df=2, p=0.938).

Cloacal protuberance (CP) volume

T stimulated the production of the CP. T males had measureable CP's by the mid-treatment recapture (Sept. 2 – Sept 21) with intermediate to low CP volumes (Fig. 5a; mean volume = 33.55 ± 3.63(SE), range 20.2-44.3; average breeding CP volume = 119.29 ± SE=2.5, N=348). In contrast, neither control nor Anti-T implanted males developed a measureable CP during the course of the experiment. However, after the implants were removed all treated males developed measureable CPs, and by the time of the post-treatment recapture these did not differ significantly in size between the treatment groups and unmanipulated red/black males (Fig. 5a; χ^2=0.436, df=3, p=0.9327). The first measureable CP found on an unmanipulated bird was

observed on October 18 (approximately 6 weeks after those seen in T males) on an age 4+ red/black male who had overwintered in the nuptial plumage and had a tiny, deflated swelling (CP volume = 18.2). Thus, the CP production stimulated in T implanted males far preceded any other such sexual development in the population.

Bill color

Bill color darkened across the treatment period for all implant groups and nonmanipulated males (Table 1; Fig. 5b). However, treatment types differed in bill coloration. The bills of T implanted males darkened earlier and more rapidly and were the darkest at both the mid and final recaptures. By the post-treatment recapture, all males had similar bill coloration. The darkening of T male bills followed a very similar trajectory to that seen in unmanipulated red/black males in the population (Table 1; Fig. 5b).

Plasma luteinizing hormone (LH)

LH concentration at the mid-treatment recapture differed between the three treatment types ($F_{2,12}$=13.44, p=0.0009; Fig 6). Consistent with our prediction, T implanted males had significantly reduced LH levels compared to control males. However, contrary to our prediction Anti-T males had intermediate concentrations of LH that did not differ from either control or T males (Tukey adjustment for multiple comparisons, α=0.05). There was no apparent difference in LH concentration between Anti-T males which had retained both ATD and FLUT implants and those with only the FLUT implant left. However, sample sizes are prohibitive to statistical analysis (N=3).

Corticosterone, body condition, and estradiol

The three treatment groups did not differ in either plasma or fecal corticosterone and the concentrations of these hormones did not change over the course of the experiment (Table 1; Fig. 7a). There was a nonsignificant difference in body condition between the treatments with a trend for better condition in T males and lower condition in Anti-T males (Table 1; Fig. 7b). Additionally, body condition but not fecal corticosterone of treated males was similar to that of unmanipulated males in the population (analysis of manipulated vs unmanipulated males: body condition χ^2=2.46, df=1, p=0.117; fecal corticosterone χ^2=6.17, df=1, p=0.013). This indicates that hormone manipulation did not negatively affect condition, but may have increased stress, albeit affecting all treatment types similarly. Fecal estrogen metabolite levels were not affected by treatment and did not change with time (Table 1; Fig..7c).

DISCUSSION

Testosterone, plumage color, and male phenotype

Our results provide strong experimental evidence that testosterone (T) is the primary mechanism controlling sexual dichromatism as well as regulating variable male breeding phenotype, including acquisition of male sexual plumage signals, in red-backed fairy-wrens. Experimental T treatment accelerated the acquisition of red/black nuptial plumage and breeding morphology. These changes closely mirrored those of unmanipulated older molting males in the population that also assumed the red/black phenotype. Both correlational analyses and experimental manipulation of social status indicate that bill color, CP volume, and plumage color are regulated by testosterone (Karubian 2008; Lindsay et al. 2009; Karubian et al. in prep),

findings that are now experimentally supported by this study. In addition, tail length, an intrasexually variable signal of male competitive ability (Karubian et al 2009), also appears to be a T-dependent trait as T treatment and subsequent removal of the implant predictably influenced the length of tail feathers (Fig. 4b). Removal of T implants led to a reduction in trait intensity and for most response variables, coincidence with character levels expressed by control males (Fig. 3a: feather color; Fig. 4a,b: feather length; Fig. 5a: CP volume; Fig. 5b: bill color). Moreover, feathers plucked when T implants were removed were replaced with longer brown feathers, indicating that the effects of T were reversible. Also, while bill color of unmanipulated males continued to increase towards the maximum darkness score, bill color of T males did not darken further once the implant was removed. Finally, feathers plucked at implant removal from control males who became breeders grew back red/black (Fig. 3a), a change that corresponded with an increase in the plasma and fecal androgen concentrations of these males (Fig. 1a,b).

The immunocompetance handicap hypothesis (Folstad and Karter 1992) posits that honesty of male sexual signals is ensured by pleiotropic actions of T, as T carries concomitant immunological costs. Although this hypothesis has received increasing support in studies of some avian breeding signals (Peters 2007b and references therein), the role of T in regulating plumage signals of male quality is less clear (Owens and Short 1995). We show here that experimentally elevated T modulates a suite of condition-dependent plumage and non-plumage traits which together comprise an intrasexually variable breeding phenotype correlated with known differences in reproductive success (Karubian 2002; Webster et al. 2008). While the costs of elevated T needed to acquire the bright male phenotype have not yet been determined for red-backed fairy-wrens, immunological (see Roberts et al. 2004 for review), metabolic (Buchanan et al. 2001), and behavioral (Wingfield et al. 2001) effects of T are well documented in other avian

species, including a congeneric species of fairy-wren (Peters 2000). Additionally, we previously documented a positive correlation between plasma T levels and condition during the critical period of molt when breeding phenotype is assumed (Lindsay et al. 2009), suggesting that only males in good body condition can afford high T production and/or withstand the costs of elevated T to produce the sexually selected bright plumage type. Thus, intrasexual variation in male red-backed fairy-wren plumage color supports honest indicator models of costly trait acquisition in that this variation reflects both T and body condition during molt.

Stress and body condition

T has been shown to increase corticosterone levels in experimental studies of other avian species (Evans et al. 2000; Duckworth et al. 2001). This effect has been speculated to lend support to the hypotheses of T production being costly for low quality individuals. However, body condition and corticosterone levels were not affected by high T in our experiment. Of the three treatment types, Anti-T treated birds had the lowest body condition (Fig 7a), a result that might be related to the infections associated with implant rejection. Also, while treatment led to increased corticosterone levels, this effect was shared by all treatment types and not confined to T treated birds (Fig 7b). As such, we have no physiological evidence to indicate a cost associated with high T. Future work should examine T effects on immune function and other possible costs of elevated T in this species.

Anti-T: an alternative explanation for bright plumage production?

Bright plumage production in male red-backed fairy-wrens is clearly not stimulated by estrogenic effects after conversion of T to estradiol by aromatase. First, our Anti-T treatment

which combined an androgen receptor blocker (FLUT) with an aromatase inhibitor (ATD) resulted in intermediate to bright plumage and not brown plumage. Second, there was no indication that the heightened levels of T measured in both T and Anti-T males (at the mid-treatment re-trap; Fig 1a) corresponded to heightened levels of excreted E2, which did not vary with either treatment or time from implant (Fig 7c). It can also be excluded that T induces molt into red/black nuptial plumage after reduction into 5α-dihydrotestosterone (DHT) because DHT acts via the androgen receptor which was blocked by FLUT.

Sexual dichromatism of passerines has been assumed to be regulated by LH (Witshi 1961; reviewed in Kimball 2006; but see Peters et al. 2000). While the production of red/black plumage by Anti-T males would be consistent with predictions of this alternative hormonal control mechanism, measured LH levels are not. Contrary to expectations, LH concentrations of Anti-T birds did not exceed those of control males, nor were they notably higher than those in T males (Fig 6). It is surprising that Anti-T males did not have high LH, given predicted elimination of T feedback on hypothalamic GnRH and pituitary LH secretion. FLUT antagonism of androgen receptors was predicted to reduce negative feedback of testicular T on the pituitary, increasing production of LH and explaining the observed increase in excreted T by Anti-T birds at the mid-treatment re-trap. Although we cannot dismiss the possibility that LH peaked prior to loss of ATD and FLUT implants, we were unable to detect any differences in LH between males with and without ATD and FLUT implants at the mid-treatment re-trap. Thus, we have no evidence that LH plays any role in acquisition of male nuptial plumage coloration in this system.

A possible explanation for both low levels of LH and bright plumage production by Anti-T birds is that FLUT acted, at least partially, as an androgen receptor agonist rather than antagonist. FLUT can have inconsistent effects such as upregulation of androgen receptor

mRNA and can cause time-dependent insensitivity to further antagonism (Chen et al. 2004; Fusani et al. 2007), but has performed reliably as an androgen receptor antagonist in other experimental studies of birds (Schwabl and Kriner 1991; Soma et al. 1999; Canoine and Gwinner 2002; Van Duyse et al. 2005). Additionally, Anti-T birds did not demonstrate other androgen-dependent responses such as the growth of the CP or the darkening of the bill (as seen in T males), suggesting that FLUT did indeed function as a T antagonist as intended. Regardless, Anti-T results call into question the simplicity of hypotheses indicating a single mechanism for control of sexual dichromatism. Although we cannot explain the effects of our anti-androgen treatment on plumage, these results hint at the possibility of multiple, mutually independent hormonal mechanisms controlling plumage color production and caution against making broad statements from results obtained using a single hormonal manipulation.

Testosterone-dependence and the timing of plumage color acquisition

Although it is often true that sexually selected variation in passerine plumage signals honestly reflects androgen levels during breeding (Saino & Møller 1994; Gonzalez et al. 2001; Duckworth et al. 2004; Peters et al. 2006), it is rarely the case that signal honesty is directly mediated by the effects of androgens at the time of trait formation (Stoehr and Hill 2001; Kurvers et al. 2008). Instead, T may be involved in organizing or maintaining intrasexual color variation without acting during molt to directly stimulate dichromatic plumage production. For example, yolk T organizes adult badge size in male house sparrows (*Passer domesticus*) (Strasser and Schwabl 2004) and nuptial plumage in male and female Black-headed gulls (*Larus ridibundus*) (Eising et al. 2006), elevated T in juvenile satin bowerbirds (*Ptilonorhynchus violaceus*) leads to early acquisition of adult plumage the year following hormone treatment

(Collis and Borgia 1992), and T treatment stimulates increased preening behavior of both the house sparrow (Møller and Erritzøe 1992; Gonzalez et al. 2001) and the blue tit (*Cyanistes caeruleus*) (Roberts et al. 2009), increasing badge size and crown UV chroma respectively. For many other species, T can delay (Schleussner et al. 1985; Stoehr and Hill 2001; Clotfelter et al. 2004; Kurvers et al. 2008) or prevent molt (Nolan et al. 1992), lead to the production of drab plumage (Stoehr and Hill 2001) or have no observable effect on plumage color (Day et al. 2006). Thus, most evidence to date suggests that, for passerine birds, T does not lead directly to acquisition of bright nuptial plumage. Instead T may indirectly influence plumage color through effects on behavior (e.g., increased preening), or the observed correlation between T and plumage color in these species may be due to correlations with other variables (e.g., body condition).

A unifying theme for many of the studies cited above is that the effects of T on plumage color were assessed during a post-nuptial, pre-basic molt, which occurs at a time when breeding has ceased, gonads are regressed, and T low (Schleussner et al. 1985; Ketterson and Nolan 1992). The results presented here, in combination with those of Peters et al. (2000), indicate that T may be causally related to acquisition of bright plumage, and therefore pivotally involved in regulating honesty of plumage signals, for species where plumage is acquired during a pre-nuptial molt just prior to breeding at a time when the gonads are recrudescing and hormonally active. Future studies on other species with a pre-nuptial molt are needed to test this hypothesis and further reveal this important mechanism of plumage color control not generally considered to function in passerine birds.

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Table 1: Results from 1) two-way nonparametric ANOVAs of treatment, time trapped (implant, mid, final), and the interaction between treatment and time and 2) one-way nonparametric ANOVAs of treatment at the post-treatment re-trap against a series of morphological and hormonal response variables.

	Treatment		**Time**		**Treatment by Time**		**Post-Treatment**	
	H*, df	p	H, df	p	H , df	p	χ^2, df	p
Molt score	6.84, 2	**0.033**	27.6, 2	**<0.0001**	9.45, 4	**0.051**	1.58, 2	0.452
% Brightness	15.8, 2	**0.0003**	20.6, 2	**<0.0001**	15.4, 4	**0.004**	6.20, 2	**0.045**
Feather color†	23.2, 2	**<0.0001**	1.29, 1	0.255	2.07, 2	0.354	0.65, 2	0.721
Bill color	22.4, 2	**<0.0001**	20.2, 2	**<0.0001**	6.17, 4	0.187	3.97, 2	0.138
Body condition	5.59, 2	*0.061*	0.32, 2	0.852	1.89, 4	0.754	3.97, 2	0.137
Fecal androgens	7.71, 2	**0.021**	4.20, 2	0.123	5.47, 4	0.242	1.80, 2	0.407
Plasma androgens!	6.89, 2	**0.032**	0.49, 1	0.480	6.52, 2	**0.032**	1.57, 2	0.456
Fecal corticosterone	0.76, 2	0.683	0.25, 2	0.884	0.36, 4	0.986	1.80, 2	0.407
Plasma corticosterone!	4.03, 2	0.133	3.05, 1	*0.081*	0.26, 2	0.876	0.70, 2	0.705
Fecal estradiol	0.87, 2	0.647	1.31, 2	0.519	4.63, 4	0.327	1.80, 2	0.407

*H~ χ^2

† Mid and Final only

! Implant and Final only

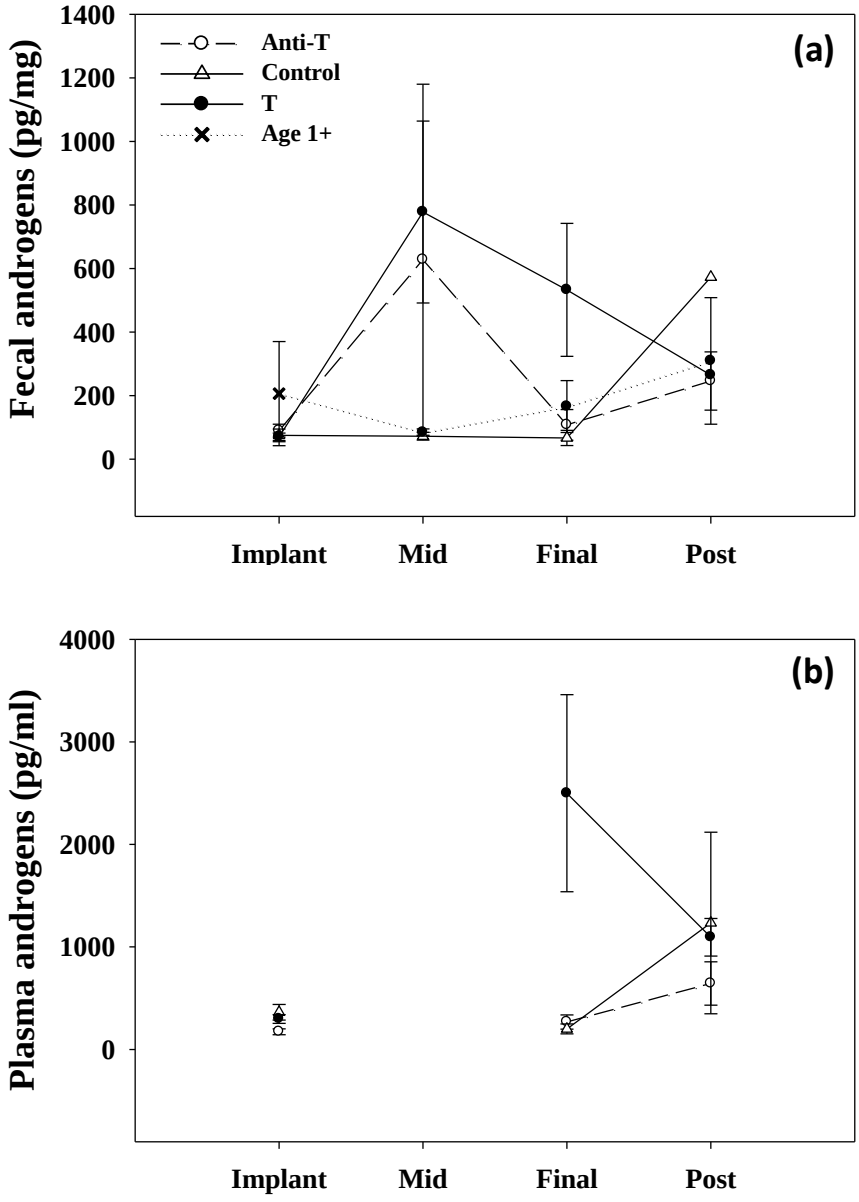

Figure 1: Fecal (a) and plasma (b) androgen concentrations for treated (T, Anti-T, control) and unmanipulated (age 1+) males across the four trapping periods of the experiment (implant, mid-treatment, final, and post-treatment).

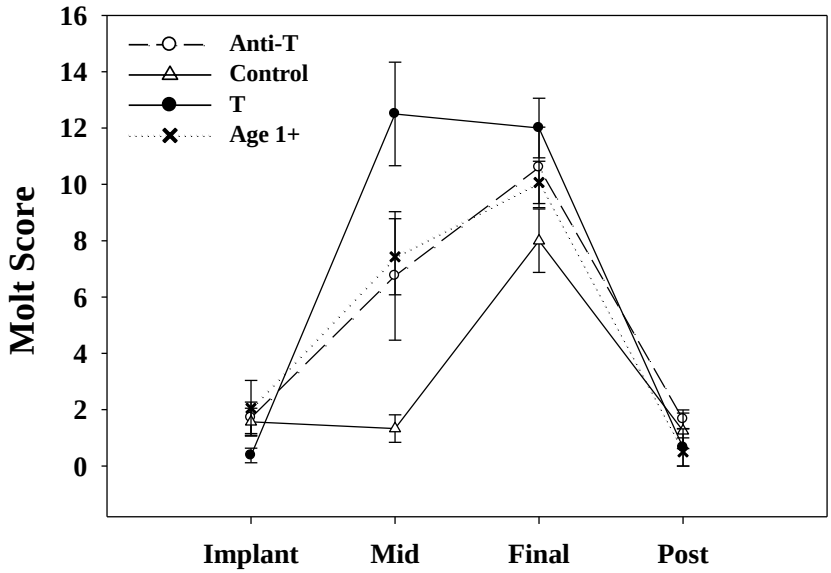

Figure 2: Cumulative molt score for treated (T, Anti-T, control) and unmanipulated (age 1+) males across the experimental season including averages from each trapping period (implant, mid-treatment, final, post-treatment).

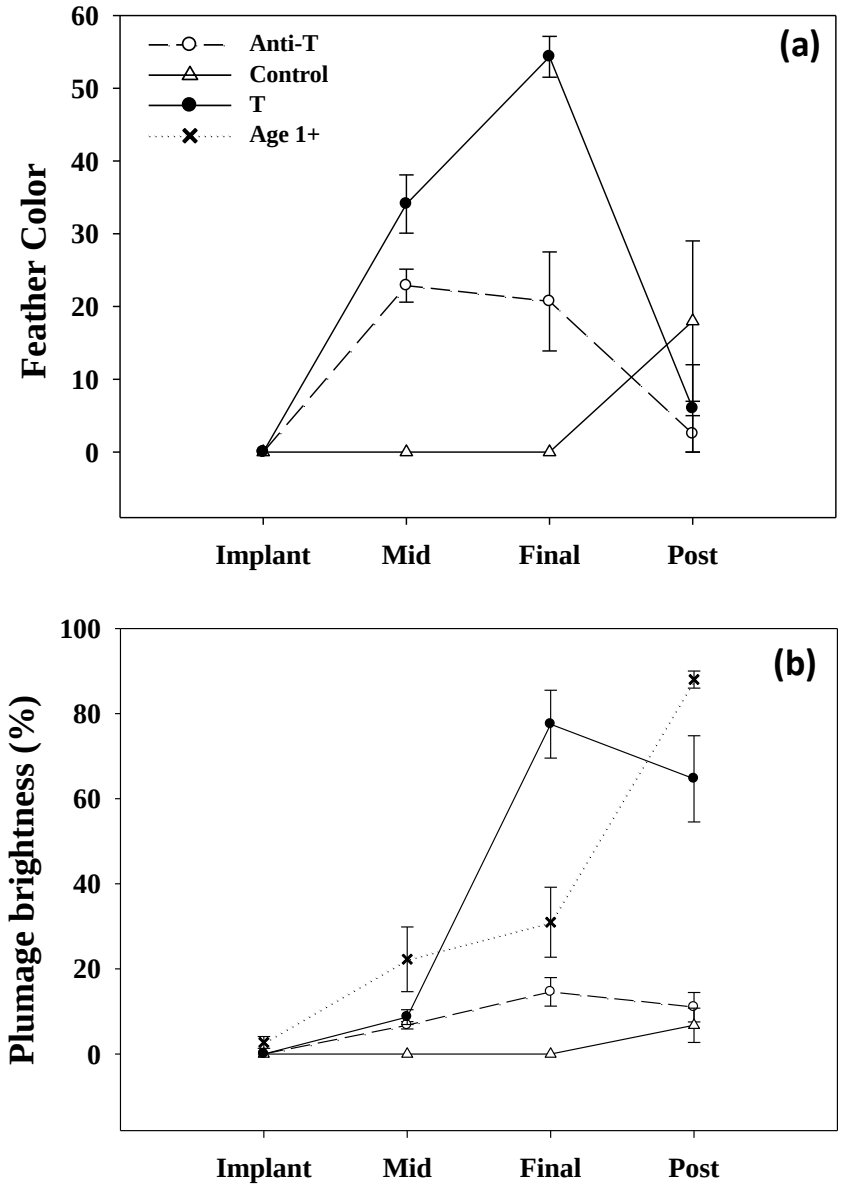

Figure 3: Color of plucked feather regrowth (a) and full body plumage brightness (b) for treated (T, Anti-T, control) and unmanipulated (age 1+) males across the four trapping periods of the experiment (implant, mid-treatment, final, and post-treatment).

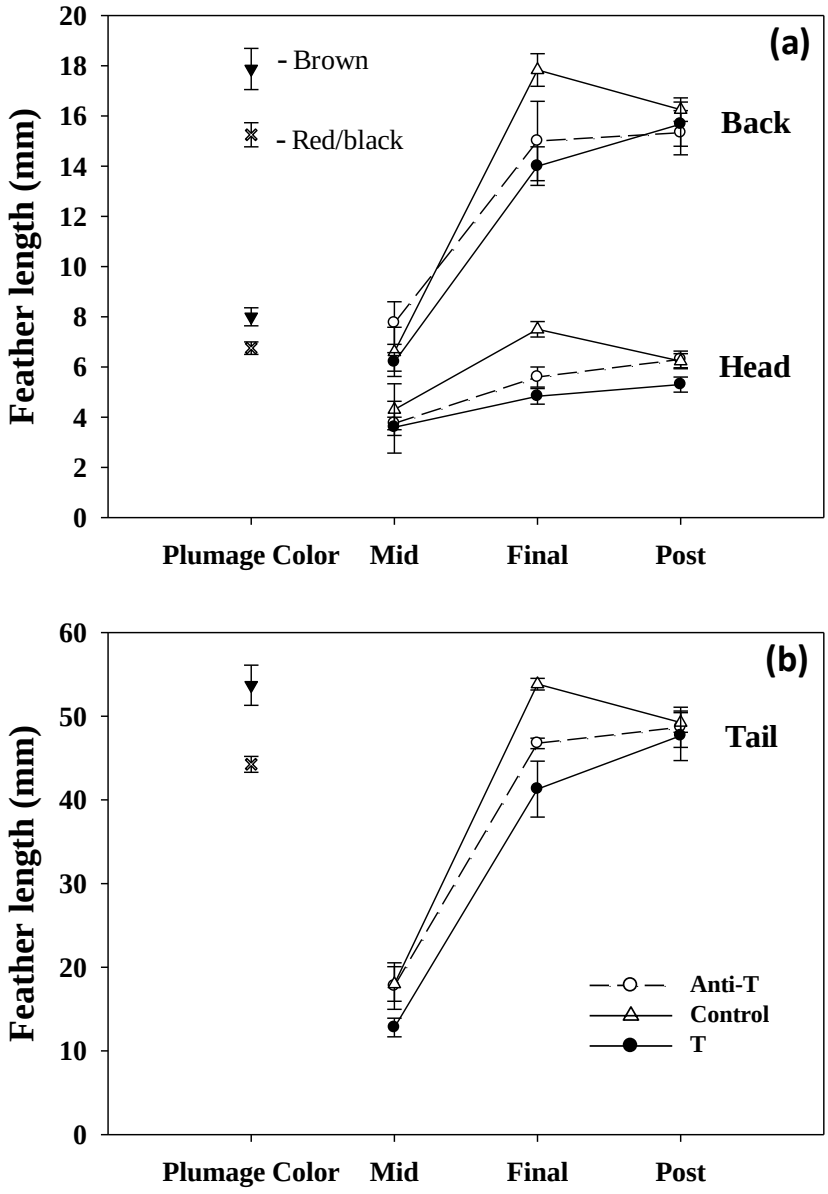

Figure 4: Variation across treatments (T, Anti-T, Control) and trapping period (mid-treatment, final, post-treatment) in the length of head and back (a) and tail (b) feathers. Mean ± SE of unmanipulated male red/black vs. brown plumage color are presented for each feather type.

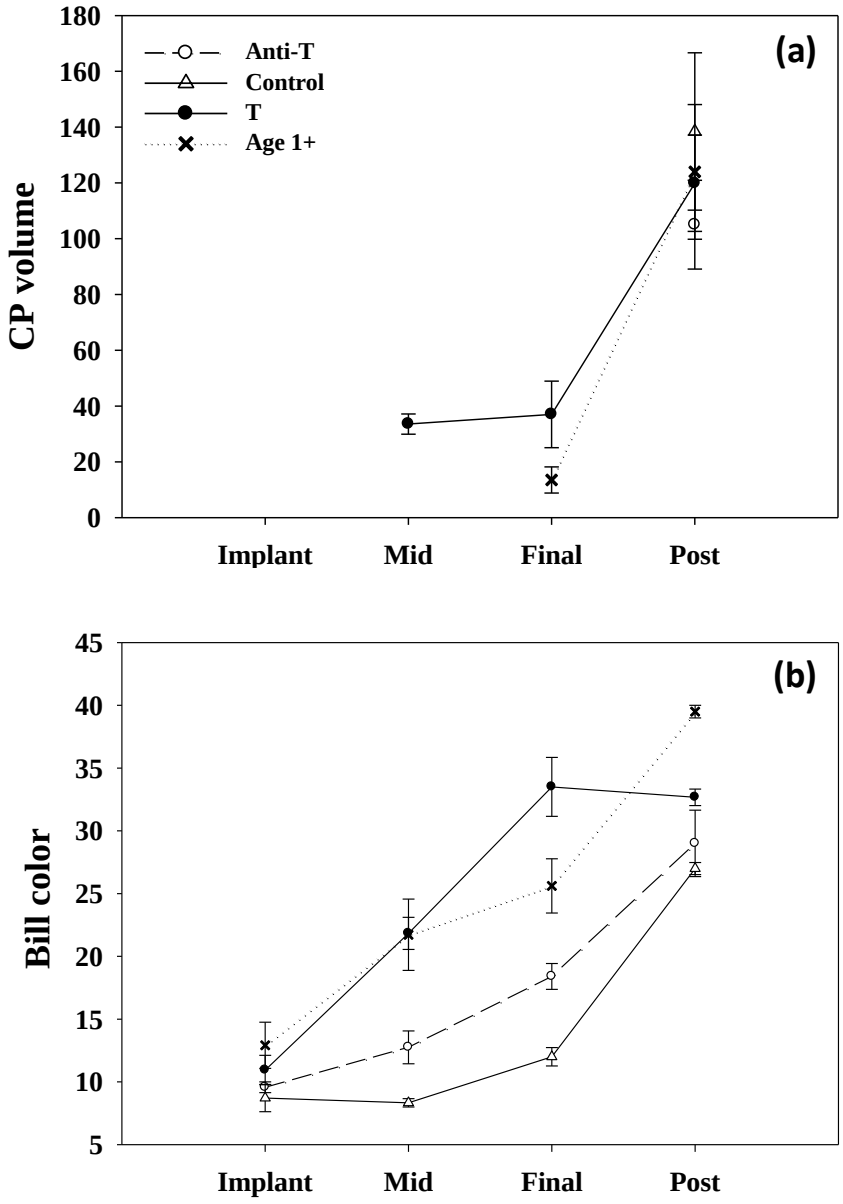

Figure 5: CP volume (a) and bill color score (b) for treated (T, Anti-T, control) and unmanipulated (age 1+) males across the four trapping periods of the experiment (implant, mid-treatment, final, and post-treatment).

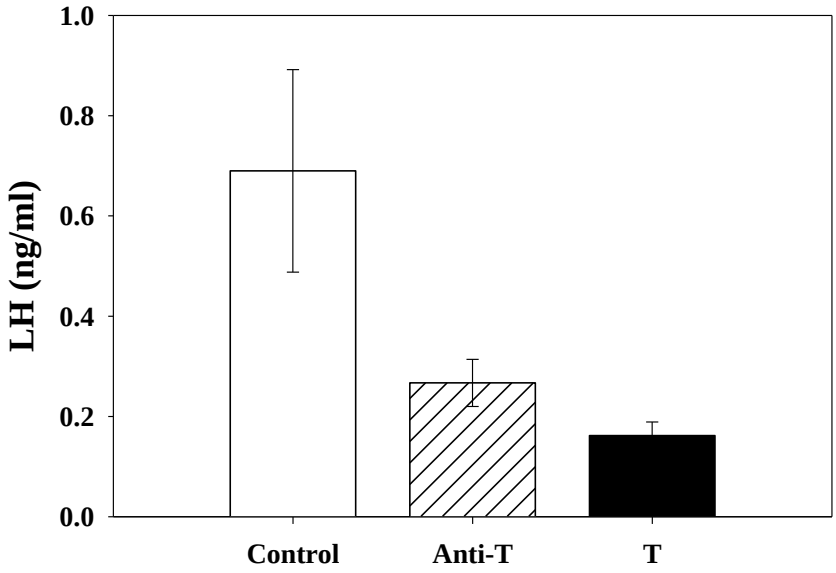

Figure 6: Mean ± SE LH concentration of the three treatment types at the mid-treatment re-trap.

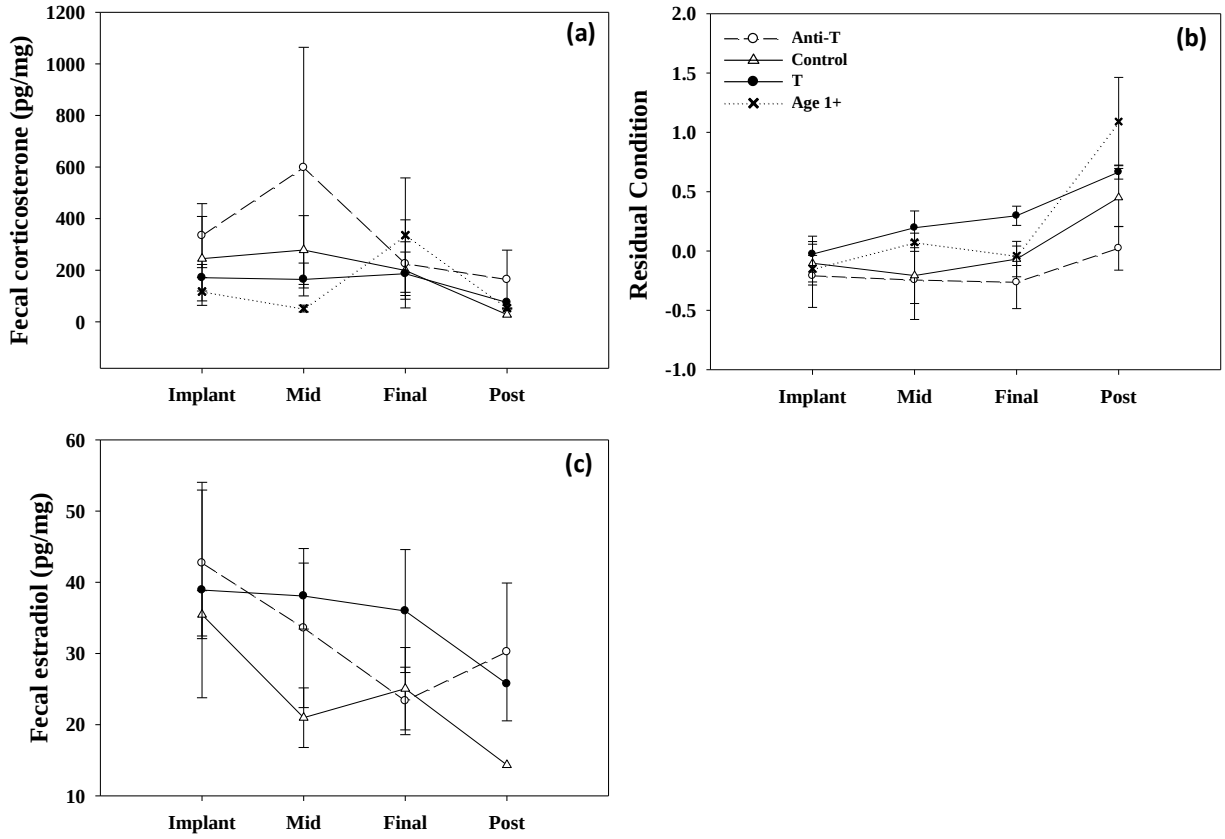

Figure 7: Fecal corticosterone (a), body condition (residuals from a regression of mass on tarsus length) (b), and fecal estradiol (c) for treated (T, Anti-T, control) and unmanipulated (age 1+) males across the four trapping periods of the experiment (implant, mid-treatment, final, and post-treatment).