

## RESEARCH ARTICLE

# Testosterone activates sexual dimorphism including male-typical carotenoid but not melanin plumage pigmentation in a female bird

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## ABSTRACT

In males it is frequently testosterone (T) that activates the expression of sexually selected morphological and behavioral displays, but the role of T in regulating similar traits in females is less clear. Here, we combine correlational data with results from T and gonadotropin-releasing hormone (GnRH) manipulations in both sexes to assess the role of T in mediating sexually dimorphic coloration and morphology in the red-backed fairy-wren (*Malurus melanocephalus*). We show that: (1) natural variation in female expression of ornamental traits (darkened bills and red back feathers) is positively associated with age and circulating androgen titres, (2) females have the capacity to express most male-typical traits in response to exogenous T, including carotenoid-pigmented body plumage, shorter feathers, darkened bill and enlarged cloacal protuberance, but (3) appear constrained in production of male-typical melanin-pigmented plumage, and (4) low androgen levels during the pre-nuptial molt, probably because of low ovarian capacity for steroid production (or luteinizing hormone sensitivity), prevent females from developing male-like ornamentation. Thus, females appear to retain molecular mechanisms for hormonally regulated male-typical ornamentation, although these are rarely activated because of insufficient production of the hormonal signal.

**KEY WORDS:** Sexual dimorphism, Testosterone, Plumage color, Bill color, Carotenoids, Melanins

## INTRODUCTION

The sexes can differ dramatically in morphology and behavior, necessitating ontogenetic mechanisms capable of overcoming constraints arising from a shared autosomal genome (Lande, 1980). Sex-limited secretion of hormones is one such mechanism, either transiently activating or permanently organizing sexually dimorphic character expression (Adkins-Regan, 2005). In males, it is typically testosterone (T) that activates secondary sex characters such as sexually selected behavioral and ornamental traits (for reviews, see Hau, 2007; Hirschenhauser and Oliveira, 2006; Oliveira, 2004; Wingfield et al., 1990). However, females also produce T, and although T generally circulates at lower levels than in males (Goymann and Wingfield, 2014; Møller et al., 2005; Wingfield and Farner, 1993; Wingfield et al., 2001) females

sometimes also produce a version of the (presumably) T-dependent male traits such as song, social aggression, bare-part coloration and elaborate plumage (Amundsen, 2000; Burley and Cooper-Smith, 1987; Odom et al., 2014; Rosvall, 2013).

It is unclear whether female ornamental trait expression relies on the same hormonal mechanisms that function in males (West-Eberhard, 2003) or whether females have evolved alternate mechanisms for expression of ornamental traits. Although exogenous T stimulates expression of some male-typical traits in females across taxa (Ketterson et al., 2005; Lahaye et al., 2013; Staub and De Beer, 1997), often only a portion of the sex-limited phenotype is produced. For example, T can increase song activity without inducing male-typical song rate (De Ridder et al., 2002), stimulate production of male-typical bill color at a lower than male-typical color intensity (Lahaye et al., 2013; McGraw, 2006), and stimulate production of male-typical feather structure without inducing male-like feather color (Peters, 2007). Therefore, although generally accepted as a primary mechanism for regulating many dimorphic characters, sex-specific T production may not be the only mechanism functioning, and the effects of T on various components of the dimorphic phenotype may be more or less sex specific.

Avian plumage coloration is a multicomponent signaling system with important functions in both sexual selection and speciation (Hill and McGraw, 2006; Price, 2007), yet the mechanisms underlying seasonal and sex-specific color expression remain elusive. Although males typically are the more ornamented sex, females often produce some elements of the male ornamental plumage that function in female–female signaling or other contexts (Amundsen, 2000). Furthermore, females are more likely than males to show evolutionary gains and losses in elaborate plumage color (Burns, 1998; Irwin, 1994; Johnson et al., 2013; Omland, 1997; Price and Eaton, 2014), indicating that sexual dichromatism is not driven solely by selection acting on mechanisms in males (Dale et al., 2015). Male-typical color expression is considered to be T dependent in Charadriiformes and luteinizing hormone (LH) dependent or genetically determined in Passeriformes (Kimball, 2006; Kimball and Ligon, 1999). However, this hypothesis does not take into account the facts that: T-treated female Charadriiform species do not produce all components of the male signal (indicating that other mechanisms may also function; Lank et al., 1999); female Passeriformes can naturally produce some components of the male-typical elaborate plumage (e.g. Morales et al., 2007), which argues against genetic fixation or strict hormonal activation; and variation in male Passeriform coloration can respond to T (Gonzalez et al., 2001; Lindsay et al., 2011; Peters et al., 2000; Roberts et al., 2009), even in species in which sexual dichromatism appears to be LH dependent (e.g. house sparrow, *Passer domesticus*; Witschi, 1961). Thus it is clear that models for hormonal control of dichromatic color expression need to be reevaluated in light of differential control of the various elements comprising the ornamental signal

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**List of symbols and abbreviations**

C	control
CP	cloacal protuberance
DHT	5 $\alpha$ -dihydrotestosterone
E2	estradiol
GnRH	gonadotropin-releasing hormone
LH	luteinizing hormone
PC1	principal component 1
PC2	principal component 2
PC3	principal component 3
PCA	principal components analysis
R	reflectance
T	testosterone
UV	ultraviolet
$\lambda$	wavelength of light

and natural variation in female as well as male coloration. Here, we investigate the function of T in mediation of sexually dimorphic plumage color and morphology of the red-backed fairy-wren [*Malurus melanocephalus* (Latham 1801)].

The red-backed fairy-wren is a seasonally and sexually dichromatic passerine bird that offers an ideal system for simultaneous assessment of mechanisms underlying sexual dimorphism and variation in both male and female ornamentation. Male red-backed fairy-wrens express discrete sexually selected (Karubian, 2002; Webster et al., 2008) and T-dependent variation in plumage and bill coloration (Karubian et al., 2011; Lindsay et al., 2011, 2009); males with low T titres during the pre-nuptial molt produce female-typical brown plumage and pale bills, whereas those with high T produce ornamented red/black plumage and black bills (Lindsay et al., 2009). Females can naturally produce some elements of the elaborate male phenotype (red feathers and darkened bill; see Results), though trait elaboration is rare and never reaches levels expressed by males despite elevated and variable T during the breeding season (Schwabl et al., 2014). We ask: (1) whether natural variation in female production of male-typical traits is associated with titres of plasma T, (2) whether exogenous T activates production of ornamental plumage and bill coloration in females as it does in males, and (3) why, if females can have elevated T and produce male-typical traits, they rarely do so. Our findings indicate that T can act on female trait expression and thus be the target of selection for mediating functionally significant variation in female ornamentation, with possible mechanistic constraints on the evolution of melanin- versus carotenoid-dependent feather pigmentation.

**MATERIALS AND METHODS****Study species and general field methods**

Male and female red-backed fairy-wrens are similar in appearance during the non-breeding season, with brown plumage and indistinguishable body morphologies. The plumage of the sexes becomes differentiated during a pre-nuptial molt occurring prior to and slightly overlapping the onset of breeding (Lindsay et al., 2009). At this time, females replace old feathers with new brown plumage, while males produce either ornamented red and black nuptial plumage (carotenoid- and melanin-pigmented, respectively; Rowe and McGraw, 2008) or female-like brown plumage. The ornamented male breeding phenotype combines red/black plumage coloration with shorter body and tail feathers, a darkened (melanin-based) bill color and an enlarged cloacal protuberance (CP) sperm storage organ, though brown breeding males also have a dark bill and enlarged CP (Karubian, 2002; Karubian et al., 2009).

We conducted long-term comparative monitoring (2003–2012), experimental hormone manipulation (T) and gonadotropin-releasing hormone (GnRH) challenges on members of a wild population of red-backed fairy-wrens in northeast Queensland, Australia (145°25'E, 17°23'S). We captured adult birds in mist nets, determined their age based on skull ossification patterns and pedigree (Lindsay et al., 2009), and banded birds for individual recognition with a numbered aluminum Australian Bird and Bat Banding Scheme band and a unique combination of three colored plastic leg bands. From each individual, we collected a small blood sample (max. 80  $\mu$ l) from the jugular vein, and took a series of morphological measurements. These included measurement of the size of the swelling posterior to the vent or CP (converted to volume using the formula  $\pi \times \text{depth}/2 \times \text{width} \times \text{length}$ ; see Karubian, 2002; Mulder and Cockburn, 1993), an objective measure of bill color using a color reference chart ranging from 1 (cream colored) to 40 (complete black; Lindsay et al., 2011), the extent of molt based on the number of feathers encased in feather shafts (scored 0–3 across six body regions – head, back, tail, wing, belly and breast – and summed to generate a maximum score of 18; Lindsay et al., 2011), and the percentage of the body showing red/black nuptial coloration [a score of 0–10 given to each of five body regions – head, back, tail, belly and breast – for a cumulative score of 50, converted to percent ( $\times 2$ ) following Webster et al., 2008]. We separated plasma from red blood cells via centrifugation and stored samples in liquid nitrogen until transport to Washington State University, where they were kept at  $-20^{\circ}\text{C}$  awaiting further analysis.

**Testosterone manipulation and GnRH challenge**

We implanted 1-year-old males and females prior to the onset of their pre-nuptial molt with either an empty (control, 'C') or testosterone-filled ('T', crystalline testosterone – Sigma T1500) silastic tube (Dow Corning) between August and October 2007 (T females  $N=4$ , T males  $N=6$ , C females  $N=3$ , C males  $N=6$ ) (see Lindsay et al. 2011 for further details). We recaptured and collected morphological measurements including the length of regrown feathers from birds mid-treatment (17.8 days post-implantation, range=15–24 days) and again approximately 6 weeks post-implantation (mean=45.6 days, range=35–56), at which time implants were removed. We determined the sex of implanted birds *post hoc* using standard molecular genetic techniques (Varian-Ramos et al., 2010).

We conducted GnRH challenges between October and December 2011, during which time we collected baseline blood samples (max. 40  $\mu$ l) from actively nesting birds before injecting them with 10  $\mu$ l of either phosphate-buffered saline (control: males  $N=7$ , females  $N=6$ ) or phosphate-buffered saline containing 500 ng of dissolved chicken GnRH (GnRH-I; American Peptide Company, 54-8-23; treatment: males  $N=39$ , females  $N=4$ ). This dosage has been shown to cause maximal LH response in other passerines (Wingfield and Farmer, 1993) and to elevate androgen production in male red-backed fairy-wrens (Karubian et al., 2011). See Barron et al. (2015) for further details. Birds were then placed in an opaque cloth bag and left undisturbed for 30 min before collecting a second blood sample (post-injection; max. 40  $\mu$ l). The majority of control and GnRH-treated females were actively molting ( $N=8$  out of 10) and all females were reproductively active, with nine females incubating and one female feeding nestlings.

All research presented in this paper was conducted under approval of the Institutional Animal Care and Use Committees of Washington State University (approval no. 03653-007) and Cornell University (approval no. 2009-0105), the James Cook University

Animal Ethics Review Committee (approval nos. A1004 and A1691) and the Queensland Government Environmental Protection Agency.

### Analysis of feather reflectance spectra

We collected six to 10 feathers at the time of T or C implant removal from each of three body regions (back, crown and breast), and used an Ocean Optics USB2000 UV-VIS spectrometer (R200-7UVVIS probe with PX2 pulsed xenon light source) to analyze reflectance ( $R$ ) across the avian visual spectrum [320–700 nm wavelength ( $\lambda$ )]. We used the program CLRv1.05 (Montgomerie, 2008) to quantify variation in: (1) brightness ( $R_{\text{total}}$ ), (2) hue ( $\lambda_{\text{Rmax}}$ ), (3) red chroma ( $R_{625-700\text{nm}}/R_{\text{total}}$ ) and (4) UV chroma (i.e. saturation in the ultraviolet range of the spectrum;  $R_{320-400\text{nm}}/R_{\text{total}}$ ) (for more details, see Lindsay et al., 2011; Rowe et al., 2010).

### Radioimmunoassay for total plasma androgen concentrations

We assayed 17–46  $\mu\text{l}$  plasma samples for total androgen concentration [testosterone and 5 $\alpha$ -dihydrotestosterone (DHT)] following a previously published protocol for this species (Lindsay et al., 2009). Following extraction with diethyl-ether, androgens were redissolved in 250  $\mu\text{l}$  phosphate-buffered saline with gelatin, and radioimmunoassays were conducted in 100  $\mu\text{l}$  aliquots using tritium-labeled testosterone (Perkin Elmer Life Sciences NET-553) and a testosterone antibody (Wien Laboratories T-3003) 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 were run in duplicate with recoveries for all (mean recovery 84%). The average intra-assay coefficient of variation across assays was 6.2% and the inter-assay variation was 5.9%. Concentrations of undetectable samples were calculated from minimal detectable levels (1.95 pg tube<sup>-1</sup>). Samples were randomly distributed throughout the six assays.

### Statistics

To analyze natural variation in male and female androgen concentrations, age and morphology, we used linear least-squares ANOVA on log-transformed hormone data or examined nonparametric Spearman rank correlations between trait values.

We utilized a principal components analysis (PCA) to describe variation in male and female production of a suite of seasonally male-specific traits under treatment with T. We extracted three factors (PC1, PC2 and PC3; Table 1) that explain 83.72% of variance, using a promax rotation that is appropriate when factors are oblique or highly inter-correlated (Brown, 2009), as was the case in this study. PC1 explained variation in parameters related to production of black, presumably melanin-pigmented body feathers (reflectance spectra of head and chest and percent of body in male-typical nuptial coloration; a completely ornamented male has black feathers on all body regions except the back). PC2 explained variation in coloration of red, presumably carotenoid-pigmented feathers (the back), bill color, morphology of the CP (CP volume and length of the CP tip), and body feather length. For ease of interpretation, we refer to PC1 as a measure of melanin pigmentation and PC2 as a measure of carotenoid pigmentation and body morphology (including melanin-dependent bill coloration). We used linear least-squares ANOVA to assess sex and treatment effects on production of PC1 and PC2. There was neither a treatment nor sex $\times$ treatment effect on PC3 (treatment effect

**Table 1. PCA of correlated phenotypic parameters responsive to testosterone in male and female red-backed fairy-wrens (explaining 83.72% of variation)**

Response variable	PC1 10.46 (65.38%)	PC2 1.64 (10.28%)	PC3 1.30 (8.1%)
Cloacal protuberance volume	0.032	<b>0.801</b>	−0.008
Cloacal tip	0.212	<b>0.815</b>	0.016
Bill color	0.045	<b>0.887</b>	0.067
Head feather length	−0.277	−0.355	<b>0.723</b>
Back feather length	0.117	<b>−0.766</b>	0.098
Tail feather length	−0.222	−0.579	0.009
Percent of body in nuptial plumage	<b>0.892</b>	0.110	0.018
Back brightness	−0.100	<b>0.862</b>	0.065
Back red chroma	0.374	<b>0.694</b>	0.049
Back hue	0.126	0.199	<b>0.913</b>
Chest brightness	<b>−0.894</b>	−0.107	−0.029
Chest UV chroma	<b>1.022</b>	−0.065	−0.019
Chest hue	<b>−1.102</b>	0.208	−0.024
Crown brightness	−0.593	−0.404	−0.021
Crown UV chroma	<b>0.964</b>	0.031	−0.024
Crown hue	<b>−0.789</b>	−0.233	−0.006

Eigenvalues and the percent of variation explained are listed for each component, and the factors with greatest contribution (factor loadings greater than 0.6) are in bold.

$F_{1,18}=0.02$ ,  $P=0.88$ ; sex $\times$ treatment  $F_{1,18}=0.02$ ,  $P=0.88$ ), and these data are not discussed further. We performed *post hoc* power analysis on data generated via PCA with  $\alpha=0.05$ , model root mean square errors generated in each analysis (PC1=0.158; PC2=0.271) and the detected differences in means for each model. Our probability of correctly rejecting our null hypothesis across our four treatment groups given our current sample size was estimated at 100%. All pairwise comparisons between treatment groups were conducted using either Wilcoxon or Kruskal–Wallis tests (rank sums) with a chi-square approximation, or univariate standard linear least-squares models, depending on data distributions.

We assessed treatment and sex differences in overall hormonal response to GnRH injection using linear mixed models. In these analyses, we log transformed androgen concentrations and sequentially removed nonsignificant ( $P>0.1$ ) covariates (date, age, time of day, nest age, molt score and mass) to leave only nest age (days since first egg laid;  $F_{1,15}=5.02$ ,  $P=0.04$ ) and time of day (minutes post-sunrise;  $F_{1,51}=4.72$ ,  $P=0.03$ ) as significant parameters in the female treatment by pre/post-injection and post-injection treatment by sex models, respectively. All analyses were conducted using JMP 7 (SAS Institute, Cary, NC, USA) or NCSS (<http://www.ncss.com/>).

## RESULTS

### Variation in female bill and plumage color is associated with age and androgens

Female red-backed fairy-wrens increasingly express male-typical plumage and bill color as they age, and this variation in trait expression is positively associated with plasma androgen concentrations. The percent of the integument producing male-typical nuptial coloration varied subtly in unmanipulated females (range 0–10%, mean $\pm$ s.e.=0.07 $\pm$ 0.02%,  $N=745$ ), and this variation was positively associated with age (ages 1–9 years;  $r_s=0.163$ ,  $P<0.01$ ,  $N=382$ ). Female expression of male-typical plumage was limited to a few red feathers on the upper back, and never included the jet-black melanin pigmentation typical of nuptial males. It was also rare, with no 1-year-old females and only 4.6% of older females

**Table 2. Summary of effects of testosterone (T) treatment on male and female phenotype in red-backed fairy-wrens**

	T male (N=6)			C female (N=3)			T female (N=4)		
	Character state	Mean±s.e.	Range	Character state	Mean±s.e.	Range	Character state	Mean±s.e.	Range
Androgens (pg ml <sup>-1</sup> )	High	2974±1023*	473–5127	Low	191±82	87–354	High	3991±407	2889–4853
Morphology									
Bill color	Black	33.50±2.35	24–40	Pale	14±3.61	9–21	Black	36.25±1.11	33–38
CP volume (mm <sup>3</sup> )	Large	36.98±11.93	11.2–81	Absent	0	0	Small	35.95±2.27	31.2–41
CP tip (mm)	Large	1.58±0.12	1.2–2.0	Absent	0	0	Small	1.48±0.12	1.3–1.8
Tail length (mm)	Short	44.50±0.96	43–47	Long	49±1.53	46–51	Short	43.50±0.87	41–51
Back length (mm)	Short	13.20±0.92	12–16	Long	16±3	10–19	Short	14.25±0.25	14–15
Head length (mm)	Short	4.80±0.37	4–6	Long	7.67±0.67	7–9	Short	6.25±0.75	5–8
Nuptial color (%)	Extensive	77.25±7.97	42–96	Absent	0	0	Partial	17.25±3.11	9–24
Plumage color									
Crown	Black			Brown			Dark brown/orange		
Tail	Black			Brown			Dark brown		
Chin	Black			White			Black/brown		
Breast	Black			White			Orange/pink		
Belly	Black			White			White/orange		
Back	Red			Brown			Orange		

All traits were measured at the time of implant removal (~1 month post-implantation). Tail, back and head lengths refer to the lengths of feathers produced in these three regions. Note: three T males lost their implants shortly before implant removal, explaining low androgen values at this time. C, control; CP, cloacal protuberance.

(i.e.  $N=12$  of 259 females aged 2 years or older) exhibiting any male-typical nuptial plumage coloration. In contrast, older females frequently had darker bills (presumably from more and/or different melanin deposition), with darkness increasing significantly with age ( $r_s=0.298$ ,  $P<0.0001$ ,  $N=373$ ).

The age-related color changes were mirrored by increasing plasma androgen concentrations with age ( $F_{1,104}=4.93$ ,  $P=0.03$ ; modeled with plasma androgens as response and both age and breeding stage as significant predictive factors), and we found a noisy but significant positive association between female bill color and circulating androgen concentrations ( $r_s=0.133$ ,  $P=0.05$ ,  $N=209$ ). Although a relationship between androgens and plumage color could not be tested as only a few ( $N=3$ ) of the 202 females with androgen measurements had any nuptial plumage (red feathers), we found that all three had plasma androgen titres that fell within the top quartile of the distribution (686.4, 731.9 and 918.7 pg androgen ml<sup>-1</sup> plasma; range of general population is 61.1–2305.6 pg androgen ml<sup>-1</sup> plasma, mean±s.e.=421.05±19.38 pg androgen ml<sup>-1</sup>,  $N=221$ ).

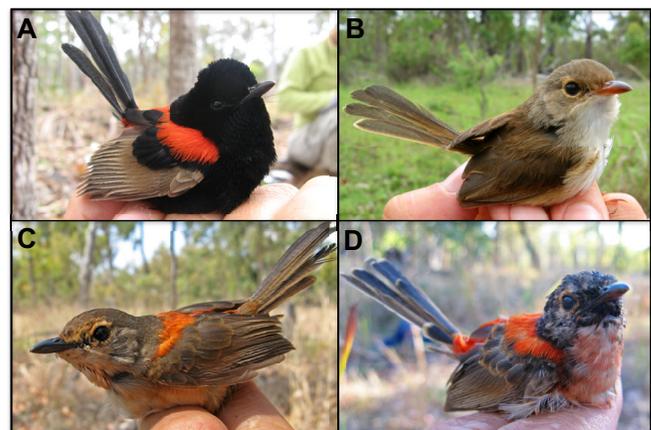
### T treatment alters female plumage color and body morphology

Prior to placement of T implants, males and females did not differ in concentrations of plasma androgens ( $\chi^2=0.67$ , d.f.=1,  $P=0.41$ ), bill color ( $\chi^2=0.33$ , d.f.=1,  $P=0.56$ ) or molt score ( $\chi^2=1.05$ , d.f.=1,  $P=0.31$ ), but did differ in body mass ( $\chi^2=7.69$ , d.f.=1,  $P=0.01$ ), with males being larger than females (mean±s.e., females: 6.67±0.12 g; males 7.15±0.11 g). The brown plumage phenotype shared between the sexes at this time was spectrally indistinguishable in all measured feather tracts (males  $N=6$ , females  $N=3$ ; brightness, hue, UV chroma, red chroma on breast, crown and back, all  $P>0.1$  excepting a trend for higher UV chroma in male breast feathers,  $P=0.07$ ).

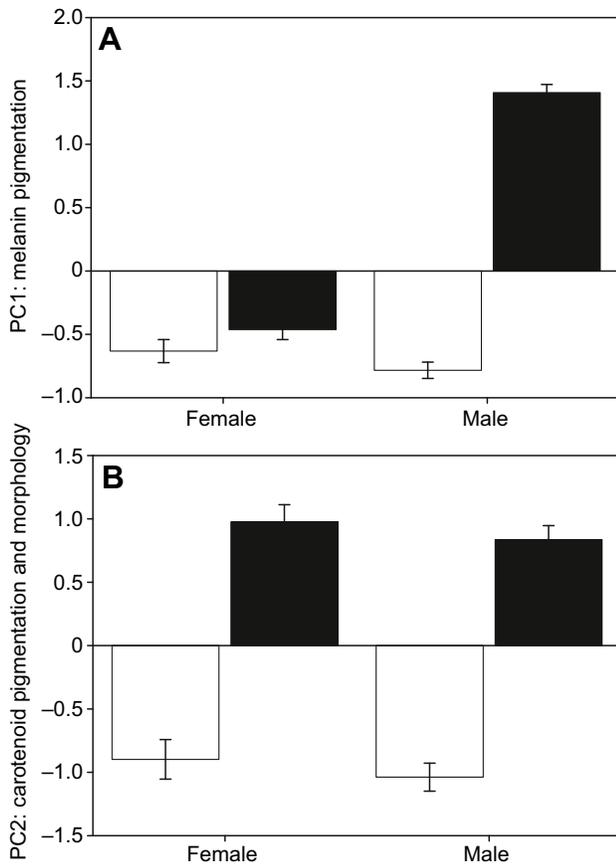
By elevating female plasma T to levels typical of breeding red/black males (Table 2), we stimulated an early onset of the pre-nuptial molt (T female versus C female molt score at mid-treatment:  $\chi^2=3.92$ , d.f.=1,  $P<0.05$ ) similar in intensity to the molt initiated in T-implanted males (T female versus T male molt score;  $\chi^2=0.01$ , d.f.=1,  $P=0.91$ ; see also Lindsay et al., 2011). T-females produced short male-like head, back and tail feathers intermediate in color to

those of T-implanted males and control females, along with a suite of other male-typical morphological traits, including classical androgen-dependent traits such as a darker (more melanized) bill and an enlarged CP with a male-like cloacal tip (Fig. 1, Table 2, see also Fig. S1).

The effects of T on female plumage color varied between feather tracts (Table 2) and between pigment types (melanins versus carotenoids). Males and females responded similarly to T, with production of carotenoid-pigmented back feathers, male-typical body morphology (darkened bill, CP and CP tip), and shortened body and tail feathers (PC2; treatment effect  $F_{1,18}=209.48$ ,  $P<0.0001$ ; sex×treatment  $F_{1,18}=0.00$ ,  $P=0.99$ ; Table 2, Fig. 2B). The only sex difference detected in the traits contributing to PC2 was a difference in the reflectance parameters of the red back feathers, which were higher in red chroma for T males than for T females ( $\chi^2=5.5$ , d.f.=1,  $P=0.02$ ; Fig. 3B). In contrast, T males and



**Fig. 1. Control and testosterone (T)-implanted male and female red-backed fairy-wrens.** Plumage color and body morphology of a red/black-plumaged T male (A) and brown-plumaged control female (B). The female shown in C is typical of three out of the four females treated with T, with a black bill, orange to red back feathers, and a pale wash of orange-tinted feathers on belly and breast. The T female shown in D produced the greatest amount of black melanin-pigmented feathers; these were confined to the chin, head and tail.

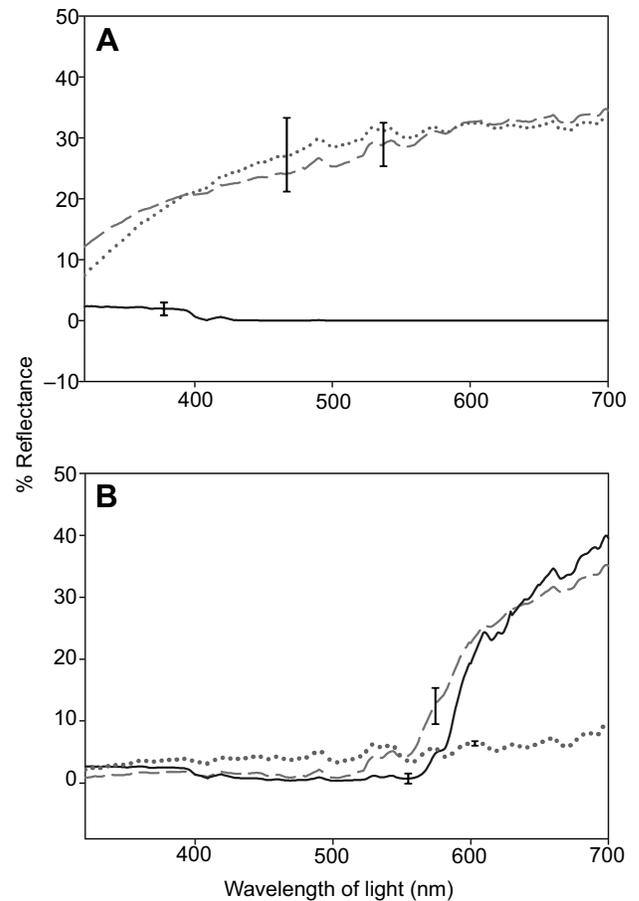


**Fig. 2. Effects of T treatment on red-backed fairy-wren phenotype as summarized via PCA.** Mean  $\pm$  s.e. of sex and treatment for (A) PC1, a measure of melanin feather pigmentation and (B) PC2, a measure of carotenoid pigmentation and classically androgen-dependent morphological traits such as bill color and cloacal protuberance volume. White bars indicate control birds and black bars T-implanted birds.

T females differed markedly in expression of melanin-pigmented plumage (PC1; Table 2, Fig. 2A), as males produced more black plumage than did females (treatment effect  $F_{1,18}=244.29$ ,  $P<0.0001$ ; sex  $\times$  treatment  $F_{1,18}=179.32$ ,  $P<0.0001$ ).

T males produced the typical nuptial black feathers on chin, crown, breast, belly and tail (see Fig. 3A for reflectance parameters of breast feathers), but only one of the T females grew a noticeable patch of male-like black feathers on the chin, head and tail, while the others grew female-typical brown feathers in these tracts, and primarily female-typical white belly feathers. Most strikingly, however, all T females produced a unique wash of pale orange-to-pink feathers on parts of the crown, belly and breast (Fig. 1C), never previously documented on a red-backed fairy-wren. This was particularly pronounced in the female with the most male-like black feathers (Fig. 1D). This orange/pink coloration was largely confined to the outermost tips of each individual feather such that while the plumage patch appeared orange, each plucked feather was largely brown to white. Hence, reflectance spectra from feathers plucked off the breast are more similar to those of control females (Fig. 3A) than may be expected.

Continued elevation of T in females was necessary for maintenance of male-typical trait expression, as removal of the T implant caused a cessation in trait elaboration for all measured characters. Dropping androgen concentrations in females corresponded with a decline in black bill coloration, atrophy of the CP and no further production of



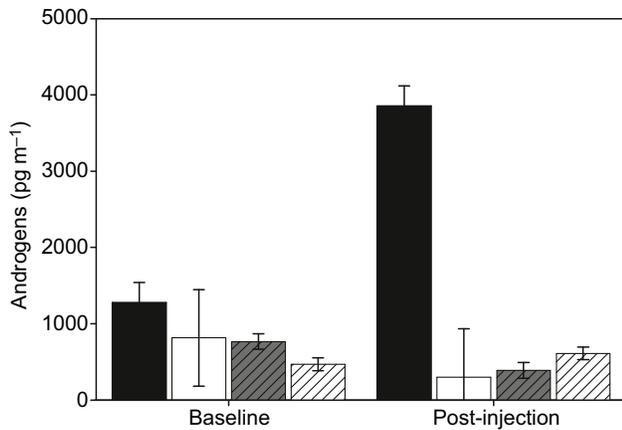
**Fig. 3. Average reflectance spectra of plumage produced by male and female red-backed fairy-wrens in response to T.** Reflectance of (A) breast (black in ornamented males, creamy white in control females) and (B) back feathers [red in ornamented males and pale brown in control (C) females] produced by T males (black lines), T females (dashed lines) and C females (dotted lines). Standard error bars for brightness (total reflectance) are indicated for each curve.

red or black feathers (traits measured  $\sim$ 122.1 days post-implant, range=115–130 days, insufficient sample sizes for statistical analysis: T females  $N=2$ , T males  $N=3$ , C females  $N=1$ ; see Fig. S1). Feathers plucked at implant removal were universally replaced with female-typical long brown or white feathers.

#### Female plasma androgen titre is low and does not increase following GnRH challenge

Naturally circulating plasma androgen concentrations differed significantly between males and females during molt ( $F_{1,335}=19.26$ ,  $P<0.0001$ ). Specifically, males molting into red/black plumage had significantly higher plasma androgen concentrations than did females ( $F_{1,210}=60.79$ ,  $P<0.0001$ ), but female and brown-plumed male androgen levels did not differ ( $F_{1,258}=2.44$ ,  $P=0.119$ ).

We used GnRH challenges to determine whether females have the native capacity to elevate androgens, as necessary for the development of male-typical traits. Whereas male plasma androgen concentrations strongly increased following GnRH injection (Barron et al., 2015), female concentrations did not (treatment  $\times$  pre/post-injection:  $F_{1,15}=5.85$ ,  $P=0.03$ ; Fig. 4), revealing sex-specific regulation of T production [post-injection (30 min) treatment  $\times$  sex:  $F_{1,51}=28.80$ ,  $P<0.00001$ ; Fig. 4].



**Fig. 4. Sex by treatment response to gonadotropin-releasing hormone (GnRH) injection in red-backed fairy-wrens.** Control male (white bars) and female (white bars with lines) and GnRH-treated male (black bars) and female (gray bars with lines) mean  $\pm$  s.e. baseline plasma androgens ( $\text{pg ml}^{-1}$ ) and as measured 30 min post-injection.

## DISCUSSION

Our results indicate that various components of sexually dimorphic plumage elaboration (i.e. melanin versus carotenoid pigmentation; feather color versus morphology) are differentially regulated (as per Grether et al., 2004). The female-like phenotype (brown plumage coloration and body morphology) appears to be the default character state that develops in both sexes when T is low (Lindsay et al., 2011, 2009; present study). Increased endogenous and exogenous T triggers molt into a more elaborate plumage type in both sexes along with production of a darkened bill. Although the mechanism(s) for elaborate trait expression seem remarkably conserved between the sexes, with females capable of producing traits that normally occur and function only in males (i.e. CP and cloacal tip), females appear constrained in their ability to produce melanin-pigmented black plumage in response to T, unlike males. This constraint may arise as a consequence of sex differences in production of other hormones, for example, estradiol (E2), a hypothesis requiring further exploration.

### Female bill and plumage color increase with age and endogenous androgens

Both age and androgen titre are associated with expression of a more male-like ornamental phenotype in female red-backed fairy-wrens. Age-dependent increases in female ornamentation are not uncommon (Martinez-Padilla et al., 2011; Morales et al., 2007; Vergara et al., 2009), and can be facilitated by changing concentrations of reproductive hormones produced throughout a female's lifespan (Owens and Short, 1995), as appears to be the case in the red-backed fairy-wren. Evidence from a comparative analysis of sexual dimorphism in the Maluridae family suggests that the evolution of female bill coloration is a product of selection on females themselves (Karubian, 2013), rather than simply a by-product of selection on the male phenotype (Lande, 1980). Female bill coloration is important in socio-sexual signaling in other avian species (Burley and Coopersmith, 1987; Murphy et al., 2009), as well as in male red-backed fairy-wrens (Karubian et al., 2011; Lindsay et al., 2009), and can be modulated by T in females (Lahaye et al., 2013; McGraw, 2006; Pham et al., 2014). Thus, androgens circulating in females at physiological rather than pharmacological levels (as induced by T implantation) can act on trait expression and may be the target of selection for mediating functionally significant

variation in female ornamentation (Goymann and Wingfield, 2014). The signaling function of female red-backed fairy-wren bill color remains to be explored.

### T-dependent carotenoid-based but not eumelanin-based dichromatism

T activates sexually dichromatic plumage production in members of the Charadriiformes (Kimball, 2006; Kimball and Ligon, 1999) while in Passeriformes sex differences in plumage coloration are thought to be either LH regulated or hormone independent (reviewed in Kimball, 2006; Kimball and Ligon, 1999; Witschi, 1961). In fact, our study is the first, to our knowledge, demonstrating T-regulated sexual dichromatism in a member of the Passeriformes. Many temperate zone passerines for which the mechanisms underlying dichromatic and intrasexually polychromatic plumage color patterns have been well studied acquire breeding plumage during a post-nuptial molt, when gonads are regressed and androgen levels are low (Gonzalez et al., 2001; Nolan et al., 1992; Roberts et al., 2009; Stoehr and Hill, 2001). In these species, females treated with T fail to produce the male-typical coloration (Keck, 1934; Witschi, 1961) and T treatment can delay molt in both sexes (Clotfelter et al., 2004; Dawson, 1994; Kurvers et al., 2008; Nolan et al., 1992; Runfeldt and Wingfield, 1985; Stoehr and Hill, 2001). In many tropical species that, like the red-backed fairy-wren, undergo a prenuptial molt just prior to breeding (when gonads become activated and androgens are elevated in males but not females), regulation of nuptial coloration by gonadal hormones seems plausible (Lindsay et al., 2011; Peters et al., 2013) and even expected. This hypothesis should be further explored in other species with a prenuptial molt.

Interestingly, T treatment in the congeneric and likewise prenuptially molting superb fairy-wren (Peters, 2007) caused females to molt and develop a cloacal tip, but not a CP nor male-typical bill or plumage coloration (excepting certain 'glossy' structural feather elements). However, unlike red-backed fairy-wrens, superb fairy-wrens have no (or at least no visible) carotenoid pigmentation and instead have only eumelanin- and microstructure-based black and blue plumage. These components may be unresponsive to T (except, perhaps, for the observed increase in 'glossiness'), as is apparently the case in female red-backed fairy-wrens. The discrepancy in T effects on bill versus feather melanization in female red-backed fairy-wrens is less surprising, given that T initiates bill melanization in species in which plumage color is unresponsive to androgens (Keck, 1934; Witschi, 1961). It is possible that the thresholds for melanization of bill and plumage differ or that different signaling molecules are involved, ideas requiring further investigation. Together, the observations above highlight the need to take into account the various pigmentary and structural components, as well as the different types of integument involved in sexual dichromatism.

While carotenoid-based bare part coloration has been induced by T in females of several avian species (De Ridder et al., 2002; Eens et al., 2000; McGraw, 2006), our result is the first indication that carotenoid-based plumage color can be induced by T in a female bird. This contrasts with a previous study suggesting that T acts as a constraint on carotenoid plumage pigmentation (Stoehr and Hill, 2001). Once again, however, this was a temperate zone species (house finch, *Haemorrhous mexicanus*) without prenuptial molt, and likely no natural T regulation of the male ornamental plumage (see above).

Although both sexes produced red carotenoid coloration in response to T, T-female back feathers were significantly less deep in chroma than those of 'wild-type' or T males (Fig. 3B). Therefore, given a similar hormonal stimulus (fixed dose of exogenous T), males are either more sensitive (e.g. possess more or different

hormone receptors) and/or have a more efficient downstream carotenoid pigmentation ‘machinery’ (i.e. uptake, metabolic conversion, transport or deposition of carotenoids), as suggested for the zebra finch (McGraw, 2006). Such cellular control mechanisms for carotenoid deposition are potentially important, yet largely unexplored, sources of color variation (but see Hill and Johnson, 2012; McGraw et al., 2006, 2002), and thus essential keys to understanding sexual selection and evolution.

T-treated female red-backed fairy-wrens showed a robust capacity for carotenoid feather pigmentation (PC2), but black eumelanin-dominated feather production (PC1) was limited and varied across body topology, with expression restricted to the chin and margins of feather tracts (Fig. 1D). Because of the presence of a small number of black feathers in T-treated females, sexually dimorphic eumelanin feather pigmentation is not likely developmentally organized or dependent on dimorphic gene expression in this species. Estradiol, which is detectable in pre-breeding female red-backed fairy-wrens (Schwabl et al., 2014), may regulate female-typical feather pigmentation. E2 inhibits production of male-typical feather coloration in other avian orders (Struthioniformes, Galliformes and Anseriformes; Kimball, 2006; Kimball and Ligon, 1999), and specifically appears to inhibit male-typical eumelanin pigment deposition in mallards (*Anas platyrhynchos*; Haase et al., 1995). E2 might also suppress male-typical plumage production in members of the Passeriformes (Witschi, 1961; Perlut, 2008). Although E2 was neither manipulated nor measured in red-backed fairy-wrens, it is possible that both low T and elevated E2 contribute to suppression of the melanin component of male-typical coloration in this and other passerine species, whereas carotenoid pigmentation may be more one-dimensionally T dependent.

Although T-treated female red-backed fairy-wrens failed to produce the black breast and belly feathers of males, they grew unique, seemingly carotenoid-pigmented orange and pink plumage in these regions, colors never observed naturally in males or females. We suggest two explanations for this novel finding. First, it is possible that the black eumelanin-dependent plumage of male red-backed fairy-wrens masks an underlying carotenoid pigmentation, with T treatment stimulating only one-half of the male-typical signal in females. Such a hidden carotenoid component in an otherwise melanin-dominated signal has been documented in at least one other avian species, the orchard oriole (*Icterus spurius*; Hofmann et al., 2007). The role of T in differential regulation of these two pigment types in a single feather remains to be explored. Alternatively, the unique orange and pink feathers produced by T-treated female red-backed fairy-wrens may contain rufous tinted pheomelanins rather than carotenoids, indicative of sexual dimorphism in the capacity for eumelanin versus pheomelanin deposition. Sex differences in eumelanin versus pheomelanin pigment content of otherwise visually monochromatic feathers have been documented in barn swallows (*Hirundo rustica*; Saino et al., 2013). Biochemical studies are needed to untangle these possibilities and shed light on mechanisms involved in regulating sex differences in carotenoid versus melanin pigmentation.

### Female ornamentation may be naturally limited by low gonadal T production

Female androgen levels are lowest during the pre-nuptial molt (Schwabl et al., 2014; present study), and are similar to those seen in males that produce brown, female-typical plumage and morphology, suggesting that females do not develop elaborate plumage because of low circulating T. In contrast to males (Barron et al., 2015), female red-backed fairy-wrens were

unresponsive to GnRH, suggesting that during at least a portion of the period in which females produce their breeding plumage they are protected from elevated T and expression of male-typical traits by ovarian insensitivity to GnRH-induced gonadotropin (LH) secretion. Because female red-backed fairy-wrens maintain low but detectable levels of circulating androgens across the phases of the nesting cycles (Schwabl et al., 2014), it is likely that ovarian LH responsiveness is stage specific, as appears to be the case in other avian systems (Rosvall et al., 2013 and references therein).

### Conclusions

The female genotype appears to include hormone-sensitive genes for maleness and male ornamentation; however, these are rarely expressed because of insufficient or time-limited production of the hormonal signal (T). The ‘hidden’ genotype of the female red-backed fairy-wren as revealed by our T manipulation appears to expose potential substrate for plumage color evolution in this species, with expression of a unique ornamental phenotype requiring only a simple change in hormonal milieu (i.e. T, DHT, E2), which may be facilitated via changes in ovarian steroid production during the pre-nuptial molt. Although a coupling of sexual dimorphism to sex differences in adult androgen secretion may explain the evolution of sex differences in many traits (Cox et al., 2015), our results implicate a role for additional actions of female hormones and non-hormonal mechanisms in regulating dimorphic expression of complex, multi-component traits.

### Acknowledgements

We are grateful to our many volunteer field assistants, to Staffan Andersson for helpful comments in the preparation of this manuscript, and to Lee Astheimer of the University of Wollongong, Brad Congdon of James Cook University and Major Tim Daniel of Kalinvale Farm for logistical support.

### Competing interests

The authors declare no competing or financial interests.

### Author contributions

W.R.L. conceived the study and wrote the manuscript; W.R.L. and D.G.B. collected the field and laboratory data and conducted the analyses; H.S. and M.S.W. provided funding and critical oversight on the project; all authors contributed to the writing.

### Funding

The research was funded by the National Science Foundation (USA) through a grant to M.S.W. and H.S. (no. 0213075) and a graduate traineeship to D.G.B. (no. 0549425).

### Data availability

Data are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.gf46s>

### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.135384.supplemental>

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