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Variation in song system anatomy and androgen levels does not correspond to song characteristics in a tropical songbird

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Variation in song structure and song production of birds are thought to relate to variation of both androgen levels and neural nuclei in the song system, as typically these nuclei are larger in males than in females, vary in size among males and are sensitive to steroid hormones. We investigated the relationships among song and note structure, singing rate, androgen levels and the sizes of two song nuclei, the higher vocal centre (HVC) and the robust nucleus of the arcopallium (RA) in male and female red-backed fairy-wrens, *Malurus melanocephalus*. Males of this duetting species express three discrete reproductive phenotypes that differ in plumage colour and behaviour. Although HVC and RA structure differed between the sexes, there were no sex differences in note structure and complexity of songs, although females differed from some male types in song rate and frequency characteristics. Both auxiliary males and females had significantly lower androgen levels than the two breeding male phenotypes. Male reproductive phenotypes had similar song characteristics and HVC and RA structure, but differed in androgen levels. Sexes and male phenotypes varied in song rate, but these differences did not correspond to differences in androgen levels. Thus, sex differences in song nuclei anatomy and androgen levels were not associated with differences in song structure and singing rate; and, the differences in androgen levels among male phenotypes were not reflected in differences in singing rate, song structure or the song nuclei. We conclude that, similar to other recent findings, the sexes of the red-backed fairy-wren can produce similar song and express similar singing behaviour despite differences in song system structure and circulating androgen levels; singing and song system anatomy appear not to be part of the suite of traits associated with differences in androgen levels in male red-backed fairy-wrens.

Avian song and the neural song system is a key model system for studies of behavioural and neural sex differences, neuroplasticity and brain–behaviour relationships, and therefore offers unique opportunities to unravel the roles of genes, hormones and the social environment in moulding brain and behaviour. The song system is a network of forebrain nuclei that functions in learning and production of song in songbirds. In this network, the nuclei higher vocal centre (HVC) and robust nucleus of the arcopallium (RA) comprise the motor pathway to muscles of the syrinx that, via medullary nuclei, control the production of song, particularly the repertoire of note types and syllable types within songs (Bolhuis & Gahr, 2006; Nottebohm & Arnold, 1976).

HVC and RA are typically larger in male songbirds than in female songbirds, but the degree of size dimorphism varies substantially among species (MacDougall-Shackleton & Ball, 1999: results from 20 species). In species in which song is restricted to males, such as the zebra finch, *Taeniopygia guttata*, HVC and RA are much larger in males than in females, whereas in species in which both sexes sing and produce songs of similar structure, sexual size dimorphism of both nuclei is reduced (Brenowitz, 1997; Brenowitz & Arnold, 1986; Gahr, 2007; MacDougall-Shackleton & Ball, 1999). These observations have led to the hypothesis that sex differences in neural space determine sex differences in structure and complexity of motor output (Nottebohm, Kasparian, & Pandazis, 1981). However, sexual

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dimorphism of song nuclei volume also occurs in species where the sexes have similar song production and sing similar or near similar song (i.e. Brenowitz, 1997; Brenowitz & Arnold, 1986; Gahr & Metzdorf, 1997; Gahr, Sonnenschein, & Wickler, 1998; Hall, MacDougall-Shackleton, Osorio-Beristain, & Murphy, 2010; Jawor & MacDougall-Shackleton, 2008; Voigt & Gahr, 2011).

Sizes of the song nuclei also vary in individuals of the same sex, probably due to the influence of environmental and hormonal factors. In males of seasonally breeding species the sizes of HVC and RA vary with reproductive stage (e.g. Nottebohm et al., 1981), photoperiod (e.g. Tramontin, Hartman, & Brenowitz, 2000) and androgen levels (e.g. Small, Brenowitz, & Moore, 2007). Social context also influences male singing and song system anatomy (Sartor & Ball, 2005; Sockman, Salvante, Racke, Campbell, & Whitman, 2009; Strand, Ross, Weiss, & Deviche, 2008), and the volumes of male song nuclei vary with social status in species with complex social organization of breeding units. For example, dominant males of the white-browed sparrow weaver, *Plocepasser mahali*, have larger HVC and RA volumes and differ from subordinate males in the cellular machinery and gene expression patterns in these nuclei (Voigt & Gahr, 2011; Voigt, Leitner, & Gahr, 2007). Variation in male song nuclei size is assumed to relate to differences in song structure and repertoire size at the individual, population and species level (Airey & DeVoogd, 2000; Canady, Kroodsma, & Nottebohm, 1984; DeVoogd, Krebs, Healy, & Purvis, 1993; Garamszegi & Eens, 2004; Kirn, Clower, Kroodsma, & DeVoogd, 1989; Leitner & Catchpole, 2004), its evolution driven by sexual selection and limited by proximate mechanisms (Gil & Gahr, 2002; Podos, Huber, & Taft, 2004).

The gonadal steroid hormone testosterone could be a common physiological denominator to explain sex differences, seasonal plasticity, social modulation and species differences in song nuclei volume, singing and song structure. First, female songbirds generally have much lower testosterone levels than males (Goymann & Wingfield, 2014; Ketterson, Nolan, & Sandell, 2005; Møller, Garamszegi, Gil, Hurtrez-Boussès, & Eens, 2005), and exogenous testosterone increases female song nuclei volumes (Brown & Bottjer, 1993; Madison, Rouse, Balthazart, & Ball, 2014; Nottebohm, 1980) and stimulates singing in females of several species (e.g. Appeltants, Ball, & Balthazart, 2003; Hausberger, Henry, & Richard, 1995; Kern & King, 1972; Kriner & Schwabl, 1991; Lahaye, Eens, Darras, & Pinxten, 2012; Madison et al., 2014; Rasika, Nottebohm, & Alvarez-Buylla, 1994; Voigt & Leitner, 2013; and references in Rosvall, 2013). Second, seasonal plasticity of singing and song structure and of song nuclei anatomy in males of photoperiodic species is, at least in part, regulated by changes in testosterone levels (e.g. Bernard & Ball, 1997; Gulledge & Deviche, 1997; Small et al., 2007; Smith, Brenowitz, & Wingfield, 1997; Tramontin, Wingfield, & Brenowitz, 2003). Third, exogenous testosterone increases song repertoire size and singing rate in seasonally nonreproductive males (Van Hout, Pinxten, Darras, & Eens, 2012) and exogenous testosterone metabolites modify song nuclei anatomy (Hall & MacDougall-Shackleton, 2012). Fourth, male social status in breeding groups is often associated with differences in androgen levels (e.g. DuVal & Goymann, 2011; Ryder, Horton, & Moore, 2011; Soares et al., 2010; Wingfield & Lewis, 1993).

We examined the relationship between singing rate, song structure, song nuclei anatomy and circulating androgen levels in male and female red-backed fairy-wrens, *Malurus melanocephalus*, a cooperatively breeding Australian songbird. In this tropical species both sexes sing, as do other members of the songbird family Maluridae (i.e. Greig, Price, & Pruett-Jones, 2013; Hall & Peters, 2008; Rowley & Russel, 1997). All group members, including auxiliary helper males, sing and all join their songs to form overlapping, polyphonic duets and choruses that appear to function in intergroup territorial interactions (Dowling & Webster, 2013). Red-backed fairy-wren songs are similar to the chatter song of the superb fairy-wren, *Malurus cyaneus* (Dalziell & Cockburn, 2008) and the type I song of the splendid fairy-wren, *Malurus splendens* (Greig & Pruett-Jones, 2008). Unlike these species, the red-backed fairy-wren sings only one song type, but varies the number, type and order of notes within songs (Dowling & Webster, n.d.-b).

Male red-backed fairy-wrens express three morphologically and behaviourally distinct reproductive phenotypes: ornamented (red/black plumage) breeders, unornamented (brown, female-like plumage) breeders, and unornamented auxiliary helpers (Karubian, 2002; Webster, Karubian, & Schwabl, 2010). Male phenotypes join mates and other group members in singing duets and choruses (Dowling & Webster, 2013), but differ in their sexual and parental behaviour (Karubian, 2002), sperm production (Rowe, Swaddle, Pruett-Jones, & Webster, 2010) and reproductive success (Webster, Varian, & Karubian, 2008). Male types vary dramatically in androgen levels: during all reproductive stages, red/black males show the highest, brown breeders intermediate and brown auxiliaries the lowest androgen levels (Lindsay, Webster, Varian, & Schwabl, 2009). The androgen testosterone (T) is both necessary and sufficient to induce the ornamented male plumage phenotype, as T levels during the prenuptial moult predict red/black versus brown breeding plumage phenotype (Lindsay et al., 2009) and T implants induce a prenuptial moult into the red/black male plumage phenotype (Lindsay, Webster, & Schwabl, 2011). Androgen levels rise when males change social status from auxiliary helper to breeder, and this transition is associated with changes in bill colour and size of the cloacal protuberance (Karubian, Lindsay, Schwabl, & Webster, 2011), both of which are androgen-regulated traits (Donham, Wingfield, Mattocks, & Farner, 1982; Laucht, Kempenaers, & Dale, 2010).

Given these behavioural and hormonal differences among male breeding types, this species is well suited to examine the relationship among androgens, brain song centres and acoustic output. The present study investigates whether singing rate, song structure and its underlying neural motor pathway (nuclei HVC and RA) are part of the suite of traits associated with variation of androgen levels in alternative reproductive phenotypes of male red-backed fairy-wrens, and whether predicted sex differences in androgen level are associated with differences in singing rate, song structure and anatomy of song control regions. Based on the hypotheses outlined above, we predicted that differences between male phenotypes in androgen levels would be reflected by differences in singing rate, song structure and anatomy of the song nuclei HVC and RA. We further predicted that lower androgen levels in females than in males would be reflected in sex differences in singing rate, song structure and song nuclei size.

METHODS

Study Species

We studied a population of colour-banded red-backed fairy-wrens near Herberton, Queensland, Australia (145°25′E, 17°23′S), which has been monitored continuously since 2003. The study sites are located in open sclerophyl forest with tall eucalypt overstory and grass understory. The reproductive biology of this nonmigratory species is described in detail by Webster et al. (2010). Breeding is seasonal, lasting from September through April (with occasional nesting thereafter), although onset varies with rainfall (Webster et al., 2010). Like other members of the Maluridae (Peters, Kingma, & Delhey, 2013), red-backed fairy-wrens moult twice per year with most birds assuming cryptic, brown plumage during the

nonbreeding season; the prenuptial moult, in which males acquire their red/black or brown and females acquire their brown breeding plumage, begins many weeks prior to breeding and can persist populationwide through December and January (Lindsay et al., 2009). Birds form large, mixed-sex groups during the nonbreeding dry season and form pairs or pairs with auxiliary males prior to breeding. We determined sex and social status (breeding versus auxiliary helper male) based on plumage colour, behaviour and morphology. We investigated singing behaviour and song structure, song system anatomy and androgen levels in different sets of subjects. While this sampling scheme did not allow us to directly correlate variables on an individual basis, it allowed us to evaluate patterns of the association of hormones, behaviour and brain structure that are predicted by various a priori hypotheses for differences between the sexes and among the male phenotypes.

General Field Methods

Birds were routinely trapped using target mist netting. From each captured bird (except those collected for song system anatomy) we collected a maximum of 80 μl of whole blood from the jugular vein using a 29 g insulin needle, and transferred the blood to heparinized microcapillary tubes. Plasma was stored in liquid nitrogen and transported frozen on dry ice to Washington State. Upon capture, we measured cloacal protuberance (CP) size, tarsus length, body mass, and scored plumage colour. We measured the length (L), width (W), depth (D) of the CP, from which we calculated CP volume using the formula $\pi \times D/2 \times W/2 \times L$ (Karubian, 2002; Mulder & Cockburn, 1993; Tuttle, Pruett-Jones, & Webster, 1996). We scored the percentage of the body in red and black plumage versus brown plumage following Karubian (2002). Because of the bimodal distribution of the percentage of 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 ($\leq$33%) of his body was covered in red or black plumage, and 'red/black' if two-thirds or more ($\geq$67%) of his body was in red or black plumage (Webster et al., 2010). Birds with intermediate plumage scores were excluded from analyses. Sexes were identified using behavioural and morphological traits. We weighed left and right testes of males and ovaries of females euthanized for brain histology (see below).

Song Recording, Metrics and Analyses

We investigated singing rate by observing breeding groups as described in Dowling and Webster (2013) between September and January of 2009/2010, 2010/2011 and 2011/2012. We conducted observations on 57 focal breeding groups (121 individuals), each of which was observed for an average ± SD of 3.74 ± 3.5 h. We conducted a total of 214 h of observation and observed each group once per stage in three breeding stages: (1) prebreeding stage, defined as the period before the female began nest building (64 h of total observation); (2) female fertile period, when the first solicitation or copulation was seen until the penultimate egg was laid (54 h of total observation); solicitations or copulations were seen on average ± SD of 6 ± 3.5 days before the first egg was laid; (3) postfertile stage, the time between completion of the clutch and fledging of young (96 h of total observation). To calculate singing rate, we tallied the number of songs an individual sang and recorded the time the bird was observed to be present on the territory (excluding time spent on the nest). We calculated song rates (songs/observation hour) for 44 females, 46 red/black males, 16 brown males and 15 auxiliary males.

We used a Marantz PMD 661 solid-state digital recorder (D&M Professional, Itasca, IL, U.S.A.) at 96 kHz sampling rate, 24-bit depth, connected to a K6 power module and a ME67 shotgun microphone capsule (Sennheiser Electronic Corporation, Old Lyme, CT, U.S.A.) to record songs. For the analysis of song characteristics we used the maximum number of songs of high recording quality available from each bird based on spectrograms digitized in Raven 1.4 (Bioacoustics Research Program, 2011).

We analysed song structure using whole-song characteristics and fine-scale note characteristics. The first approach analyses the time and frequency characteristics of entire songs, while the second provides detailed information on the frequency and shape of individual notes within songs, which also allowed us to determine note repertoire and thus song complexity (i.e. the proportion of different notes in a song). The combination of both types of analyses facilitates detection of differences between individuals in the number of note types in their repertoire, how notes are combined into songs and the time and frequency characteristics of those full songs.

For whole-song analysis we drew selection boxes around entire songs and summarized seven song metrics (see Results, Table 1): song length (the difference between the beginning and end of the song in seconds), lowest frequency (the frequency in Hertz that has 5% of the song's energy below it and 95% of the song's energy above it), highest frequency (the frequency in Hertz that has 95% of the song's energy below it and 5% of the song's energy above it), bandwidth (the difference between highest and lowest frequencies, i.e. the frequency range), peak frequency (the frequency at which the maximum power in the selection occurs), aggregate entropy (the overall disorder of a sound based on the energy distribution within the entire selection) and average entropy (an average of the disorder measures for each frame in a selection). In this analysis we used songs of 73 individuals and an average ± SD of 6.3 ± 7.1 songs per individual.

For fine-scale note analyses we measured notes within songs using Luscinia (Lachlan, 2007) and summarized nine note characteristics (see Results, Table 2): number of notes per song, note length (the difference between the start and the end of a note in milliseconds), gap length (the length in milliseconds of the gap between the end of the note of interest and the start of the next note), number of note types (the number of unique note types in the song; note classification described below), note rate (notes per second), complexity (number of note types in a song divided by the total number of notes in the song, more detail below), bandwidth (the difference between highest and lowest frequencies in the note, i.e. the frequency range in Hertz), note slope (note bandwidth divided by note length) and peak frequency (the frequency in Hertz within the note where its intensity is greatest). In this analysis we used an average ± SD of 4.9 ± 4.7 songs per individual and analysed the songs of 77 individuals.

We defined a note in the song as a distinct motor unit without a pause (i.e. a continuous trace on a spectrogram; see Results, Fig. 2), and a song as a sequence of notes separated from each other by an

Table 1
Factor loadings for seven parameters of broad-scale song analysis into the first two principal components (PC) in a principal component analysis

	PC1	PC2
Eigenvalue	2.5	2.3
% Variation	35.6	32.1
Song length (s)	−0.16	−0.63
Lowest frequency (Hz)	−0.73	0.51
Aggregate entropy	0.88	0.26
Average entropy	0.84	0.28
Bandwidth (Hz)	0.63	0.22
Highest frequency (Hz)	−0.19	0.81
Peak frequency (Hz)	−0.21	0.86

Table 2
Factor loadings for nine parameters of fine-scale note analysis into the first three principal components (PC) in a principal component analysis

	PC1	PC2	PC3
Eigenvalue	3.18	1.97	1.67
% Variation	35.40	21.9	18.6
Number of notes	−0.81	0.28	−0.44
Note length (ms)	0.61	−0.06	−0.34
Gap after note (ms)	0.72	−0.23	−0.44
Number of note types	−0.57	0.33	−0.35
Note rate (notes/s)	−0.75	0.16	0.60
Complexity[a]	0.55	−0.21	0.49
Note slope	0.44	0.89	−0.03
Note bandwidth (Hz)	0.44	0.89	−0.03
Peak frequency (Hz)	0.27	0.28	0.67

[a] Proportion of different note types within the song.

interval of 40 ms or less. We used JMP 11.0 (SAS Institute Inc., Cary, NC, U.S.A.) to classify note types using normal mixtures clustering. From this we identified an optimal number of clusters (i.e. note types) using AICc values (Burnham & Anderson, 2002) as the fit statistic to determine the optimal number of clusters for the note data set ($N = 20\,944$ notes). This yielded 31 note types, which were then visually inspected to confirm that they were classified appropriately. This note type number is consistent with a visual classification of note types performed on a subset of songs (which also yielded 31 note types, Barker & Congdon, 2010). We calculated song complexity (versatility) by dividing the number of note types in a song by the total number of notes in the song, which gave us the proportion of different note types in each song.

Brain Collection and Histology

We collected birds for brain histology using mist nets in the breeding seasons of 2006 (two red/black males, two brown males, one auxiliary male, two females), 2007 (three red/black males, two brown males, one auxiliary male, two females) and 2010 (two red/black males, one brown male, one auxiliary male, one female) between 18 December and 3 January. In these years breeding started (first egg found) on 30 August (2006), 22 September (2007) and 28 November (2010), respectively. Breeding role was determined by behavioural observation and breeding stage ranged from the prebreeding to the fledgling stage. We did not know the exact breeding stage for all individuals but all collected birds were in reproductive condition, males having enlarged testes and cloacal protuberances and females pre- and postovulatory ovarian follicles.

We euthanized birds by intraperitoneal injection of sodium pentobarbital. Heads were immediately flash-frozen using liquid nitrogen. We performed histology at the Max Planck Institute for Ornithology using brains that were removed from partially thawed skulls and fixed in formaldehyde. Fixed brains were split into hemispheres and the right hemisphere was sectioned sagitally into 30 μm sections on a cryostat. Every fourth section was mounted onto Superfrost slides for Nissl staining. HVC and RA sizes and RA cell densities were measured without knowledge of the sexes or reproductive phenotype of specimens. Areas of interest were analysed on a Zeiss microscope at 40× magnification and then digitized using a SPOT video camera. Image analysis (area size measurements and RA cell counts) was carried out with SPOT software (Diagnostic Instruments, Inc., Sterling Heights, MI, U.S.A.). To investigate the cellular structure of RA, we counted all cells in selected frames in RA (range 40 000–60 000 μm^2) of one to three sections per bird using SPOT image analysis software at 250× magnification. We counted all profiles that contained a nucleus throughout the entire section depth (30 μm) of the counting frame. We differentiated between large and small cells by the presence of a nucleus and visible soma in large cells and by the presence of only a stained nucleus without a well-defined soma in small cells. Large cells also had the typical appearance of neurons. We calculated RA cell number (large and small) from cell density measured in 30 μm sections multiplied by RA volume. All measurements refer to the right HVC and RA. In analyses of HVC and RA volume we controlled for variation in brain size by including hemisphere mass as a covariate.

Plasma Androgen Levels

Blood samples were collected from birds (other than those used for song and song system analyses) in the breeding seasons (September–January) of 2003 to 2009 covering reproduction from prebreeding through the fledgling stage. We analysed plasma androgen levels using radioimmunoassay as previously reported (Lindsay et al., 2009). Plasma samples ranged between 10 and 50 μl, and we ran assays for total androgen concentration (testosterone and 5α-dihydrotestosterone (5α-DHT)). 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) and a testosterone antibody (Wien Laboratories T-3003) that cross-reacts significantly with 5α-DHT. Samples of 30 μl or less were run as singlet assay tubes, samples of 35 μl or greater were run as duplicate tubes. Singlet samples were redissolved after extraction in 110 μl of phosphate-buffered saline with gelatine (PBSg, pH 7.1) and duplicate assays received 210 μl of PBSg. Radioimmunoassays were conducted in 100 μl aliquots according to standard techniques (Schwabl, 1993). Analyses of singlets and duplicates yielded similar results (Lindsay et al., 2009). Two recovery samples containing 2000 cpm tritium-labelled testosterone were included per assay using pooled plasma samples (mean recovery: 75%). The average intra-assay coefficient of variation across the six assays was 13.1% and the interassay variation was 22.1%, calculated according to (Chard, 1995). Samples were distributed randomly across assays.

Data Analyses

To test for differences in song rate between sexes and among male types, we used a generalized linear mixed-effect model (GLMM) with a Poisson distribution with number of songs as the response variable and time observed as an offset argument using the lme4 package in R version 3.1.2 (R Core Development Team, 2013). The model included individual as a random effect and sex/male phenotype, breeding stage and the interaction of sex/male phenotype and breeding stage as fixed effects. To determine the nature and direction of differences in the model, we conducted post hoc tests of pairwise differences between fitted means (least-square means calculated from the GLMM, using lsmeans package in R) for each sex and male type within each of the three breeding stages. Fitted means were backtransformed to show response in units of songs per hour. *P* values were adjusted for multiple comparisons using Tukey's method for a family of four means.

We examined differences in song structure by principal component analyses of metrics in the whole-song (using Raven, Table 1) and fine-scale note analysis (using, Luscinia, Table 2). For both analyses, song measurements were loaded into two or three principal components (Tables 1, 2). We assessed whether sexes or male phenotypes clustered in their scores along these axes in a scatterplot. We used ANOVA in JMP 11.0 (SAS Institute Inc., Cary, NC, U.S.A.) to test the effects of sex and male phenotype and breeding stage on these song measurement principal components in both broad- and fine-scale analyses.

We analysed differences in absolute and relative HVC and RA volume (volume/hemisphere mass), RA cell density and RA cell number by MANOVA. In comparison of absolute volumes we included hemisphere mass as a covariate. Differences between groups were tested post hoc with protected Fisher's least significant difference (LSD) tests.

We used ln-transformed androgen levels in ANOVA to test for effects of sex and male phenotype and breeding stage, controlling for season by including date as a covariate. Only samples collected within 20 min of capture were used in androgen analyses (mean ± SD delay = 8.8 ± 5.4 min).

RESULTS

Singing Rate and Song Structure

Differences in singing rates of the sexes and male types depended on breeding stage (GLMM: sex/male type∗breeding stage: $\chi^2_6 = 68.4$, $P = 8.741\text{e-}13$; Fig. 1). In both the prebreeding stage and the fertile stage (nest building and laying), brown males and auxiliaries sang more than females and red/black males, while in the postfertile stage (incubation, feeding nestling or fledglings), sexes and male types did not differ in song rate (Supplementary Table S1).

The songs of the sexes and male phenotypes (Fig. 2 for representative sonagrams) showed some differences in broad-scale song and fine-scale note characteristics (Fig. 3, Supplementary Tables S2, S3). However, clustering analyses showed no sex or male phenotype segregation along the two principal components in either of these analyses. Combined, these components explained 67.7% (whole song) and 57.3% (fine-scale) of variance (Fig. 3, Tables 1, 2).

ANOVA of song characteristics by sex and male phenotype yielded similar results, with few differences detected for whole song (PC1 (song length, entropy measures, lowest frequency and bandwidth): $F_{3,73} = 0.73$, $P = 0.54$; PC2 (highest frequency, song length and peak frequency): $F_{3,73} = 7.35$, $P = 0.0002$). The significant difference between sex and male type in PC2 was driven mainly by a higher peak frequency in songs of females and helpers than in songs of breeding male types (ANOVA: $F_{2,73} = 5.66$, $P = 0.0053$). Mean and standard deviation for each sex and male type for the seven parameters used in our broad-scale song analysis are provided in Supplementary Table S2. Similarly, we found differences in fine-scale note characteristics that were related to one measure of frequency, PC3 (peak frequency) ($F_{3,77} = 4.51$, $P = 0.0058$), but not to several other measures of frequency and song complexity (PC1 (number of notes, number of note types, complexity, note rate, note length, gap after note): $F_{3,77} = 1.92$, $P = 0.13$; PC2 (bandwidth, note slope): $F_{3,77} = 1.48$, $P = 0.23$). Mean and standard deviation for each sex and male type for the nine parameters used in fine-scale note analysis are provided in Supplementary Table S3. Post hoc tests of differences in peak frequency between sex and male type showed that note peak frequency was higher for females than for all male types (ANOVA: $F_{1,77} = 21.8$, $P < 0.0001$). Sex differences in size might explain these differences in song frequency, as females whose songs were included in our analyses weighed significantly less than males (ANOVA: $F_{1,45} = 1.98$, $P = 0.002$). Breeding stage (prebreeding, prelaying, lay, incubation, nestling, fledgling) had no significant effect for the whole-song analysis (PC1: $F_{5,107} = 2.24$, $P = 0.06$; PC2: $F_{5,107} = 0.78$, $P = 0.57$) or note analysis (PC1: $F_{5,104} = 0.42$, $P = 0.84$; PC2: $F_{5,104} = 1.76$, $P = 0.13$, PC3: $F_{5,104} = 0.42$, $P = 0.83$).

Our fine-scale note analyses revealed no difference between male phenotypes or between the sexes in several note characteristics (fine-scale note analysis PC1), including number of note types, which is a fine-scale measure of vocal diversity, appropriate for a species with one main song type.

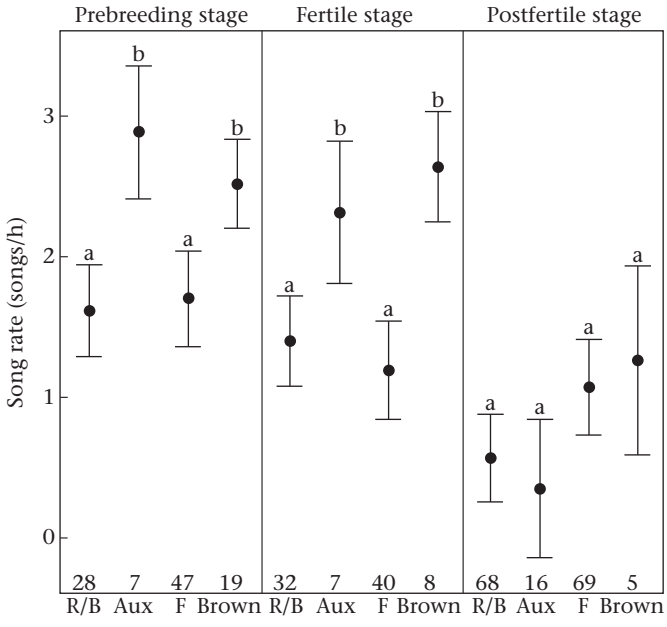

Figure 1. Singing rates (songs per hour, fitted means with 95% CI from generalized linear mixed-effect model) for male phenotypes of red-backed fairy-wrens in three breeding stages. Within each breeding stage, levels with different letters were significantly different from each other. R/B: red/black breeder male; Brown: brown breeder male; Aux: brown auxiliary males; F: female. Numbers above sex and male types give samples size of each type for each stage.

Song System Nuclei

Representative photomicrographs of HVC and RA for male types and females are shown in Fig. 4. Absolute HVC and RA volumes (Fig. 5) varied significantly with male type and sex (HVC: $F_{3,15} = 3{,}60$, $P = 0.038$; covariate hemisphere mass: $F_{3,15} = 0.06$, $P = 0.804$; RA: $F_{3,15} = 4.52$, $P = 0.019$; covariate hemisphere mass: $F_{3,15} = 0.19$, $P = 0.668$). HVC was significantly smaller in females than in red/black breeder males and auxiliary males ($P = 0.005$ and $P = 0.018$, respectively) but not different from brown breeder males (Fig. 5). RA volume was significantly smaller in females than in red/black breeder males ($P < 0.001$), but not different from brown breeder males and auxiliary males (Fig. 5). Relative HVC and RA size (volume divided by hemisphere mass) varied significantly with male type and sex (HVC: $F_{3,18} = 4.37$, $P = 0.018$. RA: $F_{3,18} = 7.155$, $P = 0.002$). Relative HVC volume was significantly smaller in females than in red/black breeder males and auxiliary males ($P = 0.005$ and $P = 0.012$, respectively) but not different from brown breeder males ($P = 0.112$). Female relative RA volume was smaller than in red/black breeder males and brown breeder males ($P < 0.001$, $P = 0.018$), but not different from auxiliary males ($P = 0.101$). Density of large but not small cells in HVC varied among male types and sexes (large cells: $F_{3,17} = 7.983$, $P = 0.002$; small cells: $F_{3,17} = 1.524$, $P = 0.244$). The female RA had a higher density of large cells than did the male RA (females versus red/black males: $P = 0.001$; females versus brown males: $P < 0.001$; females versus auxiliary males: $P = 0.054$) while density of small cells did not differ (all $P > 0.05$). The total number of large or small cells in RA did not vary across male types or sexes (large cells: $F_{3,16} = 0.540$, $P = 0.662$; small cells: $F_{3,16} = 1.663$, $P = 0.215$; Fig. 5).

Absolute and relative HVC and RA volume and density and total number of large and small cells in RA did not differ across male

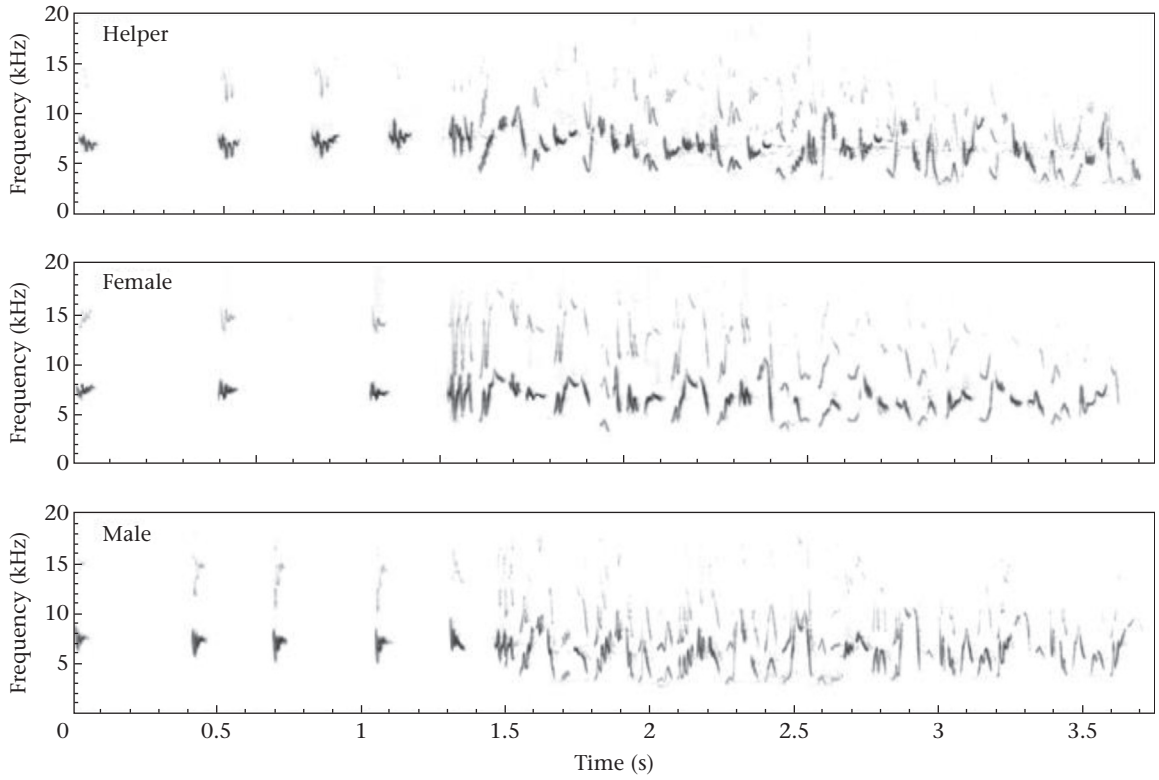

Figure 2. Representative sonagrams for songs of an auxiliary helper male, a breeding female and a breeding red/black male from separate breeding groups.

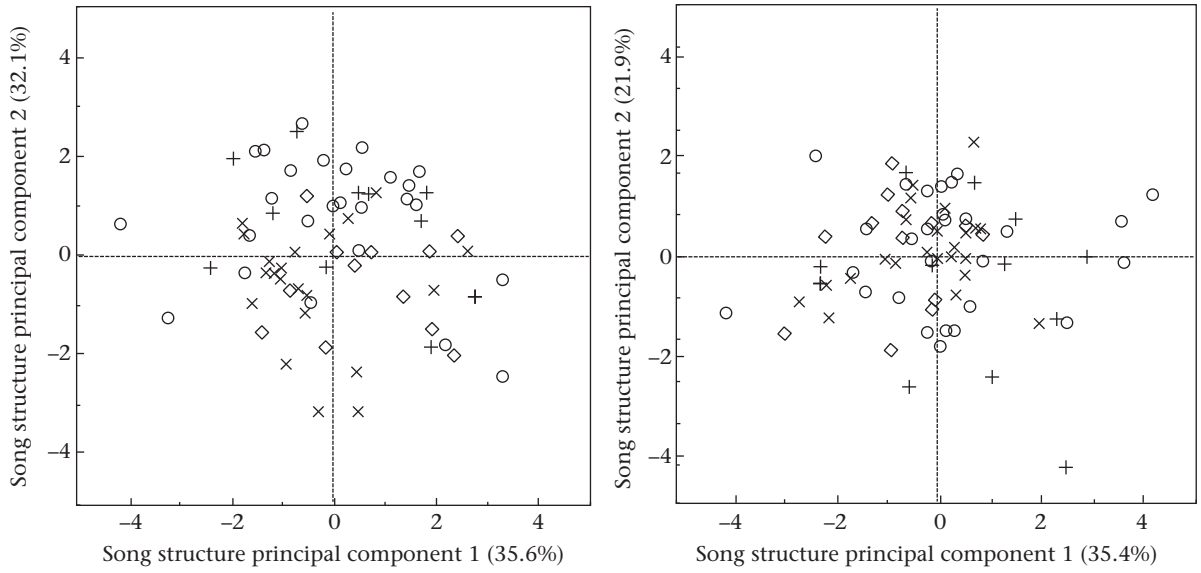

Figure 3. Clustering of scores for the first two principal components (PC1, PC2) for females (○), auxiliary helpers (+), brown breeder males (◊) and red/black breeder males (×). Left: broad-scale song measurements produced in Raven; *N*: 26 females, 11 auxiliaries, 14 brown breeder males and 22 red/black breeder males; PC1 (song length, entropy measures, lowest frequency and bandwidth) explained 35.6% of variance; PC2 (highest frequency, song length and peak frequency) explained 32.1% of variance. Right: fine-scale note analysis using Luscinia; *N*: 28 females, 12 auxiliaries, 13 brown breeding males and 24 red/black breeding males; PC1 (number of notes, number of note types, complexity, note rate, note length, gap after note) explained 35.4% of variance; PC2 (bandwidth, note slope) explained 21.9% of variance; PC3 (peak frequency, not shown) explained 18.6% of variance.

phenotypes (Fig. 5). Male phenotypes used in brain analyses did also not differ in combined testes mass and volume of the cloacal protuberance (mean ± SD testes mass: red/black males: 0.226 ± 0.045 g; brown males: 0.207 ± 0.028 g; auxiliary males: 0.208 ± 0.045 g; $F_{2,18} = 2.46$, $P = 0.117$; mean ± SD cloacal protuberance volume: red/black males: 135.2 ± 27.6 mm^3; brown males: 125.7 ± 51.4 mm^3; auxiliary males: 120.7 ± 42.2 mm^3; $F_{2,18} = 2.24$, $P = 0.138$). None of these variables were correlated with song system measures (all $P > 0.05$).

Plasma Androgens

We previously reported that male reproductive phenotypes differ dramatically in plasma androgen levels during reproduction and the

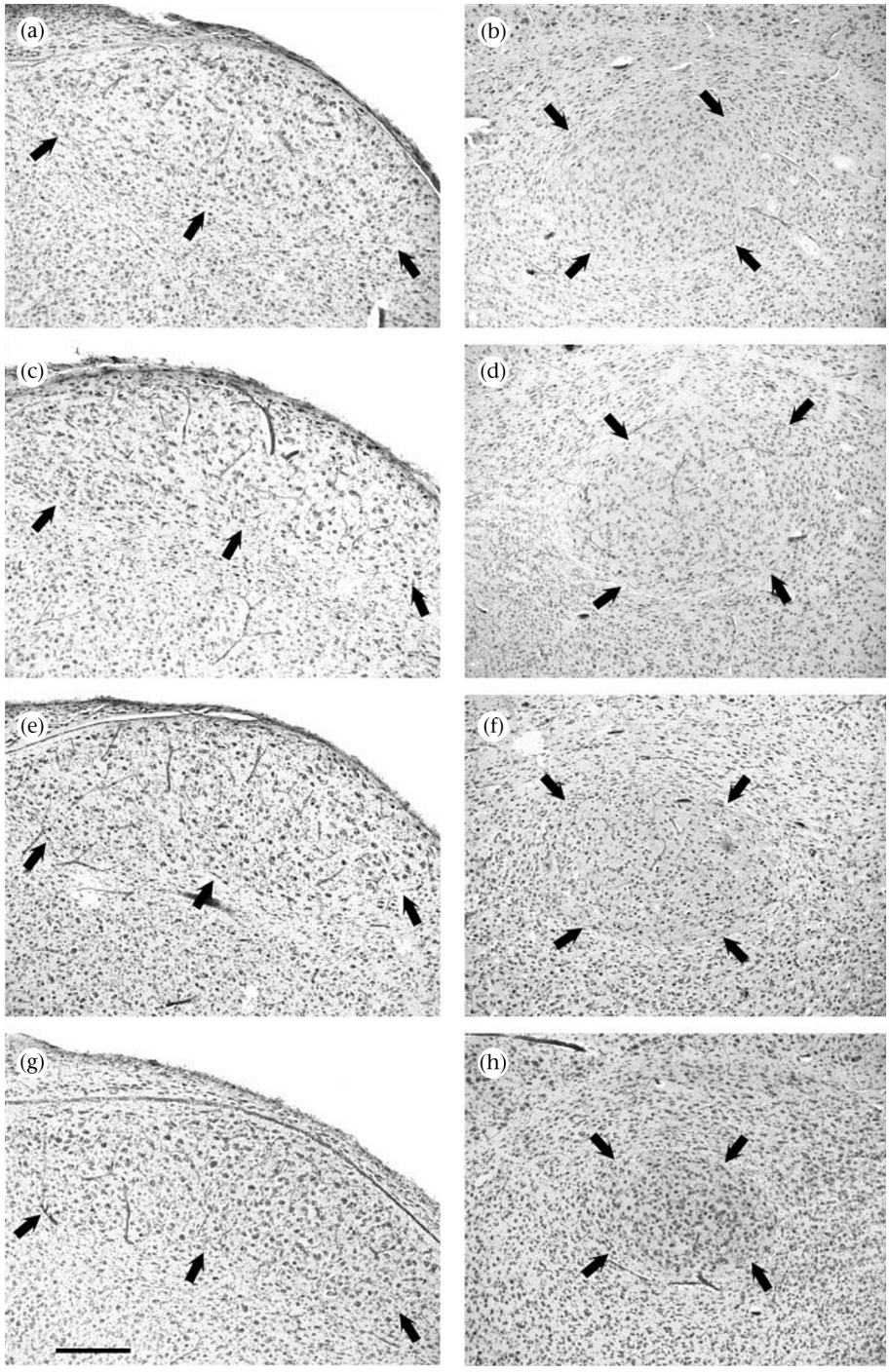

Figure 4. Representative photomicrographs of brain sections containing the higher vocal centre (HVC) and the robust nucleus of the arcopallium (RA) of a brown breeder male (a, b), red/black breeder male (c, d), auxiliary male (e, f) and female (g, h). Scale bar (in g) = 100 μm. Note the high density of large cells in female RA.

preceding prenuptial moult when they acquire their breeding plumage phenotype (Lindsay et al., 2009). Here we compare these levels with those of females. Androgen levels varied between male types, sexes and breeding stages (ANOVA, sex/male type: $F_{3,300} = 33.194$, $P < 0.001$; breeding stage: $F_{5,300} = 2.452$, $P < 0.001$; covariate date: $F_{1,300} = 0.55$, $P = 0.508$). As before, the three male types differed significantly in androgen levels across all stages (red/black versus brown breeding males: $P < 0.001$; red/black breeding males and brown breeding males versus auxiliary males: both $P < 0.001$). Red/black breeder males had the highest androgen levels during all stages of reproduction, followed by brown breeder males and then brown helper males (Fig. 6). Females had substantially lower androgen levels than red/black breeder males and brown breeder males (both $P < 0.001$) but significantly higher levels than auxiliary helper males ($P = 0.001$; mean ± SE: auxiliaries = 329 ± 52 pg/ml; females = 497 ± 27 pg/ml; Fig. 6). Androgen levels of females during breeding were not related to the plumage phenotype of their social mates (Schwabl, Lindsay, Barron, & Webster, 2014).

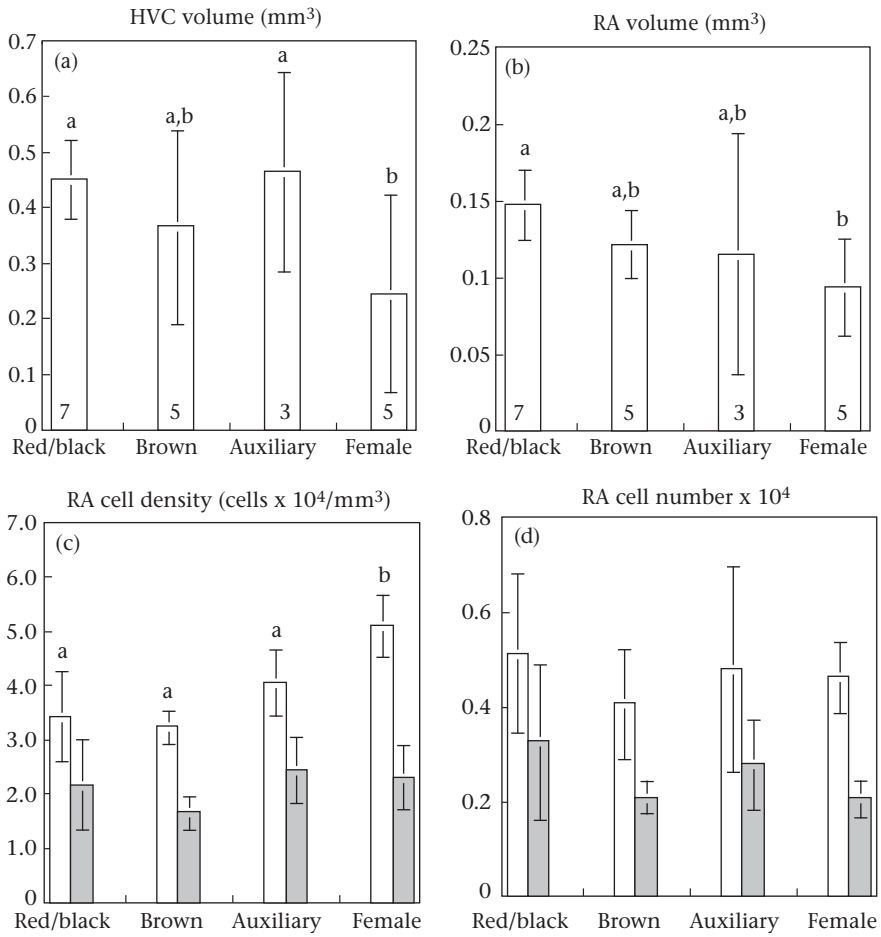

Figure 5. Mean volumes ± 95% CI for (a) the higher vocal centre (HVC) and (b) the robust nucleus of the arcopallium (RA) of male phenotypes (red/black breeding, brown breeding, brown auxiliary) and females. (c) Mean density ± 95% CI of large (open bars) and small (shaded bars) cells in the RA of male phenotypes and females. (d) Mean total number ± 95% CI of large and small cells in the RA. Different letters above columns indicate significant (LSD: $P < 0.05$) differences. Sample sizes are given in bars for HVC and RA volume.

DISCUSSION

We show that in the red-backed fairy-wren (1) the sexes appear to produce songs of similar structure and complexity, yet differ in size and architecture of the song control nuclei and in circulating androgen levels, (2) the alternative male reproductive phenotypes produce similarly structured songs and possess anatomically similar song control nuclei, (3) sexes and male types differ in song rates prior to breeding and during the period of female fertility, but differences in song centre characteristics and androgen levels do not correspond to song rate differences. These results run counter to the expected relationship between neural network space, singing behaviour and song structure and androgen levels among male phenotypes and between the sexes. Such results are inconsistent with the hypothesis that high singing rates and similar motor output (i.e. song structure) require high androgen levels and similar anatomy of the underlying neural substrates of song production.

In our study, dominant frequency was the primary song measure that differed between the sexes, with females singing at a higher pitch than males. Although it is likely that song centre nuclei influence the pitch produced by the syrinx, current empirical data suggest that characteristics of song centres are most strongly associated with the repertoire of songs and syllable types that an individual sings (Bolhuis & Gahr, 2006; Nottebohm & Arnold, 1976). The differences we see between the sexes in pitch may instead be due to differences in body size. Scaling of pitch with body size is an honesty-enforcing constraint seen across taxa (Fletcher, 2004; Hall, Kingma, & Peters, 2013).

Several other studies have reported that the sexes sing at similar rates and produce songs of similar structure despite sex differences in song nuclei volume, neuron phenotype and cell architecture. For example, male and female rufous-and-white wrens, *Thyrophilus rufalbus* (Troglotydidae) engage in duets, but HVC, RA and Area X are significantly larger in males than in females; such brain dimorphism is reduced, but not absent, in the bay wren, *Cantorchilus nigricapillus* (Troglotydidae), in which females sing more elaborate songs in their duets with males (Brenowitz & Arnold, 1986). In the bush shrike, *Lanarius funebris* (Malaconotidae), a duetting species with similar song complexity in the sexes and large sex differences in androgen levels (Schwabl & Sonnenschein, 1992), HVC and RA volumes are almost two times larger in males than in females (Gahr et al., 1998). In the forest weaver, *Ploceus bicolor* (Ploceidae), a species in which mated pairs sing a unison duet and identical songs, HVC and RA are more than 1.5-fold larger in males than in females and females have 30–70% more synapses in HVC and RA than do males (Gahr, Metzdorf, Schmidl, & Wickler, 2008). In the white-browed sparrow weaver (Ploceidae), a species in which male and female group members engage in duet and chorus song, males also have larger HVC and RA nuclei than females (Voigt & Gahr, 2011). In the northern cardinal, *Cardinalis cardinalis* (Cardinalidae), males and females also produce near monomorphic

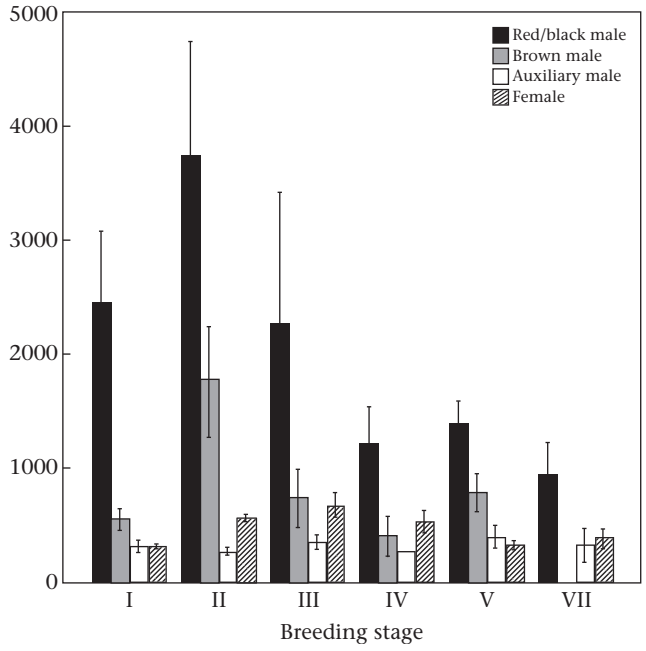

Figure 6. Mean ± SE plasma androgen concentrations of male phenotypes and females across the breeding stages. I: pre-breeding; II: nest building; III: laying; IV: incubation; V: nestlings; IV: fledglings. Sample size per stage (red/black males, brown males, auxiliary males, females): prebreeding: 8, 13, 13, 68; nest building: 13, 9, 10, 57; laying: 6, 3, 2, 14; incubation: 8, 4, 1, 21; nestlings: 11, 14, 15, 28; fledglings: 6, 0, 5, 9.

song, but the song system nuclei of males are 1.5–2 times larger than the females' nuclei (Jawor & MacDougall-Shackleton, 2008). Finally, female streak-backed orioles, Icterus pustulatus (Icteridae), sing at higher rates than males, yet despite this female bias in song rate, males have a significantly larger HVC than females (Hall et al., 2010). We add the duetting and chorusing tropical red-backed fairy-wren of the Australasian family Maluridae to this increasing number of species in which a relationship of sexual dimorphism of song system nuclei, singing and song structure breaks down.

Together, these studies suggest that males and females can produce similar motor output despite differences in size and neuronal phenotype of the underlying brain structures. Sexual dimorphism in size and structure of the song system and androgen levels in the absence of sex differences in song structure and song production warrants further investigation. We need to search for other functions of the sex differences in song nuclei size and anatomy, consider that similar acoustic output can result from different cellular composition and structure of underlying brain nuclei (Gahr et al., 2008) and investigate the entire neural network of motor output control (Moore, Székely, Büki, & DeVoogd, 2011). Comparative studies that rely on male-to-female ratios in size of song system nuclei to investigate the evolution of sex differences in brain structure and elaboration of acoustic signals (e.g. DeVoogd et al., 1993; Garamszegi, Eens, Erritzøe, & Møller, 2005; MacDougall-Shackleton & Ball, 1999) need to take these results into account and should be re-evaluated by inclusion of more studies of tropical and subtropical songbirds. Song production in female songbirds is phylogenetically widespread and is the ancestral character state (Odom, Hall, Riebel, Omland, & Langmore, 2014), and many of the species in which females sing and engage in complex duets with males are tropical or subtropical (Odom et al., 2014; Robinson, 1949; Slater & Mann, 2004), and the tropics have the highest diversity of songbirds (Rickleffs, 2006). Yet, studies of sexual dimorphism of singing and song and of their neural substrates and hormones are largely limited to north temperate taxa in which female song is more of an exception than a rule (Kroodsma, Viellard, & Stiles, 1996; Price, Lanyon, & Omland, 2009; Riebel, 2003) and, when occurring, is often less complex than in males (e.g. Arcese, Stoddard, & Hiebert, 1988; Beletsky, 1982, 1983).

The male reproductive phenotypes of the red-backed fairy-wren differ in social status, reproductive roles and expression of territorial and parental behaviour (Barron, Webster, & Schwabl, 2015; Karubian, 2002; Webster et al., 2010). Our previous research provides evidence that differences in androgen levels are associated with a suite of morphological (i.e. plumage and bill colour, feather length), reproductive (cloacal protuberance) and behavioural (sexual versus paternal investment) male traits in this species (Barron et al., 2015; Karubian et al., 2011; Lindsay et al., 2011, 2009). Here we show that the male phenotypes, despite their large differences in androgen levels, produce similar songs and have similar HVC and RA volumes and RA cell densities. These results are in contrast to studies of other songbirds with complex social breeding systems that show the influence of social status on the architecture and cellular structure of song control nuclei and song structure. For example, dominant males of the white-browed sparrow weaver have larger HVC and RA volumes than subordinate males, they differ from subordinate males in the cellular machinery and gene expression patterns in song nuclei, and only dominant males sing solo songs while all males and females engage in duet song (Voigt & Gahr, 2011; Voigt et al., 2007). Male social status, however, was not reflected in consistent differences in circulating testosterone levels in these studies (Voigt & Leitner, 2013; Voigt et al., 2007). In contrast, the three male phenotypes of the red-backed fairy-wren have song control nuclei of similar size and cellular architecture, but they clearly differ in androgen levels. Although androgens regulate a suite of morphological traits associated with the reproductive phenotypes of the red-backed fairy-wren (Karubian et al., 2011; Lindsay et al., 2011, 2009), the song control system does not appear to be a component of this suite.

There is ample evidence for song system nuclei being sensitive to androgens. HVC, RA and MAN (magnocelluar nucleus of the anterior nidipallium) contain androgen receptors (i.e. Ball, Riters, & Balthazart, 2002; Gahr, 2007) and treatment of males with androgens increases the size of song nuclei (Smith et al., 1997). Volumes of male song nuclei vary seasonally (e.g. Kirn & Schwabl, 1997; Nottebohm et al., 1981; Tramontin et al., 2000; but see Smulders et al., 2006), related, at least in part, to changes in circulating androgen levels (i.e. Tramontin et al., 2003) and changes in song structure. However, male song nuclei can also remain neuroanatomically stable (Gahr, 1990; Leitner, Voigt, Garcia-Segura, Van't Hof, & Gahr, 2001) despite seasonal changes in androgen levels and song structure (Leitner et al., 2001). Similarly, seasonal dissociation of changes in androgen levels, growth of HVC and RA and singing activity has been found in males of Mediterranean populations of blue tits, *Parus caeruleus* (Caro, Lambrechts, & Balthazart, 2005; Caro et al., 2006). Previous research detected variation in the singing rate of male red-backed fairy-wren within the breeding season (males initiate more songs during the female's fertile stage than after, Dowling & Webster, 2013; and brown males show higher overall song rates, this study), but we do not know whether singing rate and song structure vary with the annual cycle. In addition, we do not know whether size and structure of song nuclei change seasonally (i.e. with onset and termination of reproduction) in male red-backed fairy-wren, but if this is the case, our results suggest that circulating androgens probably do not play a large role, in contrast to strong evidence for androgen regulation of morphological traits like plumage and bill colour, feather length and cloacal protuberance size (Karubian et al., 2011; Lindsay et al., 2011, 2009). Our results indicate differences in song rate among male

phenotypes that are opposite to differences in androgen levels: brown breeding males and auxiliaries sang more than red/black breeding males during the prebreeding and fertile stage, but their androgen levels were consistently lower across breeding stages than those of red/black males. Experimental approaches, such as castration and hormone replacement, are required to further investigate the role of androgens in the singing behaviour of this species. We do not know why breeding brown males and auxiliaries sang at higher rates than red/black males and females during the prebreeding and fertile stage, but data on duet rate suggest that brown males duet with their mates more often than red/black males (especially in the female's fertile stage, Dowling & Webster, n.d.-a), which may contribute to this pattern.

Similar to females in most other songbirds (Goymann & Wingfield, 2014; Ketterson et al., 2005; Møller, Garamszegi, Gil, Hurtrez-Boussès, & Eens, 2005), female red-backed fairy-wrens had drastically lower androgen levels than breeding males, but their levels were significantly higher than those of auxiliary helper males. Despite these low androgen levels, female song rates and song structure were similar to those in red/black males. This indicates that high androgen levels are not required for females to engage in singing and to produce songs that are similar to those of males. It also suggests that low androgen levels are not the cause for their smaller and structurally different song nuclei because helper males had lower androgen levels than females, yet their song nuclei were similar to those of breeding males. Although androgen treatment can increase the size of female song nuclei (Brown & Bottjer, 1993; Madison et al., 2014; Nottebohm, 1980) and can induce song in females of several species (i.e. Appeltants et al., 2003; Hausberger et al., 1995; Kern & King, 1972; Kriner & Schwabl, 1991; Lahaye et al., 2012; Madison et al., 2014; Rasika et al., 1994; Voigt & Leitner, 2013; references in Rosvall, 2013), in the red-backed fairy-wren, high circulating androgen levels do not seem to be necessary for females to sing at rates and with song structure similar to males.

Androgen levels of breeding male red-backed fairy-wren varied dramatically, with levels as high in the red/black phenotype as peak levels in breeding males of northern latitude species and as low in auxiliary males as those of nonreproductive males of northern latitude species (Garamszegi et al., 2008; Goymann & Landys, 2011; Goymann, 2009; Goymann et al., 2004; Goymann & Wingfield, 2014). Androgens clearly influence phenotype of the red-backed fairy-wren: male plumage phenotypes can be predicted from androgen levels during the time when they acquire their breeding plumage and androgen levels differ throughout reproduction (Lindsay et al., 2009; this study); testosterone treatment prior to and during prenuptial moult induces the ornamented red/black male plumage phenotype (Lindsay et al., 2011); and change of the social status from helper to breeder male is associated with a rise in androgen levels and change in androgen-sensitive structures, such as bill colour and capacity to produce red/black plumage (Karubian et al., 2011). Moreover, testosterone treatment prior to and during moult activates a male-like phenotype in females that includes partially red/black plumage, dark bill, short feathers and an enlarged cloacal protuberance (Lindsay, Barron, Webster, & Schwabl, n.d.), suggesting sensitivity of females to testosterone.

In conclusion, androgens regulate and integrate a suite of sexual traits in the red-backed fairy-wren, but this trait module does not appear to include song rate, song structure or the underlying neural system. This raises the question of which sexually dimorphic and male polymorphic reproductive and behavioural traits are regulated and integrated by androgens and which are not, and how such variation relates to social and mating systems, sexual selection, seasonality and the environment across species.

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Supplementary Material

Supplementary Material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2015.03.006.

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