

Do androgens link morphology and behaviour to produce phenotype-specific behavioural strategies?



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Morphological and behavioural traits often covary with each other, and the links between them may arise from shared physiological mechanisms. In particular, androgens such as testosterone have emerged as prime candidates for linking behaviour and morphology due to the environmental sensitivity and pleiotropic effects of these hormones. In this study we investigated the hypothesis that androgens simultaneously relate to morphological and behavioural variation, thereby producing the integrated reproductive phenotypes of male red-backed fairy-wrens, *Malurus melanocephalus*. Males of this species can adopt one of three discrete breeding phenotypes: breeding in red/black plumage, breeding in brown plumage, or remaining as nonbreeding brown natal auxiliaries. Although the expression of morphological traits in this species is regulated by androgens and phenotypes differ in baseline androgen levels (red/black breeder > brown breeder > auxiliary), injection with GnRH failed to expose phenotype-specific constraints on androgen production. Observations of territoriality, nestling feeding and extra-territorial forays revealed phenotype-specific patterns of mating and parental effort, yet these were largely related to age and were not correlated with baseline or GnRH-induced androgen levels, or the androgen change between these points. While these findings support the idea that morphological and behavioural traits are linked via phenotypic correlations, they do not support the hypothesis that behavioural differences arise from variation in circulating androgens or the capacity to produce them.

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Individuals of most species show variation across morphological and behavioural traits, with selection on the collective phenotype often linking suites of related traits (Lande & Arnold, 1983; Sih, Bell, & Johnson, 2004; Sih, Bell, Johnson, & Ziemba, 2004) and even producing morph-specific behavioural strategies (Dammhahn, 2012; Nicolaus et al., 2012; Wolf, van Doorn, Leimar, & Weissing, 2007). Because inherent genetic, energetic and time constraints can limit phenotypic expression, however, suites of traits often fall along life-history continua determined by trade-offs between mating versus parental investment (Magrath & Komdeur, 2003) and current versus future reproduction (Reznick, 1992; Santos & Nakagawa, 2012; Stearns, 1992). Sexual signals represent a unique case of phenotypic variation in which exaggerated morphological and/or behavioural traits expressed by a potential mate convey information about the direct (e.g. resources, parental care) or indirect (genetic) benefits of mating with the sender (Andersson,

1994). Males often advertise their reproductive phenotype (e.g. social status, nutritional state, investment in sexual versus parental behaviour) with morphological and behavioural signals such as bright coloration and elaborate mating displays. The evolution and maintenance of such signalling systems requires that those signals honestly indicate mate quality (e.g. Hill, 1990; Jennions, Møller, & Petrie, 2001; Zahavi, 1975), and morphological and behavioural signals are hypothesized to be honestly linked and constrained via regulation by shared underlying mechanisms that result from physiological trade-offs and antagonistic pleiotropy (Cox, Stenquist, Henningsen, & Calsbeek, 2009; Hau, 2007; Lande, 1980).

Androgens such as testosterone have gained particular attention as regulators of honest signal elaboration because they transduce environmental information and their pleiotropic actions link suites of traits (Ketterson & Nolan, 1999; McGlothlin & Ketterson, 2008), plus they can carry physiological costs (e.g. immunosuppression; Folstad & Karter, 1992; Saino & Møller, 1994). Specifically, androgens are thought to promote the exaggeration of morphological signals (e.g. Gonzalez, Sorci, Smith, & de Lope, 2001; Zuk, Johnsen, & Maclarty, 1995) while shifting behaviour to increase mating effort at the expense of parental investment (reviewed in Ketterson &

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Nolan, 1999). While our understanding of these patterns has benefited greatly from studies of captive populations and hormone manipulations (e.g. Ketterson & Nolan, 1999; Lynn, Prince, Schook, & Moore, 2009; Roberts, Ras, & Peters, 2009; Van Roo, 2004), a more complete picture also requires that individual variation in androgen levels be examined in unmanipulated wild animals (Kempenaers, Peters, & Foerster, 2008; Williams, 2008); such studies often detect no relationship between androgens and phenotype, or even report patterns opposite these predictions (Adkins-Regan, 2005; Lynn, 2008).

The high variability of baseline androgen concentrations across short timescales has created hesitation regarding their usefulness in predicting individual behaviour (Adkins-Regan, 2005; Ball & Balthazart, 2008). McGlothlin et al. (2008; McGlothlin, Jawor, & Ketterson, 2007) suggested that transient increases in testosterone should be more relevant to trade-offs between mating and parental investment because these short-term 'spikes' are generated by social exchanges inherent to territorial interactions or courtship (e.g. M. C. Moore, 1983; Oliveira, 2004; Pinxten, de Ridder, & Eens, 2003; Wingfield, Hegner, Dufy, & Ball, 1990) and could allow males to minimize the costs of high testosterone without sacrificing expression of testosterone-mediated behaviour (Wingfield, Lynn, & Soma, 2001). Their research demonstrated that variation in responsiveness to a given dose of gonadotropin-releasing-hormone (GnRH), which regulates gonadal androgen production, predicts the sexual phenotype of male dark-eyed juncos, *Junco hyemalis*; birds with higher GnRH-induced testosterone concentrations were more territorial (McGlothlin et al., 2007), and birds that increased testosterone more in response to GnRH (compared to baseline) exhibited less parental care (McGlothlin et al., 2007) and more sexually selected white coloration of tail feathers (McGlothlin et al., 2008). Similarly, alternative behavioural/morphological phenotypes of male white-throated sparrows, *Zonotrichia albicollis*, also differed in their testosterone response to GnRH challenge (Spinney, Bentley, & Hau, 2006), further suggesting that activity of the hypothalamic–pituitary–gonadal (HPG) axis might integrate morphological and behavioural expression.

Here we investigate whether activity of the HPG axis relates to individual and phenotype-specific differences in morphology and behaviour through action on both phenotype components in a wild songbird, the red-backed fairy-wren, *Malurus melanocephalus*. This Australian species is particularly well suited for studies of the endocrine basis of sexual signal expression and behaviour, as males express three discrete breeding phenotypes that differ in morphology, behaviour and circulating androgen levels (Karubian, 2002; Varian-Ramos, Lindsay, Karubian, & Webster, 2012; Webster, Varian, & Karubian, 2008). During the first year of reproduction males can breed in sexually preferred red/black nuptial plumage and display frequent extraterritorial forays and low nestling feeding rates ('red/black breeders'), breed in brown female-like plumage with infrequent extraterritorial forays and high nestling feeding rates ('brown breeders'), or remain on their natal territory as a brown helper with unknown foray frequency and intermediate nestling feeding rates ('auxiliaries'). Nearly all males are red/black breeders in subsequent breeding seasons, meaning this species shows phenotypic variation within an age class (1-year-old) and age variation within a phenotypic class (red/black breeders); age differences could therefore underlie some phenotypic differences. Baseline androgen concentrations during prenuptial moult and all breeding stages vary across phenotypes with high concentrations in red/black breeders, intermediate concentrations in brown breeders and low concentrations in auxiliaries (Lindsay, Webster, Varian, & Schwabl, 2009). When males shift from an auxiliary role to a breeding role their androgen

concentrations increase, accompanied by a darkening of the bill and a capacity to produce red/black plumage (Karubian, 2008; Karubian, Lindsay, Schwabl, & Webster, 2011). Furthermore, testosterone implants prior to and during the prenuptial moult stimulated production of red/black plumage and darkening of the bill (Lindsay, Webster, & Schwabl, 2011). In support of the idea that sexual signal expression should honestly indicate mate quality, males in better condition produced more red/black plumage, although this pattern surprisingly did not appear to arise from condition-dependent androgen regulation (Barron, Webster, & Schwabl, 2013).

In this study we tested the hypotheses that circulating androgen levels, and/or the capacity for androgen production, correlate with integrated male breeding phenotypes in this species. We first aimed to determine whether foray behaviour, nestling feeding and territoriality covary across male phenotypes (Karubian, 2002), as would be expected if male behaviour is constrained by a trade-off between mating and parental investment. For this we predicted that male mating and parental behaviour would vary by phenotype, with red/black males investing more heavily in mating (territoriality and extraterritorial forays) and less in parental care (nestling feeding). Second, we sought to establish whether phenotypes differ in their physiological capacity to produce androgens, which could explain why brown breeders and auxiliary males maintain lower circulating concentrations than red/black breeders. We predicted that males with red/black plumage and/or a breeding role would have a greater capacity to increase androgens (as indicated by their response to a GnRH challenge). Finally, we investigated whether variation in androgen secretion is correlated with behavioural variation and could therefore provide a mechanistic link between morphology and behaviour to produce a correlated phenotype, predicting that males with higher baseline or GnRH-induced androgens would invest more in mating behaviour at the expense of parental care.

METHODS

Study Species and Basic Field Methods

This study was conducted in a population of colour-banded red-backed fairy-wrens near Herberton, Queensland, Australia (145°25'E, 17°23'S). Males of this species are phenotypically plastic in their first year, with discrete variation in breeding role (breeder versus auxiliary) and plumage colour (red/black versus brown), although almost all males become red/black breeders in following breeding seasons. These phenotypic differences also extend to other morphological and behavioural traits, as red/black males have shorter tails (Karubian, 2002) and darker bills (Lindsay et al., 2009) and invest in extrapair mating at the expense of parental care by foraging off their territory more frequently and feeding nestlings less than brown breeders (Karubian, 2002). The elevated mating investment of red/black males is mirrored by a female preference for red/black plumage (Karubian, 2002), resulting in higher reproductive success for red/black males through greater production of extrapair young (Webster et al., 2008). While we know that red/black males are more aggressive towards red/black intruders than they are towards brown intruders (Karubian, Sillett, & Webster, 2008), we lack an understanding of whether phenotypes differ in their territorial response to intruders.

We target-trapped birds during the breeding season between 9 October and 6 December, 2011 using mist nets by slowly walking towards the birds to push them towards the nets and/or briefly (<2 min) playing conspecific vocalizations. We collected basic morphological measurements (e.g. mass, tarsus) and determined age of unbanded birds (second year versus after-second year) using

the degree of skull ossification (Lindsay et al., 2009) before attaching an Australian Bird and Bat Banding Scheme (ABBBS) aluminium leg band and a unique combination of three coloured plastic leg bands for subsequent identification. Captures and sightings of birds were used to assess plumage colour, estimating the proportion of feathers in red/black plumage. Using this value we categorized birds as brown (<33% red/black feathers), intermediate (33–66%), or red/black (>66%). Because plumage coloration is strongly bimodal (Webster et al., 2008), few males were in intermediate plumage, and these were omitted from all analyses. We also monitored all nests and identified all members of each breeding group, consisting of the breeding male, the breeding female and up to two auxiliaries.

Blood Sampling and GnRH Injections

Upon capture we immediately removed birds from the net and collected a maximum of 40 μ l of blood from the jugular vein into heparinized microcapillary tubes (bleeding delay: mean = 3.8 min, range 1.8–8.1 min) for measurement of baseline androgen levels. Birds were then placed into an opaque bag and left undisturbed until 20 min after capture (to minimize temporal variation from capture), at which time we injected most birds with 10 μ l of 500 ng of chicken GnRH-I (American Peptide Company, 54-8-23) dissolved in phosphate-buffered saline 1x (GIBCO, 20012). Injections were made into the left pectoralis major muscle using a sterile 25-gauge needle attached to a 50 μ l syringe (Hamilton Company, Henderson, NV). This 'GnRH challenge' dose has been shown to cause a maximal LH response in other passerines (e.g. Wingfield & Farner, 1993) and to cause elevated androgen production in males from our study population (Karubian, et al., 2011). A small random subset of birds received an injection containing only 10 μ l of phosphate-buffered saline to serve as controls. Birds were again placed into an opaque cloth bag and left undisturbed until an additional 40 μ l of blood was collected 30 min after injection for measurement of GnRH-induced androgen levels (Jawor et al., 2006).

Behavioural Observations

To estimate the frequency of male forays (departures from territory), we located focal males on their territories at approximately sunrise and followed them for up to 1 h, recording the number and duration of forays. Because it was not always possible to complete a full hour of observations (due to losing the focal male and being unable to relocate him; mean \pm SE duration = 52.4 \pm 2.0 min) we calculated and report here the number of forays and the duration of time off territory per observation hour. A concurrent study with the same birds found that auxiliaries do not influence the foray behaviour of red/black males (Potticary, Dowling, Barron, & Webster, n.d.), making it unnecessary to control for helper effects in analyses of this behaviour (see below). Although these forays may not be solely for the purpose of obtaining extrapair matings (Stutchbury, 1998; Yezerinac & Weatherhead, 1997), departing birds generally visited surrounding territories that were actively occupied, suggesting they were being used to assess mating opportunities and/or competition from neighbours (see Karubian, 2002).

We estimated male response to a simulated territorial intrusion (STI) by presenting a conspecific decoy and playback 10–15 m from his nest 1–3 h after sunrise. We randomly paired one of four hand-crafted wooden decoys (similar in size, posture and colour to live red/black males) with a compilation of songs by one of four unfamiliar red/black males recorded more than 30 km from the focal population. Decoys were attached to a stick protruding 1 m above a small tripod in an otherwise open area to minimize visual

obstruction. Songs were played with a portable digital player (Naxa NM145) and an amplifying speaker (Pignose 7-100) covered in grass below the decoy at an average volume of 61 dB, measured at 2 m using a sound level meter (Extech 407730). Song playbacks consisted of six songs that were alternated with 10 s of silence to produce a rate of six songs per minute. To prevent birds from responding to the researcher during placement, set-up was performed quickly when adults were not nearby. After placement, the observer returned to a small, camouflaged blind set up approximately 15–25 m away the previous day. Following 5 min of silence, we began 15 min of song playback. We considered a bird to have responded if it approached within 20 m of the decoy; our first estimate of territoriality was whether a bird responded during the playback period. When the focal bird(s) did respond we continued the playback for an additional 10 min and recorded four variables related to intensity of response: (1) total duration of response (seconds within 20 m); (2) time spent within 5 m of the decoy (s); (3) total number of vocalizations (counted with tally meter); and (4) total number of flights past the decoy. By conducting trials near the focal birds' nests we were able to minimize responses by nonfocal males from neighbouring territories; however, if multiple males responded we discarded the observation ($N = 5$), as we were unable to separate the response to the decoy from that to other birds in such situations. We also omitted trials if we were unable to identify the responding male by its colour bands ($N = 2$).

We observed nestling feeding of focal males by monitoring nests for 1 h between 3 and 6 h after sunrise from a blind set up the previous day using binoculars and/or a spotting scope. While we tried to perform observations when nestlings were 3–5 days old, we also collected observations on nests that were found after this time (mean nestling age = 5, range 3–8 days). We ensured that observers would not influence feeding behaviour by entering the blind quickly when no birds were nearby and waiting 10 min before beginning the observation period. In addition to recording the number of feeding visits to the nest by each parent, we also estimated food load size. We gave food items a value of 1 if they were smaller than the parent's bill, 2 if they were the size of the bill, 3 if they were twice the bill size, and 4 if they were at least three times the size of the bill. Feeding index was calculated by multiplying the average prey size by the number of feeding visits.

These behavioural proxies are interpreted as being representative of the animals' natural foray, territorial and nestling feeding behaviour and are hereafter referred to as such.

Radioimmunoassay

Blood samples were kept on ice in the field and promptly centrifuged upon return to the field station. We then removed plasma for storage in liquid nitrogen until transport to Washington State University, where samples were kept in a -20°C freezer. The 17–46 μ l plasma samples were assayed for total androgen concentration (testosterone and 5 α -dihydrotestosterone (DHT); see below for antibody cross-reactivity) following a previously validated and published protocol for this species (Lindsay et al., 2009). Steroids were extracted with diethyl-ether and redissolved in 250 μ l phosphate-buffered saline with gelatine, pH 7.1 (PBSg). Radioimmunoassays were conducted in 100 μ l aliquots using tritium-labelled testosterone (Perkin Elmer Life Sciences NET-553, Waltham, MA, U.S.A.) and a testosterone antibody (Wien Laboratories T-3003, Flanders, NJ, U.S.A.) that cross-reacts with closely related steroids (100% reactivity with testosterone, 60% with 5 α -dihydrotestosterone, 5% with aldosterone, less than 15% with other androgenic steroids, and less than 0.05% with 17-beta-oestradiol and all other tested steroids: values provided by the manufacturer). Because of the substantial cross-reactivity of the testosterone

antiserum with DHT we refer to our measurements as androgen concentrations.

Samples were run in duplicate with recoveries for all (mean recovery 85%). The average intra-assay coefficient of variation across the three assays was 6.2%, and the interassay variation was 5.9% (calculated according to Chard, 1978/1995). We detected androgen concentrations between 197.6 pg/ml and 11476.1 pg/ml (mean = 2071.4 pg/ml, median = 1071.4 pg/ml). For undetectable samples ($N = 7$) we calculated androgen concentrations using the minimal detectable levels of the standard curve (1.95 pg/tube). The 92 total samples were randomly distributed across the three assays.

Statistical Analyses

We assessed treatment and phenotypic differences in overall hormonal response to GnRH challenge using linear mixed models with individual males as the random factor and time (pre/post injection) and either treatment or phenotype as fixed factors. We used a Cox proportional survival regression to look for phenotypic differences in probability to respond territorially, as this approach allowed the integration of a continuous variable accounting for time until response. All other analyses of phenotypic differences in hormones and behaviour employed an ANCOVA with phenotype as a fixed factor. In all above analyses we began with a full suite of potential covariates (hormones: date, mass, time post-sunrise, nest age since onset of laying; behaviour: date, nest age, number of young, number of auxiliaries), then sequentially removed nonsignificant covariates until all P s < 0.1. To evaluate the influence of age on behaviour and androgens we considered it alongside the above factors and covariates for each dependent variable. Comparisons of alternative foray (number of departures versus time off territory) or feeding (number of feeds versus prey size) metrics, in contrast, were conducted using linear regressions.

In instances where we compared a behaviour to a hormone concentration, each variable could be independently impacted by covariates; therefore, traditional analytical approaches controlling only for covariates of the dependent variable are inappropriate. In these instances we present two alternative approaches. We began by directly comparing the raw values using a linear regression. We then performed a multiple regression of each variable with its significant covariates (see Results) and used the resulting residuals in subsequent comparisons using linear regression. Comparisons between behaviour variables and hormones were restricted to instances where both were sampled within the same nesting stage (mean \pm SE difference: foray = 2.14 ± 0.57 days, territoriality = 1.23 ± 0.38 days, feeding = 1.70 ± 0.52 days) to avoid temporal differences or confounds related to stage-dependent androgen concentrations (see Results).

Our estimates of territoriality are based on the first principal component of a principal component analysis (PCA) of four recorded metrics of response to STI (duration of response, number of vocalizations, time spent <5 m from the decoy, number of flights past the decoy), which had an eigenvalue of 2.42 and explained 61% of the variance (Table 1). Note that the factor loadings for this principal component have been inverted in all analyses and figures to improve interpretation. The second principal component had an eigenvalue of 0.67 and explained only 17% of the variance, so did not meet the Kaiser criteria for inclusion (eigenvalue >1; Kaiser, 1960) and was not further considered. Territorial response did not vary across different decoys or playbacks, and neither time post-sunrise nor wind speed influenced any behaviour (all $P > 0.10$); therefore, these variables were excluded from all analyses. We chose to report nestling feeding patterns without controlling for number of young, because we were trying to gauge the males' total feeding effort rather than the resources received by

Table 1

Variable factor loadings for the first principal component (PC) of male response to a simulated territorial intrusion

Variable	Factor loading
Duration of response	0.77
Total vocalizations	0.70
Seconds <5 m from decoy	0.85
Flights past decoy	0.79

PC1 had an eigenvalue of 2.42 and explained 61% of the variance. PC2 had an eigenvalue of 0.67 and explained only 17% of the variance, so was not further considered. Note that factor loadings have been inverted to ease interpretation.

each nestling; however, results remained qualitatively similar if we controlled for number of young. Androgen concentrations were ln transformed in all analyses to improve normality, although figures present raw values for easier interpretation. All pre-treatment blood samples were collected within 10 min of capture, and should therefore reflect baseline androgen concentrations. On average we collected pre-treatment bloods samples from red/black birds 1.7 min faster than we did from brown birds because their plumage allowed us to prioritize them as males, whereas brown males were indistinguishable from females while in the net and thus sometimes required more time to identify and bleed. However, in support of the assumption that these samples represented baseline concentrations that were uninfluenced by capture, bleeding delay was unrelated to baseline androgen concentrations ($F_{1,30} = 0.33$, $P = 0.57$) and was therefore removed as a nonsignificant covariate from all hormonal analyses. All analyses were conducted using the program NCSS (Hintze, 2007).

Ethical Note

All animals were captured, handled and released in a safe and humane manner using procedures approved by the Institutional Animal Care and Use Committees at Washington State University (approval no. 03653-007) and Cornell University (approval no. 2009-0105), the James Cook University Animal Ethics Committee (approval no. A1691) and the Queensland Government Environmental Protection Agency. The time birds were kept away from their territories caused no negative consequences for their reproduction, and no harm came to any of the birds from trapping, handling, injecting or bleeding. Samples were approved for export from Australia by the Australian Government Department of Environment and Heritage and for import into the United States by the United States Department of Agriculture.

RESULTS

Behaviour

We observed the foray behaviour of 42 breeding males (brown = 21, red/black = 21) and the nestling feeding behaviour of 39 males (auxiliary = 4, brown = 13, red/black = 22). Additionally, we simulated territorial intrusions (STI) on 64 different territories, and recorded the response of 48 males (auxiliary = 7, brown = 17, red/black = 24).

Red/black breeders forayed more often (ANCOVA: $F_{1,40} = 4.66$, $P = 0.04$; Fig. 1a) and stayed off their territories for proportionally longer periods of time (ANCOVA: $F_{1,40} = 4.49$, $P = 0.04$) than did brown breeders. These two estimates of foray behaviour were strongly positively correlated (linear regression: $R^2 = 0.45$, $b = 2.88$, $F_{1,40} = 33.22$, $P < 0.0001$), and therefore below we report results only for foray rate. Despite their greater time off territory,

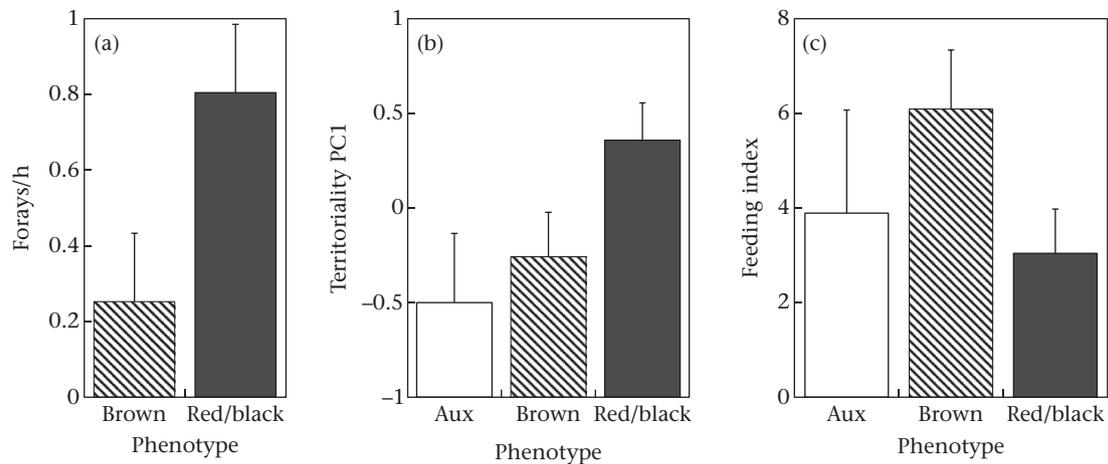


Figure 1. Differences in mean + SE (a) foray frequency, (b) territorial response and (c) nestling feeding behaviour across male phenotypes in red-backed fairy-wrens: red/black breeders, brown breeders and nonbreeding brown auxiliaries (aux). Foray behaviour was estimated from 42 males (brown = 21, red/black = 21), territoriality from 48 males (aux = 7, brown = 17, red/black = 24) and feeding from 39 males (aux = 4, brown = 13, red/black = 22). Means are corrected for significant covariates, as described in the Methods. $P = 0.58$; Fig. 1b).

red/black males were no less likely to respond to the STI (red/black = 73%, brown = 52%, auxiliary = 64%; Cox proportional survival regression: $\chi^2 = 2.64$, $N = 64$, $P = 0.27$). However, among responding birds, phenotypes differed significantly in the strength of their territorial response (ANCOVA: $F_{2,44} = 3.18$, $P = 0.05$; Fig. 1b) with red/black breeders responding more strongly than both brown breeders ($F_{1,44} = 4.03$, $P = 0.05$; Fig. 1b) and auxiliary males ($F_{1,44} = 4.27$, $P = 0.04$; Fig. 1b). No difference existed between the responses of brown breeders and auxiliary males ($F_{1,44} = 0.31$, $P = 0.58$; Fig. 1b).

These patterns appear to be related primarily to age differences, however, as foray behaviour and territoriality increased with age (ANCOVA: foray: $R^2 = 0.13$, $b = 0.23$, $F_{1,40} = 6.19$, $P = 0.02$; territoriality: $R^2 = 0.31$, $b = 0.45$, $F_{1,46} = 20.25$, $P < 0.0001$); with age included as a covariate the phenotypes did not differ in either behaviour (foray: $F_{1,39} = 0.45$, $P = 0.51$; territoriality: $F_{2,43} = 0.21$, $P = 0.82$). Within red/black breeders, age remained positively correlated with territorial response ($R^2 = 0.24$, $b = 0.43$, $F_{1,22} = 7.22$, $P = 0.01$), but not foray behaviour ($R^2 = 0.06$, $b = 0.18$, $F_{1,19} = 1.26$, $P = 0.28$). No phenotypic signature existed within 1-year-old males in either behaviour (foray: $F_{1,8} = 0.23$, $P = 0.65$; territoriality: $F_{2,8} = 1.05$, $P = 0.39$), although small samples sizes restricted power. The presence of an auxiliary did not influence the territorial response of red/black males ($F_{1,19} = 0.13$, $P = 0.73$). Nest age since onset of laying was similarly unrelated to foray behaviour ($R^2 < 0.01$, $b = -0.01$, $F_{1,39} = 0.24$, $P = 0.63$) and territorial response ($R^2 = 0.01$, $b = 0.02$, $F_{1,45} = 0.73$, $P = 0.40$), as these remained similar across incubation and nestling stages (foray: $F_{1,39} = 0.26$, $P = 0.62$; territoriality: $F_{1,45} = 0.70$, $P = 0.41$).

In contrast, male nestling feeding was unrelated to age (ANCOVA: feeding index: $R^2 < 0.001$, $b = -0.03$, $F_{1,33} < 0.01$, $P = 0.96$) but appeared to be associated with phenotype: brown breeders fed at more than twice the rate of red/black breeders, although this difference did not reach significance ($F_{1,36} = 3.37$, $P = 0.07$), and auxiliaries fed at intermediate rates that did not differ significantly from the other two male types (red/black: $F_{1,36} = 0.77$, $P = 0.39$; brown: $F_{1,36} = 0.10$, $P = 0.75$). An apparent trade-off between the number of visits to the nest and prey size (linear regression: $R^2 = 0.23$, $b = -0.16$, $F_{1,19} = 5.66$, $P = 0.03$) produced a pattern whereby red/black breeders provided significantly larger food items than auxiliary males (ANCOVA: $F_{1,15} = 5.37$, $P = 0.03$) and nonsignificantly larger items than brown breeders ($F_{1,15} = 3.28$, $P = 0.09$); the food size of auxiliary and brown

breeding males in turn did not differ ($F_{1,15} = 0.57$, $P = 0.46$). The larger food size did not appear to fully offset the difference in feeding rate, however; when feeding rate and size were combined into a feeding index, there was still a tendency for brown breeders to bring more food than red/black breeders ($F_{1,34} = 3.60$, $P = 0.07$; Fig. 1c). Auxiliary males fed at intermediate levels that did not differ significantly from the other two male phenotypes (red/black: $F_{1,34} = 0.13$, $P = 0.72$; brown: $F_{1,34} = 0.76$, $P = 0.39$; Fig. 1c). The presence of an auxiliary did not influence nestling feeding effort by red/black breeders ($F_{1,19} = 0.54$, $P = 0.47$). Across all males, nestling feeding was unrelated to nestling age ($R^2 = 0.03$, $b = 0.69$, $F_{1,36} = 1.13$, $P = 0.30$) but did decrease with advancing date in the season ($R^2 = 0.18$, $b = -0.08$, $F_{1,37} = 8.33$, $P < 0.01$).

Androgens

We captured and collected pre- and post-treatment blood samples from 46 males, 39 of which were injected with GnRH and 7 of which received control injections. Birds injected with GnRH subsequently produced higher levels of androgens than did control birds (ANCOVA: post-injection: $F_{1,43} = 64.74$, $P < 0.0001$; linear mixed models: treatment*pre/post injection: $F_{1,44.0} = 26.87$, $P < 0.0001$; Fig. 2a), and therefore control birds were only included in analyses of baseline androgens below. As expected, phenotypes differed in their baseline androgen concentrations ($F_{2,31} = 3.92$, $P = 0.03$; Fig. 2b). However, post-injection androgen concentrations did not differ significantly across male phenotypes ($F_{2,33} = 1.82$, $P = 0.18$; Fig. 2b), thereby producing phenotypic differences in the overall GnRH-induced androgen change (linear mixed models: $F_{2,30} = 3.35$, $P < 0.05$; phenotype*pre/post injection: $F_{2,34.0} = 3.67$, $P = 0.04$; Fig. 2b). Independent of differences in plumage and reproductive role, age had an influence on baseline androgen levels (ANCOVA: $R^2 = 0.10$, $b = 0.30$, $F_{1,31} = 6.11$, $P = 0.02$) and androgen change ($R^2 = 0.07$, $b = -0.24$, $F_{1,30} = 4.03$, $P = 0.05$), but not on post-injection androgen concentrations ($R^2 < 0.01$, $b = 0.05$, $F_{1,30} = 0.23$, $P = 0.63$). Nest age significantly covaried with baseline androgen concentrations ($R^2 = 0.12$, $b = -0.07$, $F_{1,31} = 7.67$, $P < 0.01$) and GnRH-induced androgen change ($R^2 = 0.17$, $b = 0.08$, $F_{1,30} = 9.74$, $P < 0.01$) but not post-treatment androgen levels ($R^2 < 0.01$, $b < 0.01$, $F_{1,30} = 0.17$, $P = 0.68$). Time after sunrise covaried with post-treatment androgen levels ($R^2 = 0.11$, $b < 0.01$, $F_{1,35} = 4.22$, $P < 0.05$) but not baseline androgen concentrations

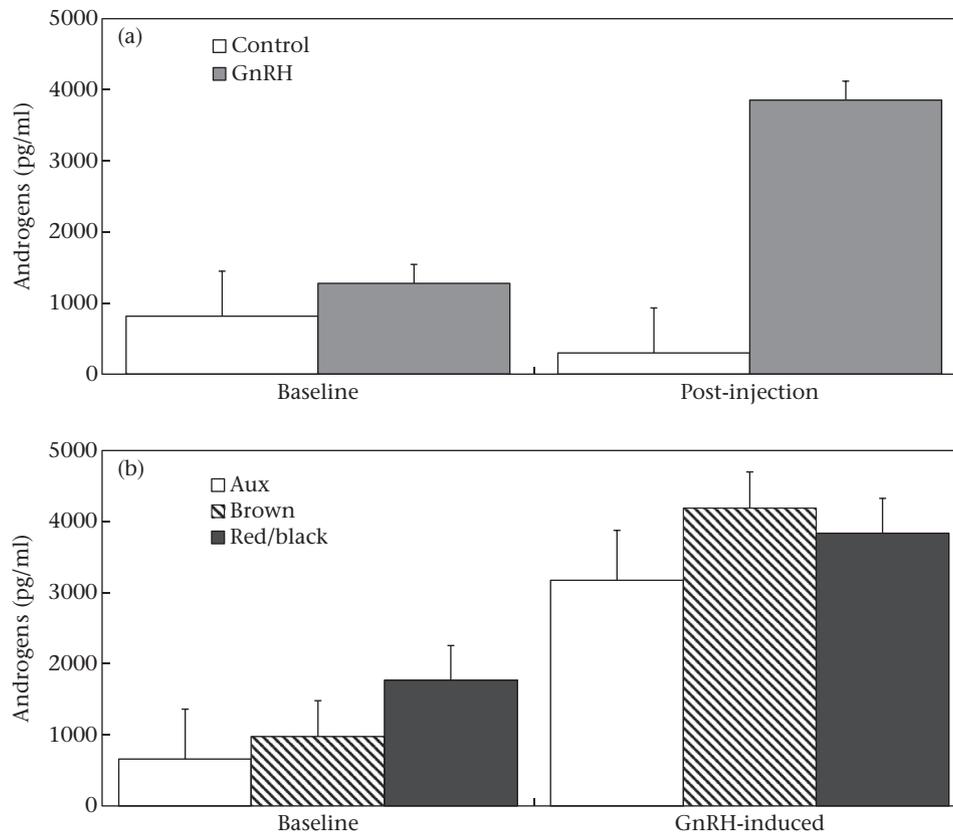


Figure 2. Differences in mean + SE baseline and post-injection androgen concentrations of red-backed fairy-wren males by (a) treatment and (b) phenotype. Phenotypes include red/black breeders, brown breeders and nonbreeding brown auxiliaries (aux). Post-injection samples were collected 30 min after injection with control or GnRH solution. Treatment effects of GnRH injection are from 46 males across all phenotypes (control = 7, GnRH = 39), whereas phenotypic comparisons omitted two males with intermediate plumage and only included males receiving the GnRH treatment (aux = 7, brown = 14, red/black = 16). Means are corrected for significant covariates, as described in the [Methods](#).

($R^2 < 0.01$, $b < 0.001$, $F_{1,30} = 0.10$, $P = 0.75$) or GnRH-induced androgen change ($R^2 = 0.05$, $b < 0.01$, $F_{1,30} = 2.80$, $P = 0.10$).

Androgens and Behaviour

No relationship existed between baseline androgen concentrations, GnRH-induced androgen concentrations, or the overall change in androgen concentrations and any measure of individual behaviour, regardless of whether covariates were statistically controlled ([Table 2](#), [Supplementary Figs S1](#), [S2](#), [S3](#)). All correlations remained nonsignificant ($P > 0.10$) if analyses were restricted to red/black or brown breeders.

DISCUSSION

Do Males Express Phenotype-specific Behavioural Strategies?

Our results provide further evidence for behavioural differences among male phenotypes of the red-backed fairy-wren ([Karubian, 2002](#)) and support the hypothesis that behaviour is shaped by phenotype-specific trade-offs between mating and parental investment. Specifically, red/black breeders appear to invest heavily in mating effort (extraterritorial forays and territorial aggression) at the expense of parental effort (nestling feeding), whereas the opposite is true of brown males. Despite more frequent departures

Table 2

Results from a linear regression between a male's foray frequency, territorial response and nestling feeding and his androgen concentrations at baseline and 30 min after GnRH injection, and the resulting androgen change between those time points

	ln baseline androgens					ln post-GnRH androgens					ln androgen change ^a					
	N	R ²	b	F	P	N	R ²	b	F	P	N	R ²	b	F	P	
Raw																
Foray	22	0.02	-0.13	0.35	0.56	17	0.06	0.20	0.93	0.35	17	<0.01	-0.06	0.03	0.87	
Territoriality	22	0.04	0.16	0.76	0.40	18	<0.01	0.03	0.03	0.87	18	0.07	-0.22	1.21	0.29	
Feeding	10	0.20	-0.08	2.05	0.19	8	0.35	-0.03	3.24	0.12	8	0.20	0.07	1.48	0.27	
Corrected																
Foray	22	0.09	-0.26	2.03	0.17	17	0.11	0.28	1.77	0.20	17	0.02	0.18	0.38	0.55	
Territoriality	22	0.06	-0.20	1.32	0.26	18	0.01	0.11	0.22	0.65	18	<0.01	0.07	0.14	0.71	
Feeding	10	<0.01	-0.01	0.05	0.83	8	0.21	-0.03	1.55	0.26	8	0.04	-0.02	0.26	0.63	

Raw values refer to the comparison of ln-transformed androgen concentrations to raw behavioural estimates, whereas corrected values refer to the comparison of the residuals of ln-transformed androgens and behavioural estimates from a linear regression with their respective covariates (see [Methods](#)).

^a Calculated as (ln post-GnRH androgens) – (ln baseline androgens).

from their territory, red/black breeders were equally effective at detecting simulated intruding males and showed a stronger territorial response. Considering that these phenotypic differences in foray and territorial behaviour appear to be more closely associated with age than with plumage or status type per se, they may be derived to some extent from social interactions that affect the age-specific fitness benefits of these behavioural strategies. Male feeding, on the other hand, was unrelated to age, and although the differences in feeding by red/black and brown breeders that we observed were not statistically significant, their match to previous findings in this population (Karubian, 2002) supports the validity of both accounts of lower parental investment by red/black breeders. Accordingly, the benefits of heavy mating investment for older red/black breeders appear to outweigh any detriment of decreased parental care, whereas limited breeding opportunities of younger brown breeders and auxiliary males could lead to a shift towards heavier parental effort (Badyaev & Hill, 2002; Karubian et al., 2008; Maynard Smith, 1977; Webster et al., 2008).

Do Phenotypes Differ in Their Ability to Produce Androgens?

Although plumage colour in this species is androgen regulated (Lindsay et al., 2011) and male phenotypes differ drastically in androgen levels during all reproductive stages (Lindsay et al., 2009), our findings do not support the hypothesis that phenotype is constrained by differences in physiological capacity to produce androgens (Spinney et al., 2006). This result is counter to the prediction that differential physiological costs of testosterone prevent low-quality males from producing the high testosterone levels necessary for sexual signal elaboration (e.g. Folstad & Karter, 1992; Saino & Møller, 1994) and indicates that androgen production is instead regulated by factors other than testicular steroidogenesis and LH secretion in response to a given endogenous GnRH dose, possibly GnRH secretion or input into the GnRH system. Although age was positively associated with baseline androgen levels and red/black plumage, it had no relationship with androgen production in response to exogenous GnRH, and phenotypic differences in baseline androgen levels were independent of age. Furthermore, age cannot explain the marked variation in androgen concentrations and plumage during the first breeding season. Rather, social cues to the local competitive environment and opportunities for mating might influence GnRH secretion and consequent androgen levels as has been suggested by other studies (Hirschenhauser & Oliveira, 2006; Maia, Brasileiro, Lacava, & Macedo, 2012; Oliveira, 2004).

Do Androgen Concentrations Predict Individual Variation in Behaviour?

Counter to predictions of the hypothesis that individual variation in androgen secretion patterns predicts behavioural variation (Ketterson & Nolan, 1992; McGlothlin et al., 2007; Wingfield, Ball, Dufty, Hegner, & Ramenofsky, 1987; Wingfield et al., 1990), individual hormonal and behavioural variation were unrelated regardless of the androgen measure or analytical approach. The lack of hormone–behaviour relationships contrasts with studies demonstrating that experimentally elevated testosterone causes birds to shift from parental to mating effort (reviewed in Ketterson & Nolan, 1999), including in the congeneric superb fairy-wren, *Malurus cyaneus* (Peters, Cockburn, & Cunningham, 2002). However, our results are mirrored by several studies demonstrating insensitivity of mating (e.g. Meddle et al., 2002; I. T. Moore et al., 2004) and parental (e.g. Lynn, Hayward, Benowitz-Fredericks, & Wingfield, 2002; Van Duyse, Pinxten, & Eens, 2002) behaviour to experimental increases in testosterone (reviewed in Lynn, 2008),

as well as studies reporting no correlation between parental or mating behaviour and endogenous circulating testosterone concentrations (e.g. Cramer, 2012; DeVries & Jawor, 2013; Levin & Wingfield, 1992; Schwabl, Flinks, & Gwinner, 2005; Silverin, Baillien, & Balthazart, 2004). Our results likewise do not support the hypothesis that ability to rapidly increase testosterone in response to GnRH pulses resulting from behavioural interactions regulates the trade-off between mating and parental behaviour (McGlothlin et al., 2007). It should be acknowledged that our sample sizes for comparing androgen levels to feeding behaviour were restricted by lower hormonal sampling in the nestling stage, and relatively large R^2 values suggest a negative relationship could emerge with larger sample sizes. However, after removing any influence of spurious covariates, these relationships weakened sharply, and the only other research to replicate McGlothlin et al. (2007) and compare GnRH-induced testosterone levels to behaviour similarly found no relationship between the GnRH-induced change in androgens and parental care in northern cardinals, *Cardinalis cardinalis* (DeVries & Jawor, 2013). Therefore, uncertainty remains regarding the generality of relationships between short-term testosterone increases and behavioural trade-offs between mating and parental investment. Cumulatively, these findings highlight the lack of a uniform testosterone-mediated trade-off between mating and parental investment across taxa (Adkins-Regan, 2005).

While it is feasible that behavioural and hormonal variation are unrelated to each other in our study species, other possibilities do exist. One explanation is that a stepwise function exists, whereby above a given concentration, behaviour is independent of activation by circulating androgen levels (Hews & Moore, 1997). The possibility that behavioural and morphological phenotypes have been permanently set by hormonal organization (M. C. Moore, 1991) can be excluded as males of this species can change phenotype in adulthood (Karubian et al., 2011, 2008). Behavioural differences could also be related to variation in concentrations of binding globulins (Pryke, Astheimer, Griffith, & Buttemer, 2012) and, more likely, the sensitivity of behaviour-related brain structures to androgens as demonstrated for other species (Ball & Balthazart, 2008; Burns, Rosvall, & Ketterson, 2013; Canoine, Fusani, Schlinger, & Hau, 2007; Rosvall et al., 2012). Finally, hormones other than androgens (e.g. glucocorticoids, prolactin, non-peptides) might play a role in linking behaviour with life-history state (Angelier, Clement-Chastel, Welcker, Gabrielsen, & Chastel, 2009).

Conclusions

In summary, our results support the hypothesis that the behavioural strategies of male red-backed fairy-wrens differ across phenotypes (as defined by plumage colour, reproductive role and age), with older red/black breeders investing more in mating than parental activities and younger brown breeders and auxiliaries showing the opposite pattern. However, despite previous demonstration that male phenotypes differ in androgen levels during all reproductive phases (Lindsay et al., 2009) and that acquisition of plumage colour type is mediated by testosterone (Lindsay et al., 2011), differences in plumage colour and reproductive role do not appear to arise from variation in the capacity to produce testosterone. Furthermore, we found no evidence that variation in circulating androgens relate to behavioural variation. While these results demonstrate individual integration of morphology and behaviour, they do not support the hypothesis that variation in circulating androgen concentrations or the capacity to produce them are responsible for activation of behavioural differences across male phenotypes.

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Supplementary Material

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