PROXIMATE CAUSES AND ULTIMATE CONSEQUENCES OF PHENOTYPIC VARIATION IN MALE RED-BACKED FAIRY-WRENS

By

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To the Faculty of Washington State University:

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Abstract

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Sexual signals are often plastic, and should therefore be adjusted to match an individual's phenotype and optimize lifetime fitness. However, little is known of physiological mechanisms permitting such phenotypic integration, or of evolutionary mechanisms maintaining sexual preferences for plastic traits. We attempted to fill these knowledge gaps through experimental and correlative investigations of the red-backed fairy-wren (*Malurus melanocephalus*), an Australian songbird in which males exhibit discrete breeding phenotypes varying in attractiveness, androgen concentrations, and fitness (high to low): red/black breeder, brown breeder, or non-breeding brown natal auxiliary.

We first experimentally manipulated male condition to test whether honesty of red/black plumage is maintained through condition-dependent androgen regulation. Experimental birds in better condition acquired more red/black plumage, as predicted. However, experimental and control birds did not differ in androgen concentrations, and long-term data from the same population revealed no correlation between condition and androgen levels.

We then investigated whether morphological and behavioral traits are correlated, and if so, whether this phenotypic integration could arise from pleiotropic actions of androgens. Red/black breeders invested more in mating behaviors and less in parental behaviors than brown breeders and auxiliaries. However, injection with GnRH failed to expose phenotype-specific constraints on androgen production, and individual behaviors were unrelated to baseline or GnRH-induced androgen levels.

Finally, we examined the fitness benefits underlying female preferences for and investment in red/black males. Females paired with red/black males began breeding earlier, fed nestlings more, and produced more offspring than those paired with brown males. Despite similar pre-breeding energy stores and markedly higher reproductive investment, females with red/black males finished breeding with greater energy stores, survived at higher rates, and had higher lifetime fitness. Moreover, females breeding earlier with red/black males had more grandchildren because their sons were more likely to display attractive plumage and sire offspring.

Collectively these results demonstrate that male sexual phenotype is honestly maintained through condition-dependent signaling, though circulating androgens likely do not mediate this link or directly underlie phenotypic differences in behavior. It is clear that females receive direct reproductive, survival, and inclusive fitness benefits by pairing with and investing heavily in red/black males.

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CHAPTER ONE

BODY CONDITION INFLUENCES SEXUAL SIGNAL EXPRESSION INDEPENDENT OF CIRCULATING ANDROGENS IN MALE RED-BACKED FAIRY-WRENS

INTRODUCTION

Sexual signal elaboration provides a striking example of phenotypic variation in fitness enhancing traits where more elaborate traits are favored due to their benefits in attracting mates or deterring rivals (Andersson 1994). The evolution of these responses to sexual signals requires that signals honestly reflect the quality of potential mates, and considerable theoretical and empirical support has arisen for condition-dependent signal elaboration (Zahavi 1975, Hill 1990, Jennions et al. 2001). A complete picture of the evolutionary forces acting on trait expression demands knowledge of the trait's mechanistic underpinnings, yet integrative research into the proximate link between condition and trait expression has lagged behind functional approaches (but see von Schantz et al. 1999, Knapp 2003, McGraw and Parker 2006).

Among male vertebrates, androgenic steroids such as testosterone often play a role in regulating physiological, morphological, and behavioral reproductive strategies (Nelson 2000, Adkins-Regan 2005) and thus appear likely regulatory signals of mechanisms integrating physiology and phenotype (Badyaev 2004, McGlothlin and Ketterson 2008). Because testosterone can impose behavioral and physiological costs it has been proposed as the critical connection between individual condition and trait expression (e.g. Folstad and Karter 1992, Saino and Moller 1994). The central role of glucocorticoids in energy allocation and its generally negative relationship with androgen secretions has also led to the proposition that these

hormones provide a mechanistic link between condition and androgens (Husak and Moore 2008).

Plumage signals of male birds provide an excellent model to study the role of hormones in sexual trait expression, but our present understanding of the causal relationship among condition, hormones, and plumage elaboration is limited for several reasons. First, most studies have been conducted during or after the breeding season (e.g. Saino and Moller 1994, Duckworth et al. 2004, Peters et al. 2006), thereby leaving uncertainty regarding mechanisms acting at the time of trait acquisition (i.e., prior to breeding). Second, the majority of studies rely on correlative analyses, and among those that do adopt an experimental approach, many bypass condition in favor of hormonal manipulations aimed at ultimate influences of testosterone on immune function and plumage elaboration (e.g. Peters 2000, Peters et al. 2000, Gonzalez et al. 2001). Finally, most studies consider only a portion of the proposed pathway between condition and signal expression, such as the relationship between condition and glucocorticoids or androgens and plumage coloration. In fact, we could only find one series of experiments on the topic that manipulated condition directly, in which Perez-Rodriguez et al. (2006, 2008) demonstrated condition-dependence in corticosterone, androgens, and sexual ornamentation of bare body parts in captive red-legged partridges. These past studies have advanced the field and collectively grant us the theoretical and empirical ability to carry out a comprehensive assessment of condition-dependent androgen regulation as the mechanism maintaining honesty of plumage signals.

In this study we provide an experimental assessment of whether condition-dependent glucocorticoid and androgen secretion during acquisition of nuptial plumage regulates sexual signal elaboration in the red-backed fairy-wren (*Malurus melanocephalus*). This small Australian

passerine is ideally suited to investigate the mechanistic basis of plasticity in sexual signals, as males exhibit three discrete, alternative breeding phenotypes that differ in plumage color and reproductive behavior (Karubian 2002): males can breed in red/black nuptial plumage ("red/black breeder"), breed in brown female-like plumage ("brown breeder"), or remain as non-breeding brown auxiliaries on the natal territory ("auxiliary"). Although phenotype is partially related to age, males are able to assume any of the three phenotypes in their first year of reproduction (Webster et al. 2008). Plumage phenotype is acquired in a prenuptial (prealternate) molt that occurs prior to breeding. Phenotype appears to be under strong sexual selection, with differences in mating investment and attractiveness combining to give red/black breeders the highest fitness (Karubian 2002, Karubian et al. 2008, Webster et al. 2008, Rowe et al. 2010), primarily as a consequence of their success at siring young outside of the pair-bond (Webster et al. 2008).

The three male reproductive phenotypes of this species show distinct differences in circulating androgen concentrations during the prealternate molt as well as during reproduction, with red/black breeders having the highest levels and auxiliaries having the lowest (Lindsay et al. 2009). Furthermore, testosterone implantation during the prenuptial molt led to production of red/black plumage (Lindsay et al. 2011), demonstrating that high testosterone levels are causally related to acquisition of male-typical nuptial plumage, in contrast to many other passerines (Kimball 2006). A previous study (Lindsay et al. 2009) also found that body condition during molt differed among the three male phenotypes (red/black > brown > auxiliary), and that androgen levels were correlated with body condition across individuals. Accordingly, we hypothesized that condition-dependent androgen secretion is responsible for maintaining signal honesty in this system. In the present study we tested the causal role of body condition in

regulating androgen concentrations during prenuptial molt and the resulting plumage coloration through experimentally manipulating body condition by trimming flight feathers prior to molt. Because previous studies have employed this method to manipulate condition (e.g., Winkler and Allen 1995, Whittingham et al. 2005), we expected trimmed males to be in poorer body condition than controls (but see Results). We recaptured birds at the peak of molt to test the prediction that better condition would lead to higher circulating androgens and, in turn, relatively more red/black plumage. We further utilized this experiment to establish whether corticosterone might play a role in these relationships through negative covariance with testosterone (Husak and Moore 2008).

METHODS

Study species and basic field methods

This study was conducted in a population of color-banded red-backed fairy-wrens near Herberton, Queensland, Australia (145°25'E, 17°23'S), which has been monitored continuously since 2003. We target trapped birds using mist-nets and a combination of herding and brief (<2 min) playback of conspecific vocalizations. Upon capture we immediately removed birds from the net and collected a maximum of 80 µl of blood from the jugular vein into heparinized microcapillary tubes (bleeding delay: mean = 5.9 min, range = 1.7-31.0). Unbanded birds received an Australian Bird and Bat Banding Scheme (ABBBS) aluminum leg band and a unique combination of three colored plastic leg bands for subsequent visual identification, then birds were aged as either second year (1 year-old) or after-second year (1+ year-old) using the degree of skull ossification (Lindsay et al. 2009). We performed basic morphometric measurements on all captured birds (mass and wing, tarsus, tail, and culmen lengths), quantified the degree of

feather molt on six body regions (head, back, wing, belly, chest, tail; scale of 0-3 each), and estimated fat stores based on fat seen in the furcular cavity on a scale from 0 (no fat) to 3 (bulging) in 0.5 increments (Lindsay et al. 2009). Fat score was chosen as the measurement of condition because its independence from body size makes it preferable to other condition indices (Gosler et al. 1998, Gosler and Harper 2000), although it is correlated with the residual of the regression of mass on tarsus length in this species (Lindsay et al. 2009).

Throughout the breeding seasons we used sightings and captures of birds to estimate the proportion of their body covered in red/black plumage. Using this value we categorized birds as brown (<33% red/black feathers), intermediate (33-66%), or red/black (>66%), although plumage coloration is strongly bimodal with few males in intermediate plumage (Webster et al. 2008).

Condition manipulation

We conducted the condition manipulation experiment before and during the 2010-2011 breeding season. We initially captured birds during the pre-breeding season between August 7 and September 14, 2010 and randomly assigned all second year birds (born previous breeding season) to one of two treatments. Control birds were released after completion of basic processing. Manipulated birds, on the other hand, had 3 primaries (numbers 5, 7, and 9) on each wing and the 4 central tail retrices trimmed at their base before being released. Because second year males cannot be differentiated from females we manipulated both male and female birds and used established molecular genetic techniques (see Varian-Ramos et al. 2010) to determine sex post-hoc. Male birds were recaptured 18-81 days post-treatment using protocols described above to obtain blood samples, take morphometric measures, and score plumage coloration.

Radioimmunoassay

Blood samples were kept on ice in the field and then and promptly centrifuged upon return to the field station. We then measured hematocrit and removed plasma for storage in liquid nitrogen until transport to Washington State University, where samples were kept in a -20° freezer. The 16 to 52 µl plasma samples were assayed using previously published protocols that allow simultaneous measurement of total androgen and corticosterone concentrations (Lindsay et al. 2011), except that we used 30 µl of redissolved extract for determination of recoveries and included 10 duplicate known samples. Corticosterone recoveries were determined for each sample (mean recovery was 88%), whereas androgen recoveries were obtained from four samples unrelated to the experiment but analyzed in the same assay (mean recovery of 86%). The single assay had an intra-assay variation of 5.40% for androgens and 9.51% for corticosterone (calculated according to Chard 1995; androgen values calculated from 10 duplicate known samples). Given our assay parameters the minimum detectable androgen concentration was 116.3 pg/ml of plasma and we measured androgen concentrations between 113.5 pg/ml and 4593.1 pg/ml (mean = 390.7 pg/ml, median = 191.6 pg/ml). The minimum detectable corticosterone concentration was 0.88 ng/ml and we detected concentrations between 1.93 ng/ml and 57.7 ng/ml (mean = 11.0 ng/ml, median = 8.2). Concentrations of undetectable samples were back calculated from minimal detectable levels (androgens = 1.95 pg/tube, corticosterone = 3.91 pg/tube). The radioimmunoassay was performed using tritium-labeled testosterone (Perkin Elmer Life Sciences NET-553, Waltham, Massachusetts USA) and corticosterone (Perkin Elmer Life Sciences NET-339) with corticosterone antibody (Esoterix Endocrinology B3-163) and a testosterone antibody (Wien Laboratories T-3003, Flanders, New

Jersey USA) that cross-reacts with closely related steroids (100% reactivity with testosterone, 60% with 5alpha-dihydrotestosterone, 5% with aldosterone, <15% with other androgenic steroids, and less than 0.05% with 17<beta>-estradiol and all other steroids: values provided by the manufacturer). Samples from the treatment types and time periods were randomly distributed throughout the single assay.

Statistical analyses

We analyzed experimental effects on fat stores, plumage coloration, molt, and hormone concentrations using an analysis of covariance (ANCOVA) with treatment as a fixed factor. We assessed effects on hormones and condition by measuring the difference between values at the time of treatment and at the final recapture to control for individual variation (i.e. differences in receptor densities). Differences in proportions of individuals in each plumage category were evaluated using chi-square tests of contingency tables and the relationship between plumage coloration and molt and circulating hormones were analyzed with a multiple regression. We employed a multiple regression with date included as a factor in analyses of the relationship between androgens and corticosterone. Because the magnitude of treatment effects may vary with time since manipulation, we included days since treatment as a covariate in fat and hormone analyses. We also included date as a covariate in analyses of plumage coloration and hormones since these variables change with progress toward the breeding season. Small sample sizes prevented statistical analyses of treatment effects on breeding role.

We used data collected between 2004 and 2011 in a multiple regression to look at the relationship between hormone concentrations and fat stores in molting males. In these analyses we included date, breeding stage, total molt score, and year as factors and eliminated

pseudoreplication from multiple captures of individual birds by only including the capture with the highest molt score. Results from this broader database remained qualitatively similar if we employed an alternative condition metric (residual of regression of mass on tarsus) and/or removed nonsignificant terms from our model; therefore we only present results using a priori models to assess the role of fat scores.

Androgen concentrations were natural-log transformed and corticosterone concentrations were square root transformed in all analyses to improve normality, although figures present raw values for easier interpretation. To ensure that hormone concentrations reflected baseline values (see Lindsay et al. 2009) we omitted any blood samples collected more than 5 minutes after capture for corticosterone (bleeding delay: mean = 3.1 min, range = 2.2-4.8 min) and 10 minutes after capture for androgens (bleeding delay: mean = 4.2 min, range = 2.2-9.8 min). With these restrictions, delay was a nonsignificant covariate in all hormonal analyses and was therefore omitted.

All analyses were conducted using the program NCSS (Hintze 2007). Animal procedures were approved by the Washington State University Institutional Animal Care and Use and the James Cook University Animal Ethics Committees.

RESULTS

Feather trimming significantly affected the body condition of males, although opposite the direction we initially predicted, as feather-trimmed birds maintained more of their pre-treatment fat reserves than did control birds ($F_{1, 16} = 4.16$, $p = 0.05$; Fig. 1).

At the time of recapture, better body condition of these birds was associated with enhanced elaboration of plumage coloration, with feather-trimmed males developing

significantly more red/black plumage than did control males ($F_{1, 17} = 4.73$, $p = 0.04$; Fig. 2). These birds also molted more heavily ($F_{1, 15} = 4.88$, $p = 0.04$), and across treatments heavier molting birds ultimately acquired more red/black plumage ($R^2 = 0.57$, b = 7.77, $F_{1, 16} = 23.21$, $p < 0.001$). At the conclusion of molt, 45% of all trimmed males (including those not recaptured) had developed some red/black plumage, three exhibited enough to be categorized as having either intermediate (>33%) or red/black (>66%) plumage, and one molted into completely red/black plumage. Conversely, only 25% of control males produced any red/black plumage and the most ornamented individual exhibited only 18% red/black plumage, leading to a difference between treatments in the proportion of birds with plumage categorized as dull ($X^2 = 3.76$, $p = 0.05$). Treatment did not appear to influence breeding role, with 60% of control and 58% of treatment birds becoming breeders.

The better condition (greater fat stores) of trimmed males did not result in altered corticosterone ($F_{1, 5} = 2.19$, $p = 0.20$; Fig. 3A-B) or androgen ($F_{1, 12} = 0.24$, $p = 0.63$; Fig. 3C-D) plasma concentrations compared to pre-treatment concentrations. Furthermore, ultimate plumage coloration was not linked to hormonal concentrations at the beginning of treatment (corticosterone: $R^2 = 0.05$, b = -11.51, $F_{1, 6} = 0.42$, $p = 0.54$; androgens: $R^2 = 0.05$, b = 11.29, $F_{1, 12} = 0.79$, $p = 0.39$) or at recapture (corticosterone: $R^2 = 0.04$, b = 9.46, $F_{1, 12} = 0.74$, $p = 0.41$; androgens: $R^2 = 0.05$, b = 6.46, $F_{1, 15} = 1.03$, $p = 0.33$). Corticosterone and androgen concentrations were unrelated to each other at the beginning of treatment (prior to molt onset; $R^2 = 0.08$, b = -0.14, $F_{1, 7} = 0.68$, $p = 0.44$) and at recapture (during peak molt; $R^2 < 0.01$, b = 0.05, $F_{1, 12} = 0.01$, $p = 0.91$).

A correlative analysis of our 2004 - 2011 database revealed a negative, but marginally nonsignificant, relationship between body condition (fat stores) and circulating corticosterone

concentrations among molting males (R^2 =0.08, b = -1.50, $F_{1, 10}$ = 4.24, p = 0.07; Fig. 4A). Conversely, no relationship existed between fat stores and circulating androgen levels among molting males (R^2 = 0.03, b = -0.33, $F_{1, 27}$ = 2.14, p = 0.16; Fig. 4B).

DISCUSSION

Our results provide experimental evidence for condition-dependence of sexual trait expression by young male red-backed fairy-wrens, as birds with experimentally enhanced condition acquired a significantly greater proportion of red/black nuptial plumage. Somewhat surprisingly, the direction of the manipulation's effect on condition was opposite to predictions based on previous studies, with birds having their flight feathers trimmed maintaining greater fat stores and hence better body condition than controls. This unexpected result could arise from increased investment in self-maintenance, but such an interpretation conflicts with the greater investment in ornamentation by these males or the lack of marked treatment effects on reproductive role. More likely it results from subtle impacts on the behavior of these birds, whose foraging habits and infrequent flight differ substantially from the habits of other birds that have been subjected to similar experimental manipulations (e.g., Winkler and Allen 1995, Whittingham et al. 2005). Behavioral changes resulting from feather trimming that might have decreased energetic expenditure or increased food consumption could include decreased time sentineling within their social group, reduced aggressive interactions, or simply less flight. Moreover, past studies employing feather-trimming protocols have been limited to the breeding season and most have focused on females; neither of the two studies we could find with males detected a significant effect of treatment on body condition (Slagsvold and Lifjeld 1990, Sanz et al. 2000). Ultimately this study is incapable of concluding the cause of this counterintuitive

experimental pattern, but it does highlight variation across species in responses to artificial 'handicaps'.

Our experimental manipulation had a clear impact on body condition, and thus enabled our study to examine the causal connection between body condition and plumage signals: experimental males, which were in better condition than controls, were significantly more likely to molt red/black plumage than were controls. Experimental males were also molting more heavily at the time of recapture, likely indicating earlier and/or faster molt. Although our sampling scheme does not allow conclusive interpretation of this molt pattern, it is consistent with work on other *Malurus* wrens showing associations between condition and molt date (Mulder and Magrath 1994, Cockburn et al. 2008). Thus, this study supports the causal relationship between condition and plumage signals, as suggested by earlier correlational analyses (Lindsay et al. 2009), and also allows us to elucidate the proximate hormonal mechanisms by which condition affects development of nuptial plumage coloration.

Condition manipulation did not generate the predicted change in corticosterone levels. While this could suggest no effects on the energetic allocation of treatment birds, it remains possible that the lack of effect arose because our condition enhancement didn't necessitate hormonal compensation as would be expected for condition reduction (Moore and Jessop 2003) or because small sample sizes limited our statistical power. Contrary to the notion that androgen levels are constrained through a negative relationship with corticosterone (Husak and Moore 2008), our results suggested no relationship between these hormones prior to or during molt. The lack of a negative relationship during molt suggests these hormones interact little during the critical window of trait determination and may explain the lack of a relationship between plumage coloration and corticosterone among non-manipulated molting birds in this population

(Lindsay et al., unpublished data). Thus, at least in this species, there is little evidence for a pathway by which condition affects androgens via glucocorticoids to determine signal expression.

Despite previous demonstration of testosterone-dependence in plumage coloration of this species (Lindsay et al. 2011), effects on trait expression in our study do not appear to be strongly tied to circulating androgen concentrations. The most parsimonious explanation for this is simply that no relationship exists between condition and androgens, and this is also suggested by the nonsignificant relationship between condition and androgens in either the experiment or the data from seasons 2004 - 2011. Nonetheless, we are hesitant to conclude strongly that there is no connection between condition and androgens, considering the demonstrated co-dependence of plumage expression on both androgens (Lindsay et al. 2011) and condition (this study) suggests some link between the two. Considering the lack of evidence for condition-dependent androgen regulation, however, we conclude that closer scrutiny may be needed to resolve the relationships among condition, androgens, and sexual signals.

Alternative explanations exist for the lack of an observed relationship between condition and androgens. First, it has been proposed that androgen secretions might be constrained by condition only when condition falls below some threshold (Perez-Rodriguez et al. 2006), and considering our feather-trimming enhanced, rather than worsened, condition, many control birds might have remained above this threshold value. Second, the causal relationship between condition and androgens could be opposite the direction predicted, with androgens influencing condition. This would not exclude the possibility that condition also influences androgens, but simply that such a pattern is obscured by the opposing relationship. This explanation is supported by the enhanced condition of young males implanted with testosterone in this species (Lindsay et

al. 2011). Finally, it remains possible that our sampling scheme was unable to detect transient yet important changes in androgen concentrations.

Regardless of which of the above scenarios (or combination of scenarios) is acting, this experiment suggests the condition-dependence of sexual signals is not necessarily mediated by a simple and linear interplay between physiological condition and androgens. The theoretical logic and simplicity of androgen-mediated condition-dependence has led to wide acceptance of this idea and produced a wealth of studies that rely on, yet rarely test, this assumption (but see Perez-Rodriguez et al. 2006). An alternative possibility is that physiological, environmental, and genetic systems interact to produce individual hormone levels and the resulting phenotype (Kempenaers et al. 2008). A complete picture of the mechanisms involved requires the simultaneous consideration in these factors in a comprehensive model, although the extensive data required for such an undertaking leaves the issue unresolved. Until the mechanisms of condition-dependence are unraveled, however, researchers should exhibit caution in seeking simple explanations and rather examine multiple proximate explanations for the maintenance of sexual signal honesty.

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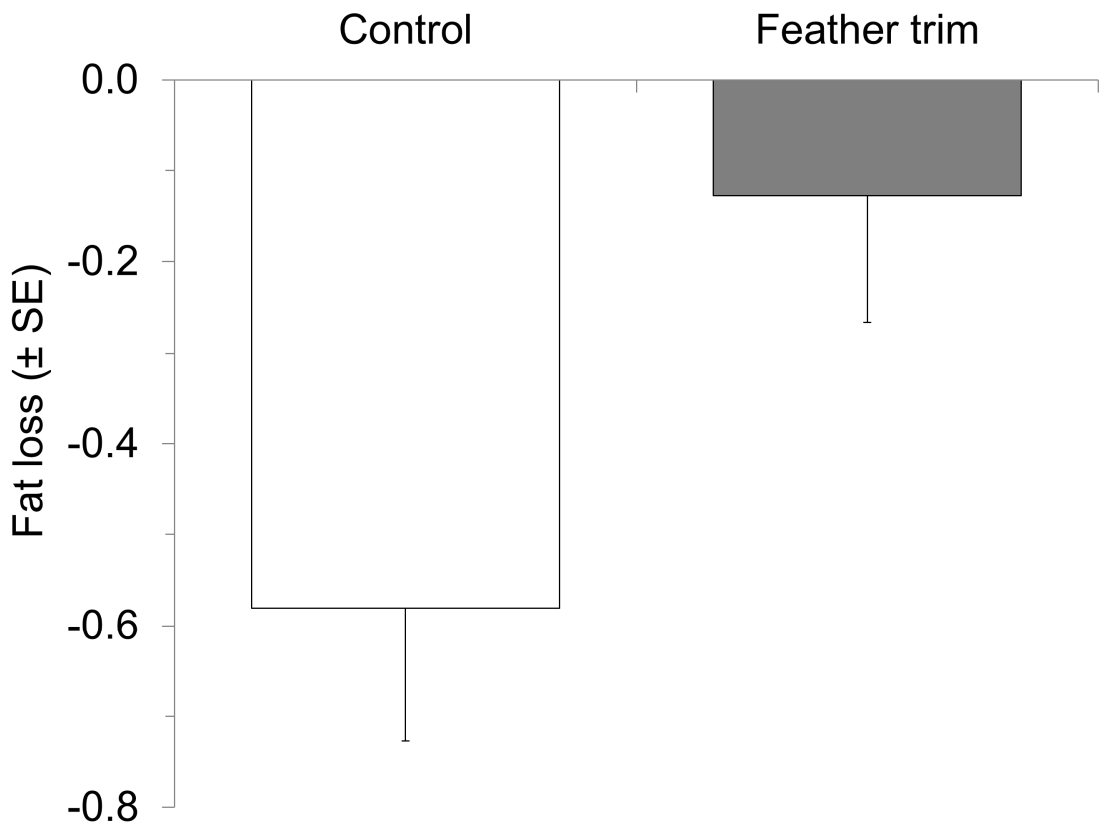

Figure 1. Mean + SE loss of fat stores between the time of treatment and recapture in control (□) and feather trim (■) males. Fat stores were estimated from fat in the furcular hollow (scale of 0-3). Sample sizes for control and feather trim birds are 9 and 10, respectively.

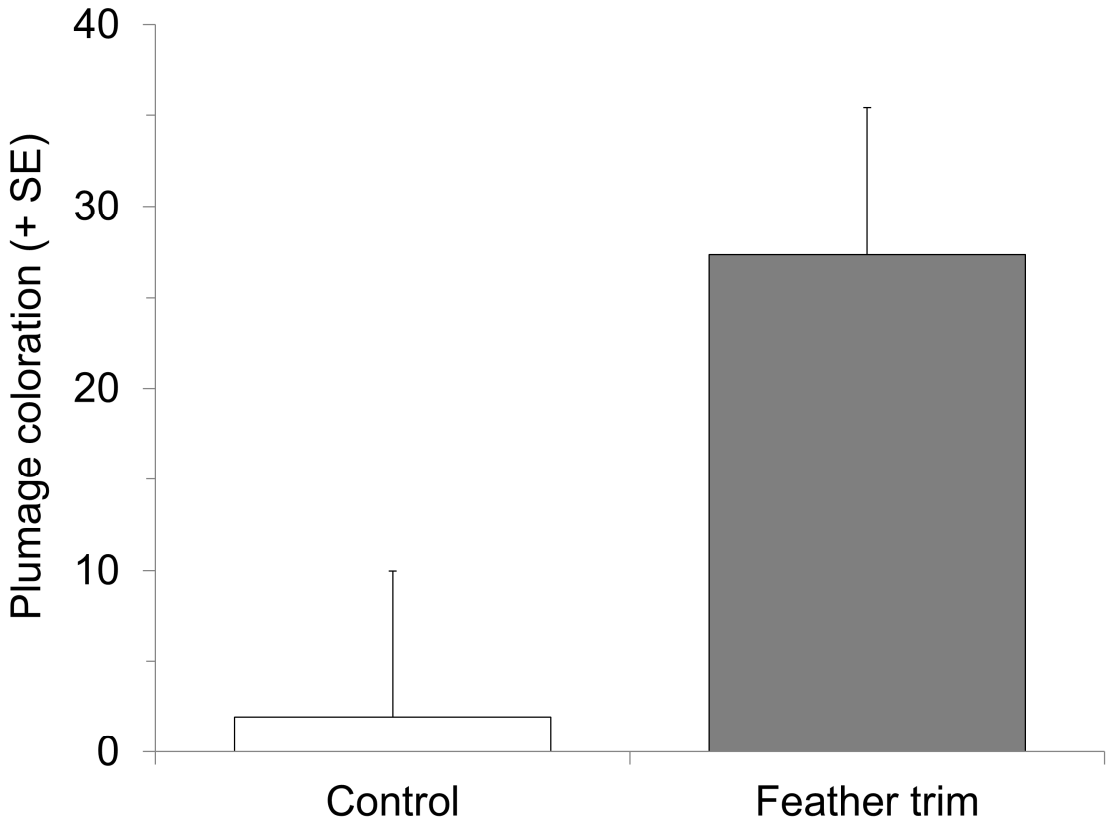

Figure 2. Mean + SE plumage coloration of captured control (□) and feather trim (■) males at the conclusion of molt. Plumage coloration is an estimation of the proportion of their body covered in red/black plumage (scale of 0-100). Values are derived from 10 birds in each treatment.

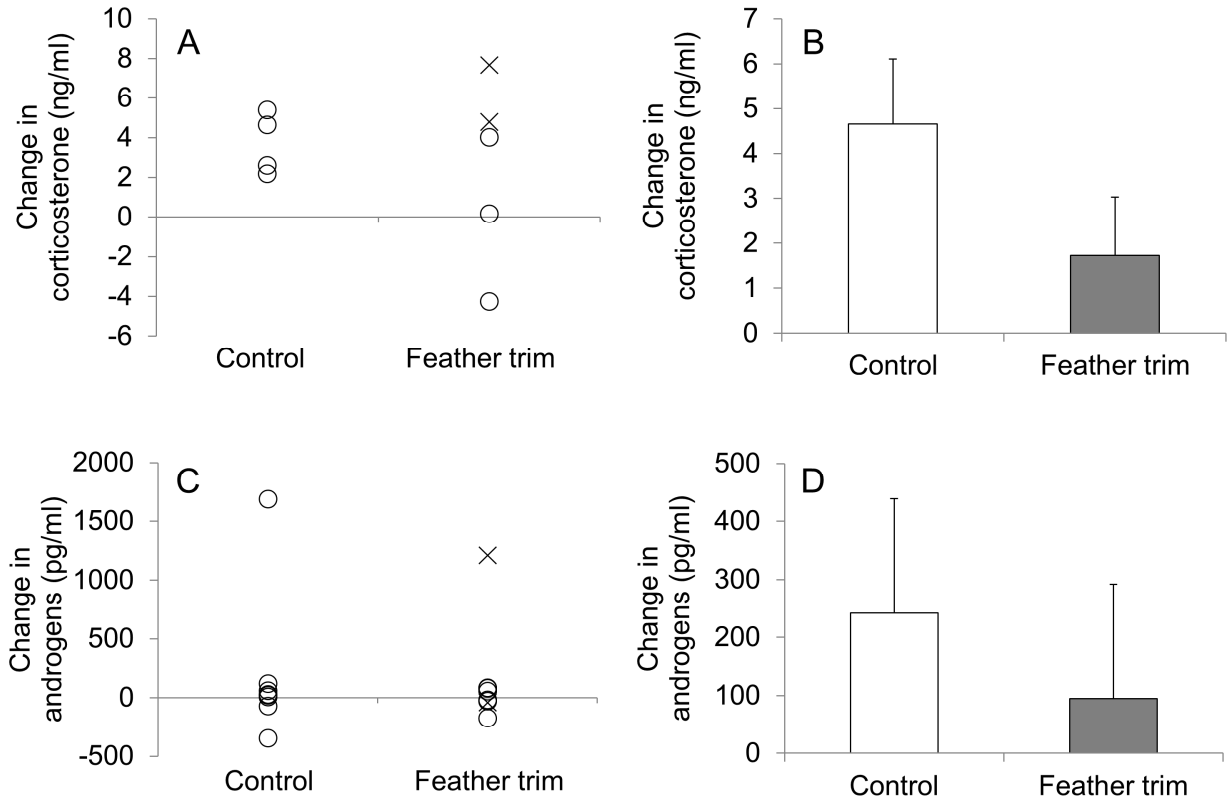

Figure 3. Changes in corticosterone (A, B) and androgen (C, D) concentrations of control (□) and feather trim (■) males between the time of treatment and recapture during the peak of molt showing raw values (A, C) and mean + SE (B, D). Corticosterone was measured in 4 control and 5 feather trim birds, whereas androgens were measured in 8 birds from each treatment. O: males with brown (<33% red/black) plumage; X: males with intermediate (33-66%) or red/black (>66%) plumage.

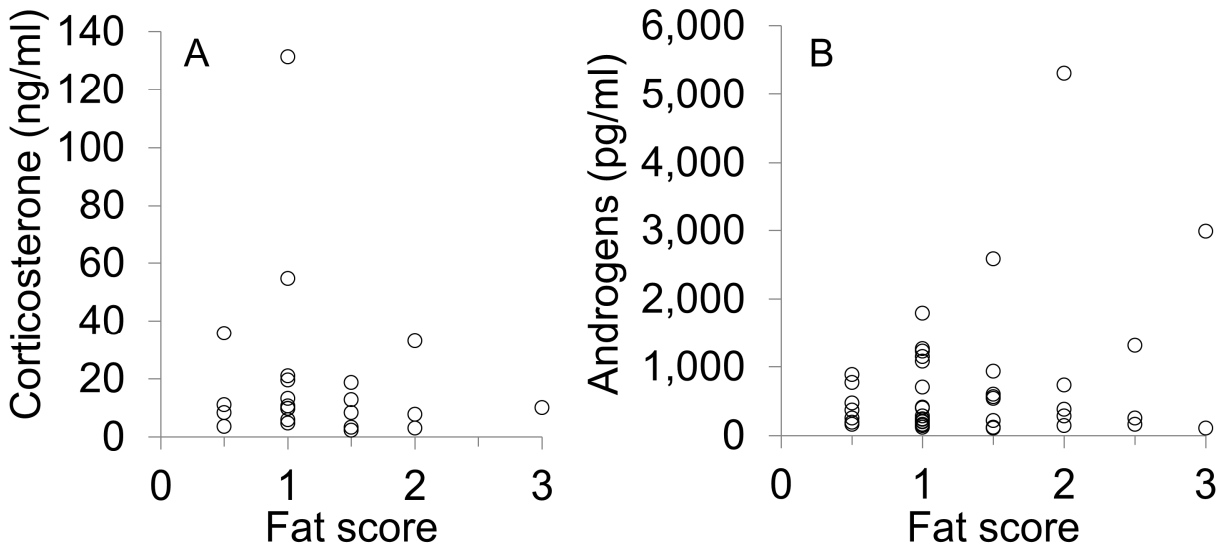

Figure 4. Relationship between fat stores and hormone concentrations among second year males studied between 2004 and 2011. Sample sizes for corticosterone (A) and androgens (B) were 23 and 41, respectively. Fat scores were estimated from fat in the furcular hollow (scale of 0-3).

CHAPTER TWO

DO ANDROGENS LINK MORPHOLOGY AND BEHAVIOR TO PRODUCE PHENOTYPE-SPECIFIC BEHAVIORAL STRATEGIES?

INTRODUCTION

Individuals of most species exhibit variation across physiological, morphological, and behavioral traits, with selection on the collective phenotype often linking suites of related traits (Lande and Arnold 1983). While naturalists have long observed the covariation of morphological traits, much recent emphasis has been placed on understanding behavioral covariation, whereby an individual's behavior is correlated across time and/or contexts (Sih et al. 2004a, Sih et al. 2004b). Furthermore, morphological and behavioral traits can become linked to produce morph-specific behavioral strategies, particularly in instances where morphological variants differ in current and future reproductive potential (Wolf et al. 2007, Dammhahn 2012, Nicolaus et al. 2012). Because inherent genetic, energetic, and time constraints can limit phenotypic expression and even prevent certain trait combinations, however, suites of traits often fall along life-history continua such as mating versus parental investment (Magrath and Komdeur 2003) and current versus future reproduction (Reznick 1992, Stearns 1992, Santos and Nakagawa 2012).

Sexual signals represent a unique case of phenotypic variation in which exaggerated morphological and/or behavioral traits exhibited 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 behavior) with morphological and

behavioral signals such as bright coloration and elaborate mating displays. The evolution and maintenance of such signaling systems requires that those signals honestly indicate mate quality, to prevent cheating (e.g. Zahavi 1975, Hill 1990, Jennions et al. 2001). One possible explanation for the covariation of honestly expressed morphological and behavioral signals is that they are linked and constrained via regulation by shared underlying mechanisms that result from physiological trade-offs and antagonistic pleiotropy (Lande 1980, Hau 2007, Cox et al. 2009).

Because steroid hormones transduce environmental information and their pleiotropic actions link suites of traits, they are frequently suggested as a mechanism behind life-history trade-offs (Ketterson and Nolan 1999, McGlothlin and Ketterson 2008). Androgens such as testosterone have gained particular attention as regulators of honest signal elaboration due to their physiological costs (e.g. immunosuppression; Folstad and Karter 1992, Saino and Moller 1994). Specifically, androgens are thought to promote the exaggeration of morphological signals (e.g. Zuk et al. 1995, Gonzalez et al. 2001) while shifting behavior to increase mating effort at the expense of parental investment (reviewed in Ketterson and Nolan 1999), and are expected to be higher in dominant males (e.g. Wingfield et al. 1991). This relationship is believed to be reciprocal, whereby androgens are themselves responsive to phenotype through its influence on behavioral interactions with conspecifics (Wingfield et al. 1990, Oliveira 2004). However, studies often detect no relationship between androgens and phenotype, or even report patterns opposite these predictions (Adkins-Regan 2005, Lynn 2008). While our understanding of these patterns has benefitted greatly from studies of captive populations and artificial hormone manipulations (e.g. Ketterson and Nolan 1999, Van Roo 2004, Lynn et al. 2009, Roberts et al. 2009), a more complete picture also requires that individual variation in androgen levels be examined in unmanipulated wild animals (Kempenaers et al. 2008, Williams 2008).

The high variability of baseline testosterone concentrations across short time scales has created hesitation regarding their usefulness in predicting individual behavior (Adkins-Regan 2005, Ball and Balthazart 2008). McGlothlin et al. (2007, 2008) recently 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. Moore 1983, Wingfield et al. 1990, Pinxten et al. 2003, Oliveira 2004). Moreover, McGlothlin et al. (2007, 2008) demonstrated that variation in responsiveness to a given dose of gonadotropin-releasing-hormone (GnRH), which regulates gonadal androgen production, is positively related to territoriality and sexual signal expression but negatively related to parental effort in male Dark-eyed Juncos (*Junco hyemalis)*. Specifically, they found that 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 behavioral/morphological phenotypes of male White-Throated Sparrows (*Zonotrichia albicollis)* also differed in their testosterone response to GnRH challenge (Spinney et al. 2006), further suggesting that activity of the hypothalamic-pituitary-gonadal (HPG) axis might integrate morphological and behavioral expression. Short term increases in testosterone could allow males to minimize the costs of high testosterone without sacrificing expression of testosterone-mediated behavior (Wingfield et al. 2001) and to alternate between mating and parental behavior (McGlothlin et al. 2007). "GnRH challenges" may therefore provide a means of testing the hypothesis that the HPG axis generates individual and phenotype-specific differences in morphology and behavior.

Here we investigate whether activity of the HPG axis links morphology and behavior 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 behavior, as males exhibit discrete breeding phenotypes that differ in morphology, behavior, and circulating androgen levels. During the first year of reproduction males can breed in sexually preferred red/black nuptial plumage and exhibit frequent extra-territorial forays and low nestling feeding rates (“red/black breeders”), breed in brown female-like plumage with infrequent extra-territorial 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"; Karubian 2002, Webster et al. 2008, Varian-Ramos et al. 2012). Nearly all males are red/black breeders in subsequent breeding seasons, meaning this species exhibits 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 pre-nuptial molt 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 et al. 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 et al. 2011). Furthermore, testosterone implants prior to and during the prenuptial molt stimulated production of red/black plumage and darkening of the bill (Lindsay et al. 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 et al. 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 behavior, nestling feeding, and territoriality co-vary within as well as across male phenotypes (Karubian 2002), as would be expected if male behavior is constrained by a trade-off between mating and parental investment. Second, we sought to establish whether phenotypes differ in their physiological capacity to produce androgens, which could explain why brown breeder and auxiliary males maintain lower circulating concentrations than red/black breeders. Third, we investigated whether variation in androgen secretion is correlated with behavioral variation and could therefore provide a mechanistic link between morphology and behavior to produce a correlated phenotype. Finally, because phenotype in this species is somewhat age-dependent we also examined whether phenotypic differences are partially a consequence of age-dependence of behaviors or androgens. These hypotheses generate the following predictions: (a) male mating and parental behavior would be negatively correlated and would vary by phenotype, with red/black males investing more heavily in mating (territoriality and extra-territorial forays) and less in parental care (nestling feeding), (b) 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), and (c) males with higher baseline or GnRH-induced androgens would invest more in mating behavior at the expense of parental care.

METHODS

Study species and basic field methods

This study was conducted in a population of color-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 vs. auxiliary) and plumage color (red/black vs. brown), although almost all males become red/black breeders in following breeding seasons. These phenotypic differences also extend to other morphological and behavioral traits, as red/black males have shorter tails (Karubian 2002) and darker bills (Lindsay et al. 2009) and invest in extra-pair mating at the expense of parental care by foraying 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 extra-pair young (Webster et al. 2008). While we know that red/black males are more aggressive toward red/black than brown intruders (Karubian et al. 2008), we lack an understanding of whether phenotypes differ in their territorial response to intruders, and it is also not known whether territorial, foray, and feeding behavior covaries across individuals within each male phenotype.

We target trapped birds during the breeding season between 9 October and 6 December, 2011 using mist-nets by slowly walking toward the birds to push them toward 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 vs. after-second year) using the degree of skull ossification (Lindsay et al. 2009) before attaching an Australian Bird and Bat Banding Scheme (ABBBS) aluminum leg band and a unique combination of three colored plastic leg bands for subsequent identification. Captures and

sightings of birds were used to assess plumage color, 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, 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 µl 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 minutes after capture (to minimize temporal variation from capture), at which time we injected most birds with 10µl of 500ng 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 25G needle attached to a 50µl syringe (Hamilton Company, Henderson, NV). This "GnRH challenge" dose has been shown to cause a maximal LH response in other passerines (e.g. Wingfield and Farner 1993) and to cause elevated androgen production in males from our study population (Lindsay, unpublished). A small random subset of birds received an injection containing only 10µl of phosphate-buffered saline to serve as controls. Birds were again placed into an opaque cloth bag and left undisturbed until an additional 40 µl of blood was collected 30 minutes after injection for measurement of GnRH-induced androgen levels (Jawor et al. 2006).

Behavioral 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 ± SE duration = 52.4 ± 2.0 min) we calculate and report number of forays and duration of time off territory per observation hour. A concurrent study with the same birds found that auxiliaries do not influence the foray behavior of red/black males (Ahvi Potticary, unpublished data), making it unnecessary to control for helper effects in analyses of this behavior (see below). Although these forays may not be solely for the purpose of obtaining extra-pair matings (Yezerinac and Weatherhead 1997, Stutchbury 1998), departing birds generally visited surrounding territories that were actively occupied, suggesting they were being used to assess mating opportunities and/or competition from neighbors (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 color to live red/black males) with a compilation of songs by one of four unfamiliar red/black males recorded >30km from the focal population. Decoys were attached to a stick protruding 1m 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 2m 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, setup was performed quickly when adults were not nearby. After placement, the observer returned to a small, camouflaged blind set up approximately 15-25m 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 20m 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 20m); (2) time spent within 5m of the decoy (sec); (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 non-focal males from neighboring 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 color bands (N = 2). Trials were conducted under comparable weather conditions.

We observed nestling feeding of focal males by monitoring nests for 1 h between 3-6 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 behavior 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 3x the

size of the bill. Feeding index was calculated by multiplying the average prey size by the number of feeding visits.

These behavioral proxies are interpreted as being representative of the animals' natural foray, territorial, and nestling feeding behavior 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° freezer. The 17 to 46 µl plasma samples were assayed for total androgen concentration (testosterone and 5α-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 µl phosphate-buffered saline with gelatin, pH 7.1 (PBSg). Radioimmunoassays were conducted in 100 µl aliquots using tritium-labelled testosterone (Perkin Elmer Life Sciences NET-553, Waltham, Massachusetts USA) and a testosterone antibody (Wien Laboratories T-3003, Flanders, New Jersey USA) that cross-reacts with closely related steroids (100% reactivity with testosterone, 60% with 5alpha-dihydrotestosterone, 5% with aldosterone, <15% with other androgenic steroids, and less than 0.05% with 17beta-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 inter-assay variation

was 5.9% (calculated according to Chard 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). Concentrations of undetectable samples were calculated from minimal detectable levels of the standard curve (1.95 pg/tube). 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 behavior employed an analysis of covariance (ANCOVA) with phenotype as a fixed factor. Comparisons of alternative foray (# departures vs. time off territory) or feeding (# feeds vs. prey size) metrics were conducted using linear regressions. To evaluate the influence of age on behavior we used a multiple regression with age included among other covariates for the dependent behavior. In all analyses we began with a full suite of potential covariates (hormones: date, age, mass, time post-sunrise, nest age since onset of laying; behavior: date, nest age, number of young, number of auxiliaries), then sequentially removed nonsignificant covariates until all $p < 0.1$.

In instances where we compared two behavior variables or a behavior and 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 linear regression of each variable with its significant covariates (see Results) and used the resulting residuals in subsequent comparisons using linear regression. Comparisons between behaviors are restricted to instances where they were sampled during the same nesting attempt (mean ± SE difference: foray vs. territoriality = 0.93 ± 0.38 days, foray vs. feeding = 9.25 ± 1.01 days, territoriality vs. feeding = 6.56 ± 0.90 days) to avoid temporal or social differences between breeding attempts. Because behaviors did not vary with nest age or across nest stages (see Results), however, we retained comparisons across incubation and nestling stages. Comparisons between behavior variables and hormones were further restricted to instances where both were sampled within the same nesting stage (mean ± SE difference: foray = 2.14 ± 0.57 days, territoriality = 1.13 ± 0.38 days, feeding = 1.70 ± 0.52 days) to avoid 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 <5m 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 behavior (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 are trying

to gauge the males' total feeding effort rather than the resources received by each nestling; however, results remain qualitatively similar if we control for number of young. Androgen concentrations were natural-log 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. In support of this assumption, bleeding delay was unrelated to baseline androgen concentrations ($F_{1, 29} < 0.01$, $p = 0.98$) and was therefore omitted from all analyses.

All analyses were conducted using the program NCSS (Hintze 2007). Animal procedures were approved by the Washington State University Institutional Animal Care and Use and the James Cook University Animal Ethics Committees.

RESULTS

We observed the foray behavior of 42 breeding males (brown = 21, red/black = 21) and the nestling feeding behavior 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 ($F_{1, 40} = 4.66$, $p = 0.04$; Fig. 1A) and stayed off their territories for proportionally longer periods of time ($F_{1, 40} = 4.49$, $p = 0.04$) than did brown breeders. These two estimates of foray behavior were strongly positively correlated ($R^2 = 0.45$, $b = 2.88$, $F_{1, 40} = 33.22$, $p < 0.0001$), and therefore below we report results only for foray rate. Despite their greater time off territory, red/black males were no less likely to respond to the STI (red/black = 73%, brown = 52%, auxiliary = 64%; $n = 64$, $X^2 = 2.64$, $p = 0.27$). However, among responding birds, phenotypes differed significantly in the strength of their territorial response ($F_{2,}$

$_{44}$ = 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 behavior and territoriality increased with age (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 behavior (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 behavior (R^2 = 0.06, b = 0.18, $F_{1, 19}$ = 1.26, p = 0.28). No phenotypic signature existed within one-year-old males in either behavior (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 behavior (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 (feeding index: R^2 < 0.001, b = -0.03, $F_{1, 33}$ < 0.01, p = 0.96) but appears associated with phenotype: brown breeders fed at more than twice the rate of red/black breeders, though 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 (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 ($F_{1, 15} = 5.37$, $p = 0.03$) 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 trend 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 ($R^2 = 0.18$, $b = -0.08$, $F_{1, 37} = 8.33$, $p < 0.01$).

Across phenotypes, measures of foray behavior, territorial response, and nestling feeding were not associated (Fig. 2A-F; Table 2) regardless of whether values were corrected for their respective covariates (see Methods). When looking within phenotypes, however, red/black breeders that fed nestlings more also exhibited stronger territorial responses, though this pattern was only clearly significant for corrected values (Fig. 2C, Fig. 2F, Table 2). These males also displayed a negative relationship between feeding and foray behavior that approached significant after correcting for covariates (Fig. 2B, Fig. 2E, Table 2), yet no relationship existed between territorial response and foray behavior within these red/black males (Fig. 2A, Fig. 2D, Table 2). In contrast, brown breeders did not exhibit correlations among any of the observed behavioral traits regardless of whether we analyzed raw (Fig. 2A-C, Table 2) or corrected (Fig. 2D-F, Table

2) behavioral estimates. Small sample size (n = 3) prevented an examination of the relationship between territorial response and nestling feeding within auxiliary males.

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 (post-injection: $F_{1, 43} = 64.74$, $p < 0.0001$; treatment*pre/post injection: $F_{1, 44.0} = 26.87$, $p < 0.0001$; Fig. 3A), 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. 3B). However, post-injection androgen concentrations did not differ significantly across male phenotypes ($F_{2, 33} = 1.82$, $p = 0.18$; Fig. 3B), thereby producing phenotypic differences in the overall GnRH-induced androgen change ($F_{2, 30} = 3.35$, $p < 0.05$; phenotype*pre/post injection: $F_{2, 34.0} = 3.67$, $p = 0.04$; Fig. 3B). Independent of differences in plumage and reproductive role, age had an influence on baseline androgen levels ($R^2 = 0.10$, $b = 0.30$, $F_{1, 31} = 6.11$, $p = 0.02$) 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 ($R^2 < 0.01$, $b < 0.001$, $F_{1, 30} = 0.10$, $p = 0.75$) or GnRH-induced androgen change ($R^2 = 0.05$, $b < 0.01$, $F_{1, 30} = 2.80$, $p = 0.10$).

No relationship existed between baseline androgen concentrations, GnRH-induced androgen concentrations, or the overall change in androgen concentrations and any measure of

individual behavior, regardless of whether covariates were statistically controlled (Fig. 4, Fig. 5, Fig. 6, Table 3). All correlations remained nonsignificant ($p > 0.10$) if analyses were restricted to red/black or brown breeders.

DISCUSSION

Our results provide further evidence for behavioral differences among male phenotypes of the red-backed fairy-wren (Karubian 2002), and support the hypothesis that behavior is shaped by phenotype-specific trade-offs between mating and parental investment. Specifically, red/black breeders appear to invest heavily in mating effort (extra-territorial forays and territorial aggression) at the expense of parental effort (nestling feeding), whereas the opposite is true of brown males. Despite more frequent departures from their territory, red/black breeders were equally effective at detecting simulated intruding males and were more aggressive in their territorial response. Considering that these phenotypic differences in foray and territorial behavior 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 behavioral 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 toward heavier parental effort (Maynard Smith 1977, Badyaev and Hill 2002, Karubian et al. 2008, Webster et al. 2008).

When we looked for evidence of individual trade-offs between mating and parental behavior, however, the support was mixed. While the negative relationship between feeding behavior and extra-territorial forays suggests a trade-off, this pattern was weak and appeared restricted to red/black males. The stronger correlation between feeding behavior and territorial responsiveness was again driven by behavioral associations within red/black males, but these behaviors were positively related. Such a positive association contrasts the general expectation that time and energetic constraints prevent an individual from investing heavily in both mating and parental behavior (Magrath and Komdeur 2003), and could arise from individuals being similarly reactive to diverse stimuli, whereby the cues they receive from nestlings and competitors initiate a similarly strong behavioral response.

The contrast between results derived from analyzing phenotypic means versus repeated individual behavior might arise because much behavioral variation exists within phenotypes and individual correlations among behaviors existed only within red/black breeders, an interesting finding that warrants further investigation to understand how and why correlated behavioral strategies could be restricted to a portion of the entire population. Regardless of the exact causes, the discrepancy between these scales of analysis does highlight the importance of accounting for individual variation within statistical means (Williams 2008) by directly comparing individual behaviors, ideally across multiple measures of investment (e.g. foray and territoriality), when evaluating evidence for a trade-off between mating and parental behavior.

Although plumage color 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 and Karter 1992, Saino and Moller 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 (Oliveira 2004, Hirschenhauser and Oliveira 2006, Maia et al. 2012).

Counter to predictions of the hypothesis that individual variation in androgen secretion patterns predicts behavioral variation (Wingfield et al. 1987, Wingfield et al. 1990, Ketterson and Nolan 1992, McGlothlin et al. 2007), individual hormonal and behavioral variation were unrelated regardless of the androgen measure or analytical approach. The lack of hormone-behavior relationships contrasts with studies demonstrating that experimentally elevated testosterone causes birds to shift from parental to mating effort (reviewed in Ketterson and Nolan 1999), including in the congeneric superb fairy-wren (Peters et al. 2002). However, our results are mirrored by several studies demonstrating insensitivity of mating (e.g. Meddle et al. 2002, Moore et al. 2004) and parental (e.g. Lynn et al. 2002, Van Duyse et al. 2002) behavior to experimental increases in testosterone (reviewed in Lynn 2008), as well as studies reporting no correlation between parental or mating behavior and endogenous circulating testosterone

concentrations (e.g. Levin and Wingfield 1992, Silverin et al. 2004, Schwabl et al. 2005, Cramer 2012, DeVries and Jawor 2013). Our results likewise do not support the hypothesis that ability to rapidly increase testosterone in response to GnRH pulses resulting from behavioral interactions regulates the trade-off between mating and parental behavior (McGlothlin et al. 2007). It should be acknowledged that our sample sizes for comparing androgen levels to feeding behavior were restricted by lower hormonal sampling in the nestling stage, and relatively large r-squared 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 behavior similarly found no relationship between the GnRH-induced change in androgens and parental care in Northern Cardinals (DeVries and Jawor 2013). Therefore, uncertainty remains regarding the generality of relationships between short-term testosterone increases and behavioral 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 behavioral and hormonal variation are unrelated to each other in our study species, other possibilities do exist. One explanation is that a step-wise function exists, whereby above a given androgen concentration behavior is independent of circulating androgen levels (Hews and Moore 1997). Behavioral differences could also be related to variation in concentrations of binding globulins (Pryke et al. 2012) and, more likely, the sensitivity of behavior-related brain structures to androgens as recently demonstrated for other species (Canoine et al. 2007, Ball and Balthazart 2008, Rosvall et al. 2012, Burns et al. 2013). Finally,

hormones other than androgens (e.g. glucocorticoids, prolactin, nonapeptides) might play a role in linking behavior with life-history state (Angelier et al. 2009).

In conclusion, our results support the hypothesis that the behavioral strategies of male red-backed fairy-wrens differ across phenotypes (as defined by plumage color, 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. Our results demonstrate that behavior within the red/black breeder phenotype is related across contexts, with those who are more parental being more territorial and possibly foraying off their territory less. In contrast, we observed no such covariation within brown breeders, suggesting that behavioral tradeoffs are associated with plumage color. Despite previous demonstration that male phenotypes differ in androgen levels during all reproductive phases (Lindsay et al. 2009), and that acquisition of plumage color type is mediated by testosterone (Lindsay et al. 2011), differences in plumage color 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 behavioral variation. While these results demonstrate individual integration of morphology and behavior, they do not support the hypothesis that variation in circulating androgen concentrations or the capacity to produce them are responsible for behavioral differences across male phenotypes.

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Table 1. Variable factor loadings for the first principal component of male response to a simulated territorial intrusion. This principal component had an Eigenvalue of 2.42 and explained 61% of the variance. The second principal component 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.

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

Table 2. Results from a linear regression between foray frequency, territorial response, and nestling feeding behavior across all phenotypes and within red/black and brown breeding males. Raw values refer to the comparison of raw behavioral estimates, whereas corrected values refer to the comparison of residuals of the behavioral measures from a linear regression with their respective covariates (see methods).

	Territoriality vs Foray					Feeding vs. Foray					Territoriality vs. Feeding				
	n	R^2	*b*	*F*	*p*	*n*	R^2	*b*	*F*	*p*	n^a	R^2	*b*	*F*	*p*
All															
Raw	28	0.03	0.17	0.68	0.42	20	0.14	-2.34	3.02	0.10	25	0.05	0.97	1.15	0.29
Corrected	28	0.04	-0.20	0.96	0.34	20	0.14	-2.50	3.02	0.10	25	0.12	1.69	3.11	0.09
Red/black															
Raw	15	<0.001	0.03	<0.01	0.93	13	0.20	-1.86	2.79	0.12	16	0.22	0.14	3.96	0.07
Corrected	15	0.03	-0.17	0.38	0.55	13	0.27	-1.96	4.06	0.07	16	0.55	0.25	16.77	0.001
Brown															
Raw	13	<0.01	0.17	0.07	0.80	7	0.12	-6.65	0.65	0.46	6	<0.01	-0.01	0.03	0.88
Corrected	13	<0.001	0.02	0.01	0.92	7	0.04	-3.24	0.21	0.67	6	<0.01	-0.01	0.03	0.86

[a]Sample size for all birds includes 3 auxiliaries, and so is greater than the sum of Red/black and Brown sample sizes

Table 3. Results from a linear regression between a male's foray frequency, territorial response, and nestling feeding and his androgen concentrations at baseline, 30min after GnRH injection, and the resulting androgen change between those time points. Raw values refer to the comparison of ln-transformed androgen concentrations to raw behavioral estimates, whereas corrected values refer to the comparison of the residuals of ln-transformed androgens and behavioral estimates from a linear regression with their respective covariates (see methods).

	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

[a]Calculated as (ln-post-GnRH androgens) - (ln-baseline androgens)

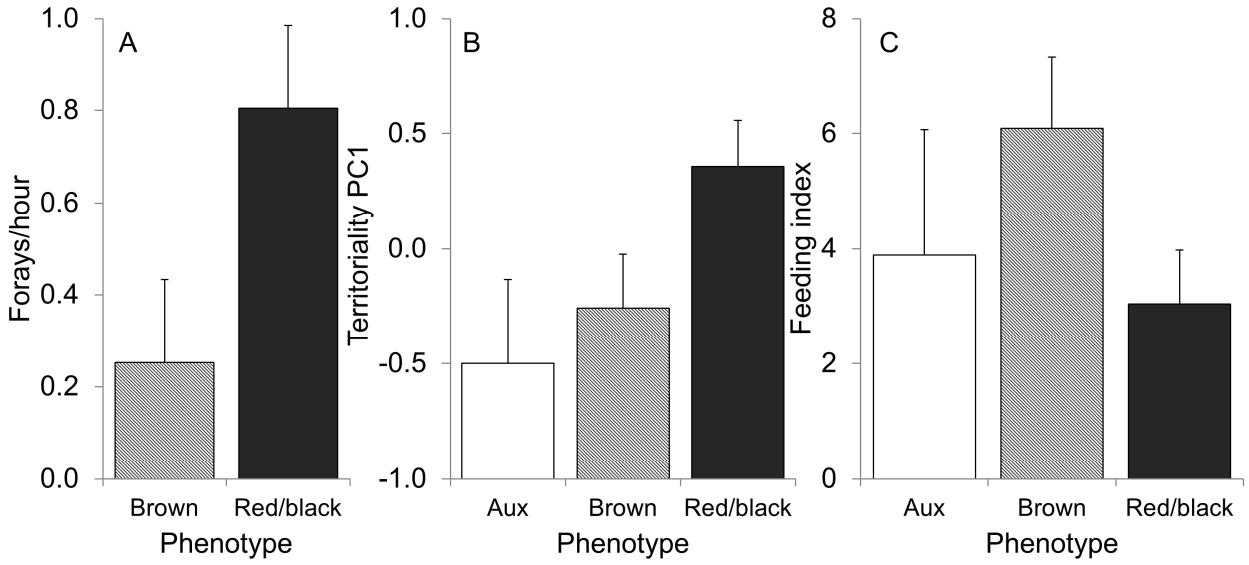

Figure 1. Differences in mean (+ SE) foray frequency (A), territorial response (B), and nestling feeding (C) behavior across male phenotypes: red/black breeders, brown breeders, and non-breeding brown auxiliaries. Foray behavior was estimated from 42 males (Brown = 21, Red/black = 21), territoriality 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 methods.

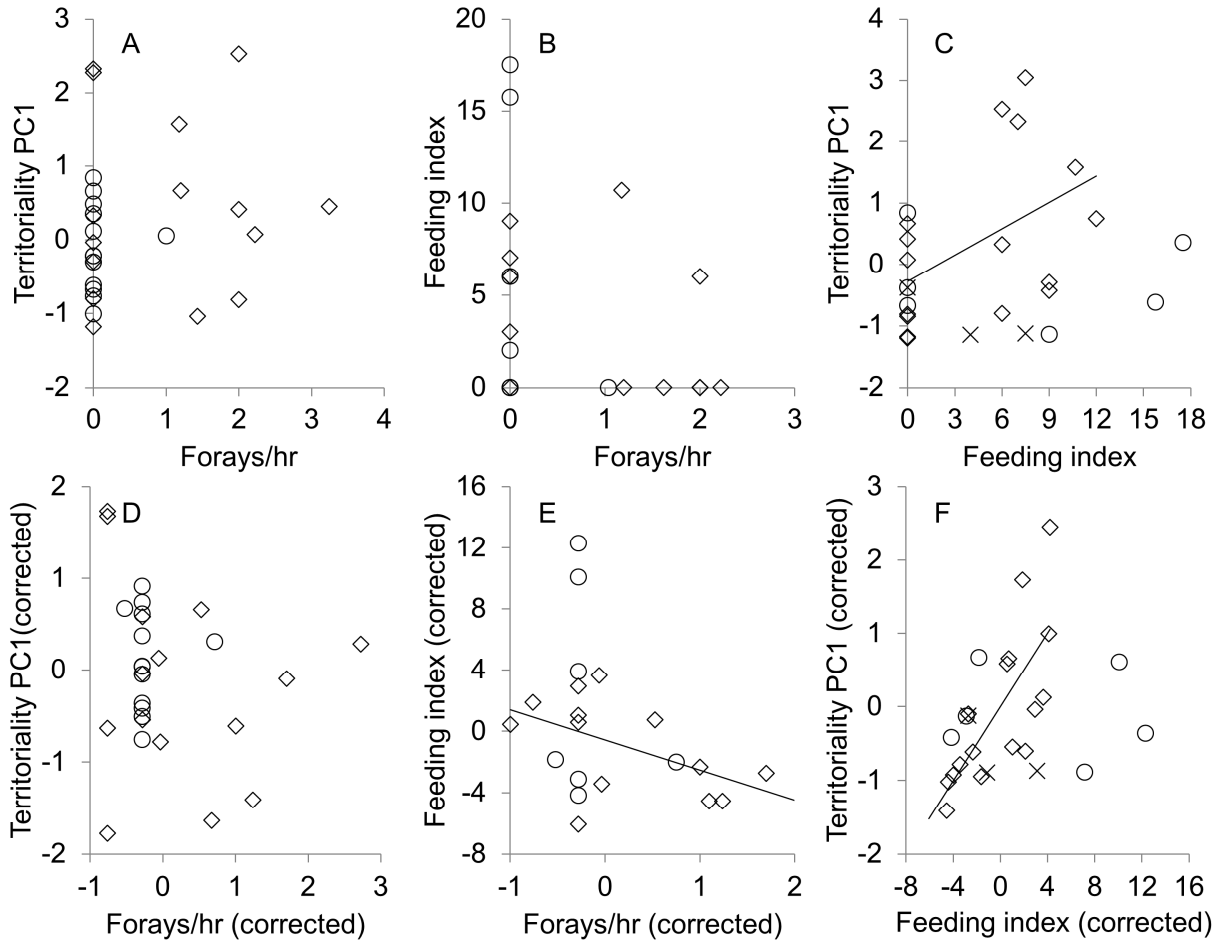

Figure 2. Relationship among male foray frequency, territorial response, and nestling feeding. The top figures (A, B, C) compare raw behavioral measures, whereas the bottom figures (D, E, F) compare the residuals of the behavioral measures from a linear regression with their respective covariates (see methods). Phenotypes are coded with different symbols: non-breeding brown auxiliaries (×), brown breeders (○), and red/black breeders (◇). Trend lines are given for patterns within red/black breeders that have considerable support ($p \leq 0.07$). No correlations existed among behavioral variables across all phenotypes or within brown breeders or auxiliaries. Sample sizes and statistics appear in Table 2.

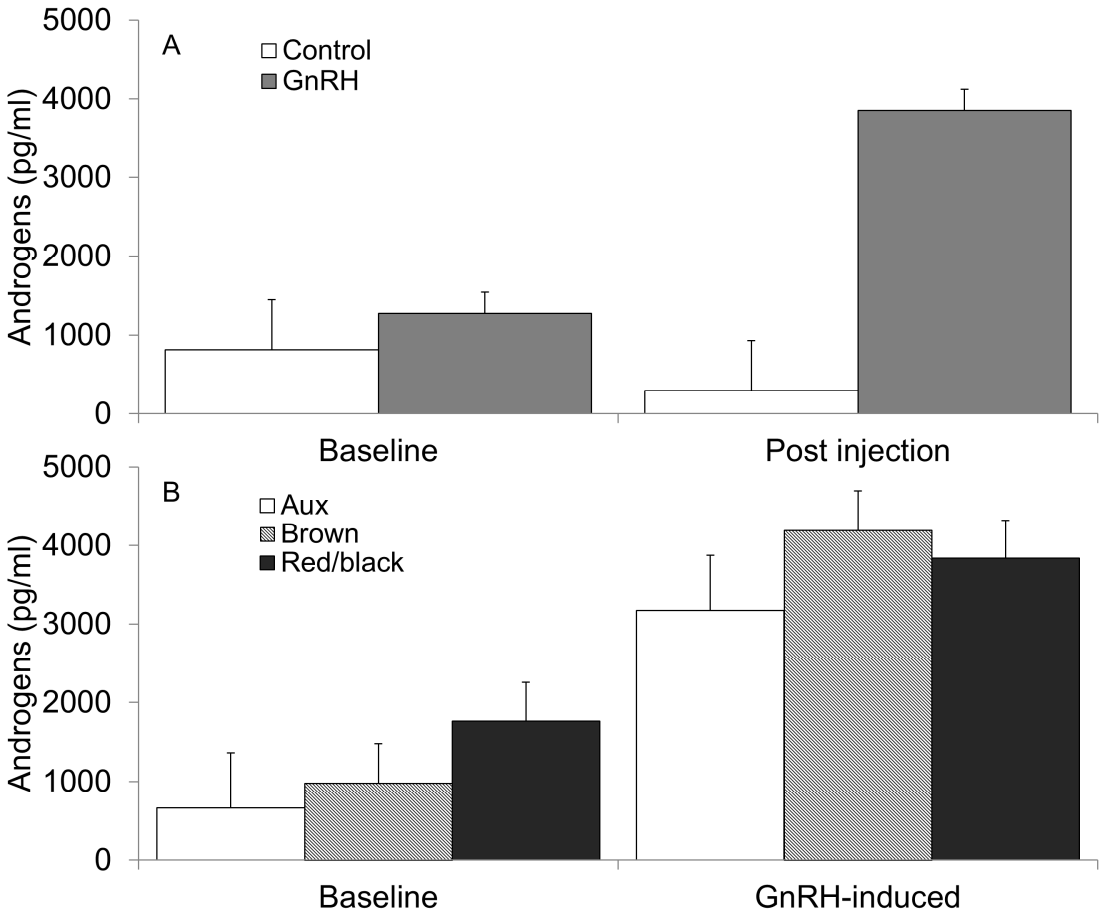

Figure 3. Differences in mean (+ SE) baseline and post-injection androgen concentrations of males by treatment (A) and phenotype (B). Phenotypes include red/black breeders, brown breeders, and non-breeding brown auxiliaries. Post-injection samples were collected 30min after injection with control or GnRH solution. Treatment effects of GnRH injection are from 46 males across all phenotypes (Control = 7, GnRH = 39), whereas phenotypic comparisons omitted 3 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 methods.

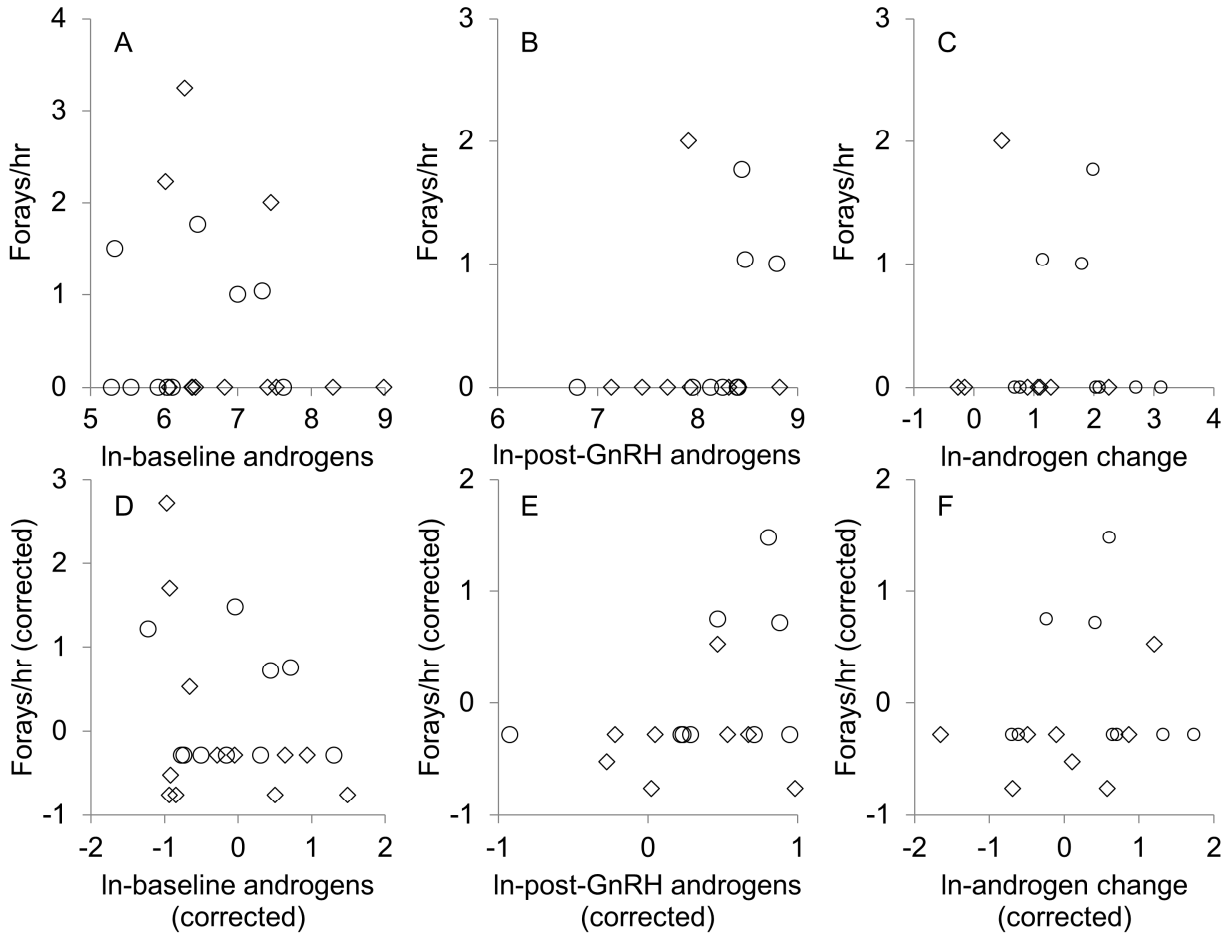

Figure 4. Relationship between a male's foray frequency and his androgen concentrations at baseline, 30min after GnRH injection, and the resulting androgen change between those time points. The top figures (A, B, C) compare ln-transformed androgen concentrations to raw behavioral estimates, whereas the bottom figures (D, E, F) compare the residuals of ln-transformed androgens and behavioral estimates from a linear regression with their respective covariates (see methods). Phenotypes are coded with different symbols: non-breeding brown auxiliaries (x), brown breeders (○), and red/black breeders (◇). Ln-androgen change is calculated as (ln-post-GnRH concentration) - (ln-baseline concentration). Sample sizes and statistics appear in Table 3.

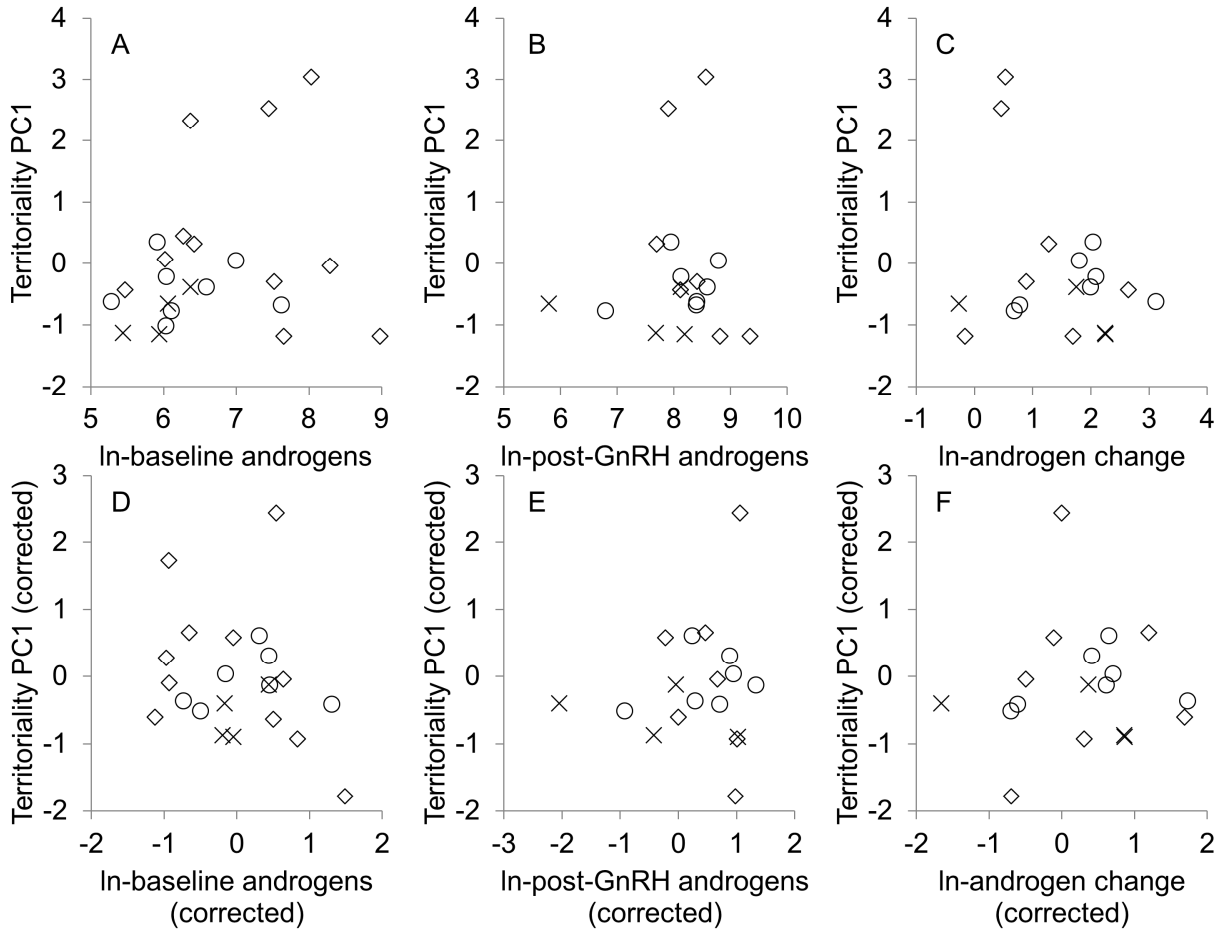

Figure 5. Relationship between a male's response to a simulated territorial intrusion (PC1) and his androgen concentrations at baseline, 30min after GnRH injection, and the resulting androgen change between those time points. The top figures (A, B, C) compare ln-transformed androgen concentrations to raw behavioral estimates, whereas the bottom figures (D, E, F) compare the residuals of ln-transformed androgens and behavioral estimates from a linear regression with their respective covariates (see methods). Phenotypes are coded with different symbols: non-breeding brown auxiliaries (x), brown breeders (○), and red/black breeders (◇). Ln-androgen change is calculated as (ln-post-GnRH concentration) - (ln-baseline concentration). Sample sizes and statistics appear in Table 3.

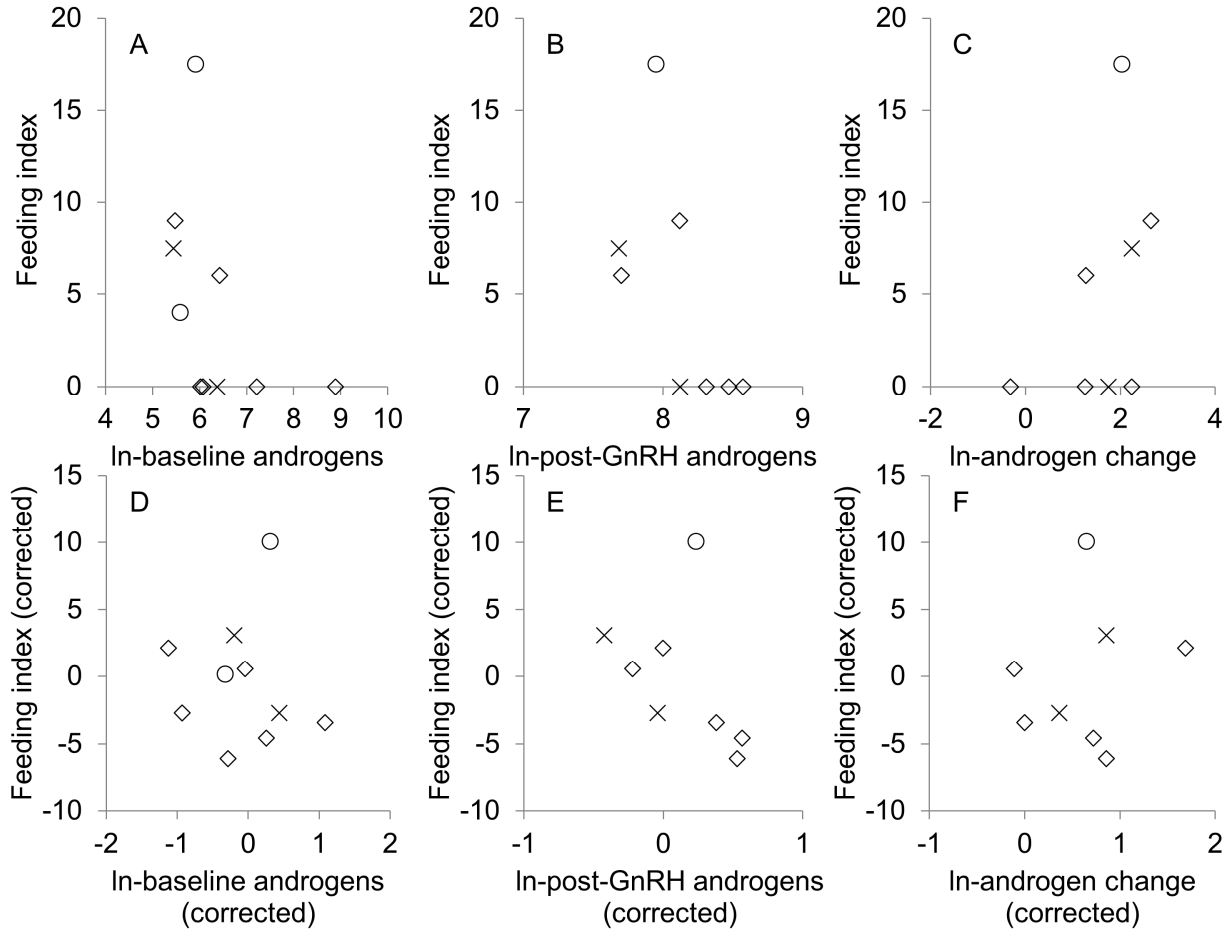

Figure 6. Relationship between a male's nestling feeding and his androgen concentrations at baseline, 30min after GnRH injection, and the resulting androgen change between those time points. The top figures (A, B, C) compare ln-transformed androgen concentrations to raw behavioral estimates, whereas the bottom figures (D, E, F) compare the residuals of ln-transformed androgens and behavioral estimates from a linear regression with their respective covariates (see methods). Phenotypes are coded with different symbols: non-breeding brown auxiliaries (x), brown breeders (○), and red/black breeders (◇). Ln-androgen change is calculated as (ln-post-GnRH concentration) - (ln-baseline concentration). Sample sizes and statistics appear in Table 3.

CHAPTER THREE

DIFFERENTIAL ALLOCATION VERSUS DIFFERENTIAL COSTS IN MATE CHOICE AND REPRODUCTIVE INVESTMENT

INTRODUCTION

Parental investment is predicted to enhance offspring fitness but come with costs to future reproduction (Trivers 1972, Catry et al. 2013), and should therefore match current environmental conditions to optimize lifetime fitness. The differential allocation hypothesis posits elevated reproductive investment of females when paired with attractive males (Burley 1986, 1988, Horvathova et al. 2012), although it remains unclear what benefits counter the costs to maintain this strategy. Indirect genetic benefits that enhance offspring fitness might help maintain differential allocation (Sheldon 2000), yet such benefits are not universal (Qvarnstrom et al. 2006, Maklakov and Arnqvist 2009) and empirical and theoretical models have suggested they are likely not sufficient to offset losses to future reproduction (Kirkpatrick and Barton 1997, Charmantier and Sheldon 2006, Kotiaho and Puurtinen 2007).

The emphasis on benefits of differential allocation has largely overshadowed the other half of the equation – the potential costs of elevated investment – even though attractive males often defend high quality territories with greater resource availability (Lampe and Espmark 2003, Ritschard and Brumm 2012) that could mitigate fitness costs of increased investment by females. For example, numerous studies have demonstrated that food availability constrains the start of egg laying (Arcese and Smith 1988, Schoech and Hahn 2007) and that early breeding promotes greater reproductive output in multi-brooded species (Martin 1987, Weggler 2006),

meaning these supposedly female traits could in fact be a (partial) consequence of her mate's quality and the environment he can provide. Insight into this idea is largely confounded, however, because most research has been restricted to migratory species where the timing of breeding is at least partially derived from migratory chronology (Cooper et al. 2011) and thus inseparable from prior non-breeding conditions (Gonzalez-Prieto and Hobson 2013). Parental care has also received considerable attention in the context of differential allocation (Horvathova et al. 2012) because it promotes offspring growth and development, is responsive to partner contribution, and is susceptible to sexual antagonism (Houston et al. 2005), yet few studies have considered that similar levels of female provisioning may carry different costs depending upon partner attractiveness and the resources he controls. A rigorous analysis of the differential allocation hypothesis therefore requires considering differential reproductive costs within and across breeding seasons according to the attractiveness of wild males with naturally varying territory qualities. Thus far such an investigation has not been conducted.

We sought to explain differential allocation through disparate costs of reproduction in red-backed fairy-wrens (*Malurus melanocephalus*) by monitoring the reproduction of two populations across 10 years (2003-2012; N = 598 females total) that varied in reproductive timing and output due to differences in the timing and intensity of monsoon rains (Webster et al. 2010). This Australian songbird is non-migratory and seasonally territorial, during which time they are socially monogamous but highly promiscuous (Karubian 2002). This species is ideally suited for such a question because females face discrete variation in the attractiveness and parental contribution of their partner depending upon his plumage, and frequent mate switching (40%) across seasons permits a longitudinal investigation controlling for differences among individual females. Females prefer males with red/black plumage over those with female-like

brown plumage as both social (Karubian 2002) and extra-pair (Webster et al. 2008) mates, and while variation in male plumage color is partially explained by age, no benefit has been identified to explain this sexual preference. Indeed, older red/black males invest heavily in extra-pair mating at the expense of parental care (Karubian 2002, Barron et al. *in review*), suggesting there may be direct reproductive costs of pairing with such males.

We began searching for fitness benefits received by females with attractive males by first determining whether females increase reproductive investment when paired to red/black males, and, if so, whether that elevated investment translates to greater reproductive success of those females. We then examined whether any higher investment by females comes at a cost to self-maintenance and survival, as generally expected, or whether females with attractive males can maintain future reproductive potential due to lowered costs of current reproduction. The former scenario would predict similar lifetime fitness of females regardless of their partners' plumage, whereas the latter would predict higher fitness for females that paired more often with attractive males.

METHODS

Study species and basic field methods

We conducted this study in two populations of color-banded red-backed fairy-wrens located near Herberton, Queensland, Australia (145°25'E, 17°23'S) that have been continuously monitored since 2003. While our estimates of female lifetime fitness (n = 258) and of the carry-over of male natal environment (n = 133) include data from 2003-2012 to permit robust sample sizes, the remainder of data in this study is restricted to the 2009-2012 breeding seasons (n = 241 females). Males of this species exhibit discrete variation in plumage color, either displaying a red/black

nuptial plumage or a brown plumage that is indistinguishable from that of a female. Plumage coloration is primarily plastic in the first breeding season, during which time approximately 15% of males molt into red/black plumage, whereas most males adopt red/black plumage during subsequent breeding seasons (Karubian 2002). Plumage color is tightly associated with variation in mating and parental behaviors, with older red/black males providing little parental care and instead investing heavily in mating activities (Karubian 2002, Barron et al. *in review*). We estimated the proportion of a male's feathers in red/black plumage each time he was captured or observed in the field, and used it to categorize them 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 captured adult birds across the prebreeding and breeding seasons each year using mist-nets, after which we collected a maximum of 70 µl of blood from the jugular vein into Lysis buffer, and quantified fat stores seen in the furcular cavity on a scale from 0 (no fat) to 3 (bulging) in 0.5 increments (Lindsay et al. 2009, Barron et al. 2013). We utilized this as our measure of female condition because it likely reflects energy stores used for self-maintenance, and its independence from body size makes it preferable to other condition indices (Gosler et al. 1998); however, it is correlated with a conventional measure of condition in this species, the residual of the regression of mass on tarsus length (Lindsay et al. 2009). We considered birds prebreeding if captured before any eggs had been laid on their site, and considered them late breeding if captured after December 1. Previously uncaptured birds were aged (second year vs. after-second year) using the degree of skull ossification (Lindsay et al. 2009) and marked with an Australian Bird and Bat Banding Scheme aluminum leg band and a unique combination of three

colored plastic leg bands. We observed all birds throughout the formation of breeding groups, which consist of a breeding male, breeding female, and in approximately 20% of cases at least one male natal auxiliary born the previous year (Varian-Ramos et al. 2010). Although past research has found little effect of auxiliary presence on female reproduction or survival in this species (Varian-Ramos et al. 2010), all results reported here have considered helper effects and controlled for them as appropriate.

We searched for and found nests using parental cues throughout the breeding seasons from August–January each year. Once found, nests were checked every 2-3 days, and when nestlings were approximately 5-8 days old we weighed them (0.01g) and collected up to 40 µl of their blood into Lysis buffer for genetic paternity analyses (see below). During the 2011 breeding season we also briefly removed all eggs to measure their mass (0.001g).

Feeding observations

During the 2012 breeding season we observed nestling feeding by monitoring nests for 1 h between 3-6 h after sunrise using binoculars and/or a spotting scope from a small, camouflaged blind set up approximately 15-25m away the previous day. We performed observations when nestlings were 3-8 days old (mean ± SE age = 4.6 ± 0.2 days) and statistically controlled for nestling age as a covariate. We ensured that researchers would not influence feeding behavior by entering the blind quickly when no adult 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 assigned 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 3x the size of the bill. We calculated a feeding index by multiplying

the average prey size by the number of feeding visits. All observations were conducted under comparable weather conditions, and observations were omitted if any of the feeding adults were not identified by their color bands (N = 4). Our analyses ultimately included feeding observations from 24 pairs (16 with red/black males, 8 with brown males).

Maternity and paternity assignments

Adults and nestlings were genotyped at seven microsatellite loci as described previously (Baldassarre and Webster 2013). When combined, the microsatellite loci were highly polymorphic and informative for paternity analysis (mean number of alleles per locus = 11.14, mean expected heterozygosity = 0.75, combined exclusion probability > 0.99). We assumed the breeding female observed at a nest was the genetic mother of all offspring in that nest, as previous studies have revealed no intra-specific brood parasitism in this species (Webster et al. 2008, Baldassarre and Webster 2013). Paternity, on the other hand, was assigned using the program CERVUS v. 3.0 (Kalinowski et al. 2007). As expected, rates of extra-pair paternity were high in our populations with 61% of offspring being sired by extra-pair males, though we observed large variation across years and sites (range = 35-90%).

Statistical analyses

We primarily assessed the influence of male plumage on female reproductive investment and output using mixed-models repeated measures analyses, which modeled male plumage as a fixed factor and included repeated measurements of individual females across nests and years. Because approximately 40% of females change partners between years (Barron, unpublished data), this analysis accounted for intrinsic variation within females and thus allowed us to better separate

the extrinsic influence of male plumage. Our analysis of the relationship between male plumage and nest initiation date relative to annual site-specific means used a similar analytical approach, although we treated the site/year as the repeated subject and included the average nest initiation day and its interaction with male plumage as fixed factors. Because we did not collect repeated measures of nestling feeding by females, we analyzed its relationship to male plumage with an analysis of covariance (ANCOVA), although when investigating sex-specific feeding responses to male plumage we did utilize a mixed-models repeated measures analysis with feeding observations of both males and females and fixed factors of sex and sex*male plumage. We employed multiple regression analyses to examine the relationship between a female's proportion of mates that were red/black and measures of her lifetime reproduction and survival (average nest initiation day, lifespan, and total fledglings), whereas female annual survival and the carry-over of natal environment to plumage were analyzed with logistic regression analyses due to their binary nature. Similarly, we used logistic and multiple regressions, respectively, to investigate whether a male's natal environment carries over to predict his adult plumage and reproductive success.

Our estimates of annual reproductive investment and output are based upon a principal components analysis (PCA) of four correlated reproductive metrics: # nesting attempts, # eggs, # nestlings, and # fledglings (Table 1). The first principal component had an Eigenvalue of 2.15 and explained 54% of the variance, while the second principal component had an Eigenvalue of 1.31 and explained 33% of the variance. The third principal component had an Eigenvalue of 0.50 and only explained 11% of the variance, so did not meet the Kaiser criteria for inclusion (Eigenvalue > 1; Kaiser 1960) and was not further considered.

We excluded any females that could have been influenced by experimental manipulations, and all analyses omitted females that changed social mates and/or auxiliary helpers during a season to ensure that male characteristics and group dynamics were consistent and could be accurately assigned. Because our analyses of annual female survival and the adjustment of breeding chronology to yearly means did not account for repeated measures on a given female we restricted data to a female's first breeding attempt to avoid pseudoreplication across years. To remove the influence of covariates that could confound male plumage, all analyses began with a full model that included site, year, and auxiliary presence as fixed factors (except no year for egg mass and feeding) and the following continuous covariates as indicated: female age (all except measures of lifetime fitness), male mate age (all except measures of lifetime fitness), day of year (capture day for fat, observation day for feeding, nest initiation day for all others), and number of eggs/nestlings (egg and nestling mass, nestling period, total feeding per nestling, fledging and recruitment rates, # fledglings, # grandchildren, natal carry-over of plumage and reproductive success). Nonsignificant covariates were sequentially removed until all $p < 0.1$, and significant covariates are only reported when relevant. For detailed descriptions of each analysis see Table 2.

All analyses were conducted using the program NCSS (Hintze 2007). Animal procedures were approved by the Washington State University Institutional Animal Care and Use and the James Cook University Animal Ethics Committees.

RESULTS

Older females paired with older males ($F_{1, 297.7} = 13.71$, $p < 0.001$; Table 2A) and more frequently obtained males with red/black plumage ($F_{1, 310.7} = 8.22$, $p < 0.01$; Table 2A), but this

assortative mating appeared unrelated to the female's energetic state since pre-breeding fat stores did not increase with age ($F_{1, 20.2} = 1.14$, $p = 0.30$; Table 2B) and did not predict the subsequent plumage color of her partner ($F_{1, 62.2} < 0.01$, $p = 0.99$; Table 2B; Fig. 1A). Older females began nesting earlier in the year, which was partly accounted for by their age ($F_{1, 192.2} = 9.70$, $p < 0.01$; Table 2C) but better explained by their partner's plumage type ($F_{1, 315.6} = 13.18$, $p < 0.001$; Table 2C). Although there was no evidence that older females bred earlier in better years (female age*avg. nest date: $F_{1, 190.0} = 0.03$, $p = 0.86$; Table 2D), females paired with red/black males further advanced nest initiation in early breeding years than did those paired with brown males (male plumage*avg. nest date: $F_{1, 191.0} = 6.63$, $p = 0.01$; Table 2D; Fig. 2). In the year with the earliest onset of reproduction, females paired with red/black males started laying nearly a full month earlier than those paired with brown males, enough time to raise one brood to fledging.

Females paired with red/black males exhibited higher levels of reproductive investment and output across all breeding stages, as indicated by the first principal component of investment ($F_{1, 290.8} = 5.94$, $p = 0.02$; Table 2E; Fig. 3A) which loads positively with the number of attempts, eggs, nestlings, and fledglings (Table 1). The greater investment of these birds results largely from their earlier breeding, as nest initiation date strongly predicted female reproduction ($F_{1, 250.4} = 39.62$, $p < 0.001$; Table 2E; Fig. 3B) and its inclusion in the model eliminated the statistically significant difference between male plumage types ($F_{1, 272.8} = 1.58$, $p = 0.21$; Table 2E). Neither female nor male age predicted female investment (female age: $F_{1, 247.7} < 2.22$, $p = 0.14$; male age: $F_{1, 276.0} < 0.01$, $p = 0.94$; Table 2E), suggesting this pattern does not arise from the older age of birds in red/black pairs. In fact, a restricted analysis of one-year-old males revealed the same pattern of greater investment by females paired with red/black males ($F_{1, 91.6} = 4.43$, $p = 0.04$; Table 2E), except that this effect was independent of the influence of nest initiation date ($F_{1, 85.1}$

= 11.85, $p < 0.001$; Table 2E). The second principal component of investment loaded heavily and positively with number of fledglings but negatively with number of nesting attempts and eggs (Table 1), and likely reflects lower renesting rates after successfully fledging young. This measure was a product of reproductive variation among years ($F_{3, 253.1} = 3.62$, $p = 0.01$; Table 2E) and sites ($F_{1, 167.6} = 4.54$, $p = 0.03$; Table 2E), and did not vary with male plumage ($F_{1, 287.0} = 0.57$, $p = 0.45$; Table 2E) or nest initiation date ($F_{1, 276.0} = 2.35$, $p = 0.13$; Table 2E).

In accordance with the disparity in investment in offspring quantity, females in 2011 laid larger eggs when paired with red/black males, although this difference was not significant ($F_{1, 47.8} = 3.00$, $p = 0.09$; Table 2F). Observations during the 2012 breeding season indicate that females did not adjust their feeding rates according to their partner's plumage ($F_{1, 23} = 0.12$, $p = 0.73$; Table 2G), but those with red/black males did provide larger food items ($F_{1, 19} = 10.96$, $p < 0.01$; Table 2H). When feeding rate and food size were combined into a feeding index, the relative contribution of the sexes clearly varied by male plumage: pairs with brown males fed equally ($F_{1, 21} = 0.58$, $p = 0.91$; Table 2I), whereas females provided the majority of feeding in pairs with red/black males ($F_{1, 21} = 25.51$, $p < 0.001$; Table 2I; Fig. 4). As a result of this compensatory feeding by females (sex*male plumage: $F_{1, 21} = 4.70$, $p = 0.04$; Table 2I), nestlings of received a similar amount of food regardless of their social father's plumage type ($F_{1, 19} = 0.19$, $p = 0.67$; Table 2J). Our long-term data (2009-2012) show that nestlings of red/black and brown males were of similar mass at the time of weighing ($F_{1, 214.4} = 0.12$, $p = 0.72$; Table 2K), although those of red/black males stayed in their nests longer before fledging ($F_{1, 159.9} = 7.22$, $p < 0.01$; Table 2L). Ultimately, nestlings raised by red/black and brown males were equally likely to fledge ($F_{1, 222.8} = 0.38$, $p = 0.54$; Table 2M) and be recruited into the population in the subsequent year ($F_{1,}$

$_{154.2}$ = 0.05, p = 0.83; Table 2N), despite reduced feeding by red/black males (Karubian 2002, Barron et al. *in review*).

Our 2009-2012 data further reveal that the reproductive benefits of breeding early with red/black males extended beyond the current year, with females that began breeding earlier having more grandchildren from their sons (the philopatric sex) in the following season ($F_{1, 88.5}$ = 5.99, p = 0.02; Table 2O). While this was partially a product of early breeding females fledging more young ($F_{1, 105.5}$ = 3.92, p = 0.05; Table 2P), those females had significantly more grandchildren even after controlling for number of fledglings ($F_{1, 10.5}$ = 10.48, p < 0.01; Table 2P). When looking at an expanded dataset (2003-2012) of young males breeding on their natal grounds it appears this inclusive fitness benefit arises because males born early in the season were much more likely to become red/black breeders in their first year (n = 133, X^2 = 31.01, p < 0.001; Table 2Q; Fig. 5A), and therefore sired more offspring than males born later in the season ($F_{1, 115}$ = 14.71, p < 0.001; Table 2R; Fig. 5A). Not only is early breeding more likely in pairs with red/black males, but independent of fledge date the males raised by red/black social fathers appeared more than twice as likely to adopt red/black plumage (brown = 8%, red/black = 18%; n = 105, X^2 = 3.62, p = 0.06; Table 2Q) and sire offspring in their first year (brown = 13%, red/black = 34%; n = 113, X^2 = 4.31, p = 0.04; Table 2S). This pattern seemed derived from paternal environment rather than genetic contribution, as offspring plumage and reproductive success (RS) were not associated with their genetic father's plumage in their natal year (plumage: n = 58, X^2 = 1.31, p = 0.25; RS: n = 59, X^2 < 0.01, p = 0.96; Table 2Q&S) or during his first breeding season (plumage: n = 61, X^2 = 1.30, p = 0.25; RS: n = 61, X^2 < 0.01, p = 0.99; Table 2Q&S).

Despite their earlier breeding season and greater production and care of offspring, females paired with red/black males increased their fat stores across the breeding season (2009-2012; $F_{1,\ 185.2} = 17.14$, $p < 0.001$; Table 2T; Fig. 1A), while those paired with brown males maintained constant fat stores ($F_{1,\ 42.1} = 0.12$, $p = 0.74$; early/late*male plumage: $F_{1,\ 169.3} = 4.60$, $p = 0.03$; Table 2T; Fig. 1A). As a result, females paired with red/black males were in significantly better energetic condition towards the end of the breeding season than those paired with brown males ($F_{1,\ 75.7} = 6.47$, $p = 0.01$; Table 2T; Fig. 1A). This skewed cost of reproduction appears to carry-over to influence probability of future reproduction, as females paired with red/black males were more likely to survive to the following season than were those paired to brown males ($n = 196$, $X^2 = 3.71$, $p = 0.05$; Table 2U; Fig. 1B). Furthermore, from 2003-2012 females that paired more often with red/black males across their lifetime bred earlier on average ($F_{1,\ 197} = 5.92$, $p = 0.02$; Table 2V), lived longer ($F_{1,\ 247} = 10.56$, $p = 0.001$; Table 2W), and fledged more young ($F_{1,\ 217} = 5.38$, $p = 0.02$; Table 2X).

DISCUSSION

In contrast to prevailing theory (Burley 1986, 1988, Sheldon 2000, Horvathova et al. 2012), these results demonstrate that females with attractive mates can better afford to allocate resources to their offspring, allowing simultaneous investment in current reproduction and survival. This pattern does not appear to originate from characteristics of the females themselves, as females paired with red/black and brown males were in similar body condition prior to pairing, and their age had no influence on annual reproductive output. Female reproductive investment, output, and costs are instead likely affected, either directly or indirectly, by the attractiveness and quality of their partner and the associated advantages of early seasonal onset

of reproduction (Weggler 2006). While it remains unclear what specific attributes of attractive males ameliorate female reproductive costs, territory quality is a likely candidate (Martin 1987, Lampe and Espmark 2003, Ritschard and Brumm 2012), as red/black males are more territorial than brown males (Barron et al. *in review*) and both red/black males and their partners provide larger prey items than do pairs with brown males (Barron et al. *in review*). Regardless of the underlying causes, these results demonstrate that the costs of reproduction can be influenced by mate attractiveness, thereby making it easier for females to invest more in the offspring of attractive mates.

We also provide rare documentation that natal environment carries over to influence sexual signal expression and reproductive success as an adult. Such environmental regulation of sexual signals is generally predicted to erode sexual preferences due to the lack of heritable advantages to the offspring (Andersson 1994), yet the inclusive fitness benefits of pairing with attractive males were nonetheless maintained through an enhanced ability to breed early and via other non-genetic paternal effects. Sexual preference for an environmentally-mediated trait therefore appears to be partially maintained in this system by a positive feedback loop between male attractiveness and fledge date (Fig. 4B), and we propose developmental programming of attractiveness can be maintained without genetic heritability when parent attractiveness underlies variation in natal environment.

Although these results provide two novel mechanisms through which differential allocation can prove beneficial to females in the absence of indirect genetic benefits, it is not contingent upon a lack of indirect benefits, nor do we necessarily advocate a lack of indirect benefits in this system. Instead, these results indicate that indirect benefits are not always necessary to explain differential investment. Indeed, indirect genetic benefits to offspring fitness

appear unlikely in this context considering over half of all offspring were sired by extra-pair males regardless of the social male's plumage type (Webster et al. 2008). Direct and indirect benefits are not mutually exclusive, however, and in fact may provide additive benefits of selecting and investing heavily in the offspring of attractive males (Kokko et al. 2006). We advocate that indirect genetic benefits must be considered in conjunction with direct benefits to survival and inclusive fitness and be balanced against varying reproductive costs in order to accurately depict the dynamics of sexual selection.

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Table 1: Variable factor loadings for the first two principal components of female reproductive investment and output. The first principal component had an Eigenvalue of 2.15 and explained 54% of the variance. The second principal component had an Eigenvalue of 1.31 and explained 33% of the variance. The third principal component had an Eigenvalue of 0.50 and only explained 11% of the variance, so was not further considered.

Variable	Factor loading	
	PC1	PC2
# nesting attempts	0.84	-0.48
# eggs	0.90	-0.35
# nestlings	0.68	0.55
# fledglings	0.42	0.80

Table 2. Detailed descriptions of analyses employed throughout this study, and the range of years included in those analyses. Repeated subjects received multiple observations and are included as a random factor within mixed models repeated measures analyses. All analyses began with a full model of fixed effects, covariates, and interaction terms. Nonsignificant terms were sequentially removed until all $p < 0.10$.

Dependent variable	**Analysis**	**Repeated subject**	**Fixed effects**	**Covariates**	**Interaction terms**	**Years included**
A) Female age	Mixed models repeated measures	Female	Male plumage Helper (Y/N) Site Year	Male age		2009-2012
B) Pre-breeding fat scores	Mixed models repeated measures	Female	Male plumage Site Year	Capture day Female Age		2009-2012
C) Nest initiation date	Mixed models repeated measures	Female	Male plumage Helper (Y/N) Site Year	Female age Male age		2009-2012
D) Nest initiation date	Mixed models repeated measures	Site/Year	Male plumage Helper (Y/N)	Female age Male age Avg. nest initiation date	Age*avg. nest initiation date Male plumage*avg. nest initiation date	2009-2012
E) Reproductive investment (PCA)	Mixed models repeated measures	Female	Male plumage Helper (Y/N) Site Year	Female age Male age Nest initiation date		2009-2012

Table 2 (continued).

Dependent variable	Analysis	Repeated subject	Fixed effects	Covariates	Interaction terms	Years included
F) Egg mass	Mixed models repeated measures	Female	Male plumage Helper (Y/N) Site	Female age Male age Nest initiation date # eggs Egg age Female mass		2011
G) Feeding rate	ANCOVA		Male plumage Helper (Y/N) Site	Female age Male age Date Time # nestlings Nestling age		2012
H) Avg. food size	ANCOVA		Male plumage Helper (Y/N) Site	Female age Male age Date Time # nestlings Nestling age		2012
I) Feeding index	Mixed models repeated measures	Pair	Male plumage Helper (Y/N) Site Sex	Age Date Time Nestling age	Male plumage*sex	2012
J) Male + female feeding	ANCOVA		Male plumage Helper (Y/N) Site	Female age Male age Date Time # nestlings Nestling age		2012

Table 2 (continued).

Dependent variable	Analysis	Repeated subject	Fixed effects	Covariates	Interaction terms	Years included
K) Avg. nestling mass	Mixed models repeated measures	Female	Male plumage Helper (Y/N) Site Year	Female age Male age Nest initiation date # nestlings Nestling age		2009-2012
L) Nestling period	Mixed models repeated measures	Female	Male plumage Helper (Y/N) Site Year	Female age Male age Nest initiation date		2009-2012
M) Proportion of nestlings fledged	Mixed models repeated measures	Female	Male plumage Helper (Y/N) Site Year	Female age Male age Nest initiation date # nestlings		2009-2012
N) Proportion of nestlings recruited	Mixed models repeated measures	Female	Male plumage Helper (Y/N) Site Year	Female age Male age Nest initiation date # nestlings		2009-2012
O) Grandchildren	Mixed models repeated measures	Female	Male plumage Helper (Y/N) Site Year	Female age Male age Nest initiation date # fledglings		2009-2012
P) Grandchildren per son	Mixed models repeated measures	Female	Male plumage Helper (Y/N) Site Year	Female age Male age Nest initiation date # fledglings		2009-2012

Table 2 (continued).

Dependent variable	Analysis	Repeated subject	Fixed effects	Covariates	Interaction terms	Years included
Q) Son plumage	Logistic regression		Male plumage Father plumage Helper (Y/N) Site Year	Female age Male age Fledge day		2003-2012
R) Son reproductive success	Multiple regression		Male plumage Father plumage Helper (Y/N) Site Year	Female age Male age Fledge day		2003-2012
S) % sons siring young	Logistic regression		Male plumage Father plumage Helper (Y/N) Site Year	Female age Male age Fledge day		2003-2012
T) Fat score	Mixed models repeated measures	Female	Male plumage Helper (Y/N) Site Year Early/late	Female age Capture day	Male plumage* early/late	2009-2012
U) Annual survival	Logistic regression		Male plumage Helper (Y/N) Site Year	Female age Male age		2009-2012
V) Avg. lifetime nest initiation date	Multiple regression		Site Birth year	% red/black partners		2003-2012
W) Lifespan	Multiple regression		Site Birth year	% red/black partners		2003-2012

Table 2 (continued).

Dependent variable	Analysis	Repeated subject	Fixed effects	Covariates	Interaction terms	Years included
X) Lifetime RS	Multiple regression		Site Birth year	% red/black partners		2003-2012

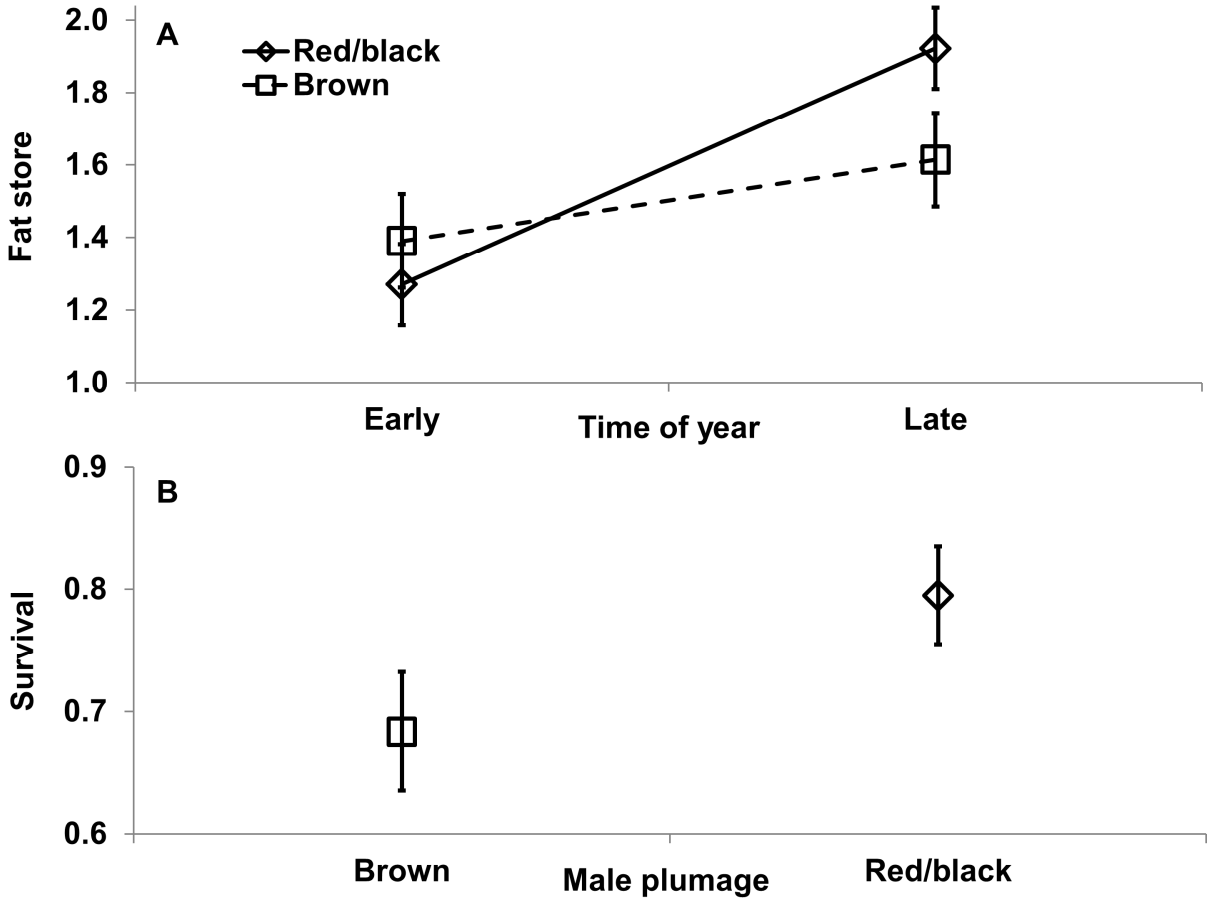

Figure 1. Differences in mean (± SE) fat stores at the beginning and end of the breeding season (A) and survival to the subsequent season (B) of females according to their male's plumage color. Fat stores were estimated from fat in the furcular hollow (scale of 0-3). Early time points were prior to the start of breeding, whereas late time points were in approximately the last month of breeding (after Dec. 1). Analyses of fat stores include 194 captures of 143 females, whereas analyses of survival are based upon the first breeding season of 196 females.

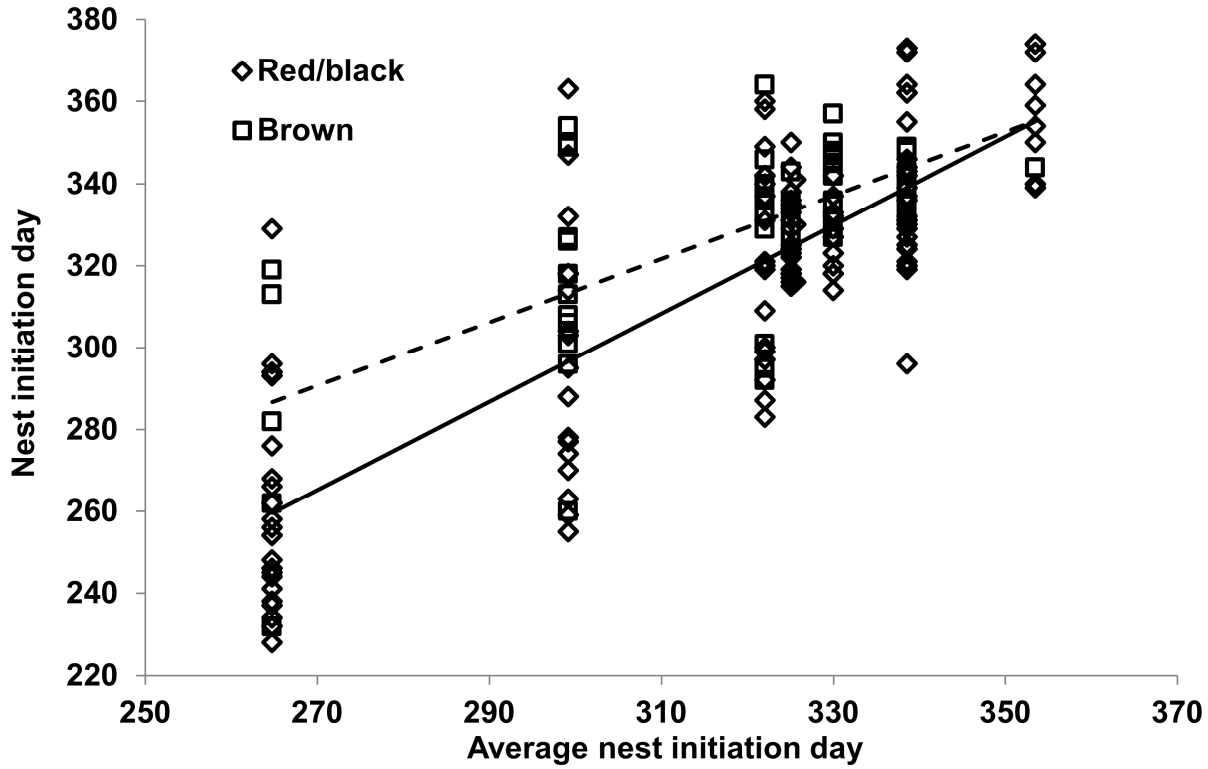

Figure 2. The relationship between a female's nest initiation date and the average nest initiation date of females at that site and year, according to whether her mate had red/black (solid line) or brown (dashed line) plumage. Note that in early breeding years females paired with red/black males initiated laying nearly a month earlier than those paired with brown males. Completion of a brood from laying to fledging requires about a month in this species, suggesting an advantage of almost one full brood (modal brood size 3) in early-breeding years. This analysis is based upon the first breeding season of 196 females.

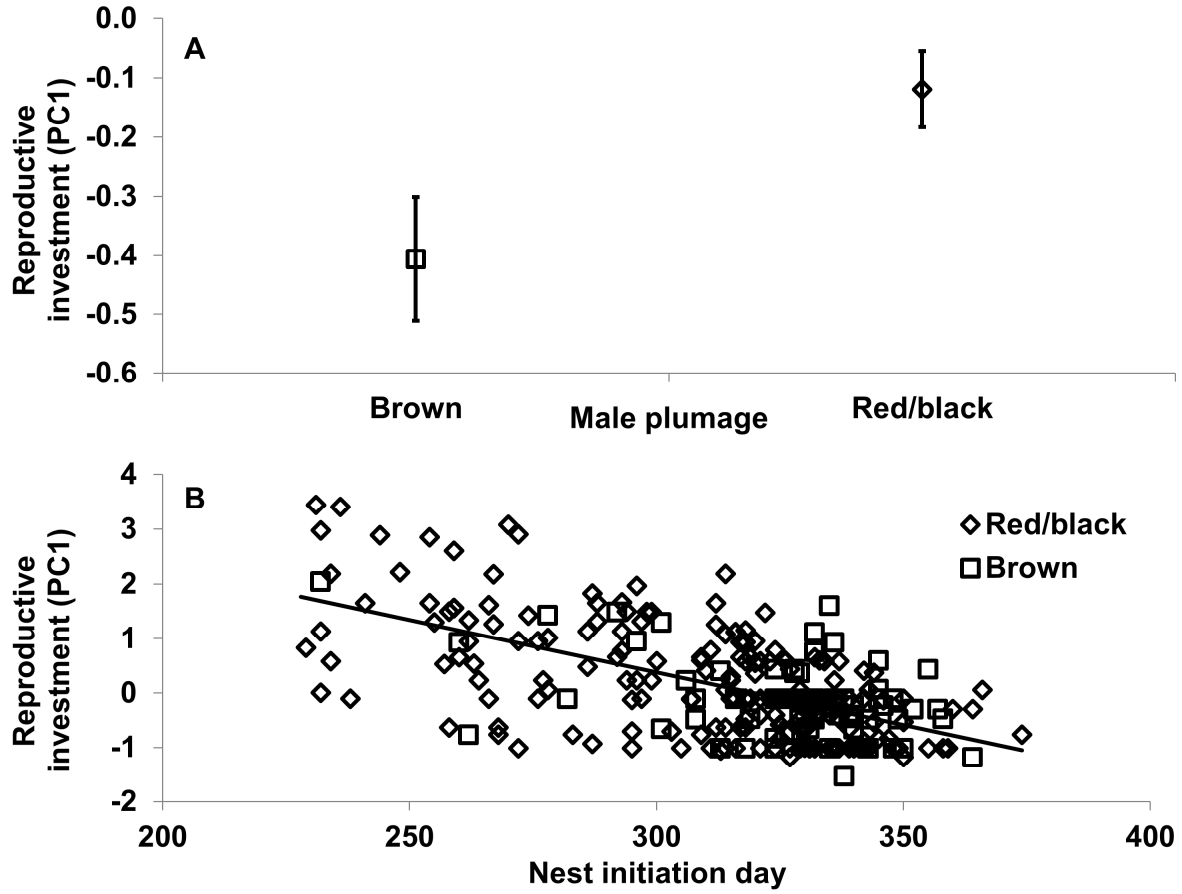

Figure 3. Female annual reproductive investment and output according to her partner's plumage (A) and the day on which she began nesting (B). This measure of reproduction is based upon the first principal component from a principal components analysis that loads positively with the number of breeding attempts, eggs, nestlings, and fledglings (Table 1). These analyses include 298 measures of annual reproduction from 206 females.

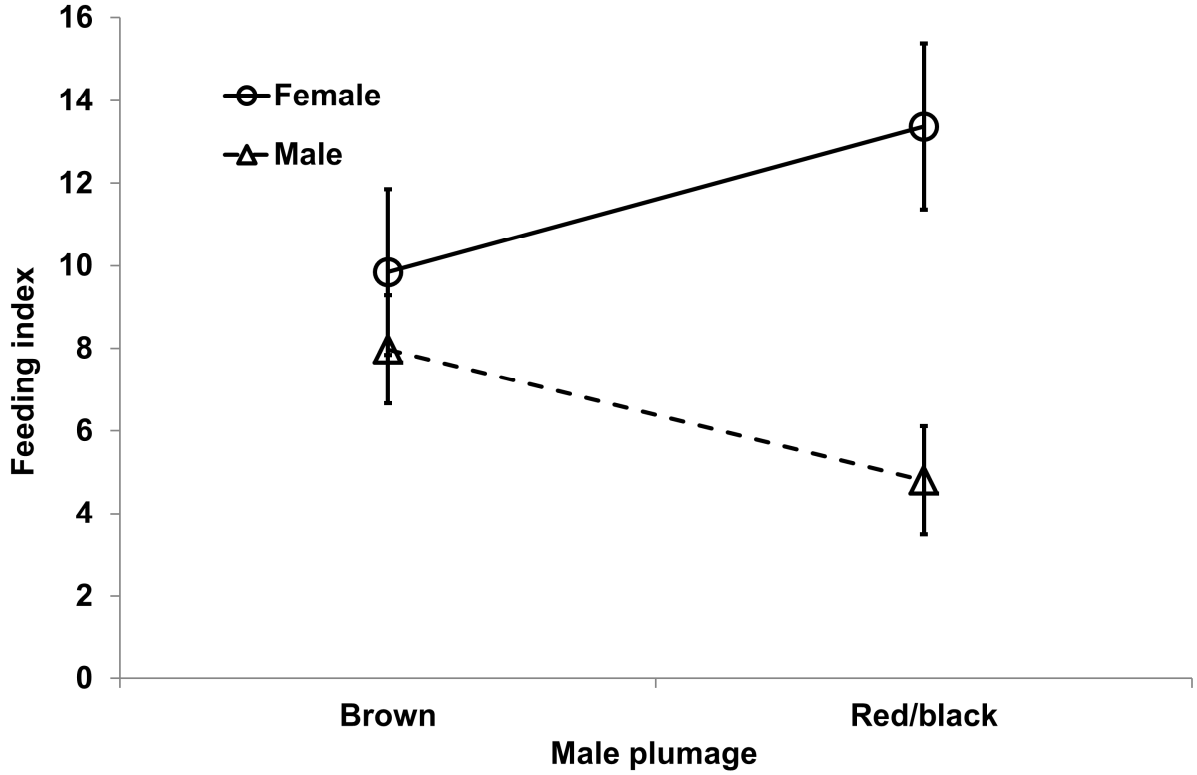

Figure 4. Nestling feeding (± SE) of males and females according to the male's plumage. Feeding index was calculated as the product of hourly feeding rate and average food size. Analyses include observations of 24 breeding pairs (16 with red/black males, 8 with brown males).

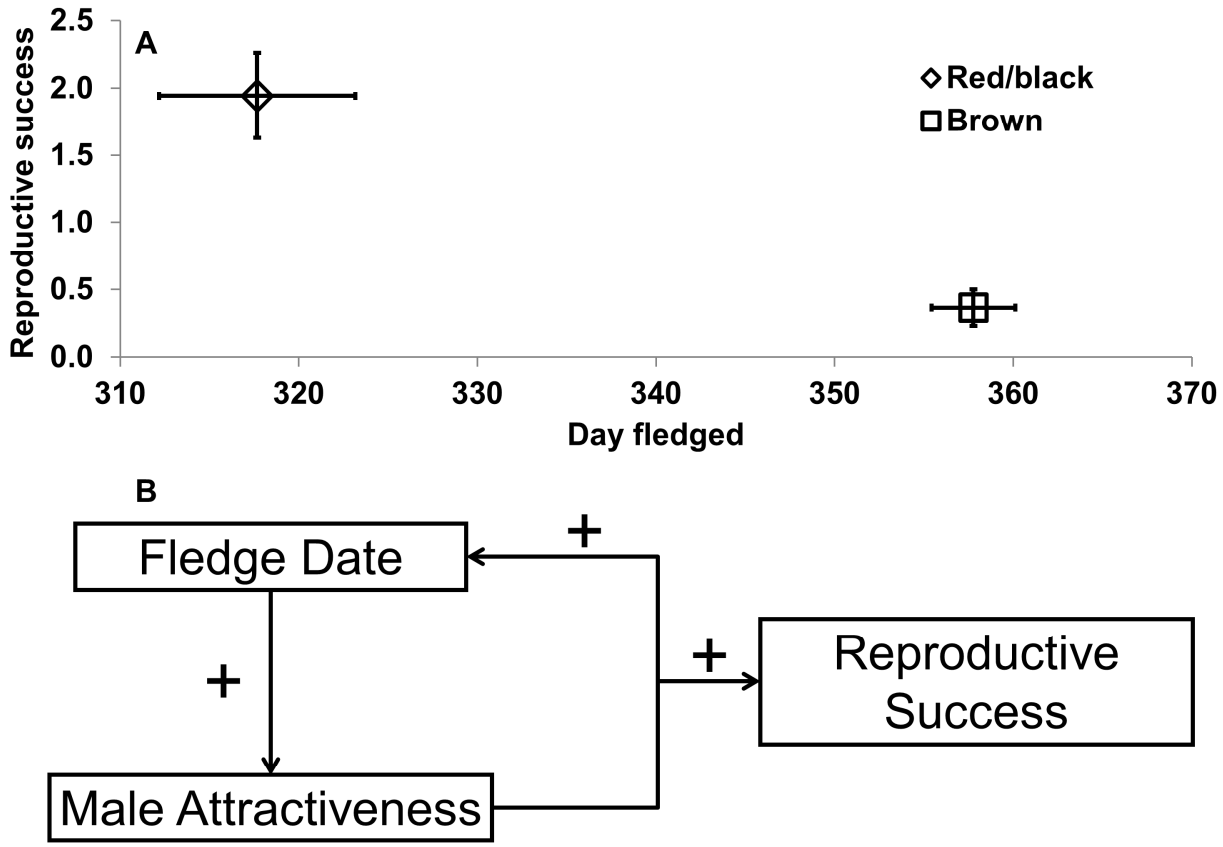

Figure 5. Male offspring that fledge earlier in the year are more likely to adopt attractive red/black plumage and to sire offspring in their first breeding season (A). Because the early breeding of attractive males simultaneously increases current reproductive success and future inclusive fitness it creates an evolutionary feedback loop that favors selection for and investment in attractive males (B). The influences of fledge day on plumage and reproductive success were analyzed using 115 and 124 males, respectively, that were monitored as both nestlings and adults.

CONCLUSION

The research presented here was conducted in collaboration with many contributors, though this dissertation and the information within it are my own. Chapter 1 is published in the journal *General and Comparative Endocrinology* (Barron et al. 2013), and Chapter 2 is currently in revision for publication in *Hormones and Behavior*. Chapter 3 is in the process of being prepared for publication. Each manuscript resulting from a chapter of this dissertation is coauthored by myself, Michael Webster, and Hubert Schwabl.

While my first two chapters began untangling the mechanisms maintaining signal honesty and integrating phenotypic traits, my proximate questions do not end with this dissertation. I am therefore currently working with additional collaborators on a number of related ventures stemming directly from data collected during my dissertation research. For example, we are examining the role of corticosterone in driving male phenotypic differences in plumage and behavior (with Willow Lindsay), and whether changes in circulating hormones could be responsible for fitness advantages of artificially reddened males (with Daniel Baldassarre). In order to better understand whether endocrine mechanisms mediate phenotypic responses to ecological conditions, we are working with an undergraduate crew leader from our field site (Jordan Boersma) to publish his independent research project on the effects of wildfire on male breeding physiology and phenotype. We also want to better understand the physiological responses of females to male plumage, considering they likely underlie the variation we observed in their reproductive effort and costs. With this aim we are investigating whether females integrate cues from male phenotype and social environment into their own endocrine regulation

(with W. Lindsay) or that of their eggs, and describing the mechanisms underlying differential effects of testosterone on the plumage of males and females (with W. Lindsay).

My final chapter has identified several benefits to females that could explain their preference for and investment in attractive red/black males. While this solves a long-standing puzzle in this system, it more importantly has broad-reaching implications in proposing unique direct benefits to females that could drive mating preferences in other systems. Just as with my proximate research above, many interesting questions remain regarding ultimate selective forces. We are presently exploring whether climatic variation leads to changes in phenotypic and reproductive skew, and are using quantitative genetic models to separate additive genetic variation from parental effects on phenotype and fitness in order to test for direct versus indirect benefits of female mate choice (with Patrick Carter, D. Baldassarre, and W. Lindsay). Considering few of the traditional explanations for cooperative breeding have been supported in this system, we are also working with another past crew leader (Ahva Potticary) to understand whether males derive benefits from retaining auxiliary helpers.

Collectively, these publications will not only further our understanding of the dynamics operating within the red-backed fairy-wren system, but will also advance evolutionary theory by clarifying the proximate and ultimate mechanisms of sexual selection.

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