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Endocrine correlates of mate choice and promiscuity in females of a socially monogamous avian mating system with alternative male reproductive phenotypes

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Abstract While our understanding of male reproductive strategies is informed by extensive investigations into endocrine mechanisms, the proximate mechanisms by which females compete for mates and adjust reproduction to social environment remains enigmatic. We set out to uncover endocrine correlates of mate choice, social environment, and reproductive investment in female red-backed fairy-wrens *Malurus melanocephalus*. In this socially monogamous, yet highly sexually promiscuous species, females experience discrete variation in the phenotype of their mates, which vary in both plumage signals and level of paternal care, and in the composition of their breeding groups, which consist of either the pair alone or with an additional cooperative auxiliary; female investment varies according to these social parameters. We found that androgen, estrogen, and glucorticoid levels varied with reproductive stage, with highest androgen and estrogen concentrations during nest construction and highest corticosterone concentrations during the pre-breeding stage. These stage-dependent patterns did not vary with male phenotype or auxiliary presence, though androgen levels during pre-breeding mate selection were lower in females obtaining red/black mates than those obtaining brown mates. We found no evidence that androgen, estrogen, or corticosterone levels during the fertile period were related to extra-pair young (EPY) frequency. This study demonstrates clear changes in steroid levels with reproductive stage, though it found little support for variation with social environment. We suggest hormonal responsiveness to social factors may be physiologically constrained in ways that are bypassed through exogenous hormone manipulations [*Current Zoology* 60 (6): 804–815, 2014].

Keywords Androgen, Estrogen, Glucocorticoid, Mate choice, Promiscuity, Alternative phenotypes

Theoretical and empirical analyses of mating systems and sexual selection have historically taken a perspective of females as the choosy and males the competitive sex. Consequently, much research has focused on variation in male traits selected by females (quality signals, ornaments) and male-male competition for mating opportunities (aggression, social dominance, armaments), which has been informed by a vast literature on underlying proximate endocrine mechanisms (i.e. Hau, 2007). In contrast, research on females has focused primarily on the direct and indirect fitness benefits females accrue from choosing (i.e. Sheldon, 2000; Maklakov et al., 2009; Forstmeier et al., 2014) and investing more in certain male phenotypes (i.e. Horvathova et al., 2012), while information remains limited regarding the proximate endocrine mechanisms that regulate female mate choice decisions, competition for mates and their resources, expression of sexual signals and behavior, and differential allocation in reproduction (i.e. Ketterson et al., 2005; Rosvall, 2013).

In complex breeding systems with bi-parental care, alternative male reproductive strategies, and/or cooperative breeding, a female faces critical decisions that influence her reproductive output and fitness. In such systems, females choose among social mates that vary in sexual and parental abilities, obtain extra-pair young from certain males, and derive benefits from helpers at the nest. Females are expected to adjust their own reproductive investment in response to these social conditions (Hatchwell, 1999; Horvathova et al., 2003, Russell

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and Lumma, 2009; Lloyd et al., 2009; Paquet et al. 2013).

The proximate endocrine mechanisms underlying variation in female mate choice, pursuance of extra-pair fertilizations, and differential allocation in reproduction have gained recent research attention. Much of this inquiry centers on the role of androgens that circulate in substantial concentrations in female vertebrates (Goymann and Wingfield, 2014) and treatment with exogenous androgens such as testosterone enhances the expression of certain male-typical traits such as aggression and courtship (e.g. song) in females (for review, see Rosvall, 2013). Whether female circulating androgens have such behavioral and morphological functions under natural conditions is, however, a contentious issue (Goymann and Wingfield, 2014; Ketterson, 2014; Groothuis et al., 2014; Buchanan and Fanson, 2014; Rosvall, 2013). Indeed, testosterone and other androgens serve different primary functions in the gonads of each sex (i.e. follicle development and proliferation in the ovary versus spermatogenesis in the testes), and their secretion profiles and dynamics differ accordingly between the sexes (Johnson, 1998; Johnson and Woods, 2007; Williams, 2012). As a consequence, coupling of gonadal state and behavior (i.e. mating, parental) by gonadal hormones likely differs between the sexes.

Our understanding of the roles of hormones other than androgens (i.e. estrogens and glucocorticoids) in regulating the breeding behavior of female birds in the wild is relatively undeveloped, as is our knowledge of how these processes are modulated by the social environment, access to resources, and energy state. For example, estrogens, one of the key hormones produced by proliferating oocytes (Johnson, 1998; Johnson and Woods, 2007) may play a role in regulating such behaviors in females, yet this possibility remains relatively unexplored. Similarly, glucocorticoids regulate energy homeostasis and adjust an animal's physiology and behavior to its environment (i.e. Goymann and Wingfield, 2004; Romero et al., 2009, McEwen and Wingfield, 2010), making them prime candidates for modulation of female reproductive behavior. The role of glucocorticoids in maintaining energy homeostasis has generated multiple hypotheses for their physiological mediation of life history tradeoffs, including reproductive investment (Wingfield et al., 1998; Wingfield and Sapolsky, 2003).

In this paper we present an analysis of the relationships among social environment, reproductive investment, and concentrations of androgens, estrogens, and glucocorticoids in female red-backed fairy-wrens (*Malurus melanocephalus*, RBFW), an Australian passerine. We ask if androgen, estrogen, and corticosterone (the major avian glucocorticoid) concentrations vary with reproductive stage, if pre-breeding hormone levels predict the phenotype of a female's social breeding partner, if female endocrine profiles during the breeding season correlate with social male phenotype, if levels of these hormones during the fertile period predict the probability of a female producing extra-pair young, and finally if hormone levels are related to the composition of the breeding unit (with and without helpers).

Red-backed fairy-wrens are facultative cooperative breeders with discrete alternative male reproductive phenotypes that differ in plumage coloration and behavior: males can breed in red-black plumage and exhibit low levels of paternal care, breed in cryptic, female-like, brown plumage with high levels of paternal care, or remain on their natal territory as brown helpers (auxiliaries) (Webster et al., 2010). Females prefer red-black males in mate choice trials (Karubian, 2002) and as extra-pair mates (Webster et al., 2008). Females adjust nestling feeding rates according to their social environment: females paired to red-black males increase their feeding efforts to compensate for low male paternal effort (Barron et al., subm.) whereas females with helpers are able to decrease their feeding efforts (Varian- Ramos et al., 2012). Thus, this species is ideal for a detailed study of the roles of various hormonal mechanisms by which females adjust their reproductive effort to match the social environment.

We use long-term data collected from a color-banded population of RBFW (see Webster et al., 2010) to address variation in female hormonal state (i) with respect to reproductive stage, ii) in response to phenotype of the social mate, iii) in relation to number of extra-pair young (EPY) in the nest, and iv) in response to presence of auxiliaries in the breeding unit. We report androgen (plasma androgens pA), estrogen (fecal estrogen metabolites fE), and glucocorticoid (fecal corticosterone metabolites fB) concentrations.

1 Materials and Methods

1.1 General field methods

We studied a wild population of red-backed fairy-wrens on the Atherton Tablelands of Far Northeast Queensland, Australia (145°25′ E, 17°23′ S) between 2003–2012. We captured adult birds in mist nets and banded birds for individual recognition with a unique combination of three colored plastic leg bands along with a numbered aluminum band (Australian Bird and

Bat Banding Scheme). We assigned age based on known history or patterns of skull ossification (aged as second year or after-second year upon first capture, Lindsay et al., 2009). From each individual, we collected a small blood sample (max 80 μl) from the jugular vein, and opportunistically collected fecal samples deposited during processing. We unambiguously determined group composition via repeated field observations and monitored breeding attempts for all identified groups, finding nearly all nests that received eggs each season. We monitored nests by visiting them every third day and banded nestlings on approximately the 6th day after hatching, at which time we collected a small (30 μL) blood sample from the tarsal vein. Blood samples were centrifuged and separated red blood cells were stored in lysis buffer until genetic analyses. Plasma samples were stored in liquid nitrogen along with fecal samples until transport to Washington State University, where they were kept at -80°C.

1.2 Radioimmunoassay (RIA)

We assayed 17–46 μl plasma samples for total androgen concentration (pA: testosterone and 5α-dihydrotestosterone) using a previously published protocol for this species (Lindsay et al., 2009). Following extraction with diethyl-ether, androgens were re-dissolved in 250 μl phosphate-buffered saline with gelatin and RIAs were conducted in 100 μl aliquots using tritium-labelled testosterone (Perkin Elmer Life Sciences NET-553) and a testosterone antibody (Wien Laboratories T-3003) that cross- reacts with closely related steroids (100% reactivity with testosterone, 60% with DHT, 5% with aldosterone, < 15% with other androgenic steroids, and < 0.5% with estradiol and all other steroids; values provided by the manufacturer). Samples were run in duplicate with recoveries for all (mean recovery 84%). The average intra-assay coefficient of variation across the six assays was 6.2% and the inter-assay variation was 5.9%. Concentrations of undetectable samples were back calculated from minimal detectable levels (1.95 pg/tube). Samples were randomly distributed throughout assays.

Steroid metabolites were extracted following Goymann et al. (2002) and Goymann (2005) with 1 ml of 75% ethanol in double-distilled water from lyophylized, pulverized and weighed (± 1 mg) feces. 500 μl of ethanol supernatant were incubated for 16–18 hr at 39°C with 200 μl of sodium acetate buffer containing β-glucuronidase/arylsulfatase to hydrolyse β-glucuronides and sulfate esters. We measured glucocorticoid metabolite and estradiol metabolite concentrations using RIA. Assays were performed according to standard techniques (Schwabl, 1993) and the following respective radio-labelled hormones and hormone antibodies: corticosterone label; PerkinElmer Life Sciences NET 399, corticosterone antibody: Esoterix Endocrinology B3-163; according to manufacturer cross-reacts to less than 5% with androgens and progestins, including progesterone; estradiol label: NET-517; estradiol antibody: Biogenesis AR1702, cross-reacts to a low percentage with estrone and estriol. We ran duplicate assay tubes for each sample containing 20 μl hydrolyzed extract and 80 μl of phosphate-buffered saline with gelatine, pH 7.1 (PBSg). We determined recovery using 5 samples for each hormone to which we added 2000 cpm tritium labelled corticosterone or estradiol and mean recoveries were 82.2% for corticosterone and 83.9% for estradiol. Glucocorticoid and estradiol metabolite concentrations are expressed as pg/mg dry feces. The average intra-assay (WACV) and inter-assay (BACV) coefficients of variation for 6 corticosteroid assays were WACV = 12.1%, BACV = 22.0% and 6 estradiol assays were WACV = 13.6%, BACV = 24.1% (calculated according to Chard 1995). The few undetectable samples (fE n = 8 of 160 samples; fB n = 5 of 174 samples) were calculated from minimal detectable levels (fE 1.95 pg/tube; fB 3.91 pg/tube).

1.3 Fecal hormone metabolite validation

The use of fecal radioimmunoassay for androgens (fA) has been validated for male red-backed fairy-wrens (Karubian et al., 2011) and we expand this validation here to include use of feces for detection of estradiol and corticosterone metabolites in females. We demonstrate dilutional parallelism for estradiol (fE) but not corticosterone (fB) metabolites. We serially diluted pooled high concentration fecal estradiol and corticosterone metabolite extract and assessed if they would perform similar to a standard dilution of stock hormone. We then compared the linear portions of these dilution curves. We found that the linear portions of our pooled sample dilutions (plotted using log transformed values of hormone metabolite concentration) were similar in slope to the slopes of the diluted estradiol but not corticosterone standards (Fig. 1A, B; ANOVA of interaction between sample type (fecal pool or hormone standard) and log concentration (pg/tube); estradiol $F_{1,11}$ = 0.63, P = 0.450; corticosterone $F_{1,11}$ = 8.73, P = 0.018). Across all fecal hormone metabolite assays included in this analysis, the majority of samples fell on the linear portion of the standard curve (estradiol 83.4%; glucocorticoids 64.7%). We conclude that our RIA is specific for

the metabolites of estradiol, and while female fB did not behave similar to stock hormone under serial dilution (Fig. 1B), we provide biological evidence to indicate that excreted corticosterone metabolites in female red-backed fairy-wrens are similar in pattern across phases of the breeding cycle to patterns detected for circulating corticosterone in females of other studied avian species (Pereyra and Wingfield, 2003; Love et al., 2004; Horton and Holberton, 2010). Additionally, our patterns of fE excretion are highly congruent with known plasma estradiol patterns across breeding stage in other studied species (Schwabl and Sonnenschein, 1992; Horton et al., 2014).

1.4 Genetic parentage analyses

We genotyped adults and nestlings at seven microsatellite loci following published protocols (Baldassarre and Webster, 2013). The microsatellite loci were highly polymorphic when combined, and were informative for paternity analysis (mean number of alleles per locus = 11.14, mean expected heterozygosity = 0.75, combined exclusion probability > 0.99). Because previous studies have found no brood parasitism in this species (Webster et al., 2008; Baldassarre and Webster, 2013), we assumed the breeding female observed at a nest was the genetic mother of all offspring in that nest. We assigned paternity using the program CERVUS v. 3.0 (Kalinowski et al., 2007). Rates of extra-pair paternity were high in our populations with 61% of all offspring being sired by extra-pair males, though much variation existed across years and sites (range = 35%–90%).

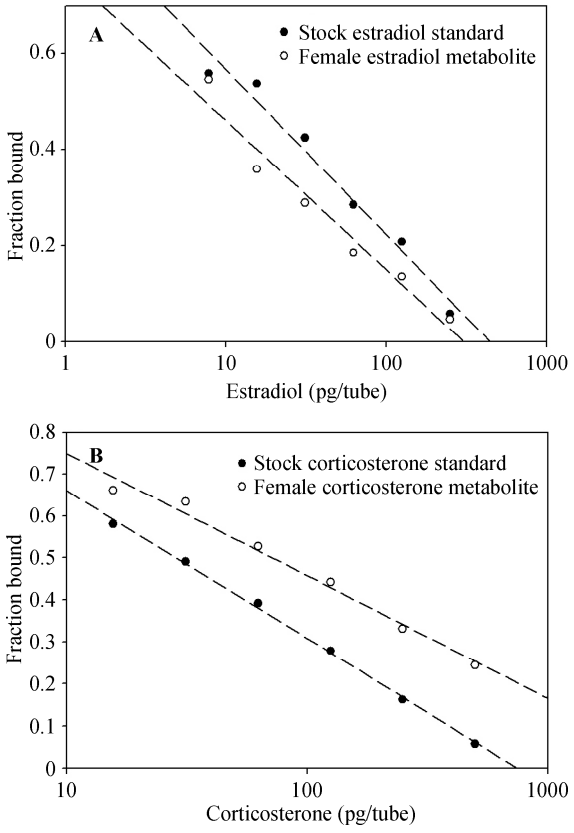

Fig. 1 Linear portions of displacement curves for (A) pool dilutions of fecal estradiol (fE) and (B) corticosterone (fB) metabolites

The dotted regression lines are for serially diluted standard (stock) hormone and the solid lines indicate serially diluted high fE and fB concentration pooled female fecal sample extract.

1.5 Statistical analyses

All statistical analyses were conducted using JMP 9.0.1 (SAS Institute Inc. Cary, NC, U.SA). We constructed separate general linear models (GLMs) for each hormone measurement: plasma androgens (pA), fecal estradiol (fE), and fecal corticosterone (fB). Hormone concentrations were log-transformed for normality.

We conducted analyses on three subsets of data to determine effects of predictive social and reproductive factors on female hormone concentrations: 1) all plasma and fecal samples in a breeding-stage "cross-seasonal" analysis, 2) only those samples collected during the "pre-breeding" phase when pair formation occurs, and 3) samples collected during the "fertile" periods of nest construction and egg-laying.

In our breeding stage "cross-seasonal" models we included factors of breeding stage, group male plumage ("male phenotype": red/black or brown), and presence or absence of cooperative auxiliaries ("auxiliaries": yes or no). We separated breeding stage into six discrete categories that are characterized by unique physiological, behavioural, and social processes. 1) *Pre-breeding:* occurs prior to onset of ovarian follicle growth and is characterized by breakup of non-breeding flocks, social mate assessment / selection, pair formation, and territory establishment. As such, this is a period of social instability and competition among females for social mates. This stage can last over an extended period of time depending on the onset of the monsoon rains that trigger reproduction. 2) *Nest construction:* this is a phase of ovarian follicle growth, sexual behavior (courtship, copulation), territorial defense, female receptivity, and within- and extra-pair mating. 3) *Laying:* this phase is similar to nest construction with the addition of egg laying. 4) *Incubation:* in incubation there is no ovarian follicle proliferation and low sexual activity. 5) *Nestling feeding*: characterized by lack of follicle proliferation and low sexual activity. 6) *Fledgling rearing*: characterized by ovarian recrudescence (follicle proliferation for subsequent clutch), and either resumption of sexual activity for a subsequent clutch or termination of re-

productive activity towards the end of the breeding season.

In our analysis of the pre-breeding phase, we examined whether female hormone titer was related to the phenotype of her breeding partner and/or the presence or absence of auxiliaries. For samples collected during the fertile phase, we assessed the relationship between hormones and female extra-pair mating behavior (the ratio of EPY resulting from extra-pair matings), male phenotype, presence / absence of auxiliaries, and day in the breeding cycle ("day in cycle"). We defined rates of extra-pair fertilizations categorically as nests containing none (0%, n = 15) or some (1%–100%, n = 14) EPY as the percentage of EPY in a given nest is bimodally distributed across the population. Clutch size in this subset of nests for which we have EPY data ranged from 2–4 eggs (mean = 3 eggs ± 0.05 (*SE*)). Day in cycle was calculated as the number of days a sample was collected either before or after laying of the first egg and we restricted our analysis to include only samples collected less than twenty days prior to lay of first egg (lay date = day 0; range -12 to +3 days, mean = -4.19 ± 0.5 days (*SE*)). While we do not know the exact time of fertility and duration of sperm storage in the RBFW this time window likely covers the period of follicular proliferation and egg formation and thus also the phase in which females are fertile.

For all statistical analyses we included potential covariates and cofactors including year (2003–2012), the day of the year on which the sample was taken (Aug 22–Feb 10), female age (age 1, 2, 3, and 4+ years), and delay from capture to completion of blood sampling (range 3–105 min., mean = 18.97 ± 1.42 min. (*SE*)). We used backwards elimination to remove non-significant factors, interactions, and covariates; final models contain only those terms and interactions with $P < 0.10$. Delay between capture and completion of blood sampling did not significantly covary with hormone titre and was removed from all final models. For the purposes of evaluation, we present exclusion p-values and F-statistics for eliminated factors in Table 1, but unless significant do not report p-values for covariates eliminated from each final model. We used Bonferonni correction to adjust for multiple comparisons for those terms and interactions analysed in multiple overlapping datasets (eg. male phenotype, auxiliary, female age, year, day of year) considering significance at $\alpha<0.025$ (datasets utilized in pre-breeding and fertile periods do not overlap with one another but both overlap with the cross-seasonal analysis).

1.6 Ethics Statement

All captured birds were treated in a safe and humane manner. Procedures were approved by the Institutional Animal Care and Use Committee of Washington State University, the James Cook University Animal Ethics Review Committee and the Queensland Government Environmental Protection Agency. Export of samples from Australia was approved by the Australian Government Department of Environment and Heritage.

2 Results

2.1 Cross-seasonal breeding stage patterns

Breeding stage was significantly correlated with plasma hormone and fecal hormone metabolite concentrations (Fig. 2). Female pA varied with stage such that fertile females in the nest construction phase of the breeding cycle had significantly higher concentrations than pre-breeding females; pA during remaining nesting stages did not differ significantly from either pre-breeding or nest-construction concentrations (Fig. 2A: Tukey adjustment for multiple comparisons, $\alpha = 0.05$). Female fE was significantly higher during nest construction than during the parental phases of egg incubation and nestling rearing (Fig. 2B, Tukey adjustment for multiple comparisons, $\alpha = 0.05$). fE concentrations were intermediate during egg laying, low during nestling feeding and pre-breeding, and intermediate during fledgling rearing when females are ramping up to initiate or engage in a subsequent breeding bout.

Female fB varied with breeding stage such that fB was significantly elevated prior to breeding (pre-breeding phase) relative to the remaining nesting phases, intermediate during the fledgling rearing phase, and uniformly low during the fertile and parental phases (Fig. 2C, Tukey adjustment for multiple comparisons, $\alpha = 0.05$).

Neither social mate phenotype nor presence / absence of auxiliaries were related to pA, fE, or fB across all phases of the nesting cycle (Fig. 2, 3). However, we show a significant effect of the interaction between breeding stage and presence / absence of auxiliaries on female fB (figure 3c), with females from groups containing auxiliaries showing high fB titers during the fledgling stage compared to those from groups lacking auxiliaries.

2.2 Hormonal correlates during the pre-breeding phase

During the pre-breeding phase of the nesting cycle, females that eventually paired with brown males had significantly higher pA titers than those that paired with

Table 1 Results from GLMs of predictive factors influencing female plasma androgen (pA) and fecal estradiol (fE) and corticosterone (fB) metabolite concentrations analyzed across all sampling periods (cross-seasonal), during the pre-breeding phase, and during the fertile period

Analysis	Factor	Plasma androgens (pA)			Fecal estradiol (fE)			Fecal corticosterone (fB)		
		F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Cross-seasonal	Model	10.6	16,154	**0.000**	12.5	6,144	**0.000**	4.42	11,127	**0.000**
	Breeding stage	3.26	5, 154	**0.008**	14.3	5,144	**0.000**	3.95	5,127	**0.002**
	Male phenotype	0.39	1, 154	0.536	0.41	1,122	0.522	1.79	1,117	0.184
	Auxiliaries	0.00	1,153	0.061	1.31	1,131	0.254	1.43	1,127	0.234
	Interactions / cofactors	*Year*			*Day of Year*			*Stage X auxiliaries*		
		18.3	5, 154	**0.000**	3.09	1,144	0.081	2.99	5,127	**0.014**
		Stage X male type								
		2.06	5, 154	0.074						
Pre-breed	Model	4.99	9,47	**0.000**	7.37	2,26	**0.003**			
	Male phenotype	7.82	1,47	**0.008**	0.11	1,14	0.751	0.00	1,15	0.979
	Auxiliaries	0.65	1,46	0.424	1.45	1,14	0.248	0.00	1,15	0.927
	Interactions / cofactors	*Age*			*Year*					
		2.59	3,47	0.064	7.37	2,26	**0.003**			
		Year								
		7.48	3,47	**0.000**						
		Day of year								
		4.92	1,47	0.032						
		Day of year X male type								
		3.89	1,47	0.054						
Fertile	Model	8.45	5,47	**0.000**	4.58	4,44	**0.004**			
	Day in cycle	3.37	1,47	0.073	11.5	1,44	**0.002**	0.52	1,47	0.472
	Male phenotype	0.09	1,43	0.772	0.00	1,39	0.958	2.54	1,54	0.116
	Auxiliaries	0.68	1,46	0.415	2.49	1,43	0.122	2.04	1,58	0.159
	Extra pair mating	0.04	1,18	0.849	0.44	1,18	0.514	1.37	1,22	0.254
	Interactions / cofactors	*Year*			*Year*					
		9.96	4,47	**0.000**	2.37	3,44	0.084			

For each analysis we present exclusion p-values and F-statistics for eliminated factors but unless significant, do not report p-values for covariates eliminated from each final model.

red/black males (Table 1; Fig. 2A). As 60% of females aged 2 and older enter the breeding season already paired (Barron, unpublished data) whereas one-year old females must actively seek mates, it is possible that the hormonal dynamics related to social mate phenotype vary with female age. While we show a positive trend for increasing pA with female age during the pre-breeding phase (table 1), there was no effect of the interaction between female age and male phenotype on pA ($F_{1,46}$ = 0.68, P = 0.413) indicating that pre-breeding females of all ages respond similarly to social mate phenotype. There was, however, a trend for an effect of the interaction between male mate phenotype and capture date (day of year) on pre-breeding female pA levels (Table 1) such that differences in pA associated with social mate phenotype were restricted to the earliest portion of the population-wide pre-breeding phase. Females pairing with brown males showed high pA early in the season with declining concentrations as the breeding season approached ($F_{1,21}$ = 6.01, P = 0.0230) while pA of females pairing with red/black males remained steady throughout the entire pre-breeding phase ($F_{1,23}$ = 1.80, P = 0.187).

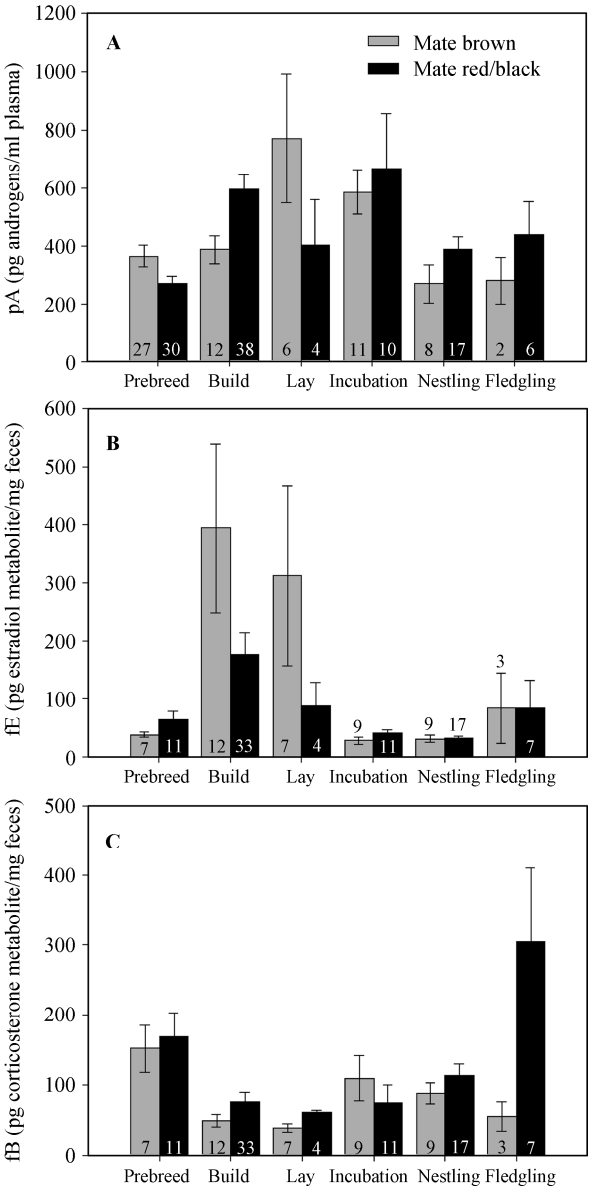

Fig. 2 Mean ± *SE* (A) plasma androgen (pA), (B) fecal estradiol (fE), and (C) corticosterone (fB) metabolite concentrations of females paired to red/black (black bars) and brown males (grey bars) across all stages of the nesting cycle

Sample sizes are indicated for each category.

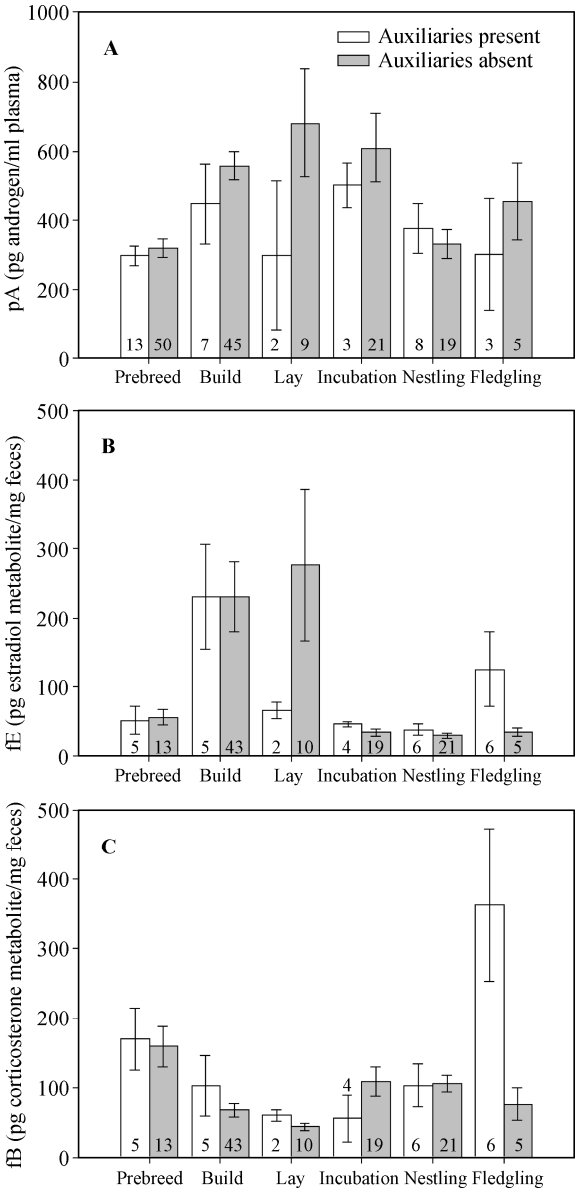

Fig. 3 Mean ± *SE* (A) plasma androgen (pA), (B) fecal estradiol (fE), and (C) corticosterone (fB) metabolite concentrations of females from groups with auxiliaries (white bars) and groups lacking auxiliaries (grey bars) across all stages of the nesting cycle

Sample sizes are indicated for each category.

Neither pre-breeding fE nor fB titers were related to male phenotype (Fig. 2B, C), and none of the hormonal measures were related to presence or absence of auxiliaries in the breeding group (Table 1; Fig. 3). Additionally, pre-breeding fB, pA, and fE were all unrelated to body condition (measured as the residuals of a regression of body mass on tarsus length: pA: $F_{1,43} = 0.66$, $P = 0.421$; fE: $F_{1,24} = 0.31$, $P = 0.584$; fB: $F_{1,9} = 0.01$, $P = 0.911$).

2.3 Hormonal correlates during the fertile phase

Day in the breeding cycle relative to egg lay date significantly predicted fE but was not related to either pA, or fB (table 1). fE increased as females approached egg lay date. Neither male phenotype nor presence / absence of auxiliaries were associated with pA, fE or fB. Likewise, none of the hormone measurements were related to female propensity to engage in extra-pair mating behavior.

3 Discussion

We show that 1) levels of both ovarian steroids, an-

drogens (pA) and estrogens (fE), varied significantly with reproductive stage and fE was highest near the time of egg laying; 2) neither plumage/behavioral phenotype of the social pair mate (red/black versus brown) nor presence/absence of auxiliaries were related to these stage-dependent pA and fE profiles; 3) pA titers during the pre-breeding phase were higher in females pairing to brown than red-black males; 4) EPY rate in a brood (absent versus present) was not related to pA, fE or fB levels during the fertile period; and 5) levels of fB also varied with reproductive stage but were unrelated to mate phenotype and presence/absence of helpers. Year of sampling had a significant effect on pA in all analyses and on fE during the pre-breeding stage but not on fB. The effect of year cannot be explained by sampling bias in breeding stage and instead may be related to some unmeasured social or environmental factor (i. e., temperature or rainfall).

Across stage, the pA and fE profiles in female RBFW are similar to androgen and estrogen profiles seen in females of other avian taxa (e. g. plasma T and E: Schwabl and Sonnenschein, 1992; Horton et al., 2014; plasma T: Geslin et al., 2004; Schwabl et al., 2005; fecal T: Schwabl, 1996; fecal E: Sockman and Schwabl, 1999). Elevated fE levels were tightly coupled to the stages of egg formation and laying, whereas pA levels were elevated both here as well as during other stages of the breeding cycle (i.e. incubation). pA levels peaked during the sexual stages (nest-building and laying) and declined to lower levels during the parental stages (nestling feeding and fledgling care).

Classic neuroendocrine studies have demonstrated mutual interactions of endocrine state and behavior between males and females in a mated pair to coordinate reproduction. Male courtship behavior (i.e. song) stimulates female reproductive development (e. g., Lehrman, 1961; Cheng, 1973, 1974; Hinde and Steel, 1976; Kroodsma, 1976) and is reflected in rapid increases of estrogen levels (e.g.. Tschernichowski et al., 1998). Similarly, female T production is also sensitive to social environment, in particular male courtship song (e.g. Marshall et al., 2005; Tanvez et al., 2004), male plumage characteristics (e. g. Kingma et al., 2009; Remes, 2011), and female-female competition (e. g. Schwabl, 1996; Langmore et al., 2002; Pilz et al., 2004; Whittingham and Schwabl, 2001; Mazuc et al., 2003). However, in this study the differences in sexual, territorial, and paternal behavior (Karubian, 2002; Barron et al., in prep.) and androgen levels (Lindsay et al., 2009) between the red-black and brown male phenotype of the RBFW were not reflected in differences of androgen and estrogen levels of their female partners during the sexual or parental phases of the breeding cycles. This result, however, may not be surprising in the light of the primary physiological roles of these ovarian steroids in egg and clutch formation (Johnson, 1998; Williams, 2012). In sum, our study provides little evidence for a relationship of social mate phenotype and the levels of the key ovarian hormones, E and T, during the sexual and parental phases. Rather, female endocrine state was associated with mate type during the pre-breeding phase, prior to initiation of reproduction.

We specifically inspected the pre-breeding phase for a relationship between androgen levels and male phenotype because this is a time when non-breeding flocks dissociate into social breeding units and young females entering their first breeding season seek mates. Although little is known about the interactions between the sexes during this time, we expect strong competition among females for access to the desired red-black male phenotype. Female RBFW gain significant fitness benefits from pairing with red/black males as they begin breeding much earlier, produce more young during a breeding season, end the season in better condition, and are more likely to survive to the next season (Barron et al., in prep.).

Research across taxa on endogenous and exogenous T effects support a role for T in regulation of female aggression and female/female competition over reproductive resources and mates (e. g., Cristol and Johnson, 1994; Sandell, 2007; Langmore et al., 2002; Rosvall, 2013 for review of manipulation). We therefore predicted elevated pA levels during pre-breeding, expecting females that obtained a red-black male as their pair partner to have higher pA levels than those that subsequently were paired with a brown male. Surprisingly, the opposite was supported; females paired to brown males had higher pA levels than those paired to red-black males. This pattern could not be explained by female age, a tempting prediction given that young females, entering their first breeding season, must compete for their first social mate, whereas many older females are already paired to desirable red-black males. An alternative explanation for our counterintuitive findings may be that those females that pair with red-black males do so earlier in the season, leaving the remaining unpaired females competing for a limited number of potential mates and ultimately settling for the less desirable male phenotype. The difference in pA levels between females paired to brown versus red-black males

decreased as the breeding season approached, suggesting that the very early pre-breeding phase might be a crucial time for female-female competition. Questions concerning the nature of male/female interactions and female/female competition during the pre-breeding phase require further investigation.

As expected, fE during pre-breeding was very low and did not differ between females based on partner type. Pair formation occurs well before the onset of reproduction, which can be delayed by several months, depending on the onset of favorable monsoon rains (Webster et al., 2010). Therefore, hormones other than gonadal steroids (T and E) such as adrenocortical steroids (i. e., dehydroepiandrosterone (DHEA)) might regulate female competition for mates and pair formation as has been proposed for males and females of taxa that express competition outside the reproductive period (e. g., Logan and Wingfield, 1990; Kriner and Schwabl, 1991; Soma and Wingfield, 2001; Gill et al., 2007; Schlinger et al., 2008). In sum, the role of hormones in RBFW female competition needs further study, paying attention to social and ecological context as well as reproductive stage.

We found no evidence that levels of pA, fE, or fB of fertile females are related to the number of EPY produced in the associated breeding attempt. If variation in endogenous T and E levels is unrelated to EPY number, manipulative studies showing mixed effects of exogenous hormones on female extra-pair copulation behavior may need to be re-evaluated for their validity in a natural context. For example, T treatment had no effect on EPY rate in the dark-eyed junco *Junco hyemalis* (Gerlach and Ketterson, 2013) but in both spotless starlings *Sturnus unicolor* and blue tits *Cyanistes caeruleus*, T implantation reduced EPY rates with the long lasting effects on female sexual behavior differing between the latter two systems (Garcia-Vigon et al., 2008; de Jong, 2013). The carry-over effects of T treatment on rates of EPY in subsequent breeding seasons reported for spotless starlings (Garcia-Vigon et al., 2008) casts doubt on how far these results can be applied to natural variation in endogenous T expressed during the fertile phase. While exogenous estrogens can enhance female sexual receptivity (Searcy, 1992), estrogens may be permissive for mating but not involved in seeking extra-pair copulations. Given such mixed evidence from experimental manipulations and the absence of a correlation of endogenous T and E levels with EPY rates in a species such as the RBFW, noted for substantial and variable EPY rates (Karubian, 2002; Webster et al., 2008), it seems unlikely that these hormones play a significant role in promiscuity and mate choice.

In our study, glucocorticoid (fB) concentrations varied with reproductive stage, with levels decreasing from pre-breeding to nest construction and egg laying, and slightly increasing during the parental stages. This pattern is consistent with patterns of plasma corticosterone reported in other species (e. g., Pereyra and Wingfield, 2003; Horton and Holberton, 2010; Love et al., 2004), In contrast to predictions for an association between levels of B and maternal care effort (e. g., Miller et al., 2009; Davis and Guinan, 2014) fB levels did not differ between females paired to red-black and brown males, despite females adjusting for the low feeding effort of red-black males (Karubian, 2002; Barron et al., in prep.). Thus, differential maternal care costs of mate choice in terms of variation in B are not detected in RBFW. Food availability during the nestling phase correlates with baseline B levels in other studied systems (e. g., Kitayski et al., 1999; Jenni-Eiermann et al., 2008), and it is possible that red/black males provide females with higher quality territories that offer abundant food resources for rearing young, thus explaining why these females do not show elevated B compared to females paired to brown males.

Presence of auxiliaries had no significant effect on stage-related changes of pA and fE levels, but did relate to fB titres during the fledgling stage. Females engaged in fledgling rearing from social groups containing auxiliary helpers had higher fB levels than those without. Thus, our results provide no evidence in support of a reduction in costs associated with maternal care in the presence of auxiliaries (see also Varian-Ramos et al., 2012). The higher female fB levels in groups with helpers might be related to limited food availability associated with a large group size. These possibilities require further behavioral investigation.

In conclusion, our study shows clear patterns of gonadal steroid and glucocorticoid hormone variation with reproductive stage but relatively little association with male phenotype or auxiliaries. We also found no evidence for a relationship between androgens, estrogens, or corticosterone and promiscuity. We did, however, detect unexpected differences in androgen levels during the pre-breeding period with partner phenotype which warrant further investigation. Overall, the patterns of estrogens and to lesser extent androgens, may be constrained by their primary functions in reproduction, allowing little scope for modification by social factors. Thus it is possible that endocrine systems other

than those that regulate basic ovarian functions like egg production such as nonapeptides (Goodson et al., 2012) and neurosteroids (Soma, 2006) modify female reproductive behavior in response to social environment. Other explanations for the lack of a relationship between circulating androgen and estrogen levels and behavior may come from the dynamic nature of hormone secretion, behavioral variation and decisions based on mechanisms functioning at hormonal targets in the brain, as well as the possibility that these relationships function via a stepwise threshold rather than in a linear dose response fashion (Ball and Balthazart, 2008).

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