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Plumage coloration, ejaculate quality and reproductive phenotype in the red-backed fairy-wren

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Understanding how pre- and postcopulatory sexually selected traits covary can provide insight into the evolution of male ornamentation and female mate choice. In this study, we examined ejaculate quality and investment in testicular tissue in relation to plumage colour and social status in the genetically promiscuous red-backed fairy-wren, *Malurus melanocephalus*. In this species, males exhibit one of three alternative reproductive phenotypes during the breeding season: males can breed in red and black plumage, breed in brown plumage, or act as brown-plumed, nonbreeding auxiliaries. We found that red/black breeders invested more heavily in spermatogenic tissue, had larger sperm reserves, and tended to have greater numbers of sperm in ejaculate samples, when compared to brown breeders and auxiliaries. Within red/black breeders, plumage redness and saturation (i.e. long wavelength hue and increased red chroma) were negatively correlated with ejaculate sample sperm density. In addition, ejaculate motility appeared to be related to variation in plumage coloration such that, overall, males with less elaborate ornamentation showed greater ejaculate quality. These results suggest that pre- and postcopulatory traits negatively covary in red/black plumed red-backed fairy-wrens and indicate a possible trade-off between investment in plumage ornaments and investment in ejaculates.

In many animals, males possess elaborate traits, such as weapons and ornaments, that are considered to be a consequence of sexual selection (Andersson 1994). When females mate with multiple males, sexual selection can continue beyond copulation in the form of sperm competition (Parker 1970). Consequently, in genetically promiscuous species, paternity success is affected by traits that influence precopulatory mate acquisition and post-copulatory fertilization success. When both pre- and post-copulatory mechanisms of sexual selection contribute to variance in paternity success, these mechanisms have the potential to amplify or counteract one other (Møller 1998; Danielsson 2001; Evans et al. 2003; Andersson & Simmons 2006).

The conspicuously coloured plumage of male birds has become a model system for understanding the mechanisms and function of male sexual ornamentation (Hill & McGraw 2006a, b), and in many species, females are known to select the most elaborately plumaged males as reproductive partners (Andersson 1994; Hill 2006). Under conditions of sperm competition, ejaculate quality (i.e. sperm number and quality) determines fertilization success in birds (Birkhead 1998; Birkhead & Pizzari 2002). Sperm numbers are influenced by testis size (larger testes produce more sperm; Amann 1970; de Reviers & Williams 1984), and relative testis size provides a measure of relative investment in testicular tissue and sperm production (Gage et al. 1995). Plumage coloration in birds may therefore represent a typical precopulatory sexually selected trait, while ejaculate quality represents a suite of postcopulatory sexually selected traits.

The red-backed fairy-wren, *Malurus melanocephalus*, is a cooperatively breeding Australian passerine in which males show alternative reproductive phenotypes that appear to be determined by differences in androgen production (Karubian et al. 2008; Webster et al. 2008; Lindsay et al. 2009): 'red/black breeders' are paired males that breed in red and black plumage, 'brown breeders' are also paired males but breed in brown, female-like plumage, and

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'auxiliaries' are unpaired males that have brown, female-like plumage and remain as helpers on their natal territory. The red back feathers of red/black breeding males are carotenoid based (Rowe & McGraw 2008) and are incorporated into courtship displays (Rowley & Russell 1997). All three phenotypes are capable of siring offspring, but red/black breeders are socially dominant, preferred by females, appear to invest more in behavioural strategies related to mating effort, and achieve greater reproductive success compared with brown breeders and auxiliaries (Karubian 2002; Karubian et al. 2008; Webster et al. 2008). In contrast, brown breeders appear to invest more in parental effort (Karubian 2002). Red-backed fairy-wrens are socially monogamous, however, extrapair paternity is common in this species and accounts for approximately 50% of all offspring (Karubian 2002; Webster et al. 2008). Consequently, both plumage coloration and ejaculate quality are likely to contribute to variance in paternity success among male red-backed fairy-wrens.

In this study, we examined the relationship between ejaculate quality and plumage coloration in male red-backed fairy-wrens at two levels. Because plumage coloration and reproductive behaviour appear to be androgen dependent in this species (Lindsay et al. 2009), and because androgens are essential for testis maturation and sperm production (Froman 1995), and have been linked to higher ejaculate quality (Folstad & Skarstein 1997; Hillgarth et al. 1997; Liljedal et al. 1999), we first predicted that relative testis size and ejaculate quality would vary in association with reproductive phenotype. Specifically, we predicted that these traits would follow the pattern of variation observed for plasma androgen levels (Lindsay et al. 2009), being largest in red/black breeders, smallest in auxiliaries, and intermediate in brown breeders. We then investigated the relationship between ejaculate quality and the spectral properties of red feathers among red/black breeders. In this instance, predicting the directionality of the associations was difficult because two distinct hypotheses suggest different patterns of association: the phenotype-linked fertility hypothesis suggests that male fertility and ornament elaboration positively covary (Sheldon 1994), whereas sperm competition theory predicts a negative association between investment in the ejaculate and investment in other reproductive traits, such as those that influence mate acquisition (Parker 1998). Furthermore, empirical studies examining the relationship between male phenotype and ejaculate quality have reported mixed results (e.g. Matthews et al. 1997; Liljedal et al. 1999; Peters et al. 2004; Malo et al. 2005; Simmons & Emlen 2006; Pitcher et al. 2007).

METHODS

Study Species and General Field Methods

The red-backed fairy-wren is found throughout northern and eastern Australia (Rowley & Russell 1997). We studied two populations near Herberton, Queensland, Australia (145°25′E, 17°22′S) for 1 month during the peak of the breeding season in 2005 and again in 2006. These populations were colour banded and monitored to determine social groupings, individual social status and breeding activity (for details see Webster et al. 2008). Males were classified according to reproductive phenotype based on social status and plumage scores following Karubian (2002; see also Webster et al. 2008; Lindsay et al. 2009).

Upon capture, we measured body mass, tarsus and wing lengths, and the length (L), width (W) and height (H) of the cloacal protuberance (CP). All CP measurements were taken by one of us (M.R.) to minimize sampling error. We estimated the volume of the CP as, volume $= \pi\,(H/2 \times W/2) \times L$ (Tuttle et al. 1996). The CP is the site of sperm storage in male passerines (Wolfson 1952; Birkhead et al. 1993), and CP size reflects both total sperm reserves (Birkhead et al. 1993) and the number of sperm in ejaculate samples collected via cloacal massage (Tuttle et al. 1996). Finally, we defined male condition as the residuals of a linear regression of body mass on tarsus length. Although the use of residuals as a measure of avian body condition has been questioned (Green 2001), mass/tarsus residuals have been shown to accurately reflect the size of fat energy stores in the red-backed fairy-wren (Lindsay et al. 2009).

Plumage Coloration in Red/Black Breeders

In addition to distinguishing between brown and red/black breeders, we used spectrographic analysis to quantify the coloration of red/black breeding males. At the time of capture, we removed six to eight red feathers from the back of each red/black breeder and stored these feathers in dry, sealed plastic bags. Feathers from individual males were mounted in an overlapping pattern on standard black cardboard. We measured reflectance using an Ocean Optics USB2000 UV–VIS spectrometer with a R200-7 UV–VIS probe and PX2 pulsed xenon light source emitting a continuous strobe light. The probe was mounted in a metal block that excluded all ambient light from a standardized measurement area (approximately 3 mm^2) and maintained the probe perpendicular to the feather surface. We obtained three readings from each feather sample and averaged the three spectra to generate one reflectance spectrum for each male's red back feathers. Every 10 samples, we recalibrated the spectrometer against a dark and a white (Ocean Optics WS-1) standard.

For each male, we summarized plumage data by calculating three standard colour metrics: brightness, red chroma and hue. Brightness was calculated as the sum of reflectance from 300 to 700 nm, and red chroma was calculated as the proportion of total reflectance occurring in the range of 600–700 nm. We calculated hue as the wavelength at which there was the steepest slope in the reflectance curve (when the curve was assessed at 1 nm increments) as opposed to the wavelength of peak reflectance, as almost all feathers had peak reflectance at our maximal value of 700 nm. Principal components analysis (PCA, analysing the correlation matrix among the three variables) was used to collapse these colour variables into fewer independent variables (principal components) of colour.

Testes Investment

Seven red/black breeders, six brown breeders and four auxiliaries were collected for dissection. We quantified fresh testes weight for both the left and right testis and calculated the gonadosomatic index (GSI) to provide a measure of relative testes size, where GSI = (combined gonad weight/body weight) × 100 (Taborsky 1998). Although the use of the GSI is not without problems (Tomkins & Simmons 2002), the three male phenotypes did not differ in body mass (ANOVA: $F_{2,48} = 0.69$, $P = 0.5$) and there was no relationship between log testes mass and log soma mass ($r^2 = 0.13$, $N = 17$, $P = 0.16$). Therefore, the simple comparison of GSI values provides an appropriate measure of the relative investment in spermatogenic tissue made by each phenotype (Stoltz et al. 2005).

Ejaculate Quality

Ejaculate samples were collected using a cloacal massage technique (Wolfson 1960; Tuttle et al. 1996; Gee et al. 2004). Specifically, the region surrounding the CP was stimulated by stroking, and gentle pressure was repeatedly applied to both sides of the cloaca to express semen stored in the seminal glomera. The cloaca was massaged until semen expression stopped. Only one person

(M.R.) collected sperm from males and made every effort to use exactly the same technique with each male. Ejaculate quality was assessed in terms of sperm numbers (i.e. ejaculate sample sperm density and total sperm count) and sperm quality (i.e. sperm viability, the proportion of morphologically normal sperm in ejaculates, the proportion of motile sperm in ejaculates) using a range of standard methods suitable for use under remote, field-based conditions.

Semen was collected in 10 μl microcapillary tubes and the volume of ejaculate samples was determined by measuring the length of the semen column against a calibrated rule (to the nearest 0.1 μl). Semen was then immediately mixed with 200 μl of Lago Formulation Avian Semen Extender (Hygieia Biological Laboratories, Woodland, CA, U.S.A.). We quantified ejaculate sample sperm density and total sperm count using a calibrated Makler counting chamber (Irvine Scientific, Santa Ana, CA, U.S.A.). A subsample of the diluted ejaculate sample was used to quantify sperm viability and sperm morphology. We quantified sperm viability by recording the proportion of live (versus dead) sperm using eosin–nigrosin staining techniques (Cooper & Rowell 1958; Bakst & Cecil 1997; see also Birkhead & Petrie 1995; Wishart et al. 2002). Values for sperm density and sperm viability were based on the average of two replicate aliquots from each ejaculate and showed high repeatability across males ($r = 0.94$ and 0.84, respectively). At the same time, sperm were categorized as either normal or abnormal (e.g. macrocephalic, bent head, multiple tails) to quantify the proportion of morphologically normal sperm in each ejaculate sample (Gee et al. 2004). A separate subsample of the diluted ejaculate sample was used to quantify the proportion of motile sperm (to the nearest 0.05) in ejaculates using the hanging drop technique (Wishart & Wilson 1997; Penfold et al. 2000). Specifically, 10 μl of the diluted ejaculate was placed on a glass slide and the proportion of motile sperm was assessed by examining several fields of view using phase contrast microscopy (20× magnification). Lago Formulation Avian Semen Extender is designed to maintain sperm integrity and motility for a period of 6 h or more. However, we assessed sperm quality traits within 3 h of sample collection to increase the likelihood that our measures provided an accurate evaluation of sperm quality.

Statistical Analysis

Because several measures of ejaculate quality were expressed as proportions, we applied arcsine-root transformation to the following variables: proportion of viable sperm, proportion of morphologically normal sperm and proportion of motile sperm in an ejaculate sample. Additional data transformations were conducted where appropriate, but when transformations failed to normalize distributions, nonparametric analyses were applied. Shapiro–Wilk tests were used to test for normality throughout. All statistics were performed using the R (2.7.0) software package (R Development Core Team 2006) and SAS 9.2 statistical analysis software (SAS Institute 2009).

We tested whether the three reproductive phenotypes differed in body size, CP volume, GSI and ejaculate quality using ANOVA and post hoc pairwise comparisons with Tukey adjustment for multiple comparisons. Because the presence of red/black plumage is loosely associated with age (Webster et al. 2008), we wanted to exclude the possibility that any observed patterns were the result of age-related variation. Therefore, we included both reproductive phenotype and age (1-year-old males versus males 2 years of age or older) in a two-way ANOVA to examine variation in body size, CP volume and ejaculate quality. All the two-way interactions were also included in these models and were subsequently removed if not significant. Male age was unknown for sacrificed individuals, so we were unable to control for age in our analysis of GSI.

To determine how male ejaculate quality relates to plumage coloration among red/black breeders, we performed Pearson pairwise correlation analyses between all ejaculate traits and the two colour PCs. As the initial correlation analyses indicated that certain independent variables could explain variation in ejaculate quality (sperm density, proportion of motile sperm), we further explored how colour PCs could explain ejaculate quality using an information theoretic approach of multimodel selection, using Akaike's Information Criterion (AIC) according to methods described by Burnham & Anderson (2002). We also incorporated body condition and age in these models. As exact age was known for only a subset of males, individuals were classified as either 1 year of age or 2 or more years of age. For each dependent variable (sperm density, proportion of motile sperm), we selected suites of models based on Δ-AIC values and the relative Akaike weight of each model (Burnham & Anderson 2002; Bolker 2008). Following this model selection, we computed average models of those selected by multiplying individual predictor coefficients (β) within each model by the respective Akaike weight for the entire model (Burnham & Anderson 2002). This gave us a way of inspecting which predictor variable was most explanatory for each dependent variable across a set of selected models, while also associating a normalized relative likelihood (i.e. sum of Akaike weights for the selected models) with these averaged interpretations. We produced average models to explain sperm density and proportion of motile sperm separately.

Ethical Note

All animals were handled in a safe and humane manner and all procedures were approved by the Institutional Animal Care and Use Committee of the University of Chicago (approval no. 71453), the James Cook University Animal Ethics Review Committee (approval no. A1004) and the Queensland Government Environmental Protection Agency. The low number of individuals euthanized in this study was specifically chosen to minimize our impact upon the populations, while still allowing us to examine variation in reproductive anatomy among males. Individuals were euthanized by anaesthetic overdose (Sodium Pentobarbital 100 mg/kg).

RESULTS

Testes Investment, Ejaculate Quality and Reproductive Phenotype

We obtained ejaculate samples for 90 individuals. When multiple ejaculate samples were collected from the same male within a single year, we included only the first sample collected for that individual. Summary statistics of body size, reproductive anatomy and ejaculate characteristics for each of the three male phenotypes are presented in Table 1. Structural size (mass, wing and tarsus lengths) was not associated with either reproductive phenotype or male age (ANOVA: reproductive phenotype: all $P > 0.1$; age: all $P > 0.1$). In contrast, relative investment in testes tissue differed significantly between male types: being largest in red/black breeders, intermediate in brown breeders and smallest in auxiliaries (ANOVA: $F_{2,14} = 3.78$, $P = 0.048$). More specifically, GSI was significantly greater in red/black breeders compared to auxiliaries (Tukey HSD: $P = 0.04$), but did not differ significantly between red/black breeders and brown breeders (Tukey HSD: $P = 0.51$), or brown breeders and auxiliaries (Tukey HSD: $P = 0.24$). Cloacal protuberance volume was also associated with reproductive phenotype (ANOVA: $F_{2,84} = 21.53$, $P < 0.001$; see also Karubian 2002; Lindsay et al. 2009) but not with male age (ANOVA: $F_{1,84} = 0.02$, $P = 0.87$). As for GSI, CP volume was largest in red/black breeders, intermediate in brown breeders and smallest in

Table 1
Body mass, relative testes size (GSI), cloacal protuberance (CP) volume and ejaculate quality for each of the three male reproductive phenotypes in the red-backed fairy-wren

	Red/black breeders	Brown breeders	Auxiliaries
Mass (g)	7.60±0.1 (52)	7.47±0.1 (21)	7.51±0.1 (16)
Tarsus length (mm)	22.57±0.1 (52)	22.51±0.1 (21)	22.43±0.2 (16)
Wing length (mm)	41.99±0.2 (52)	41.53±0.2 (21)	41.86±0.2 (16)
Gonadosomatic index (GSI)	3.24±0.27 (7)	2.86±0.14 (6)	2.2±0.34 (4)
CP volume (mm^3)	144.15±4.7 (52)	106.43±8.9 (22)	82.79±8.1 (16)
Sperm density ($\times 10^6/\mu l$)	6.86±0.4 (52)	6.62±0.5 (21)	8.43±1.1 (15)
Ejaculate volume (μl)	6.13±0.5 (52)	4.84±0.7 (22)	2.53±0.4 (16)
Total sperm count ($\times 10^6$)	41.01±3.8 (52)	32.55±4.7 (21)	21.47±3.7 (15)
Proportion of viable sperm	0.81±0.02 (52)	0.85±0.03 (21)	0.84±0.03 (15)
Proportion of motile sperm	0.17±0.02 (52)	0.17±0.04 (21)	0.14±0.05 (14)
Proportion of normal sperm	0.88±0.01 (52)	0.93±0.01 (21)	0.92±0.01 (15)
Total count of normal sperm ($\times 10^6$)	35.38±3.1 (52)	30.09±4.4 (21)	19.85±3.5 (15)

Values are means ± SE, with sample sizes in parentheses.

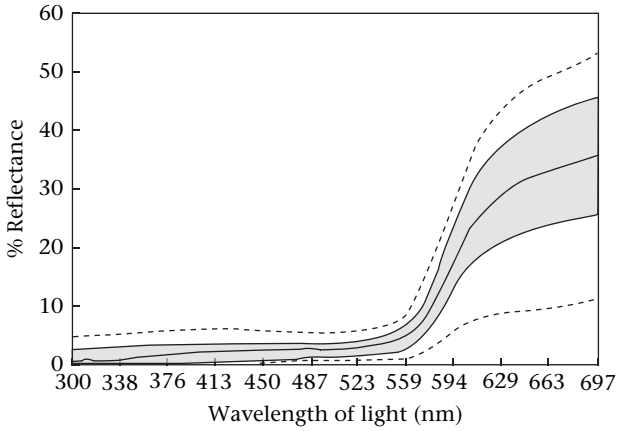

Figure 1. Reflectance spectra for red back feathers from red/black breeders of male red-backed fairy-wrens. Mean reflectance spectrum for 51 males (solid black line) ± 1 SD (grey shaded area). Upper and lower dashed lines represent the population maximum and minimum reflectance at each wavelength, respectively. Therefore, this graph gives an indication of population average, variance and range of reflectance from 300 to 700 nm of light. As expected, there is much greater variance in reflectance and, hence, colour properties, above 600 nm (i.e. in the orange–red end of the spectrum).

auxiliaries. Specifically, the volume of the CP was significantly higher in red/black breeders compared to brown breeders (Tukey HSD: $P = 0.0002$) and auxiliaries (Tukey HSD: $P < 0.0001$), but did not differ between auxiliaries and brown breeders (Tukey HSD: $P = 0.12$).

The three male phenotypes also differed in total sperm numbers (ANOVA: $F_{2,82} = 3.91$, $P = 0.02$) and in ejaculate volume ($F_{2,84} = 7.80$, $P < 0.001$), but not in ejaculate sperm density (ANOVA: $F_{2,82} = 1.91$, $P = 0.15$). Both sperm number and ejaculate volume were greatest in red/black breeders, intermediate in brown breeders, and lowest in auxiliaries, however, only the comparison between auxiliaries and red/black breeders was statistically significant (Tukey HSD: $P = 0.0005$ and $P = 0.02$, respectively). In contrast, male age was not associated with sperm number, ejaculate volume or ejaculate sample sperm density (ANOVA: sperm number: $F_{1,84} = 0.54$, $P = 0.47$; ejaculate volume: $F_{1,86} = 2.27$, $P = 0.14$; sperm density: $F_{1,84} = 0.70$, $P = 0.40$).

In regard to sperm quality, neither male phenotype nor age was associated with the proportion of viable sperm (ANOVA: phenotype: $F_{2,82} = 0.82$, $P = 0.44$; age: $F_{1,82} = 2.24$, $P = 0.14$) or the proportion of motile sperm (ANOVA: phenotype: $F_{2,81} = 0.43$, $P = 0.65$; age: $F_{1,81} = 0.05$, $P = 0.82$). In contrast, the proportion of morphologically normal sperm in ejaculates was associated with reproductive phenotype (ANOVA: $F_{2,82} = 5.86$, $P = 0.004$) but not with male age (ANOVA: $F_{1,82} = 0.01$, $P = 0.96$). Specifically, post hoc tests showed that red/black breeders had a lower proportion of morphologically normal sperm in their ejaculates compared with brown breeders (Tukey HSD: $P = 0.008$). Despite this difference, the relative number of morphologically normal sperm in ejaculates (i.e. total sperm count × proportion of morphologically normal sperm) remained greatest in red/black breeders (Table 1).

Plumage Coloration and Ejaculate Quality in Red/Black Breeders

Among red/black breeders, the averaged spectra from the red feathers showed very low reflectance from 300 to approximately 550 nm, a steep increase in reflectance from 550 to 600 nm, and then a shallower increase in reflectance above 600 nm (Fig. 1). These spectra also showed considerable variation among males in reflectance at higher wavelengths, and PCA of feather colour metrics generated two principal components that explained 83.0% of the total variation in the three measures (Table 2). Plumage colour PC1 loaded highly positively with both hue and chroma, and we therefore interpret increasing PC1 as indicating particularly saturated, redder feathers (i.e. increased red chroma and longer wavelength hue). Hue and chroma have been shown to depend upon carotenoid consumption during moult and are generally thought to indicate feather carotenoid content (Hill 1992; Inouye et al. 2001; Saks et al. 2003). Therefore, colour PC1 may represent a carotenoid signal, with higher scores representing more saturated, redder feathers due to a higher carotenoid content of the feathers. Feather colour PC2 loaded highly positively with brightness, and hence we interpret increased PC2 scores to indicate brighter feathers.

There were two significant correlations between ejaculate traits and plumage colour PC scores for red/black males (Table 3): sperm density was negatively related to plumage redness and saturation (colour PC1; Fig. 2) and the proportion of motile sperm in an ejaculate was positively related to plumage brightness (colour PC2; Fig. 3). Using AIC model selection, plumage redness and saturation (colour PC1) was the strongest and most consistent predictor of ejaculate sample sperm density (Table 4). Inspection of the four models selected by AIC revealed a high relative likelihood (summed Akaike weights of selected models = 0.977) that males with redder, more saturated plumage (i.e. lower colour PC1 scores) (weighted $\beta = -1.14$) that were marginally older (weighted $\beta = 0.150$) and in somewhat better condition (weighted $\beta = 0.706$) had higher sperm densities (Table 4).

Plumage brightness (colour PC2) was the best predictor of the proportion of motile sperm, as males with brighter plumage (i.e. higher colour PC2 scores) values also had a larger proportion of

Table 2
Summary of loading factors generated from the feather colour PCA for the red-backed fairy-wren

Colour metric	Colour PC1	Colour PC2
	55.65% of variance	27.33% of variance
Brightness	−0.595	0.804
Red chroma	0.809	0.303
Hue	0.813	0.287

Table 3
Pearson correlation analysis of plumage coloration and ejaculate quality among male red/black breeders

Colour PC	Ejaculate trait	Effect size (Pearson's *r*)	*N*	*P*	95% CI (for effect size)
PC1	Sperm density	−0.396	50	0.0044	(−0.607, −0.132)
	SQRT (sperm count)	−0.238	50	0.096	(−0.485, 0.043)
	Viability	0.157	50	0.28	(−0.127, 0.417)
	Proportion of motile sperm	−0.147	50	0.31	(−0.409, 0.137)
	Proportion of morphologically normal sperm	−0.018	50	0.90	(−0.295, 0.262)
PC2	Sperm density	0.135	50	0.35	(−0.149, 0.396)
	SQRT (sperm count)	0.125	50	0.39	(−0.159, 0.390)
	Viability	0.024	50	0.87	(−0.256, 0.301)
	Proportion of motile sperm	0.315	50	0.026	(0.040, 0.545)
	Proportion of morphologically normal sperm	−0.066	50	0.65	(−0.338, 0.216)

motile sperm (Table 5). Age and condition were weaker and less consistent predictors of motile sperm, with older males in better condition having a slightly greater proportion of motile sperm. The selected models indicated that, at a moderately high relative likelihood (0.890), males with proportionally more motile sperm had greater colour PC2 scores (weighted $\beta = 0.061$) and were marginally older (weighted $\beta = 0.007$) and in somewhat better condition (weighted $\beta = 0.030$) (Table 5).

DISCUSSION

We found that relative testis size and some components of ejaculate quality varied in association with reproductive phenotype in male red-backed fairy-wrens in the predicted pattern: red/black breeders > brown breeders > auxiliaries. Specifically, red/black breeders tended to invest more heavily in spermatogenic tissue than both brown breeders and auxiliaries. In addition, red/black breeders had larger CPs and tended to have greater numbers of sperm in ejaculate samples. Finally, while brown breeders showed a greater proportion of morphologically normal sperm in ejaculate samples relative to red/black breeders, this difference was not sufficient to devalue the numerical dominance of sperm exhibited by red/black breeders. Thus, overall, red-black breeders tended to show greater investment in sperm production and higher ejaculate quality when compared to brown breeders and auxiliaries.

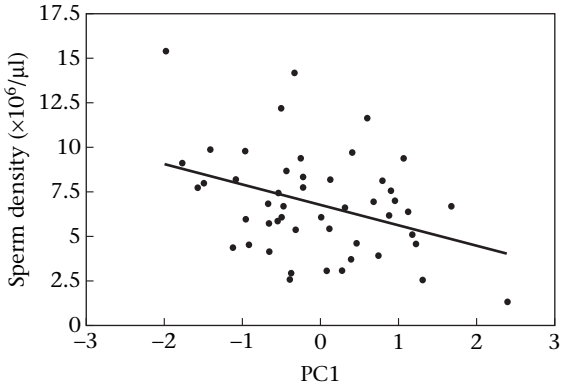

Figure 2. Relationship between colour PC1 (plumage redness and saturation) and density of sperm in ejaculate samples of male red-backed fairy-wrens. Data are for red/black breeders only.

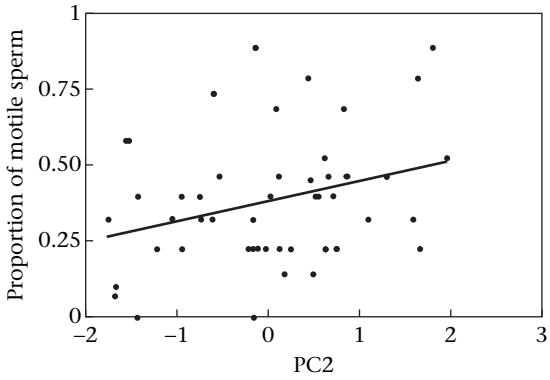

Figure 3. Relationship between colour PC2 (plumage brightness) and proportion of motile sperm in ejaculate samples of male red-backed fairy-wrens. Data are for red/black breeders only.

Observations from this study combined with previously published studies of the red-backed fairy-wren (Karubian 2002; Webster et al. 2008; Lindsay et al. 2009) suggest that plumage development, reproductive behaviour and reproductive anatomy are linked. An association between plumage development and testis size, with less ornamented males having significantly smaller testes relative to males in full nuptial plumage, has also been reported in the black-headed grosbeak, *Pheucticus melanocephalus* (Hill 1994), the long-tailed manakin, *Chiroxiphia linearis* (Foster 1987), and the red-winged blackbird, *Agelaius phoeniceus* (Wright & Wright 1944). In the red-backed fairy-wren, plumage development and reproductive behaviour appear to be determined by variation in plasma androgen levels among the reproductive phentoypes (Lindsay et al. 2009). As testis development and sperm production are also influenced by androgens (Froman 1995), it seems likely that androgens provide a proximate mechanism for the association between reproductive phenotype and investment in gonadal traits in the red-backed fairy-wren.

Androgen levels are also associated with bright male nuptial plumage in the superb fairy-wren, *Malurus cyaneus*. In this species, however, testosterone levels are not associated with either the presence or volume of the CP (Peters et al. 2000). Moreover, there is no difference in CP size between socially dominant males and auxiliary males in either the superb fairy-wren (Mulder & Cockburn 1993) or the splendid fairy-wren, *M. splendens* (Rowe & Pruett-Jones 2006). In both of these species, however, all males acquire colourful nuptial plumage during the breeding season (Rowley &

Table 4
Sperm density of red/black breeders in relation to plumage colour (PC1), age and condition

Model	Variables (standard coefficients, β)	AIC	Δ-AIC	Weight
M1	PC1 (−1.16)	99.276	0.0	0.356
M2	PC1 (−1.17), condition (1.47)	99.370	0.094	0.340
M3	PC1 (−1.16), age (0.51)	101.114	1.838	0.142
M4	PC1 (−1.18), age (0.56), condition (1.49)	101.167	1.891	0.138
M5	Condition (1.41)	106.005	6.729	0.012
M6	Age (0.36)	107.399	8.123	0.006
M7	Age (0.41), condition (1.42)	107.916	8.64	0.005

Information theoretic model selection, using Akaike Information Criterion (AIC), with ejaculate sample sperm density as the response variable and colour PC1, age and condition as explanatory variables. Parenthetical values following explanatory variables are standardized coefficients. Models were selected according to Δ-AIC and the Akaike weights of each model. As the first four models (M1–4) had Δ-AIC values of <2 and carried the greatest weights, we interpret these to be the most appropriate models to explain variation in sperm density in male red-backed fairy-wrens. Colour PC1 was an explanatory variable in all four of these selected models.

Table 5
Proportion of motile sperm in ejaculates of red/black breeders in relation to plumage colour (PC2), age and condition

Model	Variables (standard coefficients, β)	AIC	Δ-AIC	Weight
M1	PC2 (0.07)	−149.862	0.0	0.394
M2	PC2 (0.07), condition (0.09)	−148.937	0.925	0.248
M3	PC2 (0.06), age (0.03)	−147.968	1.894	0.153
M4	PC2 (0.07), age (0.03), condition (0.08)	−147.019	2.843	0.095
M5	Age (0.09)	−145.785	4.077	0.051
M6	Condition (0.04)	−145.144	4.718	0.037
M7	Age (0.09), condition (0.04)	−144.031	5.831	0.021

Information theoretic model selection, using AIC, with proportion of motile sperm as the response variable and colour PC2, age and condition as explanatory variables. Parenthetical values following explanatory variables are standardized coefficients. Models were selected according to Δ-AIC and the Akaike weights of each model. As the first three models (M1–3) had Δ-AIC values of <2 and carried the greatest weights, we interpret these to be the most appropriate models to explain variation in proportion of motile sperm in male red-backed fairy-wrens. However, model M4 had a Δ-AIC just outside this range, so it also needs to be considered. Colour PC2 was an explanatory variable in all of the selected models.

Russell 1997). Why androgens and reproductive anatomy (i.e. CP) are associated in the red-backed fairy-wren but appear to be decoupled in its congener, the superb fairy-wren, remains unclear. However, in the red-backed fairy-wren, the association between plumage development, reproductive behaviour and investment in gonadal traits may reflect a general, hormonally mediated developmental pattern related to delayed investment in first-year reproduction (Ficken & Ficken 1967; Hill 1994).

In passerines, variation in primary sexual characters may be associated with male age (Laskemoen et al. 2008). In the red-backed fairy-wren, however, it is unlikely that the observed differences between the reproductive phenotypes are simply age-related characteristics for several reasons. First, in the current study, male age was not significantly associated with any of the measured morphological or sexual traits. Second, both paternity success and variation in androgen levels appear to be primarily associated with reproductive phenotype and not age in this species (Webster et al. 2008; Lindsay et al. 2009). Thus, while it is possible that age may contribute to variation in sexual characteristics, age alone cannot fully explain the variation in male reproductive anatomy and ejaculate characteristics identified in this study.

Among red/black breeders, we found a negative relationship between plumage redness and saturation (colour PC1) and sperm density in ejaculate samples. We also found a positive correlation between plumage brightness (colour PC2) and the proportion of motile sperm in ejaculate samples. Thus, males with less red, less saturated (low PC1) and brighter (high PC2) plumage had greater ejaculate quality: specifically, these males had both a higher ejaculate sample sperm density and more motile ejaculates. Because darker, more saturated coloration and a redder hue is considered a more elaborate form of ornamentation (Hill 1996, 2002), our results suggest that males with more elaborate ornamentation have lower-quality ejaculates.

The phenotype-linked fertility hypothesis proposes that sexual ornamentation and ejaculate quality are positively associated and that females benefit from choosing to mate with the most elaborately ornamented males either directly, via fertility assurance, or indirectly, via sons inheriting high-quality ejaculates (Sheldon 1994; see also Trivers 1972; Keller & Reeve 1995; Pizzari et al. 2004). Blount et al. (2001) suggested that antioxidants, such as carotenoids, might link male ornamentation and sperm quality if both demand antioxidant pigments. In birds, exposure of sperm to reactive oxygen species inhibits sperm–oocyte fusion and decreases motility (Fujihara & Howarth 1978; Wishart 1984). Therefore, antioxidant protection appears to be vital in maintaining the fertilizing capacity of avian sperm (Surai et al. 2001). If carotenoids contribute to antioxidant defence of sperm, ornamentation and sperm quality may be linked if there is a trade-off in terms of allocation of carotenoid resources between the two biological functions. In the red-backed fairy-wren, red ketocarotenoids (astaxanthin, canthaxanthin and adonirubin) are present in both semen and plumage (Rowe & McGraw 2008), suggesting that ornamentation in this species has the potential to signal semen antioxidant defence and superior sperm quality. Contrary to this, however, our results reveal a negative association between plumage elaboration and ejaculate quality, suggesting that plumage redness and saturation could not be a signal of superior fertility in red/black breeders. It is possible that plumage colour does reveal information regarding ejaculate quality, but that the putative signal is opposite to what we would predict (i.e. males with less red, less saturated (low PC1) and brighter (high PC2) plumage, which have higher ejaculate quality, are preferred by females). However, such a signal seems unlikely based upon our knowledge of carotenoid-dependent sexual traits: females prefer redder males and males with maximum carotenoid colour expression in several avian species (Hill 1999). Note, however, that the dynamics of plumage acquisition in these species differ from that of the red-backed fairy-wren.

The negative relationship between ornamentation and ejaculate quality in our study may result from differential sperm depletion, whereby more elaborately ornamented males show lower sperm numbers and quality due to more frequent copulation events (Birkhead & Fletcher 1995; Birkhead et al. 1995; Liljedal et al. 1999). However, in other *Malurus* species, males show only a slight reduction in ejaculate sample sperm counts with successive sampling (Tuttle & Pruett-Jones 2004). Furthermore, high sperm production rates and rapid recovery from depletion suggest that male fairy-wrens have near-maximal numbers of sperm available for ejaculation at all times (Tuttle et al. 1996; Tuttle & Pruett-Jones 2004). Temporary sperm depletion is also more likely to be important in populations with high copulation frequencies (Petrie & Kempenaers 1998), but fairy-wrens appear to copulate at relatively low frequencies (Mulder & Cockburn 1993; Rowley & Russell 1997). Consequently, differential sperm depletion is unlikely to explain the negative association between coloration and ejaculate quality observed in the red-backed fairy-wren.

Theoretical models and empirical data suggest that males that are less likely to secure additional matings should increase ejaculate investment in order to maximize paternity success under sperm competition (Parker 1998; Cornwallis & Birkhead 2006). Furthermore, increasing investment in ejaculates is predicted to reduce investment in traits that influence mate acquisition (Parker 1998). Both sperm numbers and the proportion of motile sperm in ejaculates are influential in determining the outcome of sperm competition in birds (Birkhead 1998; Pizzari & Parker 2009). In the red-backed fairy-wren, ejaculate sample sperm density is strongly associated with the total number of sperm in ejaculates (Rowe & Pruett-Jones 2006). Therefore, our results suggest that males with less red, less saturated plumage (i.e. orange-hued males) show increased investment in ejaculate traits that confer a fertilization advantage. We do not know whether female red-backed fairy-wrens prefer to copulate with males showing redder coloration. Nevertheless, a redder hue has been shown to be sexually selected in many avian species (Zuk et al. 1995; Hill 1999). In the red-backed fairy-wren, such a preference may occur because redder males are likely to be higher quality due to the higher costs associated with red colour displays compared to orange colour displays (Hill 1996) or because of a general sensory bias for more exaggerated ornaments (Møller 1988; Ryan & Keddy-Hector 1992). Under such an assumption, the negative relationship between ornamentation and

ejaculate quality observed in this study may reflect a trade-off in investment in mate acquisition (plumage ornaments) versus investment in ejaculates.

In conclusion, we found that ejaculate quality was associated with male reproductive phenotype and variation in carotenoid-dependent plumage coloration in male red-backed fairy-wrens. If plumage redness and saturation are indeed sexually selected traits, males with redder, more saturated plumage may gain paternity success via precopulatory mating success, while males with less red, less saturated and brighter plumage may gain paternity success via postcopulatory fertilization success. Although this hypothesis requires further study in the red-backed fairy-wren system, the results of the current study suggest that examining the simultaneous contributions of pre- and postcopulatory mechanisms to reproductive success is crucial to understanding the evolution of male plumage coloration by sexual selection.

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