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Offspring sex ratios in relation to mutual ornamentation and extra-pair paternity in the Black Swan *Cygnus atratus*

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In sexually dichromatic birds, females may adaptively adjust the sex ratio of their offspring prior to hatching in relation to male ornamentation, for example, by producing more sons when paired to a highly attractive partner. However, to our knowledge no studies have investigated offspring sex ratio modification in species in which both sexes are ornamented, and it is unknown whether such a process would be adaptive. Here we examine variation in offspring sex ratio in the mutually ornamented Black Swan *Cygnus atratus*. Brood sex ratio was not related to the degree of ornament elaboration in either parent, or to extra-pair paternity. We suggest that parental attractiveness may not be inherited in a sex-linked manner, or may be largely non-heritable. Thus, females may not benefit from biasing the sex ratio of their offspring in relation to parental attractiveness.

There is growing evidence that female birds are able to manipulate the sex ratio of their offspring prior to hatching (Krackow 1995, Emlen 1997, Komdeur *et al.* 2002, West & Sheldon 2002). Numerous adaptive benefits to sex ratio manipulation have been proposed (reviewed in Oddie 1998, Sheldon 1998, Komdeur & Pen 2002). Among these, the role of sexual selection in adaptive sex ratio manipulation has been particularly controversial. Under classic sexual selection (i.e. male–male competition and female choice), male reproductive success will tend to be more variable than female reproductive success (Trivers & Willard 1973). In such populations, a few very attractive males will mate with many females and achieve higher reproductive success than any female. Unattractive males, by contrast, will mate with few females and their reproductive success will be lower than that of most females. Ellegren *et al.* (1996) suggested that, if sons inherit attractiveness from their fathers, then sons of attractive males might be of higher reproductive

value than the daughters of such males. It would then be adaptive for females paired to attractive males to bias the sex ratio of their offspring towards males. In accordance with this prediction, a number of studies have found that brood sex ratio correlates positively with the level of expression of a sexually selected trait in males (Ellegren *et al.* 1996, Sheldon *et al.* 1999, West & Sheldon 2002). Similarly, it has been suggested that because extra-pair offspring tend to be fathered by attractive males (Westneat *et al.* 1995, Sheldon *et al.* 1997, Westerdahl *et al.* 1997), it should be adaptive for females to bias the sex ratio of extra-pair offspring in favour of males (Sheldon & Ellegren 1996).

In many species, both males and females are ornamented. Such mutual ornamentation may be the result of a genetic correlation between the sexes (Lande 1980), in which case predictions in relation to sex ratio adjustment would be the same as for sexually dimorphic species. However, there is growing evidence that mutual ornamentation may have adaptive value in both sexes (Amundsen 2000). For example, Jones and Hunter (1993) showed that in Crested Auklets *Aethia cristatella*, males and females are both attracted to highly ornamented members of the opposite sex. If mutual ornamentation reflects

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heritable attractiveness of both parents, the level of ornamentation of both father and mother could be expected to influence the sex ratio of the offspring.

In this study we examine the factors influencing offspring sex ratios in the Black Swan *Cygnus atratus*, a mutually ornamented waterbird. Both sexes in this species possess curled ornamental feathers, which play a role in mate choice and social competition (Kraaijeveld *et al.* 2004b). Black Swans are socially monogamous and even though extra-pair paternity (EPP) is relatively common (40% of broods have at least one extra-pair cygnet), the means and variances in annual reproductive success are very similar for males and females (Kraaijeveld *et al.* 2004a). An attractive male is thus unlikely to gain higher reproductive success than an attractive female, which would suggest that attractive parents would not benefit from overproducing male offspring. Instead, the optimal sex ratio could be dependent on the relative attractiveness of the male and female of the pair.

Ornament expression is correlated with parental quality among pairs in the study population (Kraaijeveld *et al.* 2004b). Parental quality variation may have differential effects on the relative costs and benefits of sons and daughters. For example, if sons have higher survival than daughters but are more costly to produce, all pairs should overproduce sons, but only high-quality pairs may be able to do so.

To identify potential sex biases among Black Swan offspring, we calculated population sex ratios from egg-laying to independence. We estimated (1) relative costs of male and female offspring to their parents (by examining differences between the sexes in size and weight at the egg and cygnet stages), (2) relative benefits to parents of producing male or female offspring (by measuring survival to independence of males and females) and (3) whether brood sex ratios correlated with parental characteristics (ornamentation and body condition) and EPP. If ornamentation is heritable and sex-linked, we expected highly ornamented males to produce more sons and highly ornamented females to produce more daughters. Last, if heritable male traits increase the likelihood of obtaining EPP, we expected a bias towards males among extra-pair offspring.

METHODS

Study site and data collection

Data on offspring sex ratios were collected at Lake Wendouree in Ballarat, central Victoria, Australia

(37°33'S, 143°49'E), between 1999 and 2000. Lake Wendouree is a permanent lake of 238 ha supporting a population of around 170 Black Swans year-round. The Swans at this site are habituated to people and readily leave the water to accept bread morsels, allowing them to be captured by hand. Adults and full-grown juveniles were banded with metal leg bands (provided by the Australian Bird and Bat Banding Scheme) and individually coded plastic leg bands that could be read with binoculars from a distance of up to 30 m. The following measurements were taken from captured birds: tarsus length (with callipers to the nearest mm), bill and total head length (callipers, nearest 0.1 mm), wing length (butt-ended ruler, nearest cm) and body mass (spring balance, nearest 100 g). From each captured adult or full-grown immature a blood sample of 50–200 µL was taken from a tarsal vein, transferred into lysis buffer (Longmire *et al.* 1991) and stored at –20 °C for molecular analysis.

All banded birds (> 80% of the adult population, including all that attempted breeding) were censused twice per week during the breeding season (July–November) and once per month outside the breeding season by walking around the perimeter of the lake (6 km) and marking the location of each banded individual on a map.

Nests, eggs and cygnets

The Swans bred mainly in beds of Tall Spike-rush *Eleocharis sphacelata* and nests were found by searching this vegetation in a canoe once a week. Parents defended the nest aggressively during these visits. To avoid breakage of the eggs by the incubating adult, eggs were not measured at Lake Wendouree. Data on characteristics of male and female eggs were instead collected at two other Victorian populations, where the adults were shy: Reedy Lake, near Geelong (38°11'S, 144°26'E), and French Island (38°23'S, 145°16'E). At these sites, eggs were measured (maximum length and width to the nearest 0.1 mm using callipers) and weighed (to the nearest g, using a spring balance). The volume of eggs was calculated as length × width, which correlates closely with actual volume (Braithwaite 1977). Eggs were marked with indelible marker. Nests at these sites were checked weekly and some were visited while the clutch was hatching. In these cases, partially hatched cygnets were blood sampled and related to an individual egg number.

First-egg date was determined from the weekly nest checks if the nest was found before clutch completion, assuming 1.5 days to lay an egg

(Braithwaite 1977). When the nest was found after clutch completion, first-egg date was calculated from the hatching date:

$$\text{first-egg date} = \text{hatching date} - (\text{incubation period} + 1.5 \times (\text{clutch size} - 2)).$$

In this calculation, the incubation period is clutch-size specific, ranging from 39.9 to 42.3 days for clutches of four and seven eggs, respectively, time to lay one egg averages 1.5 days and steady incubation commences after the second to last egg is laid (Miers & Williams 1969, Braithwaite 1977). Hatching date was defined as the day the last egg hatched and was either determined through direct observation of hatching, or was estimated as the day before the first sighting of the brood.

After hatching, cygnets at Lake Wendouree stayed in the nest for about 24 h. Brood size was determined as the number of cygnets present when the brood was first seen. Broods were caught on shore, generally within the first week after hatching, by rounding up a family and catching all cygnets simultaneously. Because families were caught one at a time, cygnets could be assigned to a family unambiguously. To prevent cygnets from becoming separated from their parents, at least one of the parents was always caught and retained while the cygnets were being handled. All cygnets were weighed (to the nearest g, using a spring balance) and blood-sampled (about 50 μL from the brachial or tarsal vein). Tarsus and bill lengths of cygnets in 18 broods were measured. Cygnets were marked with a unique combination of small punches in the webs between their toes with a standard leather punch (Braithwaite 1981). As the small holes closed up when the cygnets grew to adult size, this procedure was unlikely to have a harmful effect on the cygnets' health.

Molecular sex and parentage determination

The phallus of male cygnets does not become evident until the bird is > 10 months old (Marchant & Higgins 1990) and could not be used to sex cygnets reliably. Cygnets were therefore sexed using molecular methods (Griffiths *et al.* 1998). Briefly, DNA was extracted from the blood samples using a standard salt-extraction protocol (Sunnucks & Hales 1996) and stored in $1\times$ TE buffer. PCR reactions comprised about 10 ng/ μL DNA, 200 μM of each dNTP (Promega), $1\times$ PCR buffer (Promega), 5 mM MgCl_2 ,

1 μM each of primers P2 and P8 and 0.75 units/15 μL Taq polymerase (Promega) in a total volume of 15 μL . Each reaction was overlaid with a drop of mineral oil. PCR conditions were as follows: initial denaturation at 94 $^{\circ}\text{C}$ for 2 min, 35 cycles of denaturation at 94 $^{\circ}\text{C}$ for 1 min, annealing at 55 $^{\circ}\text{C}$ for 1 min and extension at 72 $^{\circ}\text{C}$ for 1 min, followed by a final extension at 72 $^{\circ}\text{C}$ for 10 min. PCR products were separated by electrophoresis at 120 V for 2 h on 2% agarose gels. Gels were stained with ethidium bromide and visualized by UV transillumination. Birds were sexed according to the presence of the PCR products of the CHD-Z and CHD-W genes (Griffiths *et al.* 1998).

To verify the reliability of the molecular technique, adults of known sex were also sexed using PCR. Although male and female Black Swans are extremely similar in plumage, pairs of adults can be reliably sexed on relative size as well as behaviour. With few exceptions, adult males are larger, heavier and more aggressive than adult females. All pairs used in this study were first sexed according to these criteria and subsequently using the molecular markers. In all cases the two results were identical ($n = 60$ males, 60 females).

Parentage was assessed using a set of eight microsatellite markers as described in Kraaijeveld *et al.* (2004a). Briefly, we first inspected progeny arrays for allelic mismatches with the social mother. If a cygnet mismatched its social mother at more than one locus it was considered a case of intraspecific brood parasitism and was excluded from further analysis ($n = 11$ cygnets). We then compared the genotypes of the cygnets for which maternity was assigned to their social mother with those of their social father. Cygnets that mismatched their social father at more than one locus were considered cases of EPP. For these, we searched for the extra-pair sire among all adult males for complete matches.

Terminology

All surviving cygnets were recaptured as juveniles and banded with individually numbered metal and plastic leg bands after 8 months. The scars left by the web punches were used for individual identification. All measurements used in the analysis of offspring survival were obtained during the same breeding season as the survival data. Cygnets that had disappeared during their first 8 months of life were assumed to have died. This assumption is supported by a study in New Zealand, in which 95–100% of

Black Swans that were found during their first year of life ($n = 1792$) were recovered on their natal lake, compared with 68–82% of birds in their second year (Williams 1977).

We used body mass as an index of body condition rather than the residuals from the regression of body mass on tarsus length, as this regression was not always significant (see discussion in Green 2001).

We scored the degree of ornamentation by counting the number of curled wing-feathers (tertials and wing coverts). A feather was defined as curled when part of at least one of the vanes reached above the central feather-shaft (rachis) when viewed from the side (Kraaijeveld *et al.* 2004b).

Statistical analyses

Because most broods were caught away from the nest and a few days after hatching, not all cygnets from each brood could be sampled. The number of broods for which we could be certain that all cygnets were sampled was small ($n = 25$) and we therefore included incompletely sampled broods in our analysis (total $n = 71$). To assess whether the missing cygnets might have obscured a pattern in our main independent variable of interest (ornamentation), we constructed a 'best-case' dataset in which all unsampled cygnets were assigned the sex expected if ornamentation was heritable. Thus, unsampled offspring of highly ornamented males (number of curled feathers > mean) were assigned as male, unsampled offspring of poorly ornamented males (number of curled feathers < mean) were assigned as female, and vice versa for females. Similarly, we constructed a 'best-case' dataset for extra-pair offspring, in which a random selection of 15.1% (mean rate of EPP in our study population; Kraaijeveld *et al.* 2004a) of the unsampled cygnets were assigned EPP and as male. The remaining unsampled cygnets were assigned within-pair paternity and a 1 : 1 sex ratio.

We compared the observed frequency distribution of brood sex ratios with that expected under a binomial distribution with a mean of 0.5. Expected frequencies were calculated as follows: for each brood size in the dataset, the probability of each possible number of males was calculated, using a binomial distribution with a mean of 0.5. These probabilities were multiplied by the number of broods of that size in the sample. The frequency distribution of these expected values (bin size = 0.1) was then compared with the observed frequencies, using a G-test with Williams's correction (Fowler *et al.* 1998). The analysis

of sexual dimorphism compares brothers and sisters from the same brood, to control for cygnet age and environmental effects. When two or more individuals of the same sex were present in a brood, their measurements were averaged. Brood sex ratios were analysed using generalized linear models (GLMs) with a binomial error distribution and a logit link function. Brood size was used as the binomial denominator and the number of males as the response variable. The effect of each fitted term was assessed from the change in deviance when the term was removed from a fuller model. This change in deviance approximates a chi-squared distribution (Beath 2001). For many broods, measurements for only one of the parents were available for the same year. To ensure maximum power, we split this analysis into separate models for the effects of characteristics of the pair, those of the biological mothers and those of the biological fathers. Because Black Swans mate assortatively with respect to ornamentation (Kraaijeveld *et al.* 2004b), we also include a model of brood sex ratios in relation to mid-pair values for ornamentation and weight. Similar models were used to analyse brood sex ratios at 8 months of age (binomial denominator: total number of young surviving per brood; response variable: number of males surviving per brood) and the difference in ornamentation between the social father and the extra-pair sire. Last, we investigated brood sex ratios in relation to EPP using a generalized linear mixed model (GLMM) with a binomial error distribution and a logit link function, with brood number as random effect (Krackow & Tkadlec 2001). GLMM was calculated in R 1.9.1, using the function `glmmPQL` from the MASS library. Significance of fixed effects was assessed using penalized quasi-likelihood (Venables & Ripley 2002). GLMs were performed in GLMStat (Beath 2001). G-tests were calculated manually. All other statistical tests were performed in SYSTAT 7 (Systat 1997).

RESULTS

General patterns in offspring sex ratios

The mean clutch size at Lake Wendouree was 4.35 ± 1.43 sd. On average, we sampled 81% of cygnets per clutch. Population-level offspring sex ratios are summarized in Table 1. To estimate the sex ratios of eggs, Table 1 includes the sexes for broods for which we could be certain that all eggs had been sampled. Sex ratios of eggs and cygnets did not differ significantly from parity (Table 1). All further analysis

Table 1. Population-level sex ratios of cygnets produced in 1999 and 2000 at three stages of development: egg, cygnets at 0–2 weeks of age and juveniles of 8 months of age.

Year	Males	Females	<i>n</i> (broods)	Proportion male	<i>P</i> *
Eggs†					
1999	8	11	4	0.42	0.65
2000	57	45	21	0.56	0.28
Overall	65	56	25	0.54	0.47
Cygnets					
1999	40	44	25	0.48	0.74
2000	102	79	46	0.56	0.10
Overall	142	123	71	0.54	0.27
Juveniles					
1999	23	12	15	0.66	0.09
2000	42	30	33	0.58	0.19
Overall	65	42	48	0.61	0.03

*Two-tailed binomial tests, no significant differences at a table-wide $\alpha < 0.05$, using sequential Bonferroni analysis (Rice 1989).
†Broods for which the number of sampled cygnets equalled the clutch size at the last nest check and for which adoption was excluded using microsatellite analysis.

Table 2. Brood sex ratios for young cygnets and juveniles at 8 months of age in relation to characteristics of the pair (first-egg date and mid-pair values for ornamentation and weight), the biological mother and the biological father. Year was included as a factor in all models.

	Cygnets		Juveniles	
	Δ Deviance	<i>P</i>	Δ Deviance	<i>P</i>
Pair characteristics ($n_{\text{cygnets}} = 51, n_{\text{juveniles}} = 35$)				
Year	0.05	0.83	0.16	0.69
First-egg date	0.22	0.64	2.77	0.10
Mid-pair characteristics ($n_{\text{cygnets}} = 22, n_{\text{juveniles}} = 14$)				
Year	0.72	0.40	0.007	0.93
Ornamentation	1.13	0.29	0.07	0.79
Weight	3.28	0.07	0.36	0.55
Maternal characteristics ($n_{\text{cygnets}} = 40, n_{\text{juveniles}} = 23$)				
Year	0.31	0.58	0.05	0.82
Ornamentation	0.0008	0.98	0.43	0.51
Weight	5.05	0.02	1.54	0.21
Paternal characteristics ($n_{\text{cygnets}} = 36, n_{\text{juveniles}} = 27$)				
Year	0.06	0.81	0.002	0.97
Ornamentation	0.08	0.77	0.0007	0.98
Weight	1.26	0.26	0.01	0.92

uses the data for all clutches, including those that were incompletely sampled. The frequency distribution of brood sex ratios of cygnets did not differ significantly from that expected under a binomial distribution with a mean of 0.5 in either year (1999: $G = 4.34$,

$P = 0.88$; 2000: $G = 8.68$, $P = 0.47$). Juvenile sex ratios showed a near-significant bias towards males (Table 1). GLMs revealed no significant effects of year or first-egg date on the brood sex ratios of cygnets or juveniles (Table 2).

Sex differences in growth and survival

Table 3 summarizes size and weight of male and female eggs, cygnets and juveniles. All comparisons were within broods to control for age and environmental factors. There was no significant difference between male and female eggs in weight or volume. Likewise, there was no significant difference in mean body mass and tarsus length between male and female cygnets, but bill length was significantly longer in males than in their female siblings. Juvenile males were significantly heavier and larger in all three measurements than their sisters. However, as cygnets feed themselves, this should not represent a differential cost to the parents.

Parental effects on offspring sex ratio

Mid-pair values of ornamentation and body mass were not significantly related to brood sex ratios for either cygnets or juveniles (Table 2). However, maternal body mass was significantly positively related to the proportion of male offspring within broods of cygnets (Table 2). However, further inspection revealed a strong influence of two data points (both heavy females producing all-male broods). After removal of these two points the maternal condition effect was no longer significant. Maternal ornamentation had no significant effects on brood sex ratios (Table 2). To assess the impact of the unsampled cygnets, we repeated the analysis for paternal and maternal ornamentation using our 'best-case' dataset. Again, no significant results were detected for either paternal (Δ Deviance = 1.37, $n = 36$ broods, $P = 0.24$) or maternal ornamentation (Δ Deviance = 0.74, $n = 40$ broods, $P = 0.39$).

Brood sex ratios of cygnets and juveniles were not significantly associated with paternal (within-pair or extra-pair) weight or ornamentation (Table 2). Extra-pair cygnets were not more likely to be male than within-pair cygnets (GLMM $t_{187} = 0.24$, $P = 0.81$). This lack of effect was not due to the unsampled cygnets, because no significant effect was found in the 'best-case' dataset (GLMM $t_{228} = 0.60$, $P = 0.55$). The 28 broods that contained at least one extra-pair cygnet included 60 male and 57 female

Table 3. Sexual dimorphism in size and mass for eggs, cygnets and juveniles (8 months of age). Comparisons are made between the means for each sex within broods, using paired *t*-tests.

	Males (mean)	Females (mean)	Percentage difference	<i>n</i> (broods)	<i>t</i>	<i>P</i>
Eggs						
Volume ($10^{-2} \times \text{length} \times \text{breadth}$)	70.60	69.54	1.52	7	-1.30	0.24
Mass (g)	253.13	245.50	3.11	6	-1.68	0.15
Cygnets						
Tarsus length (mm)	34.22	33.75	1.39	6	-0.52	0.62
Bill length (mm)	22.35	21.79	2.57	6	-3.97	0.01*
Mass (g)	205.12	202.04	1.52	48	-0.86	0.39
Juveniles						
Tarsus length (mm)	96.71	92.03	5.09	19	-3.32	0.004*
Bill length (mm)	66.57	62.35	6.77	20	-4.25	< 0.001*
Wing length (mm)	470.31	452.84	3.86	18	-4.54	< 0.001*
Mass (g)	494.07	429.21	15.11	21	-3.31	0.004*

*Significant differences at a table-wide $\alpha < 0.05$, using sequential Bonferroni analysis (Rice 1989).

cygnets, not significantly different from 50 : 50 ($\chi^2_1 = 0.157$, $P = 0.69$). Furthermore, there was no evidence that the difference in ornamentation rate between the social father and the extra-pair sire influenced the sex ratio of the brood (Δ Deviance = 0.46, $n = 8$ broods, $P = 0.50$).

DISCUSSION

The population sex ratio among young Black Swan cygnets did not deviate significantly from the expected 50 : 50 ratio in either year, and the frequency distribution of brood sex ratios did not differ significantly from that expected under a binomial distribution. There is therefore no evidence for a population-wide bias in offspring sex ratio. The population sex ratio at the termination of parental care was 61% male, which approached statistical significance. We assume that this sex ratio bias at the termination of parental care was due to differential survival because published evidence suggests limited dispersal during the first year in Black Swans (Williams 1977), although this finding has not yet been validated in our study population. If this assumption holds, male offspring may thus have been of greater indirect fitness value to their parents than females.

We also found no evidence to suggest that parents biased the sex ratio of their broods facultatively in relation to parental ornamentation or EPP. In Black Swans, males and females signal their quality (in terms of social dominance) through their ornamental curled feathers (Kraaijeveld *et al.* 2004b). Highly

ornamented males did not produce more sons and highly ornamented females did not produce more daughters. One possible explanation for this is that inheritance may not be sex-linked. Thus, if a highly ornamented male produces highly ornamented daughters as well as sons, there would be no selective advantage to biasing the sex ratio, as the variances in male and female reproductive success are very similar (Kraaijeveld *et al.* 2004a). Alternatively, social dominance status and the degree of ornamentation could be largely non-heritable traits (Griffith *et al.* 1999). For example, if the sons of high-quality males do not inherit their father's traits, there is no fitness advantage to biasing brood sex ratio in relation to paternal quality.

Extra-pair sires in this population are not more ornamented than social fathers (Kraaijeveld *et al.* 2004a). However, it could be argued that males successful at obtaining EPP have other, heritable characteristics that were not detected in this study. Females fertilized by such males may benefit from biasing the sex ratio of the extra-pair offspring to males (Ellegren *et al.* 1996). However, extra-pair cygnets were equally likely to be male or female in the Black Swan, and there was no evidence that females that engaged in EPP biased the sex ratio of their offspring. One possible explanation for the lack of any correlation may be that females use EPP mainly for fertilization assurance and are not particularly selective about their extra-pair mates (Kraaijeveld *et al.* 2004a).

In summary, we found no evidence for a population-level bias in the offspring sex ratio in the Black Swan.

Furthermore, no relation was evident between offspring sex ratio and parental ornamentation or EPP.

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