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Subtle manipulation of egg sex ratio in birds

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Mothers are predicted to overproduce male or female eggs when the relative fitness gains from one sex are higher and outweigh the costs of manipulation. However, in birds such biases are often difficult to distinguish from differential embryo or chick mortality. Using a molecular technique to identify the sex of early embryos, we aim to determine the effect of maternal nutrition on zebra finch (*Taeniopygia guttata*) egg sex ratios after 2 days of incubation, which is as close to conception as is currently possible. We found no overall bias in the sex ratio of eggs laid and sex did not differ with relative laying order under any diet regime. However, mothers on a low-quality diet did produce a female bias in small clutches and a slight male bias in large clutches. On a high-quality diet, mothers produced a male bias in small clutches and a female bias in large clutches. Those on a standard diet produced a roughly even sex ratio, irrespective of clutch size. These observed biases in egg sex are partly in line with predictions that, in this species, daughters suffer disproportionately from poor rearing conditions. Thus, when relatively malnourished, mothers should only rear daughters in small broods and vice versa. Sex-ratio patterns in this species therefore appear to be subtle.

Keywords: sex allocation; *Taeniopygia guttata*; molecular sexing; diet quality

1. INTRODUCTION

The discovery that birds can vary the proportion of sons or daughters that they produce has stimulated considerable recent research. Theory predicts that mothers should overproduce male or female eggs when the prospective fitness of one sex is relatively greater (Trivers & Willard 1973) and outweighs the fitness costs associated with manipulation. Concomitantly, mothers need to be able to overcome the opposing selection pressures on gametes and offspring for whom the optimal sex ratio might conflict with that of the mother (Krackow 2002). Some avian studies show sex biases that appear to support one or more hypotheses, but many others show no adaptive patterns or indeed show the opposite from what is predicted by existing theory (reviewed in Cockburn *et al.* 2002). Even within species and populations, there can be a great deal of variability in observed sex ratios. Some of the disparity in results may be due to the stochastic environmental variation that is inherent in field studies. Therefore, some authors have tried to reduce this by breeding birds in captivity. In particular, this allows greater control over experimental manipulations of parental condition.

In captive Australian zebra finches (*Taeniopygia gutatta*), it has been predicted that mothers should bias clutches towards daughters under good conditions and towards sons under poor conditions. Neonatal conditions are thought particularly to impact upon female nestlings because of their need to build up body reserves prior to breeding at just three months of age (Kilner 1998). While showing, in some cases, that they can vary the sex ratio of their broods in a fashion consistent with adaptive explanations, different studies have not produced consistent results (Burley 1986; Clotfelter 1996; Bradbury & Blakey 1998; Kilner 1998; Rutkowska & Cichon 2002). These studies can be difficult to interpret because, as with many avian sex-allocation studies, offspring sex was identified late in development, usually at the chick or adult stage. As a result, it is often not possible to tease out whether the biases arose at 'conception', i.e. due to maternal manipulation of the primary sex ratio, or were due to factors outside of maternal control. For example, the bias could arise as a result of differential embryo or chick mortality due to chance environmental effects. Furthermore, small sample sizes and/or clutch sizes can mean that the assumptions of the statistical analyses used are violated (Wilson & Hardy 2002). In addition, it has recently been highlighted that many studies treat the sex of offspring from the same mother as statistically independent, again violating the assumptions of analytical models (Krackow & Tkadlec 2001).

We present the results of an experiment in which we manipulated the pre-breeding condition of parents using experimental diets known to influence investment in reproduction (Selman & Houston 1996) and measured, for the first time in zebra finches, the sex ratio of recently laid eggs using a molecular technique. We were, thus, able to consider sex-ratio bias close to conception while minimizing the confounding effect of later embryo or chick mortality. This paper focuses on two specific questions. First, do pairs of birds on diets of differing quality bias egg sex ratio in different directions? In particular, do mothers on high-quality diets and/or those laying small clutches produce an excess of daughters who will differentially benefit from good rearing conditions, as has been predicted (De Kogel 1997; Kilner 1998)? Second, does parental quality influence which sex is laid at advantageous positions in the laying order?

2. METHODS

We developed three experimental diets that differed in quality: one diet improved condition (high quality, HQ), another that reduced condition (low quality, LQ) and a standard, medium-quality diet (MQ) (see electronic Appendix A, available on The Royal Society's Publications Web site). The experimental diets differed in the quality and quantity of protein, fat and antioxidants available, as well as other nutrients known to induce changes in female quality and fecundity (Monaghan *et al.* 1996; Selman & Houston 1996). Each bird was randomly assigned to a diet treatment. All individuals stayed on the experimental diets for four weeks prior to pairing, and then all treatment groups received the same diet (see electronic Appendix A).

(a) *Breeding trials*

Two identical breeding trials were run using experienced (45) and virgin (45) pairs but the clutch sizes and sex ratios produced did not differ so the data were pooled (see electronic Appendix A). At the start of the breeding trial, each pair was moved to a breeding cage

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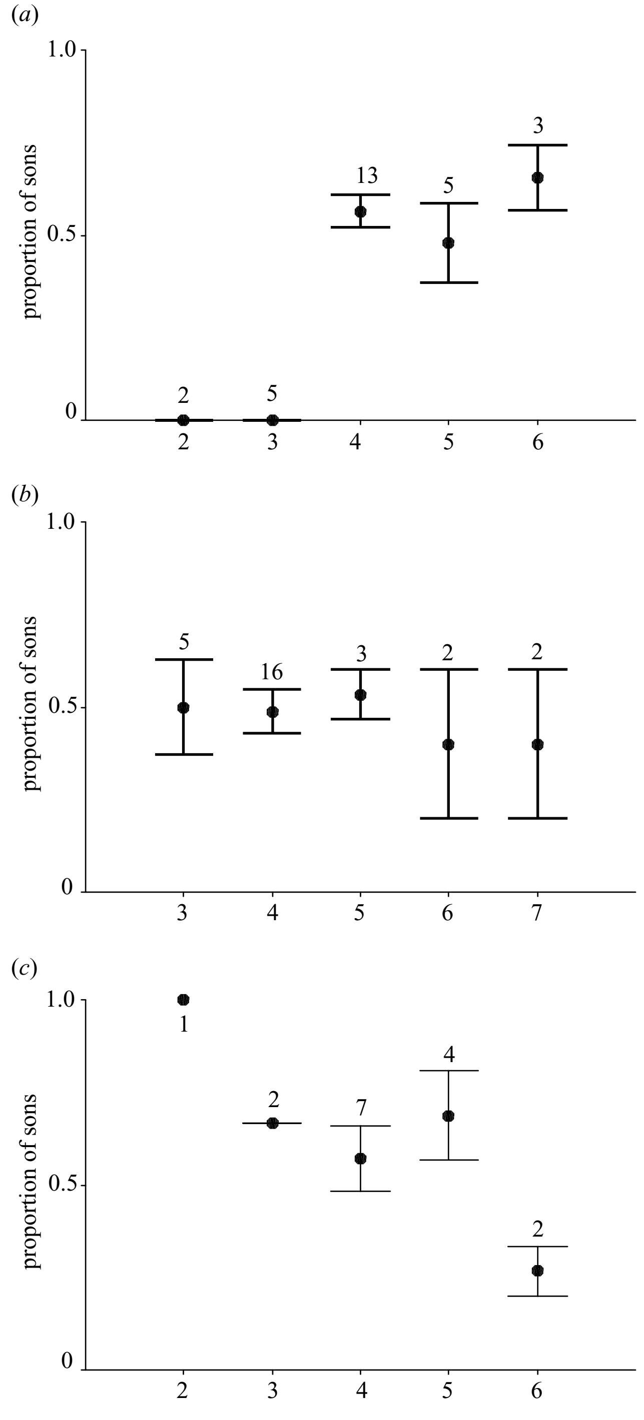

Figure 1. Mean sex ratio (proportion of sons) for different clutch sizes produced by mothers on (*a*) low-quality (LQ), (*b*) medium-quality (MQ), or (*c*) high-quality (HQ) pre-breeding diet. Sample sizes are shown above the standard error bars.

with a nest-box and nesting material. Each egg was marked sequentially and weighed on the day that it was laid. Eggs were collected 2–8 days after laying (depending on the clutch size and order of laying) to ensure that all eggs received at least 2 days of incubation, the minimum necessary to produce enough cells for reliable molecular sexing (Arnold *et al.* 2003). The egg was then opened and the developing embryo removed for molecular sexing. The sexing technique followed Griffiths *et al.* (1998), but instead of primer P8, we used our newly developed primer P17, which functions better for zebra finches (see electronic Appendix A). When the last egg in a clutch had been removed, pairs were split up.

A total of 10.3% (31/302 eggs) could not be sexed for a variety of reasons. Fifteen eggs (5.0%) were damaged or eaten so that a DNA sample could not be taken, and were not considered to be subject to maternal sex-allocation manipulation. The rest of the unsexed eggs (5.3%) were excluded because only a blastodisc was visible and molecular sexing techniques are unreliable for eggs at such early stages of development (Arnold *et al.* 2003). Microscopic examination of the germinal disc (Romanoff 1960) and analysis of the DNA concentration indicate that they were likely to have been infertile (see electronic Appendix A). The number of unsexed eggs per clutch did not differ between diets (ANOVA diet: $F_{2,68} = 2.15$, $p > 0.1$). We created a worst-case replacement scenario in which missing egg samples were of the sex predicted by the model, and then the opposite of that predicted (see electronic Appendix A for models). In neither case did the inclusion of these unsexed eggs alter our results, so these 5.3% of eggs were excluded from the main analyses.

(b) *Statistical analyses*

Clutch sex ratios were analysed in relation to parental pre-breeding diet and the clutch size by generalized linear model (GLM) in SAS (SAS Institute 1998; see electronic Appendix A). The number of sons was used as the dependent variable with the number of sexed offspring in the clutch as the binomial denominator. Egg sex was analysed as a function of laying position and parental diet using a generalized linear mixed model (GLMM) with a binomial error distribution (Krackow & Tkadlec 2001; see electronic Appendix A). Sample sizes vary among analyses because not all data were available for all eggs and all mothers.

3. RESULTS

Mothers on the LQ diet significantly decreased in condition during breeding, but those on the other two diets did not (electronic Appendix A). There were no significant differences in mean clutch sizes among treatment groups. However, there were significant differences in the mean total weight of all clutches of eggs ($F_{2,88} = 3.61$, $p = 0.033$) with HQ clutches (3.40 ± 0.84 g) being 65% heavier than LQ clutches (2.05 ± 0.41 g).

(a) *Diet manipulation and egg sex ratio*

Overall, 28 (out of 35) LQ, 28 (out of 30) MQ and 16 (out of 25) HQ pairs produced a clutch size of two or more eggs. Overall, 165 male and 161 female eggs were produced. The sex ratios on each diet were LQ 49M : 53F, MQ 55M : 57F and HQ 32M : 25F. None of these ratios was significantly biased based on *G*-tests ($p > 0.4$ in all cases). However, a GLM analysis revealed that the interaction between diet and clutch size had significant effects on the brood sex ratio (table 1*a*; figure 1). Mothers on a LQ diet produced a female bias in small clutches (less than or equal to 3) rising to an even sex ratio in medium-sized clutches and a small male bias in large clutches (six eggs). This increase in sex ratio with clutch size in LQ clutches was significant ($t = 3.27$, d.f. = 66, $p = 0.002$). On a MQ diet, offspring sex ratio was largely independent of clutch size and hovered around a 50 : 50 sex ratio ($p > 0.2$). Out of those clutches produced by HQ mothers, small ones were male-biased decreasing to an even then a female bias in large clutches, but the sample size is small and the variation was not significant ($p > 0.2$). A GLMM revealed that sex was not influenced by interactions between diet and relative laying order (table 1*b*) or diet and actual laying order (see electronic Appendix A).

4. DISCUSSION

Manipulating parental diet altered the total investment that mothers made in eggs but did not result in overall

Table 1. (*a*) Results from a general linear model of clutch sex ratio (number of sons/number offspring sexed) in relation to diet and clutch size. (*b*) Results from a GLMM of egg sex in relation to diet and relative laying order.
(Maternal identity was entered as a random effect. Non-significant effects were removed in a stepwise fashion starting with non-significant interactions. The mean intercept ± the standard error and the deviance are shown for each model.)

term	intercept	deviance	d.f.	*F*	*p*
(*a*) clutch sex ratio	2.32 (1.19)	59.85	69		
diet			2, 66	6.83	0.0020
clutch size			1, 66	0.00	0.9773
diet × clutch size			2, 66	6.53	0.0026
(*b*) egg sex	0.69 (0.44)	377.32	272		
diet			2, 276	0.93	0.3963
laying order			2, 278	2.07	0.1278
diet × laying order			4, 272	1.45	0.2170

biases in embryo sex ratio. Non-random sex-allocation patterns in response to diet were found in our study, but only in clutches that were larger and smaller than the modal size (four eggs). Mothers kept on a LQ pre-breeding diet produced an excess of daughters in small clutches (less than or equal to three eggs) and marginally more sons in large clutches (greater than or equal to six eggs). By contrast, there was a male-biased embryo sex ratio in the small clutches of parents on a HQ diet and a female-biased sex ratio in large clutches (but the sample sizes were small). The strongest biases were produced by these HQ mothers, who were best able to pay any potential costs associated with manipulating offspring sex. These biases are partly in line with adaptive explanations for sex allocation in this species. Daughters have been shown to benefit disproportionately from being reared in small broods (De Kogel 1997) and under good nutritional conditions (Kilner 1998) compared with sons. Thus, if parents are in prime condition then they will accrue relatively higher fitness gains from producing many large fecund daughters rather than sons. Conversely, when parents are relatively malnourished, their best ploy is to rear only daughters in small broods. More clearly defined theoretical predictions need to be developed concerning sex allocation in response to food availability and family size, because energetic requirements and competitive ability might have conflicting effects on the sexes, for example.

This study did not find that mothers were favouring one sex over another by laying them in a better position in the sequence, as has been found in some species (Ryder & Termaat 1987; Nager *et al.* 1999; Badyaev *et al.* 2002; but see Cordero *et al.* 2000; Arnold & Griffiths 2003). Although significant laying-order and diet interactions have been found in zebra finches, the patterns are not consistent between studies (Clotfelter 1996; Kilner 1998; Rutkowska & Cichon 2002).

Approximately 5% of eggs seemed to be infertile and could not be sexed. We tried to quantify the influence of all missing samples on our interpretation of the data by creating worst-case replacement scenarios, but this did not alter our results. In addition, the proportion of infertile eggs did not differ between diet treatments. Thus, we did not have any evidence that either sex-specific fertilization of ova (Krackow 2002) or early embryo mortality were occurring. By comparison with many avian studies, we measured sex ratio at an earlier stage in development, thus minimizing the impact of differential mortality on the primary sex-ratio estimates. Embryo mortality can occur throughout incubation and can be sex-biased (Whittingham & Dunn 2000), although this has not been found in zebra finches (Gorman 2002). Additionally, in a number of species, including zebra finches (Bradbury & Blakey 1998; Kilner 1998), nestlings show sex-differential mortality under different nutritional conditions. Collecting eggs soon after laying and using a molecular technique enhances the likelihood that the observed sex ratio is that at conception. While we have demonstrated that zebra finches can modify egg sex ratio, this occurs only in certain situations. Egg sex-ratio variation in this species appears to be much subtler than previously suggested.

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