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Maternal testosterone affects the primary sex ratio and offspring survival in zebra finches

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Female birds have repeatedly been reported to adjust the primary sex ratio of their offspring to environmental, social and physiological cues. However, the mechanism behind sex adjustment remains unknown. It has been suggested that maternal hormones may constitute an important mediator in this mechanism, as androgen levels differ between eggs bearing male and female embryos. To evaluate whether the level of maternal androgens affects the offspring sex ratio, we injected female zebra finches, *Taeniopygia guttata*, with testosterone during egg laying. The sex ratio of eggs laid after testosterone administration became significantly male biased, compared to eggs laid by control females that received a vehicle injection. However, sons of testosterone-treated females suffered lower hatching success. In contrast, daughters seemed to benefit from elevated androgen level in terms of future survival prospects. The opposite effects on male and female offspring may constitute an important constraint on maternal androgen allocation to the eggs and reduce the benefits of biasing the sex ratio towards males by increasing the testosterone level.

In birds, females are the heterogametic sex, bearing sex chromosomes Z and W, while males have ZZ; thus, females have the potential ability to control offspring sex (Pike & Petrie 2003). Follicles developing in the ovary contain both sets of chromosomes until the first meiotic division during which one of them is consigned to the polar body and the other remains in the ovum (Johnson 2000). In several bird species, chromosome segregation has been shown to occur 0.5–3 h before ovulation (Warren & Scott 1935; Olsen & Fraps 1944; Birenkott et al. 1988; Johnson 1996). Thus, fine-tuned adjustment of offspring sex should be possible even at the within-clutch level. For example, offspring sex covaries with laying order in several bird species (reviewed in Krackow 1995), so that the more valuable or more demanding sex is placed in the more advantageous position in the laying order (Kilner 1998; Badyaev et al. 2002). Furthermore, there is some evidence that females may respond to an immediate change in the environment, for example in food quality during egg laying, by adjusting offspring sex accordingly (Rutkowska & Cichoń 2002).

Such a precise mechanism of sex adjustment may be mediated by maternal hormones (Krackow 1995), especially androgens as eggs containing male embryos have higher yolk androgen concentrations (Petrie et al. 2001). The relation between maternal androgens and offspring sex might, however, depend on the females' social status (Müller et al. 2002) and diet (Rutstein et al. 2005). Evidence for a potential role of androgens in sex determination comes from studies showing that females mated to attractive partners lay eggs with a high androgen content (Gil et al. 1999, 2004; von Engelhardt 2004; but see Rutstein et al. 2004) and that such females produce male-biased clutches (Sheldon et al. 1999). However, evidence that experimentally manipulated maternal androgen level affects offspring sex has been lacking. A recent study on the free-living spotless starling, *Sturnus unicolor*, showed that females implanted with testosterone prior to egg laying overproduced sons, but this male-skewed sex ratio persisted in clutches laid in subsequent breeding seasons, long after the implants had depleted (Veiga et al. 2004).

We studied the potential role of androgens in determining offspring sex in a more rigorous laboratory experimental set-up. In our study, females were reared individually with randomly assigned partners, which controlled for potentially confounding effects of male quality and social interactions on egg androgen content and sex allocation. We manipulated maternal hormonal status by injecting zebra finch, *Taeniopygia guttata*, females with testosterone on the day the first egg in a clutch was laid (cf. Hackl et al. 2003). This ensured that females

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were manipulated at exactly the same stage of a breeding cycle. Our previous study showed that administration of 20 μg of testosterone to the female after the first egg was laid resulted in a significant increase in androgen concentration in the yolk of the third, fourth and fifth eggs in comparison to the first egg of a clutch (Rutkowska et al. 2005). In a control group, androgen concentrations in the eggs decreased with laying order (Rutkowska et al. 2005; see also Gil et al. 1999, 2004; von Engelhardt 2004; Rutstein et al. 2005). In the present study, we administrated testosterone after the first egg in a clutch had already been laid and when the second ovum had already been ovulated, so the testosterone should have an effect, if any, in the third and subsequently laid eggs. We predicted that testosterone-treated females should have a higher proportion of males in eggs laid later in the laying sequence than control females. We also followed offspring survival to independence to see how maternal androgens affect viability of sons and daughters. To date, only one study (von Engelhardt et al. 2004) that manipulated maternal hormone levels has looked at the survival of resulting offspring.

METHODS

Zebra finches originating from our laboratory colony were kept in an air-conditioned room at $21 \pm 2^{\circ}C$, under a 13:11 h incandescent light:dark regime, with lights on at 0700 hours. Birds were fed ad libitum with a standard mixture of seeds (Megan, Krakow, Poland), along with a mixture of hardboiled egg chopped with finely grated carrot. Birds also received a cuttlebone, grit and vitamins: C, A, B_1, B_6, B_{12}, D_3, K (Multivit, Tropical, Katowice, Poland). Housing conditions were kept constant during the experiment. All birds were initially maintained in a common aviary, where they could mate freely and rear one brood. Sexes were then separated for 5 months and randomly paired again in visually separated, individual cages (75 × 30 cm and 40 cm high) equipped with external nestboxes and nesting material. No negative effects of separation and regrouping were noted.

We inspected nestboxes of paired birds every morning between 0900 and 1000 hours to record nest building and egg laying, as well as to label new eggs with a nontoxic marker. In two clutches, laying was apparently interrupted for a day. However, since we could not be sure that an egg had not been laid, we numbered the subsequent eggs after the interruption as though an egg had been laid. Every second female that started a clutch received a subcutaneous injection of 20 μg of testosteronum enanthanum (Jelfa S.A., Jelenia Góra, Poland) dissolved in 50 μl of oil (liquid paraffin) in the inguinal region between 1000 and 1300 hours on the day when the first egg was laid. Every other female received only vehicle (50 μl of oil) as a control. Females in the experimental and control groups did not differ in body mass (ANOVA: $F_{1,\ 32} = 0.04$, $P = 0.83$) or in the mass of the first egg in a clutch (ANOVA: $F_{1,\ 32} = 0.58$, $P = 0.45$). To minimize stress related to manipulations, we prepared syringes in advance and the female was injected within 3 min of capture, always by the same person experienced with this procedure. We used sterile insulin syringes with integrated 29-gauge needles (Becton Dickinson, Dublin, Ireland) under licence from the Local Ethical Committee. Females did not show any visible reaction to the injections and showed no signs of abnormal behaviour when returned to their home cage. Birds were also monitored for a few hours and after the injection showed no signs of stress or ill-effects. Injection of testosterone elevates yolk androgen concentrations in the third, fourth and fifth eggs by an average of 17% in comparison to the first egg of a clutch (Rutkowska et al. 2005). This increase is within the range of yolk androgen concentrations observed among eggs laid by nonmanipulated females. Among eggs laid by control females, androgen content decreases with the laying order: in the third, fifth and sixth eggs androgen concentration is on average 6% lower than in the first egg of a clutch (Rutkowska et al. 2005). The injection per se does not affect the androgen content of the eggs nor the females' behaviour (Rutkowska et al. 2005). We do not have data on of testosterone levels in the females after injection, because taking blood samples (200 μl) from the females during clutch formation would interfere with egg production, but according to Hackl et al. (2003) injected testosterone disappears from females' circulation within a day.

On the day of expected hatching, we checked nests hourly and at night (between 2000 and 0800 hours) we transferred eggs to separate compartments in an incubator chamber (humidity ca. 70%, temperature 36.4°C). This enabled us to determine which hatchling came from which egg. We marked newly hatched chicks by clipping one of their nails, then returned them to the nest. We selected pairs of nests with similar clutch sizes (±1 egg), in which hatching started on the same day: one from the experimental group and one from the control group. To control for any effect of differences in rearing environment caused by the treatment, we cross-fostered two nestlings, between each pair at hatching: one from eggs 1 or 2 and the other from eggs 3 or 4. (Swapped nestlings were matched for the position of the egg in the laying sequence.) Experimental and control foster females did not differ in their mean brood size after manipulation (4.4 for testosterone-treated females versus 4.7 for control females; $F_{1,\ 32} = 0.33$, $P = 0.570$) and the mean proportion of males is the brood (0.58 for testosterone-treated females versus 0.48 for control females; $F_{1,\ 32} = 1.10$, $P = 0.302$). Survival did not differ between cross-fostered and noncross fostered chicks ($F_{1,\ 131} = 1.20$, $P = 0.275$). At the age of 2 weeks, chicks were ringed with individually numbered aluminium rings and their survival was followed until 50 days of age, when they were separated from the parents.

In each group, 17 females laid fertile clutches consisting of 187 eggs in total. We determined the sex of offspring by plumage characteristics ($N = 133$). Embryos that failed to hatch, and chicks that died before we could sex them by plumage, were sexed by molecular techniques ($N = 34$). DNA was extracted with Chelex and the CHD-W and CHD-Z genes from the sex chromosomes were amplified using PCR with primers P2 and P8 (Griffiths et al. 1998). The protocol was as described in Rutkowska & Cichoń

(2002). We were not able to sex 20 eggs: six were broken or missing, 14 were clearly infertile or repeated trials did not give PCR products. Twelve of the unsexed eggs were laid by control and eight by testosterone-treated females.

We analysed the probability that an egg contained a male embryo and the probabilities of hatching and survival by fitting a generalized linear mixed model using the GLIMMIX macro in SAS version 8 (SAS 2000) with logit link function and binomial error variance (Krackow & Tkadlec 2001). In the statistical models we introduced experimental treatment as a class variable and laying sequence as a covariate. The assumption of a monotonic relation between covariate and response variable in GLIMMIX might be violated, so we also analysed the experimental data by dividing eggs into two categorical groups: initial eggs (numbered 1 and 2) that were not influenced by the treatment, and late-laid eggs (numbered 3, 4, etc.) that were affected by the treatment. All analyses took into account random factors: female identity was included in all models and foster female identity was also included in analyses of offspring survival. Interaction of experimental treatment and laying sequence was the central interest of the study, because we predicted that the proportion of male offspring should increase with laying sequence in the testosterone-treated group and it should differ from that observed in the control group. In the analyses of survival probability, offspring sex was introduced as a class variable. Nonsignificant interactions were sequentially excluded from the model to increase the power of the test.

The study was carried out under licence from the Local Ethical Committee at the Jagiellonian University.

Table 1. Results of the GLIMMIX analyses in which egg sex, hatching success and survival probability were examined in relation to experimental treatment and laying sequence

Source of variation	*df*	*F*	*P*
Egg sex			
Experimental treatment	1, 158	3.69	0.056
Laying sequence	1, 141	0.31	0.581
Experimental treatment*laying sequence	1, 163	5.54	0.020
Female ID: Estimate±SE=0.04±0.23; Z=0.17, P=0.432			
Male hatching success			
Experimental treatment	1, 79.9	5.74	0.019
Laying sequence	1, 95.2	8.46	0.004
Experimental treatment*laying sequence	1, 95.2	9.83	0.002
Female ID: Estimate±SE=4.45±1.73; Z=2.58, P=0.005			
Survival			
Experimental treatment	1, 29.2	1.04	0.317
Laying sequence	1, 133	13.00	<0.001
Sex	1, 145	0.94	0.333
Experimental treatment*sex	1, 145	6.62	0.011
Female ID: Estimate±SE=0.45±0.77; Z=0.59, P=0.28			
Foster female ID: Estimate±SE=4.39±1.73; Z=2.39, P=0.008			

All analyses took into account random factors: female identity (ID) was included in all models and foster female identity (ID) was included in analyses of offspring survival. Hatching success was analysed only for male offspring, because all female offspring hatched.

RESULTS

Experimental and control females did not differ in duration of incubation (ANOVA: $F_{1,\ 32} = 2.56$, $P = 0.1$) or clutch size ($F_{1,\ 32} = 0.06$, $P = 0.8$). Egg mass increased with the laying order in both groups ($F_{1,\ 32} = 58.41$, $P < 0.001$), but did not differ between groups ($F_{1,\ 32} = 0.09$, $P = 0.8$).

Overall, eggs laid by testosterone-treated females were more likely to contain male embryos than eggs of the control females, and the difference was close to significance (Table 1, Fig. 1a). As predicted, eggs laid late in the laying sequence after administration of testosterone were more likely to contain male embryos than those laid by control females, as indicated by the significant interaction between treatment and egg-laying sequence (Table 1, Fig. 1a). Within the control group the sex ratio did not change with the laying sequence (GLIMMIX accounting for female identity, laying sequence: $F_{1,\ 67} = 2.19$, $P = 0.14$), whereas it became significantly more male biased in late-laid eggs of testosterone-treated females (GLIMMIX accounting for female identity, laying sequence: $F_{1,\ 81.3} = 4.09$, $P = 0.046$). In the analysis in which eggs were divided into two categorical groups (two initial eggs that were not influenced by the testosterone injection, and late-laid eggs that were affected by the treatment), the interaction between experimental treatment and egg category was not significant (GLIMMIX accounting for female identity, experimental treatment: $F_{1,\ 163} = 0.02$, $P = 0.902$; egg category: $F_{1,\ 138} = 0.01$, $P = 0.923$; treatment * egg category: $F_{1,\ 152} = 2.93$, $P = 0.089$).

All eggs containing female embryos hatched successfully in both groups (Fig. 1b). In contrast, eggs containing male embryos that were produced by testosterone-treated mothers had significantly lower hatching success than those laid by control females (Table 1, Fig. 1b). The significant interaction between treatment and laying sequence indicates that male hatching success varied with laying sequence in the two groups (Table 1, Fig. 1b): it increased in the control group (GLIMMIX accounting for female identity, laying sequence: $F_{1,\ 46.5} = 14.81$, $P = 0.0004$) but not in the testosterone-treated group (GLIMMIX accounting for female identity, laying sequence: $F_{1,\ 46.9} = 0.03$, $P = 0.9$). Testosterone treatment also had different effects on the survival of male and female offspring, as indicated by the significant interaction between treatment and sex (Table 1, Fig. 1c). Female chicks that hatched from late-laid eggs in the testosterone-treated group survived better than those hatched from late-laid eggs in the control group (GLIMMIX accounting for female identity and foster female identity: treatment: $F_{1,\ 68.7} = 6.33$, $P = 0.014$; laying sequence: $F_{1,\ 66.4} = 0.01$, $P = 0.9$; treatment * laying sequence: $F_{1,\ 66.4} = 8.76$, $P = 0.004$; Fig. 1c). All females that hatched from eggs laid after testosterone treatment of the mothers survived until independence, whereas survival of females in the control group decreased with the laying order. Testosterone treatment had no effect on survival of sons (in the same statistical model all $P > 0.1$).

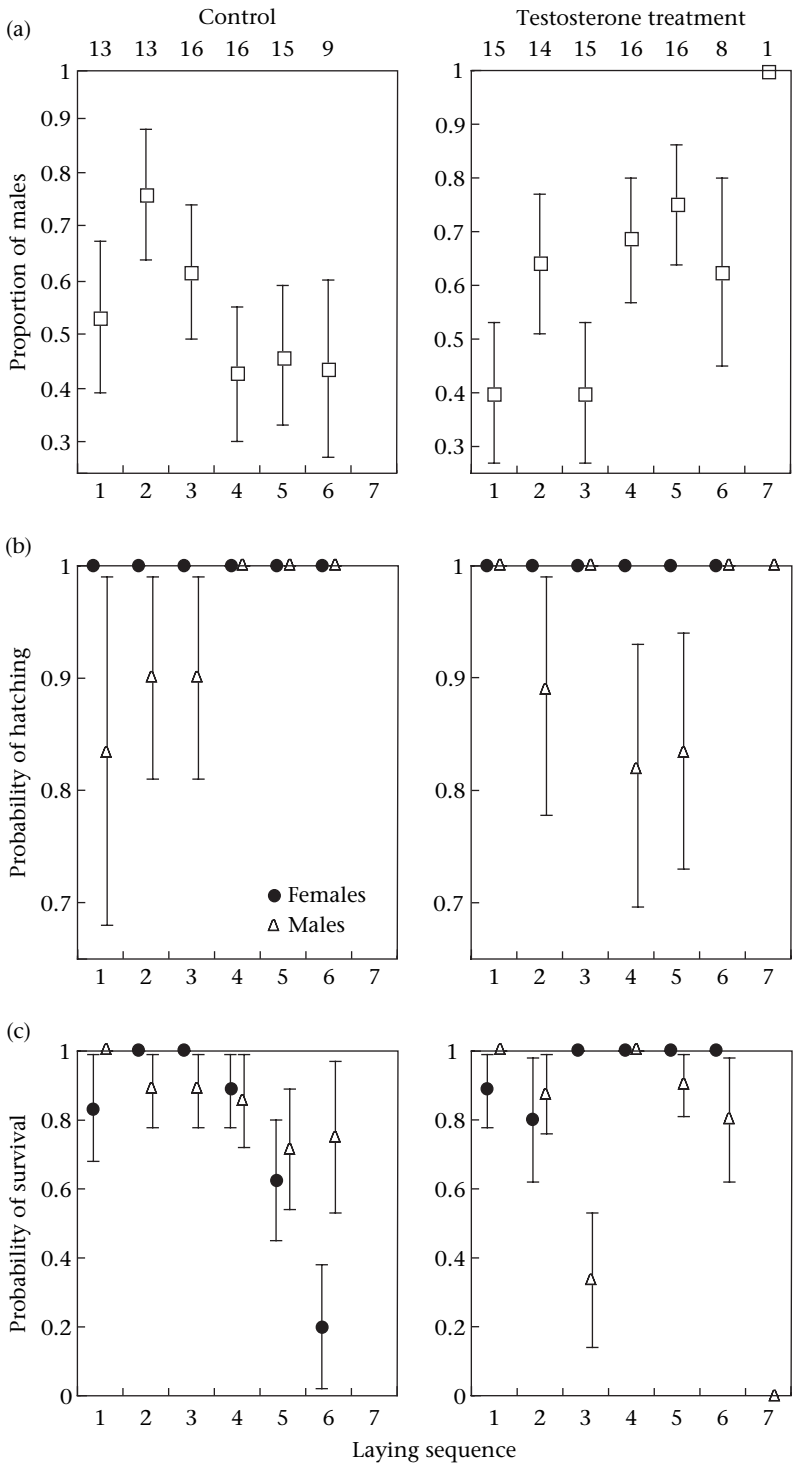

Figure 1. (a) Primary sex ratio (numbers denote sample sizes); (b) hatching success of female and male eggs; (c) probability of survival from hatching to 50 days of age of female and male offspring laid by control and testosterone-treated females. Proportions ± SE are presented. Exclusion of the single seventh egg laid by a testosterone-treated female does not change the results of the analyses.

DISCUSSION

We found that the proportion of sons in consecutively laid eggs increased for females treated with testosterone, but not for control females. In our population of zebra finches, the sex ratio of eggs laid by nonmanipulated females typically does not change with the laying order (Rutkowska & Cichoń 2005, unpublished data). However, in the more conservative analysis, in which eggs were divided into two categories, although there was a trend in the same direction, the expected interaction between the treatment and egg category was not significant. We also

found that elevated levels of androgens in eggs were associated with low hatching success of males but with improved survival of females.

A few studies have already manipulated maternal hormones to investigate their potential role in the determination of the primary sex ratio of offspring. In the domestic chicken, *Gallus gallus domesticus*, females injected with progesterone produced more female offspring (Correa et al. 2005). In the Japanese quail, *Coturnix japonica*, females implanted with corticosterone also produced more daughters, whereas implantation of testosterone and 17-β-oestradiol had no significant effect on the primary sex ratio (Pike & Petrie 2006). Similarly, no distortion of the primary sex ratio was observed in female zebra finches injected with 17-β-oestradiol (von Engelhardt et al. 2004). Our experiment seems to corroborate the finding on the spotless starling (Veiga et al. 2004), that elevated maternal androgens may indeed result in a male-biased primary sex ratio.

Thus, androgens might constitute an important mediator of the mechanism of offspring sex determination. Exposure of the oocyte to elevated androgen levels may affect chromosome segregation during meiosis by influencing, for instance, the elasticity and movements of the meiotic spindles (Olsen 1942; Yoshimura et al. 1993a, b). Alternatively, it may affect levels of other hormones, both in the mother and in the egg, which could result in nonrandom chromosome segregation. However, testosterone implants in Japanese quail do not alter the females' corticosterone or oestradiol levels (Pike & Petrie 2006).

Whether offspring sex is influenced more by hormones circulating in the bloodstream of the mother or by hormones deposited in the yolk during egg production is also unknown. In female Japanese quail, testosterone is apparently metabolized within a day of administration, whereas it is present in eggs laid on the next few days (Hackl et al. 2003). We can assume that in our experiment circulating testosterone was also metabolized soon after injection, but it was significantly elevated in the yolk (Rutkowska et al. 2005). This may indicate that elevated yolk, rather than circulating androgens, are responsible for the meiotic drive leading to a biased offspring sex ratio.

A number of studies have shown positive effects of experimentally elevated androgen levels in the eggs on offspring performance, such as increased growth rate, begging rate and social status (Schwabl 1996; Lipar & Ketterson 2000; Eising et al. 2001; but see Sockman & Schwabl 2000 for negative effects of androgens in the American kestrel, *Falco sparverius*). In consequence, elevated androgen deposition in eggs has often been considered to reflect allocation of resources by the female to the offspring (reviewed in Gil 2003). However, in these studies offspring sex was not investigated. In our study, we have shown that elevated androgen level had a positive effect on daughters but a negative one on sons. However, the effects of androgens on male and female offspring that we observed should be interpreted with caution because offspring sex was dependent on the hormonal treatment. Nevertheless, our finding corroborates a recent study in which in-ovo injection of testosterone in zebra finches had opposite effects on the offspring of the two sexes, which was expressed as impaired growth of male offspring but enhanced growth of females (von Engelhardt et al. 2006). Such sex-specific effects of androgens on juvenile birds was further supported by in-ovo injection of Flutamide, which blocks androgen receptors, so that offspring perceive reduced levels of androgens; the treatment resulted in enhanced growth of male offspring, but reduced growth in female offspring in the black-headed gull, *Larus ridibundus* (Müller et al. 2005). Altogether, these findings show that elevated androgens seem to be beneficial for female but detrimental for male offspring. It suggests the existence of an important constraint on maternal hormone allocation. If an increase in yolk androgens within a range of natural concentrations has positive effects in one sex and negative effects in the other, this would shape an optimal, probably sex-specific, maternal allocation of androgens. Given that an elevated level of androgens has detrimental effects on male offspring, we conclude that if androgens mediate the determination of offspring sex, the benefits of biasing the sex ratio towards males by increasing the level of testosterone may be limited.

In summary, our study shows that androgens may constitute an important maternal effect which may mediate the determination of offspring sex. However, if androgens were the sole factor responsible for a male-biased sex ratio we would expect a much stronger effect of our treatment. We therefore suggest that some other factors, such as other hormones, must be involved in offspring sex adjustment. Furthermore, as androgens had negative effects on male offspring we conclude that an androgen-mediated mechanism of sex determination seems to be costly and thus it may generally constrain maternal hormone allocation.

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