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ORIGINAL PAPER

Androgen-dependent maternal effects on offspring fitness in zebra finches

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Abstract There is accumulating evidence that maternal hormones may play a role in offspring sex adjustment, but little is known about the costs of such hormone-mediated mechanisms. Recent studies have reported sex-specific effects of hormones on offspring viability. Specifically, we previously found that elevating the plasma androgen level in mothers results in a male-biased offspring primary sex ratio, but it affects the viability of sons negatively and daughters positively in zebra finches (*Taeniopygia guttata*; Rutkowska and Cichoń, Anim Behav, 71:1283–1288, 2006). In this study, we studied further fitness consequences of exposure to elevated yolk androgen levels in zebra finches. We measured growth rate and cellular immune response of nestlings that hatched from eggs laid by females injected with testosterone during egg laying and nestlings of unaffected control females. We found that sons of testosterone-treated females grew slower in comparison to sons of control females. The significant interaction between experimental group and offspring sex indicates that sons of testosterone-treated mothers suffered impaired immune responsiveness while daughters seemed to benefit from elevated androgen level in terms of enhanced immune responsiveness. We found no effects of androgens on offspring performance at adulthood—neither fecundity of females nor attractiveness of males was affected. We conclude that the benefits of biasing sex ratio towards males by increasing androgen level in the yolk may be limited due to negative effects on male offspring performance early in life.

Keywords Growth rate · Cellular immune response · Fecundity · Attractiveness · Sex-specific effects · Testosterone · *Taeniopygia guttata*

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Introduction

Maternal effects are genetic and nongenetic factors that affect offspring (Mousseau and Fox 1998). In birds, several substances that are present in the eggs even in small concentration can have profound influence on offspring fitness. Those factors include hormones (Schwabl 1996; Eising et al. 2001), immunoglobulins (Saino et al. 2001), and antioxidants (Royle et al. 2001). Among them, androgens have gained particular interest. Male sexual hormones are produced in female birds in small concentrations by the adrenal gland and theca cells (Staub and De Beer 1997). Theca interna cells are specialized in steroid production—they are situated at the surface of follicles and are probably the main source of androgens in the egg (Johnson 2000; Hackl et al. 2003). Because the androgen concentration in the yolk of freshly laid eggs is several times higher than the concentration of androgens in the female's bloodstream (Groothuis et al. 2005b), their deposition may not be passive, and deposited androgens may have important functions. Indeed, there is substantial evidence that maternal androgens play a role in offspring sex adjustment and affect nestling performance in birds.

The evidence for androgen-mediated male-biased offspring primary sex ratio comes from correlative data on the relationship between high yolk or female plasma androgen levels and nestling sex ratio found in the peafowl (*Pavo cristatus*; Petrie et al. 2001; Pike and Petrie 2005) and the house finch (*Carpodacus mexicanus*; Badyaev et al. 2005), and from studies in which female androgen level was experimentally manipulated before egg laying in the spotless starling (*Sturnus unicolor*; Veiga et al. 2004) or during egg laying in the zebra finch (*Taeniopygia guttata*; Rutkowska and Cichoń 2006). However, the observed effect of androgens seems to be rather weak, indicating that there might be other important factors explaining variation in sex ratio. The limited applicability of androgen-mediated mechanisms of offspring sex determination may also stem from the negative effects of androgens on the female (e.g., Searcy 1988; Rutkowska et al. 2005) or her offspring.

A number of studies have manipulated androgen levels in the yolk and found that at early life stage androgens have positive effects on hatching time, begging rate, and growth (e.g., Schwabl 1996; Lipar and Ketterson 2000; Eising et al. 2001; but see Sockman and Schwabl 2000 for negative effects in the American kestrel, *Falco sparverius*). Recent studies have pointed to the fact that enhanced performance in a given trait may constrain other physiological functions (Andersson et al. 2004; Groothuis et al. 2005a; Navara et al. 2005). For example, androgens accelerate growth rate at the cost of lower immune response in the black-headed gull (*Larus ridibundus*; Groothuis et al. 2005a,b) and in the eastern bluebird (*Sialia sialis*; Navara et al. 2005). In our previous study (Rutkowska and Cichoń 2006), we showed that hatching success of males coming from eggs laid by testosterone (T)-treated zebra finch females was reduced. T-treatment had no effect on post-hatching survival of sons but it significantly improved survival of daughters. In fact, all female nestlings that hatched from eggs laid after T-treatment of the mothers survived until independence, while survival of females in control group decreased with laying order (Rutkowska and Cichoń 2006).

Thus, any benefits from overproduction of sons due to androgen-mediated mechanisms may be outweighed by reduced performance of sons caused by elevated androgen levels. In the present paper, we further study phenotypic effects of androgens. We looked at offspring growth and cell-mediated immune response. We also assessed reproductive success of females and attractiveness of males coming from eggs laid by T-treated females.

Methods

Zebra finches used in the present study originated from the either experimental clutches laid by females injected with testosterone after laying the first egg or from control clutches laid by unaffected females. Briefly, every second female that started egg laying received a subcutaneous injection of 20 μg T of prolonged activity (*testosteronum enanthanum*, Jelfa SA, Poland) dissolved in 50 μl of oil (*paraffinum liquidum*) in the inguinal region between 1000 and 1300 h at the day when the first egg was laid. Every other female received only vehicle (50 μl of oil) which constituted a control (Rutkowska and Cichoń 2006). In T-treated females, the injection results in ca 17% increase in yolk androgen content in late laid eggs in relation to the first eggs of a clutch, while in control females yolk androgens decreased with the laying sequence (Rutkowska et al. 2005). We therefore expected that effects of androgens should appear as the significant interactions of the experimental group and egg laying sequence. The injection per se does not affect androgen content of the eggs nor females' behavior (Rutkowska et al. 2005).

Newly hatched chicks were weighed to the nearest 0.01 g, marked by nail clipping and returned to the nest. There were no differences in hatchling mass between the groups; however, because our experiment was based on manipulation of androgen levels in the female, this might have caused differences in egg quality (such as yolk size) and/or alteration of female behavior that we could not detect. In our previous studies, we did not find any effects of manipulation on female behavior (Rutkowska et al. 2005; Rutkowska and Cichoń 2006), but we can not exclude the possibility that some subtle differences, such as incubation pattern, influenced growth of offspring coming from the first two eggs of a clutch and this might have, in turn, affected nestlings from later laid eggs. To control for such differences, we cross-fostered chicks and included foster female ID in the analyses.

Nests of the two experimental groups which started hatching on the same day and had similar brood size (±1 egg) were matched as pairs. Two nestlings were cross-fostered between each pair at hatching: one hatched from eggs 1 or 2 and the other one from the eggs 3 or 4 (swapped nestlings were matched for the position of the egg in the laying sequence; see Rutkowska and Cichoń 2006, for more details). Nestlings were weighed every second day after hatching with an electronic balance: on day 2 to the nearest 0.01 g, and later on to the nearest 0.1 g until they were 12 days old. At the age of 2 weeks, nestlings were ringed with individually numbered aluminum rings.

To assess nestling immunocompetence, 12-day old nestlings were injected with a nonpathogenic antigen, phytohemagglutinin (PHA; Sigma). PHA is a lectin of mitogenic action on T cells, and its subcutaneous injection results in infiltration of macrophages and accumulation of lymphocytes. The solution of 0.2 mg PHA in 0.04 ml saline was injected subcutaneously into the wing web. Before the

injection and 24 h later, the thickness of the wing web was measured with a pressure sensitive calliper. The difference between wing thickness prior to and 24 h after the injection is a measure of immune response to the antigen. This method is widely used for the assessment of immunocompetence in birds (e.g., Groothuis et al. 2005a; Navara et al. 2005).

Fecundity of daughters of T-treated females was assessed for 1-year-old individuals. We randomly paired females with sexually experienced males from our stock population and we followed their reproductive performance. We recorded clutch size and number of fledglings produced during the first breeding attempt of the pairs.

Sons of T-treated females were subjected to preference tests in a two-way choice situation similar to the one used by von Engelhardt (2004). Stimulus males tested together were from opposite treatments and were matched for egg laying position. The test females were randomly chosen from our stock population and were not related to the stimulus males. The test female was placed in the cage (Fig. 1) for half an hour for acclimation, after which the stimulus males were released into the cage. After 10 min, observations were started. During the 20-min test, we noted the time that a female spent at the side of each stimulus male. The preference score was expressed in minutes spent at the side of a given stimulus male.

Statistical analyses

Changes in body mass in 2-day intervals (i.e., growth rate) of the offspring were analyzed using repeated measures Anova, after log-transformation of the data. The canonical value (a linear combination of a set of original variables in which the within-set correlation has been controlled) resulting from this analysis described the growth rate. This value and the immune response were analyzed using General Linear Mixed Model (GLMM). In the statistical models, we introduced experimental treatment and offspring sex as class variables and position of the egg in laying sequence from which the bird hatched as a covariate. Behavior of adult birds was analyzed separately for each sex, and the statistical model included experimental treatment, laying sequence and their interaction. In all analyses, we controlled for female and foster female identities defined as random factors. Nonsignificant interactions were sequentially excluded from the model to increase power of the tests. All analyses were performed in SAS version 9.

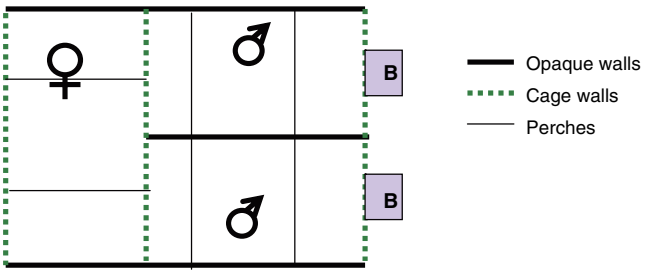

Fig. 1 Schematic drawing of the cage used for preference tests. Two stimulus males were placed in 30×40×40 cm high cages with a nest box and two perches each and could not see each other. The test female could see both males and had one perch at each side of her cage that measured 60×30×40 cm high

Results

We found that offspring growth rate did not differ between the experimental and control group and was not related to the offspring sex or position of the egg in the laying sequence (Table 1). However, the significant interactions of experimental group × offspring sex and experimental group × offspring sex × laying sequence (Table 1) suggests that T administration to the mothers differentiated growth of male and female offspring originating from subsequently laid eggs. Thus, we performed separate analyses for the two sexes. Among female offspring, there were no significant differences in growth rate and the interaction of group × laying sequence was not significant (GLMM, all $P>0.2$). Growth rate of males was higher in the T-treated than in the control group but was not related to the egg laying sequence (GLMM controlling for female and foster female IDs, experimental group: $F_{1,\ 39.8}=7.15$, $P=0.011$; egg laying sequence: $F_{1,\ 59.6}<0.001$, $P=0.96$). However, there was a significant interaction of experimental group × laying sequence ($F_{1,\ 60.3}=6.71$, $P=0.012$), which results from the fact that males coming from late laid eggs of T-treated mothers grew slower than males from late-laid eggs of control mothers (Fig. 2a), indicating that maternal androgens suppress growth rate of male offspring.

There were no significant differences in immune response between the two experimental groups or sexes, and the immune response was not related to egg laying sequence (Table 1). Yet, the significant interaction between experimental group and offspring sex suggests that T administration to the mother had a different effect on female and male offspring—positive on daughters and negative on sons (Fig. 2b). Analyses performed separately for the two sexes did not show significant differences between the groups, probably due to small sample size.

We found no effects of androgen treatment on daughters' reproductive success (number of eggs laid: GLMM, all $P>0.35$; number of fledglings: GLMM, all $P>0.40$). Similarly, attractiveness of sons tested in the mate-choice trials was not affected by the treatment (GLMM, all $P>0.2$).

Discussion

Maternal androgens in eggs are suggested to be important factors affecting sex allocation. In our previous study, we

Table 1 Results of the general linear mixed model analyses in which growth rate and cellular immune response of male and female offspring were examined in relation to experimental group and egg laying sequence

Source of variance	Degrees of freedom	*F*	*P* value	Variance ± SE	Z
Growth rate					
Group	1, 80.9	0.25	0.615		
Laying order	1, 101	0.57	0.450		
Sex	1, 112	0.05	0.818		
Group × sex	**1, 109**	**11.81**	**0.0008**		
Group × laying order	1, 107	0.13	0.724		
Laying order × sex	1, 114	0.04	0.849		
Group ×laying order × sex	**1, 110**	**10.87**	**0.0013**		
Female ID			0.263	0.0007±0.001	0.63
Foster female ID			0.028	0.0035±0.002	1.91
Cellular immune response					
Group	1, 32	0.50	0.484		
Laying order	1, 113	0.00	0.971		
Sex	1, 130	1.39	0.534		
Group × sex	**1, 129**	**4.16**	**0.043**		
Female ID			0.163	155.03±158.01	0.98
Foster female ID			0.387	41.41±114.2	0.29

Female and Foster female IDs were introduced as random terms. Significant results are in bold.

demonstrated that elevated yolk androgens enhanced viability of females compared to male offspring (Rutkowska and Cichoń 2006). In the present study, we provide further confirmation of the negative effects of androgens on male offspring. We show that exposure to elevated levels of yolk androgens reduced growth rate of the male nestlings. It also

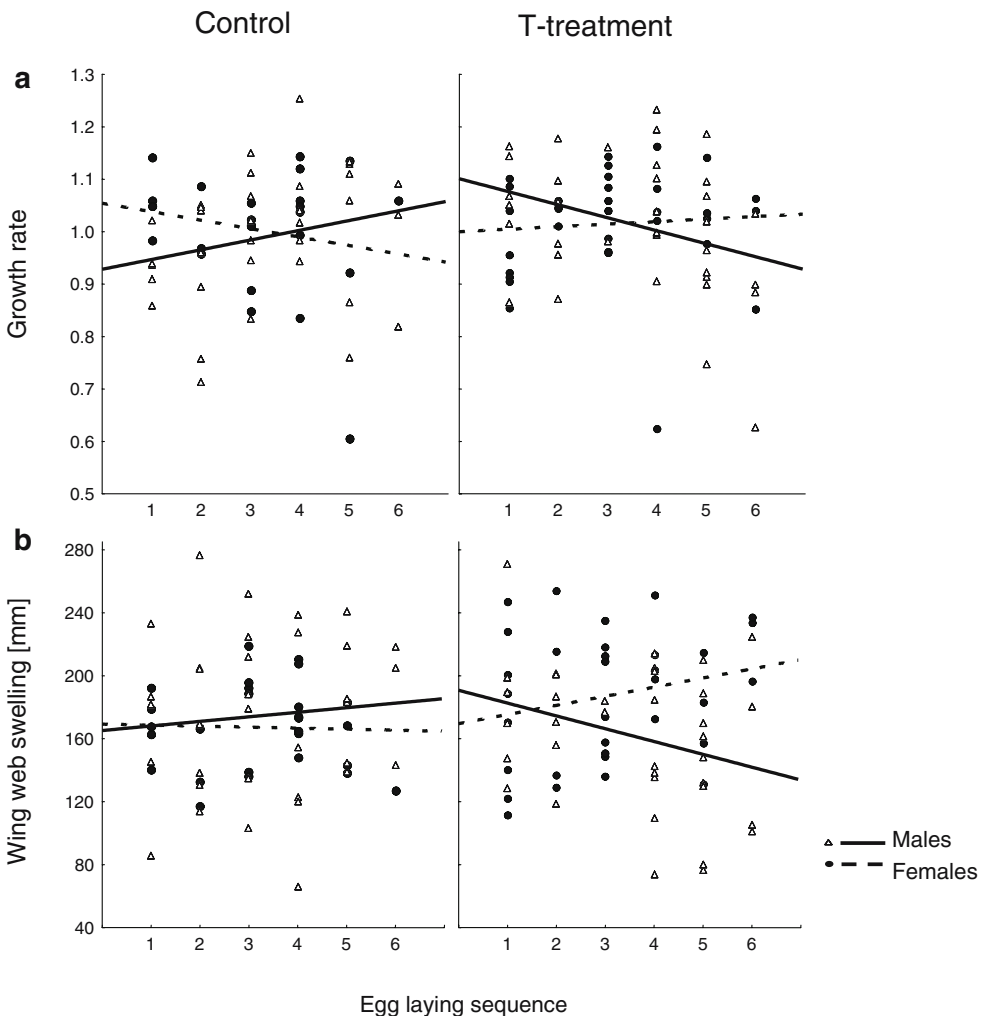

Fig. 2 **a** Growth rate and **b** cellular immune response of male and female offspring produced by control and testosterone-treated females. In the control group, females were injected with oil while in the experimental group, they received testosterone injection after laying the first egg of a clutch which resulted in elevation of yolk androgens in late-laid eggs

differentiated immune response of the offspring: the significant interaction between experimental group and offspring sex indicates that sons of T-treated mothers suffered impaired immune responsiveness while daughters seemed to benefit from elevated androgen level in terms of enhanced immunocompetence. Differences in growth rate could have affected offspring survival via their influence on within-brood competition, while altered immune function could have constituted an intrinsic source of mortality. We also found no effects of androgen exposure on female reproductive performance or male attractiveness at adulthood.

Our results contribute to studies showing the ambivalent effects of androgens. In particular, we corroborate a recent study in which in ovo injection of T in the zebra finch had opposite effects on offspring of the two sexes, which was expressed as impaired growth of male offspring but enhanced growth and begging of females (von Engelhardt et al. 2006). The sex-specific effects of androgens on juvenile birds were also demonstrated by in ovo injection of Flutamide—a substance that blocks androgen receptors. The treatment resulted in enhanced growth rate of male offspring, but reduced growth and cellular immune response in female offspring in the black-headed gull (Müller et al. 2005). Thus, elevated yolk androgens produced by three different methods, testosterone injection to the laying female (present study), to the egg (von Engelhardt et al. 2006) and injection of an antiandrogen to the egg (Müller et al. 2005), provide evidence that androgen exposure may be disadvantageous for male offspring. This may result from the fact that, in male offspring, elevated maternal androgens add to the already higher (compared to female offspring) endogenous production of these hormones or from feminizing effects of estrogens, to which testosterone can be easily aromatized. The negative effect of androgens on male immune response can be attributed to their immunosuppressive function (Folstand and Karter 1992; Ketterson and Nolan 1999). In daughters, elevated maternal androgens could have enhanced their competitive ability (von Engelhardt et al. 2006), which helped them gain more resources and raise a higher immune response. Contrary to the above findings, the injection of androgens to eggs of barn swallows (*Hirundo rustica*) reduced body size of females while it enhanced body size in males (Saino et al. 2006), and in yellow-legged gulls (*Larus michahellis*) androgen administration to the egg reduced female survival (Rubolini et al. 2006a). These sex-specific and species-specific reactions to elevated yolk androgen levels need to be explained. Two complimentary mechanisms have been put forward to explain the observed effects of yolk androgens on nestlings after hatching: (1) exposure to different steroid hormone levels may have a priming effect on synthesis and secretion of hormones later in life and (2) variation in steroid levels can affect number and distribution of receptors and thus sensitivity of target tissue to the effects of those hormones during growth and adulthood (discussed in Badyaev 2002). Studies at the physiological level are required to explain the opposite direction of response to androgens of the two sexes in different, sometimes closely related, species.

Yolk androgens have been reported to also affect adult males. Positive effects were found in the house sparrow (*Passer domesticus*; Strasser and Schwabl 2004) and in the black-headed gull (Eising et al. 2006). Negative effects were reported in the Chinese quail (*Coturnix chinensis*; Uller et al. 2005) and in the ring-necked pheasant (*Phasianus colchicus*; Rubolini et al. 2006b). Our study did not reveal any effects of yolk androgen exposure on the attractiveness of male birds, which is in contrast to the finding of von Engelhardt (2004). He showed that testosterone positively affected attractiveness of the both sexes in zebra finches. In line with the study of von Engelhardt (2004), in our study female fecundity was also not affected by the treatment.

Our results suggest that maternal androgens seem to have larger effects at the early life stages when they presumably affect within-brood competition. A possible explanation for the lack of long-term consequences of androgens could be that the observed differential mortality of nestlings reduced the variance in traits measured in later life stages. Variance could be reduced for example via lower hatching success of males that might have not withstand elevated androgen levels (Rutkowska and Cichoń 2006). Yet, our data set does not provide evidence of differences in variance between the groups.

If elevated androgen levels leads to male-biased offspring primary sex ratio (Veiga et al. 2004; Rutkowska and Cichoń 2006), and eggs bearing males contain more androgens than those bearing females (Petrie et al. 2001; but see Pilz et al. 2005), then selection should favor positive influence of yolk androgens on male fitness. In light of present results and some previous ones (Uller et al. 2005; Rubolini et al. 2006b; von Engelhardt et al. 2006), this expectation is, however, not confirmed and we even find the negative effect of androgens on fitness-related traits in males. This could be due to the fact that in natural circumstances the level of yolk androgens is attuned with other substances in the eggs (Badyaev et al. 2006), so that offspring sex and androgen levels are simultaneously adjusted to the ecological context, such as availability of resources (Rutstein et al. 2005) or female social status (Müller et al. 2002). It is debated whether elevated androgen levels can be regarded as a preferential maternal investment to the eggs sired by high-quality mate (Gil et al. 1999, 2004; von Engelhardt 2004; but see Gwinner and Schwabl 2005; Navara et al. 2006). If females indeed deposit androgens in relation to mate quality, then the

genetically good background of the male offspring that are exposed to higher yolk androgen levels might allow them to withstand or even benefit from the elevated maternal androgens. If maternal investment to the eggs is experimentally altered (i.e., not in relation to mate quality), the negative effects of elevated androgen levels come into play (this study, von Engelhardt et al. 2006).

In conclusion, our findings suggest that androgens may mediate a crucial trade-off underlying optimal sex allocation in vertebrates, i.e., the choice between offspring sex and its quality. Because of the sex-specific optima in maternal hormone concentrations in eggs, females have to tune androgen levels and offspring sex simultaneously. Otherwise, androgens may not be an effective sex determination mechanism. Further studies should explore the idea of context-dependent effects of androgens by simultaneously manipulating maternal hormones and controlling for parental quality, so that various hormone levels meet contrasting genetic backgrounds. The mechanism underlying opposite direction of response to androgens of the two sexes in different species also calls for explanation in the near future.

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