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Negative effects of elevated testosterone on female fecundity in zebra finches

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Abstract

Although factors influencing androgen deposition in the avian egg and its effects on nestling fitness are recently receiving considerable attention, little is known about the potential costs of high testosterone levels in the females. Our study aimed at determining the effect of injections of testosterone (T) in female zebra finches (*Taeniopygia guttata*), on clutch size, egg mass, yolk mass, and yolk androgen content. Females were given a single bolus injection of T in a range of doses after laying the first egg. Results show that administration of T negatively affected clutch size; the strength of this effect increased with increasing doses of T. Females injected with the highest testosterone dose showed suppressed oviposition of the third and the fourth eggs. Interestingly, testosterone administration made females produce eggs with relatively large yolks, suggesting that T may mediate the trade-off between number and size of eggs. Testosterone injection resulted in elevated levels of androgen in the eggs, in contrast to control clutches, which showed a decreasing pattern of androgen concentration along the laying sequence. We conclude that high androgen investment in eggs may be limited by physiological requirements of the ovulatory process.

Keywords: Maternal investment; Trade-off; Egg size; Androgens; *Taeniopygia guttata*

Introduction

The role of androgens in female reproduction is a neglected research area (Staub and De Beer, 1997). Although several androgens have been identified in processes of follicle maturation and ovulation (Staub and De Beer, 1997), little is known about the functional significance of seasonal and individual variation in female androgen circulating levels. Recent interest in female androgen has been spurred by the discovery that females could influence offspring development and behavior by varying androgen deposition in avian yolks (Schwabl, 1993). Although factors influencing androgen deposition to the avian egg and their effects on nestling fitness are receiving increasing attention, little is known about the costs of such allocation in females and offspring (Gil, 2003).

The increased amount of yolk testosterone in eggs laid by females mated to attractive partners (Gil et al., 1999, 2004a) or in eggs of females encountering social stress (Groothuis and Schwabl, 2002; Reed and Vleck, 2001; Schwabl, 1997; Whittingham and Schwabl, 2002) suggests adaptive androgen allocation to eggs. These patterns make adaptive sense because of the positive effects of testosterone (thereafter: T) on offspring development, such as shortened embryo development (Eising et al., 2001), more vigorous begging behavior, faster growth rate, and higher social status once the bird achieves adulthood (Lipar and Ketterson, 2000; Schwabl, 1993, 1996a). However, such increased investment may also bring about some costs. Firstly, some offspring may not be able to bear high T levels. Indeed, in one study, high androgen levels inhibited nestling growth and survival (Sockman and Schwabl, 2000). The negative effects of elevated T level could also include increased oxidative stress (von Schantz et al., 1999), suppressed immune function (Da Silva, 1999; Folstad and Karter,

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1992), or elevated sibling aggression that may lead to non-adaptive brood reduction (Mock and Parker, 1997). Secondly, these costs may also be paid by the female if increased biosynthesis of egg androgens results in increased maternal levels of circulating androgens (Schwabl, 1996b) which may in turn have negative consequences for the female. Thus, females should optimize androgen deposition into the eggs taking into account their ability to cope with high androgen levels. Indeed, several studies reported the importance of female quality on yolk T allocation. For instance, androgen allocation increases with female age in the European starling (*Sturnus vulgaris*; Pilz et al., 2003) and decreases with increasing levels of developmental stress in the zebra finch (*Taeniopygia guttata*; Gil et al., 2004b).

Although several studies have shown a close relationship between female plasma and yolk T (Schwabl, 1996b; Whittingham and Schwabl, 2002), recent studies provide negative evidence, showing a much more confusing pattern. For instance, Mazuc et al. (2003) found a negative correlation between egg and plasma T in house sparrows, and Verboven et al. (2003) found that food supplementation of female gulls increased androgen levels in the plasma, whereas in another experiment food supplementation decreased yolk androgens.

One of the few studies that has experimentally manipulated T circulating levels in females showed that female red-winged blackbirds (*Agelaius phoeniceus*) with T implants presented impaired reproduction, with disruption of nest building and egg laying (Searcy, 1988). In the recent study on the spotless starling (*Sturnus unicolor*), females implanted with T showed 2-week delay in egg laying compared to control females (Veiga et al., 2004). The negative impact of exogenous androgens has also been reported in chickens (Brahmakshatriya et al., 1969). This evidence suggests that elevated androgens in females may have a negative effect on their fitness. While it is unlikely that those costs mentioned above occur naturally, subtler fecundity costs could be expected in females subjected to high T levels within the natural range. These fecundity costs could include reduction in clutch size or egg size. So far, correlative data do not provide support for these predictions, e.g., mean androgen level was positively related to clutch size in European starling (Pilz et al., 2003) and in barn swallows (*Hirundo rustica*, Gil et al., submitted for publication). However, life history theory predicts positive correlations among individuals in traits that are subject to trade-offs, since individual quality may mask expected negative correlations (van Noordwijk and de Jong, 1986). Experimental manipulations are necessary to uncover trade-offs among life history traits.

Our study aimed at determining the effect of a bolus injection of increasing amounts of T in females on clutch size, egg and yolk mass, and yolk-androgen content in birds. For our study, we chose zebra finches, a species that easily breeds in laboratory conditions, laying clutches that average 5–6 eggs. To ensure that T administration took place at the same precise stage of the reproductive cycle, we injected females on the day the first egg was laid. This allowed us to compare the effect of the treatment on androgen levels in subsequently laid eggs with respect to the first egg. If high levels of T are costly for the female, we expected that our treatment would negatively affect clutch size, egg mass, or yolk mass. Because at the time of injection the yolks of the third and forth eggs would be undergoing the most extensive growth (Christinas and Williams, 2001), we expected to detect the effects of T treatment mainly in the third and subsequent eggs.

Methods

Experimental design

Zebra finches originating from the laboratory colony were kept in a climatized room at 21 ± 2°C, under a 13:11 h light/dark photoperiod, lights on at 0700 h. Birds were fed ad libitum with a standard mixture of seeds (Megan, Poland), along with a mixture of hard-boiled egg chopped with finely grated carrot. Birds also received a cuttlebone and grit. Rearing conditions were kept constant during the experiment.

Initially, all birds were maintained in a common aviary, where they could mate freely and rear one brood. Sexes were then separated for 3 months and paired again in visually separated, individual cages (75 × 30 cm and 40 cm high) equipped with external nestboxes and nesting material.

Following pairing, nestboxes were inspected every morning between 0900 and 1000 h to record nest building and egg laying, as well as labeling new eggs. Freshly laid eggs were removed and replaced with clay models. The removed eggs were weighed (±0.01 g) and the yolk was separated from the albumen, weighed (±0.01 g), and frozen at −20°C. As we were not always able to separate entire yolk from the albumen, 16 yolks were not weighed. Laying gaps seldom occurred. Eggs laid after a gap (up to 3 days) were numbered as though the missing egg had been laid.

After laying the first egg, females received a subcutaneous T injection (*testosteronum enanthanum*, Jelfa S.A., Poland) in the inguinal region between 1000 and 1300 h. Females were randomly assigned to seven experimental groups. Sample sizes for each group are presented in Fig. 1. T-treated females received 2.5, 5, 10, 20, or 40 μg T dissolved in 50 μl of oil *(paraffinum liquidum)*, respectively. Control females received vehicle only. Sham controls were only caught and removed from their cages for a few minutes. Females assigned to different experimental groups did not differ in body mass ($F(6,35) = 0.55$, $P = 0.77$) or in the mass of the first egg in a clutch ($F(6,35) = 0.92$, $P = 0.49$).

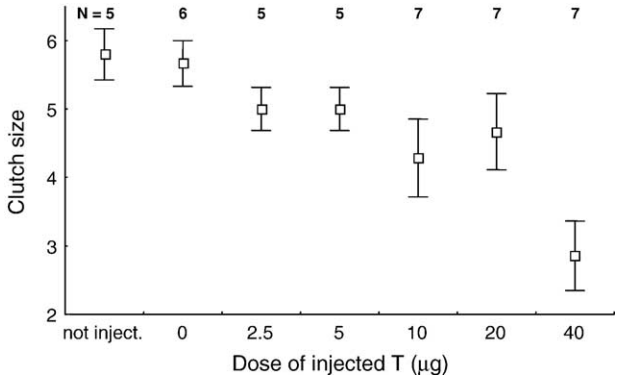

Fig. 1. Clutch size of females treated with different doses of testosterone. Numbers donate sample size for each dose. Mean and SE are presented.

Androgen assay

Yolks were defrosted and homogenized by mixing with a metal spatula. A small fraction of the yolk (average 30 mg) was weighed to the nearest milligram, placed in a glass tube, and homogenized by vortexing in 1 ml of distilled water using glass beads. Steroids were extracted by adding 3 ml of diethyl ether to the sample, vortexing for 1 min, and centrifuging for 5 min (4°C, 3400 × *g*). After snap freezing the tube in a bath of alcohol and dry ice, the ether phase was decanted in a fresh tube and evaporated in a warm bath at 30°C. The dried extract was redissolved in 1 ml of phosphate buffer and frozen at −20°C until hormone assay. This extraction protocol has been shown to be reliable and provides very high levels of steroid recovery in avian yolks (Gil et al., 2004a, 2004b). Yolk androgen concentrations were determined using a commercially available enzyme immunoassay (Cayman Chemicals, USA) following the manufacturer's protocol. The assay is 100% specific for testosterone, 36.6% for 5α-dihydro-testosterone, and 20.4% for androstenedione. Samples were run in duplicates, the intra-assay coefficient of variation was 7.72 and the inter-assay coefficient of variation was 9.36. The range of detectability of the assay calculated as the interval between 20% and 80% of maximum binding was 82.5–8.9 pg/ml per tube.

Statistical analyses

The effects of testosterone administration on egg mass, yolk mass, androgen concentration, and total androgens were analyzed with Mixed Proc in GLM with treatment (=log-transformed T-dose) as linear factor and egg number as a covariate reflecting the day since injection. The interaction between the two was of main interest in this study because it would indicate transmission of T to the egg as a function of laying order and injected T-dose. Female identity was included in the analyses as a random factor and had always a significant effect. Analyses were performed with SAS v.8 (SAS, 2000).

The research was carried out under approval of The Local Ethical Committee at the Jagiellonian University.

Results

Clutch size, egg mass, and yolk mass

Clutch size differed between experimental treatments (ANOVA, $F(6,34) = 4.85$, $P = 0.001$); females that received the highest dose of T laid significantly smaller clutches than females in the two control groups and females receiving the two lowest T doses (Tukey post hoc: difference with sham-control females: $P = 0.0017$, injected with oil: $P = 0.0016$, injected with 2.5 μg T: $P = 0.04$, injected with 5 μg T: $P = 0.04$; Fig. 1). We also examined the relation between T dose and clutch size by means of regression analysis. Clutch size decreased with increasing doses of T injection (effect of log-transformed dose: $\beta = -0.63$, $F(1,39) = 25.73$, $P = 0.00001$). This effect was stronger in the group of females receiving the highest dose, in which 6 out of 7 females stopped laying eggs at least for a day. After exclusion of this group, the relation between injected T-dose and clutch size was still significantly negative (effect of log-transformed dose: $\beta = -0.43$, $F(1,32) = 6.67$, $P = 0.014$).

Egg mass increased with increasing T dose and slope of the relationship between laying order and egg mass changed with T dose (GLM controlling for female ID, T dose: $F(1,148) = 5.30$, $P = 0.023$; Egg number: NS; T dose × Egg number: $F(1,148) = 6.82$, $P = 0.010$; Fig. 2a). Similarly, yolk mass increased with T dose and slope of the relationship between laying order and yolk mass changed with T dose (GLM controlling for female ID, T dose: $F(1,134) = 10.01$, $P = 0.002$; Egg number: NS; T dose × Egg number: $F(1,134) = 21.16$, $P < 0.0001$, Fig. 2b). There was no relationship between yolk mass and laying order in the two control groups, but this pattern changed with increasing T-dose. In females injected with T, yolk mass increased with the egg laying sequence and the late-laid eggs of females treated with the highest T-dose contained exceptionally big yolks (Fig. 2b).

Androgens

Variation of androgen concentration in yolks of subsequent eggs differed between the experimental groups, as indicated by the significant interaction of egg number and treatment (GLM controlling for female ID, T dose: NS; Egg number: $F(1,148) = 20.85$, $P < 0.0001$; T dose × Egg number: $F(1,148) = 6.77$, $P = 0.010$, Fig. 3). The effect was even more pronounced for total androgen content (GLM controlling for female ID, T dose: $F(1,134) = 4.48$, $P = 0.036$; Egg number: $F(1,134) = 19.12$, $P < 0.0001$; T dose × Egg number: $F(1,134) = 17.45$, $P < 0.0001$), but the pattern is very much the same in both measurements. Androgen concentration and total androgen content decrease with laying order after the second egg in both control groups and also in those treated with 2.5, 5, and 10 μg T. In clutches of females subjected to 20 μg T dose, androgen content in subsequent eggs increased significantly, showing a distinct

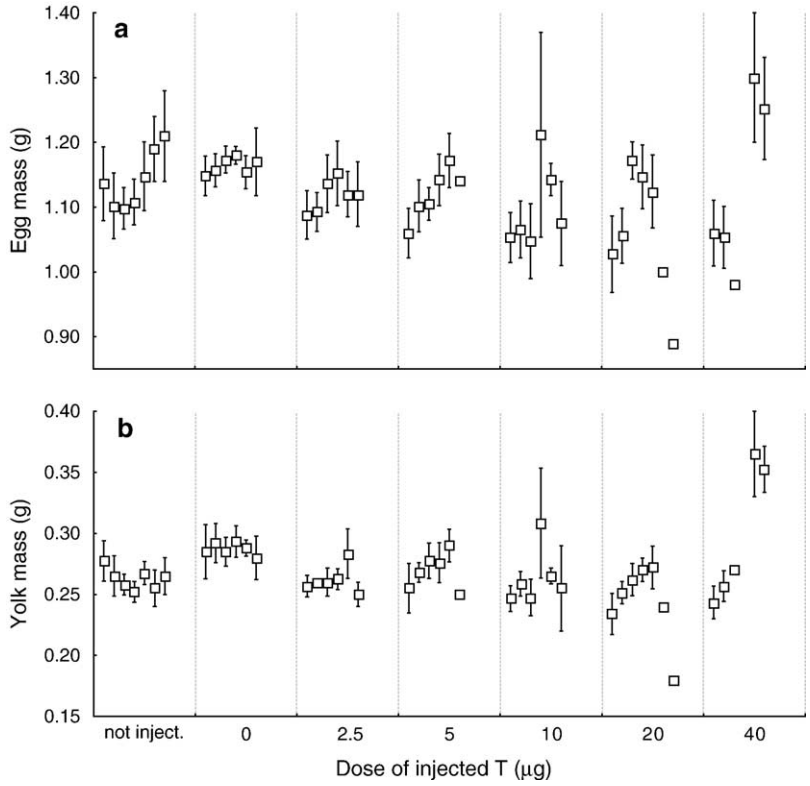

Fig. 2. (a) Mass of subsequent eggs and (b) corresponding egg yolk mass in clutches laid by females treated with different doses of testosterone. The first data point in each panel represents the first egg in a clutch. Mean and SE are presented. 4th egg laid by a female injected with 10 μg T contained 2 yolks.

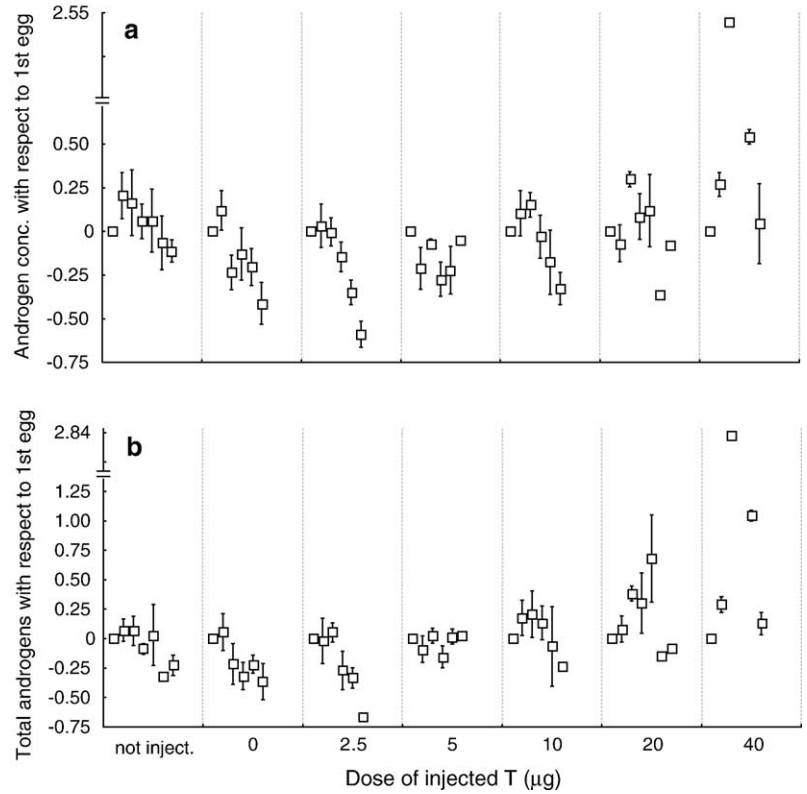

Fig. 3. (a) Androgen concentration and (b) total androgen content of subsequent eggs laid by T-treated females. The first data point in each panel represents the first egg and values for subsequent eggs are standardized with respect to the amount of androgens in the first egg of a given clutch. Mean and SE are presented.

different pattern from controls, with an increase of at least 40% in the third, fourth, and fifth eggs (Fig. 3). An exceptionally high level of androgens was detected in the group receiving the highest T dose (40 μg T): the only third egg that was laid had 40 pg/mg androgens. This concentration was 255% higher than in the first egg, but still within natural levels: average androgen concentration in control groups was 17.7 pg/mg (range: 6.5–36 pg/mg) and the highest natural concentration detected in the study was 47 pg/mg (in the first egg laid by female receiving the dose of 2.5 μg T).

The increase of androgens in egg number 3 in the only female that laid it after receiving the highest dose of T was equivalent to 0.025% of the amount injected. In females receiving 20 μg T, the amount deposited in the third and the fourth egg was 0.01% and 0.006% of the amount injected, respectively. These values were 0.001% and 0.002% in females receiving 10 μg T.

Discussion

Our experiment shows that administration of T to zebra finch females after laying the first egg induces a reduction in clutch size, it decreased as a function of T dose (Fig. 1). The highest T-dose inhibited oviposition of the 3rd and 4th eggs. On the other hand, injections of high T levels resulted in females laying eggs with relatively large yolks (Fig. 2), suggesting that T may mediate trade-offs between number and quality of offspring in passerines.

A negative effect of high exogenous androgen levels on egg laying has been reported in the chicken (Brahmakshatriya et al., 1969), in the spotless starling (Veiga et al., 2004), and in a study on red-winged blackbirds in which 11 out of 12 females treated with permanent T-implants failed to lay eggs (Searcy, 1988). In zebra finches, the same effect was observed in females implanted with T before pairing with males (Rutkowska and Cichoñ, 2005). In the present study, a bolus injection of a high T dose interfered with oviposition. These findings suggest that elevations of T levels in females may interfere with naturally occurring cycles of this or other hormones in females. In the chicken, peak T levels in plasma usually occur 10–6 h before ovulation, suggesting a role of this hormone in stimulating ovulation (Johnson, 2000). The bolus injection that our animals received probably increased plasma androgens levels to a higher concentration than those found naturally and during a more sustained period of time, thus hiding the circadian fluctuations of T occurring during laying. In turn, this may have masked feedback mechanisms with the central nervous system, probably disrupting preovulatory LH peaks (Johnson, 2000).

Our results may indicate that elevated circulating T stimulates higher nutritional investment in eggs. This suggests that maternal androgen level may be a physiological factor behind the trade-off between egg size and clutch size, reported in some passerine species including zebra finches (Williams, 2001), other vertebrate species (Sinervo and Svensson, 1998), and expected by life history theory (Stearns, 1992). Increase in T levels may increase resource allocation to eggs (reflected in higher yolk mass), but at the expense of reductions in clutch size. Alternatively, if high T levels inhibit ovulation, more resources would be deposited in fewer eggs.

Our study shows that injections of T in laying females increased yolk steroid content, albeit to a very limited extent. This is in line with a previous study on Japanese quail (*Coturnix japonica*) in which experimental elevation of another maternal steroid, estradiol, resulted in an increase of this hormone in egg yolk (Adkins-Regan et al., 1995). Female T level may influence yolk androgen concentration by direct transfer of circulating hormones to egg yolk or alternatively may regulate the activity of theca cells. Our results suggest that direct transfer of hormones is unlikely to play a major role in the determination of yolk androgen levels, since the largest increase of yolk T we found was 0.025% of injected dose. Similarly, Hackl et al. (2003) found that, after injecting radioactively labeled T to Japanese quail females, only 0.1% of radioactivity could be found in yolks of subsequently laid eggs. The two studies suggest that steroid biosynthesis by the follicles is probably the main source of T that is deposited in the eggs. However, our estimation of direct transfer of T is rough and such calculations should be treated with caution.

Until recently, studies showing a correlation between female T levels at the time of yolk formation and yolk T levels (Schwabl, 1996a), and effects of environmental and social cues in yolk testosterone levels (Gil et al., 1999; Schwabl, 1997), have led researchers to believe that female plasma androgen levels have a direct effect upon yolk levels. However, our experiment, together with evidence from Hackl et al. (2003), suggests that this might be unlikely. A more plausible explanation is that social and behavioral cues activate androgen production at higher levels that in turn modify androgen secretion in different glands. It remains to be shown why in some studies there is a close correspondence between plasma and yolk T (e.g., Schwabl, 1996b), and in others a lack of pattern or even a negative correlation (Mazuc et al., 2003; Verboven et al., 2003). Importantly, it needs to be considered that female androgens originate both in the ovary and in the adrenal tissue (Freking et al., 2000). In contrast to humans (Longcope, 1986), we do not know the relative contribution of the specialized follicle cells to total androgen production in female birds. Given that follicles secrete androgens during a very restricted period of time, we can assume that most androgen-dependent behavior and physiology in females (e.g., aggression, muscle development, CNS organization, etc. (Staub and De Beer, 1997)) are largely based in adrenal activity. This begs the question of whether stimuli that modify androgen adrenal levels also have a similar impact

in follicle activity. If females can adaptively modify yolk androgen levels, we would not expect a close correlation between T activity in these two systems, because it is unlikely that adaptive patterns of testosterone levels would be equal in the two targets: female and yolk. Rather, we would expect some kind of filter that would protect the yolk from extreme high levels that can be detrimental to embryo development (cf. Painter et al., 2002 for such buffering mechanism in viviparous lizards).

Clutches of the two control groups do not differ significantly in any measured trait, showing that the effect of the injected vehicle itself is negligible. Within the two control groups, egg mass increases with laying order, confirming earlier findings in this species (Royle et al., 2003; Rutkowska and Cichoñ, 2005; Williams, 2001). However, yolk mass does not change with the laying order, indicating that changes in egg mass may not necessarily reflect increased allocation to late-laid eggs. Our data also confirm that the concentration of yolk androgens decreases with egg laying order in zebra finches (Gil et al., 1999, 2004b). This negative relationship observed in control broods disappeared in experimental groups.

To our knowledge, this is the first experimental study that shows how injections of a bolus of a range of increasing T doses administrated to female birds affect clutch size, yolk mass, and the concentration of androgens in the eggs. We provide evidence that T may potentially mediate trade-off between clutch size and egg size, but above a certain threshold, increased T level might be costly for the female. Additionally, our results may provide a novel technique, less invasive than direct injection of T to the egg, to investigate how variation in yolk androgens influences offspring performance.

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