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Negative effects of early developmental stress on yolk testosterone levels in a passerine bird

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Summary

Female birds incorporate in the yolks of their eggs significant concentrations of a number of different androgens. Yolk androgen has been shown to positively affect several fitness components at the embryo, nestling and juvenile stages. Previous experiments have shown that females lay eggs with higher androgen concentrations when they are paired with highly ornamented males. This pattern suggests that yolk androgens are costly to females. In this study, we experimentally manipulated adult female condition in zebra finches *Taeniopygia guttata* by modifying the level of developmental stress they suffered as nestlings. This was achieved by cross-fostering nestlings to broods of varying brood size. Subsequently, we measured the yolk testosterone contents of the female offspring that resulted from the experimental manipulation. As predicted, females deposited decreasing concentrations of testosterone with increasing brood sizes experienced as nestlings: testosterone concentration (mean ± S.E.M.) of eggs laid by females from small broods, 20.66±2.08 pg mg^{-1}; medium broods, 15.32±1.94 pg mg^{-1}; and large broods, 14.51±1.66 pg mg^{-1}. Additionally, testosterone concentration decreased with laying order, and varied with clutch size in a complex way. Differences in egg testosterone between females exposed to different brood sizes are in line with previous findings in showing that early developmental stress can affect adult reproductive performance, although our study did not detect an effect in other breeding parameters, such as latency to breed or clutch size. Furthermore, the results are consistent with the hypothesis that there is a cost associated with yolk testosterone. However, it is still unclear what the nature of this cost may be, and whether it is paid by females, offspring, or both.

Key words: yolk testosterone, developmental stress, androgens, maternal effects, reproductive investment, differential allocation, zebra finch, *Taeniopygia guttata*.

Introduction

Conditions experienced during early development can have profound effects on reproductive performance later in life (Lindström, 1999). Early development is defined as the time period from conception to sexual maturity, and thus encompasses a number of life stages. However, effects have been shown to be stronger at the earliest stages of development (Lindström, 1999). These early effects can be the result of maternal adaptive manipulation (Mousseau and Fox, 1998), or else may be considered side-effects that constrain reproductive performance and survival (Haywood and Perrins, 1992).

Several studies in birds have experimentally manipulated the amount of resources available to altricial nestlings by brood-size manipulations. This approach has allowed researchers to detect early condition-dependent patterns in a number of variables, such as clutch size (Haywood and Perrins, 1992), adult size and mortality (De Kogel, 1997b) or the development of sexual ornaments (De Kogel and Prijs, 1996). It has recently been shown that the amount of androgen deposited in the egg yolk is an important component of reproductive investment in birds (Schwabl, 1993). Egg yolk androgen has positive effects on the embryo and on the development of nestlings, such as increased begging, growth and muscular development (Eising et al., 2001; Lipar and Ketterson, 2000; Schwabl, 1993), although a study has also identified survival costs of high yolk androgen levels (Sockman and Schwabl, 2000). Females have been shown to vary their allocation of yolk androgen according to the attractiveness of their male, thus suggesting that there are costs associated with this investment (Gil et al., 1999, 2004; D. Gil, P. Ninni, A. Lacroix, F. De Lope, C. Tirard, A. Marzal and A. P. Møller, manuscript submitted for publication). There is some evidence that this may be the case, such as a positive covariance between yolk-testosterone and arrival date in the barn swallow *Hirundo rustica* (D. Gil, P. Ninni, A. Lacroix, F. De Lope, C. Tirard, A. Marzal and A. P. Møller, manuscript

submitted for publication), or with female age in the starling *Sturnus vulgaris* (Pilz et al., 2003). However, a recent study has failed to detect increased egg androgen concentrations in the eggs of food-supplemented black-backed gulls *Larus fuscus* (Verboven et al., 2003).

In this study, we manipulated early development in zebra finches *Taeniopygia guttata* by cross-fostering nestlings to small, medium and large brood sizes. This manipulation typically influences early developmental stress, resulting in strong effects in nestling and adult morphology and fitness (De Kogel, 1997b; Naguib et al., 2004). Subsequently, we allowed these birds to sexually mature, and paired females randomly with non-experimental males from the same population. The hypothesis was that, if yolk testosterone allocation is costly, females should lay eggs with lower concentrations of testosterone with increasing levels of developmental stress experienced as nestlings.

Materials and methods

Material and breeding methods

We conducted the experiment on zebra finches *Taeniopygia guttata* Vieillot 1817 (wild Australian origin, F_5 generation) at the University of Bielefeld, Germany, in 2002. Females originated from a cross-fostering experiment in which we raised zebra finches in different brood sizes within the natural range (Naguib et al., 2004). We cross-fostered birds at the age of 2±1 days, by distributing chicks from each of the natural broods among 1–5 different experimental broods. Within-brood variation in nestling age or laying order did not differ between experimental groups. Experimental broods consisted of 2–6 cross-fostered chicks coming from 1–4 different original broods. We divided broods into three experimental groups: (1) small broods (2–3 nestlings), (2) medium broods (4 nestlings) and (3) large broods (5–6 nestlings). We made sure that original brood sizes were evenly distributed among experimental treatments, to avoid confounding effects of maternal quality. In a previous paper, in which we describe the methods used in bird rearing in more detail (Naguib et al., 2004), we showed that experimental manipulation affected nestling growth, testosterone and immunocompetence levels, as well as adult body size. With increasing experimental brood sizes, both male and female nestlings grew less, and had increased testosterone levels at day 11 and a lower T-cell response. Adult body size was also affected, with significantly decreasing mass, tarsus and wing length with increasing experimental brood size (Naguib et al., 2004).

Breeding of the second generation

When female offspring from the above experiment were 9 months old, we paired them up with unrelated males that had been raised in non-manipulated broods. Overall, we used 14 females coming from 10 small manipulated broods, 19 females from 10 medium broods, and 19 females from 8 large broods. Females were significantly lighter and had significantly shorter wing lengths as rearing brood size increased, while tarsus length also showed a non-significant trend in the same direction (Table 1). Males that had not been in visual or direct contact with these females were randomly allocated to females, and did not wear colour rings that could bias their attractiveness to females (Burley, 1988).

Pairs were kept in individual cages (83 cm×30 cm×40 cm) and supplied daily with both dried and germinated senegal, plata and red millet seeds and fresh water (plus additional vitamins three times a week). Each cage was provided with a wooden nest box attached to the side (12.5 cm×12 cm×14 cm) and with coconut fibres on the floor, to be used as nesting material. Cages were distributed over three rooms and arranged on rows of shelves placed on walls so that birds had visual contact with birds on the opposite side of the room. Rooms had a temperature of 23°C and a L:D regime of 16 h:8 h, with lights on at 06:00 h. Nests were checked daily between 10:00 and 12:00 h and eggs were labelled with a felt pen on the day of laying, allowing us to keep track of egg laying order. Eggs were removed on day 2 after laying and frozen at –20°C. We kept females in the experiment until they had not laid an egg for 5 days, after which we transferred them back to a larger aviary.

Hormone assay

Yolks were dissected after allowing the eggs to thaw for 10 min. After this time, egg white is defrosted while the yolk still remains compact and can be removed with a needle. Yolks were homogenised by stirring using a yellow pipette tip. A small sample (around 50 mg) was taken and weighed in a pre-tared glass tube. We added 0.5 ml of distilled water and several

Table 1. *Measurements at 9 months of age of the females used in the experiment, divided by experimental groups*

	Brood size			
	Small	Medium	Large	ANOVA
Mass (g)	12.7±0.3	12.2±0.2	11.6±0.3	$F_{2,48}$=2.56, P=0.03
Tarsus length (mm)	14.3±0.2	13.9±0.1	13.7±0.2	$F_{2,48}$=1.79, P=0.06
Wing length (mm)	56.4±0.6	55.4±0.2	54.9±0.3	$F_{2,48}$=3.02, P=0.02

Values are means ± 1 S.E.M.

The statistical test corresponded to a one-way ANOVA, with P values corrected following a two-tailed ordered heterogeneity test (Rice and Gaines, 1994).

Please note that these data (males and females together) have been published before (Naguib et al., 2004).

glass beads to the tube and vortexed it until the yolk was completely homogenised. Steroids were extracted by adding 3 ml of a 30:70 mixture of petroleum/diethyl ethers and vortexing the mixture for 1 min. We separated the ether phase by decanting the contents of the tube after having snap-frozen them in a bath of alcohol and dry ice. The ether phase was then dried under a stream of nitrogen and dry extracts reconstituted into 0.5 ml of a 0.01 mol l^{-1} phosphate buffer with bovine serum albumin (1 g l^{-1}). Although we have already shown the reliability of this extraction method (Gil et al., 2004), we tested it again for this species by re-extracting ten samples using the more thorough method, which involves ethanol precipitation and hexane washing (for details, see Schwabl, 1993). Although concentrations using the latter method were larger than those obtained using the simplified method (16.9±7.37 *vs.* 10.45±4.12 pg mg^{-1}, means ± S.D.), the regression between the two extractions was highly significant ($F_{1,10}$=70.9, P<0.001, r^2=0.87), showing that the simplified extraction can provide an accurate assay of yolk androgens. We assayed the concentration of testosterone using a commercially available radioimmunoassay (DSL-4000, made by Diagnostic Systems Laboratories, Texas, USA), with high specificity with testosterone (cross-reactivity with other androgens <6%). Samples were assayed in duplicate, with an intra-assay coefficient of variation of 3.13%. The between-assay coefficient of variation was 10.02%.

Statistical analysis

We analysed egg data with repeated-measures mixed lineal models using Proc Mixed (SAS V. 8.1). Experimental brood size was declared as a fixed factor. In order to control for random effects of females sharing a common environment as nestlings or being genetically related, data were firstly cross-classified by entering as random factors the original and experimental broods where the females had been raised as nestlings. However, none of these factors explained a significant part of the variance in any of the tests, and were removed from the models. Individual egg data were cross-classified within females (Littell et al., 1996), and the effect in this case was always highly statistically significant (all measures P<0.001). Therefore, in all models we conserved female as a random factor. We used the Satterthwaite correction to approximate the degrees of freedom (Littell et al., 1996). The statistical significance of the treatment (brood size experienced by females) was adjusted following a two-tailed ordered heterogeneity test, in the expectation that the groups would show progressive responses to the manipulation (Rice and Gaines, 1994).

Results

The final model showed that yolk testosterone concentration was simultaneously affected by brood size experienced by females as nestlings, laying order and clutch size (brood size: $F_{2,31}$=2.87, P<0.03; laying order: $F_{1,24.2}$=6.35, P<0.01; clutch size: $F_{4,30.6}$=4.5, P<0.01), with no significant interactions among these variables. Increasing brood sizes during development resulted in decreasing concentrations of yolk testosterone in the eggs laid by the adult females (Fig. 1). Yolk testosterone concentration was negatively related to egg laying order (Fig. 2), and showed a non-directional complex pattern with clutch size (Fig. 3).

Brood size experienced by females as nestlings did not affect the time interval between pairing and laying ($F_{2,45}$=0.11, P=0.89; small broods, 16.27±3.64 days; medium broods, 14.88±3.42 days; large broods, 17.18±3.54 days; means ± S.E.M.), clutch size ($F_{2,45}$=0.37, P=0.69; small broods, 2.2±0.33 eggs; medium broods, 2.58±0.31 eggs; large broods, 2.44±0.32 eggs) or egg mass ($F_{2,32.6}$=1.09, P=0.34; small broods, 809.94±37.37 mg; medium broods, 838.71±29.27 mg; large broods, 777.51±29.25 mg).

We obtained data for a sub-sample of females on nestling plasma testosterone levels at 11 days of age (Naguib et al., 2004), and tested whether these levels would positively correlate with mean yolk testosterone concentrations in

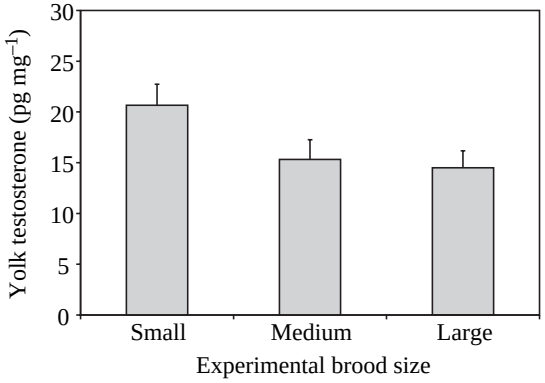

Fig. 1. Differences in yolk testosterone concentration between zebra finch females raised as nestlings in small, medium and large brood sizes. Data shown are least-square means ± 1 S.E.M. from the full model, which includes female brood size, laying order and clutch size.

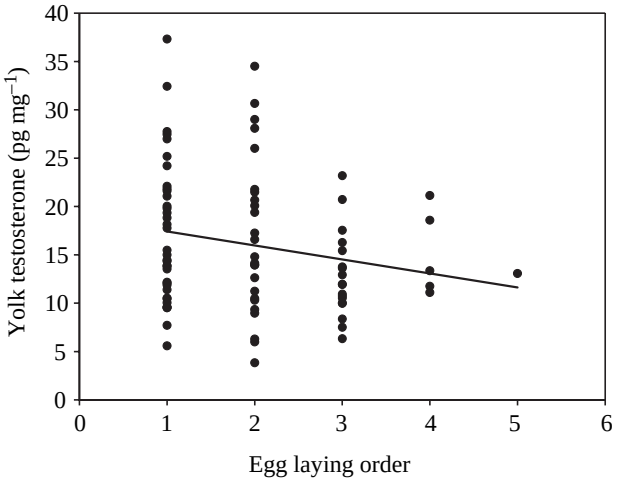

Fig. 2. Relationship between laying order and yolk testosterone concentration.

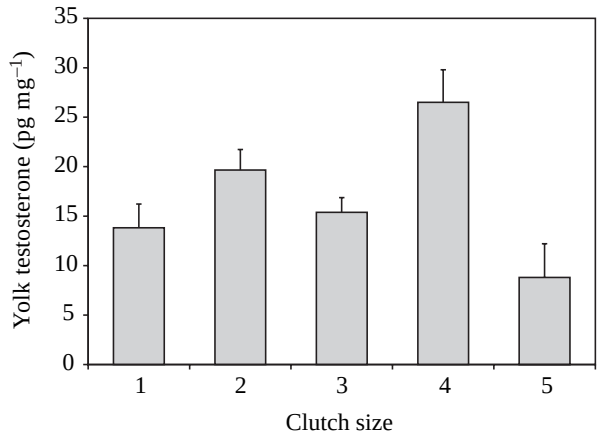

Fig. 3. Relationship between clutch size and yolk testosterone concentration. Data shown are least-square means ± 1 S.E.M. from the full model, which includes female brood size, laying order and clutch size.

adulthood, but found no significant relationship (Spearman's r=–0.104, N=19, P=0.67).

Discussion

Our experiment shows that yolk testosterone levels are affected by conditions experienced by females during early development: females laid eggs containing decreasing concentrations of testosterone as the brood size experienced as nestlings increased. Manipulation of brood size is a powerful means of identifying life-history constraints (Stearns, 1992). A revision of the literature shows that 82% of published studies find offspring traits to be negatively affected by increased brood sizes (Stearns, 1992). These studies include several examples of reductions in female offspring fecundity as a result of increased brood sizes experienced as young (Gustafsson and Sutherland, 1988; Schluter and Gustafsson, 1993). Our results parallel these findings, suggesting that yolk testosterone deposition is a costly component of maternal reproductive investment.

This finding has important implications for the differential allocation of resources in reproduction (Burley, 1988; Sheldon, 2000). Life history theory predicts that reproductive investment in iteroparous organisms should increase with increasing fitness value of a given reproductive attempt (Stearns, 1992; Trivers, 1972). Male ornament size can provide females with an honest signal of male quality (Møller and Alatalo, 1999), and females have been shown to vary their reproductive investment according to male ornamentation in a wide array of organisms (Sheldon, 2000). We have previously shown that the concentration of yolk androgens in three different bird species increases with increasing size of manipulated male ornamentation (Gil et al., 1999, 2004; D. Gil, P. Ninni, A. Lacroix, F. De Lope, C. Tirard, A. Marzal and A. P. Møller, manuscript submitted for publication). As yolk androgens have been shown to positively affect embryo and nestling development (Eising et al., 2001; Lipar and Ketterson, 2000; Schwabl, 1993; but see Sockman and Schwabl, 2000), this investment may represent an example of differential allocation by reproducing females. However, it needs to be shown that yolk androgen is costly to females (Sheldon, 2000). Our results are in line with this hypothesis, by identifying a constraint in egg testosterone allocation set up in early development.

Alternatively, we need to consider whether the differences in yolk testosterone that we found are determined by other factors. For instance, females fledging from enlarged broods may have attained lower social ranks, and experienced different levels of aggression, which in turn may have influenced yolk testosterone levels (Schwabl, 1993; Whittingham and Schwabl, 2002). This seems to be a less likely possibility, because females were allowed to breed in separate cages, without competition for mates or nest sites. Moreover, de Kogel (1997a) did not find any significant effect of brood size manipulation on later competitive behaviour. Alternatively, as female condition may bias sex ratio (Nager et al., 1999), it could be argued that differences in yolk testosterone between treatments could be due to differences in sex ratio between treatments, and females investing higher concentrations of testosterone in male than in female-bearing eggs (Petrie et al., 2001). Even though we cannot rule out this possibility, the evidence for sex-specific differential allocation of testosterone still remains controversial. Recent research has shown that reported differences in yolk androgen levels between male and female eggs in the peacock *Pavo cristatus* (Petrie et al., 2001) may have been an experimental artefact, possibly because of sexual differences in embryo steroid production and uptake (Eising et al., 2003; Müller et al., 2002).

A recent study has used food supplementation to test the cost of androgen deposition in eggs in the black-backed gull (Verboven et al., 2003). The results showed that food-supplemented females had higher androgen levels than controls, but laid eggs containing lower androgen concentrations. This evidence does not support a cost of yolk androgen to the female, and suggests that investment in egg testosterone is not a linear function of maternal condition. The discrepancy between the results of the present study with those of the gull experiment (Verboven et al., 2003), may suggest that limitations of androgen deposition are more dependent on variation in early development than in the adult condition.

Although our results suggest a constraint in the production of egg androgens, they do not identify where the cost may lie. Several possibilities exist (Gil, 2003), although so far no research has tackled this problem directly. One possibility is an inhibitory effect of circulating androgens on immune defence (Duffy et al., 2000; Folstad and Karter, 1992; Hasselquist et al., 1999), possibly through increases in oxidative stress (Chainy et al., 1997; von Schantz et al., 1999). Immunocompetence constraints could act in either the female or the offspring. In the case of the female, it would need to be shown that higher androgen biosynthesis results in higher circulating levels. Recent evidence challenges this seemingly

straightforward assumption (Mazuc et al., 2003; Verboven et al., 2003). Immunocompetence may, on the other hand, constrain egg androgen allocation in the offspring, even at the embryo stage. Although there is some positive evidence along these lines from studies using pharmacological levels of androgens (Al Afaleq and Homeida, 1998; Henry and Burke, 1999), it is unclear whether this effect would still be present within the natural range of androgen yolk levels.

Another possibility is that the cost of androgen allocation may be brought about by nestling behaviour. Levels of yolk androgen have been shown to determine begging intensity in offspring (Eising and Groothuis, 2003; Schwabl, 1993). This suggests that that females may benefit by using yolk androgens to manipulate offspring demands as a function of their own condition.

In a previous paper (Naguib et al., 2004) we showed that testosterone levels in nestlings at day 11 increased with manipulated brood size, both for males and females. We interpreted this pattern as suggesting a functional link between testosterone and offspring competition. The results of the present paper, showing a pattern in the opposite direction in the case of yolk testosterone, and no correlation between nestling and adult yolk testosterone, suggest that the hormonal milieus of the nestling and adult stage are functionally independent of one another.

We failed to find any effects of early development on other indicators of reproductive investment, such as clutch size or egg mass, as reported in previous studies (Haywood and Perrins, 1992; Schluter and Gustafsson, 1993). One possible explanation for the lack of effect of early condition on clutch size in our study may be that we used the first clutch laid by the females, whereas Haywood and Perrins used later clutches, and were thus more likely to obtain larger variation among females. Additionally, the large heritability of clutch size (Christians, 2002) may provide more limited scope for variation than plastic allocation of egg components, and thus may require larger sample sizes to detect any influence of maternal effects.

Yolk-testosterone concentration decreased with laying order, confirming previous results reported in this species (Gil et al., 1999). This pattern strongly contrasts with those of a considerable number of studies that have reported increasing androgen concentrations with increasing laying order in other passerine species (Schwabl, 1999). Decreasing levels of testosterone with laying order suggest that zebra finches hatching from the last eggs in a clutch will suffer from a double handicap in sibling competition: to hatch later than their siblings (Zann, 1996), and to develop with a lower testosterone concentration. Future research should address whether these within-brood differences in testosterone levels affect the outcome of sibling competition within a brood, and are related to patterns of brood reduction.

To summarise, our experiment has detected negative effects of early developmental stress in yolk-testosterone concentration in adulthood. This suggests that egg androgens are directly or indirectly costly for the female, and that differences in yolk-androgen between broods with respect to male attractiveness represent patterns of adaptive differential allocation.

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