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Elevated yolk androgen levels benefit offspring development in a between-clutch context

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The field of androgen deposition in avian eggs and its consequences for offspring development has received a lot of attention in recent research. However, although variation within clutches in yolk androgens is relatively well understood, the adaptive significance of patterns of variation between clutches remains rather unclear. Furthermore, it has been hypothesized that yolk androgens act as a means of an adaptive maternal effect to adjust offspring to a given posthatching environment. Thus, the consequences of maternal yolk androgens for offspring development are likely to depend on the specific environment of a given brood. We experimentally manipulated yolk androgen concentrations in spotless starling eggs, using a between-brood design in which full broods were manipulated applying either an androgen or sham treatment, in order to test the effects of between-brood variation in yolk androgen levels. We also included in the analysis several female characters that have been shown to affect androgen deposition. Androgen-chicks tended to gain more mass, a similar effect to that shown in previous studies where direct competition between chicks belonging to different treatments was allowed, but did not gain a survival benefit. Androgen-chicks had wider beak flanges, an effect that has not been described previously and could play an important role in food acquisition. In addition, androgen-chicks had higher endogenous plasma levels of androgens, which could induce higher begging intensity. We hypothesize that these effects are an important mechanistic link for our understanding of how yolk androgens exert their effects on offspring development after hatching. Contrary to our predictions, we found no evidence that the observed effects depended on the environment under which it was investigated. Because high yolk androgen levels seem to be beneficial for nestlings in this species, we hypothesize that yolk hormone deposition could be costly for females or alternatively that potential negative effects later in adulthood may constrain maternal hormone allocation. *Key words:* conflict, maternal effects, parental investment, starling, *Sturnus unicolor*, testosterone. *[Behav Ecol]*

INTRODUCTION

Maternal hormones in avian eggs have received a great deal of attention in behavioral and evolutionary ecology, since the pioneering work by Hubert Schwabl in the early 1990s (e.g., Schwabl 1993, 1996). They are thought to represent an example of a hormone-mediated maternal effect, where the offspring phenotype is influenced by the maternal phenotype by means of specific egg components. This possibility has been investigated in a number of experimental studies, which revealed that comparatively small changes in the hormonal (especially androgen) environment of an embryo induced a wide range of effects (reviewed by Gil 2003; Groothuis, Müller, et al. 2005). In short, yolk androgens stimulate the development of the hatching muscle during embryonic development (Lipar and Ketterson 2000) and modify the length of the embryonic period (Sockman and Schwabl 2000; Eising et al. 2001; Eising and Groothuis 2003; von Engelhardt et al. 2005). The exposure to maternal yolk androgens promotes begging vigor (e.g., Schwabl 1996; Eising and Groothuis 2003) and may enhance or decrease posthatching growth (e.g., Schwabl 1996; Sockman and Schwabl 2000; Eising et al. 2001). Some studies have shown that prenatal exposure to maternal androgens can also be detrimental for the survival of the offspring (e.g., Sockman and Schwabl 2000). Accelerated growth such as that induced by maternal yolk androgens (e.g., Schwabl 1996; Eising et al. 2001) may be at the cost of the immune system (Andersson et al. 2004; Groothuis, Eising, et al. 2005; Navara et al. 2005) because both are energetically costly (reviewed by Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000). Yolk androgens may mediate this trade-off and have also been shown to have direct negative effects on the immune system (Müller, Groothuis, et al. 2005; see also Tschirren et al. 2005). Therefore, the costs and benefits of maternal yolk androgens may depend on the likelihood of an infection versus the necessity of competitiveness in relation to the degree of hatching asynchrony and the associated risk of starvation-mediated mortality. These findings and the reported extensive within-clutch variation of yolk androgens related to the position of the egg in the laying sequence have led to the hypothesis that differential deposition of maternal yolk androgens within the laying sequence plays an important role in mediating sibling competition (e.g., Schwabl 1993; Eising et al. 2001).

However, maternal yolk androgen concentrations not only vary systematically across the laying sequence within clutches but also there is an even larger variation of maternal yolk androgen concentrations between clutches (Gil 2003; Groothuis, Müller, et al. 2005). Between clutches, yolk androgen concentrations are modulated by the maternal environment, such as the nutritional conditions (Verboven et al. 2003), the presence of ectoparasites, and the immune status of the female (Tschirren et al. 2004; Gil et al. 2006), and in particular by social stimulation both by unrelated conspecifics and the perceived attractiveness of the mate (reviewed by Gil 2003;

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Groothuis, Müller, et al. 2005). However, the functional consequences of between-clutch variation have been studied to a lesser extend (Navara et al. 2005; Tschirren et al. 2005; von Engelhardt et al. 2005; Rubolini et al. 2006), despite the intriguing patterns of hormone deposition that have been described (reviewed by Gil 2003; Groothuis, Müller, et al. 2005). It is therefore still unknown whether all costs and benefits established in a within-clutch context can apply in a between-clutch context. For instance, in a within-clutch context, a chick hatching from the egg with the highest androgen concentrations may grow faster and thus gain a benefit in sibling rivalry, most likely at the cost of its siblings (Eising et al. 2001). In a between-clutch context, however, yolk androgens could benefit all chicks similarly, if parents respond to the increase in overall begging levels of the whole brood. This would lead to a net increase in parental investment, thus increasing parental costs. Whether parents are able to match this increased feeding demand would depend on their quality, and females may thus benefit by adjusting the amount of hormones deposited in the yolk in relation to parental quality. This is in line with the general idea of an adaptive maternal effect. Adaptive maternal effects are thought to have evolved to translate environmental conditions such as those experienced by the mother and her physiological state into adaptive phenotypic variation of the offspring (Mousseau and Fox 1998). Thus, females may try to prepare an offspring phenotype for a given environment after hatching. The adaptive significance of maternal yolk androgens is therefore likely to depend on the environmental circumstances under which it is studied, and future studies should clearly devote further attention to this topic.

Here, we experimentally manipulated yolk androgen levels by in ovo injection of androgens in eggs of spotless starlings (*Sturnus unicolor*) and studied the effects on offspring development and survival. We assigned all eggs of a clutch to the same treatment to study the adaptive significance of between-clutch variation in maternal yolk androgens. In order to investigate the importance of the context on the outcome of this study, we included in the analysis 3 measures of maternal quality that have previously been shown to affect yolk androgen deposition: maternal ectoparasite load (parasitized females deposited less androgens, Tschirren et al. 2004), maternal age (younger females deposited less androgens, Pilz et al. 2003), and female body mass as an estimate of nutritional condition (food-supplemented females deposited less androgens, Verboven et al. 2003).

If yolk androgens have similar effects on immunity, food solicitation–related traits, and growth in a between-clutch context, such as that shown in a within-clutch context, we hypothesize that all chicks in the brood will be affected likewise by the treatment. However, this may happen in different ways: first, our treatment may shift the yolk hormone concentrations away from the optimum typically deposited by the female, creating a mismatch with the current situation. Thus, potential effects of yolk androgens on immunity, food solicitation–related traits, and growth would have an overall negative effect on chick development and survival. Second, if the effects are beneficial for chick development, but costly for the parents, for example, through an enhanced food demand, we predict that the benefits will depend on parental quality. The strength of the effect will therefore depend on the context such as shaped by those maternal characteristics that also affect yolk hormone deposition. Third, yolk androgens may be beneficial for chick development in general but costly for the female during deposition, and yolk androgen–mediated effects would have an overall positive effect. This could be the case if the female has to expose herself to the hormones she is going to deposit in the yolk (Groothuis, Müller, et al. 2005).

MATERIAL AND METHODS

Study area and study species

The study was conducted in a nest-box colony of spotless starlings in central Spain (Soto del Real, Madrid, 900 m above sea level). The study area consists of pastureland and open woodland with mainly ash (*Fraxinus angustifolius*) and oak trees (*Quercus pyrenaica*). The first nest-boxes ($N = 140$) were placed in 2003–2004 at variable distances (between 5 and 25 m), and the population has been under study since then. The colony was extended with some additional 70 boxes in March 2005 separated by about 10 m from each other. The latter set of boxes were used for the present study.

The spotless starling is a medium-sized facultatively polygynous passerine, which is closely related to the European starling (*Sturnus vulgaris*). It is commonly double brooded, and females lay the first clutch around early April and the second clutch toward mid-May. Loss of the first clutch due to predation or intraspecific competition is typically followed by a replacement clutch that is laid about 10 days later. Incubation usually does not start before the third egg is laid. Chick feeding is mainly done by females (Moreno et al. 1999; Veiga et al. 2002).

Hormone manipulation

From the beginning of April onward, nest-boxes were checked every 2 days to control nest building and daily once laying approached to determine laying date and laying order. Eggs were marked with a nontoxic marker as they were laid. Two days after the first egg was laid, we injected them with either a mixture of 5.6 ng testosterone and 16.8 ng androstenedione dissolved in 10-μl sesame oil (androgen-eggs) or 10-μl sesame oil only (sham treatment, i.e., control-eggs). The injected dose was equivalent to 1 standard deviation (SD) of the population mean (testosterone: 9.79 pg/mg yolk [SD = 4.34], androstenedione: 36.27 pg/mg yolk [SD = 12.35], Gil D, unpublished data), adjusted for yolk mass (average yolk mass 1.4 g). All subsequent eggs were measured and injected as they were laid. All eggs within a clutch received the same treatment, either androgen or control, and we inverted the treatment with every new experimental clutch. The injections were performed in the field using a 25-μl Hamilton syringe and 25-G needles, and needles were replaced with every new egg. Eggs were cleaned with 100% ethanol around the injection site at the middle of the egg. During the injection procedure, the eggs were illuminated from beneath to ascertain that the tip of the needle penetrated the yolk. The hole in the eggshell was sealed with a tiny strip of flexible wound dressing (Opsite Incise, Smith & Nephew, Hull, UK). Eggs were maintained at ambient temperature during the experimental procedure and returned to the nest within 30–45 min.

General experimental procedure

Eight days after the last egg was laid, we counted the number of blood spots on 1 cm^2 eggshell on each egg of the clutch. The blood spots are droppings of an ectoparasite (*Carnus hemapterus* [Diptera: Carnidae], López-Rull I, Gil D, unpublished observations) and provide an estimate of the parasite load that the female is exposed to (Feare 1984, personal observation). We use the mean number of spots by calculating the average number of spots of all eggs measured as an estimate for the ectoparasite load that females are exposed to during incubation.

At hatching (day 1), chicks were weighed on a digital balance to the nearest 0.01 g. We measured flange width (the distance between the outer tips of the soft yellow flanges at

both sides of the bill, Clark 1995), beak length (distance between the tip of the bill and the proximal end of the narines), and head width (the skull at the base of the beak) with a digital sliding caliper to the nearest 0.01 mm. We clipped parts of down feathers on wing, back, and/or head to allow an individual identification until metal rings could be placed. Two days after hatching of the first chick (day 3), we measured all chicks of a nest again (mass, flange width, beak length, and head width). Two days after hatching of the last chick, all unhatched eggs ($N = 68$) were taken to the laboratory and frozen for further molecular sexing. Out of those eggs, a total of 39 (20 androgen-eggs [from 17 broods] and 19 control-eggs [from 12 broods]) could be successfully sexed. Measurements were repeated on days 6, 10, 14, and 15 and in most of the broods also on day 17. In addition to the measurements mentioned above, from day 6 onward tarsus and wing lengths were measured, whereas on day 17 only mass and tarsus and wing lengths were measured. Furthermore, a small blood sample was taken for sex determination at day 6 when also the metal bands were placed. A large blood sample (about 500 µl) was taken either on day 15 or 17 for hormone analysis.

On day 14, all nestlings were injected with a phytohemagglutinin (PHA) challenge to measure the cell-mediated immune response (Smits et al. 1999). We injected 0.05 ml of a 5 mg/ml solution of PHA dissolved in phosphate-buffered saline in the center of the left wing web. Three repeated measurements of the thickness of the wing web were taken with a micrometer to the nearest 0.01 mm prior to the injection and a further three 24 h later, except for 9 chicks where, for logistic reasons, we failed to record the second measurement. We used the mean value of the 3 measurements for analysis because the repeatability was high (initial $F_{126,254} = 287.96$, $r = 0.98$; final $F_{135,272} = 280.13$, $r = 0.99$; Lessells and Boag 1987). The difference in the thickness of the wing web before and after PHA injection was used as response estimate for the cell-mediated immune response, subsequently called CMI (Smits et al. 1999).

In order to obtain detailed information on maternal condition and age, we caught the female on day 8 during chick rearing. We measured body mass, and we aged individuals on the basis of the presence/absence of white spots on their belly, throat, and back. Females with no spots on the back were assigned to be older than 1 year and females with spots as first-year breeders (Svensson 1992).

Experimental nests and hatching

First clutches were initiated between the 21st and the 28th of April, replacement clutches between the 4th and the 14th of May. Only first clutches and replacement clutches were used in the experiment because 2005 was an extremely dry year and only a few pairs successfully laid a second clutch.

In total, 82 clutches were injected (40 control-clutches, 42 androgen-clutches). Out of these, 33 clutches did not produce any hatchlings because of predation, destruction by other females, or infertility. From the remaining successful clutches, 21 control-clutches and 28 androgen-clutches, a total of 162 chicks hatched (71 control-chicks from 101 control-eggs, 91 androgen-chicks from 129 androgen-eggs). The hatchability was 70% for both treatment groups (Pearson chi-square, $P < 0.97$). All chicks of a brood hatched within 2 days, except for 2 nests. Because hatchlings from 3 control-clutches and 3 androgen-clutches did not survive until the third measurement, all subsequent analyses except survival will be based on 18 control-clutches and 25 androgen-clutches (28 first clutches, 16 replacement clutches). There were no differences in hatching date (maydate [mean ± standard error {SE}] control-group 17.22 ± 1.80, androgen-group 14.23 ± 1.29; Mann–Whitney U test, $P = 0.24$) or brood size between treatments (number of chicks [mean ± SE] control-group 3.33 ± 0.24, androgen-group 3.35 ± 0.17; Mann–Whitney U test, $P = 0.91$). Females belonging to each group did not differ in body mass (mass [mean ± SE] control-group 80.13 ± 1.07 g, androgen-group 82.27 ± 1.30 g; t-test, $P = 0.25$) or age (control-group 44% first-year females, androgen-group 45% first-year females; Mann–Whitney U test, $P = 0.85$) between treatments.

Hatching sex ratio was 0.45 in the control-group and 0.55 in the androgen-group, which was not statistically different ($\chi^2 = 1.38$, degrees of freedom [df] 1, $P = 0.24$). The sex ratio of unhatched eggs was not different from the nestling sex ratio ($\chi^2 = 0.08$, df 1, $P = 0.78$) and not different between treatments ($\chi^2 = 0.78$, df 1, $P = 0.38$).

Sex determination and hormone measurement

DNA was extracted from blood and in case of dead nestlings or unhatched eggs from a small piece of muscle using either an ammonium acetate–based salt extraction or Chelex resin–based extraction. Two microliters of the resulting DNA solution was used in a polymerase chain reaction to amplify a part of the *CHD-W* gene in females and the *CHD-Z* gene in both sexes (Griffiths et al. 1998). This method was validated with adult birds of known sex.

The blood samples for hormone analysis were taken from the jugular vein with a 25-G needle into a syringe containing traces of heparin to avoid clotting. Samples were kept cool for the rest of the day (maximum 6 h) and subsequently centrifuged at 10 000 r.p.m. for 10 min. The separated plasma was stored in eppendorff tubes at −20 °C until analysis. Steroid was extracted from 0.2-ml plasma samples with 1.5-ml diethyl ether. The ether phase was decanted out and dried in a bath of 37 °C after immersing the tube on a bath of ethanol and dry ice to freeze the plasma. Extract was resuspended in 0.2 ml of assay buffer (Cayman Chemicals, Ann Arbor, MI). Recoveries of 5 spiked pools were high and homogenous (92% ± 0.8% SE), and thus final concentrations were not corrected. Hormone concentrations were determined in duplicate using a commercially available enzyme immunoassay (Cayman Chemicals) following the manufacturer's protocol. The assay is 100% specific for testosterone, 27.4% for 5α-dihydro-testosterone (5α-DHT), and 3.7% for androstenedione. The intraassay coefficient of variation was 9.0%, and the interassay coefficient of variation was 3.9%. 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. Linearity was checked by serial dilutions of a pool, which provided a slope that did not differ from the expected value (data not shown). Hormone levels were not normally distributed and were therefore log transformed.

Statistical procedures

Body mass, CMI, and all skeletal measurements were analyzed using hierarchical linear models in MLwiN (Bryk and Raudenbush 1993) to test the effect of treatment in a nested design. This method allows analyses of variance and covariance to be performed using unbalanced data, taking into consideration the nested relationship and repeated measurements of chicks and controlling for multiple (independent) variables. The following variables were included in the main models: treatment, sex, hatching order, hatching date (of the first chick), brood size, and all possible interactions. Only variables that contributed significantly ($\alpha \leq 0.05$) to the model were retained. To model the growth curve, we included age and the square of age as predictors in the model; the cube of age was also included when considering body mass and beak flange width and wing length (see Eising et al. 2001; Von Engelhardt et al. 2005). Significance

Table 1
Hierarchical linear model analysis of nestling body mass gain

Factors	Estimate	Error	Δdeviance	*P*
Constant	5.68	1.80		
Age	4.77	0.35	165.12	<0.0001
Square of age	0.35	0.05	53.25	<0.0001
Cube of age	−0.02	0.002	149.60	<0.0001
Hatching date	−0.13	0.06	4.39	0.04
Hatching order	−0.84	0.24	11.40	<0.001
Brood size	−1.04	0.45	4.94	0.03
Sex	−0.99	0.76	1.68	0.19
Sex × age	0.21	0.06	11.76	<0.001
Treatment	−0.31	0.99	0.10	0.75
Treatment × age	0.12	0.06	3.47	0.06

Treatment is coded 1 for control-chicks and 2 for androgen-chicks; sex is coded 0 for females and 1 for males.

was tested using the increase in deviance (Δdeviance) when a factor was removed from the model, which follows a χ^2 distribution.

We repeated the analysis for the subset of broods where the female had been caught and included female body mass and female age in the model. Furthermore, because blood spots that estimate female ectoparasite load were nearly absent on eggs of replacement clutches (mean number of spots [±SE]—first clutch: 29.67 ± 4.0; replacement clutch: 3.30 ± 1.56), we analyzed the possible effect of maternal ectoparasite load on chick growth for first clutches only.

Data on offspring sex were transformed by the logit link function and analyzed assuming an extrabinomial error distribution at the individual level (Goldstein 1995). Significance was tested using the Wald statistic, which follows a χ^2 distribution and accepted at $P < 0.05$ (2-tailed). Survival data were analyzed using the Wilcoxon–Gehan statistics in the life-tables option in SPSS.

RESULTS

Growth

Body mass gain

Offspring body mass was negatively affected by hatching date ($P = 0.04$), brood size ($P = 0.03$), and position in the hatching order ($P < 0.001$) (Table 1). There was a significant positive effect of the interaction of sex and age ($P < 0.001$), with males gaining more weight compared with females. There was a tendency for a positive interaction effect of androgen treatment and age ($P = 0.06$): androgen-chicks tended to gain more mass compared with control-chicks (Figure 1).

Two chicks (one control- and one androgen-chick, measured at the age of 4, 8 and 12 days; Figures 1 and 2) hatched 2 days after the first chick; however, omitting these 2 chicks did not change the outcome of this or any of the following analyses.

Body mass gain in relation to female characteristics

Females were caught in 16 control-broods and 22 androgen-broods. The effect of the interaction between female body mass and treatment tended to be significant (estimate 0.38, error 0.20, Δdeviance = 3.49, $P = 0.06$). When analyzing the treatment groups separately, the effect of female body mass on offspring weight tended to be positive in the androgen-group (estimate 0.18, error 0.10, Δdeviance = 2.80, $P = 0.09$), but there was no such effect in the control-group (estimate −0.18, error 0.15, Δdeviance = 1.42, $P = 0.23$). Older females had heavier offspring (estimate −2.27, error 0.86, Δdeviance = 6.31, $P = 0.01$), but this effect was not different between treatments.

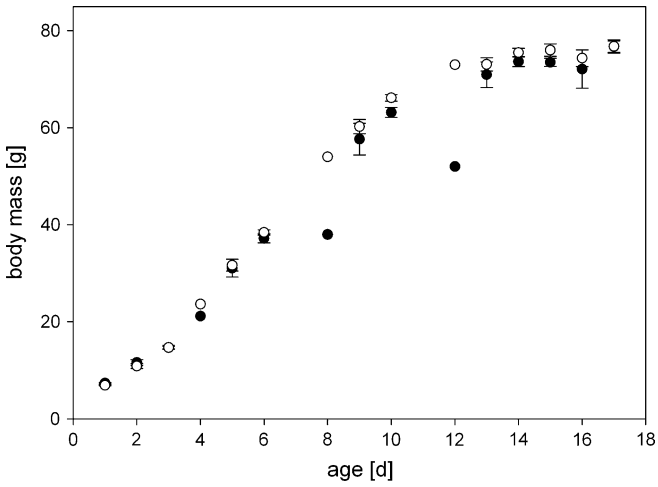

Figure 1
Body mass (mean ± SE) of control-chicks (closed symbols) and androgen-chicks (open symbols) in relation to their age. Those chicks measured at the age of 4, 8, and 12 days (one control- and one androgen-chick) hatched 2 days after the first chick of their brood.

There was no significant effect of the mean number of spots on the eggs on offspring weight (estimate −0.02, error 0.05, Δdeviance = 0.10, $P = 0.75$) and no significant interaction between the mean number of spots and treatment (estimate 0.04, error 0.03, Δdeviance = 1.52, $P = 0.22$), analyzed for the first clutches.

Skeletal growth

"Tarsus length" was negatively affected by the position in the hatching order (estimate −0.20, error 0.05, Δdeviance = 14.44, $P = 0.0001$). There was a significant effect of offspring sex, with males having longer tarsi (estimate 0.29, error 0.12, Δdeviance = 5.23, $P = 0.02$) and a nearly significant positive trend of hatching date (estimate 0.02, error 0.01, Δdeviance = 3.83, $P = 0.05$). There was no significant interaction effect of treatment and age (estimate −0.02, error 0.02, Δdeviance = 1.60, $P = 0.21$).

"Beak flange width" was positively affected by hatching date (estimate 0.03, error 0.01, Δdeviance = 5.64, $P = 0.02$). There was a significant effect of the interaction of treatment and age (estimate 0.05, error 0.02, Δdeviance = 10.59, $P = 0.001$) (Figure 2), in the sense that T chicks developed wider flanges.

"Beak length" was larger for males than for females (estimate 0.20, error 0.08, Δdeviance = 6.18, $P = 0.01$). There was no significant interaction effect of treatment and age (estimate 0.01, error 0.01, Δdeviance = 0.84, $P = 0.36$). The effect of the interaction of treatment and age was significant for "head width" (estimate 0.22, error 0.01, Δdeviance = 4.08, $P = 0.04$). There was a negative effect of hatching order on "wing length" (estimate −0.47, error 0.19, Δdeviance = 6.09, $P = 0.01$) but no significant interaction effect of treatment and age (estimate 0.05, error 0.04, Δdeviance = 1.39, $P = 0.24$).

Cell-mediated immunity

Cell-mediated immunity (CMI) could be measured for 136 chicks (from 44 broods) that survived until day 15. There was no significant effect of treatment on CMI (control-chicks 1.14 ± 0.05 [mm], androgen–chicks 1.22 ± 0.04 [mm]; estimate −0.02, error 0.08, Δdeviance = 0.05, $P = 0.82$). Offspring that was heavier at the day of injection tended to

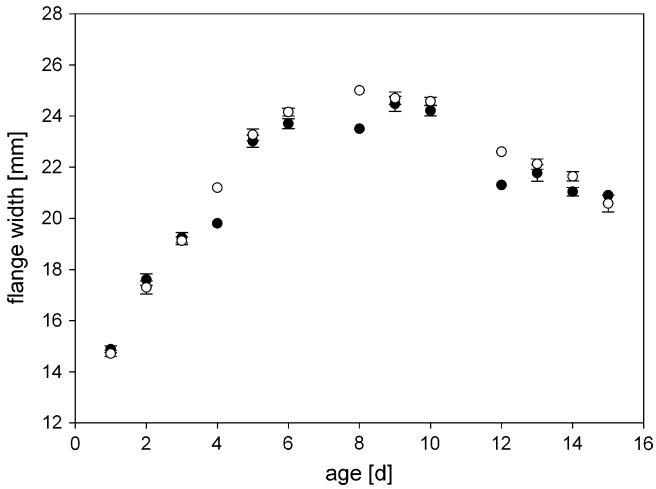

Figure 2
Beak flange width (mean ± SE) of control-chicks (closed symbols) and androgen-chicks (open symbols) in relation to their age. Two chicks (one control- and one androgen-chick, measured at the age of 4, 8, and 12 days) hatched 2 days after the first chick.

have a higher CMI (estimate 0.008, error 0.004, Δdeviance = 3.39, $P = 0.07$). Because androgen-chicks tended to have a greater body mass, we analyzed the possibility whether androgen-chicks would have a lower CMI for a given body mass by correcting CMI for body mass at the day of injection. However, even after correction, there were no significant differences between experimental groups (residual CMI: estimate 0.039, error 0.084, Δdeviance = 0.22, $P = 0.64$).

Survival

Nine chicks disappeared before they could be sexed. These chicks are only included in the survival analysis in relation to treatment and are not included in Figure 3. There was no overall difference in nestling survival between control-chicks (84%) and androgen-chicks (79%) (Wilcoxon–Gehan statistic 0.288, $P = 0.59$). When analyzing the sexes separately, we did not find an effect of treatment either on male (Wilcoxon–Gehan statistic 0.175, $P = 0.68$) or on female survival (Wilcoxon–Gehan statistic 2.49, $P = 0.11$). However, the pattern of mortality was different. When only those chicks that subsequently died before fledging were included in the analysis ($N = 30$), treatment significantly affected survival (Wilcoxon–Gehan 4.50, $P = 0.03$). This is due to the fact that mortality occurred earlier in control-chicks (mean mortality age ± SE: 6.45 ± 1.44 days) compared with androgen-chicks (mean mortality age ± SE: 11.16 ± 1.16 days).

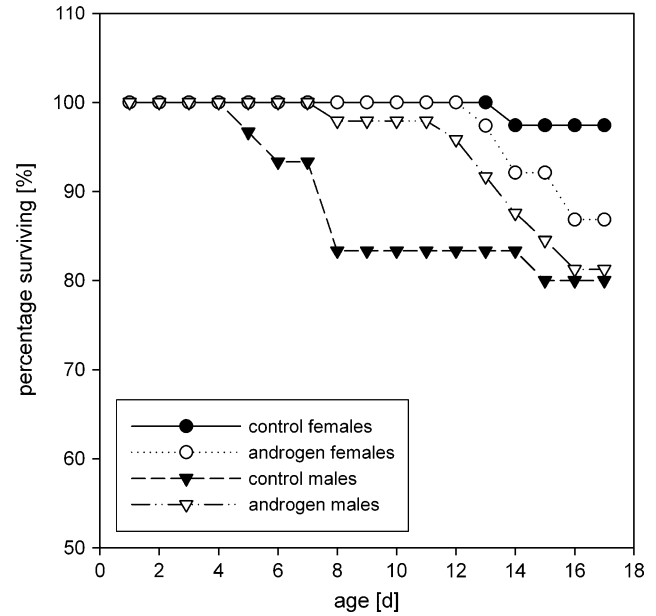

Figure 3
Distribution of mortality for all chicks that could be sexed, separated for sex (females are represented by circles, males by triangles) and treatment (control-chicks, filled symbols; androgen-chicks, open symbols).

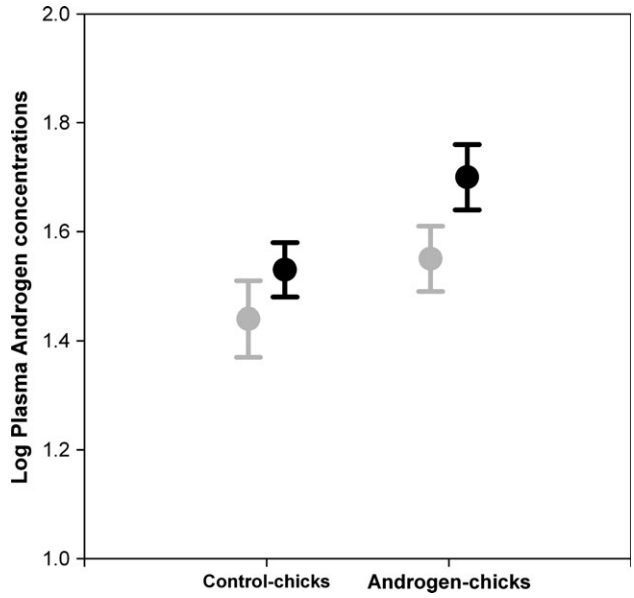

Figure 4
Plasma androgen concentrations (pg/ml) (log transformed) for control-chicks and androgen-chicks at about 15 days of age (females, gray symbols; males, black symbols).

Hormones

Plasma androgen concentrations could be measured for 97 chicks out of 43 different broods (37 control-chicks, 60 androgen-chicks) that were sampled at about 15 days of age. The reduction in sample size is due to either nestlings dying before sampling or an insufficient blood sample. We excluded from the final analysis 3 extreme values because they were identified as outliers (one control-chick 922.47 pg/ml and 2 androgen-chicks, 1066.77 and 14747.64 pg/ml). Including these values did not change the outcome of the analysis.

Control-chicks had an average plasma androgen concentration of 36.29 pg/ml (SE 4.04) whereas that of androgen-chicks were 60.35 pg/ml (SE 7.32). This difference was significantly different (estimate 0.14, error 0.07, Δdeviance = 3.94, $P < 0.05$; Figure 4). Male chicks had higher plasma androgen concentrations than female chicks (estimate 0.14, error 0.06, Δdeviance = 4.68, $P = 0.03$; Figure 4), but the interaction between sex and treatment was not statistically different (average ± SE—control-males: 37.71 ± 6.33, control-females: 35.08 ± 6.33; androgen-males: 72.50 ± 11.25, androgen-females: 44.46 ± 7.47) (estimate 0.07, error 0.13, Δ deviance = 0.26, $P = 0.61$).

DISCUSSION

Maternal yolk androgen concentration in bird eggs varies to a much larger extent between clutches than within clutches (Gil 2003; Groothuis, Müller, et al. 2005). Nevertheless, most studies on the functional consequences of androgen deposition have focused on a within-clutch approach, where experimental nests contained chicks from eggs with elevated androgen concentrations as well as sham-treated eggs (e.g., Eising et al. 2001; Pilz et al. 2004; Tschirren et al. 2005). However, the costs and benefits of yolk androgens are likely to be affected by direct competition between chicks of different treatments. Therefore, not all costs and benefits established in a within-clutch context may be generalized to a between-clutch context, when direct competition is excluded. Furthermore, maternal effects have been hypothesized to allow adaptive maternal adjustment of the offspring phenotype to the prevailing conditions after hatching. The consequences of embryonic androgen exposure for offspring development should thus depend on the environment experienced by the offspring.

In a between-clutch approach, we found that embryonic androgen exposure had a number of positive effects on offspring development, which was similar for both sexes (see also Saino et al. 2006; von Engelhardt et al. 2005; Rubolini et al. 2006).

Food acquisition

One of the most intriguing results of this study is the positive effect of yolk androgens on the beak flange width, with androgen-chicks developing larger beak flanges (Figure 2). Nestlings with larger flanges would display a larger gape area, which is a prominent feature of the begging display. Androgen-chicks did not have longer bills, which one would have expected if the effect would have been a simple consequence of androgen-chicks being larger in general (Clark 1995), or a growth-enhancing effect of the head in particular. The modification of the flange size may represent one pathway that allowed androgen-chicks to gain more mass compared with control-chicks. Increase of the gape size through yolk androgens adds a new element to the suite of yolk androgen–dependent modifications of traits that play an important role in food acquisition (alertness [Eising and Groothuis 2003], begging frequency [Schwabl 1996; Eising and Groothuis 2003; von Engelhardt 2005], and neck muscle [Lipar and Ketterson 2000]).

Furthermore, our study shows that embryonic exposure to maternally derived androgens increased endogenous androgen production after hatching (see Daisley et al. 2005 for a similar tendency in a precocial bird). Androgen levels in passerine nestlings have been shown to positively correlate with begging effort (Goodship and Buchanan 2006) and to increase with experimentally elevated nestling competition (Naguib et al. 2004). Thus, a direct relationship between embryonic androgen exposure and posthatching endogenous androgen production is likely to represent the underlying mechanism for the observed effects of maternal yolk androgens on begging as reported previously (e.g., Schwabl 1996; Eising and Groothuis 2003). This finding is highly important for our understanding of the costs and benefits of hormone-mediated maternal effects.

Male chicks had higher plasma levels of androgens (Figure 4), which may relate to their higher food demand and the need for competitiveness (Naguib et al. 2004; see also Goymann et al. 2005). Alternatively, males may in general produce higher levels of androgens from early ages onward, although previous studies did not show consistent sex differences in plasma androgen concentrations during the nestling period (e.g., males higher levels [black coucal]: Goymann et al. 2005, females higher levels [zebrafinch]: Adkins-Regan et al. 1990, no difference [zebrafinch]: Schlinger and Arnold 1992). Because our androgen assay cross-reacted significantly with 5α-DHT, a testosterone metabolite that has been found in some passerine nestlings (Schlinger and Arnold 1992), it is possible that our results could be due to an increase of 5α-DHT and not of T.

Because both wider gapes and high androgen levels promote the effect of begging, parents returning to their nest to feed androgen-nestlings are confronted with a greater feeding stimulus. This will, very likely, influence their feeding decisions. However, because we applied a between-clutch manipulation of the yolk androgen contents, the effect will not result in a decision about which chick to feed within the brood but rather on how much to feed the whole brood. Our results suggest that, in addition to the role of yolk androgens in sibling competition, yolk androgens may play an important role in parental feeding decisions and therefore in parent–offspring conflict. The tendency of androgen-chicks to gain more mass when they had a heavy mother may indicate that they were better at exploiting the resources of their mothers, to the extent that heavy starling mothers are better providers (Wendeln and Becker 1999).

Growth, immunity, and survival

Chicks hatching from eggs with elevated yolk androgen levels gained slightly more mass compared with chicks in control-nests. The reason for this difference not attaining statistical significance may be partly due to the fact that the brood size was reduced by 30% due to hatching failure as a consequence of the injection procedure, improving in this way the food conditions. Because it was an extremely dry year, the unfavorable environmental circumstances may have partly offset the positive effects of smaller brood sizes. Nevertheless, we also found effects of other variables such as hatching order, offspring sex, or hatching date on growth that have been reported to affect offspring body mass in a number of other studies (e.g., hatching date: Siikamäki 1998, hatching order: reviewed in Krebs 1999, sex: Müller, Kalmbach, et al. 2005, brood size: Naguib et al. 2004).

The growth-enhancing effects of yolk androgens did not depend on the position in the hatching sequence or offspring sex (see also, e.g., Tschirren et al. 2005; Rubolini et al. 2006). Interestingly, Pilz et al. (2004) injected European starlings eggs exclusively with testosterone using a concentration that was 10 times higher in terms of testosterone than in our experiment and more than 2 times higher in terms of total androgen concentration than ours, but did not find a stronger effect of their treatment on growth (see Navara et al. 2005 for evidence of dose-dependent effects). Further studies on dose dependency and manipulations of androstenedione versus only testosterone are needed to investigate this topic further.

We did not find a negative effect of yolk androgens on the CMI (Tschirren et al. 2005; Rubolini et al. 2006, see also Andersson et al. 2004; Groothuis, Eising, et al. 2005; Müller, Groothuis, et al. 2005; Navara et al. 2005). Chicks hatching from eggs with elevated yolk androgen levels died later but did not gain an overall survival advantage (Figure 3). However, only limited mortality occurred in this experiment.

Adaptive adjustment of offspring phenotype

We hypothesized that our in ovo androgen treatment could create a mismatch within the context in which the chicks were raised because our treatment shifted the androgen concentrations

away from the optimum deposited by the female. However, we found that embryonic exposure to elevated androgen levels had positive effects overall on offspring development irrespective of offspring sex, hatching order, and female quality. Higher yolk androgen levels than deposited seem to be beneficial for offspring, although we cannot exclude the existence of long-term costs (Strasser and Schwabl 2004; Eising et al. 2006). This raises the question as to why all eggs do not contain high amounts of yolk androgens. We hypothesized that, if the effects on offspring development are positive, the potential costs for the parents would limit the amount of hormones deposited. These costs may relate to the parental effort during nestling period or the hormone deposition itself. In spotless starlings, both will be mostly paid by the female because not only egg laying but also the major part of chick feeding is performed by the female (Moreno et al. 1999; Veiga et al. 2002). If the first is true, we would expect that the consequences of the androgen treatment for offspring development will depend on the maternal quality (such as her age, body mass, or ectoparasite load), which determines how well she can cope with an enhanced parental effort. However, we found little evidence to support this hypothesis.

Based on the findings by Pilz et al. (2003) showing a positive relationship between maternal age and the amount of yolk hormones deposited, we expected that older females would be better at raising androgen-chicks than younger females, the latter having difficulties raising androgen-chicks. Although older females had heavier offspring in general, we did not find evidence for an interaction with our androgen treatment. We did not find evidence that the maternal ectoparasite load affected the ability to raise androgen-nestlings, although only first clutches were analyzed. This is in line with previous studies on great tits (*Parus major*) showing that experimental elevation of the embryonic androgen exposure did not increase the parasite susceptibility of the nestlings (Tschirren et al. 2005), although exposure to ectoparasites by females during egg laying has been shown to affect yolk androgen deposition (Tschirren et al. 2004). Finally, we found a nonsignificant tendency for the mass gain of androgen-chicks to be greater when the females were heavier, which was not the case in control-broods. This is contrary to our expectation, given that food-supplemented female lesser black-backed gulls (*Larus fuscus*) deposited less androgens in their eggs (Verboven et al. 2003).

In conclusion, we did not find strong evidence for a context dependency. However, it has to be taken into account that we could not manipulate the context itself in this study, which would be the most powerful approach (Groothuis and von Engelhardt 2005).

Finally, costs relating to the deposition itself may for instance prevent younger females from depositing higher amounts of androgens (Pilz et al. 2003). This hypothesis is supported by this study, given the overall positive effects on offspring development and the lack of a strong context dependency. However, until now little is known of the mechanistic aspects of hormone deposition and potential physiological costs for the female that can occur if the female cannot independently regulate hormone levels in the yolk and her plasma (Groothuis, Müller, et al. 2005; Rutkowska et al. 2005). Alternatively, raising a clutch of chicks that hatched from eggs with high androgen content may carry costs through enhanced parental effort in terms of future reproductive success. These hidden costs were not investigated in this study. Given the outcome of this study, we feel that addressing these 2 questions could be a promising avenue for further research.

In conclusion, we found that yolk androgens increased the flange size, a trait that plays a role in food acquisition. Furthermore, chicks that hatched from eggs with elevated yolk androgen concentrations had higher endogenous levels of androgens at 15 days after hatching, which may promote begging. We hypothesize that, as a consequence of this, androgen-chicks gained slightly more mass, although this difference was not statistically significant. Both results point toward an increase in parental feeding effort, indicating that yolk androgens play an important role not only in mediating sibling competition but also in parent–offspring conflict. This issue needs further attention, and more studies are needed to investigate the relationship between parental effort and yolk androgen contents of the clutch. We found no evidence of a context dependency. This raises the question of whether and how yolk androgens prepare offspring to the prevailing posthatching conditions. We hypothesize that either the deposition of yolk hormones is costly for the female or long-term costs for the parents, which were not investigated in this study, may explain why not all clutches contain high androgen levels.

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