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Maternal yolk testosterone does not modulate parasite susceptibility or immune function in great tit nestlings

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Summary

1. Maternal yolk hormones can enhance the development and phenotypic quality of nestling birds. Nevertheless, within species large differences in yolk androgen concentrations among clutches are observed. This differential allocation of maternal yolk hormones might be explained by a trade-off between beneficial effects of yolk androgens and their associated costs.
2. Potential costs include an increased susceptibility to parasites in nestlings exposed to high concentrations of yolk androgens during embryonic development, weaker immune response or increased levels of circulating corticosterone that indirectly reduce immune function.
3. In a field study, we manipulated yolk testosterone in great tit (*Parus major*) eggs and tested the nestling's susceptibility to ectoparasites as measured by the parasites' effect on growth, the cellular immune response, and the levels of circulating corticosterone.
4. At the end of the nestling period, nestlings originating from testosterone-injected eggs were heavier than control nestlings. This effect was strongest in nestlings at the end of the size hierarchy, as shown by a significant interaction between hormone treatment and the nestlings' size rank within nests.
5. High levels of yolk testosterone promoted growth of the nestling's body mass similarly in parasite-infested and parasite-free nests, and neither affected the levels of plasma corticosterone, nor the nestling's cell-mediated immune response.
6. In summary, our results do not show negative short-term effects of high concentrations of yolk testosterone on immune function or parasite susceptibility, but emphasize that maternal investment via deposition of yolk testosterone can promote fitness-related growth and development of nestlings.

Key-words: *Ceratophyllus gallinae*, corticosterone, host–parasite interactions, maternal effects, maternal hormones.

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Introduction

Maternally derived androgenic hormones in vertebrate eggs can have profound effects on the offspring's development and behaviour later in life. High concentrations of yolk androgens increase, for example, the post-hatching growth of nestling birds (Schwabl 1996; Eising *et al*. 2001; but see Sockman & Schwabl 2000). Yolk androgens can also increase the nestlings' competitiveness in sibling interactions (Schwabl 1996; Lipar & Ketterson 2000; Eising & Groothuis 2003), and can enhance social status after fledging (Schwabl 1993). Maternal investment in yolk androgens can thus increase the reproductive value of the offspring by their direct influence on nestling traits. Despite these beneficial effects of yolk androgens, large variation in the deposition of maternal androgens between clutches of a species can be observed (e.g. Schwabl 1993; Reed & Vleck 2001; Verboven *et al*. 2003; Gil *et al*. 2004; Tschirren, Richner

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& Schwabl 2004). It has been suggested that costs for the nestlings or the mother associated with high levels of yolk androgens might counterbalance the beneficial effects of hormone deposition in the eggs. Yolk androgens might impair the development or function of the immune system (e.g. Grossman 1985; Alexander & Stimson 1988), enhance parasite susceptibility (Zuk & McKean 1996; Schalk & Forbes 1997; Poiani, Goldsmith & Evans 2000) or indirectly exert an immunosuppressive effect via an increase in circulating glucocorticoids (Gross, Siegal & DuBose 1980; Ketterson *et al*. 1991; De Besedovsky & Rey 1996; Apanius 1998; Padgett & Glaser 2003). Immunosuppressive effects of androgens are well known in both juvenile and adult mammals (e.g. Grossman 1985; Olsen & Kovacs 1996; Gaillard & Spinedi 1998) and birds (e.g. Gause & Marsh 1986; Saino, Møller & Bolzern 1995; Peters 2000; but see Hasselquist *et al*. 1999). However, the effect of androgen exposure during embryonic development on immune function later in life is, to our knowledge, unknown for natural bird populations. Prenatal exposure to steroids can modulate the immune system in mammals (Martin 2000), and in chicken high concentrations of yolk androgens inhibit the development of the bursa of Fabricius, an immunological organ in birds, and impair antibody production (e.g. Glick 1961; Glick & Sadler 1961; Verheul *et al*. 1986; Schuurs *et al*. 1992; see Andersson *et al*. 2004 for Chinese quails). However, Norton & Wira (1977) showed that there is a dose-dependent effect of yolk androgens on immune function in chicken. While *in ovo* injection of pharmacological concentrations of testosterone propionate was immunosuppressive, an *in ovo* injection of high physiological concentrations had immunostimulating effects. Thus, the very high doses of androgens often used in poultry studies may not be comparable to the effect of yolk androgens on immune function under natural conditions. If high physiological concentrations of yolk androgens impair the development or function of the immune system under natural conditions – either directly or indirectly by increasing the circulating levels of corticosterone (i.e. the main glucocorticoid in birds) – females have to trade off the costs and benefits associated with high levels of yolk androgens. They are expected to modulate the deposition of maternal androgens depending on the nestling's genetic resistance against infections (Gil *et al*. 1999) or the anticipated parasite load of the offspring. Indications for ectoparasite-modulated deposition of maternal androgens were found in a previous study on great tits (Tschirren *et al*. 2004). Females exposed to nest-based ectoparasites prior to egg laying deposited lower concentrations of testosterone and its precursor androstenedione in the eggs compared to unexposed females. The differential allocation of maternal resources in relation to ectoparasite abundance might be adaptive if high concentrations of yolk androgens are immunosuppressive or increase the nestling's parasite susceptibility.

Here we present an experimental field study investigating the effect of yolk testosterone on growth and development, susceptibility to parasites and cellular immune response in nestling great tits (*Parus major* L.). The great tit is a small, hole-nesting passerine and one of the main hosts of the ectoparasitic hen flea (*Ceratophyllus gallinae* Schrank) (Tripet & Richner 1997). Hen fleas are nest-based parasites that produce two overlapping generations during the host's nesting period (Tripet & Richner 1999). Hen fleas suck blood from nestlings and adult birds, impair the nestling's quality (Richner, Oppliger & Christe 1993) and reduce the host's lifetime reproductive success (Fitze, Clobert & Richner 2004a; Fitze, Tschirren & Richner 2004b). We experimentally manipulated yolk testosterone and exposed nestlings to hen fleas or kept them free of parasites after hatching. We measured body mass, body size and the nestling's circulating levels of corticosterone, and assayed their cell-mediated immunity by an *in-vivo* hypersensitivity response to an injection of phytohaemagglutinin (PHA). Beneficial effects (e.g. faster growth or higher body mass) of experimentally increased concentrations of yolk testosterone were predicted under parasite-free conditions, but negative effects for nestlings exposed to parasites. Further, we expected a reduced cellular immune response in nestlings hatched from testosterone-injected eggs, due either to the direct immunosuppressive effect of high concentrations of yolk androgens or indirectly due to increased levels of circulating glucocorticoids.

Materials and methods

YOLK ANDROGEN MANIPULATION AND GENERAL EXPERIMENTAL PROCEDURE

The study was performed in a great tit population breeding in nest boxes in the Forst, a forest near Bern, Switzerland (46°54′N 7°17′E/46°57′N 7°21′E). We regularly visited the nest boxes from the beginning of the breeding season onwards to determine the start of nest building and egg laying. After the clutch was completed we injected all eggs of a clutch with either 30 ng of testosterone (17β-hydroxy-4-androsten-3-on) (Fluka, Switzerland) dissolved in 5 μL of sesame oil, or with 5 μL of sesame oil as a control. The injected dose of testosterone was similar to the highest concentrations of yolk testosterone found in great tit eggs in our population (Tschirren *et al*. 2004). The injections were perfomed in the field using a cold light source (Intralux 4000, Volpi, Switzerland), a 25-μL syringe (Hamilton 702LT) and a 25-G needle. After the injection the hole in the eggshell was sealed by applying a small drop of tissue adhesive (Vetseal, B. Braun Medical, Sempach, Switzerland). We injected a total of 623 eggs from which 500 nestlings hatched. The overall hatching success was 80·3% and it was not significantly different between testosterone-injected (81·4% hatched) and control eggs (79·2% hatched) ($\chi^2 = 0{\cdot}473$, $P = 0{\cdot}492$, $N = 623$).

One day after hatching, nestlings of a control and a testosterone-injected clutch with the same hatching date and a similar brood size were partially exchanged. Nestlings were ranked in their original nest according to body mass and then assigned alternately to stay in their nest or to be transferred to the partner nest. For later identification nestlings were marked individually by clipping down feathers. After cross-fostering, all nests contained nestlings hatched from both testosterone-injected and control eggs. The nest material was then heat-treated in a microwave oven (Richner *et al.* 1993) to kill all parasites naturally present in the nest. Thereafter one nest of each cross-foster pair was assigned randomly to be infested with 40 female and 20 male hen fleas while the other nest remained free of parasites. The hen fleas used for the infestation were extracted from old nests collected in the study area at the start of the breeding season.

Eight days post-hatching nestlings were ringed with aluminium rings and 15 days post-hatching we measured body mass and length of the metatarsus (Svensson 1992).

CELL-MEDIATED IMMUNE RESPONSE

The cell-mediated immune response of the nestlings was measured using the phytohaemagglutinin assay (e.g. Smits, Bortolotti & Tella 1999). Phytohaemagglutinin (PHA-P, Sigma Chemicals, Buchs, Switzerland) is a lectin from *Phaseolus vulgaris* that has a strong mitogenic effect on T lymphocytes (Goto *et al.* 1978; McCorkle, Olah & Glick 1980; Cheng & Lamont 1988) and is commonly used to assess the cell-mediated immune response of birds in ecological studies (e.g. Lochmiller, Vesley & Boren 1993; Sorci, Soler & Møller 1997; Fargallo *et al.* 2002; Tschirren, Fitze & Richner 2003). Nestlings were injected subcutaneously with 0·1 mg of PHA-P dissolved in 0·02 mL of sterile phosphate-buffered saline (PBS) in the centre of the left wing-web (patagium) 13 days post-hatching. The thickness of the patagium at the injection site was measured with a micrometer (Mitotuyo, Tokyo, Japan, Type 2046FB-60) to the nearest 0·01 mm prior to and 24 h (± 1 h) after injection. The micrometer applies a constant pressure on the wing web and the measure stabilizes within time. The thickness of the wing web 5 s after applying the micrometer was used as a standardized measurement. The difference between the wing-web thickness before and after PHA injection was used as measure of the cell-mediated immune response (Smits *et al.* 1999).

CORTICOSTERONE ASSAY

Nine days post-hatching we took a blood sample (approx. 60 μL) from the tarsal vein using heparinized microhaematocrit capillaries. Blood of each nestling was sampled within less than 5 min and the sampling order was noted. Blood samples were taken randomly with respect to the treatment of the nestling. The samples were stored in a cooling bag in the field, and centrifuged for 5 min at 825 ***g*** the same evening. Plasma was separated from the red blood cells and stored at –20 °C for corticosterone analyses. Total corticosterone was assayed in 186 nestlings from 29 randomly chosen unparasitized and 175 nestlings from 27 randomly chosen parasitized nests.

Tritiated corticosterone (NET-399 PerkinElmer Life Sciences Inc., Boston, USA) was added to 20 μL of plasma (10 samples had a volume of less than 20 μL) and allowed to equilibrate overnight at 4 °C. Samples were then extracted twice with 4 mL of 30 : 70 petroleumether : diethylether on Extrelute NT (Merck KGaA, Darmstadt, Germany) columns. The extracts were dried under a stream of nitrogen and redissolved in 550 μL of phosphate-buffered saline (PBSg). Volumes of 200 μL were transferred to duplicate assay tubes and 100 μL were transferred to a scintillation vial to measure recovery rates. The radioimmunoassay was according to published protocols (Schwabl, Bairlein & Gwinner 1991) with corticosterone antiserum B3-163 (Esoterix Inc., Austin, USA) used in the assay. Mean recovery was 73·6 ± 0·53%. All samples were analysed in a single assay. The corticosterone detection limit for a 20 μL plasma sample with mean recovery of 73·6% was 0·272 ng/mL plasma. In eight nestlings hatching from control-injected eggs and 14 nestlings hatching from testosterone-injected eggs plasma corticosterone concentrations were undetectable (6% of the assayed birds) and the detection limit value of the assay was assigned to these individuals.

Corticosterone concentrations increased significantly with the order of blood sampling within a nest ($F_{1,288}$ = 12·866, $P < 0·001$, $r = 0·111$). We therefore controlled for disturbance during handling by including the order of blood sampling in the analysis. There were no significant seasonal or diurnal changes in corticosterone concentrations (all $P > 0·29$). We used log-transformed corticosterone concentrations in our analysis to allow for parametric testing.

STATISTICAL ANALYSES

Nested ANOVAs were used to analyse the effects of the parasite and the hormone treatment on morphological traits, cell-mediated immune response and plasma corticosterone levels. Parasite treatment and hormone treatment were included as fixed factors into the model, and the nest of rearing, nested within parasite treatment, was included as a random effect. One day after hatching nestlings were ranked according to body mass within the nest where they grew up (heaviest nestling = rank 1) and rank was included into the analyses as a measure of size hierarchy. Seasonal changes in the measured traits were accounted for by including the date of the measurement into the analyses. Non-significant interactions were backward eliminated, except for the interaction between the hormone and parasite treatment and the interaction between the hormone treatment and nestling rank. All tests were two-tailed with a

significance level set at $P = 0{\cdot}05$. Residuals of the models were tested for normality and homoscedasticity. Means ± 1 SE are given. Statistical analyses were performed using JMP IN 4·0 (Sall & Lehmann 1996).

Results

MORPHOLOGY

One day after hatching, nestlings originating from testosterone-injected eggs did not differ significantly in body mass from nestlings originating from control-injected eggs ($F_{1,402} = 0{\cdot}059$, $P = 0{\cdot}809$). At the end of the nestling period, however, nestlings hatched from testosterone-injected eggs were heavier than control nestlings ($F_{1,272} = 3{\cdot}987$, $P = 0{\cdot}047$). Body mass was significantly different between broods ($F_{60,272} = 8{\cdot}064$, $P < 0{\cdot}0001$) and was influenced by the rank of the nestling within the nest of rearing 1 day post-hatching ($F_{7,272} = 12{\cdot}510$, $P < 0{\cdot}0001$). The significant interaction between hormone treatment and nestling rank ($F_{7,272} = 2{\cdot}495$, $P = 0{\cdot}017$, Fig. 1a) shows that testosterone manipulation in the egg influenced body mass differently depending on the rank of the nestlings. Nestlings at the end of the size hierarchy grew faster in the testosterone-injected group (Fig. 1a, *post-hoc* contrast rank eight: $F_{1,272} = 9{\cdot}148$, $P = 0{\cdot}022$ after Bonferroni correction). In contrast, the nestlings at the beginning or in the middle of the rank order did not differ significantly in body mass between the treatment groups (Fig. 1a, *post-hoc* contrast, all $P > 0{\cdot}37$ after Bonferroni correction). Nestlings growing up in a flea-infested nest tended to be lighter 15 days post-hatching ($F_{1,60} = 2{\cdot}777$, $P = 0{\cdot}101$). There was no significant interaction effect between parasite and hormone treatment on nestling body mass 15 days post-hatching ($F_{1,272} = 0{\cdot}315$, $P = 0{\cdot}575$).

Metatarsus length was significantly different between broods ($F_{60,272} = 1{\cdot}578$, $P = 0{\cdot}008$) and was influenced by the nestling rank ($F_{7,272} = 4{\cdot}225$, $P < 0{\cdot}001$). We did not find significant effects of the parasite ($F_{1,60} = 2{\cdot}108$, $P = 0{\cdot}152$) or the hormone treatment ($F_{1,272} = 1{\cdot}024$, $P = 0{\cdot}312$) on metatarsus length, but again a significant interaction between hormone treatment and nestling rank ($F_{7,272} = 2{\cdot}124$, $P = 0{\cdot}041$, Fig. 1b, *post-hoc* contrast rank eight: $F_{1,272} = 8{\cdot}399$, $P = 0{\cdot}033$ after Bonferroni correction, all other contrasts $P > 0{\cdot}80$ after Bonferroni correction). Fitting nestling rank as a quadratic term revealed that metatarsus length followed a convex function in nestlings hatched from control eggs ($\beta = -0{\cdot}027 \pm 0{\cdot}009$), while it followed a concave function in nestlings hatched from testosterone-injected eggs ($\beta = 0{\cdot}003 \pm 0{\cdot}009$, hormone treatment × rank^2: $F_{1,283} = 10{\cdot}542$, $P = 0{\cdot}001$). As found for body mass, there was no significant interaction effect between parasite and hormone treatment on metatarsus length ($F_{1,272} = 0{\cdot}008$, $P = 0{\cdot}927$).

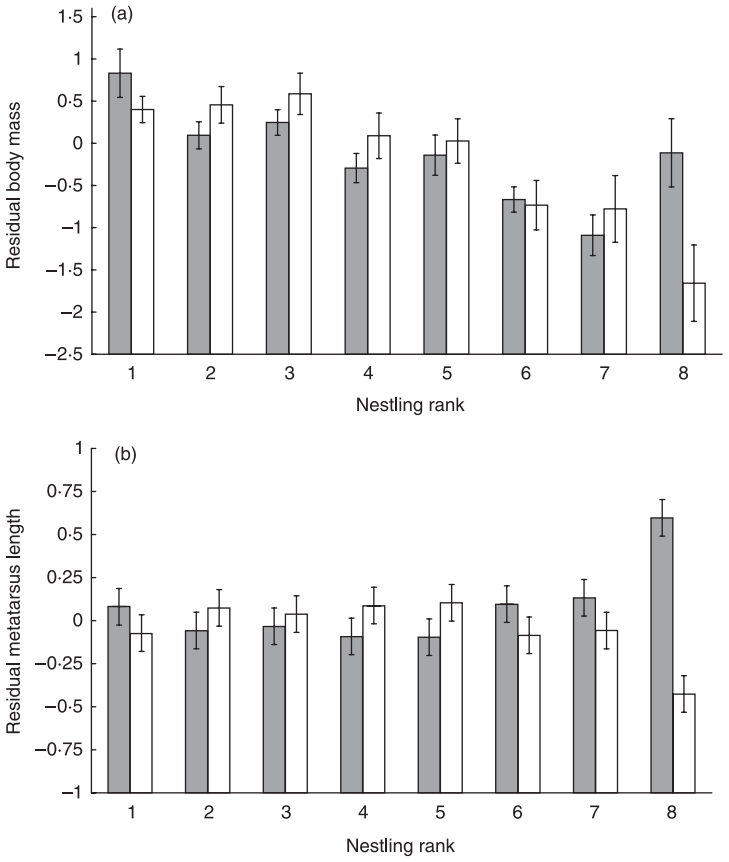

Fig. 1. Residual body mass (a) and residual metatarsus length (b) (± 1 SE) of nestlings hatched from testosterone-injected (filled bars) and control-injected (open bars) eggs in relation to their rank within the nest.

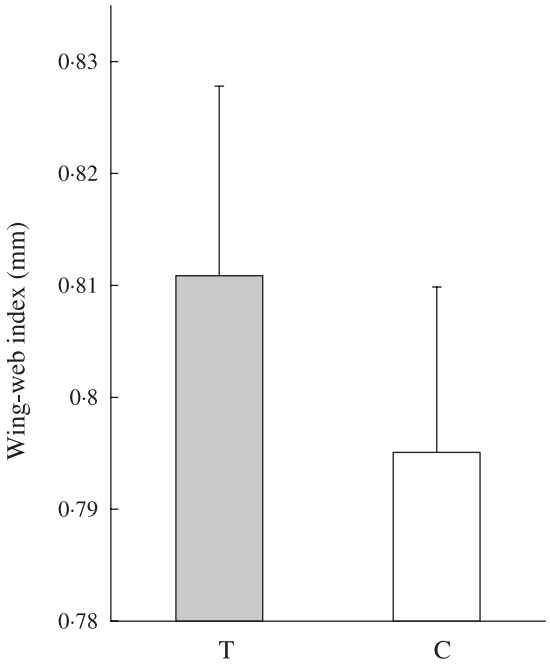

Fig. 2. Cell-mediated immune response (+ 1 SE) of nestlings hatched from testosterone-injected (T, filled bar) and control-injected (C, open bar) eggs 14 days post-hatching. Immune response controlled for body condition gave similar results (see Results section).

CELL-MEDIATED IMMUNE RESPONSE

Response to PHA injection was significantly different between broods ($F_{60,272} = 1{\cdot}902$, $P < 0{\cdot}001$), but not significantly influenced by the parasite treatment ($F_{1,60} = 1{\cdot}856$, $P = 0{\cdot}178$), the hormone treatment ($F_{1,272} = 0{\cdot}521$, $P = 0{\cdot}471$, Fig. 2), the rank of the nestling ($F_{7,272} = 1{\cdot}322$, $P = 0{\cdot}240$), the interaction between hormone treatment and nestling rank ($F_{7,272} = 1{\cdot}605$, $P = 0{\cdot}134$) or the interaction between hormone and parasite treatment ($F_{1,272} = 0{\cdot}320$, $P = 0{\cdot}572$). These results did not change if body condition, defined as the residuals of the regression of body mass 15 days post-hatching on metatarsus length ($F_{1,271} = 29{\cdot}396$, $P < 0{\cdot}0001$), was included as a covariate into the analysis. None of the interactions with condition explained a significant proportion of the variation in PHA response (all $P > 0{\cdot}356$).

CORTICOSTERONE

Mean plasma corticosterone concentrations of nestlings 9 days post-hatching was $6{\cdot}92 \pm 0{\cdot}35$ ng/mL plasma (range: 0·27–53·07 ng/mL plasma). Plasma corticosterone levels were significantly different between broods ($F_{54,288} = 2{\cdot}894$, $P < 0{\cdot}0001$), but not significantly influenced by the nestling rank ($F_{7,288} = 1{\cdot}163$, $P = 0{\cdot}324$), the parasite treatment ($F_{1,54} = 0{\cdot}343$, $P = 0{\cdot}561$), the hormone treatment ($F_{1,288} = 0{\cdot}575$, $P = 0{\cdot}449$), the interaction between hormone and parasite treatment ($F_{1,288} = 0{\cdot}147$, $P = 0{\cdot}701$) or the interaction between nestling rank and hormone treatment ($F_{7,288} = 0{\cdot}795$, $P = 0{\cdot}592$). Corticosterone concentrations were not significantly related to the cellular immune response of the nestlings (Pearson correlation: $r = 0{\cdot}053$, $P = 0{\cdot}342$, $n = 324$).

Discussion

Female birds deposit yolk testosterone into the eggs, and despite the beneficial effects on growth and competitiveness of young after birth, a large variation in the concentration of yolk testosterone is observed among clutches. It suggests either that some females are limited in the production and deposition of yolk androgens, or that females have to trade-off the beneficial and associated negative effects of yolk androgens for the offspring such as compromised immune functioning or parasite resistance and that different females arrive thereby at different optimal solutions. In this study we manipulated both yolk testosterone and ectoparasite load after hatching to investigate this trade-off. Nestling great tits hatching from eggs with experimentally increased yolk testosterone concentrations were heavier but not larger at the end of the nestling period compared to control nestlings. As fledgling body mass is related to the chance of an individual to be recruited to the breeding population the following year (e.g. Tinbergen & Boerlijst 1990) maternal yolk testosterone increases the reproductive value of the offspring, and hence maternal fitness.

In the great tit, we find a moderate hatching asynchrony of up to 5 days (Glutz von Blotzheim & Bauer 1993) between hatching of the first and the last nestling. Hatching asynchrony among siblings is suggested to be a maternal strategy to reduce the number of nestlings when parents cannot care adequately for a full brood, e.g. when food is limited (Lack 1947; Amundsen & Slagsvold 1998). However, when food is abundant and parents can rear the full brood, hatching asynchrony reduces phenotypic quality of late-hatching nestlings (Forbes 1994; but see Laaksonen 2004). In our study, the positive effect of yolk testosterone on body mass was found mainly in nestlings at the end of the size hierarchy. These nestlings normally have low competitive abilities and have access to less food compared to their bigger siblings. High concentrations of yolk testosterone may increase the nestling's competitiveness, as found in canaries (Schwabl 1996) and black-headed gulls (Eising & Groothuis 2003). Our study suggests therefore that high concentrations of yolk androgens might mitigate the consequence of asynchronous hatching in great tit broods.

Hen fleas are nest-based ectoparasites that survive inside the nest cavity until the breeding period of the following year, and produce up to two generations during the host's breeding cycle. Thus if fleas are already present during the prelaying period, high flea abundance during the nestling stage can be anticipated. A previous study showed that female great tits exposed to ectoparasitic hen fleas prior to egg-laying deposit

lower concentrations of testosterone and its precursor androstenedione into the eggs compared to parasite-free females (Tschirren *et al*. 2004). A reduced deposition of maternal androgens into the eggs might thus reflect an adaptive female strategy to reduce the nestlings susceptibility to hen fleas, or to hen flea-transmitted pathogens if exposure to high physiological levels of yolk androgens during embryonic development has immunosuppressive effects later in life. However, counter to our expectations, we did not find a significant interaction effect between the hormone and the parasite treatment on nestling morphology. In both parasite-free and parasite-infested broods high concentrations of yolk androgens increased the body mass and body size of the nestlings similarly, with strongest effects in nestlings at the end of the size hierarchy. There is thus no indication that high concentrations of yolk testosterone increase the nestling's susceptibility to ectoparasitic hen fleas under natural conditions. Similar susceptibility to parasites of nestlings hatching from control and testosterone-injected eggs might be explained by overall good weather and feeding conditions (Merino & Potti 1996) potentially mitigating the negative effects of parasites. However, the flea effect on nestling body mass was similar or even stronger in the year of the experiment compared to previous experimental studies (Fitze *et al*. 2004a) within the same great tit population (mean body mass 15 days post-hatching in this study: flea-infested nests: 15·01 ± 0·14; parasite-free nests: 15·62 ± 0·13, flea effect: −0·61 g; in 1997: flea-infested nests: 14·00 ± 0·23; parasite-free nests: 14·97 ± 0·23, flea effect: −0·97 g; in 1998: flea- infested nests: 14·93 ± 0·15; parasite-free nests: 15·39 ± 0·21, flea effect: −0·46 g; in 1999: flea-infested nests: 16·01 ± 0·28; parasite-free nests: 16·41 ± 0·25, flea effect: −0·40 g; in 2000: flea-infested nests: 15·31 ± 0·24; parasite-free nests: 15·31 ± 0·27 flea effect: 0·00 g).

There was also no significant negative effect of high concentrations of yolk testosterone on the nestling's cell-mediated immune response. This result contrasts with findings in adult male European starlings (*Sturnus vulgaris*), where manipulation of circulating testosterone in the high physiological range led to a 50% decrease of the cell-mediated immune response, assessed by the same method as in our study (Duffy *et al*. 2000). Compared to Duffy *et al*. (2000), the mean overall difference in cell-mediated immune response between nestlings hatching from testosterone-injected eggs and control eggs was small (3%) and it was higher – rather than lower – in nestlings hatched from testosterone-injected eggs compared to control nestlings. Our result also contrasts with laboratory studies on poultry where exposure to high concentrations of androgens during embryonic development led to lower immune function later in life, e.g. by inhibition of the development of the bursa of Fabricius and by a reduction or absence of antibody production (Glick 1961; Glick & Sadler 1961; Verheul *et al*. 1986). However, the immunosuppressive effects of embryonic exposure to androgens in chicken were dose-dependent (Glick 1961; Norton & Wira 1977; Verheul *et al*. 1986), and were found mainly when very high concentrations of androgens were applied. Thus, these laboratory studies might not be comparable with the effect of yolk testosterone on nestling immune function under natural conditions.

In house sparrows (*Passer domesticus*), implantation of testosterone in adult males led to lower secondary immune response (Evans, Goldsmith & Norris 2000). At the same time, the testosterone implantation also increased circulating levels of corticosterone. When correcting for the increase in corticosterone, the testosterone implantation increased rather than decreased the immune function, indicating that androgens can be immunostimulating, and that reduced immunocompetence might be the result of a correlated increase in corticosterone (see Møller & Saino 1994 for a review). In our study, we found no evidence for increased levels of corticosterone in nestlings hatched from eggs with increased concentrations of yolk testosterone. This result contrasts to a study on American kestrels (Sockman & Schwabl 2001), where nestlings hatching from androgen-injected eggs showed higher levels of plasma corticosterone later in life. Our results thus suggest that the higher corticosterone levels found in kestrel nestlings hatched from androgen-injected eggs might not be a direct effect of the yolk androgens per se but may have arisen by the severely reduced growth of the hormone manipulated nestlings.

Parasites harm their hosts and are thus expected to induce stress. Consistent with this prediction, infection with the ectoparasitic martin bug (*Oeciacus hirundinis*) led to increased levels of heat-shock proteins in the blood of nestling house martins (*Delichon urbica*) (Merino *et al*. 1998). However, experimental infestation of nestling great tits with ectoparasitic hen fleas did not significantly influence the nestling's plasma corticosterone levels in our study. Further, plasma corticosterone levels were not related to the PHA response at the end of the nestling period. This result contrasts with findings in nestling barn swallows, where the PHA response was correlated negatively with levels of corticosterone (Saino *et al*. 2003; but see Saino *et al*. 2002 for adult barn swallows). This difference might, however, be explained by the time lag of 4 days between blood sampling and assaying the nestling's immune response in our study.

In conclusion, our study reveals that nestlings exposed to high concentrations of yolk testosterone during embryonic development show higher body mass at the end of the nestling period, due probably to a higher competitiveness of the nestlings hatched from testosterone-injected eggs. The effect of the hormone treatment was dependent on the rank of the nestlings, and small nestlings benefited most of the extra testosterone in the yolk. Our results provide no evidence for increased susceptibility to parasites or suppression of the cell-mediated immune response in nestlings hatched from testosterone-injected eggs, but emphasize that maternal

yolk testosterone can positively affect fitness-related traits of nestlings. To understand the large variation observed in the deposition of yolk androgens in natural populations, we require studies investigating the long-term costs of exposure to high androgen concentrations during embryonic development, and studies on the costs arising from the deposition of high concentrations of yolk androgens for the female.

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