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Yolk androgens and the development of avian immunity: an experiment in jackdaws (*Corvus monedula*)

Maria I. Sandell*, Michael Tobler and Dennis Hasselquist
Department of Animal Ecology, Lund University, SE-223 62, Sweden
*Author for correspondence (e-mail: maria.sandell@zooekol.lu.se)

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SUMMARY

Maternally derived androgens have been shown to influence offspring phenotype in various ways. In birds, the benefits of prenatal androgen exposure, such as increased competitiveness and accelerated growth in nestlings, have been suggested to be balanced by costs, such as reduced immune function. In this study, we used an integrative approach to examine the influence of yolk androgens on the development of immune function in jackdaw (*Corvus monedula*) nestlings. Specifically, we tested whether the effects of yolk androgens on offspring immunity may extend over the first few days of life and be detectable even after several weeks. We manipulated yolk androgen concentrations in jackdaw eggs and estimated immune responsiveness by challenging the young with different pathogens at different stages of the nestling period. Six-day-old chicks hatched from eggs with elevated yolk androgen levels had lower pre-challenge antibody titres against lipopolysaccharide (LPS) than control chicks. However, antibody titres against LPS did not differ between treatment groups eight days after a challenge with LPS. During the late nestling phase, both humoral (towards diphtheria/tetanus antigens) and cell-mediated (towards phytohaemagglutinin) immune responsiveness were lower in chicks from yolk androgen-treated eggs compared with control chicks. Our experimental study on jackdaw chicks shows that elevated yolk androgen levels result in a general immunosuppression in offspring; this conclusion was based on results for several immunological tests of both humoral and cell-mediated immunity conducted at 1–2 and 3–4 weeks of age.

Key words: development, immunocompetence, life-history, maternal effects, androgens.

INTRODUCTION

A major objective of evolutionary biology is to relate phenotypic variation to fitness and, thus, to understand how variation is generated and lost. The prevailing view that an individual's phenotype reflects the interaction between its own genotype and the environment has recently been expanded to include the impact of the social environment on phenotypic expression, particularly as it acts through maternal effects (Mousseau and Fox, 1998). Maternal effects are the direct influences on offspring fitness that is a result of the phenotype or environment of its mother (Falconer and Mackay, 1996).

In egg-laying vertebrates, maternal effects may act through differential transfer of nutrients, maternal antibodies and hormones (Schreck et al., 1991; Williams, 1994). Maternal hormones influence the development of offspring in a wide range of vertebrate species (Schreck et al., 1991; Schwabl, 1993; Adkins-Regan et al., 1995; Schwabl, 1996; Conley et al., 1997). In birds, it has been argued that females may enhance their reproductive success by mediating sibling competition and growth of offspring by means of differential hormone transfer to the egg yolk (Schwabl, 1993; McNabb and Wilson, 1997; Eising et al., 2001). For example, differential transfer of steroids to eggs within the same clutch may mitigate or increase the effect of hatching asynchrony as yolk steroids enhance nestling growth and competition (Sockman and Schwabl, 2000; Eising et al., 2001; Groothuis et al., 2006)

Although several studies have shown how nestlings benefit from increased yolk androgens, e.g. in terms of increased growth and survival (for a review, see Groothuis and Schwabl, 2008), high levels of yolk androgens also involve costs. Exposure to elevated androgen levels in developing chicks can increase metabolism (Tobler et al., 2007) (but see Eising et al., 2003) and may possibly increase susceptibility to oxidative stress (Royle et al., 2001; Groothuis et al., 2006). Several studies have also shown that yolk androgens can influence nestling immune capacity (Andersson et al., 2004; Groothuis et al., 2005a; Mueller et al., 2005; Navara et al., 2005; Navara et al., 2006) (but see Tschirren et al., 2005). However, the majority of these studies involve a single immunological test, namely the test of cell-mediated immunity by injection with phytohaemagglutinin (PHA) (e.g. Lochmiller and Deerenberg, 2000). Only one study has also investigated the effect of yolk androgens on humoral immunity in combination with cell-mediated immunity (Mueller et al., 2005). In this study on black-headed gulls (*Larus ridibundus*), however, immune tests were performed at a very early stage of the chick phase (seven days of age), i.e. when the immune system of the young is immature and chicks rely on maternally transferred antibodies for protection against antigens. Hence, it is not known whether the effect of yolk androgens on immunity extends beyond the first days of life and whether it involves other parts of the immune system than PHA-induced cell-mediated immunity. There is, therefore, a need for studies that investigate the effect of yolk androgens on several lines of immunity during an extended part of the chick period when the chick is reliant on its own immune defence. An integrative approach investigating several lines of immunity is essential for understanding the trade-offs between immunocompetence and other life history traits (e.g. Norris and Evans, 2000; Adamo, 2004).

During the first phase of life, young birds have a less effective defence against parasites and infections than adult birds. Birds are born with an incomplete immune system and young chicks have to rely on maternal antibodies and the innate immune defence system

to fight off pathogens (Apanius, 1998). It has been shown that activating or maintaining immune functions can be metabolically costly and resource demanding (Saino et al., 1997; Lochmiller and Deerenberg, 2000; Alonso-Alvarez and Tella, 2001; Råberg et al., 2003) (but see Svensson et al., 1998), which indicates a trade-off between immune function and life-history traits (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000). Similar trade-offs may also exist between different parts of the immune system, e.g. if investment in cell-mediated immune function impairs concomitant investment in humoral immune function (Salvante, 2006) and these trade-offs may be different during different life stages. Maternal steroids may prime the development and expression of various components of the immune system such as cell-mediated and humoral immune function differently.

In this study, we used an integrative approach to examine the influence of yolk androgens on the immune function of jackdaw (*Corvus monedula* L.) nestlings. This species shows strong hatching asynchrony with brood reduction of up to 50% (Gibbons, 1987; Heeb, 1994). Within-clutch levels of yolk androgens (testosterone and androstendione) decrease with laying sequence (M.I.S., unpublished data) and may, hence, promote size differences in hatching asynchrony. Moreover, jackdaws have a relatively long nestling period of over 30 days and are, therefore, well suited to study the development of the immune system. This species is also a suitable contrast with studies on yolk hormones and immunity in the black-headed gull [*Larus ridubundus* (Muller et al., 2005)], which also has a long nestling period, hatching asynchrony but an increase of yolk androgens with laying order. Specifically, whether humoral immunity may be affected by yolk androgens even long after hatching, at a time when the offspring is reliant on its own immune system for protection against pathogens. We assessed humoral immune function of chicks hatched from either androgen-treated or control eggs towards different immune system activators; the mitogen lipopolysaccharide (LPS) when chicks were 1–2 weeks old and two types of antigens (diphtheria toxoid and tetanus toxoid) when chicks were 3–4 weeks old. In three-week-old chicks, we also assessed cell-mediated immune response by injection with PHA. Repeated exposure to different antigens and a mitogen allowed us to test whether and how the influence of yolk steroids on adaptive immune function changes over the developmental phase.

MATERIALS AND METHODS

The experiment was carried out during 2003 and 2004 in the Revinge area in southern Sweden (*ca*. 55 deg.41′N, 13 deg.27′E). The jackdaws used in this study were breeding in two colonies with 39 and six nest boxes, respectively. In 2003, we used only pairs in the large colony whereas in 2004 both colonies were used. The distance between the colonies was 400 m. Nest boxes were checked daily before laying to determine the start of breeding. When egg laying was initiated, each egg was replaced with a plastic egg on the day of laying. All eggs were transported to the field station (1.5 km away) and stored outside in a closed nest box. Egg mass (to nearest 0.1 g) was measured.

When the fourth egg in a clutch was laid (all clutches contained at least four eggs), we manipulated yolk androgen concentration in the collected eggs. We assigned eggs to two groups depending on laying order – early (the two first eggs laid) and late group (egg number three and four in the laying sequence). One egg in each group was randomly injected with 70 ng testosterone and 300 ng androstendione suspended in 25 µl sesame oil (androgen chicks). The remaining eggs received an injection of 25 µl sesame oil only (control chicks). The holes were sealed with OpSite (Smith & Nephew, Mölndal, Sweden), a transparent adhesive dressing. The dose of injected androgens corresponds to approximately one standard deviation of the mean yolk androgen levels found naturally in our population (M.I.S., unpublished data) and is, therefore, assumed to be within the physiological range of the species.

After injection, eggs were returned to the nests in the colonies. Eggs from two nests were combined in a dyad so that one nest received only early laid eggs (one control egg and one androgen-injected egg from own nest and one control egg and one androgen-injected egg from another nest) or only late-laid eggs (one control egg and one androgen-injected egg from own nest and one control egg and one androgen-injected egg from another nest). This enabled us to analyse the effect of androgen manipulation and, at the same time, separate genetic effects (genetic nest) and environmental effects during the nestling rearing period (foster nest) as well as analyse potential laying order effects (early or late eggs). In 2003, all eggs laid after the fourth egg were collected and frozen. Due to the low hatching success and subsequent small clutches in 2003, we changed the experimental setup during 2004 and we returned any eggs laid after the fourth egg to the nest. The fifth and/or sixth egg were treated as control eggs (N=14) or received yolk androgen manipulation (N=9) and they were added to the nest one day later than the first four eggs. Due to brood reduction during the first week, only four chicks from eggs five or six were alive during the immune challenge. These chicks were not included in analyses of nestling growth or immune response but the ‘extra’ eggs were accounted for in analyses by including actual brood size at different periods of the nestling cycle in the models (see below).

Hatching success, fledgling success and nestling growth were monitored during nest visits with 1–4 day intervals. Body mass was measured eight times; on day 0, 2, 6, 10, 14, 20, 24 and 28, tarsus length on day 10, 14, 20, 24 and 28 and wing length on day 14, 20, 24 and 28. Brood size was measured at hatching and when the oldest chick was 6, 14 and 28 days old.

Testing humoral immunity

We tested humoral immunocompetence using one mitogen and two types of antigens during two stages of the nestling period. During the early developmental period of the nestlings, we injected six-day-old chicks with LPS. LPS is derived from cell walls of Gram-negative bacteria, *Escherichia coli*, and mimics a natural infection with bacteria (e.g. Kuby, 1998; Bonneaud et al., 2003). Immune response towards LPS involves both the innate defence (such as behavioural responses, fever, inflammatory response and production of acute phase proteins) and the adaptive defence (Janeway and Travers, 1996). We injected nestlings with 7.5 µg LPS suspended in 100 µl PBS (0.1 mg of LPS per kg of body mass) (Grindstaff et al., 2006). We avoided measuring aspects of the acute phase response to avoid disturbance at the nest when jackdaw nestlings were less than 10-days-old. Prior to injections, we took a blood sample (50 µl) from the jugular vein for analyses of pre-injection concentrations of antibodies against LPS. We expected these six-day-old nestlings to have anti-LPS antibodies as a consequence of maternal antibody transfer (most probably including anti-LPS antibodies because exposure to Gram-negative bacteria should be common among adult birds) but possibly also because these nestlings already had functional B-cells. Our rationale for the LPS injection of six-day-old chicks was to ensure that all chicks had a similar (moderate) exposure to LPS at a time when their own B-cells are beginning to mature and, hence, to be able to measure the T-cell-independent activation of their endogenous B-cells based on a (as far as practically possible) similar level and timing of LPS challenge.

Because B-cells need to be activated to produce antibodies against LPS and antibodies have a half-life of 5–7 days in newly hatched chicks (Davison et al., 2008), we decided to measure anti-LPS antibody titres eight days after LPS injection. Hence, when nestlings were 14-days-old we took a second blood sample for analyses of the nestlings' anti-LPS antibody response (100 µl). This response measures the T-cell-independent activation of B-cells to produce IgM antibodies (Janeway and Travers, 1996) and should reflect the ability of 14-day-old nestlings to produce a first-line antibody defence to an infection with Gram-negative bacteria. Maternally transferred antibodies (*via* egg yolk) should be very low or non-existent in the circulation in 14-day-old nestlings (Hasselquist and Nilsson, 2009). Moreover, the response in 14-day-old nestlings should be relatively independent of difference in exposure to Gram-negative bacteria before LPS injection at day six, because LPS is a T-cell-independent antigen that does not induce formation of memory B-cells and, thus, does not induce secondary antibody responses (Janeway and Travers, 1996).

When nestlings were 14-days-old, we challenged other aspects of their humoral immunity using a diphtheria/tetanus vaccine (DTV) containing two antigens. Diphtheria toxoid and tetanus toxoid are potent antigens that activate antigen-specific B-cells resulting in a T-cell-dependent primary antibody response (i.e. first production of IgM followed by IgG antibodies peaking *ca*. 14 days post-injection as well as the formation of memory B-cells) (Janeway and Travers, 1996). Individuals were vaccinated with 100 µl human DTV (2 Lf diphtheria toxoid, 5 Lf tetanus toxoid adsorbed in aluminium phosphate; Aventis Pasteur, Toronto, Canada) in the pectoral muscle. Prior to the injection, a blood sample of 100 µl was taken from the jugular vein. The sample was used to analyse post-injection analyses of LPS treatment (see above) as well as measurement of background values of antibodies to diphtheria/tetanus. Primary antibody responses towards diphtheria/tetanus peak around 9–15 days after vaccination in small to medium sized passerines (Hasselquist et al., 1999; Owen-Ashley et al., 2004) and *ca*. 14 days after vaccination in, e.g. pheasants (Ohlsson et al., 2002). We, therefore, collected a blood sample after 14 days, when the young were 28-days-old (100 µl). All blood samples were centrifuged for 10 min at 1000 ***g*** and the separated plasma was stored at –50°C until analysis. The fact that jackdaw nestlings stay for about a month in the nest box, which is a much longer period than nestlings of other nest box breeding passerine birds, which leave the nest when *ca*. two weeks old (e.g. Grindstaff et al., 2006), allowed us to measure primary antibody responses of nestlings

Antibody titres were determined using enzyme-linked immunosorbant assays (ELISA). Protocols followed those previously developed for passerines [LPS (Grindstaff et al., 2006); diphtheria/tetanus (Owen-Ashley et al., 2004)]. An individual's pre- and post-vaccination plasma samples in duplicate were placed on the same ELISA plate. Pre- and post-injection plasma samples were diluted 1:100 for LPS antibodies, 1:300 for the diphtheria antibodies and 1:900 for the tetanus antibodies. The strength of the humoral immune response was estimated as the difference between post- and pre-immunisation antibody titres. For comparison of samples between plates, a serially diluted standard (pooled serum from jackdaw chicks immunised with the antigens) was run on all plates. However, in diphtheria/tetanus assays, we used two different standards in 2003 and 2004 and, therefore, we cannot distinguish between-year assay variation from other between-year variation due to, e.g. environmental factors. We, therefore, controlled for year in all statistical analyses of antibody titres against diphtheria and tetanus toxoids.

Cell-mediated immunity: PHA challenge

The mitogen PHA is commonly used in passerine birds as a T-cell stimulant. Injection of PHA (Cat. L-8754, Sigma Chemical Co., St Louis, MO, USA) in the wing web produces a swelling approximately 24 h after injection. PHA activates the cell-mediated immunity in a specific way but it also stimulates parts of the innate immunity, e.g. macrophages and inflammatory responses (e.g. Martin et al., 2006).

On day 19, chicks were injected with PHA dissolved in PBS in the wing web on both wings. The injection volume differed between years although the concentration was the same. In 2003, we used 0.5 mg PHA in 0.1 ml PBS and in 2004, 0.25 mg in 0.05 ml PSB. Because chicks were injected on both wings, the total amount of PHA was 1 mg and 0.5 mg for 2003 and 2004, respectively. Granbom and colleagues showed that the spatial repeatability of PHA swellings between both wings is relatively low and to improve the accuracy of the test, both wings should be injected with PHA (Granbom et al., 2005). Smits and colleagues showed that it is not required to use a control injection of saline to accurately assess the response to PHA (Smits et al., 1999). Prior to injection, the injection point was marked and three measurements of the wing web were taken with a digital micrometer to the nearest 0.001 mm. 24 h later (±20 min), three new measurements were taken on each wing. Median values were used in all analyses.

Statistics

Statistical analyses were conducted with SAS System for Windows 9.1 (SAS Institute Inc., Cary, NC, USA). We used repeated mixed-model analysis of variance (ANOVA) (PROC MIXED) (Littell et al., 2004) with foster nest and genetic mother as random factors, egg treatment, year and sex as fixed factors and hatching date, body mass and brood size as covariates. Random effects were estimated with the likelihood ratio test as described in Littell et al. (Littell et al., 2004). Non-significant ($P>0.1$) fixed factors and covariates as well as interactions were sequentially backward eliminated from the models. The Sattherthwaite approximation was used to calculate the denominator degrees of freedom (Littell et al., 2004). The significance level was set at $P<0.05$.

There is a sex-specific growth and mortality in jackdaw nestlings (Table 1) (Arnold and Griffiths, 2003). 82% of all nestlings were sexed with molecular methods (Fridolfsson and Ellegren, 1999). We ran statistical tests both including sex as a factor in all models of nestling growth, nestling size and immune response (i.e. with reduced sample size) and with the complete dataset excluding sex. However, models with and without sex produced qualitatively the same results and we, therefore, present only the results from analyses with sex included.

RESULTS

Hatching success

We injected 245 eggs in 59 nests (23 nests in 2003 and 36 nests in 2004), including nine last-laid eggs not included in the analyses. Hatching success was 68.0% in 2003 and 73% in 2004. There was no significant difference in hatching success between control eggs and androgen-injected eggs (chi-squared test, $\chi^2=1.00$, d.f.=1, $P=0.32$) and there was no effect of laying order on hatchability ($P=0.45$).

Clutch size and brood size

Clutch size declined from 4.83±0.13 chicks (means ± s.e.m.) at hatching to 2.26±0.12 chicks at day 14 and 1.5±0.16 chicks at day

Table 1. Nestling growth in jackdaw chicks from control-injected and androgen-injected eggs

	Body mass				Tarsus length				Wing length			
Source	χ^2	F	d.f.	P	χ^2	F	d.f.	P	χ^2	F	d.f.	P
Year		1.48	1, 45.1	0.23		3.2	1, 32.5	0.082		3.40	1, 34.8	0.074
Experiment		0.04	1, 89.3	0.84*		0.07	1, 33.8	0.79*		0.11	1, 29	0.74*
Sex		4.18	1, 10.7	0.043		2.50	1, 37.8	0.27*		0.68	1, 34.5	0.41*
Hatching date		9.12	1, 43.8	0.004		3.85	1, 40.8	0.056		4.12	1, 40.4	0.049
Number of nestlings		6.15	1, 47.4	0.016		31.6	1, 31.6	0.021		5.57	1, 35.3	0.024
Age		868.15	7, 35.8	<0.0001		22.8	4, 13.1	<0.0001		225.2	4, 14.1	<0.0001
Age×year		7.75	7, 35.7	<0.0001		8.26	2, 13.7	0.0004		8.26	2, 14.7	0.0004
Foster nest	3.6		1	0.058	4.5		1	0.033	9.6		1	0.002
Genetic parent	1.3		1	0.27	0.2		1	0.65	0.8		1	0.37

Repeated mixed-model analyses of body mass (day 0, 2, 6, 10, 14, 20, 24 and 28), tarsus length (day 10, 14, 20, 24 and 28) and wing length (day 14, 20, 24 and 28). The full model included year, sex and experiment as fixed factors, genetic nest and foster nest as random factors and hatching date, body mass and brood size at hatching and day 14 as covariates. Presented are the reduced models with non-significant effects (P>0.1) backward eliminated from the models. *Results for experiment and sex from full models.

28. There was a significant difference in clutch size at hatching between years due to the difference in experimental design (2003, 2.75±0.14 chicks; 2004, 3.8±0.13 chicks; t-test, t_{54}=5.45, P<0.001) but this difference disappeared during the nestling period and there was no significant difference in brood size at day 14 (2003, 2.42±0.17; 2004, 2.17±0.19, t_{52}=0.85, P=0.40), indicating that the degree of brood reduction was higher in 2004, presumably due to larger broods.

Mortality

Mortality rate during the nestling period was relatively high (37.5%) and was due to either starvation or predation. Predation (18.4%), which was most probably due to pine martens (*Martes martes*), did not differ between egg treatments (chi-squared test, χ^2=2.10, d.f.=1, P=0.14).

Mortality not related to predation is assumed to be due to starvation/sickness and when excluding predated nestlings, mortality was still not significantly related to egg treatment (χ^2=0.34, d.f.=1, P=0.56). The timing of nestling mortality due to starvation did not differ between treatments (mean age: control chicks, 5.23±0.96 days; chicks from androgen-treated eggs, 7.23±0.96 days; t-test, t_{24}=0.98, P=0.33).

Nestling growth

Hatching mass was not influenced by yolk androgen treatment ($F_{1,40.7}$=1.45, P=0.23 with egg mass as a covariate). Nestling growth rate from hatching until day 28 was significantly different between years with a faster growth rate in 2003 but overall size did not differ significantly between years (Table 1). There was a significant effect of sex on body mass (males were heavier than females) but there was no significant sex difference in tarsus length or wing length (Table 1). However, there was no effect of egg androgen treatment on nestling growth measured as increase in body mass, tarsus length or wing length (Table 1). Final body mass at day 28 was also unrelated to egg treatment (body mass, $F_{1,36.5}$=0.001, P=0.96 with effect of sex, $F_{1,44.9}$=11.24, P=0.0016 and year, $F_{1,35}$=12.14, P=0.0013).

Nestlings that hatched early during the breeding season grew faster than those hatched late resulting in a significant effect of hatching date on body mass and wing length and a near significant effect on tarsus length (Table 1). However, the effect of hatching date was independent of androgen treatment. Rearing environment was important as indicated by the significant effect of foster nest (Table 1).

LPS challenge

Jackdaw chicks had plasma antibodies against LPS already prior to LPS challenge at day six [mean 3.15±0.35 (log: 1+mOD min^{-1})]. There was a significant difference in antibody concentrations between control and androgen chicks in the pre-injection samples; control chicks had higher antibody titres than androgen chicks [control chicks, 3.48±0.48 (log: 1+mOD min^{-1}); androgen chicks, 2.82±0.14 (log: 1+mOD min^{-1}); $F_{1,52.2}$=6.16, P=0.016] (Fig. 1). There was also a significant interaction between egg treatment and hatching date ($F_{1,46.7}$=5.71, P=0.021) but no overall effect of hatching date (P=0.45). The interaction was due to a trend towards a seasonal decline in LPS antibody titres among control chicks ($F_{1,38.9}$=3.54, P=0.06) compared with no seasonal effect among androgen chicks (P=0.29). There was no effect of brood size or body mass on antibody concentration prior to injection. There was a significant effect of foster nest (χ^2=9.8, d.f.=1, P=0.0017) but not of the genetic parents (χ^2=2.8, d.f.=1, P=0.091) on pre-injection values.

The majority of chicks (87%) increased LPS antibody concentrations after the injection and the difference between pre- and post-injection concentration was significant (paired t-test, t_{88}=6.48, P<0.0001). Pre- and post-injection concentrations were significantly, albeit weakly, correlated (r_{59}=0.30, P=0.02). However, antibody concentrations in chicks eight days after the challenge with LPS did not differ significantly between treatment groups ($F_{1,39.5}$=0.02, P=0.89) (Fig. 1). Hatching date was significantly related to antibody response with a larger response in early broods irrespective of egg treatment (Table 2).

Diphtheria/tetanus challenge

Pre-injection concentrations against diphtheria and tetanus were low [diphtheria toxoid, 0.85±0.08 (log: 1+OD min^{-1}); tetanus toxoid, 0.71±0.78 (log: 1+mOD min^{-1})] and significantly correlated (r_{70}=0.79, P<0.0001). There was no significant effect of egg treatment, body mass, hatching date or brood size on pre-injection values (P>0.2 in all cases). The diphtheria/tetanus challenge produced significantly higher antibody titres than pre-injection values (diphtheria, t_{69}=4.42, P<0.0001; tetanus, t_{69}=6.62, P<0.00019). The antibody titres on average showed an increase that was 24-fold for tetanus toxiod and 5-fold for diphtheria between pre-injection and post-injection samples (14 days later at the presumed peak of the primary antibody response), showing that nestlings 2–4 weeks old can already produce a normal T-cell-dependent antibody response. A high correlation between the antibody production towards diphtheria toxoid and tetanus toxoid

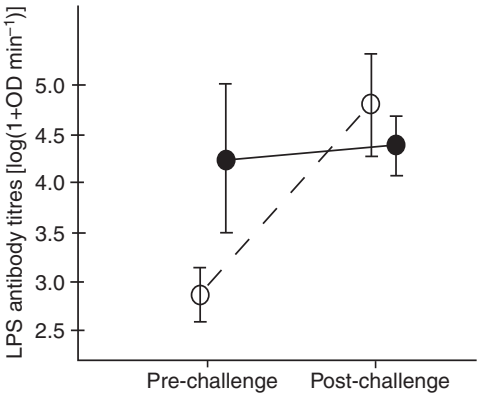

Fig. 1. Mean (±1 s.e.m.) titres of antibody against lipopolysaccharide (LPS) prior to challenge and eight days after the challenge in jackdaw chicks hatched from eggs with elevated yolk androgens (open circles) and control eggs (filled circles).

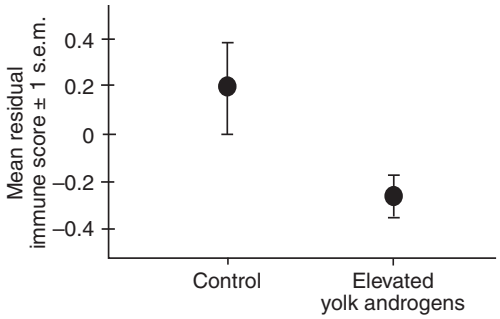

Fig. 2. Mean residual immune score (±1 s.e.m.) in response to a challenge of diphtheria/tetanus antigens of jackdaw chicks hatched from eggs with elevated yolk androgen or control eggs. The *y*-axis reflects residuals of the statistical model (see Materials and methods) with year as a fixed factor.

(r_{70}=0.54, P<0.0001) showed that these two responses were congruent and we combined these two responses into one humoral immune score with principal component analysis. Androgen egg treatment had a significant effect on humoral immune responses. Control chicks had significantly higher antibody titres against diphtheria/tetanus than androgen chicks (Table 2; Fig. 2). There was also a trend towards stronger immune responses early in the breeding season (Table 2) but no effect of sex, body mass or brood size (day 14).

PHA challenge

T-cell-mediated immune response differed significantly between years probably due to variation in injection volume (see Materials and methods) (2003, 1.03±0.45 mm; 2004, 0.45±0.30 mm; $F_{1,37.1}$=51.16, P<0.001). However, after controlling for year, there was a significant difference in response to PHA injection between treatment groups. Nestlings from androgen-injected eggs had a significantly lower cell-mediated immune response than control chicks (Table 2; Fig. 3). There was a marginally significant effect of foster nest on the intensity of response (χ^2=3.9, d.f.=1, P=0.047) but there was only a weak tendency for a genetic effect (χ^2=2.9, d.f.=1, P=0.09). None of the covariates (hatching date, body mass or brood size) was correlated with T-cell-mediated immune response (all P-values>0.25).

Table 2. Results of repeated mixed-model analyses testing for differences in cell-mediated and humoral immune responses after challenges with PHA, LPS and tetanus/diphtheria vaccine with egg androgen manipulation

Effect	d.f.	F	χ^2	P
PHA				
Year	1, 28.7	37.84		<0.0001
Androgen treatment	1, 28.2	5.61		0.025
Mother identity	1		2.9	0.084
Foster nest	1		3.9	0.047
LPS				
Year	1, 14.5	17.51		<0.0001
Hatching date	1, 19.9	4.65		0.043
Body mass	1, 48.8	2.94		0.092
Mother identity	1		5.5	0.015
Foster nest	1		3.8	0.051
Diphtheria/tetanus (PCA)				
Year	1, 11.1	30.7		0.0002
Androgen treatment	1, 25.7	8.9		0.0062
Hatching date	1, 4.05	5.60		0.059
Mother identity	1		2.1	0.14
Foster nest	1		2.9	0.084

The full model included year, sex and experiments as fixed factors, genetic nest and foster nest as random factors and brood size at injection, hatching date, body mass at injection and pre-vaccination titres as covariates. Presented are the reduced models with non-significant effects (P>0.1) backward eliminated from the models.

Correlations between different immune responses

To analyse correlations between immune responses we used residuals from models including year as dependent variable because all three measurements of immune function were significantly different between years (see Table 1 and Materials and methods for a potential explanation of between-year differences). There were no significant correlations between any of the three tests of immunity (LPS *versus* PHA, r_{50}=0.06, P=0.69; LPS *versus* diphtheria/tetanus, r_{60}=0.07, P=0.58; PHA *versus* diphtheria/tetanus, r_{41}=0.03, P=0.89). Within each experimental category, the result was similar with no significant correlations (P>0.1 in all cases).

DISCUSSION

The vertebrate immune system is a complex organisation of different lines of defence against pathogens. Although many studies have argued that an integrative approach is essential for understanding the relationship between immunocompetence and life-history strategies when studying an individual's ability to resist disease and pathogens (Norris and Evans, 2000; Adamo, 2004; Salvante, 2006), few studies have analysed several lines of immunity simultaneously [but see Faivre et al. (Faivre et al., 2003; Forsman et al., 2008)]. Immunosuppression caused by exposure to elevated yolk steroid levels has been found in studies of other bird species but the majority of these studies have used a single immunological test, that is the test for (unspecific) cell-mediated immunity by injection with PHA (Andersson et al., 2004; Groothuis et al., 2005a; Navara et al., 2006). One of the few studies that has assessed the effect of yolk androgens on avian immunity with an integrative approach is the study by Mueller and colleagues (Mueller et al., 2005) on black-headed gulls (see also Navar et al., 2005). However, this study focused only on the early stage of the chick phase, that is the first week of life when the chicks endogenous immune system has just begun to develop and it is not clear whether the effect of yolk androgens may extend

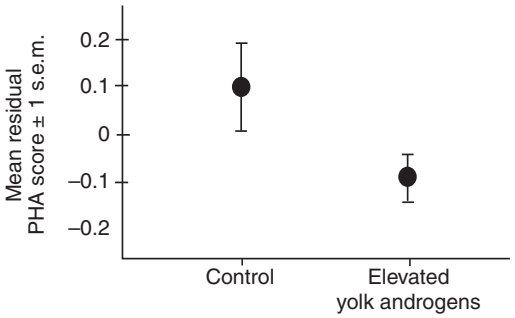

Fig. 3. Mean residual scores (±1 s.e.m.) in wing web swelling in response to a phytohaemagglutinin (PHA) challenge in jackdaw chicks hatched from eggs with elevated yolk androgens or control eggs. The *y*-axis reflects residuals from the statistical model including year as a fixed factor (see Materials and methods).

beyond this first period of life but whether they also affect the chick when its endogenous immune system is further developed. Our experimental study on jackdaw chicks shows that elevated yolk androgen levels result in a general immunosuppression in nestlings and this conclusion was based on congruent results for three different immunological tests of both humoral and cell-mediated immunity conducted at 1–2 and 3–4 weeks of age.

The bird's immune system begins developing before hatching and is complete by sexual maturity. Our understanding of the ontogeny of the avian immune system originates almost exclusively from studies on poultry and is, therefore, biased towards precoccial birds. Little is known about potential differences between altricial and precoccial birds (Apanius, 1998). One exception is the American kestrel (*Falco sparverius*) where studies demonstrate that chicks acquire antibody responsiveness to antigens when approximately 1–2 weeks old (Apanius, 1993). Because young jackdaws remain in the nest for almost five weeks, it was possible for us to follow potential effects of elevated yolk androgen exposure over a relatively long period. Androgen exposure during embryonic development resulted in lower antibody titres during the early phase (six-day-old chicks) when the chicks' own immune system just has begun to develop. Moreover, we also found that in fully grown chicks (i.e. 3–4 weeks old chicks) both cell-mediated and humoral immune responsiveness, measured as immune responses to injected non-pathogenic antigens, were suppressed in chicks from androgen-treated as compared with control eggs. These results clearly show that both arms of the adaptive immune defence were negatively affected by yolk androgens. At the age of 3–4 weeks, the immune system of the jackdaws is still under development and we can therefore not conclude that the adaptive immune system has been permanently suppressed by the yolk androgen treatment. Still, suppressed adaptive immune responsiveness when *ca*. one month old, i.e. at an age when juvenile jackdaws become fledged, should potentially have a huge impact on fitness. The first period after fledgling is a stressful period for passerine birds, when the young have to learn to feed and avoid predators and mortality is typically high at this stage (Newton, 1998). A newly fledged bird will also be exposed to a broader range of pathogens than when inside the nest; therefore, the need for a well-developed, efficient immune system should be crucial for survival.

Antibody titres towards LPS differed between chicks from androgen-treated eggs and control chicks already prior to the injection at day six. LPS contains antigens that trigger a T-cell-independent B-cell response and at high concentrations a non-specific antibody response (Janeway et al., 2005). LPS antibodies found in six-day-old nestlings could be of maternal origin or produced by the nestlings. Maternal transfer of antibodies reflects the pathogens that mothers have been exposed to and they may be present 1–2 weeks after hatching, although in decreasing concentrations (Smith et al., 1994; Lung et al., 1996; Grindstaff et al., 2006). Maternal transfer of antibodies against LPS was not manipulated in this study and, hence, unrelated to yolk androgens. If antibody levels in six-day-old chicks were mainly of maternal origin, this would indicate that maternal antibodies are catabolised or used in immune defence at different rates in control and androgen chicks. However, the chicks may have started to produce endogenous antibodies already before they were six-days-old and the antibody titres we measured would then been a mixture of maternal and endogenous antibodies. If the latter explanation is true, then our results may also reflect a difference in the timing of the onset of endogenous antibody production caused by the yolk androgen treatment. This would then indicate that the elevated yolk androgen could delay the development of neonatal immune function so that androgen chicks started their endogenous production later than control chicks. This is supported by the observation that androgen-treated chicks increase their LPS-specific antibody titres between day six and 14, resulting in no difference between treatment groups at day 14. This could also be explained if both treatment groups had reached their limit of production of LPS-specific antibodies at day 14. In the experiment with black-headed gulls it was found that embryonic androgen exposure had negative effects on antibody production towards LPS (Mueller et al., 2005). In the latter study, chicks were challenged at day seven and antibody response was measured after 48 h. The pre-injection antibody titres were not significantly different but post-injection titres were lower in androgen chicks. These results were also interpreted as a delayed response in androgen chicks because production of LPS antibodies was still in its early phase at this time. Our result on jackdaw chicks are in contrast with these results since we found that elevated yolk androgens in jackdaws had a negative effect already prior to the LPS challenge.

In the present study, the cell-mediated response to PHA was tested during a period when the nestlings were mounting a humoral immune response. PHA response is thought to be a trade-off against other life-history components and varies with individual condition (Martin et al., 2006). Although both PHA response and diphtheria/tetanus antibody titres were lower in androgen chicks compared with control chicks, there was no direct correlation between the two responses. Similarly, there was no correlation between responses to LPS and diphtheria/tetanus. Exposure to elevated androgen levels during embryonic development seems to have an overall negative effect on the development of the adaptive immune system in jackdaw chicks. However, other environmental factors, such as food availability and rearing conditions, also influenced the immune responsiveness of jackdaw chicks. If these effects vary within the nestling period, this could explain the lack of clear correlations between the different tests.

The mechanisms by which yolk steroids influence immune function are not yet known. One of the important stages of the development of the avian immune system occurs during the first weeks of the chick's life when the diversification of antibody repertoire through gene conversion takes place in the bursa of Fabricius (Glick and Sadler, 1961). Studies have shown that high doses of testosterone administrated *in ovo* early in embryonic development caused regression of the bursa in chickens resulting in a reduction in IgG production (Erickson and Pincus, 1966; Lerner et al., 1971). Experiments with mibolerone, an androgen analogue

of testosterone, showed negative dose-dependent effects on the embryonic development of the bursa and the maturation and differentiation of B lymphocytes (Bhanushali et al., 1985). Hence, androgens transferred from the mother to the egg may have negative consequences on nestling immunity by acting on the development of or the antibody repertoire diversification in the bursa of Fabricius. We suggest that such an effect could be long-lasting influencing immunocompetence even in adulthood. There could also be other potential explanations for why yolk androgen-treatment resulted in suppressed immune responsiveness. It has been suggested that differences in prenatal exposure to androgens between males and females can explain sex-specific differences in immune function in mammals, in which females generally have greater humoral and cell-mediated immunity than males (Martin, 2000). These prenatal effects in mammals are the result of differential steroid production by the embryos, not by exposure to maternal hormones. The link between yolk androgens and circulating endogenous androgens in nestlings is not yet clear but if embryonic exposure to yolk androgens influences circulating levels of testosterone in bird nestlings, the negative effects of yolk treatment could be explained by direct or indirect negative effects of circulating testosterone. Immunosuppression as a result of high circulating levels of testosterone has been shown in adult birds (e.g. Duffy et al., 2000; Owens-Ashley et al., 2004) (but see Hasselquist et al., 1999).

In conclusion, our study demonstrates that there are immunological costs associated with increased levels of yolk androgens that are detectable even after several weeks of chick development. The potential for long-lasting effects on offspring fitness is large and may be mediated both through organisational effects on immunocompetence and indirect ways through trade-offs with other life-history traits (Carere and Balthazart, 2007).

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