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RESEARCH ARTICLE

Experimentally increased *in ovo* testosterone leads to increased plasma bactericidal activity and decreased cutaneous immune response in nestling house wrens

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SUMMARY

Maternally derived testosterone in the eggs of birds may benefit nestlings by increasing various aspects of their growth, condition and behavioral development, but these benefits may come at a cost, including suppression of immune responsiveness. Experiments on a variety of species in which *in ovo* levels of testosterone have been experimentally increased have produced mixed results; some have found increased growth and suppressed immune function of nestlings whereas others have found the opposite. In an attempt to clarify the relationship between *in ovo* testosterone and nestling size, mass, health state and immune responsiveness, we experimentally increased levels of testosterone in the eggs of house wrens (*Troglodytes aedon*). We simultaneously determined the size, mass, hematocrit (a measure of health state), cutaneous immune response to phytohaemagglutinin and plasma bactericidal activity of nestlings near the time of fledging. We predicted that nestlings hatching from testosterone-injected eggs would exhibit lower immune responsiveness, but achieve greater mass, size and condition, than nestlings hatching from vehicle-injected control eggs. Instead, we found that nestlings hatching from testosterone-injected eggs had a weaker cutaneous immune response but greater bactericidal activity than those hatching from control eggs. They did not, however, differ significantly in mass, size or hematocrit from controls. These results suggest that experimentally increased *in ovo* testosterone induced a trade-off between bactericidal activity and the cutaneous immune response. The opposite responses by two different measures of immune function to experimentally increased *in ovo* testosterone underscore the importance of including multiple immune assays when investigating the potential for trade-offs with the immune system and other physiological functions.

Key words: yolk testosterone, phytohaemagglutinin assay, bactericidal assay, PHA, trade-off, *Troglodytes aedon*.

INTRODUCTION

The discovery that female birds regularly deposit steroids, such as testosterone and other androgens, into the yolk of their eggs (Schwabl, 1993) raised the question of how these hormones affect embryonic development and subsequent nestling growth. The benefits of yolk testosterone have been well documented and include positive correlations between the amount of testosterone in the egg and intensity of begging behavior, growth rate and aggressive behavior (e.g. Lipar and Ketterson, 2000; Groothuis et al., 2005; Gil, 2008; Groothuis and Schwabl, 2008). However, such benefits may also come at a cost, with many adverse effects of yolk testosterone and other androgens also reported (reviewed in Gil, 2003; Gil, 2008; Navara and Mendonça, 2008). Such effects include suppression of immune responsiveness, at least in the short term, when *in ovo* testosterone is experimentally increased (e.g. Müller et al., 2005; Navara et al., 2005; Sandell et al., 2009) and when natural variation in nestling testosterone is examined (López-Rull et al., 2010). The underlying cause of immune suppression by yolk testosterone may be a trade-off between resources allocated to two energetically expensive physiological tasks: growth (increased by androgens) and immune-system use and maintenance (decreased by androgens) (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000); however, this has by no means been clearly established as the sole cause (reviewed in Groothuis et al., 2005; Gil, 2008). In fact, experimentally increased *in ovo* testosterone does not always lead to increased nestling growth or size (e.g. Müller et al., 2005; Sandell et al., 2009), and there are reports of enhanced immune function (Navara et al., 2006) or no change in immune function in response to experimentally increased testosterone in eggs (Tschirren et al., 2005; Pitala et al., 2009).

In this study we investigated the effects of experimentally increased levels of testosterone in the egg on the size, mass, health state and immune response of nestling house wrens (*Troglodytes aedon*). We measured two aspects of immune responsiveness. The first, cutaneous immune response, involves both adaptive and innate components of the immune system and was determined for each nestling using a standard *in vivo* phytohaemagglutinin (PHA) assay (McCorkle et al., 1980; Smits et al., 1999; Martin et al., 2006b). The second, bactericidal activity of blood plasma, measures components of the innate immune system (Matson et al., 2006), including natural antibodies, lysozyme, nitric oxide, complement proteins and antimicrobial peptides (Forsman et al., 2010). We also measured the mass, size and hematocrit (a health-state condition measure) (Ots et al., 1998) of individual nestlings near the time of fledging. We predicted that nestlings hatching from testosterone-injected eggs would exhibit lower immune responsiveness, but achieve greater mass, size and health-state condition, than nestlings hatching from vehicle-injected control eggs.

MATERIALS AND METHODS

Study species

House wrens, *Troglodytes aedon* (Vieillot 1809), are small (10–12 g) migratory passerines with a breeding range extending over much of the continental USA. Egg laying extends from late April–early May to early August, with two distinct peaks, the first in May (early season) and the second in late June–early July (late season). Clutch size ranges from four to eight eggs (early-season mode, seven eggs; late-season mode, six eggs) (Finke et al., 1987). Females alone incubate the eggs for 11–13 days. After hatching, the female broods the nestlings, and both the male and female provision the nestlings until they leave the nest 14–17 days later. Additional details of the biology of the house wren are provided by Johnson (Johnson, 1998).

This research was conducted according to all applicable federal and state regulations, and with the approval of the Illinois State University Institutional Animal Care and Use Committee (protocol no. 15-2006).

Study site and field methods

This study was conducted during the 2008 breeding season on the 130 ha Mackinaw study area in McLean County, IL, USA (40°40′N, 88°53′W). The site contains 700 nestboxes, whose dimensions and construction materials are described in appendix 1 of Lambrechts et al. (Lambrechts et al., 2010). Nestboxes are aligned 30 m apart on north–south lines, with 60 m between lines, in upland and floodplain secondary deciduous forest (density=5.4 nestboxes ha^{-1}). For this study, we used nests only from the northeastern portion of the study area (see Eckerle and Thompson, 2006) to minimize any effect that local-scale differences in habitat might have on the outcome of the experiment.

Egg injection

Each nestbox was checked every 1–3 days for nest-building activity. Once egg laying had begun in a nest, we selected clutches for injection by identifying pairs of nests with similar numbers of eggs and clutch-initiation dates. Each whole clutch was randomly assigned to a treatment, a testosterone-injected (2 ng testosterone in 5 µl sterile sesame oil) experimental treatment (N=30 clutches) or a vehicle-injected (5 µl sterile sesame oil) control (N=27 clutches). We removed the freshly laid eggs and replaced them with fake, plastic eggs so that the number of eggs would be the same if the female returned to the nest during the injection. The eggs were taken away from the nest and a betadine solution (10% providone-iodine, Purdue Pharma, Stamford, CT, USA) was applied to the acute pole of the egg before injection. Eggs were laid on their side and the yolk was visualized using an LED light source. We then made a hole at the acute pole of the egg with a sterilized 27 gauge needle through which we inserted the needle of a 100 µl Hamilton syringe (Hamilton Company, Reno, NV, USA) to inject either the vehicle or testosterone dissolved in the vehicle. We injected into the albumen to avoid damaging the yolk (Navara et al., 2005; Paitz et al., 2011), and the testosterone dose was chosen to be approximately two standard deviations above mean levels (mean±s.d.=4.17±2.92 ng g^{-1} yolk) previously found in eggs from the study population (Grana, 2009). All eggs were injected prior to the beginning of incubation. Additional details on the injection procedures are provided in Barnett et al. (Barnett et al., 2011). Not all of these eggs or clutches were included in the analyses because of hatching failures, nest depredation, nestling disappearance or difficulties in obtaining blood samples. There was no difference between treatments, however, in the proportion of nests that produced nestlings surviving to at least brood-day 11 (control: 21 of 27; testosterone-injected treatment: 26 of 30; continuity-adjusted χ^2_1=0.28, P=0.59). Sample sizes for each analysis are given in Table 1.

Mass, tarsus and hematocrit

We weighed nestlings to the nearest 0.1 g (Acculab, Pocket Pro 250-B) on brood-day 9 (the first egg hatches on brood-day 0) and brood-day 11 (final mass). On brood-day 11, we also banded nestlings with a numbered US Fish and Wildlife Service aluminum band and measured their tarsus length to the nearest 0.1 mm using dial calipers. On brood-day 11, an area of the left wing of each nestling was sterilized with 70% ethanol before blood samples (~50 µl) were taken from the brachial vein and collected in sterilized, heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA, USA), which were sealed and stored on ice in the field. Immediately upon our return from the field the same day, we measured hematocrit (mean of three measures of percentage of whole blood occupied by packed red blood cells) with a Hematastat II (Separation Technologies, Sandord, FL, USA) after centrifugation of the tubes at 1610 ***g*** for 60 s. Separated plasma was then transferred from the capillary tube to a 1.5 ml centrifuge tube using a Hamilton syringe and stored in a refrigerator at ~4°C until used in the bactericidal assay later the same day.

Assessing immune response

To measure the cutaneous immune response, the wing-web (patagium) was injected on brood-day 11 with a single 50 µl dose of 5 mg ml^{-1} PHA (catalog no. L8754, Sigma Aldrich, St Louis, MO, USA) dissolved in sterile phosphate buffered saline. The wing-web thickness of each nestling was measured to the nearest 0.01 mm using a digital thickness gauge (no. 547–500, Mitutoyo America Corp., Aurora, IL, USA) in triplicate both immediately before injection and ~24 h later. Swelling response was the difference between the mean of the pre-injection measurements and the mean of the post-injection measurements.

To assess bactericidal activity of blood plasma, 5 µl of each plasma sample was incubated in 100 µl sterile CO_2-independent media (catalog no. 18045, Gibco-Invitrogen, Carlsbad, CA, USA) containing ~5% fetal bovine serum and 4 mmol l^{-1} L-glutamine (Matson et al., 2006) with ~200 colony-forming units of *Escherichia coli* (ATTC strain 8739) at 41°C for 45 min, after which they were immediately plated in duplicate on sterile tryptic soy agar plates. For each round of the assay, duplicate controls consisting of only media and *E. coli* were run in the same manner as the samples.

Table 1. Summary of morphological, physiological and immunological measures

	Testosterone injected		Vehicle injected	
Variable	No. broods	Least square mean ± s.e.m.	No. broods	Least square mean ± s.e.m.
Mass (g)	26	9.93±0.125	21	9.95±0.136
Tarsus (mm)	26	18.77±0.080	20	18.83±0.088
Hematocrit (%)	22	41.99±0.933	19	42.06±0.949
log Cutaneous immune response (mm)	24	0.169±0.006	18	0.187±0.007
Bactericidal activity (%)	23	34.7±2.6	18	26.6±2.9

Table 2. Mixed-model ANOVA of the effect of testosterone treatment on the cutaneous immune response induced by phytohaemagglutinin injection and plasma bactericidal activity of nestling house wrens

	$F_{d.f.}$	P
Cutaneous immune response		
Treatment	$4.20_{1,25.2}$	0.056
Season	$4.90_{1,29.9}$	0.022
Time of injection	$3.59_{1,30.5}$	0.018
Number of nestlings	$0.65_{1,29.7}$	0.077
Season × time of injection	$6.69_{1,29.1}$	0.016
Bactericidal activity		
Treatment	$4.12_{1,35.3}$	0.050
Season	$7.55_{1,36.2}$	0.009

Type 3 tests of fixed effects.
For cutaneous immune response, N=24 testosterone-injected broods and N=18 vehicle-injected broods; for bactericidal activity, N=23 testosterone-injected broods and N=18 vehicle-injected broods.

Samples and controls were then incubated overnight at 37°C (Forsman et al., 2008). The number of colonies on each plate was determined, and percent killing was calculated as [1–(duplicate sample mean number of colonies/duplicate control mean number of colonies)]×100.

Statistical analyses

We used SAS 9.1 statistical software for all analyses (SAS Institute, 2004). We employed mixed-model ANOVA in PROC MIXED to examine the effect of testosterone on cutaneous immune response (log transformed), bactericidal activity, final mass, tarsus and hematocrit. Nest was included as a random effect to account for the statistical non-independence of nestlings within a brood. Treatment was included as a fixed effect, and number of nestlings on brood-day 11 (brood size) and brood-day 0 (season) and time of injection [PHA only; see effect of time of day on response to PHA injection in Forsman et al. (Forsman et al., 2010)] were included as covariates. Parameter estimates were obtained using restricted maximum likelihood with a variance-components covariance structure, and degrees of freedom were estimated using the Kenward–Roger correction (Littell et al., 2006). To obtain minimal adequate models, we employed sequential backward elimination to remove non-significant terms (P>0.15), beginning with all two-way interactions (Crawley, 1993). To assess within-brood correlations between the cutaneous immune response and bactericidal activity, we employed multivariate ANOVA (MANOVA) in PROC GLM, with nest as a fixed effect and cutaneous immune response and bactericidal activity as the dependent variables.

RESULTS

Nestling mass, tarsus and hematocrit

There was no significant effect of treatment on nestling final mass, tarsus length or hematocrit (all P>0.15; Table 1). Nestling final mass decreased as brood size increased (estimate + s.e.m.=–0.125+0.062; $F_{1,50.6}$=4.10, P=0.048), and also decreased over the course of the breeding season (estimate+s.e.m.=–0.011+0.004; $F_{1,46.1}$=7.00, P=0.011). Hematocrit increased significantly as the breeding season progressed ($F_{1,38}$=4.68, P=0.037).

Immune response

The cutaneous immune response did not vary significantly among broods (Wald Z=1.11, P=0.13). There was an almost-significant difference (P=0.056) between treatments in cutaneous immune response, with nestlings hatching from testosterone-injected eggs tending to produce a weaker response than those from vehicle-injected eggs (Tables 1, 2). Cutaneous immune response decreased as brood size increased (estimate±s.e.m.=$–5.66\times10^{-3}\pm3.09\times10^{-3}$), and there was a significant interaction between time of injection and season in their effect on cutaneous immune response.

Bactericidal activity varied significantly among broods (Wald Z=2.50, P=0.0001). There was a significant difference between treatments in bactericidal activity, with the plasma of testosterone-injected nestlings killing more bacteria than that of vehicle-injected nestlings (Table 2). Bactericidal activity also increased with season (estimate±s.e.m.=$2.70\times10^{-3}\pm0.98\times10^{-3}$).

Among-brood variation in immune responsiveness

Overall, nestling immune responsiveness varied significantly among broods (MANOVA, Pillai's trace=0.9279, $F_{72,234}$=2.81, P<0.0001), with two significant dimensions of variation in immune response detected among broods. The first canonical variate (59% of variation explained) was influenced most strongly by bactericidal activity and, to a lesser extent, by cutaneous immune response, whereas the opposite was true for the second canonical variate (41% of the variation explained; Table 3). Furthermore, the standardized canonical coefficients of the first canonical variate were both positive, but were of the opposite sign for the second canonical variate (Table 3).

DISCUSSION

Nestling mass, size and condition

Nestlings hatching from testosterone-injected eggs did not attain greater final mass or structural size or end in a better health state (as measured by hematocrit) than nestlings from vehicle-injected eggs, despite the possibility that they increased their rate of begging (see Differences in immune response). This lack of difference was not the result of a trade-off between nestling number and quality, because broods hatching from testosterone- and vehicle-injected clutches in our study did not differ in any measure of nest or hatching success or in the number of nestlings [data presented in Barnett et al. (Barnett et al., 2011)]. It was also unlikely to be attributable to the use of a physiologically inadequate or an excessive (pharmacological) amount of testosterone, as we used a physiological dose that was at the upper end of the range of testosterone levels previously measured in eggs from the study population (see Materials and methods).

Table 3. MANOVA summary statistics for differences among broods in immune responsiveness and within-brood correlations between the cutaneous immune response and bactericidal activity

					Standardized canonical coefficients	
Source	Eigenvalue	Variance explained (%)	$F_{d.f.}$	P	Cutaneous immune response	Bactericidal activity
Canonical variate 1	1.0442	59.3	$2.81_{72,232}$	<0.0001	0.4967	1.1054
Canonical variate 2	0.7154	41.7	$2.39_{35,117}$	0.0003	1.0557	–0.5442

Studies that have experimentally increased *in ovo* testosterone have frequently reported increased nestling mass, size or condition compared with controls (reviewed in Groothuis et al., 2005; Navara and Mendonça, 2008). The lack of detectable differences that we found may appear surprising, but is likely explained by the failure of parents to increase their provisioning to nestlings hatching from testosterone-injected eggs (see Differences in immune response). However, the documented effects of the experimental increase of testosterone and other androgens on final nestling mass are complex and varied, including reports of no effects on nestling mass and decreases in nestling mass (e.g. Sockman and Schwabl, 2000; Navara et al., 2005; Pitala et al., 2009; Tobler et al., 2010).

Differences in immune response

Increased *in ovo* testosterone led to changes in immune responsiveness, with lower cutaneous immune response in nestlings hatching from testosterone-injected eggs than in those hatching from vehicle-injected eggs. Surprisingly, however, the bactericidal response was higher. The treatment effect on the cutaneous immune response cannot be attributed to a simple trade-off between immune-system maintenance or use and growth, because nestlings in the two treatments did not differ in mass or other morphological or condition measures. The differences reported here and in the literature on the relationship between experimentally manipulated levels of yolk testosterone and subsequent immune responsiveness of nestlings hatching from those eggs could have many causes. For example, when food is abundant and there is no need for a trade-off in allocating resources between growth and maintenance, immune suppression in nestlings may not occur (Navara et al., 2006). Embryos may even alter their hormonal environment, resulting in differential modulation of the effects of maternally or experimentally derived steroids on nestling immune function (von Engelhardt et al., 2009; Paitz et al., 2011). Finally, failure of other studies to detect a relationship between yolk hormone levels and a particular type of nestling immune response may be a consequence of measuring only one type of immune response, because different branches of the immune system may not respond in a positively correlated manner (Adamo, 2004). What, then, led to the decrease in one measure of immune response and an increase in the other in our study?

One possible explanation is that adaptive components of the immune system are not only more energetically and nutritionally costly to develop than innate components but also develop more slowly than innate components because they require the production of specific receptors that recognize a broad range of potential antigenic threats (Palacios et al., 2009). Another possibility is that there are trade-offs between different branches of the immune system itself (Martin et al., 2006a; Salvante, 2006; Forsman et al., 2008; Tobler et al., 2010). Results from other studies suggest several possible causes for suppression of the cutaneous immune response. One is that the presence of testosterone leads to an increased metabolic rate in the embryo or nestling (e.g. Tobler et al., 2007), which in turn diverts energy away from the cutaneous immune response in older nestlings. Another possibility is that any contribution made by leukocytes to the cutaneous immune response is reduced in nestlings hatching from testosterone-injected eggs because high levels of testosterone result in the redistribution of leukocytes, as proposed by the immunoredistribution hypothesis (Braude et al., 1999). A third possibility is that increased *in ovo* testosterone leads to chronic stress in embryos or nestlings, and adaptive components of the cutaneous immune response may be among the first components of the immune system to be adversely affected by the stress-induced response (Kuhlman and Martin, 2010). Finally, there may be trade-offs between the cutaneous immune response and other physiological or behavioral processes, or within the immune system itself between the cutaneous immune response and bactericidal activity.

It is possible that differences in the begging behavior of nestlings hatching from testosterone- and vehicle-injected eggs contributed to the differences in response to PHA injection. In a later experiment carried out on the same population used in this study, young (brood-day 4–5) nestlings hatching from testosterone-injected eggs begged more than those from vehicle-injected eggs; however, this did not lead to increased parental rates of provisioning (Barnett et al., 2011). Therefore, the testosterone-induced increase in begging by young house wren nestlings may have come at an immunological cost, diverting energy away from the immune system and depressing their cutaneous immune response. Consistent with our findings, Moreno-Rueda (Moreno-Rueda, 2010) recently reported that experimentally increased begging by nestling house sparrows (*Passer domesticus*) produces a marked decline in their cutaneous immune response but not in their growth, when the amount of food provided is the same as that provided to controls. Alternatively, it is possible that there was a simple trade-off between the two immune responses, with the enhancement of the bacteria-killing capacity of the blood plasma coming at the expense of mounting a response to the injection of PHA (e.g. Martin et al., 2006a; Tobler et al., 2010). Our analysis showed significant differences among broods in overall nestling immune responsiveness, consistent with an earlier study (Forsman et al., 2010). Standardized canonical coefficients revealed that both the cutaneous immune response and bactericidal activity contributed positively to the first canonical variate, suggesting that this dimension reflects the overall immune robustness of nestlings. More importantly, however, the second canonical variate was strongly positively related to the cutaneous immune response and negatively related to bactericidal activity, which is consistent with a trade-off between these two components of nestling immunity. This possibility is bolstered by a similar apparent trade-off among the cutaneous, humoral and innate axes of the immune system documented in a previous study of this house wren population (Forsman et al., 2008). This trade-off may be central to the differential effect of increased testosterone on the cutaneous immune response and bactericidal activity revealed by our analyses. Specifically, increased testosterone may mediate increased investment in one branch of immunity at the expense of another. How might this be brought about?

Opinion varies on the relative costs of different components of the vertebrate immune system (reviewed in Lochmiller and Deerenberg, 2000; Klasing, 2004; Martin et al., 2008), and costs associated with maintaining robust bactericidal capacity have not, to our knowledge, been explored. Any attempt to understand testosterone-mediated upregulation of the bactericidal response in our study should focus on the non-cellular component of the response, as most of the capacity of avian blood to kill the *E. coli* strain we used (8737) resides in the plasma (Millet et al., 2007). These constitutive antimicrobial agents in plasma include lysozymes, nitric oxide, antimicrobial peptides, complement proteins and natural antibodies (Forsman et al., 2010). It is important to note that both the cutaneous immune response, induced by injection of PHA, and bactericidal activity involve components of both the innate and adaptive axes of the immune system (Juul-Madsen et al., 2008; Forsman et al., 2010; Vinckler et al., 2010); additionally, components of the bactericidal response, such as complement proteins, are themselves quite complicated and have multiple functions in vertebrates (e.g. Ricklin et al., 2010). Although production of

molecules associated with innate humoral immunity may not be viewed as costly at a single point in time, sustained constitutive production may represent a substantial investment. Therefore, we think it is premature to argue that one axis or the other is more costly and more likely to be involved in trade-offs.

We conclude that the simplest explanation for our results is that experimentally increased *in ovo* testosterone induced a trade-off between bactericidal activity and the cutaneous immune response. However, because nestlings hatching from testosterone-injected eggs probably begged more than those hatching from vehicle-injected eggs, we cannot rule out the possibility that the cutaneous immune response might also have been involved in a trade-off with begging activity. Our finding that two different measures of immune response produced opposite outcomes highlights the need for regularly employing multiple immune assays, as recommended by Adamo (Adamo, 2004) and others, as well as measurement of behavioral traits that might influence growth and maintenance.

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