Yolk Testosterone Stimulates Growth and Immunity in House Finch Chicks

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ABSTRACT

Female birds deposit variable amounts of androgens, such as testosterone, into the yolks of their eggs. Evidence suggests that yolk androgens play an important role in the determination of offspring phenotype. While androgens are generally regarded as anabolic and immunosuppressive, studies of the behavioral and physiological effects of yolk androgens on offspring of several avian species have been conflicting, leaving the adaptive significance associated with deposition patterns of yolk androgens unclear. We injected either a physiological dose of testosterone or a control vehicle into house finch (*Carpodacus mexicanus*) eggs and examined the effects of these injections on offspring growth and immunity. Two days after hatching, nestlings from eggs treated with testosterone were significantly larger than nestlings from eggs treated with a control injection, suggesting a stimulatory effect of yolk androgens in early development. By 8 d after hatching, however, this effect disappeared, and chicks from the two treatment groups were similar in size. Nestlings in the testosterone treatment group showed a significantly larger swelling response to phytohemagglutinin than control nestlings 15 d after hatching, which is close to fledging. Overall, our observations show that when food resources are abundant, testosterone stimulates both early growth and immunity in developing house finches.

Introduction

Avian and reptilian eggs have been shown to contain variable amounts of physiologically relevant substances, including carotenoids, antibodies, and hormones. Females deposit these substances in patterns according to a number of environmental and social stimuli. Specifically, yolk androgens, including testosterone, dihydrotestosterone, and androstenedione, vary according to position in a clutch, photostimulatory cues, and breeding density (Schwabl 1993, 1996*a*, 1997; Reed and Vleck 2001). The effects of yolk androgens on the growth and development of offspring have been the focus of a few studies but remain poorly understood.

It has been hypothesized that the deposition of yolk hormones is a proximate means by which female birds can manipulate offspring phenotype. For example, *in ovo* injections of androgens increased begging behavior and growth rates in canary (*Serinus canaries*) and black-headed gull (*Larus ridibundus*) chicks (Schwabl 1996*b*; Eising and Groothuis 2003) and decreased the chance of starvation during a drought year in European starling (*Sturnus vulgaris*) chicks (Pilz et al. 2004). Additionally, yolk testosterone levels were positively correlated with growth of the hatching muscle in red-winged blackbirds (*Agelaius phoeniceus*; Lipar and Ketterson 2000). Because many of the measured effects of yolk androgens on offspring appear positive, yolk androgens have generally been characterized as resources that, when allocated to eggs, benefit offspring and increase reproductive success.

The characterization of androgens as resources, however, ignores the negative effects of androgens that have been observed. For example, injections of androgens into the yolks of American kestrel (*Falco sparverius*) eggs resulted in offspring that hatched later and had a higher mortality rate (Sockman and Schwabl 2000). In eastern bluebirds (*Sialia sialis*), *in ovo* testosterone injections produced a stimulatory effect on growth but a simultaneous inhibitory effect on the cell-mediated immunity of the resulting chicks (Navara et al. 2005). Androgens are better viewed as mediators of energy allocation in growing vertebrates that enhance some aspects of growth and development at a cost to others. Under conditions of abundant food resources, we might expect to see more benefits than costs to high levels of androgens.

To better understand the role of testosterone in the growth and development of passerine birds, we manipulated the testosterone levels in the eggs of house finches (*Carpodacus mexicanus*). Female house finches often begin incubating before the last egg is laid, resulting in offspring that hatch asynchronously. Females lay five eggs per clutch and two to three clutches over the course of the breeding season each year. Previous work on this species has shown that females deposit higher levels of yolk

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androgens into later-laid eggs (Navara et al. 2006), which is consistent with patterns found in canary (Schwabl 1993), American kestrel (Sockman and Schwabl 2000), and house sparrow eggs (Schwabl 1997). Additionally, female house finches deposit significantly more yolk androgens into eggs sired by less attractive males (Navara et al. 2006), which is the opposite of yolk androgen deposition patterns found in zebra finch eggs (Gil et al. 1999). A full understanding of these patterns of androgen deposition requires the examination of the physiological effects associated with yolk androgen deposition.

We injected either a physiological dose of yolk testosterone or a control vehicle into house finch (*C. mexicanus*) eggs and examined the resulting effects on offspring growth and immunocompetence in the offspring. Based on work done in other species, we predicted that yolk androgens would exert a stimulatory effect on growth and an inhibitory effect on the immunity of house finch offspring.

Material and Methods

Egg Injections

We monitored a breeding population of house finches in Lee County, Alabama, for the onset of nest building and egg laying. After the appearance of the first egg, we noted the first day on which the female was flushed from the nest and the eggs were warm to the touch to establish the date of incubation onset. Previous experiments using nest temperature measurements showed that these visual assessments consistently and accurately estimated the day of incubation onset (Badyaev et al. 2003). Eggs were injected on the day that they were laid with one of two injection treatments: (1) 200 ng of testosterone (T) in 5 μL peanut oil or (2) a control injection of 5 μL peanut oil. This injection dose was chosen based on the natural variation of yolk testosterone found in house finch eggs, ranging from 0 to 840 ng/yolk. Very few eggs contained yolk testosterone concentrations at the highest end of the range (eggs in the first clutch position generally ranged from 0 to 22.5 ng/yolk, while eggs in the fifth clutch position generally ranged from 0 to 50 ng/yolk; K. Navara et al., unpublished data), and our injection treatment was calculated such that it raised the yolk testosterone levels of most eggs to above-average levels but significantly below the highest levels found in nature. Testosterone was chosen as the injected androgen because it is the most highly concentrated androgen in house finch egg yolks, on average making up approximately 80% of all androgens in the yolks of house finch eggs. Injections were performed using a 50-μL Hamilton syringe, and the injection site was sealed with brush-on liquid Krazy Glue. Injection treatments were alternated between eggs in the nest, and the sequence of injection treatments was alternated between nests to avoid confounding effects associated with the position of an egg in the clutch. Using the date of incubation onset, we were then able to estimate the hatch date for each individual egg.

Hatching Success and Growth Measurements

The hatching success of eggs in each treatment group was calculated by dividing the number of nestlings hatching in a treatment group by the total number of eggs injected with that treatment, thus giving us a percentage for each clutch. Nestlings were measured on days 2, 8, and 14 posthatch. Morphological measurements taken on each of these days included mass, using a 20-g spring scale (accuracy = 0.2 g), and tarsus length, using manual dial calipers (accuracy = 0.01 mm). Sample sizes for the different measurement dates differed slightly because of occasional offspring mortality or premature fledging.

Cell-Mediated Immunity

Phytohemagglutinin (PHA) is a known T-cell stimulant in passerine birds (Goto et al. 1978). Injection of this antigen results in swelling around the injection site within 24 h. On day 15 posthatch, a 1-cm patch on the left midpatagium was cleared of feathers. Two measures of thickness were taken using a pressure-sensitive digital micrometer (accuracy = 0.05 mm). The bare skin was swabbed with alcohol, and 20 μg of PHA in 50 μL phosphate-buffered saline was injected subcutaneously using a 27-gauge needle. Injection dosages were extrapolated according to weight from the amounts used in a variety of passerine species in a study by Smits and Williams (1999), who showed that a control injection is not necessary to accurately assess the swelling response to PHA in passerines, so no control injection was performed. Two measurements of wing-web thickness were taken after 24 h to assess swelling. A PHA index was computed as the thickness of the wing-web postinjection minus the thickness of the wing-web preinjection. The PHA index was indicative of the T-cell responsiveness and, thus, cell-mediated immunocompetence. Sample sizes for these measurements were slightly lower than for growth measurements because offspring occasionally fledged before our final patagium measurement. Nestlings were handled under a state of Alabama permit (no. 12), under a federal permit (no. 784373), and according to the guidelines of the Auburn University Institutional Animal Care and Use Committee (PRN no. 2003-0466).

Statistical Analyses

The effect of *in ovo* treatment on hatching success was analyzed using an unpaired *t*-test. To avoid potential complications associated with varying levels of hatching asynchrony in this species, we used only chicks hatching first in their broods for our analyses. We also tested for brood size effects on each measured variable using an ANOVA and found that brood size does not contribute significantly to any measure of offspring size or immunity. Thus, brood size was left out of our analyses. Hatching success values were normalized using an arcsine transformation, and the effects of *in ovo* injection treatments on hatch-

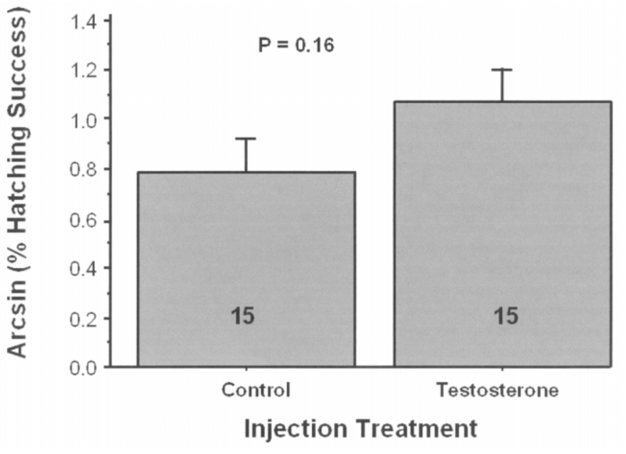

Figure 1. The effects of *in ovo* testosterone and control injection treatments on mean (±SE) hatching success of house finch nestlings. Hatching success was calculated by dividing the number of nestlings hatching in a treatment group by the number of eggs injected with that treatment. Injection treatments included a high-dose injection (200 ng T in 5 μL peanut oil) and a control injection (5 μL peanut oil). The number located in each bar indicates the number of clutches included in the analysis.

ing success were then analyzed using an unpaired *t*-test. The effects of *in ovo* androgen treatment on offspring mass and tarsus length at each developmental stage were analyzed using unpaired *t*-tests. The PHA indices, calculated as described above, were analyzed in relation to *in ovo* treatment group and body condition on day 14 using an ANCOVA. Body condition was calculated as the residuals of the regression of mass to tarsus length of the chicks on day 14 posthatch. All statistical tests used here were two tailed.

Results

Hatching success did not differ between treatment groups ($t = 1.395$, $P = 0.17$), suggesting that above-average levels of yolk T do not have an effect on embryonic mortality (Fig. 1). Mean hatching success for this experiment was 66%, and mean brood size was two nestlings from a mean of 2.9 eggs per nest. Since no nests contained eggs that were not injected with a control or testosterone treatment, we were unable to determine whether the injection itself had an effect on hatching success. Brood sizes for the two treatment groups were statistically similar ($\chi^2 = 0.04$, $P < 1.0$).

On day 2, tarsus length differed significantly between control and testosterone treatment groups ($t = -2.39$, $P = 0.02$), with nestlings in the testosterone treatment group measuring significantly larger than nestlings in the control group (Fig. 2). At this time, there was no significant difference in the weight of the nestlings between the two treatment groups ($t = -1.388$, $P = 0.17$; sample sizes for control and testosterone treatments are $n = 11, 17$, respectively).

On days 8 and 14, there was no effect of *in ovo* treatment group on either tarsus length or weight of the nestlings (day 8, tarsus, $t = 0.506$, $P = 0.62$, weight, $t = -0.181$, $P = 0.88$; day 14, tarsus, $t = 0.126$, $P = 0.90$, weight, $t = 0.659$, $P = 0.52$; sample sizes for control and testosterone treatment groups, respectively, are: day 8, $n = 11, 18$; day 14, $n = 10, 16$). Nestlings in the testosterone treatment group produced a significantly larger swelling response to an injection with PHA than did control nestlings ($F_{1,20} = 6.27$, $P = 0.02$; sample sizes for control and testosterone treatments are six and 15, respectively; Fig. 3).

Discussion

As predicted, *in ovo* testosterone injections exerted a stimulatory effect on growth during early development in house finch chicks: 2 d after hatching, chicks in the testosterone treatment group had significantly larger tarsi than those in the control group. This size difference disappeared by day 8 posthatch, and chicks from both treatment groups remained statistically similar in size through day 14 posthatch, which is our measurement closest to fledging. Contrary to our predictions, chicks in the testosterone treatment group exhibited a larger swelling response to presentation with PHA on day 14 posthatch, indicating a larger T-cell immune response, than did control chicks.

Testosterone has a well-defined suite of anabolic effects on muscle and bone in many species, which could have been responsible for the observed stimulatory effect of *in ovo* testosterone treatment on skeletal growth in our birds. For example, bone and cartilage contain androgen receptors, making them androgen

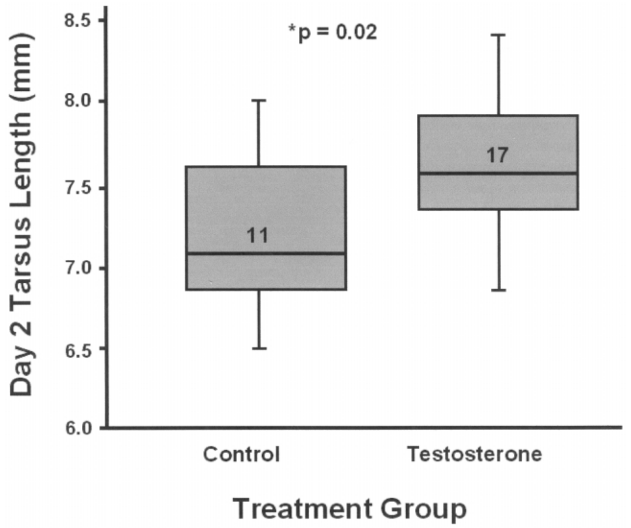

Figure 2. The effects of *in ovo* testosterone and control injection treatments on mean (±SE) tarsus length of nestlings 2 d after hatching. Injection treatments included a high-dose injection (200 ng T in 5 μL peanut oil) and a control injection (5 μL peanut oil). Sample sizes are indicated by numbers located in the box plots.

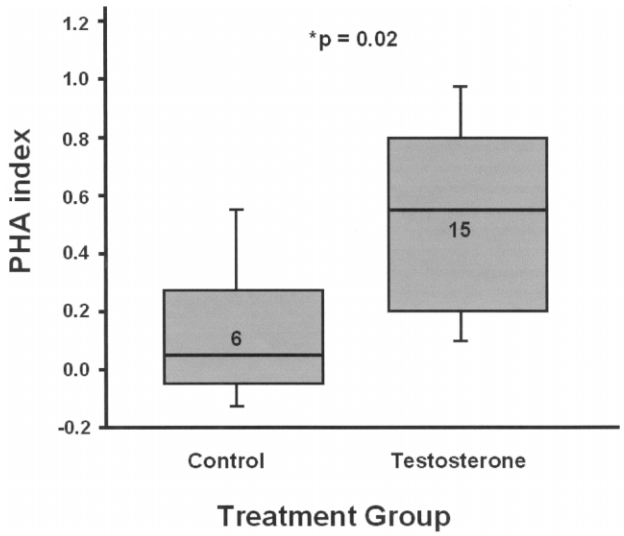

Figure 3. The effects of *in ovo* testosterone and control injection treatments on mean (±SE) swelling response (PHA index) to phytohemagglutinin (PHA) for testosterone versus control chicks. The PHA index was calculated using the following formula: PHA index = (postinjection − preinjection)/[(preinjection + postinjection)/2]. Injection treatments included a high-dose injection (200 ng T in 5 μL peanut oil) and a control injection (5 μL peanut oil). Sample sizes are indicated by numbers located in the box plots.

target tissues (Corvol et al. 1992). Androgens have been found to stimulate the release of bone growth factors (Kasperk et al. 1990) as well as cartilage cell proliferation (Fischer et al. 1995). While these studies have been largely conducted in mammals, it is possible that androgens absorbed from the yolk exerted similar effects on bone and cartilage during embryonic development in these birds.

The fact that size measures were similar between control and testosterone chicks by day 8 posthatch is not surprising because 2003, the year in which our study was conducted, was a record rainfall year in Alabama (National Oceanic and Atmospheric Administration, National Weather Service, Southern Region, http://www.srh.noaa.gov/ffc/html/pis803.shtml), which likely increased the food availability in the study area. Additionally, feeding stations located throughout our study site gave birds consistent access to food while they were breeding. Perhaps during a year when food is more limited, offspring that are larger early in the developmental period would outcompete the smaller offspring in the brood. By depositing more yolk androgens into later-laid eggs in the clutch, as we have shown in house finches, females may be giving offspring that hatch later a developmental boost that could help to protect them from potentially detrimental competition over food during a time where food is limited. This idea has previously been demonstrated in European starlings during a drought year (Pilz et al. 2004).

Our observations that testosterone chicks produced a larger swelling response to PHA than control chicks was the opposite of what we predicted. Previous studies in avian species have documented a suite of immunosuppressive effects associated with testosterone. Androgens are generally regarded as immunologically inhibitory, decreasing thymic and bursal sizes in birds (Olsen and Kovacs 1996). Additionally, testosterone has been shown to directly induce oxidative stress in many tissues (von Schantz et al. 1999), which could damage lymphocytes involved in the immune response and result in immunosuppression (Raberg et al. 1998). Our results, however, are consistent with those found in Siberian hamsters (*Phodopus sungorus*), where testosterone treatments enhanced the responses to PHA (Bilbo and Nelson 2001); in free-living superb fairy wrens (*Malurus cyaneus*), where testosterone was positively correlated with immune function (Peters 2000); and in mice (*Mus musculus*), where androgen deprivation resulted in a decrease in the number of mature T lymphocytes in circulation (Viselli et al. 1995). In fact, while androgens are generally regarded as immunosuppressive, several studies have documented either no relationship between androgens and immunity or an enhancing effect of androgens on immune function (reviewed by Owen-Ashley et al. [2004]). Thus, the stimulatory effects of *in ovo* testosterone injections on the responses of house finch chicks to PHA injections are not surprising.

Androgens are often considered mediators of energy allocation, diverting the use of energy to activities associated with growth, dominance, or sexual display instead of immune function (Penn and Potts 1998). Because there was an abundance of food available for birds during the course of our study season, it is likely that the energy trade-off that might normally exist between growth and immunity in developing offspring was absent. Thus, we might have expected to see similar immunological responses to PHA between nestlings in the two treatment groups. In this case, however, nestlings in the testosterone treatment group exhibited a significantly larger swelling response than controls, a result that is difficult to explain in the context of energy allocation.

Instead, we must examine the suite of interrelated physiological effects associated with yolk androgens before we can fully understand the reasons behind our observed results. Androgen receptors are located throughout several major body systems, resulting in widespread physiological changes on exposure to androgens. For example, testosterone has been shown to interact with the hypothalamic-pituitary-thyroid axis, potentially altering basic metabolic processes (Esposito et al. 2002); the hypothalamic-pituitary-adrenal axis, potentially affecting the allocation of energy within the body (Csaba et al. 1988; McCormick et al. 1998); and the growth hormone axis (Painson et al. 2000), altering the regulation of growth processes. In many cases, there is reciprocal "cross talk" between hormone systems that results in a web of physiological manipulation and control (Esposito et al. 2002). It is clear that we must take into account several measures of offspring quality to get a more complete idea of the physiological effects associated with the deposition of yolk androgens.

In this study, we measured only one aspect of immunity, the T-cell-mediated immune response. In reality, the immune response is complex, utilizing a three-tiered attack system when a foreign agent is encountered. Upon exposure to a pathogen, an organism elicits an innate phagocytic response, a T-cell-mediated inflammatory response, and a B-cell-mediated production of antibodies to the pathogen. It is possible that, while yolk androgens had a stimulatory effect on the response of nestlings to a PHA challenge, the same treatment might have a different effect on other aspects of immunity. Additionally, many aspects of the immune system can interact with one another when challenged with a pathogen, and androgens can have effects on this interaction at many different levels. Thus, it is important to use caution when interpreting immune responses solely through a PHA challenge, and, while we can conclude that yolk androgens have a stimulatory effect on T-cell immunity in house finch chicks, more work needs to be done to assess overall immunocompetence in these birds. Future studies should address both the antibody-mediated immune response and the innate phagocytic response to novel challenges.

Our results demonstrate that the effects of yolk androgens on any one aspect of growth and development are species specific. Because of physiological differences between the offspring of different species and different sexes, androgens deposited into the yolk can have a variety of effects on any particular physiological system. Yolk androgen injections have been found to increase begging behavior in canaries (Schwabl 1996*b*) and black-headed gulls (Eising and Groothuis 2003) but not in European starlings (Pilz et al. 2004). *In ovo* injections of yolk androgens increased nestling mortality in American kestrels (Sockman and Schwabl 2000) and eastern bluebirds (Navara et al. 2005) but did not alter embryonic mortality above control injection mortality levels in our study with house finches. Finally, *in ovo* androgen administration resulted in a suppression of T-cell-mediated immunity in eastern bluebirds (Navara et al. 2005) and black-headed gulls (Groothuis et al. 2005), while the same measure of immunity was stimulated by *in ovo* testosterone injections in our house finch chicks. Thus, while it is likely that yolk androgens serve as powerful maternal effects that can potently alter the fitness of females and their offspring, the formation of hypotheses regarding the adaptive significance of yolk androgens must be particularly characterized using species-specific information concerning the physiological effects of yolk androgens, and by taking into account environmental and social changes that could alter the outcomes associated with this potentially powerful form of maternal investment.

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