YOLK ANDROGEN DEPOSITION IN TWO PASSERINE SPECIES:

DO FEMALES PLAY FAVORITES?

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YOLK ANDROGEN DEPOSITION IN TWO PASSERINE SPECIES: DO FEMALES PLAY FAVORITES?

Kristen J. Navara

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DISSERTATION ABSTRACT

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DO FEMALES PLAY FAVORITES?

Kristen J. Navara

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Eggs of oviparous species contain variable amounts of androgens, such as testosterone (T), dihydrotestosterone (DHT), and androstenedione (A4). Previous studies in birds show that yolk androgens of maternal origin have both immediate and long-term effects on offspring growth, immune function, behavior, and mortality. Additionally, female birds alter patterns of androgen deposition into yolk in a species-specific manner according to social interactions, environmental conditions, and incubation patterns.

Thus, the deposition of yolk androgens has been proposed as an adaptive mechanism by which female birds can promote the survival of certain offspring, and thus maximize reproductive success.

I assessed deposition patterns of yolk androgens as well as the effects of high yolk androgen concentrations on offspring in two passerine species with very different life history traits. House finches *(Carpodacus mexicanus*) hatch asynchronously and females choose mates based on plumage coloration. In a correlational study, female house finches deposited significantly more androgens into eggs sired by unattractive males, and into eggs laid later in the laying sequence, but only when mated to unattractive males. Injections of testosterone into house finch eggs resulted in offspring that were larger at hatch and had a larger T cell immune response compared to control nestlings.

The eastern bluebird (*Sialia sialis)* differs from the house finch in that individuals of both sexes are extremely aggressive and territorial. Presentation of an "intruder challenge" drove females to deposit significantly more androgens into eggs, but concentrations of androgens in female plasma were significantly lower in stimulated females than in controls. Additionally, hormone profiles of egg yolks did not simply reflect the content of female plasma during follicular development. Finally, injections of testosterone into bluebird eggs increased embryonic mortality, stimulated weight gain during the nestling period, and decreased T cell immunity of offspring. Strategies involving the deposition of yolk androgens as well as the effects of such strategies on offspring are likely adaptive but are complex and species-specific.

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INTRODUCTION TO DISSERTATION

Females of oviparous species have very little direct control over the development of offspring during the embryonic period because the embryo is separated from the female by a physical barrier – the eggshell. Thus, the deposition of physiologically relevant materials into eggs during follicular development is one of the only ways in which females can direct the quality of offspring during the sensitive time of embryogenesis. Androgens, such as testosterone (T), dihydrotestosterone (DHT), and androstenedione (A4) are potent regulators of physiological and developmental processes, and exposure to even low levels of androgens during the embryonic period can significantly alter the quality and survival of offspring. Female birds and reptiles deposit androgens into the yolks of their eggs, and deposition patterns of these androgens vary according to social and environmental conditions. Further, studies on several avian species have shown that yolk androgens significantly alter the quality and survival of offspring. Thus, the deposition of androgens into eggs may be an adaptive mechanism by which females can guide the development of offspring, and selectively favor certain offspring over others to maximize reproductive success.

WITHIN AND AMONG-CLUTCH PATTERNS OF YOLK ANDROGEN

In recent years, there has been an explosion of studies examining patterns of androgen deposition in the yolks of avian eggs. This work began with a study by Schwabl (1993)

reporting that androgen content in the yolks of canary eggs increased with laying order. Canaries hatch asynchronously, meaning females begin incubation before the last egg is laid resulting in a temporal hatching range and thus a size gradient among offspring in a brood (Schwabl 1993). Schwabl (1993) suggested that the deposition of yolk androgens may be a strategy employed by canary females to stimulate androgen-related aggressive behaviors and growth in offspring hatching later in the brood, thus compensating for the detrimental size gradients associated with hatching asynchrony. Further studies showed a similar pattern of androgen deposition in the eggs of other species in which offspring hatch asynchronously, including the red-winged blackbird *Agelaius phoeniceus* (Lipar et al. 1999), the European Starling *Sturnus vulgaris* (Pilz et al. 2003), the American Kestrel *Falco sparverius* (Sockman and Schwabl 2000), the black-headed gull *Larus ridibundus* (Eising et al. 2001), the lesser black-backed gull *Larus fuscus* (French et al. 2001), and the house sparrow *Passer domesticus* (Mazuc et al. 2002). This was not the case, however, for all species exhibiting hatching asynchrony. For example, the cattle egret *Bubulcus ibis* (Schwabl 1997), the American Coot *Fulica americana* (Reed and Vleck, 2001), and the zebra finch *Taeniopygia guttata* (Gil et al. 1999) exibited a reverse deposition pattern, where androgen concentration was highest in the eggs laid earlier in the clutch. Thus, it became clear that the driving forces behind the deposition of yolk androgens are more complicated than previously thought and may be species-specific as well. As a result, researchers began examining other potential environmental and social variables that may have an effect on how females deposit androgens into eggs.

Several avian species also vary the androgen content of yolk based on the surrounding competitive environment during the nesting period. For example, house sparrows *Passer domesticus* increased levels of yolk T with colony size (Schwabl 1997) and, in European starlings, yolk T was positively correlated to breeding density (Pilz and Smith, 2004). Additionally, in tree swallow *Tachycineta bicolor* eggs, yolk T correlated positively with the number of nest intrusions experienced by the females (Whittingham and Schwabl 2002). Adaptive hypotheses suggest that female birds may deposit androgens into yolk as a mechanism to increase androgen-related growth and aggression in offspring, thus preparing those offspring for a more competitive environment. The only experimental test of this hypothesis, however, did not demonstrate a link between aggressive interactions and the concentrations of androgens deposited into eggs (Mazuc et al. 2003).

Yet another potential influence on the deposition of yolk androgens was identified by Gil et al. (1999), who demonstrated that female birds alter androgen deposition patterns according to mate quality. That is, zebra finch females deposited significantly more androgens into eggs when mated to males wearing red leg-bands (which increased the perceived attractiveness of males) as opposed to green leg-bands (which decreased the perceived attractiveness). Similarly, canary females deposited significantly more androgens into eggs when exposed to a song that was experimentally manipulated to include several attractive song elements (Gil et al. 2004, Tanvez et al. 2004). Gil et al (1999) suggested that, if yolk androgens are resources that are both beneficial to offspring and costly for females to produce and/or deposit, female birds may deposit androgens according to a strategy in which females invest more in

offspring sired by more attractive, better quality males, preferentially promoting the survival of better quality offspring. As a result, females allocate more androgens to eggs sired by more attractive males.

HOW DO YOLK ANDROGENS AFFECT OFFSPRING?

Despite the hypotheses that yolk androgens have an adaptive significance in terms of reproductive success, the available evidence suggests that yolk androgens are not simply resources allocated by females to some offspring over others. It is, in fact, impossible to categorize androgens as resources because of their multiple, far-reaching effects within several physiological systems. Androgens impact the organization and activation of several major body systems, including the adrenal axis (McCormick et al. 1998), the thyroid axis (Pathak and Chandola-Saklani 1988, Esposito et al. 2002), and the somatotrophic axis (Bondanelli et al. 2003, Corvol et al. 1992, Jansson et al. 1985). Experimental studies examining effects of yolk androgens on avian offspring have demonstrated stimulatory effects on growth (Schwabl 1996, Eising et al. 2001, Pilz et al. 2004) but also an inhibitory effect on immune function (Groothuis et al. 2005) and post-hatch survival (Sockman 2000) of nestlings. Even more complex is the fact that effects of yolk androgens on offspring vary among species: While high levels of yolk androgens increased mortality in American Kestrels, offspring mortality was decreased in European Starlings. Additionally, while yolk androgens increased growth rates of most avian species, growth rates decreased in American Kestrel offspring receiving *in ovo* androgen injections (Sockman 2000). The pleiotropic and species-specific natures of androgenic effects on offspring make it difficult to categorize the deposition of yolk

androgens as either beneficial or detrimental. Thus, hypotheses concerning the adaptive significance associated with the deposition of yolk androgens must be made based on a comprehensive examination of the patterns and effects of yolk androgens in each individual species.

HOW DO THEY DO IT?

Finally, the mechanisms involved in the deposition of androgens in eggs have yet to be elucidated. Many of the above-mentioned studies in which yolk androgen levels varied with the social environment assume that the deposition of high levels of yolk androgens stem from high levels of circulating androgen in the female. This assumption is based on a correlational study of common canaries *Serinus canaria*, in which circulating levels of plasma androgens during follicular development correlated positively to androgen levels in the eggs (Schwabl 1996) and on studies in which experimental elevation of maternal estradiol resulted in a corresponding increase in levels of estradiol measured in the yolks of eggs both in zebra finches *Taeniopygia guttata* (Williams*, et al.* 2004) and Japanese quail *Coturnix japonica* (Adkins-Regan*, et al.* 1995). In a study of house sparrows, however, circulating plasma androgen levels correlated negatively with androgen concentration in the eggs (Mazuk*, et al.* 2003), and, in European starlings *Sturnus vulgaris* patterns of sex steroid levels found in the plasma were different from patterns previously found in the yolks of starling eggs (Pilz & Smith 2004, Williams*, et al.* 2004). Instead, an experimental injection of radiolabelled hormone illustrated that >99% of yolk steroids originate in the follicular cells surrounding the oocyte (Haekl et al. 2003).

PROPOSED STUDIES

My work examines patterns of androgen deposition in the eggs as well as the effects of physiologically high levels of yolk testosterone on offspring growth, survival, and immune function in two passerine species with very different life history traits. The house finch *Carpodacus mexicanus* is small, non-territorial, sexually dimorphic passerine. Female house finches often begin incubating before the last egg is laid, resulting in offspring that hatch asynchronously. As a result, house finch nestlings hatching from eggs in the fifth clutch position have been shown to be significantly smaller and significantly less likely to survive (Dervan, 2000). Male house finches express carotenoid-based plumage coloration ranging from bright red to drab yellow, with females showing a mating preference for males with redder, more saturated plumage (Hill 1990; Hill 1991). In this species, redder males provision at a higher rate, providing direct benefits to females and their offspring (Hill 1991). Male color has also been positively correlated with breeding success (McGraw et al. 2001) and overwinter survival (Hill 1991) and it has been shown that brighter red males pair with older, more attractive females (Hill 1993). I examine patterns of androgen deposition in relation to both mate attractiveness and laying order in this species, as well as the effects of high doses of exogenously administered androgen on nestling growth and immunity.

Eastern bluebirds *Sialia sialis* are socially monogamous passerines that breed over much of eastern North America. This species is an obligate secondary cavity nester (they depend on nest cavities to reproduce, but cannot excavate their own) and nest cavities are a limited resource (Gowaty & Plissner 1998). Members of both sexes

are extremely territorial and aggressive interactions are routinely observed at nest boxes, perhaps as a protective mechanism against cavity usurpation. Further, bluebird offspring exhibit a relatively high level of hatching synchrony, because females generally begin incubation after the last egg in the clutch is laid. I experimentally examine the effects of territorial intrusion and aggressive interaction on deposition patterns of yolk androgens in this species. Additonally, I attempt to shed some light on the mechanisms behind the deposition of yolk androgens by examining the profiles of yolk steroids in bluebird eggs in relation to circulating plasma steroids in the female bluebird during follicular development. Finally, using *in ovo* injections of either testosterone or a control vehicle, I examine the effects of yolk androgens on growth, survival, and immune function in bluebird offspring. Overall, I hope to provide comprehensive analyses of yolk androgens and shed some light on their adaptive significance in two very different passerine species.

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CHAPTER ONE:

YOLK ANDROGEN DEPOSITION AS A COMPENSATORY STRATEGY

Introduction

Discussions of maternal investment often focus on patterns of resource allocation to offspring. Many behavioral measures of maternal investment, such as incubation, provisioning, and protection of offspring, have been well characterised. Female birds and reptiles also have the ability, however, to invest in offspring before they hatch, through the deposition of physiologically relevant substances, such as carotenoids (Surai et al. 2001; Dierenfeld et al. 2002), antibodies (Saino et al. 2003), and hormones (Schwabl et al. 1997; Lovern and Wade 2001) into eggs. Specifically, yolk androgens have become the subject of much discussion, and the effects of androgens on offspring, as well as the adaptive significance associated with deposition patterns of yolk androgens remain unclear.

Androgens are mediators that, when deposited into eggs by female birds and reptiles, can have potent effects on offspring growth and survival. For example, *in-ovo* injections of androgens into the yolks of canary (*Serinus canaria)* and black-headed gull (*Larus ridibundus*) eggs increased begging behavior and growth rates of chicks (Schwabl 1996b; Eising and Groothuis 2003) and decreased the chance of starvation during a drought year in European starling chicks (*Sturnus vulgaris*) (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). On the other hand, injections of androgens into eggs were associated with decreases in T-cell immunity (Groothuis et al. 2005; Navara et al. 2005) as well as increases in offspring mortality (Sockman and Schwabl 2000; Navara et al. 2005). Because many of the measured effects of yolk androgens appear positive, however, androgens have been frequently described as resources that, when allocated to eggs, benefit offspring and increase reproductive success.

Previous research has shown that the strategies of androgen deposition in yolk differ according to environmental and social contexts. For example, females of some avian species deposit more androgens into eggs laid later in the clutch, potentially negating the offspring size gradient caused by hatching asynchrony (Schwabl 1993; Lipar et al. 1995; French et al. 2001; Royle et al. 2001; Sockman et al. 2001; Groothuis and Schwabl 2002). In addition, androgen concentrations in eggs have been found to vary according to mate quality; Female zebra finches (*Taeniopygia guttata*) deposited more yolk androgens into eggs sired by males wearing 'more attractive' red legbands than those sired by males wearing 'less attractive' green legbands (Gil et al. 1999), and female canaries deposited more yolk androgens into eggs after being exposed to 'more attractive' songs (Gil et al. 2004; Tanvez et al. 2004). These latter studies, in which androgens appear to be allocated as a resource with more androgens going to offspring of higher value, have caused researchers to describe yolk androgen distribution in the framework of the differential allocation hypothesis (DAH) – the preferential allocation of resources to offspring sired by more attractive, higher quality males (Burley 1988). On the other hand, female collared flycatchers (*Ficedula albicollis)* deposit more

androgens into eggs sired by younger males (Michl et al. 2005). Because older males may provide more direct benefits and may be of better genetic quality, female flycatchers may instead use yolk androgens in a compensatory strategy, to counterbalance the potential detriments associated with a young, and thus lower-quality, father (Michl et al. 2005).

The DAH has three underlying assumptions: (1) Attractive males must provide a greater opportunity for production of high-quality young than unattractive males, making the offspring of the former males worthy of greater investment, (2) differential resource investment (in this case yolk androgens) must be costly to the female in terms of future reproductive effort, and (3) the resource must be beneficial to the offspring of the current reproductive attempt (Sheldon 2000). A compensatory strategy, on the other hand, would also have three underlying assumptions: The first two assumptions are similar to the DAH in that (1) attractive males must provide a greater opportunity for production of high-quality young than unattractive males (eg. providing more direct benefits or better genes), and (2) the differential deposition of the mediator in question (in this case yolk androgens) must be costly to the female or the offspring in terms of reproductive success. The third assumption (3), however, differs from the DAH in that the deposition of the mediator must in some way mitigate the disadvantages experienced by an offspring sired by a lower quality male. Thus, the mediator need not be a resource that is always beneficial to offspring, but exposure to such a mediator must provide certain benefits to offspring that are lacking as a result of the current breeding situation.

We examined the idea that yolk androgens are resources deposited according to the DAH or according to a compensatory strategy in a wild population of house finches. Female house finches often begin incubating before the last egg is laid, resulting in offspring that hatch asynchronously. As a result, house finch nestlings hatching from eggs in the fifth clutch position have been shown to be significantly smaller and significantly less likely to survive (Dervan 2001). Male house finches express carotenoid-based plumage colouration ranging from bright red to drab yellow, with females showing a mating preference for males with redder, more saturated plumage (Hill 1990; Hill 1991). In this species, redder males provision at a higher rate, providing direct benefits to females and their offspring (Hill 1991). Male colour has also been positively correlated with breeding success (McGraw et al. 2001) and overwinter survival (Hill 1991) and it has been shown that brighter red males pair with older, more attractive females (Hill 1993a). Because female house finches paired with more attractive males gain more direct (Hill 1991) and, perhaps, indirect benefits (Hill and Farmer in press) than females paired to less attractive males, we predicted that, if yolk androgens are deposited according to the DAH, females paired to more attractive males would deposit more androgens into their eggs. Alternatively, if yolk androgens are deposited according to a compensatory strategy, we predict that females will deposit more androgens into eggs sired by less attractive males. Additionally, due to the level of hatching asynchrony exhibited in this species, we predicted that female house finches would deposit more androgens into eggs laid later in the clutch.

Materials and Methods

Male colour and Female Condition

We monitored a nesting population of house finches in Lee County, AL. The field site contained approximately 150 nest boxes within a 25mi^2 area, most of which were occupied by nesting house finches. House finches are extremely non-territorial, however all nests were located at least 10m from one another, minimizing breeding density. We examined allocation patterns of yolk androgens at each nest in relation to the colour of the attending male as well as in relation to female condition. Nesting male and female house finches were captured using either potters traps specially altered to enclose the nest or basket traps surrounding feeders throughout the study site. For males, three colour measurements were taken from each of three locations on the body, including the head, breast, and rump, using a Colortron© reflectance spectrophotometer (Hill 1998). This device measures three separate aspects of colour: hue, saturation, and brightness. All measurements of separate body parts were averaged to provide an overall average measurement for each colour variable. Also upon capture, female tarsus length was measured using manual dial calipers (accuracy = 0.01mm) and mass was measured using a digital scale (accuracy = 0.05g). The residuals of mass:tarsus length were calculated as a measure of condition in these females. Despite suggestions by Green (2001) that mass:tarsus length is a generator of spurious results, we feel that a measurement that incorporates both skeletal size and mass of the animal generates an excellent estimation of individual avian condition, and the residuals of these measurements provide a clean method of separating the effects of condition from the effects of body size (Reist 1985).

Egg collection and yolk androgen analysis

After the appearance of the first egg, we used visual assessments to determine the date of incubation onset. Previous experiments using nest temperature measurements showed that visual assessments consistently and accurately estimated the day of incubation onset (Badyaev et al. 2003). All eggs were retrieved after 36 hours of incubation, prior to the development of embryonic gonadal tissue (K.Navara, unpublished data), and frozen at -20°C. In cases where incubation began before the last egg was laid, each egg within the nest was collected after 36 hours of its incubation onset, resulting in a similar incubation time for all eggs. Because previous studies have shown that concentrations of yolk androgens decrease after the onset of incubation (Elf and Fivizzani 2002; Rutstein et al. 2005), we were careful to collect all eggs after the same period of incubation, thus eliminating variation resulting from the period of incubation itself. We were able to obtain full data, including yolk androgen concentrations, male color, and female condition on 15 nests. In two cases, we captured only the male parent. Finally, clutch sizes often vary among individuals and among nest attempts, resulting in unequal sample sizes among clutch positions in our analyses.

The albumin, yolk, and the small embryo were separated by thawing. Embryos in collected eggs were of comparable sizes at this stage of development. Yolks were homogenized and 20-40mg of the homogenate was diluted in 1ml of water for the analysis. Yolk testosterone (T), androstenedione (A4), and dihydrotestosterone (DHT) were separated by celite column chromatography according to methods described by Schwabl (1993). T and A_4 were quantified using a standard competitive binding

radioimmunoassay, using a specific antibody (Endocrine Science, USA) according to methods outlined in Mendonça et al. (1996). DHT was quantified using a commercial I^{125}-labeled radioimmunoassay kit from Diagnostics Systems Laboratories (Webster, TX). Interassay variation was 20% for A_4, 3% for T, and 14% for DHT and intrassay variation was 6% for A_4 and 4% for T. Assay lower detection limits were 20pg/ml for A_4 and T, and 25pg/ml for DHT. Because all androgen concentrations were highly correlated with one another ($p < .0001$ in all cases), and in most cases all androgens showed similar patterns when analysed separately, we used the sum of all three androgens in our analyses.

Statistical Analyses

The average values for the three male colour variables were analysed using a principal components analysis (PCA) and all three variables were significantly explained by one principal component (PC1) (Variance: Hue=0.83, Brightness = 0.60, Saturation = -0.70). The Colortron© measures hue on a continuous color scale; lower hue values are indicative of a red color while higher hue values are indicative of a yellow color. Thus, as we would expect, when the hue value is lower (red), plumage is more saturated as well while, when the hue value is higher (yellow), plumage is less saturated but is brighter. For our comparisons, we divided males into two groups at a natural break in the variation of the principal component values (which appeared at zero): males with a PC1 below zero, which tend to be more red, were labeled as "more attractive" males, and males with a PC1 above zero, which tend to be less red, were labeled as "less attractive". The relationship between yolk androgens and male attractiveness was tested

using a nested analysis of variance (ANOVA), using male ID as the nested variable within treatment group. This allowed the inclusion of potentially important within-clutch variation while preventing the replication that may occur using eggs within the same nest and sired by the same males as independent samples. All nests included in these analyses were sired by individual pairs of birds that were not repeated in the analyses.

Total yolk androgens were examined in relation to female condition using the residuals of the regression of female mass:tarsus length, and comparisons were made using a simple regression. Variation in total yolk androgen content in relation to the month in which the eggs were laid was analyzed using an ANOVA. Clutch averages of total yolk androgens were used in these analyses to avoid using the same females in duplicate analyses.

Patterns of yolk androgen content across clutch positions were analysed in relation to male attractiveness using a two-way ANOVA. Additionally, we conducted a linear regression to examine the within-clutch patterns of yolk androgens content, and split those analyses based on male attractiveness. Using an F-test, we tested for equality of the slopes for the two regression lines created for attractive versus unattractive males (Sokal and Rohlf 1995). Finally, the relationship between yolk androgens and male attractiveness was analysed in each individual clutch position using unpaired t-tests. While the use of a repeated measures ANOVA to analyse yolk androgen content in relation to clutch position would be preferable, we were not able to utilize such analyses because clutch size was not constant for these birds. Statistical analyses were conducted using JMP software (SAS Institute, 1993).

Results

House finch females deposited significantly higher concentrations of total yolk androgens overall into eggs sired by less attractive males ($F_{19,52} = 4.53$, $p < 0.001$; Fig 1). Unpaired t-tests showed that this pattern of increased yolk androgens in eggs sired by unattractive males persisted across all laying positions (egg#1 – t = 1674, p <0.001, egg#2 – t = 1.794, p = 0.055, egg#3 – t = 2.449, p = 0.05, egg#4 – t = 2.175, p = 0.025, egg#5 – t = 2.064, p = 0.03; Fig 2). Average yolk androgen content did not vary with female condition ($R^2 = 0.107$, p = 0.23; Fig 3) or according to the month in which the eggs were laid ($F_{2,30} = 0.656$, p = 0.56; Fig 4). Additionally, the number of days between the appearance of the first egg and the onset of incubation was statistically similar between females mated to attractive and unattractive males ($F_{1,12} = 2.031$, p = 0.18).

Total yolk androgens increased significantly with clutch order ($F_{4,70} = 2.680$, p = 0.04). Post-hoc tests indicated that, overall, eggs in the fifth clutch position contained significantly higher levels of total yolk androgens than those in the first (p = 0.004) and second (p = 0.03) clutch positions. When clutches of males differing in attractiveness were analysed separately, the same pattern remained for eggs sired by less attractive males, with eggs in the fifth clutch position containing significantly more total yolk androgens than those in the first (p = 0.02). In eggs sired by more attractive males, however, there was no difference in total yolk androgens among eggs in any of the clutch positions (p = 0.30; Fig. 2). Additionally, the slopes of the regression lines of within-clutch patterns of yolk androgens were significantly different between eggs sired

by attractive and unattractive males ($F_{1,73} = 150.04$, $p < 0.001$). As a result, offspring sired by less attractive males and hatching from eggs in the fifth clutch position received the highest levels of yolk androgens.

Discussion

Contrary to the predictions of the DAH, house finch females deposited more yolk androgens into eggs sired by less attractive males. Additionally, females deposited more androgens into eggs laid later in the clutch, but only when paired with less attractive males. This suggests that yolk androgens are not resources deposited by female house finches according to the DAH. Alternate factors that may have influenced these patterns could include the occurrence of extra pair copulations (EPCs), seasonal effects, or limitations associated with female quality. Less than 9% of house finch offspring, however, result from EPCs and the number of extra-pair young in a nest is not related to the colour, age, or condition of the social male at that nest (Hill et al. 1994). Additionally, while males of higher quality tend to pair earlier in the reproductive season (Hill 2002), yolk androgen content did not vary by the month in which the eggs were laid, suggesting that the observed pattern is not due to a seasonal effect (Fig 3). Female quality could potentially affect allocation strategies of yolk androgens, and previous research has shown that more attractive male house finches tend to mate with older, more attractive females. We found no link, however, between female condition and average yolk androgen content of a clutch (Fig 4). Thus, our observations suggest that females altered their patterns of androgen deposition according to the colour of their social mates.

Patterns of yolk androgen deposition in house finches do not follow the DAH for several possible reasons: First, there is still no definitive evidence that the deposition of yolk androgens is adaptive. Thus, from a non-adaptive standpoint, yolk androgen content may simply reflect steroid concentrations in females as a result of other physiological processes. Additionally, because male color and female quality are often correlated in house finches (Hill 1993b) it is impossible to completely separate the effects of female condition from male color when examining a potential deposition strategy. Thus, perhaps the observed patterns of yolk androgen deposition are not related to male quality at all, but result from the physical quality of the female. If this were true, however, we would expect females mated to more attractive males to be of better quality and thus to deposit more androgens into eggs than females mated to unattractive males, the opposite of what we found in this study. We suggest, instead, that the deposition of yolk androgens in this species may not meet assumptions 1-3 (above) of the DAH, and that female house finches distribute yolk androgens according to an entirely different strategic model.

Attractive male house finches provide more food to females and offspring, so the first assumption of the DAH, that attractive males contribute in some way to the current reproductive effort, is met (Hill 1991). The second assumption, (i.e. that producing and transferring androgens to the yolk by females is costly) has yet to be shown in this or any species. Although it has been shown, in general, that high levels of plasma androgens can have detrimental physiological effects (Olsen and Kovacs 1996; Klukowski et al. 1997), and that, at the time of follicular development in the canary, plasma androgens are positively correlated with yolk androgen levels (Schwabl 1996a),

there has been no direct demonstration of a cost to the mother associated with the allocation of yolk androgens. If testosterone levels required for the deposition of yolk androgens were costly to females, we would predict that females in better condition would be better able to withstand any costs associated with depositing higher levels of androgens into eggs. Pilz et al. (2003b) showed that yolk androgen concentration in European starling eggs correlated to age and clutch size, but not to female body condition. Similarly, in the house finch, female condition did not relate to yolk androgen content (Fig 4), challenging the idea that females incur a cost related to the deposition of yolk androgens.

This does not mean, however, that the offspring do not incur a cost as a result of exposure to high levels of yolk androgens. Although yolk androgens have been shown to advantageously alter growth and developmental patterns, their effects are not universally beneficial. For example, injections of androgens into American kestrel (*Falco sparverius)* eggs resulted in offspring that hatched later and had a higher mortality rate (Sockman and Schwabl 2000). Additionally, in the eastern bluebird (*Sialia sialis)*, in-ovo androgen injections had both a stimulatory effect on offspring growth while exerting a suppressive effect on immune function at the same time (Navara et al. 2005). These studies suggest that yolk androgens are not simply beneficial resources, but are mediators that can have a multitude of effects on offspring quality, perhaps providing a set of costs along with the benefits previously observed. The documented immunological costs experienced by offspring, as well as other costs that have yet to be identified, associated with exposure to high yolk androgen levels potentially satisfy the second assumption of both the DAH and the compensatory

deposition strategy. Because yolk androgens are not simply beneficial resources, however, the third assumption of the DAH, that the investment is a beneficial resource to offspring, is not satisfied. Instead, the stimulatory effects of androgens on growth of avian offspring may help to mitigate the lack of direct benefits received from males of lower quality, and, while those benefits come with an immunological cost, they satisfy the third assumption of the compensatory deposition strategy. Thus, while the deposition of yolk androgens by female house finches does not satisfy the assumptions of the DAH, all three assumptions associated with a compensatory strategy are satisfied, suggesting that female house finches deposit yolk androgens in a compensatory manner.

Our observation that within-clutch patterns of yolk androgens only existed in clutches sired by unattractive males can also be explained by a compensatory deposition strategy. Because attractive male house finches provide more food to offspring (Hill 1991), it is likely that size gradients resulting from hatching asynchrony would be more pronounced in broods sired by less attractive males and receiving less food than in broods sired by attractive males and receiving more food. In fact, male provisioning behavior has been shown to have direct effects on offspring recruitment in house finches (Badyaev and Hill 2002). Pilz et al. (2004) showed, in European starling chicks (*Sturnus vulgaris*), that injections of yolk androgens decreased the chance of starvation during a drought year, but had no detectable effects during a year in which water and food was abundant. Perhaps only house finch nestlings that are sired by an unattractive male and receive a suboptimal amount of food through the nestling period require compensation through exposure to yolk androgens, while nestlings sired by an attractive male and receiving more than enough food can overcome the size gradients associated

with hatching asynchrony without any assistance. This idea has yet to be tested in this or any species.

Despite the uncertainties concerning the effects of yolk androgen deposition, we continue to see striking within and among-clutch patterns of yolk androgen content that lend support to the idea that yolk androgens play an important role in offspring development. It is less likely, however, that yolk androgens are "costly resources" that are traded off between reproductive attempts than that they are utilized as modulators to alter the growth and development of the offspring in a more immediate sense. Many asynchronously hatching species, including the house finch, deposit more androgens into eggs laid later in the clutch, a distribution pattern of yolk androgens consistent with the idea of counterbalancing the effects of hatching asynchrony (Schwabl 1993; Lipar et al. 1995; French et al. 2001; Royle et al. 2001; Sockman et al. 2001; Groothuis and Schwabl 2002). Additionally, females of many species deposit more yolk androgens when breeding under more crowded conditions (Schwabl 1997; Whittingham and Schwabl 2001; Groothuis and Schwabl 2002; Pilz et al. 2003a), a strategy that could potentially prepare offspring for the impending competitive environment resulting in areas where conditions are more crowded. In the house finch, the role of yolk androgens appears to be consistently compensatory, with females depositing more androgens into eggs sired by less attractive males, and into later laid eggs, but only in clutches sired by less attractive males. The observed allocation pattern targets those offspring that are smaller at hatch and exist in a nest where the male provisions less, a compensatory form of hormone distribution that may help to negate both the size

gradient caused by hatching asynchrony as well as the consequences associated with the lower quality and/or the lack of direct benefits received from a less attractive male.

In most cases of sexual selection by female choice, there exists a spectrum of males based on the quality of the sexually selected trait. The DAH predicts that females mated to less attractive males would 'cut their losses' and save their investment potential for future reproductive attempts (Sheldon 2000). We propose that females might attempt to alter the condition of lower quality offspring to salvage an otherwise unsuccessful breeding attempt. This compensatory strategy would require that the overall effect of the observed allocation pattern be beneficial in terms of current reproductive success, and that the observed investment strategy not be costly to the female in terms of future reproductive success. Instead, differential patterns of investment may persist due to a range of costs incurred by some offspring as a result of the allocation strategy. Whether or not yolk androgen allocation meets those assumptions is still unclear. It is possible, however, that the compensatory allocation hypothesis is an alternative strategy of investment adopted by females of some species when distributing yolk androgens.

The premise of the compensatory distribution hypothesis is flexible, and allows for the contradictory patterns and effects found in relation to the deposition of yolk androgens. For example, in some asynchronously hatching species, yolk androgen concentrations are higher in later laid eggs (Schwabl 1993) while in other species, androgen levels are higher in eggs laid earlier in the clutch (Gil et al. 1999). In the offspring of some species, yolk androgens exhibit beneficial effects (Schwabl 1996b; Lipar and Ketterson 2000), while in others, they evoke clearly detrimental effects

(Sockman and Schwabl 2000; Navara et al. 2005). These data suggest that the basis behind the deposition of yolk androgens is not simple, and that females do not always use these mediators in one directional way. Instead, it is likely that the optimal patterns of yolk androgen deposition are determined by a mixture of plastic environmental, social, and physiological circumstances, and that distribution patterns may differ according to a variety of adaptive allocation strategies adopted by different species. A more comprehensive examination of the costs and benefits associated with the strategies of yolk androgen distribution must be completed before we can understand the adaptive significance of this potentially powerful maternal effect.

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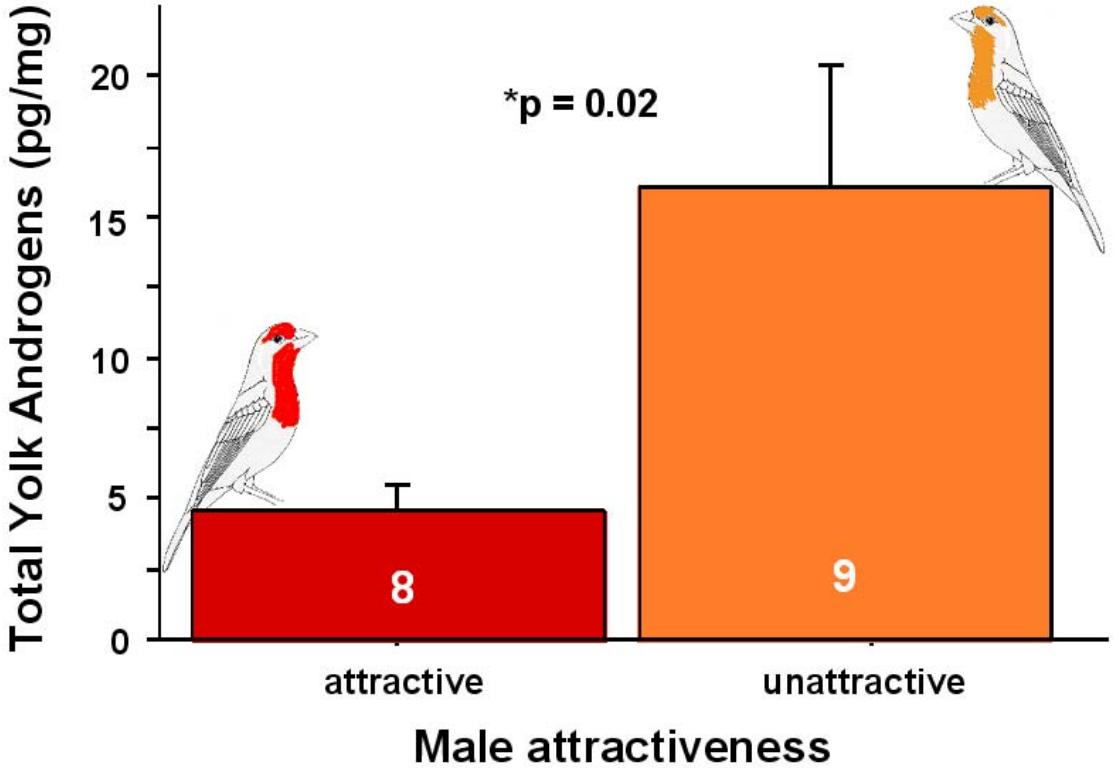

Fig. 1. Mean contents per milligram of yolk of total yolk androgens, defined as the sum of testosterone (T), androstenedione (A_4), and dihydrotestosterone (DHT) (error bars show standard errors) in eggs sired by less attractive male house finches vs. eggs sired by more attractive male house finches. Numbers located inside the bars indicate the number of males analysed in each attractiveness group.

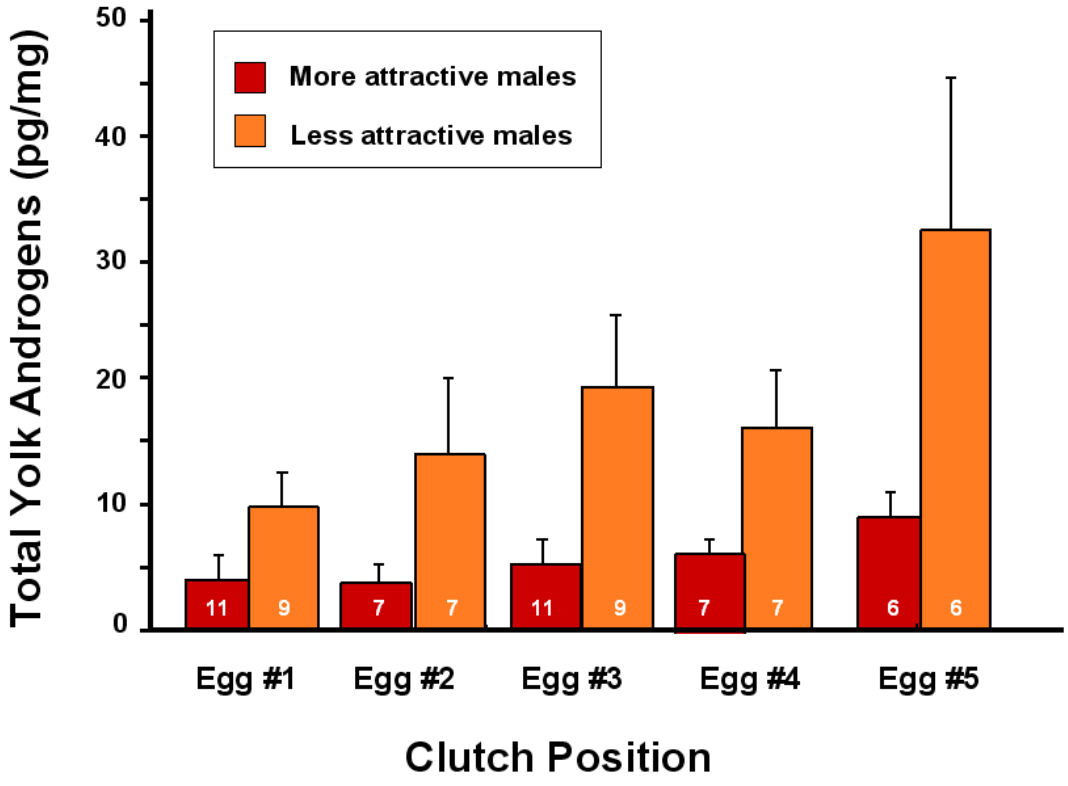

Fig. 2. Mean contents per milligram of total yolk androgens, defined as the sum of testosterone (T), androstenedione (A_4), and dihydrotestosterone (DHT) (error bars show standard errors) in eggs of different clutch positions. Numbers located inside the bars indicate the number of eggs in each clutch position and sired by males in each attractiveness group.

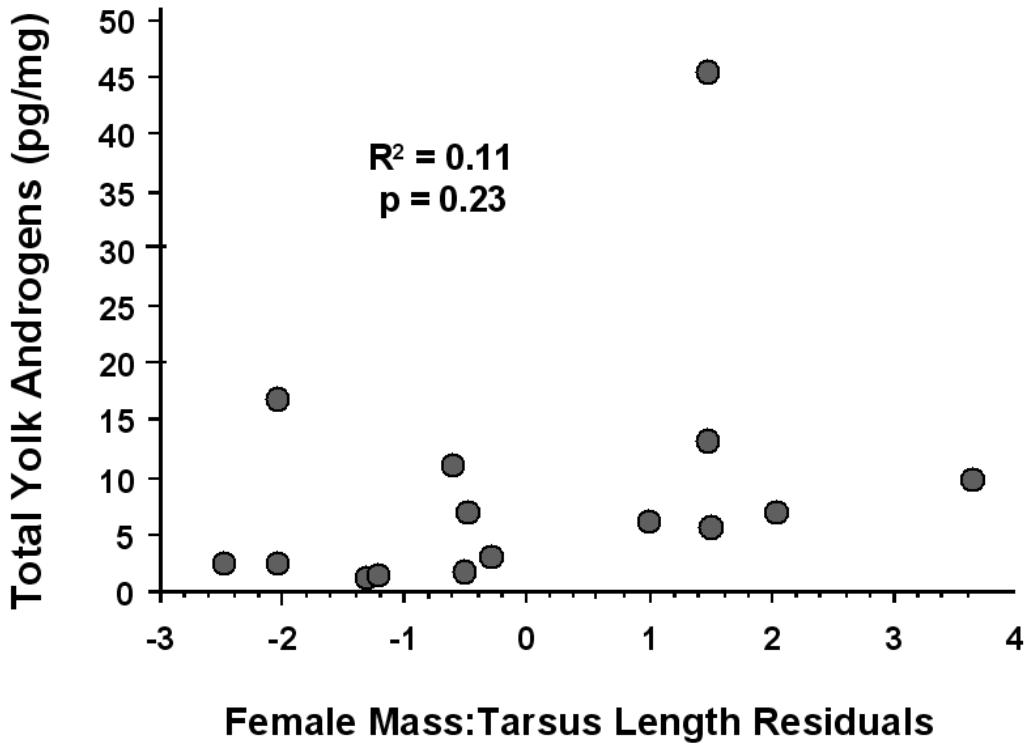

Fig 3. Female condition, as defined by the residuals of mass:tarsus lengths, analysed according to average clutch total yolk androgens using a simple regression.

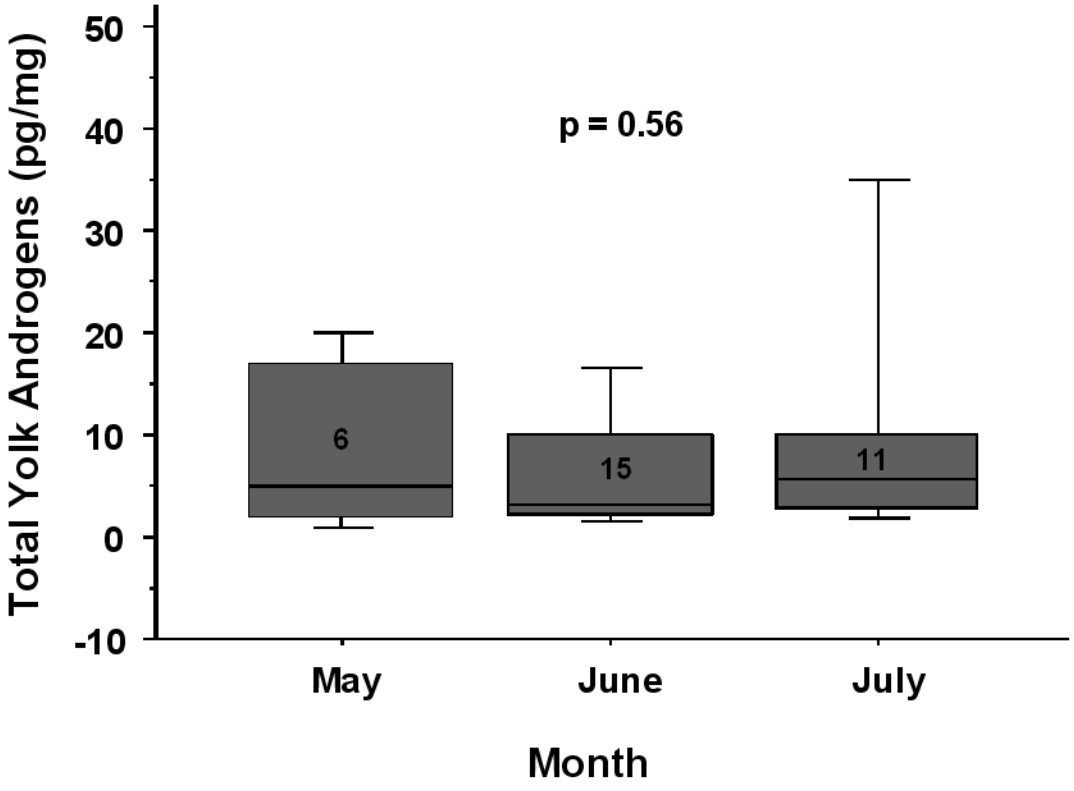

Fig. 4. Mean contents per milligram of total yolk androgens, defined as the sum of testosterone (T), androstenedione (A_4), and dihydrotestosterone (DHT) (error bars show standard errors) in eggs laid in May, June, and July. Numbers located inside the boxes indicate the number of clutches (used for clutch average yolk androgen analyses) in each month of the breeding season.

CHAPTER TWO:

YOLK TESTOSTERONE STIMULATES GROWTH AND IMMUNITY IN HOUSE FINCH CHICKS

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, Schwabl 1996, Schwabl 1997, Reed, et al. 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, Eising, et al. 2003) and decreased the chance of starvation during a drought year in European starting (*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, et al. 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, et al. 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 enhancing 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 5 eggs per clutch and 2 to 3 clutches over the course of the breeding season each year. Previous work on this species has shown that females deposit higher levels of yolk androgens into later-laid eggs (K. Navara, unpublished data), 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., in press), the opposite of yolk androgen deposition patterns found in the zebra finch (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 (*Carpodacus 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 immunity of house finch offspring.

Materials and Methods

Egg Injections

We monitored a breeding population of house finches in Lee County, AL 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 they were laid with one of two injection treatments: (1) 200ng of testosterone (T) in 5ul peanut oil or (2) a control injection of 5ul peanut oil. This injection dose was chosen based on the natural variation of yolk testosterone found in house finch eggs, ranging from 0 to 840ng per yolk. Very few eggs contained yolk testosterone concentrations at the highest end of the range (eggs in the first clutch position generally

ranged from 0-22.5ng/yolk, while eggs in the fifth clutch position generally ranged from 0-50ng/yolk) (K. Navara, 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 50ul Hamilton syringe, and the injection site was sealed with brush-on liquid Krazy glue©. Injection treatment was 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

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.

Nestlings were measured on days 2, 8 and 14 post-hatch. Morphological measurements taken on each of these days included mass using a 20g spring scale (accuracy = 0.2g), and tarsus length, using manual dial calipers (accuracy = 0.01mm). Sample sizes for the different measurement dates differed slightly due to occasional offspring mortality or premature fledging.

Cell Mediated Immunity

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 post-hatch, a 1-cm patch on the left mid-patagium was cleared of feathers. Two measures of thickness were taken using a pressure-sensitive digital micrometer (accuracy = 0.05mm). The bare skin was swabbed with alcohol and 20ug of PHA in 50ul PBS 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). Smits and Williams (1999) 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 hours to assess swelling. A PHA index was computed as the thickness of the wing-web post injection minus the thickness of the wing web pre-injection. 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 prior to our final patagium measurement.

Nestlings were handled under a State of Alabama permit (no. 12) and 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 only used 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 was calculated as the number of hatched eggs per clutch divided by the number of eggs laid per clutch and those values were normalized using an arcsin transformation. The effects of *in ovo* injection treatments on hatching 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 analysis of covariance (ANCOVA). Body condition was calculated as the residuals of the regression of mass to tarsus length of the chicks on day 14 post-hatch. 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 (Figure 5). 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 (Figure 6). 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 respectively are n=11,17).

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 control nestlings ($F_{1,20} = 6.27$, $p = 0.02$; Sample sizes for control and testosterone treatments are 6 and 15 respectively) (Figure 7).

Discussion

As predicted, *in ovo* testosterone injections exerted a stimulatory effect on growth during early development in house finch chicks: Two days 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 post-hatch, and chicks from both treatment groups remained statistically similar in size through day 14 post-hatch, 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 post-hatch, indicating a larger T-cell immune response, than 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 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 post-hatch is not surprising because 2003, the year in which our study was conducted, was a record rainfall year in Alabama (NOAA National Weather Service, Southern Region - http://www.srh.noaa.gov/ffc/html/pis803.shtml). These conditions likely increased 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 were larger early in the developmental period would out-compete 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, et al. 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, et al. 2001), in free-living superb fairy wrens (*scientific name)* where testosterone was positively correlated to immune function, 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 in 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, et al. 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 upon exposure to androgens. For example, testosterone has been shown to interact with the hypothalamo-pituitary-thyroid axis, potentially altering basic metabolic processes (Esposito, et al. 2002), the hypothalamo-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; 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 as well as the innate phagocytic response to novel challenges.

Our results demonstrate that effects of yolk androgens on any one aspect of growth and development are species-specific. Due to physiological differences between 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) 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 formulation of a hypothesis regarding the adaptive significance of yolk androgens must be characterized using species-specific information concerning the physiological effects of yolk androgens, with consideration given to environmental and social changes that could alter outcomes associated with this potentially powerful form of maternal investment.

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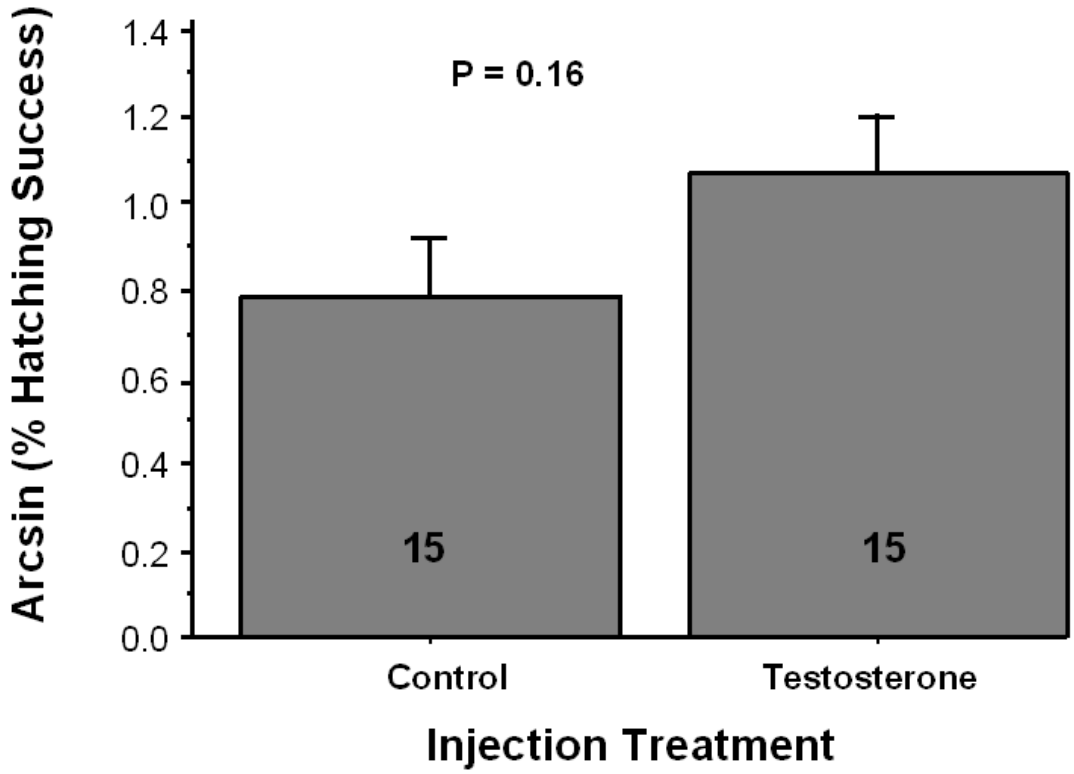

Fig. 5. The effects of *in ovo* testosterone and control injection treatments on mean (± SE) hatching success of house finch nestlings. Hatching success was calculated by dividing the number of nestlings hatching in a treatment group by the number of eggs injected with that treatment. Injection treatments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

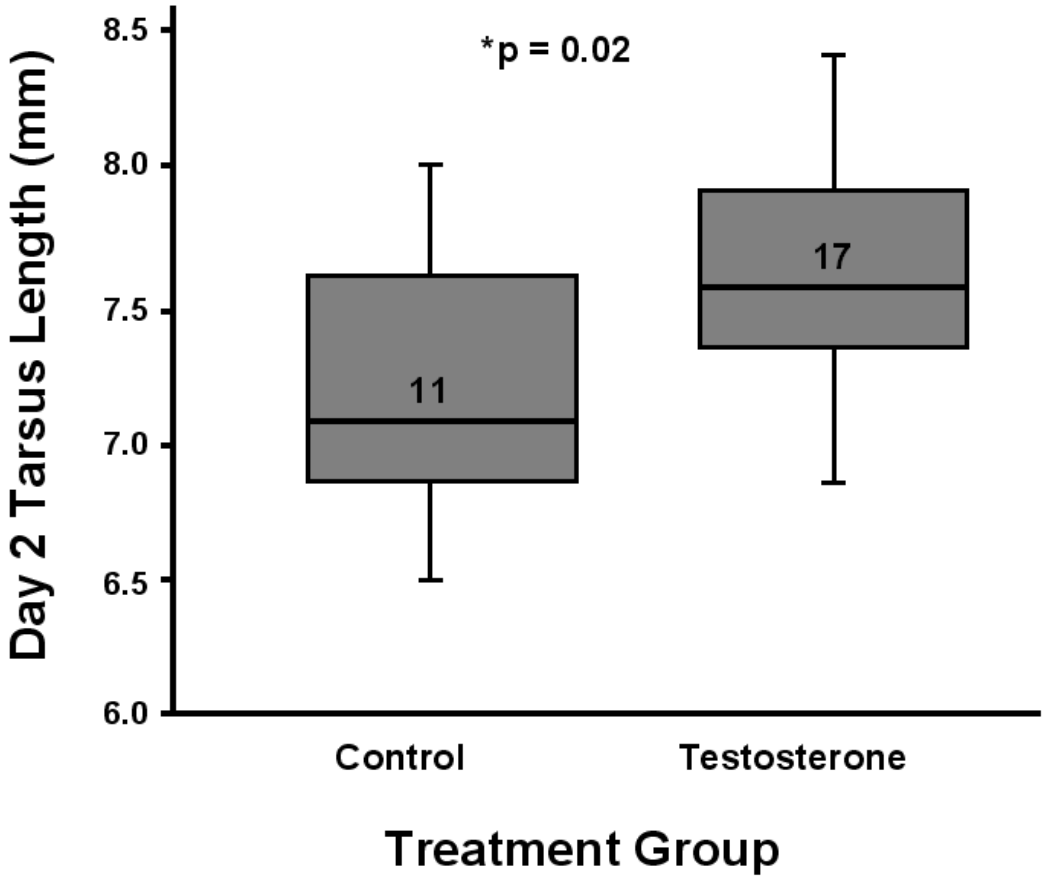

Fig. 6. The effects of *in ovo* testosterone and control injection treatments on mean (± SE) tarsus length of nestlings two days after hatching. Injection treatments included a high-dose injection (200ng T in 5ul peanut oil) and a control injection (5ul peanut oil). Sample sizes are indicated by numbers located in the box plots.

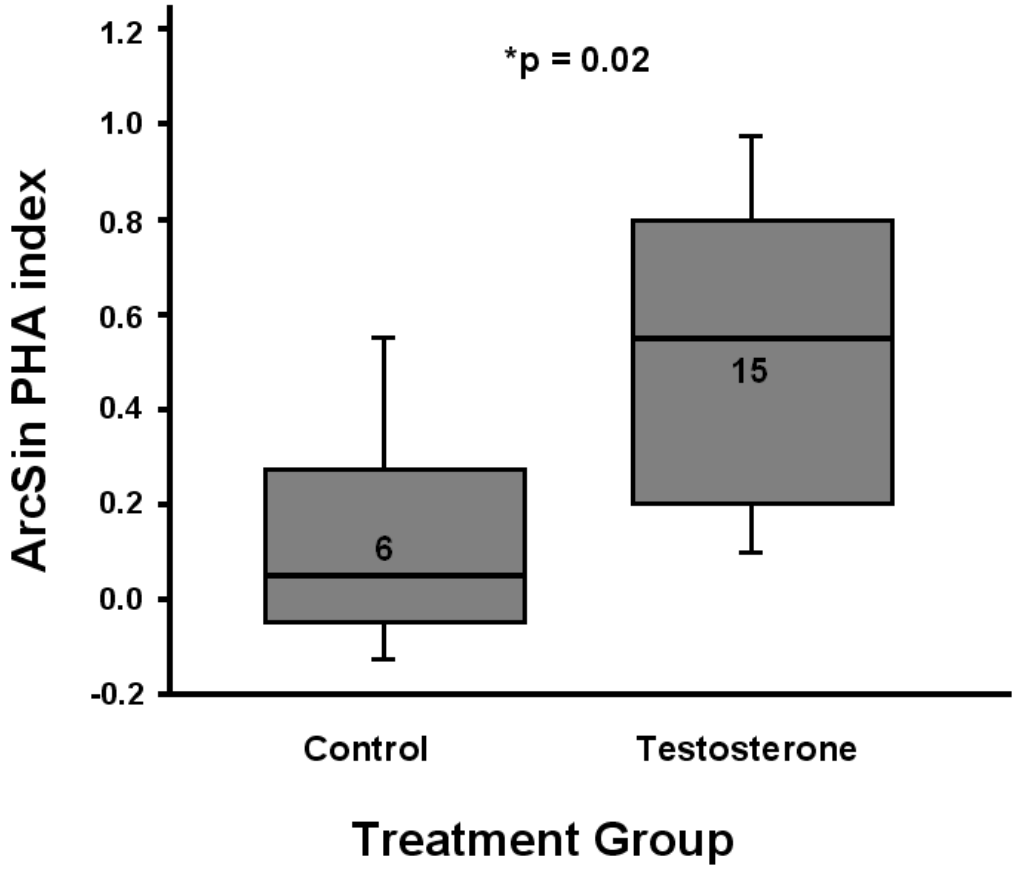

Fig. 7. The effects of *in ovo* testosterone and control injection treatments on mean (± SE) swelling response (PHA index) to phytohemagglutinin (PHA) for testosterone vs. control chicks. The PHA index was calculated using the following formula:

$$\text{PHA index} = \frac{\text{post-injection} - \text{pre-injection}}{(\text{pre-injection} + \text{post-injection})/2}$$

CHAPTER THREE:

YOLK ANDROGENS VARY INDEPENDENTLY OF MATERNAL ANDROGENS IN EASTERN BLUEBIRDS: AN EXPERIMENTAL STUDY

Introduction

Maternal effects can have potent impacts on offspring quality and survival (Mousseau &Fox 1998). One such maternal effect - the deposition of androgens into the yolks of eggs by females of many oviparous species - has an array of physiological and behavioural effects on offspring. Specifically, high levels of yolk androgens are associated with increases in offspring growth (Schwabl 1996, Eising*, et al.* 2001, Groothuis &Schwabl 2002, Pilz*, et al.* 2004, Navara*, et al.* 2005), increases in muscle development (Lipar &Ketterson 2000), decreases in T-cell mediated immune function (Andersson*, et al.* 2004, Groothuis*, et al.* 2005, Navara*, et al.* 2005) and increases in both embryonic and post-hatch mortality (Sockman &Schwabl 2000, Navara*, et al.* 2005). Of particular relevance to the current study, exposure to high levels of yolk androgens also results in higher begging rates in nestlings and more competitive behaviour in adulthood (Schwabl 1993). It has thus been suggested that the deposition of yolk androgens by the females is an adaptive mechanism to increase levels of aggression in their offspring, to prepare offspring for a competitive environment.

In birds, concentrations of yolk androgens vary both within and among clutches. In some species in which offspring hatch asynchronously, yolk androgens are significantly higher in eggs laid later in the laying sequence (Schwabl 1993, Lipar &Ketterson 2000, Sockman &Schwabl 2000, Eising*, et al.* 2001, French*, et al.* 2001, Lipar 2001), and adaptive hypotheses propose that females deposit yolk androgens to facilitate the survival of offspring that hatch from later-laid eggs by stimulating androgen-related growth and aggressive behaviours in those offspring. Along the same lines, yolk androgen levels correlated positively with breeding density in house sparrows (*Passer domesticus)* (Schwabl 1997), number of territorial intrusions in tree swallows (*Tachycineta bicolor)* (Whittingham &Schwabl 2001), relative competitive environment in black-headed gulls (*Larus ridibundus)* (Groothuis &Schwabl 2002), and breeding density in European Starlings (*Sturnus vulgaris)*(Pilz &Smith 2004). In each of these cases, the deposition of yolk androgens was identified as a potential mechanism for stimulating offspring performance in a competitive environment.

While an increasing amount of work has focused on the effects of yolk androgens on offspring, very few studies have considered the interaction between the physiological state of the female and the concentration of yolk androgens in her eggs. Many of the above-mentioned studies in which yolk androgen levels varied with the social environment assume that the deposition of high levels of yolk androgens stem from high levels of circulating androgen in the female. This assumption is based on a correlational study of common canaries (*Serinus canaria*), in which circulating levels of plasma androgens during follicular development correlated positively to androgen levels in the eggs (Schwabl 1996) and on studies in which experimental elevation of

maternal estradiol resulted in a corresponding increase in levels of estradiol measured in the yolks of eggs both zebra finches (*Taeniopygia guttata*) (Williams*, et al.* 2004) and Japanese quail (*Coturnix japonica*) (Adkins-Regan*, et al.* 1995). In a study of house sparrows, however, circulating plasma androgen levels correlated negatively with androgen concentration in the eggs (Mazuk*, et al.* 2003), and, in European starlings (*Sturnus vulgaris*) patterns of sex steroid levels found in the plasma were different from patterns previously found in the yolks of starling eggs (Pilz &Smith 2004, Williams*, et al.* 2004). These studies suggest that the relationship between androgen levels in females and their eggs may be more complicated than previously thought and may be species-specific as well. It remains unclear whether the deposition of androgens into the yolks of eggs is a passive side-effect of high circulating androgen levels in the female or an active shuttling of androgens directly into eggs, perhaps to avoid raising plasma androgen concentrations above basal levels.

To better understand the proximate controls of yolk androgen deposition in relation to levels of circulating androgens in the laying female, we experimentally tested the effects of both laying order and aggressive social interactions on androgen concentrations in both egg yolks and female plasma in the eastern bluebird (*Sialia sialis*). The eastern bluebird is an excellent study species in which to examine effects of environmental and social interactions on both yolk and female plasma androgen levels because individuals of both sexes are extremely aggressive and territorial during the nesting period (Gowaty 1981).

Eastern bluebirds are socially monogamous passerines that breed over much of eastern North America. This species is an obligate secondary cavity nester (they

depend on nest cavities to reproduce, but cannot excavate their own) and nest cavities are a limited resource (Gowaty &Plissner 1998). Aggressive interactions are routinely observed at nestboxes, perhaps as a protective mechanism against cavity usurpation. Thus, we could manipulate the perceived competitive environment of females by presenting nesting females with a simulated intrusion by an unfamiliar female.

The effects of increased yolk androgens are well-studied in eastern bluebirds. Experimentally elevated T levels in the eggs of eastern bluebirds increased posthatch growth rates in nestlings, but decreased embryonic survival and suppressed T-cell immunity (Navara*, et al.* 2005). Thus, the deposition of yolk androgens could serve as a method for controlling offspring quality and survival by eastern bluebird females.

We experimentally altered the perceived social environment of breeding pairs during the period of rapid yolk deposition using an intruder presentation and compared the steroid hormone concentrations of the resulting eggs to those from a set of control breeding pairs. A subset of control and stimulated females were captured either immediately after stimulation or one day prior to the first-egg date, and blood samples were taken to examine effects of the intruder presentation on circulating plasma androgen levels during the time of follicular development. We predicted that exposure of breeding females to an intruder presentation would cause an increase in circulating plasma androgens and consequently lead to an increase in the quantity of the androgens in the yolks of eggs that they laid.

Materials and methods

Presentation of an intruder female

The experiment was conducted in Lee County, AL on portions of a large population of eastern bluebirds where a majority of individuals are colour-banded under Federal License # 21661, collecting permit # MB784373-1 and Institutional Care and Use Committee # 0309-R-2321. Nestboxes were checked daily for signs of nest building. Previous observations indicate that the time period between the completion of the nest lining and the appearance of the first egg is approximately two days in length (K. Navara and L. Siefferman, unpublished data). Thus, we timed the experiment such that the intruder presentations and blood sample collections occurred on the day the nest lining was completed. Intruder presentations were given for 30-min periods on two consecutive days, and all developing follicles in the sequence were in the stage of rapid yolk deposition during the both intruder presentations (Fig. 8). In cases where the predicted first-egg date was not correct, the timing of follicular development was determined after the first egg was laid, and eggs resulting from follicles that were not in stages of rapid yolk deposition during the intruder presentation were excluded from the analyses.

To stimulate aggression towards female bluebirds, a captive female bluebird was presented in a cubical wire cage placed one meter from the nest box. One of five stimulus females (captured within 2 weeks of the trial from a population located > 30 miles away) was randomly assigned to each trial. All stimulus animals were active during the trials and their behaviour did not differ noticeably among different trials or

among females. A tape recorder that played bluebird chatter was placed beside the stimulus animal throughout the course of the intruder presentation. Control pairs were exposed briefly to the tape recorder with bluebird chatter to draw the initial attention of the pair, but were never exposed to the presence of a stimulus female.

During the entire period of intruder presentation, resident females were observed (from a blind) and several behaviours suggestive of female agitation and aggression (Plissner &Gowaty 1995) were quantified, including (1) the time between the beginning of the intruder presentation and the arrival of the female to the nest box, (2) the number of times the female approached and entered the nest box, and (3) the number of times the female attempted to attack the intruder (see Gowaty 1981, Gowaty &Wagner 1988). All females displayed all of the quantified aggressive behaviours during intruder presentations, suggesting that they were, in fact, agitated by the presence of the intruder.

Collection of female blood samples

A subset of females was captured for blood sampling and quantification of circulating plasma androgens. Stimulated females were captured using a mist net immediately after the second intruder presentation. Blood was sampled from the brachial vein within five minutes of capture to avoid complications associated with capture stress (Romero, *et al.* 1997). Control females were captured in mist nets one day prior to the appearance of the first egg. During this time, a tape recorder playing bluebird chatter (as mentioned above) was used briefly to draw the attention of the nesting pair after which the female was captured and a blood sample was taken as described above.

Hormone assays in plasma and eggs

Eggs were collected on the day of laying and were replaced with dummy eggs to prevent nest abandonment. Because eggs had not yet been incubated, all yolk hormones measured were of maternal origin. Upon collection, eggs were frozen at -20°C until the time of extraction for hormone analyses, when yolks were separated by thawing and 35mg of yolk was weighed for extraction. The procedures for extraction and radioimmunoassays of testosterone (T), androstenedione (A4), 17ß-estradiol (E), and corticosterone (B) from yolk homogenates have been described previously by Schwabl (1993). Intra-assay coefficients of variation were A4 – 2.2%, T – 3.7%, E – 5.6%, and B – 7.4%. Extraction and radioimmunoassay of plasma T, A4, E, and B were completed in one set and procedures have been described previously by Mendonça*, et al.* (1996) and Wingfield & Farner (1975).

Statistical Analyses

All data except for corticosterone (B) measures were non-normally distributed and were logarithmically transformed. Parametric tests (including ANOVAs and simple regressions using Statview© software) were used for all statistical analyses.

Results

Hormone profiles in eggs and plasma

First, we examined the differences between yolk and female plasma hormone profiles, regardless of treatment group because both the overall yolk and plasma hormone profiles were similar between stimulated and control groups. None of the four yolk

hormone concentrations varied significantly across the laying order order (A4: $F = 0.91$, $p = 0.46$, T: $F = 1.89$, $p = 0.11$, E: $F = 0.62$, $p = 0.65$, B: $F = 1.34$, $p = 0.26$). Thus, respective concentrations of each hormone in egg yolks were determined using average values for each clutch. Androstenedione was the predominant hormone in bluebird yolks followed by T. Both E and B were found in relatively low concentrations in yolks (Fig. 9a) Female plasma showed a very different hormone profile. Corticosterone was the predominant hormone in female plasma while A4, T, and E existed in relatively low levels in female plasma (Fig. 9b).

Behavioural responses to intruder presentation

Twenty-eight females were exposed to intruder presentations while 20 were assigned to the control group. All but one female arrived at the intruder site within minutes of the intruder presentations. This female did not arrive at the box during the entire duration of the first intruder presentation and was excluded from all analyses. All remaining females responded by flying to their nest boxes, entering their nest boxes, and attacking the caged intruder. Over the course of the experiment, some nests were lost to predators and are not included in these analyses.

Yolk hormone levels in response to intruder challenge

Presentation of an intruder to nesting females resulted in a significant increase in levels of A4 and T in yolks compared to controls ($A4_{2,26}$: $F = 17.89$, $p < 0.001$, T: $F_{2,26} = 15.42$, $p < 0.001$; Fig 10). Levels of E and B in yolks did not differ with intruder presentations (E: $F_{2,26} = 1.04$, $p = 0.32$, B: $F_{2,26} = 1.18$, $p = 0.29$). Although females

showed variation in aggressive behavioural responses to intruding females (time to respond to intruder, number of nest checks, and number of attacks on intruder), simple regressions showed no significant relationship between aggressive behaviour and levels of any of the yolk androgens (for all hormones: time to respond, $R^2 < 0.15$, $p > 0.10$, # of nest checks, $R^2 < 0.12$, $p > 0.15$, # of attacks, $R^2 < 0.27$, $p > 0.15$).

Female plasma hormone levels in response to intruder challenge

The presentation of an intruder had a significant effect on levels of circulating A4 and T in females: both hormones were significantly lower in stimulated females as compared to controls (A4: $F = 4.37$, $p = 0.04$, T: $F = 5.11$, $p = 0.03$) (Fig 11). Levels of E and B circulating in female plasma, however, did not differ between stimulated and control females (E: $F = 0.40$, $p = 0.53$, B: $F = 0.03$, $p = 0.86$). Again, simple regressions showed that none of the four hormones varied in concentration with degree of aggressive behaviour (for all hormones: time to respond, $R^2 < 0.04$, $p > 0.60$, # of nest checks, $R^2 < 0.04$, $p > 0.61$, # of attacks, $R^2 < 0.33$, $p > 0.10$).

Discussion

Hormone profiles and concentrations in both egg yolks and female plasma were first examined regardless of treatment group to determine if hormone levels in the yolk varied across the laying order and how yolk hormone levels related to circulating hormone levels in female plasma at the time of follicular development. Our study is one of the few that sampled hormone levels circulating in female plasma during the critical time frame of rapid yolk deposition (but see Schwabl 1996, Williams*, et al.*

2004). All four measured hormones, A4, T, E, and B were detected in the yolks of eastern bluebird eggs. Androstenedione was the most prominent androgen in bluebird eggs as was also the case with the eggs of canaries (Schwabl 1993), the American kestrels (*Falco sparverius)* (Sockman &Schwabl 2000), and black-headed gulls (Groothuis & Schwabl 2002). On the contrary, testosterone was the predominant androgen in the eggs of both zebra finches (*Taeniopygia guttata)* (Gil*, et al.* 1999) and house finches (*Carpodacus mexicanus*) (Navara*, et al.* in press). Yolk hormone concentrations did not vary according to laying order. The relative constancy of levels of yolk androgens across the laying sequence is not surprising given that bluebird eggs hatch more synchronously and offspring exhibit less of a size gradient compared to other passerine species.

Hormone profiles found in the plasma of females at the time of follicular production were substantially different from the profiles of the eggs that were being yolked at that time. B was the most prominent hormone in female plasma, while A4, T, and E were each found in relatively low levels. These data demonstrate that yolk hormone content is not simply a reflection of circulating plasma content during the time of follicular development, and are consistent with a study on Japanese quail in which the authors concluded that >99% of yolk steroids come directly from the cells of the follicular wall (Hackl*, et al.* 2003).

Further evidence that yolk androgens are not simply a reflection of female circulating plasma content can be seen when examining the results of our intruder presentation experiment. Yolk androgen concentrations were significantly higher in eggs yolked during an intruder presentation as compared to control, suggesting that the

presence of an intruder female stimulates an increase in the deposition of androgens into eggs. Yet, plasma androgen levels in stimulated females were significantly lower than in control females.

Our study is the first to experimentally demonstrate changes in the concentrations of androgens in yolk and female plasma in response to an aggressive challenge. A similar experimental test in which a male intruder was presented to nesting house sparrow pairs did not show a change in yolk androgens or in female plasma androgens in response to the intrusion (Mazuk*, et al.* 2003). In the house sparrow study, however, the intruder was male, presenting little threat of territorial invasion for a female bird, and was presented for five-hour periods, which may greatly exceed the duration of a normal aggressive interaction between birds. In such an extended time period, nesting pairs may have dismissed the intruder as a threat, in which case females may not alter deposition patterns of yolk androgens. Additionally, female blood samples were taken after clutch completion, long after the critical time during which hormone levels would have changed in response to the intruder presentation, and during a time in which androgen levels are known to decrease with the onset of incubation (Wingfield*, et al.* 2001).

A large body of previous work has suggested a role for androgens in aggressive behaviours; aggressive behaviours in both males and females may be associated with a rise in circulating testosterone levels (Petrie 1983, Wingfield*, et al.* 1987, Silverin 1990, Staub & De Beer 1997, Eens & Pinxten 2000, Nelson 2000). Elekonich & Wingfield (2000) showed, however, that plasma testosterone levels are significantly lower in female song sparrows (*Melospize melodia)* experiencing aggressive interactions

compared to control females. Similarly, Cristol & Johnsen (1994) showed that testosterone levels decrease significantly before the annual period of aggressive and territorial behaviour ends in red-winged blackbirds (*Agelaius phoeniceus)* and proposed that this decrease in testosterone is adaptive, protecting against potential interruptions in the reproductive cycle that can result from high testosterone levels. Elevated androgen levels have, in fact, been shown to interrupt processes associated with ovulation and reproductive cyclicity in both birds and mammals (Harper 1969, Searcy 1988). Additionally, aggressive behaviours associated with an increase in testosterone may interrupt behaviours necessary for a successful breeding attempt (Hegner & Wingfield 1987, Oring*, et al.* 1989, Wingfield*, et al.* 2001)

We suggest that eastern bluebirds regulate hormone levels in an adaptive manner in response to environmental change by actively shuttling androgens into eggs. This could help females to maintain androgens circulating in the plasma at basal levels, preventing androgen-driven interruptions of the reproductive cycle during aggressive interactions. As such, eggs act as a "sink", absorbing androgens produced by the follicular cells in response to aggressive interactions before the sex steroids are secreted into circulation. Such a phenomenon has been proposed for the protective isolation of pollutants in eggs; fish, amphibian, and avian eggs may act as an excretion site for pollutants, protecting females from the potentially harmful, disruptive effects associated with pollutant exposure (Kleinow *et al.* 1999). Thus it is not hard to imagine that a similar excretion method may exist for potentially disruptive hormones.

Our hypothesis is further supported by the differing profiles of sex and adrenal steroid hormones in bluebird plasma and egg yolks: That is, circulating levels of sex

steroids in female plasma were relatively low, perhaps resulting from the shuttling of those steroids into the yolks of developing follicles, while B was found in relatively higher concentrations within female circulation. Given that sex steroids are produced by follicular cells surrounding the developing oocyte and B is produced at an entirely different location within the body (the adrenal glands), it is likely that the androgen content of yolk is representative of steroids that never made it into circulation, perhaps due to active shuttling across the oocyte plasma membrane.

An increasing body of work addresses the question of the adaptive significance of yolk androgens by examining the effects of high doses of androgens on developing offspring. Previous work has shown that high levels of yolk testosterone have significant effects on the size, immunocompetence, and embryonic survival of eastern bluebird nestlings (Navara*, et al.* 2005), suggesting that yolk androgens may be adaptive tools utilized by female eastern bluebirds as a means of altering offspring quality and reproductive success. The current study, however, is one of the few that questions the significance of how yolk androgens may alter the female's physiological state and is the first to identify the deposition of yolk androgens as a potentially protective mechanism against the disruptive effects of androgens during a sensitive period in the reproductive cycle. Presumably, the ability to deposit androgens in variable amounts has arisen through a combination of physiological constraints on both the female and the offspring. There is, however, a need for more experimental studies before we will fully understand the adaptive significance of and the constraints associated with the deposition of yolk androgens.

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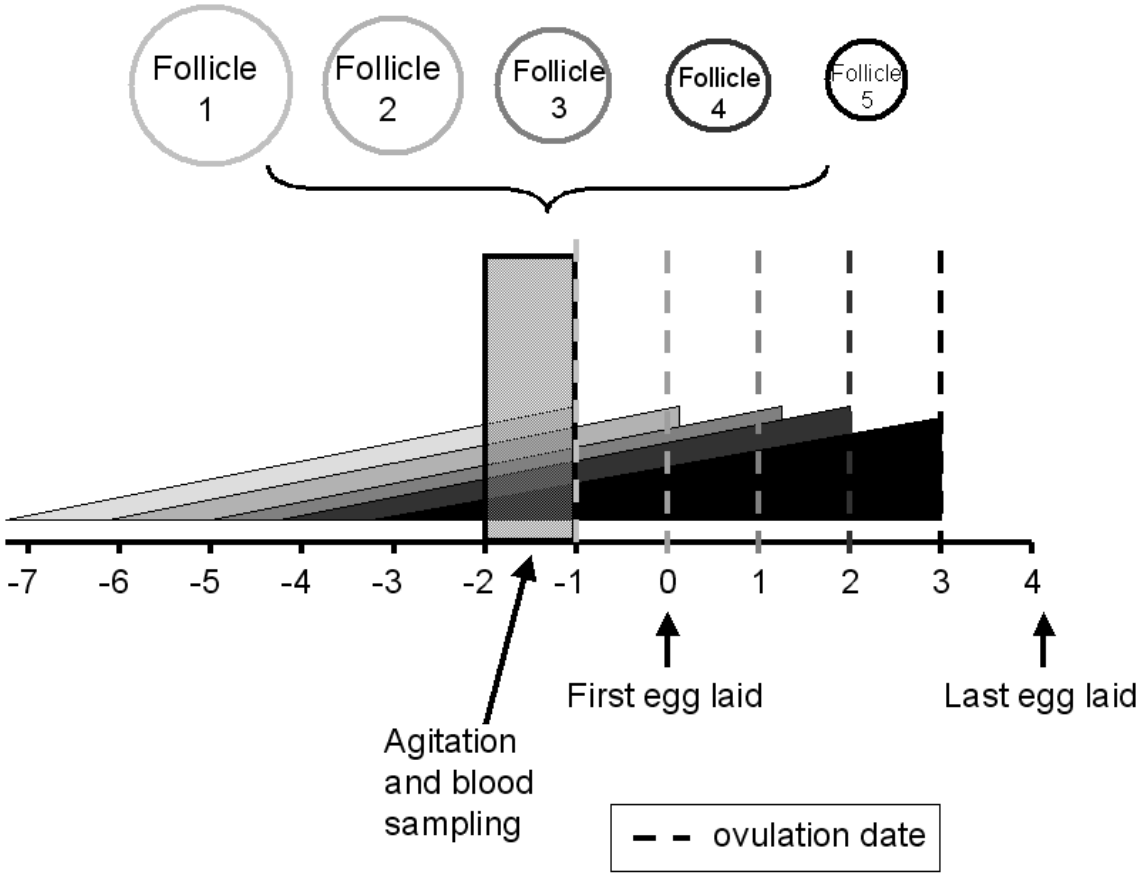

Fig. 8.Diagram representing timing of intruder presentations and blood sampling of female eastern bluebirds in relation to phase of rapid yolk deposition for all developing follicles in a clutch. Triangles represent the six-day period of rapid yolk deposition for each follicle. Dotted lines represent the ovulation dates. Follicles develop at a 24-hour time lapse from one another and are thus laid at a rate of one egg per day. Intruder presentation was conducted on days –2 and –3 (as indicated by the shaded rectangle), a time when all five follicles in the sequence were undergoing rapid yolk deposition.

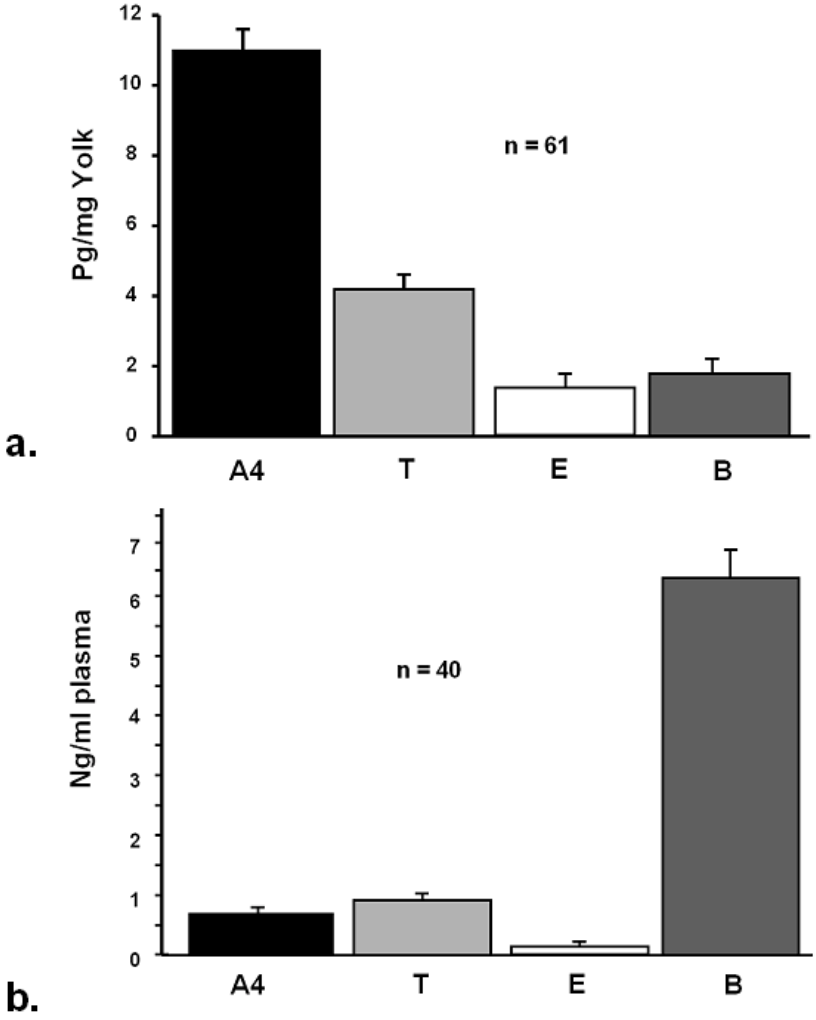

Fig. 9. Hormone profiles including androstenedione (A4), testosterone (T), estradiol (E), and corticosterone (B) found in eastern bluebird (a) yolks (average clutch values) and and (b) female circulating plasma (± SE) collected during the period of rapid yolk deposition and follicular development (b). Values of n represent the (a) number of clutches and the (b) number of females included in the analyses.

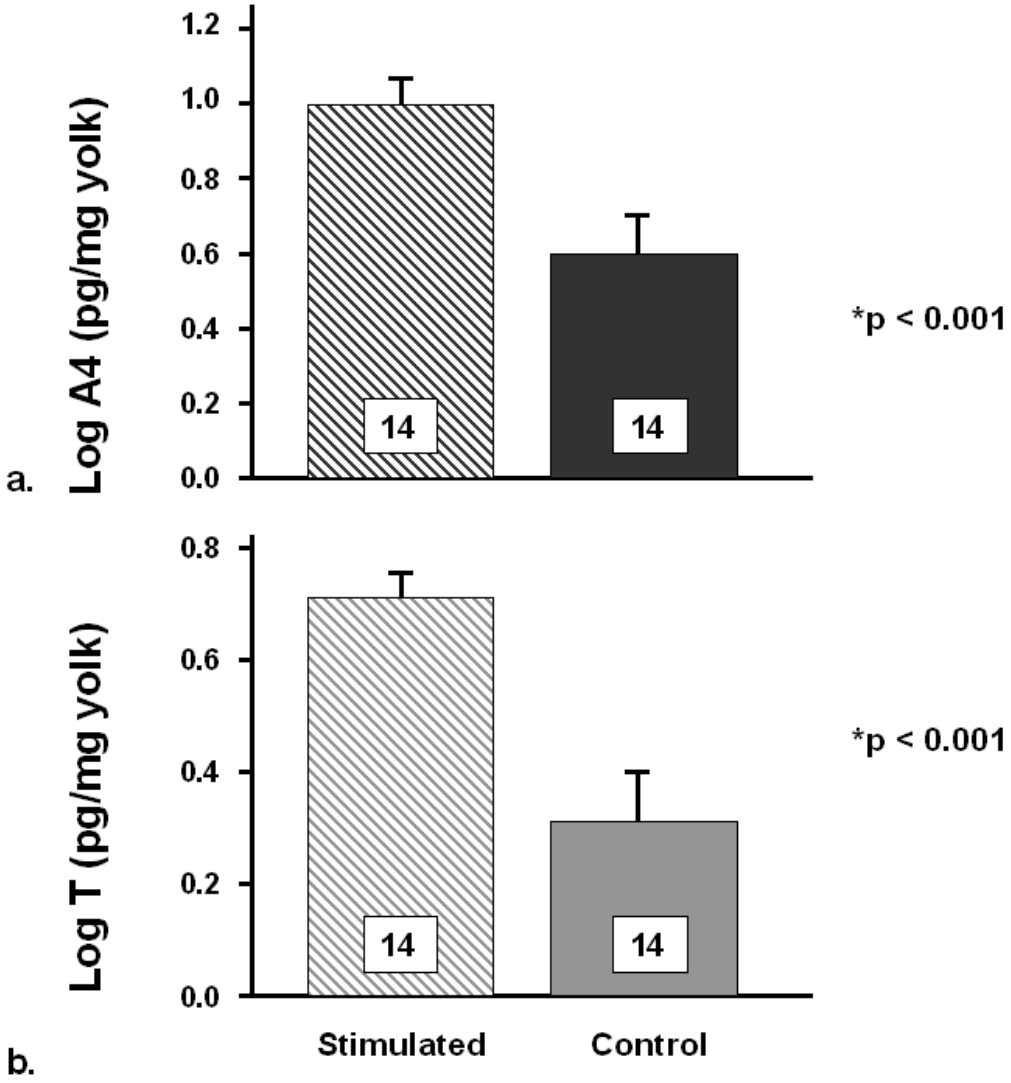

Fig.10. Log yolk (a) androstenedione (A4) and (b) testosterone (T)(± SE) values for eggs laid by stimulated and control females. Hatched bars represent eggs in the stimulated groups while solid bars represent eggs in the control groups. Clutch means were used for these analyses. Actual mean hormone values (in pg/mg) were: Stimulated A4 – 10.99, Control A4 – 5.44, Stimulated T – 5.17, Control T – 2.63.

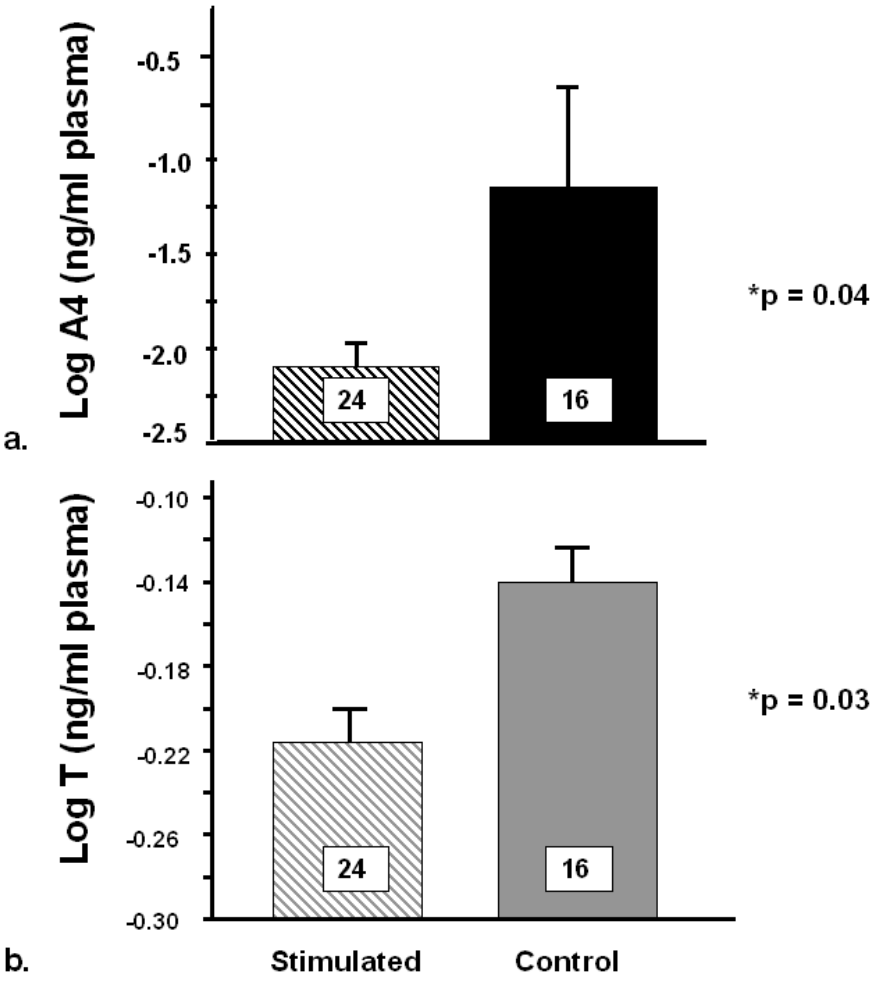

Fig.11. Log female plasma (a) androstenedione (A4) and (b)testosterone (T)(± SE) values for stimulated (hatched bars) and control (solid bars) females captured during experiment 2. Values located in the bars represent the number of females in the analyses. Actual mean hormone values (in ng/mg) were: Stimulated A4 – 0.045, Control A4 – 0.364, Stimulated T – 0.604, Control T – 1.433.

CHAPTER FOUR:

VARIABLE EFFECTS OF EXOGENOUS YOLK TESTOSTERONE ON GROWTH AND IMMUNITY IN BLUEBIRD NESTLINGS

Introduction

Maternal investment can have a profound impact on the growth, development and, ultimately the fitness of offspring. Many behavioral measures of maternal investment, such as incubation, provisioning, and the protection of offspring have been well studied, however, female birds have the ability to invest even more directly in their offspring by altering the hormone content of the yolk of their eggs (Schwabl 1993, Janzen, et al. 1998, Lovern and Wade 2001).

It has recently been shown that androgens, such as testosterone (T), dihydrotestosterone (DHT), and androstenedione (A_4) are available to embryos in the yolks of avian eggs (Schwabl 1993). In many species of birds, yolk androgen concentrations vary within and between clutches, and it has been hypothesized that they can have major fitness effects on the developing offspring (Winkler 1993, Schwabl 1996, Schwabl 1997, Lipar and Ketterson 2000, Sockman and Schwabl 2000, Reed and Vleck 2001). Attempts to understand the differential allocation of yolk hormones have focused on environmental and social contexts in relation to yolk hormonal content. For example, several studies have examined patterns of yolk androgen deposition in relation

to breeding condition. In the American coot (*Fulica Americana*), black-headed gull (*Larus ridibundus*), and house sparrow (*Passer domesticus*), females deposit more yolk androgens when breeding under more crowded conditions (Schwabl 1997, Reed and Vleck 2001, Groothuis and Schwabl 2002) and when the female experiences more aggressive interactions (Whittingham and Schwabl 2002). Additionally, female zebra finches (*Taeniopygia guttata)* deposit more androgens into yolk when mated to a more attractive male (Gil, et al. 1999). Females of some species also adjust the amount of hormone allocated to eggs in different positions in the laying sequence, typically allocating more androgens into later eggs (Schwabl 1993, Lipar, et al. 1995, French, et al. 2001, Royle, et al. 2001, Sockman, et al. 2001, Groothuis and Schwabl 2002). Because androgens have been known to increase aggression (Ketterson, et al. 1992) and begging behavior (Schwabl and Lipar 2002), chicks exposed to more yolk androgens may do better in competition with siblings. In this way, females may be able to compensate for the offspring size gradient caused by hatching asynchrony and potentially increase their reproductive success (Schwabl 1993, Schwabl, et al. 1997).

While these results suggest a strategy behind the allocation of yolk androgens, there remains little detailed information on the effects of yolk androgens on the development of songbirds. Steroid hormones deposited into the yolk by female birds and reptiles have been found to have a mélange of positive and negative effects on offspring fitness. Injections of androgens into the yolks of canary eggs (*Serinus canaria)* increased begging behavior and growth rates of chicks (Schwabl 1996), and yolk testosterone levels were positively correlated with growth of the hatching muscle in red-winged blackbirds (*Agelaius phoeniceus)* (Lipar and Ketterson 2000). On the

other hand, 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).

The current literature tends to characterize the allocation of androgens to the yolk as either physiologically beneficial or detrimental. There is growing evidence, however, that testosterone in the yolk has complex and often conflicting physiological effects. In addition to the documented effects of yolk androgens on young birds, testosterone increases song rate, aggression, nest defense, home range sizes, and mating success (Wingfield, et al. 2001, Nolan, et al 1992, Ketterson and Nolan 1999) but also decreases overwinter survival (Nolan, et al. 1992) and has immunosuppressive effects in adults (Olsen and Kovacs 1996, Casto, et al. 2001, Duckworth, et al. 2001) . Yolk androgens may have similar types of effects in developing nestlings as well. Thus, it seems more instructive to view yolk T as a mediator of developmental events, affecting partitioning of resources between different demands such as the energetic demands of hatching, growth, competition for food, and immunological defenses. The optimal dose of yolk androgens provided by the female will, therefore, be dependent upon the integration of many androgenic effects within the body. While offspring exposed to high levels of yolk androgens will experience both positive and negative physiological consequences, presumably the environmental and social conditions in which the female is producing offspring and in which the offspring develop in the first days after hatching will determine the optimal level of T to be allocated. Thus, rather than discussing the adaptive significance of a single optimum allocation strategy, we should think in terms of a range of adaptive responses to a range of environmental challenges.

To test the effects of T on a developing songbird, we injected the eggs of wild Eastern bluebirds (*Sialia sialis)* with one of three treatments: a high dose of T (high-dose), a low-dose of T (low-dose), or a control injection (control). We measured the hatching success of eggs and growth of resulting nestlings every three days until fledging. All nestlings were also subjected to challenges with phytohemagglutinin (PHA) to measure cell-mediated immune response and sheep red blood cells (SRBC) to measure humoral immune response. Finally we measured differential white blood cell (WBC) counts. In this way, we monitored a variety of developmental and physiological parameters that may be affected by yolk testosterone. Based on previously published observations of other songbird species, we hypothesized that T would have an overall stimulatory effect on nestling growth while having a suppressive effect on nestling immunocompetence. To our knowledge, this is the first study to consider both the growth and immunological consequences of yolk androgen allocation in the same individuals.

Materials and Methods

The Eastern bluebird is a socially monogamous, sexually dichromatic passerine. During the spring of 2003, we monitored 115 Eastern bluebird nest boxes located in Lee County, AL for signs of nest-building and egg laying. The treatment of nestlings was approved by the Institutional Animal Care and Use Committee (PRN no. 2003-0466).

Egg Injections.

Immediately after the completion of each clutch, all eggs within that clutch were assigned to one of three treatment groups and injected with either (1) 3ug T in 5ul peanut oil (high-dose); (2) 0.3ug T in 5ul peanut oil (low-dose); or (3) 5ul peanut oil (control). These injection amounts were based on yolk levels found in bluebirds eggs collected from the study site in the previous year, which varied from 2.9ng/yolk to 240ng/yolk (Navara, unpublished data). These injection amounts were slightly above physiological levels to compensate for degradation or incomplete incorporation of the hormone into the yolk. Clutches were assigned at random to one of the three treatment groups. Treatment was injected into the small end of the egg using a 5ul Hamilton syringe prior to the onset of embryonic development.

To test if the vehicle containing the treatments actually reached the yolk, we injected 5ul of peanut oil stained with Sudan B into two eggs and retrieved them after two days. Yolks were frozen and separated from the albumin. Yolks of all injected eggs contained a homogeneous amount of blue dye throughout (albumin contained none), suggesting that the treatments do, in fact, diffuse uniformly into the yolk within two days time. Further, previous poultry studies used this egg-injection method successfully for *in-ovo* androgen manipulations (Henry and Burke 1999).

Nestling Growth Measurements.

Nestlings hatching from treated eggs were measured on days 2, 5, 8 and 14 post-hatch. Morphological measurements taken on each of these days included mass using a 30g spring scale (accuracy = 0.2g), right tarsus length, right wing length, and bill length

using manual dial calipers (accuracy = 0.01mm). Using the residuals of body mass and tarsus length, we calculated the condition index as body mass:tarsus length ratio, which is often used as a measure of condition in avian nestlings (Richner, et al. 1993, Yom-Tov 2001). Additionally, we used the residuals of body mass and wing length to calculate maturity as body mass:wing length ratio, a measure that is often used in avian nestlings (Hario 2001). On day 14, we were also able to assess the sex of many of the nestlings due to the development of sexually dimorphic color patterns.

Nestling Hormone Levels.

On day 10, the first day post-hatch when a sufficient blood sample could be obtained, 60ul of blood was taken from the brachial vein of nestlings using a 26-gauge needle and plasma was separated through centrifugation. Steroid hormones were isolated from plasma using liquid column chromatography according to Schwabl (1993) and quantified using one radioimmunoassay as described in Mendonça, et al. (1996). Lower detection limit of this assay is 10pg. Average recoveries for testosterone after extraction and separation by column chromatography were 54% and intra-assay variation was 3.3%.

Cell Mediated Immunity.

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 post-hatch, a 1-cm patch on the left mid-patagium was cleared of feathers. Two measures of thickness were taken using a pressure-sensitive digital micrometer (accuracy =

0.05mm). The bare skin was swabbed with alcohol and 20ug of PHA in 50ul PBS was injected subcutaneously using a 27-gauge needle. As a control, 50ul of PBS was injected into the right patagium. 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). Two measurements of wing-web thickness were taken after 24 h to assess swelling. A PHA index was computed according to Fair and Myers (2002) as the thickness of the PHA-inoculated wing-web minus the thickness of the control-inoculated wing-web, standardized by the average patagium thickness before inoculation:

$$\text{PHA index} = \frac{\text{postPHA} - \text{postPBS}}{(\text{prePBS} + \text{prePHA})/2}$$

This formula requires that left and right wing webs do not differ in pre-injection thickness, which is in fact the case here ($df = 43$, $t = -0.271$, $p = 0.78$). The PHA index was indicative of the T-cell responsiveness and thus cell-mediated immunocompetence.

Humoral Immunity.

To assess the humoral immune response, all chicks were inoculated intraperitoneally on day 5 post-hatch with 0.2ml of a 10% SRBCs (Colorado Serum Company, Denver, CO) in phosphate buffered saline (PBS). Cells were washed twice in PBS and resuspended to the desired concentration. Antibodies produced in response to the SRBCs were quantified after 10 days (on day 15 post-hatch) by taking 50ul of blood from the brachial vein and performing a standard hemagglutination assay (Hay and Hudson 1989). In short, 20ul of plasma was serially diluted in 20ul of PBS

(1:2…1:1024) in 96-well v-bottom plates. Wells 11 and 12 served as negative and positive controls and did not contain any plasma. Instead, these wells were loaded with 20ul of PBS or 20ul of anti-sheep hemolysin (Colorado Serum Company, Denver, CO), respectively. Next, 20ul of a 2% SRBC suspension in PBS was added to each well. The plates were incubated at room temperature for 24 h. Finally, wells containing plasma samples were compared to positive and negative controls. Antibody titers were expressed as the $\log_2$ of the highest dilution of plasma containing hemagglutination (Lochmiller, et al. 1993). Because the skin covering the abdomen is translucent in bluebird nestlings, it was easy to visually confirm the correct injection location.

Differential WBC Counts.

A blood smear was made on day 15 post-hatch from the blood sample taken for the hormone analysis. These smears were stained with Wright-Giemsa stain and one hundred white blood cells were classified according to Dein (1986) after which leukocyte percentages were calculated, and grouped differentially as lymphocytes, heterophils, and eosinophils. The heterophil:lymphocyte ratio, often used as an overall measure of immunity, was also calculated (Fair and Myers 2002).

Statistical Analysis.

Differences in hatching success between groups was analyzed using a chi square test that included the number of eggs that hatched (surviving nestlings) and the number of eggs that did not hatch (indicative of embryonic mortality) in each treatment group. Measures of nestling growth, including wing length, tarsus length, bill length, and body

mass, were analyzed using principal components analysis. Resulting principal components were compared using an ANOVA. Post hoc comparisons were used to examine differences between individual treatment groups. The effects of treatment on swelling response to PHA were analyzed using an ANOVA. Individual leukocyte percentages were arcsin transformed and analyzed using an ANOVA. To control for the potential effects of brood size in this experiment, we initially used brood size as a covariate in all analyses, but because the size of the brood did not contribute significantly to the variation in any measure of size or immunocompetence, it was eliminated as a covariate in all analyses ($p > 0.16$ in all cases). Additionally, because our experimental design did not control for genetic contributions to the growth and immunocompetence of these offspring, brood averages were taken for each measurement, rather than treating nestlings as individuals in these analyses.

Results

Hatching Success.

Injection of treatments had a significant effect on hatch rate. Eggs that were injected with the control treatment displayed a significantly lower hatching success than a separate set of eggs that were not injected ($\chi^2 = 13.23$, $p < 0.001$), illustrating a detrimental effect of the injection itself on the developing embryo.

Chi square tests between eggs from the three injection treatments illustrated that treatment of eggs with both a low dose of T and high dose of T significantly decreased hatching success over controls (low dose: $\chi^2 = 4.06$, $p < 0.05$, high dose: $\chi^2 = 5.38$, $p < 0.025$; Fig. 12). All eggs that did not hatch were checked for overt signs of bacterial

infection (a dark mass surrounding the injection site on the inside of the egg) resulting from the injection itself, and only those without bacterial infection were retained in the analysis.

Nestling Growth.

For the three measures of skeletal growth, including right tarsus length, right wing length, and bill length, PC1 explained 88.5% of the variance in size on day 2 post-hatch, 84.4% on day 5, 61.3% on day 8, and 52.4% on day 14. Therefore, PC1 was used as a measure of skeletal size at all stages.

On day 2 post-hatch, there was no overall difference in PC1 among treatment groups ($F = 2.00$, $df = 2, 12$, $p = 0.18$), however unpaired t-tests indicated that the PC1 of low dose nestlings approached a significant increase over controls ($p = 0.07$; Fig 13). Nestling weight did not differ among the treatments at this stage ($F = 0.76$, $df = 2,12$, $p = 0.48$). Size differences among treatment groups disappeared by day 5 and there were no differences in any measure of size among the treatment groups for days 5 and 8 post-hatch (Day 5: PC1,$F = 0.425$, $df = 2,18$, $p = 0.66$, mass, $F = 0.442$, $df = 2,18$, $p = 0.65$, Day8: PC1, $F = 0.103$, $df = 2,19$, $p = 0.90$, mass, $F = 0.184$, $df = 2,19$, $p = 0.83$). At day 14, however, weight differed significantly among the treatment groups ($F = 5.108$, $df = 2,18$, $p = 0.018$). Post hoc comparisons indicated that high-dose nestlings were significantly heavier than nestlings in the control ($p = 0.013$) and low-dose ($p = 0.008$) treatment groups (Fig 14). Skeletal size, as measured by PC1, did not differ among nestlings in the different treatments at this stage of development ($F = 1.127$, $df = 2,19$, $p = 0.34$).

Our measure of condition (residuals of body mass/tarsus ratio) on day 14, just prior to fledging, varied significantly among treatment groups ($F = 5.88$, $df = 2,18$, $p = 0.01$). Post hoc comparisons indicated that the condition index of chicks in the high-dose treatment group was significantly larger than nestlings in the control ($p = 0.009$) and low-dose ($p = 0.005$) treatment groups (Fig. 15). Additionally, our measure of maturity (residuals of body mass/tarsus ratio) varied significantly among treatment groups on day 14 ($F = 6.38$, $df = 2,18$, $p = 0.008$). Post hoc comparisons indicated that chicks from the high-dose treatment group were significantly more mature than those in the control ($p = 0.004$) and low-dose ($p = 0.008$) treatment groups (Fig. 16).

Nestling Hormone Levels.

On day 10 post-hatch, testosterone (T) levels did not vary among the treatment groups ($DP = 2,15$, $F = 0.513$, $p = 0.61$).

Nestling Cell-Mediated Immunity.

There was a significant difference in swelling response among the treatment groups ($F = 3.15$, $df = 2,30$, $p = 0.03$). Nestlings hatching from eggs injected with a high-dose of T had a significantly smaller swelling response to PHA than controls ($p = 0.01$). Low dose nestlings had a smaller swelling response than controls, but this difference was not significant ($p = 0.08$)(Fig 17).

Nestling Humoral Immunity.

The results of the hemagglutination titration assays on plasma from days 10 and 15 post-hatch showed a lack of a humoral immune response, and further tests could not be done because nestlings had fledged. On day 10, only 5 chicks out of 64 showed a positive antibody response, all of which had a titer of 1 out of a possible 10. Although all of the antibody-positive chicks hatched from control eggs, this number of responding nestlings was too small for any conclusions to be drawn. Further, on day 15, only 2 chicks showed a positive antibody response, both of which had a titer of 1, and these were not the same chicks that showed a positive response on day 10.

Differential WBC counts.

Percentages of all leukocyte types were similar among treatment groups (heterophils: $F = 1.35$, $df = 2,67$, $p = 0.27$, lymphocytes: $F = 1.22$, $df = 2,67$, $p = 0.30$, basophils: $F = 0.51$, $df = 2,67$, $p = 0.60$, eosinophils: $F = 0.985$, $df = 2,67$, $p = 0.38$, monocytes: $F = 0.03$, $df = 2,67$, $p = 0.97$). Additionally, the heterophil:lymphocyte ratio was similar among treatment groups as well ($F = 0.179$, $df = 2,67$, $p = 0.84$).

Discussion

We observed that yolk androgens have a variety of effects on the growth and development of young bluebirds, some potentially positive, others potentially negative. Hatching success significantly decreased with both a low and a high dose of yolk T. This observation is not surprising given that T has been shown to cause developmental arrest of embryonic crustaceans (Mu and LeBlanc 2002) and is associated with higher

levels of apoptosis in human vascular endothelial cells (Ling, et al. 2002). Additionally, T has been shown to directly induce oxidative stress in many tissues (von Schantz, et al. 1999) which could retard embryonic growth. The low survival of high-dose nestlings through the embryonic period could result from one or more of these effects.

Low doses of yolk T had a stimulatory effect on skeletal growth during the embryonic period, resulting in larger offspring at hatch. While this difference at hatch was not significant ($p = 0.07$), when we used nestlings as individuals in our analysis (instead of using brood averages of size measures), nestlings receiving a low dose were significantly larger than control nestlings ($p = 0.02$).

The fact that offspring in the low-dose treatment group tended to have larger measures of skeletal size (PC1) on day 2 than offspring in the other two treatment groups is interesting because it suggests that yolk T in moderate amounts has a stimulatory effect on embryonic growth of the resulting offspring. These results are consistent with mechanistic studies of the effects of androgens on the growth of several body tissues. For example, bone and cartilage contain androgen receptors, making them androgen 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). Thus, it is not surprising that in-ovo testosterone injections had a stimulatory effect on skeletal growth during the embryonic period. All differences in skeletal size, however, disappeared over the nestling period. Additionally, there was no variation in plasma T levels among treatment groups by the middle of the nestling period.

At two weeks post-hatch, high-dose chicks were significantly heavier, more mature, and in better condition than chicks in the other two treatment groups. In contrast, high doses of T had a clearly inhibitory effect on cell-mediated immunity.

Our observation that high-dose nestlings were significantly heaver, more mature, and in better condition two weeks after hatching is consistent with results found by Schwabl (1996), in which high yolk androgen levels resulted in canary chicks that begged more and grew faster. We did not quantify begging behavior in these nestlings, but it is possible that offspring in the high-dose group begged more, received more food, and thus gained more weight than offspring in the other two groups.

Because we did not see differences in plasma T levels among treatment groups on day 10 post-hatch, it is likely that the weight differences that we saw were due to organizational effects on the embryo caused by high levels of yolk T. In other words, early exposure to T might permanently change the way an individual physiologically and behaviorally responds, ultimately affecting processes associated with weight gain. For example, in rats, treatment with sex steroids during the neonatal period has been found to be responsible for permanently altering patterns of glucocorticoid secretion (McCormick, et al. 1998) as well as 'imprinting' sexually dimorphic patterns of growth hormone release (Jansson, et al. 1985, Painson, et al. 2000). Finally, early exposure to testosterone has been shown to permanently alter sex steroid receptor concentrations in the rat (Kuhnemann, et al. 1995) as well as testosterone-metabolizing enzymes in the zebra finch (Vockel, et al. 1990), altering the way an individual responds to hormonal stimuli during adulthood. While most of these effects have been demonstrated in mammals and have yet to be examined in birds, the major axes that control the release

of growth hormone, glucocorticoids, and sex steroids are highly conserved and likely to be similar in birds and mammals. As a result, yolk T can have permanent effects on processes associated with weight gain, such as lipid mobilization, muscle breakdown, and behavioral responses through adulthood, which may explain the body mass differences we saw among treatment groups.

Our observation that yolk T had a suppressive effect on the T-cell immune responses in these nestlings are consistent with the findings of many studies examining the effects of androgens on immunity. Androgen receptors have been found in the thymus (the site of T-cell maturation and differentiation) in densities comparable to those found in the reproductive tract (Viselli, et al. 1995) and the stimulation of these androgen receptors causes a shift in the thymic cell population towards apoptosis while castration shifts the population towards maturation as helper or cytotoxic T-cells (Olsen, et al. 1991, Olsen and Kovacs 1996). It has been suggested that, overall, androgens generally suppress cytotoxic/suppressor T-cell function, but the effects seen in the peripheral immune system are most likely due to alterations within the thymocyte population (Olsen & Kovacs 1996). While numbers of circulating leukocytes did not differ among treatment groups in this study, chicks exposed to high levels of T during the embryonic period may have developed an immune system with a smaller number of mature, functional T-cells. Additionally, T has been shown to directly induce oxidative stress in many tissues (von Schantz, et al. 1999), which damages lymphocytes involved in the immune response and results in immunosuppression (Raberg, et al. 1998). This phenomenon could explain the decreased swelling responses of high T nestlings.

Because previous experiments show that T has an immunosuppressive effect on the development of humoral immunity (Glick 1961, Norton and Wira 1977, Deyhim, et al. 1992), we also expected chicks in the high-dose group to exhibit lower antibody titers than chicks in the other two groups. However, the number of nestlings that responded to our SRBC challenge was minimal. We believe that because we were only able to wait 10 days after the initial antigen injection (due to the length of the nestling period) the time period may not have been long enough for us to witness the production of antibodies to the SRBCs. In other avian studies, researchers have waited two weeks after SRBC injection before testing for antibodies to SRBCs (Smits and Williams 1999). Therefore, our results concerning the humoral immune response are inconclusive.

We attempted to encompass several aspects of the immune system in this study in an effort to assess overall immunocompetence. While our PHA challenge detected treatment differences in T lymphocyte responsiveness, it will be important in future studies to assess humoral immune function. Additionally, a good general assessment of the overall immune response would be a challenge with a novel antigen normally encountered in the environment, and we encourage the inclusion of such a test in future studies.

Our results suggest that yolk androgens have a variety of flexible context-dependent effects on the development and survival of avian nestlings. When breeding density is great and competition is high, offspring may benefit from high levels of yolk T because they will be able to beg more and/or gain weight more efficiently during the nestling period. The immunosuppressive effects of high yolk T, however, leave the

resulting offspring vulnerable to infection by pathogens, so the likelihood of parasitism will trade-off against excelled growth and development. In this way, environmental and social conditions mediate the fitness effects of the allocation of yolk androgens.

Testosterone acts as a signal used to control and coordinate the allocation of resources to different processes within the body. The alteration of reproductive success through the allocation of yolk T is an extremely complicated phenomenon that involves the integration of environmental, social, and physiological effects. The optimal level of yolk T will most likely vary between male and female offspring as well as among offspring at different positions within the clutch order and offspring reared in different environments. Therefore, we must think of the adaptive significance of differential yolk T allocation in terms of a range of adaptive responses to a range of environmental challenges.

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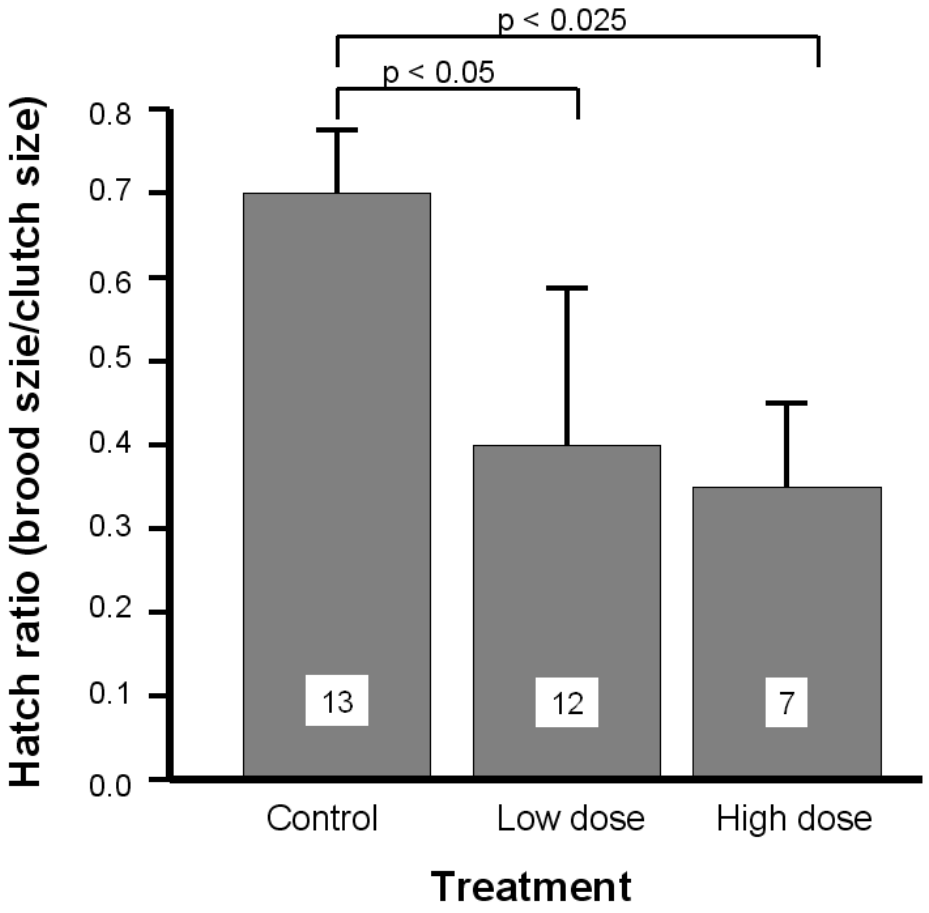

Fig. 12. The effects of *in ovo* testosterone and control injection treatments on mean (± SE) hatch rate of Eastern bluebird nestlings. Differences in hatching success between groups was analyzed using a chi square test that included the number of eggs that hatched (surviving nestlings) and the number of eggs that did not hatch (indicative of embryonic mortality) in each treatment group. Injection treatments included a high-dose injection (3000ng T in 5ul peanut oil), a low-dose injection (300ng T in 5ul peanut oil), and a control injection (5ul peanut oil). The number located in each bar indicates the number of clutches included in the analysis.

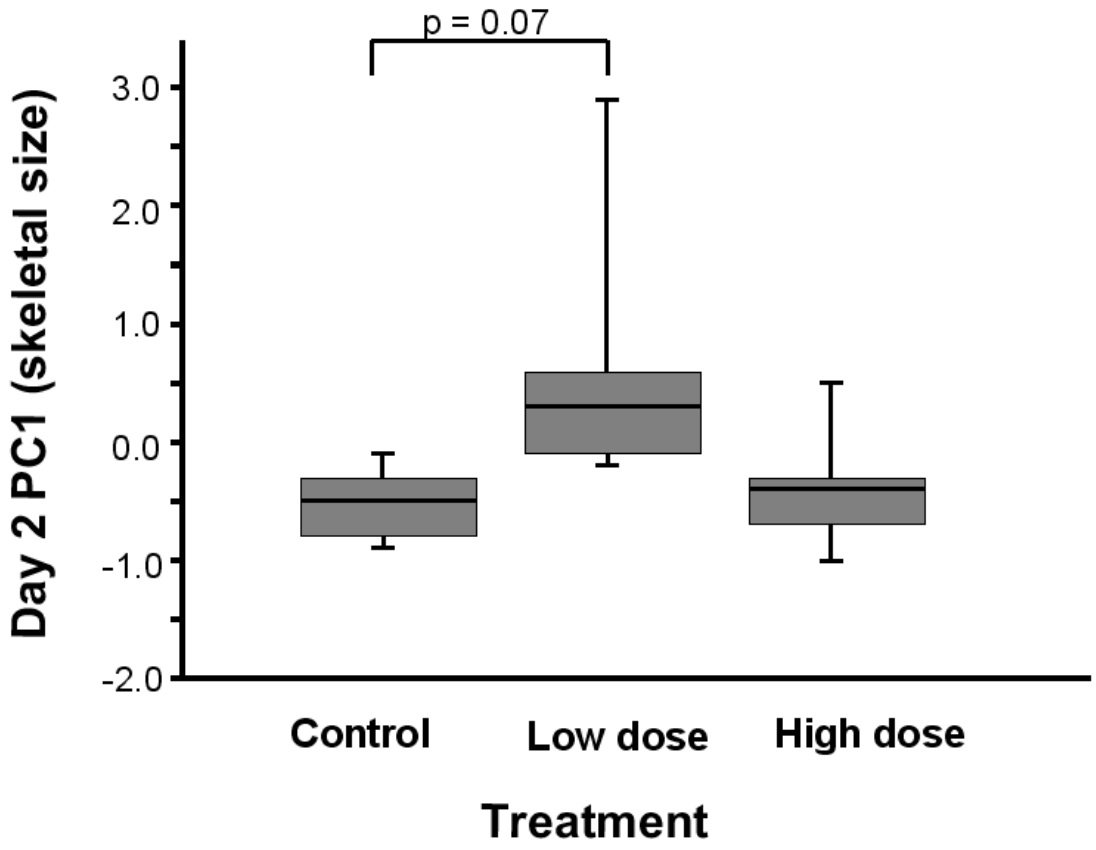

Fig. 13. The effects of *in ovo* testosterone and control injection treatments on mean (± SE) PC1, a principal component of skeletal growth (including right tarsus length, right wing length, and bill length) on day 2 post-hatch. Injection treatments are the same as those described in Figure 12.

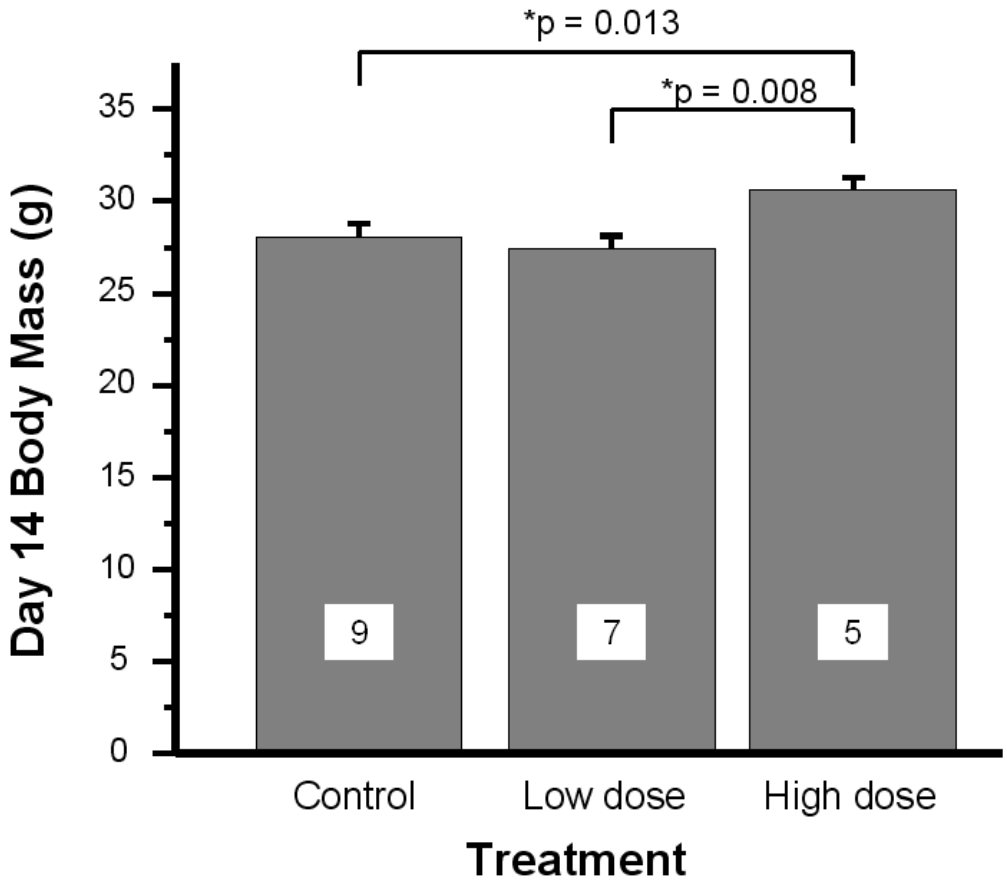

Fig. 14. The effects of *in ovo* testosterone and control injection treatments on mean (± SE) body mass on day 14 post-hatch. Injection treatments are the same as those described in Figure 12.

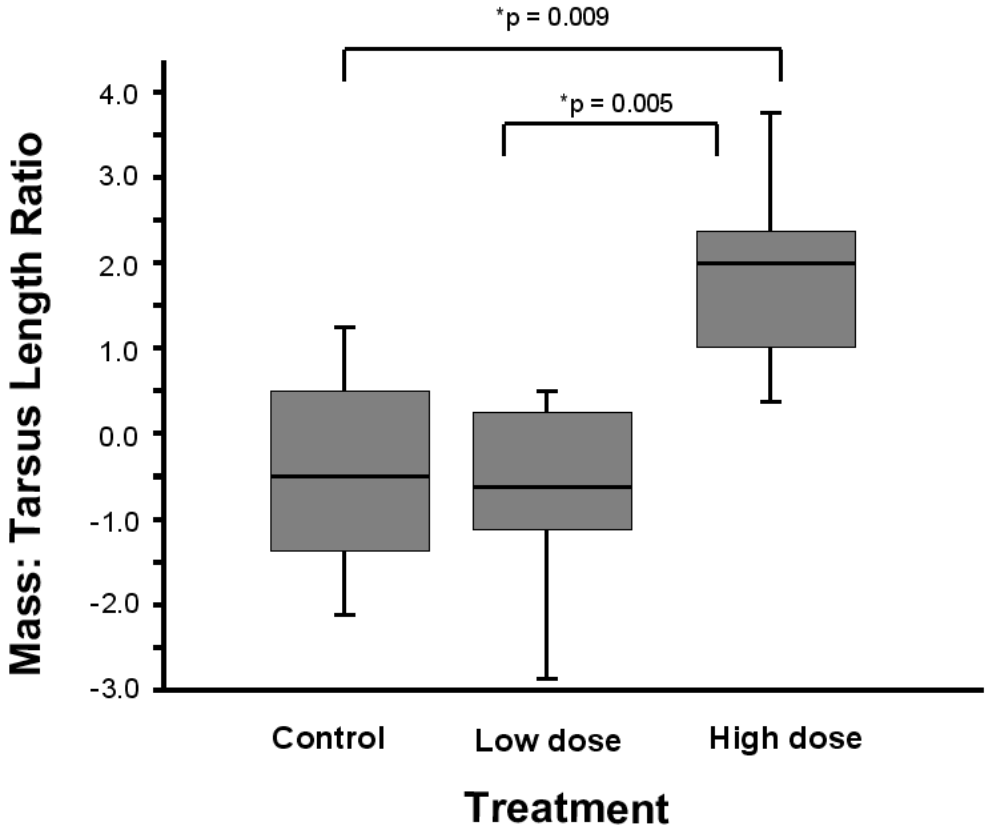

Fig. 15. The effects of *in ovo* and control injection treatments on mean (± SE) condition (measured as the ratio of the residuals of body mass : tarsus length). Injection treatments are the same as those described in Figure 12.

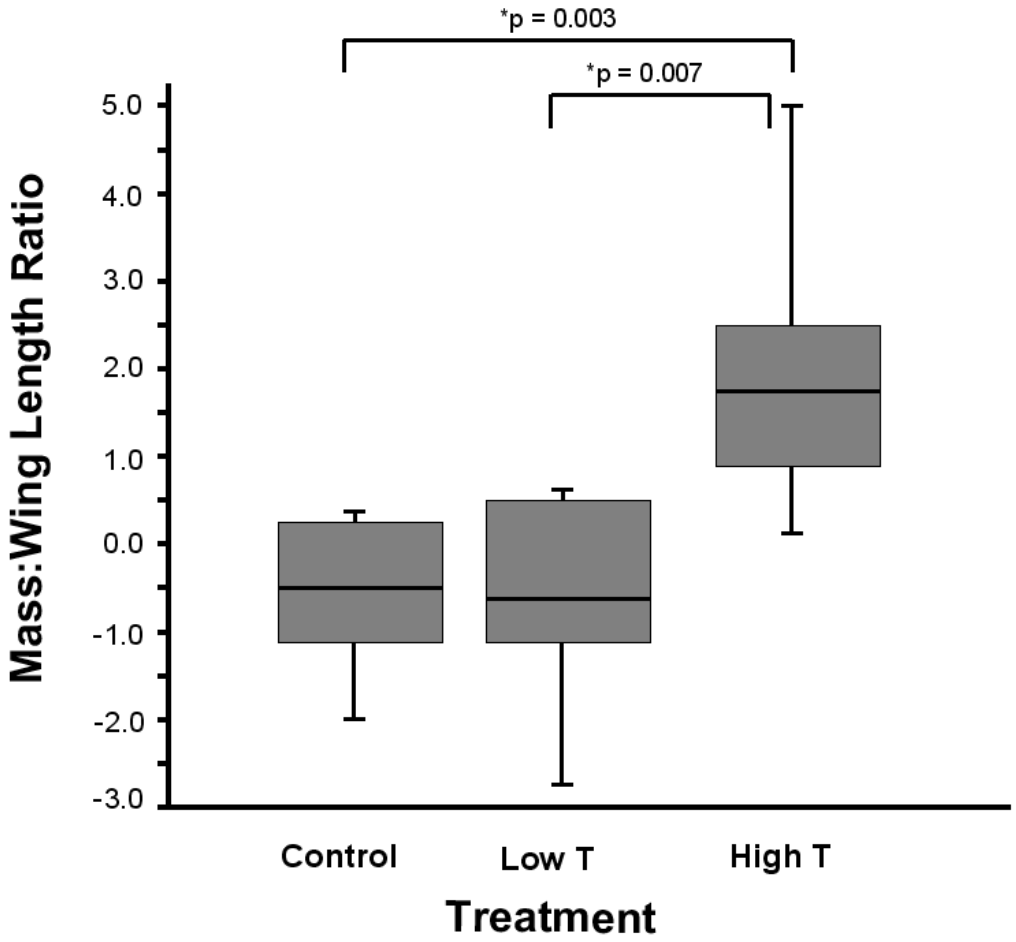

Fig. 16. The effects of *in ovo* testosterone and control treatments on mean (± SE) maturity (measured as the ratio of the residuals of body mass : wing length). Injection treatments are the same as those described in Figure 12.

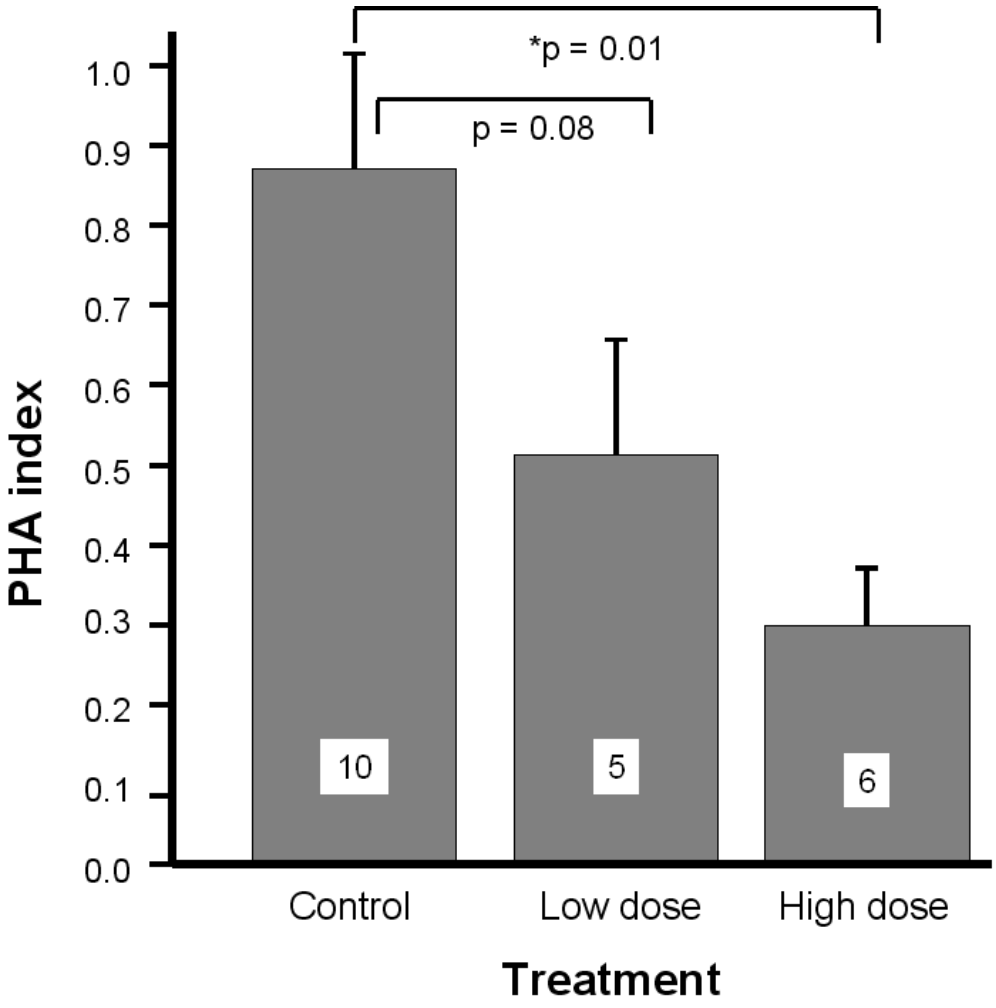

Fig. 17. The effects of *in ovo* testosterone and control injection treatments on mean (± SE) PHA index (calculated as described in Fair (2002)). Injection treatments are the same as those described in Figure 12.

CONCLUSIONS

The deposition of androgens into egg yolks is generally considered an adaptive mechanism utilized by female birds to boost the quality and survival of certain offspring over others. My work, however, suggests that androgens are not simply resources allocated by female birds according to a universal strategy. Instead, there are trade-offs associated with the exposure of embryos to yolk androgens, and while the patterns of yolk androgens observed within and among avian clutches may very well represent adaptive strategies, the overall beneficial or detrimental nature of such a strategy is determined by several environmental and social variables.

House finch females deposited significantly more androgens into eggs sired by less attractive males, and into eggs laid later in the clutch, but only when sired by less attractive males. Taken together with our finding that *in ovo* injections of yolk androgens stimulated embryonic bone growth as well as immune function in offspring around the time of fledging, these data suggest that female house finches may utilize yolk androgens in a compensatory strategy, to salvage what would otherwise have been a suboptimal breeding opportunity with a less attractive, lower quality male. Since male house finches that are less attractive also provision less (Hill 1991), and offspring hatching later in the clutch are significantly smaller as a result of hatching asynchrony, we might expect that a deposition strategy that increases the growth rates and immune function of offspring hatching later in the clutch and sired by unattractive males might be selected for, and thus could represent an adaptive strategy.

Eastern bluebird females, on the other hand, deposited yolk androgens in patterns that were entirely different than we observed in house finch clutches. Androgen content of bluebird yolk did not differ significantly across the laying order, as would be expected based on the relatively synchronous hatching patterns displayed by bluebird offspring. Additionally, despite the fact that male color has been also implicated as a sexually selected trait in this species, female bluebirds did not deposit yolk androgens differentially based on male color (K. Navara, unpublished data). Instead, our experimental intruder presentation study demonstrated that bluebird females increase the androgen content of eggs in response to an aggressive intrusion at the nest box. Taken together with our findings that *in ovo* injections of testosterone increased weight gain over the nestling period, these data support the idea that female bluebirds may deposit androgens into yolk as a mechanism of increasing size and thus competitive ability of offspring hatching in a competitive environment, a potentially adaptive strategy.

Finally, we found that the steroid hormone profiles found in bluebird eggs did not directly reflect the steroid hormone profile of female plasma during the period of follicular development. These data suggest that yolk content is not simply a reflection of circulating plasma content, and, along with the finding by Hackl et al. (2003) that >99% of steroid hormones in the yolk originate from the surrounding follicular cells, suggest that the deposition of androgens into yolk represents a shuttling of hormones from the follicles of the cells directly into invaginating vesicles in the oocyte membrane and into the oocyte where the yolk is concentrically deposited in layers (Fig. 18) (Sturkie 2000). This idea is further supported by our finding that circulating androgens

in female plasma were significantly lower in females exposed to intruder stimulation as compared to controls. Androgens produced in the follicular cells may be actively shuttled directly into the yolks of developing follicles as an alternative to being released into circulation where they may be harmful and disruptive during a sensitive time in the reproductive cycle. The mechanism by which social and environmental variables affect the amount of androgen deposited into yolk, however, remains to be elucidated and we still have very limited information about how the deposition of androgens into the yolk affects the female physiologically. Thus, more experiments examining the mechanisms involved in the deposition of yolk androgens as well as the effects of such potential strategies on female physiology are necessary.

Despite the fact that our observations generally support the idea that exposure to androgens during the embryonic period may have beneficial effects on offspring, a cautionary note is warranted: We observed immunosuppressive effects on bluebird offspring in response to *in ovo* injections of testosterone compared to control injections, which is one of the many potential costs associated with the deposition of androgens into eggs. A general trend of physiological studies in evolutionary and ecological literature is that a single result, for example the stimulation of growth by yolk androgens, is interpreted as evidence towards a universal aphorism, for example that yolk androgens are resources, and thus the deposition of androgens into eggs is adaptive. It is important to emphasize that androgens are not resources, but are instead mediators of physiological processes, with far-reaching effects in almost every body system. Exposure to androgens during the embryonic period has the potential to permanently organize and imprint the major brain axes as well as the immune and

endocrine systems (Fox 1995, Goy 1980, Jansson et al. 1985, McCormick et al. 1998). Thus, the effects measured as growth and immunocompetence in our bluebird and house finch nestlings may actually be associated with permanent organizational effects that could have even more potent impacts later in life. Thus, it is imperative that we conduct more in-depth studies examining the multitude of effects that androgens may have on embryonic physiology, nestling growth and immune function, and health and survival during adulthood. Only then can we draw more concrete conclusions about how the deposition of yolk androgens affects the quality of offspring and thus the reproductive success of females.

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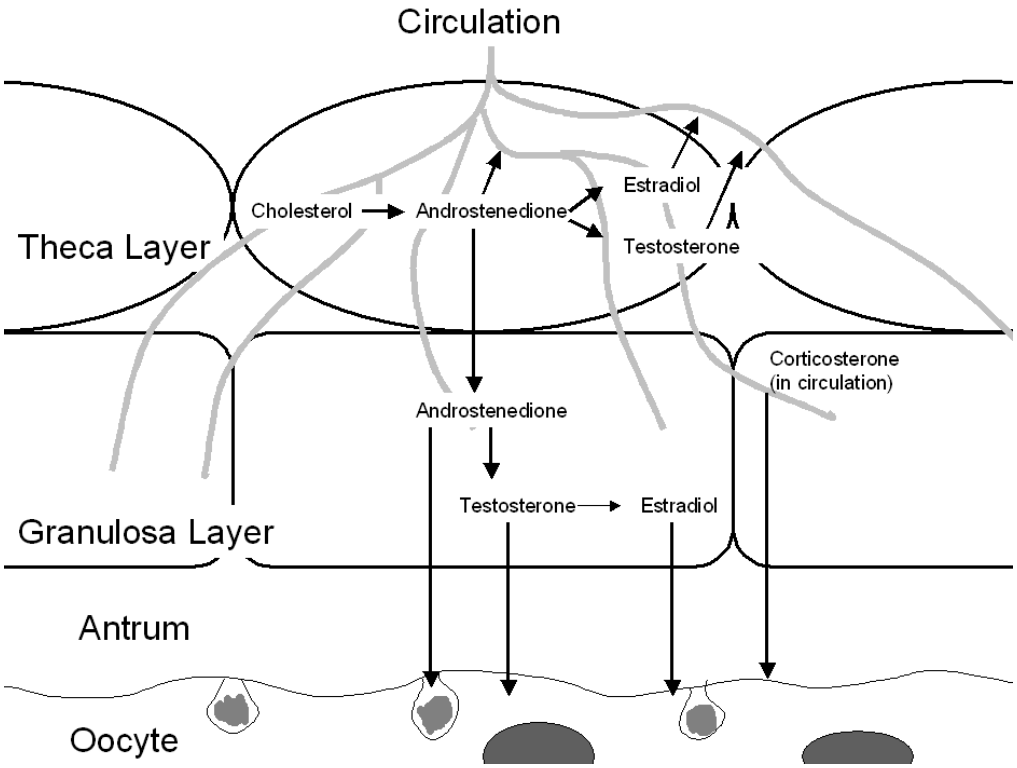

Fig. 18. Diagram of a hypothetical mechanism for the deposition of androgens into egg yolks. Cholesterol is converted to androstenedione (A4) in the theca layer of the developing follicle. Androstenedione can be shuttled directly into invaginating vesicles within the oocyte membrane, converted to estradiol (E) or testosterone (T) in the theca layer, or shuttled to the granulosa layer where it is metabolized into E or T. Hormone metabolized in the granulosa layer is likely shuttled into the yolk due to the proximity of the granulosa cells to the oocyte membrane. Alternatively, androgens located in the theca layer may pass into a network of capillaries that penetrate all developing follicles, and pass into circulation.