Effect of Prenatal and Natal Administration of Testosterone on Production of Structurally Based Plumage Coloration

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Accepted 3/2/2013; Electronically Published 4/12/2013

ABSTRACT

Testosterone has been implicated as a developmental mechanism involved in the organization and expression of sexually dimorphic traits, such as plumage coloration, in birds. Although research findings relating testosterone levels to plumage expression is equivocal, few studies have investigated how testosterone may influence the expression of structurally based plumage coloration. Here, we use experimental and correlational evidence to test the hypothesis that testosterone influences the development and maintenance of structurally based plumage coloration in a wild-breeding population of eastern bluebirds (*Sialia sialis*). First, we experimentally manipulated yolk testosterone and measured the effect on the development of plumage coloration of nestlings. Second, we implanted juvenile bluebirds with testosterone and measured the effect on nestling growth, body condition, and plumage coloration of nestlings. Third, we measured covariation between circulating testosterone and plumage coloration of breeding males. Yolk testosterone injections had no significant effect on nestling plumage coloration. Testosterone implantation, however, caused a reduction in plumage brightness, elevated corticosterone, and slower growth in nestlings. Finally, in breeding adult males we found no significant relationship between structural coloration and testosterone; however, males with higher testosterone levels exhibited duller chestnut (melanin-based) plumage. Our observations lead us to reject the hypothesis that testosterone increases structural plumage coloration in male eastern bluebirds.

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Physiological and Biochemical Zoology 86(3):323–332. 2013. 1522-2152/2013/8603-3005$15.00. DOI: 10.1086/670383

Introduction

Bright and showy plumage coloration has been proposed to serve as a condition-dependent indicator of individual condition (reviewed in Hill and McGraw 2006*a*, 2006*b*; but see Prum 2010). Ornamental coloration of feathers can result either from pigments deposited in feathers or from the microstructures of feathers interacting with ambient light. A large and growing number of studies have shown that pigment-based plumages of birds commonly function as honest indicators of quality (Hill 2006); however, the mechanisms that link ornamentation to condition remain poorly understood for many types of ornaments (Hill 2011). Particularly contentious has been whether and how structurally based blue and ultraviolet (UV) feather coloration link to individual condition (Prum 2006). Empirical studies show that hue and brightness of structural coloration can serve as signals of nutritional state (McGraw et al. 2004; Siefferman and Hill 2005*a*, 2007) and parasite load (Hill et al. 2005) during feather growth and that structural coloration is associated with resource-holding potential (Siefferman and Hill 2005*c*) and parental care (Siefferman and Hill 2003). The mechanisms by which parasites or nutrition impact production of structural coloration and hence the mechanisms that underlie associations between structural coloration and performance remain speculative (Fitzpatrick 1998; Keyser and Hill 1999; Prum 2006).

The influence of testosterone on male sexual signaling is one of the main assumptions of the immunocompetence handicap hypothesis of sexual selection theory (Folstad and Karter 1992). Testosterone is expected to have a positive impact on ornamentation but to suppress the immune system (Roberts et al. 2004) while increasing corticosterone levels (Ketterson et al. 1991) and metabolic rate (Buchanan et al. 2001); thus, sexual signaling may be kept honest if only high-quality males can tolerate these costs. Another proposed mechanism is that when plumage traits are associated with testosterone, honesty of signaling may occur through social enforcement (the badge of status hypothesis; Maynard Smith and Harper 1988). Only the most aggressive males should be able to bear the costs of being constantly tested by conspecifics with agonistic interactions. To fully understand what is being signaled by ornamental traits, it is important to determine whether testosterone affects their production.

What little is known about the effects of testosterone on plumage coloration indicates that the interactions between testosterone and color production is complex and varies dramatically among avian taxa and across different types of color displays (Owens and Short 1995). For example, in some avian orders—but not most songbirds in the order Passeriformes—ornamental plumage develops normally in castrated males (Witschi 1961; Kimball 2006), indicating that testosterone has no effect on ornamental coloration in these taxa. Most songbirds molt their plumage in the autumn, when levels of sex steroids are low (Wingfield et al. 1990), and treatment with testosterone during fall molt can dull sexually selected plumage coloration (Stoehr and Hill 2001). Indeed, a comparative phylogenetic analysis indicates that, with the exception of shorebirds in the order Charadriiformes, bright plumage is unlikely to be testosterone dependent in birds (Kimball and Ligon 1999).

Several recent studies, however, have reported positive correlations between individual variation in ornamental plumage and testosterone in male songbirds. In male house sparrows (*Passer domesticus*), in which the melanin-based breast badge is an important sexually selected trait, three studies have found that badge size correlated positively with plasma testosterone levels during prebasic molt (Evans et al. 2000; Buchanan et al. 2001; Gonzalez et al. 2001; but see Laucht et al. 2010), when the new badge is formed. During the breeding season after ornament production is complete, circulating plasma testosterone is positively associated with the extent of tail white (structural coloration) in dark-eyed juncos (*Junco hyemalis*; McGlothlin et al. 2007) and carotenoid-based red coloration in house finches (*Carpodacus mexicanus*; Duckworth et al. 2004). Furthermore, fecal testosterone covaries positively with forehead patch size (structural coloration) in collared flycatchers (*Ficedula albicollis*) during the breeding season (Garamszegi et al. 2004). Testosterone is positively correlated with structurally based blue coloration of yearling blue tits (*Cyanistes caeruleus*; Peters et al. 2006); however, the direct influence of testosterone on preening activity is likely the mechanism behind this relationship (Roberts et al. 2009). Taken together, these studies leave open the possibility that some ornamental coloration in some species of songbirds could be enhanced by elevated testosterone levels.

The activational and organizational actions of steroids make manipulations of exogenous testosterone early in development particularly informative. Most studies of yolk androgens in birds have focused on the growth, behavior, and immunocompetence of the young. In general, studies have found positive effects of testosterone on growth and begging and negative effects on the immunocompetence of young nestlings (reviewed in Groothuis et al. 2005). In contrast, only in a few species have researchers addressed long-term organizational actions of yolk and juvenile hormones on the development of sexually selected traits. Testosterone treatment in egg yolks influences the expression of melanin traits, including the black hood of black-headed gulls (*Larus ridibundus*; Eising et al. 2006), the breast badge size of male house sparrows (Strasser and Schwabl 2004), and the black breast stripe size in great tits (*Parus major*; Galván and Alonso-Alvarez 2010). In ring-necked pheasants (*Phasianus colchicus*), administration of yolk testosterone decreases spur length but does not impact size or the carotenoid-based color of the wattle (Rubolini at al. 2006), while testosterone manipulation during the juvenile period enhances the size of wattles but not of spurs (Briganti et al. 1999). However, administration of egg testosterone to the pheasants caused wattles to be less colorful (Bonisoli-Alquati et al. 2011).

In this study, we used both experimental and correlational approaches in a study of eastern bluebirds (*Sialia sialis*) to test the hypothesis that testosterone influences the production of structurally based ornamental plumage coloration. First, we experimentally manipulated yolk testosterone using two concentrations of testosterone and measured the effect on the development of plumage coloration of 15-d-old nestlings. Second, we implanted juvenile bluebirds with testosterone and measured the effect on growth and plumage coloration of 15-d-old nestlings. Bluebirds grow the ornamented rectrices and remiges that they will retain through their first breeding season in the 30 d after hatching, so manipulation of nestling and fledgling hormone levels can directly affect this sexually selected trait. Third, we measured covariation between circulating testosterone and plumage coloration of breeding males.

Material and Methods

Study Species

Male bluebirds have UV-brilliant blue plumage on their heads, backs, rumps, wings, and tails and dark chestnut coloration on their breasts. Females have the same color pattern as males but with drabber blue and chestnut coloration. The UV-blue coloration is derived from the noniridescent reflecting nanostructure (Shawkey et al. 2003), while the chestnut breasts coloration is derived from a combination of eumelanin and phaeomelanin pigments (McGraw et al. 2004). More colorful males and females feed chicks more often and gain higher reproductive success (Siefferman and Hill 2003, 2005*a*). Males with the most ornamented UV-blue coloration are better able to compete successfully for limited nest sites (Siefferman and Hill 2005*c*). Experiments demonstrate that UV-blue plumage coloration is a condition-dependent trait in both female and male adult bluebirds (Siefferman and Hill 2005*a*, 2005*b*).

The UV-blue feather coloration displayed by both male and female bluebirds during their first breeding season is grown during the nestling and fledgling period, while they are still dependent on parental care. Colorful rectrices and remiges begin to emerge by age 11 d, and the color of remiges can be quantified at age 14 d (Siefferman and Hill 2007). Young bluebirds retain these juvenile wing and tail feathers as part of their first nuptial plumage, while only the contour feathers are replaced during the first prebasic molt (Gowaty and Plissner 1998). Experimental manipulations of food availability to nestlings demonstrate that male nestling bluebirds reared in poor natal conditions grow more slowly and display duller wing color than those reared in better natal environments (Siefferman and Hill 2007).

Table 1: Linear mixed model test statistics showing effects of testosterone injection and sex on concentration of brightness and ultraviolet (UV) chroma

Trait, factor	Estimate	SE	df	*F*	*P*
Brightness:					
Implantation			39.86	1.48	.24
Control[a]	.97	.86			
Low T[a]	.06	.86			
Sex[b]	−3.82	.50	37.80	57.64	<.001
UV chroma:					
Implantation			38.29	.13	.88
Control[a]	.003	.007			
Low T[a]	.004	.008			
Sex[b]	−.05	.004	40.55	123.67	<.001

Note. Nests are controlled for as random factors in all analyses. All interaction terms were not significant ($P > 0.05$).

[a]Estimates are relative to nestlings receiving high-testosterone injections.

[b]Estimates are relative to males.

Field Methods

We studied a population of eastern bluebirds breeding in nest boxes in Lee County, Alabama (32°35′52″N, 85°28′51″W; elevation = 216 m) in 2003, 2006, and 2007. The study site included pasture and edge habitat. We monitored nests of eastern bluebirds every other day during the nest-building stage. During the laying period, we visited the nests each day and marked new eggs with a permanent marker (Sharpie) to establish laying order. During the hatching period, we determined which chick hatched from each egg by visiting the nest every 3 hr (0600–1900 hours) during daylight until all eggs hatched. We identified individual nestlings by marking their tarsi with a unique color of permanent marker. We defined the age of the brood by the hatching date of the first hatched nestling (day 1 = hatch day). We then measured the mass of nestlings to the nearest 0.1 g at ages 2, 10, and 15 d. At age 15 d, we measured the right tarsus and wing to the nearest 0.1 mm. Nestlings increase rapidly in mass from the time they hatch until they are about age 11 d, but by age 13 d the mass of nestlings begins to asymptote (Pinkowski 1975). Hence, the mass of a nestling at age 15 d is an accurate estimate of fledging mass. Nestlings generally fledge from the nest between ages 15 and 18 d. All breeding adults were captured by using either mist nets or nest traps and banded with a unique combination of one US Fish and Wildlife Service and three color bands. Birds were handled according to the guidelines of the Auburn University Institutional Animal Care and Use Committee (PRN 2003-0466).

Nestlings at age 8 d have feather sheaths; at age 11 d feathers begin to emerge from the feather sheaths, and at age 15 d 2 cm of the wing feathers have emerged from the sheaths (see detailed methods in Siefferman and Hill 2007). The fifth primary is the longest feather at this age, and remiges are less than 1 cm long. At age 15 d we cut 2-cm feather samples from the right fifth primary for spectrophotometric plumage analysis. We determined sex using sexually dichromatic plumage coloration; past comparison of sex determination with plumage coloration and molecular sexing showed that 95% of offspring could be properly classified using plumage coloration (L. Siefferman, unpublished data).

Egg Injection Experiment

We injected eggs of first broods during the breeding season of 2003 as part of another study (Navara et al. 2005). Immediately after the completion of each clutch, we assigned all eggs within that clutch to one of three treatment groups and injected eggs with (1) 3 mg of T in 5 μL of peanut oil (high dose), (2) 0.3 mg of T in 5 μL of peanut oil (low dose), or (3) 5 μL of peanut oil (control). These concentrations were based on yolk levels found in bluebird eggs collected from the study site that varied from 2.9 to 240 ng/yolk (K. J. Navara, unpublished data). To compensate for degradation or incomplete incorporation of the hormone into the yolk, these concentrations were slightly above physiological levels. We randomly assigned clutches to one of the three treatment groups. Before the onset of embryonic development, we injected the treatment into the small end of the egg with a 5-mL Hamilton syringe. Before injection, the small end of the egg was cleaned with alcohol. After injection, the hole was sealed with superglue. We injected 5 μL of peanut oil stained with Sudan B into two eggs and retrieved them after 2 d to verify whether the vehicle containing the treatments actually reached the yolk. All injections were performed in the field. Yolks were frozen and separated from the albumin.

Manipulation of Nestling Testosterone

In 2006, we implanted 133 nestlings subcutaneously along the flank with one 5-mm length of Silastic tubing packed with crystalline testosterone (Sigma Chemical) and sealed with Silastic glue. The inner diameter was 1.44 mm, and each end had ~1 mm of glue. Sham-treated nestlings ($n = 88$) were implanted with one empty 5-mm tube, while true control nestlings ($n = 419$) received no treatments. The implantation did not cause bleeding, was done without anesthetic, and was sealed with superglue. Broods were assigned at random to one of the three treatment groups. At day 15 we collected a blood sample (~80 μL obtained from the wing vein) within 3 min of opening the nest box. Plasma was separated and frozen (−20°C) for later hormone analysis (see detailed methods below). Before collecting the blood samples, the testosterone implants were removed.

Adult Coloration and Circulating Testosterone Levels

In May 2007, males ($n = 37$) feeding nestlings were captured in nest boxes. To control for the idiosyncrasies of capture and to obtain robust individual estimates of testosterone production, we captured adult males using nest traps while they fed 4-d-old nestlings. We watched males enter the nest box and, once captured, collected blood samples from them (~150-μL blood sample obtained from the wing vein) within 3 min of

Table 2: Linear mixed model test statistics showing effects of testosterone implantation and sex on concentration of testosterone and corticosterone

Trait, factor	Estimate	SE	df	*F*	*P*
Testosterone:					
Treatment[a]	−3.06	.95	25	10.31	.004
Sex[b]	−.39	1.03	25	.15	.71
Corticosterone:					
Treatment[a]	−34.43	13.83	46	6.20	.02
Sex[b]	18.25	12.81	46	2.03	.16

Note. Nests are controlled for as random factors in all analyses. All interaction terms were not significant ($P > 0.05$).

[a]Estimates are relative to testosterone-implanted nestlings.

[b]Estimates are relative to males.

entering the nest box. Plasma was separated and frozen (−20°C) for later hormone analysis. We banded the adults if not previously captured and estimated the age of birds as either yearling (having undergone only one postnestling molt) or being in their second or subsequent year on the basis of the shape of the tenth primary feather (Pitts 1985). We collected eight rump and eight breast feathers for spectrometric analyses.

Plumage Measurements

Feathers were taped to matte black paper (Canson) such that feather swatches mimicked how feathers naturally lay on birds. Feathers were stored in a climate-controlled environment until spectrophometric analyses were conducted. We measured the reflectance of the UV-blue plumage of nestlings from a point 2 cm below the distal end of the right fifth primary and measured plumage reflectance of adults within the feather swatch. Following the protocols of Siefferman et al. (2005) and Siefferman and Hill (2007), one researcher (L. Siefferman) recorded spectral data with an S2000 spectrometer (range = 250–880 nm; Ocean Optics, Dunedin, FL) using a micron fiber-optic probe at a 90° angle held 2 mm from the feather surface. We took five measurements from different locations on each color patch and averaged the data.

We calculated three standard descriptors of reflectance spectra for UV-blue coloration: mean brightness, UV chroma, and hue. Mean brightness was calculated as the mean of the summed reflectance from 300 to 700 nm, while UV chroma was the proportion of the total reflectance that was in the UV range (∫300–400 nm/∫300–700 nm). Hue is the principal color reflected by the feather and was calculated as the wavelength at peak reflectance. We do not report hue for nestling plumage because the reflectance curve of UV-blue plumage of nestlings shows no clear peak and hue measurements are prone to error. We calculated two standard descriptors of reflectance spectra for chestnut coloration of breast of adult males: mean brightness and red chroma. Red chroma was the proportion of the total reflectance that was in the red range (∫575–700 nm/∫300–700 nm). Because the hue of the chestnut breast feathers expressed very little variation among males, we do not report hue for breast coloration.

Testosterone Assays

We determined testosterone levels using a commercial enzyme immunoassay (EIA; 901-065; Assay Designs, Ann Arbor, MI) following the detailed protocol of Clotfelter et al. (2004). We added ~2,000 cpm of titrated testosterone to each sample and calculated recoveries after extraction (4 mL of diethyl ether). We resuspended extracts in 50 μL of ethanol and diluted to 350 μL with assay buffer from the kit. From each reconstituted sample, we used 100 μL to determine recoveries and used duplicate 100-μL quantities in the EIA. We determined testosterone concentrations with a four-parameter logistic curve-fitting program (Microplate Manager; BioRad Laboratories, Hercules, CA) and corrected for incomplete recoveries. Intra-assay variation was 2.93%. Within each data set, we corrected for interplate variation by multiplying each measurement by the grand mean of assay standards across all plates within the data set and dividing by the plate mean of these standards. We report hormone levels as nanograms per milliliter.

Corticosterone Assays

We measured plasma corticosterone using standard radioimmunoassay techniques, following the detailed protocols of Ketterson et al. (1991) and Wingfield and Farner (1975). We extracted samples with 4 mL of diethyl ether evaporated under nitrogen gas and resuspended them in phosphate buffer. Samples were then assayed in duplicate, and assay values were cor-

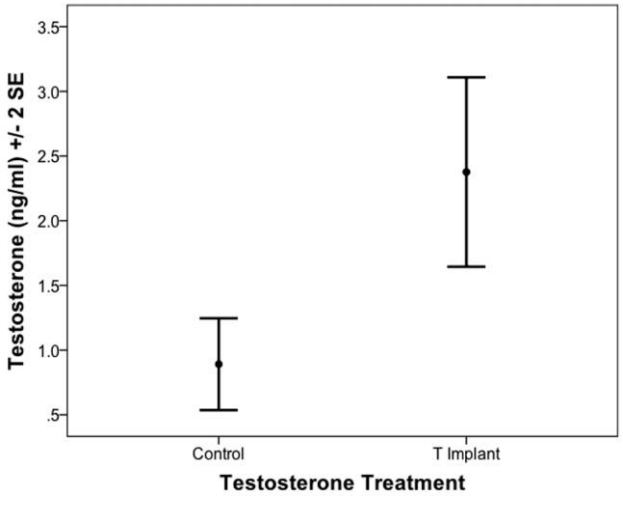

Figure 1. Effects of testosterone implantation treatment and control on mean (±SE) circulating testosterone of eastern bluebird nestlings at age 15 d. At age 5 d nestlings underwent subcutaneous implantation on the flank with one 5-mm length of Silastic tubing (1.44-mm inner diameter) packed with crystalline testosterone. Male and female data are combined.

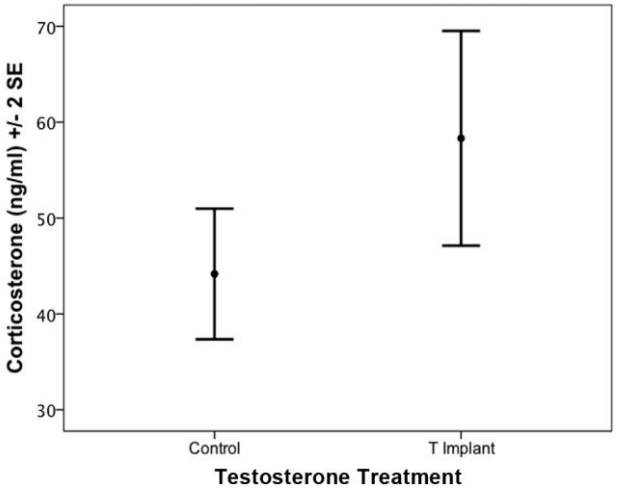

Figure 2. Effects of testosterone implantation treatment and control on mean (±2 SE) circulating corticosterone of eastern bluebird nestlings at age 15 d. Implantation treatments are the same as those described in figure 1. Male and female data are combined.

rected for plasma volumes and individual recoveries after extraction. Intra-assay variation was 1.91%. We report hormone levels as nanograms per milliliter.

Statistical Analyses

Shapiro-Wilk tests revealed that data did not deviate significantly from normality (all $P > 0.05$). To analyze the effect of yolk testosterone injection and sex on juvenal plumage coloration, we used general linear mixed-effects models with nest identification as the random effect. To analyze the effects of testosterone implantation and sex on (1) testosterone level, (2) corticosterone level, (3) body size, and (4) plumage coloration of nestlings, we used general linear mixed-effects models with nest identification as the random effect. In all analyses, we tested two-way interactions between the fixed factors; however, because no interaction terms were significant ($P > 0.05$), interactions were removed from the models. To measure the relationship between treatment and nestling mass at age 10 d and plumage coloration, we used general linear mixed-effects models with nest identification as the random effect. We found a significant interaction between treatment and nestling mass on color ($P < 0.01$), suggesting that the relationship between condition and color varied with treatment. Thus, we separated our data into testosterone-treated and control nestlings and into male and female nestlings and measured the relationship between body mass and coloration using nest as the random effect. To measure the relationship between circulating testosterone and plumage characteristics in adult male bluebirds, we used Pearson correlations. SPSS software (ver. 19.0) was used to analyze data, and all tests were two-tailed.

Results

We detected no significant effect of the in ovo testosterone treatment on nestling plumage coloration; however, males were significantly brighter and more UV chromatic than females (table 1). In the testosterone implant study, we found no significant difference between sham-implanted and control nestlings for any dependent variable (*t*-tests: all $t < 1.14$, all $P > 0.25$); thus, we pooled data for those two groups and refer to them collectively as control nestlings. We found that testosterone treatment significantly heightened both circulating testosterone and corticosterone in 15-d-old nestlings (table 2; figs. 1, 2). Moreover, testosterone-treated offspring weighed significantly less at age 10 and 15 d (table 3; fig. 3) and exhibited significantly shorter tarsi at age 15 d (table 3). At age 15 d the plumage coloration of testosterone-treated male and female offspring was significantly duller (less bright; table 3; fig. 4), but UV chroma was not significantly influenced by the experimental treatment (table 3). Overall, males were significantly brighter and more UV chromatic than females (table 3; fig. 4). In control males and females, we found significant positive relationships between nestling mass at age 10 d and coloration (heavier offspring were brighter); this relationship, however, was not evident in testosterone-treated nestlings (table 4).

Analyses of adult males revealed that testosterone concentration did not differ significantly between second-year and after-second-year males ($t = 0.37$, $P = 0.71$; $n = 11$ and 33, respectively), nor did we find an interaction between age and any plumage variables on testosterone concentration (all $P > 0.10$). We found no evidence of a correlation between circulating testosterone and structural plumage coloration in adult males. The only significant relationship between circulating testosterone and plumage coloration showed that males with less

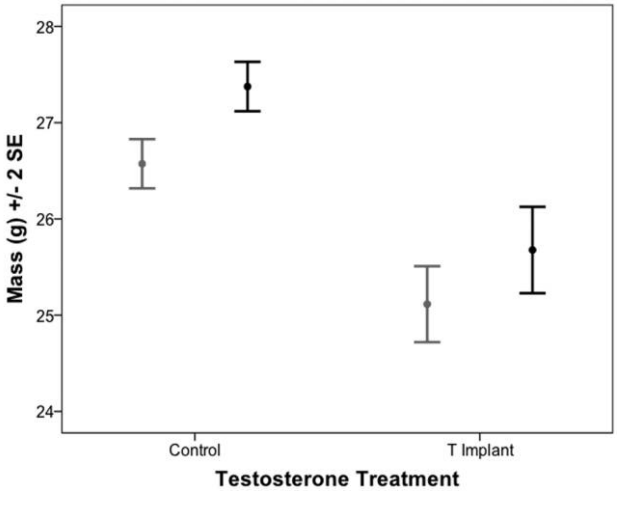

Figure 3. Effects of testosterone implantation treatment and control on mean (±2 SE) mass of eastern bluebird nestlings at age 15 d. Implantation treatments are the same as those described in figure 1. Males are in black, and females are in gray.

Table 3: Linear mixed model test statistics showing effects of testosterone implantation and sex on body size and plumage coloration traits

Trait, age, factor	Estimate	SE	df	*F*	*P*
Mass (g), 10 d:					
Treatment[a]	2.081	.425	215.75	23.96	<.001
Sex[b]	−.073	.206	551.06	.25	.62
Mass (g), 15 d:					
Treatment[a]	1.357	.309	239.62	19.30	<.001
Sex[b]	−.505	.122	623.85	17.22	<.001
Tarsus length (mm), 15 d:					
Treatment[a]	.478	.096	216.06	24.88	<.001
Sex[b]	−.076	.047	670.07	2.61	.11
Brightness (%), 15 d:					
Treatment[a]	.656	.258	201.31	6.45	.01
Sex[b]	−4.419	.147	650.81	898.26	<.001
UV chroma (%), 15 d:					
Treatment[a]	.005	.005	223.04	1.02	.31
Sex[b]	−.061	.002	552.95	1,270.58	<.001

Note. Nests are controlled for as random factors in all analyses. All interaction terms were not significant ($P > 0.05$). UV = ultraviolet.

[a]Estimates are relative to testosterone-implanted nestlings.

[b]Estimates are relative to males.

ornamented melanin breast coloration (lower red chroma) exhibited higher levels of testosterone (table 5).

Discussion

We found no support for the hypothesis that testosterone enhances ornamental plumage coloration; elevation of testosterone before or during feather growth did not increase the UV-blue structural coloration of male eastern bluebirds. Injections of testosterone into the yolk had no effect on plumage coloration despite previous research demonstrating a positive impact of the high-dose treatment on nestling structural size and negative impacts on nestling immunocompetence (Navara et al. 2005). Moreover, implanting nestlings for 10 d with testosterone produced a negative effect on brightness of UV-blue coloration in both male and female offspring. Overall, our data fail to support the hypothesis that elevated testosterone positively influences structural plumage ornamentation in male eastern bluebirds.

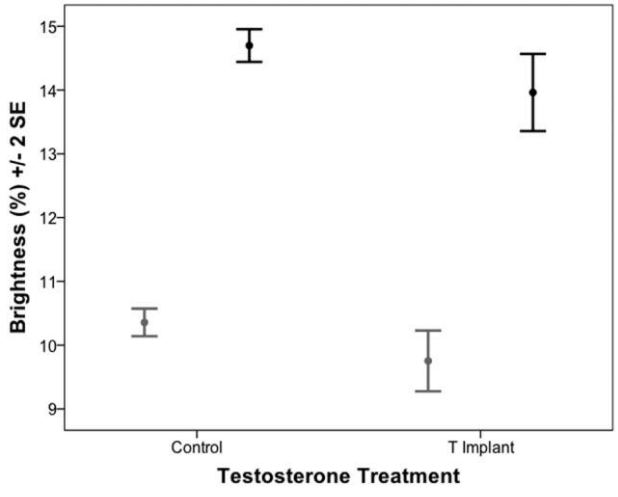

Figure 4. Effects of testosterone implantation treatment and control on mean (±2 SE) brightness of wing feathers of eastern bluebird nestlings at age 15 d. Implantation treatments are the same as those described in figure 1. Brightness was calculated as the mean of the summed reflectance from 300 to 700 nm determined using a reflectance spectrometer. Males are in black, and females are in gray.

We observed not only that circulating testosterone levels at the time of color formation do not promote more elaborate ornamentation but also that feather coloration of adults during breeding was not positively associated with levels of circulating testosterone. As a matter of fact, we observed that both structural blue and melanin-based chestnut coloration were negatively correlated with testosterone; the more ornamented animals had lower circulating testosterone. Many studies have revealed positive relationships between testosterone and the size of melanin-based black plumage badges (Evans et al. 2000; Buchanan et al. 2001; Gonzalez et al. 2001; Strasser and Schwabl 2004; Eising et al. 2006; Galván and Alonso-Alvarez 2010). Thus, we are puzzled by the negative relationship between the chroma of the melanin-based chestnut breast of the adult male bluebirds and circulating testosterone. Eastern bluebirds with lower red chroma have a lower ratio of eumelanin to phaeomelanin pigments (McGraw et al. 2004), are less ornamented, and look more similar to females. Our past research with this population of bluebirds suggests that males with more ornamented breast patches are better mates (Siefferman and Hill 2003) and tend to be older (Siefferman et al. 2005). Age is not likely responsible for the positive covariation between breast

Table 4: Test statistics showing that correlations between nestling mass at age 10 d and plumage coloration vary with testosterone implantation treatment

Sex, treatment, trait	Estimate	SE	df	*F*	*P*
Female:					
Control:					
Brightness	3.47	.30	266	8.56	.004
UV chroma	.001	.0001	266	1.10	.29
Testosterone:					
Brightness	3.33	.61	60	.19	.67
UV chroma	.0016	.003	60	.02	.90
Male:					
Control:					
Brightness	4.29	.38	252	6.18	.01
UV chroma	.0041	.00004	252	.02	.89
Testosterone:					
Brightness	5.68	1.07	56	.65	.43
UV chroma	.00040	.0008	56	.13	.72

Note. Nests are controlled for as random factors in all analyses.

ornamentation and circulating testosterone because we found no difference in testosterone levels of first year or older birds.

Few studies have investigated the relationships between testosterone and mechanisms of plumage coloration other than melanin pigmentation. Exceptions include two studies of carotenoid-based plumages and two studies of structurally based plumages. Exogenous testosterone caused a reduction in ornamentation of red carotenoid-based plumage coloration in house finches (Stoehr and Hill 2001) and ring-necked pheasants (Bonisoli-Alquati et al. 2011). To date, the effect of testosterone on plumage variation produced by reflectance of light from feather nanostructure has been studied only in blue tits and eastern bluebirds. Correlations have been found between circulating testosterone and plumage chroma of blue tits, but the direction and strength of the relationship varied between older and younger males; the relationship was strong and positive for yearling males and weaker and negative for older males (Peters et al. 2006). Only one manipulative study of structural ornaments has shown that exogenous administration of testosterone during molt led to an increase in chroma, but this relationship was apparent only in spring and not apparent in autumn directly after molt (Roberts et al. 2009). The testosterone-treated birds preened more often in the spring than control birds; thus, preening is likely the mechanism behind this positive covariation. Overall, our data demonstrating that nestlings implanted with testosterone exhibit duller blue color corroborates the findings of a recent study of eastern bluebirds breeding in Oklahoma; adult males with less ornamented blue plumage (UV chroma and hue) had significantly lower androgen levels and higher corticosterone levels (Grindstaff et al. 2012). The negative relationship that we found between testosterone treatment and the brightness of structurally based blue plumage could be either a direct consequence of testosterone on the development of feather nanostructure or an indirect effect of the negative relationship between either testosterone or corticosterone and body condition. Finally, it is important to point out that the levels of testosterone in treated chicks may have reached pharmacological levels.

The nestling hormone manipulation may have caused duller plumage as a consequence of stress. Indeed, the natural covariation between body size during development and plumage coloration (this study; Siefferman and Hill 2007; Soley et al. 2011) was decoupled in the testosterone-treated birds. Additionally, the testosterone implantation in nestlings caused a concurrent rise in circulating corticosterone. These data are corroborated by studies demonstrating that testosterone increases circulating corticosterone in several avian species (e.g., Evans et al. 2000; Casto et al. 2001; Owen-Ashley et al. 2004). Our testosterone-treated nestlings also exhibited slower growth near fledging age. Several other studies have documented negative effects of elevated testosterone on avian body condition (Ros 1999; Wikelski et al. 1999; Clotfelter et al. 2004; Mougeot et al. 2004), while other studies have demonstrated that increased corticosterone levels covary negatively with body condition (Schwabl 1995; Hood et al. 1998; Kitaysky et al. 2001; Sockman and Schwabl 2001; Perfito et al. 2002; Breuner and Hahn 2003; Pereyra and Wingfield 2003). Indeed, elevated yolk androgen reduces growth rates of male nestling collared flycatchers (Pitala et al. 2009).

Brightness and mass positively covaried among control nestlings, while there was no significant association among testosterone-implanted nestlings. Similar relationships have been found in nestling ring-neck pheasants and superb fairy wrens (*Malurus cyaneus*); after experimental administration of testosterone, the natural covariance between ornamentation and immunocompetence was decoupled (Peters 2000; Bonisoli-Alquati et al. 2011). These observations suggest that the response of nestlings to implantation may have depended on the condition (sensu Hill 2011) of nestlings. Perhaps only nestlings in good condition, with highly functional cellular processes, could afford to express brighter structural plumage while withstanding the reduction in body condition associated with the testosterone implants. Two previous studies suggest that plumage coloration is condition dependent in nestling bluebirds; experimentally induced poor natal condition reduced color brightness (Siefferman and Hill 2007), and nestling growth was positively related to blue brightness (Soley et al. 2011).

In adult males, we failed to detect any significant relation-

Table 5: Test statistics showing correlations between circulating testosterone concentration and plumage characteristics in adults

Trait	*r*	*P*
Breast brightness	−.02	.91
Breast chroma	−.41	.01
Rump brightness	.06	.73
Rump chroma	.26	.12
Rump hue	−.11	.53

ships between the structurally based UV-blue coloration and circulating testosterone. Yet Grindstaff et al. (2012) detected a negative relationship in another population of breeding bluebirds. We may have failed to detect a true relationship between testosterone secretion and plumage ornamentation—indeed, our sample size would have enabled us to find only a strong effect. Moreover, testosterone levels are not static but often show variation on long-term and short-term scales in response to social stimuli, seasonal changes (Wingfield et al. 1990), and diurnal changes (Laucht et al. 2011). During the breeding season, socially modulated elevated testosterone is associated with reproductive behaviors, including male-male aggression and female courtship (Harding 1981; Wingfield 1985; Wingfield et al. 1990, 2001; Pinxten et al. 2003; Goymann et al. 2007). Sexual ornaments (not circulating testosterone levels) are more strongly associated with the ability to produce large changes in testosterone (McGlothlin et al. 2008). We measured circulating testosterone when males were feeding offspring and not during territory formation, and measuring testosterone during territory formation would have been ideal. However, eastern bluebirds produce multiple broods per breeding season in Alabama, and aggressive behaviors remain consistent throughout the breeding season (L. Siefferman, unpublished data). Furthermore, Grindstaff et al. (2012) detected a negative relationship between ornamentation and androgen levels, and they also measured males during the nestling stage but with a larger sample size. Moreover, recent evidence suggests that measuring testosterone levels after administration of gonadotropin-releasing hormone (Jawor et al. 2006) or while birds sleep (Laucht et al. 2011) allows researchers to measure an individual's maximum testosterone level. Thus, our study, like others (Owens and Short 1995; Kimball 2006), may have failed to detect a true relationship between plumage and testosterone.

Three lines of evidence revealed no support for the hypothesis that high levels of testosterone lead directly to brighter structural plumage coloration in eastern bluebirds. First, the injection of yolk testosterone did not enhance structural coloration regardless of the negative effect on nestling immunity (Navara et al. 2005). Second, implantation of nestlings with testosterone did not enhance structural coloration. Third, adult males with higher circulating testosterone did not express brighter structural coloration. Instead, we found evidence that higher testosterone was associated with duller nestling structural and adult melanin ornamentation. From these observations we do not support the hypothesis that testosterone influences structural coloration in eastern bluebirds. Nonetheless, the negative effect of testosterone implantation on blue structural color of nestlings may translate into a reduction in individual reproductive success if coloration influences the outcome of male-male agonistic encounters.

Acknowledgments

We thank Amanda Bessler and Laura Beard for help with fieldwork, Jodie Jawor and Danielle Whittaker for help with hormone assays, Joel McGlothlin for help with statistical analyses, and Alex Bentz and two anonymous reviewers for suggestions to the manuscript. This work was supported by the National Science Foundation (DEB-0075388, DEB-0218313, DEB-0077804, IBN-0235778, and IBN-0235778) and the National Institute of Allergy and Infectious Diseases (R01AI049724). Nestlings were handled under a state of Alabama permit (97181) and a federal permit (PRT 784373).

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