

## Consequences of elevating plasma testosterone in females of a socially monogamous songbird: evidence of constraints on male evolution?

Ethan D. Clotfelter,<sup>a,\*</sup> Dawn M. O'Neal,<sup>b</sup> Jacqueline M. Gaudio,<sup>a</sup> Joseph M. Casto,<sup>c</sup>  
Ian M. Parker-Renga,<sup>c</sup> Eric A. Snajdr,<sup>c</sup> Deborah L. Duffy,<sup>c</sup>  
Val Nolan Jr.,<sup>c</sup> and Ellen D. Ketterson<sup>c</sup>

<sup>a</sup>Department of Biology, Providence College, Providence, RI 02918, USA

<sup>b</sup>Department of Biology, Washington University, St. Louis, MO 63130, USA

<sup>c</sup>Department of Biology and Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington, IN 47405, USA

Received 20 November 2003; revised 1 March 2004; accepted 2 March 2004  
Available online 8 June 2004

### Abstract

To explore whether selection for testosterone-mediated traits in males might be constrained by costs of higher testosterone to females, we examined the effects of experimental elevation of plasma testosterone on physiological, reproductive, and behavioral parameters in a female songbird, the dark-eyed junco (*Junco hyemalis*). We used subcutaneous implants to elevate testosterone (T) in captive and free-living female juncos. In captive birds, we measured the effects of high T on body mass, feather molt, and brood patch formation. In the field, we monitored its effects on the timing of egg laying, clutch size, egg size, egg steroid levels, incubation, and nest-defense behavior. Females implanted with testosterone (T-females) had significantly higher circulating levels of testosterone than did control females (C-females). Captive T-females had lower body mass, were less likely to develop brood patches, and delayed feather molt relative to C-females. Among free-living females, the interval between nest completion and appearance of the first egg was longer for T-females than for C-females and egg yolk concentrations of testosterone were higher, but there were no significant differences in estradiol levels, clutch size, or egg size. Incubation and nest defense behavior were also similar between T- and C-females. Our results suggest that selection on males for higher testosterone might initially lead to a correlated response in females producing changes in body mass and feather molt, both of which could be detrimental. Other possible female responses would be delayed onset of reproduction, which might reduce reproductive success, and higher yolk testosterone, which might have either positive or negative effects on offspring development. We found no reason to expect reduced parental behavior by females as a negative fitness consequence of selection for higher testosterone in males.

© 2004 Elsevier Inc. All rights reserved.

**Keywords:** Dark-eyed junco; Testosterone; Egg yolk steroids; Incubation; Nest defense; Evolutionary constraint; Antagonistic selection

In general, when selection favors traits in one sex that are harmful to the other sex, the correlated response to selection in the second sex slows the rate of evolution and can be viewed as a constraint (Shuster and Wade, 2003). Thus, an important question facing behavioral endocrinologists and evolutionary biologists is how natural and sexual selection produce dramatic sex differences in morphology and behavior when their force ought to be weakened by correlated selection (Shuster and Wade, 2003). Sex-limited expression is one means to overcome constraints (West-

Eberhard, 2003), and sex-specific trait expression is often organized or activated by steroid hormones (Balthazart and Adkins-Regan, 2002). Organizational effects prepare one sex for later exposure to the activating effects of a hormone while rendering the other sex insensitive; adult sex differences may be attributable to the presence of activating levels of the hormone in only one sex and/or to insensitivity to that level of hormone in the other sex (Balthazart and Adkins-Regan, 2002).

In vertebrates, testosterone (T) often activates male-specific behavior, but its presence and action are clearly not confined to males. A recent review reports significant levels of T in females of several bird species (Wingfield et al., 2000), and females with naturally higher levels of T are

\* Corresponding author. Department of Biology, Amherst College, Amherst, MA 01002. Fax: +1-413-542-7955.

E-mail address: [edclotfelter@amherst.edu](mailto:edclotfelter@amherst.edu) (E.D. Clotfelter).

more likely to win territorial interactions with other females (Langmore et al., 2002; but see Elekonich, 2000). Evidence from some species suggests that male and female maximal T levels are positively correlated (Sandell et al., in preparation). Several authors have pointed out the need for greater understanding of the role of T in female vertebrates (Staub and De Beer, 1997), and we stress here the need to understand how its action in females might affect initial response to altered selective pressures within species.

One way to assess how selection on one sex might affect the other is to manipulate hormones and measure the consequences and their associated costs and benefits in both sexes. In the sex role-reversed spotted hyena (*Crocuta crocuta*), for example, Drea et al. (2002) found that blocking prenatal exposure to naturally circulating androgens widened the birth canal in females so as to increase survival of neonates, but produced a shorter, squatter penis in males that was ineffectual at intromission. In birds, the use of testosterone manipulations to address the question of correlated selection is likely to be fruitful because levels of this hormone are directly related to numerous sexually selected traits in males (Balthazart, 1983; Hillgarth and Wingfield, 1997) and because hormone levels for both sexes are known for several species (Wingfield et al., 2000). Previous studies have shown benefits of high testosterone (T) in male birds in the form of extra-pair fertilizations (Raouf et al., 1997), but also revealed countervailing costs to males, such as reduced survival or reproductive success with their social mates (Raouf et al., 1997; Reed et al., submitted for publication; reviewed in Ketterson et al., 2001). Far less is known about the effects of T in females in general, but recent studies have begun to address them (Ketterson et al., 2001; Langmore et al., 2002; Staub and De Beer, 1997). For example, T activates vocal behavior in starlings (*Sturnus vulgaris*; Hausberger et al., 1995), increases the size of the vocal control region in Psittaciformes (Nespor et al., 1996), increases aggressive behavior in red-winged blackbirds (*Agelaius phoeniceus*; Searcy, 1988), and increases courtship behavior in budgerigars (*Melopsittacus undulatus*; Nespor et al., 1996) and ruffs (*Philomachus pugnax*; Lank et al., 1999). But to our knowledge, only De Ridder et al. (2002) have systematically addressed whether the detrimental effects of experimentally elevated T are confined to males or whether they might be borne by females as well.

In the current study, we manipulated testosterone levels in a female songbird, the dark-eyed junco (*Junco hyemalis*), to determine whether elevated T produces similar effects in females and males and whether the effects in females might constrain the evolution of higher testosterone levels in males. Juncos are a good species for this study because the consequences of elevated T in males are relatively well understood (reviewed in Ketterson et al., 2001). Further, female juncos, like other songbirds in temperate latitudes, produce testosterone early in the breeding season during pair formation (Ketterson et al., 2001; Wingfield et al., 2000). We used subcutaneous implants to elevate plasma

T levels in captive and free-living females and monitored changes in the following traits: body mass, feather molt (replaces worn feathers), brood patch formation (brood patch facilitates heat transfer during incubation), timing of egg laying, clutch size, egg size, egg steroid levels (affects offspring growth and behavior), incubation, and defense of the nest against predators. For each phenotypic measure, we compared T-females to C-females, asking whether differences observed were likely to be beneficial or detrimental. Our null hypothesis is that effects of elevated T in males and females are unrelated and the sexes are free to evolve independently, perhaps because past selection has made females insensitive to T when its effects are detrimental (Lynn et al., 2002; Shuster and Wade, 2003). The two alternative hypotheses are the antagonistic selection hypothesis, in which hormonal effects are beneficial in one sex and detrimental in the other, and the concordant selection hypothesis, in which the consequences are the same in both sexes, that is, beneficial in both or detrimental in both.

## Materials and methods

### *Study species and sites*

Dark-eyed juncos are socially monogamous, ground-nesting songbirds. Only females incubate, but both sexes defend the nest and feed the young. Nolan et al. (2002) give a detailed account of their breeding biology. We have studied juncos at the Mountain Lake Biological Station (MLBS) in southwestern Virginia, USA, since 1983. We began implanting male and female juncos with testosterone in 1987 and 2001, respectively. In 1997, we began studying captive juncos at the Kent Farm Bird Observatory (KFBO) in Bloomington, IN, USA, using wild-caught birds from MLBS (descriptions in Clotfelter et al., 2001, Duffy et al., in preparation). This research was conducted in compliance with NIH standards and with the approval of the Institutional Animal Care and Use Committees of Providence College, Indiana University, and the University of Virginia (MLBS).

### *Testosterone implants*

Our implant techniques have been reported previously (Ketterson et al., 1991, 1992). We caught free-living females in traps or nets in the period of 15 April–15 May (before most had begun nesting), randomly determined their treatment (testosterone or control), and anesthetized them with methoxyflurane (Metofane<sup>®</sup>, Pitman-Moore, Inc.). For T-females, a 7-mm Silastic<sup>®</sup> tube (Dow Corning, 1.47 mm i.d., 1.96 mm o.d.) packed with 5 mm of crystalline T (Sigma-Aldrich, Inc.) was inserted subcutaneously along the left flank. This dose produces T levels similar to natural early season peaks in female juncos (Ketterson et al., 2001). Control birds were given an empty tube but were otherwise

handled identically. Implants maintain elevated T levels for several months (males: Ketterson et al., 1991; females: Ketterson et al., unpublished data) and are removed from birds 15 July–15 August each year.

#### *Captive birds*

Beginning 10 January 2002, captive females that had previously been maintained indoors on day lengths similar to ambient photoperiod began to be subjected to artificially lengthening days. On 2 February, day length reached 15L/9D, where it was held until late April. On 14 February, these females were anesthetized and implanted as described above. At this point, each implanted female was housed with another, non-implanted female as part of a separate study measuring the effects of social status on mate choice (Duffy et al., in preparation). On 1 May, we transferred females to outdoor aviaries on natural photoperiod, which caused them to experience a phase shift and a 30-min decrease in day length. During this time, females could hear males but had no physical contact with them. On 27 May, after approximately 3.5 weeks on natural day lengths, females were weighed, examined for the presence of a brood patch and the onset of postnuptial molt, and their implant removed.

#### *Free-living birds*

We searched for nests daily from 1 May to 15 July, 2001 and 2002. Once found, nests were marked and the attending adults were identified. We checked nests daily until they were completed (fully lined with deer hair) and all eggs had been laid; we deemed a clutch as complete when no new eggs were added for 24 h. To compare clutch size by treatment, we averaged clutches for each female. In 2002, we weighed eggs (in nests found during egg laying) and measured lengths (L) and widths (W) with dial calipers to calculate egg volume ( $L \times W^2 \times 0.524$ ; Nolan, 1978). Twenty-four hours after clutch completion, we collected the third egg (or the largest egg in clutches for which laying order was not certain) and stored it at  $-20^\circ\text{C}$  for yolk steroid analysis.

In 2001, we videotaped incubating females twice each (once 0600–1100 EDT and once 1300–1700 EDT); in 2002, we videotaped them four times (twice each during same periods). Cameras concealed 2–3 m from the nest were used to videotape females for 60-min sessions. We began videotaping upon clutch completion; for nests found during incubation, we videotaped them the day they were found. To standardize our observations, females were flushed from their nests just before we began videotaping. We analyzed the time (seconds) females took to resume incubation and the proportion of time (arcsin transformed) they spent incubating as a function of their hormone treatment. To reduce interobserver error and bias, all videotapes were analyzed by J.M.G. without knowledge of the focal female's hormone treatment.

We tested a female's response to a simulated predator by placing a taxidermic mount of a chipmunk (*Tamias striatus*) within 1 m of her nest. These observations were conducted on the third day after clutch completion or as soon as the nest was discovered (in the case of nests found during incubation). The observer (D.M.O.) waited until the focal female was off her nest, placed the chipmunk under a camouflaged cloth tied to a string, and then concealed herself under another camouflaged cloth approximately 10–15 m away. Once the female resumed incubation (i.e. no longer appeared disturbed), the chipmunk was uncovered by pulling the string. We recorded the following responses by the female and her mate for the next 10 min: number of swoops at the predator (dives with no contact), number of hits on the predator, and number of nest checks (bird enters nest or stands on rim). These methods and response categories were based on Cawthorn et al. (1998). Behavioral data were collected by D.M.O. while blind to the female's hormone treatment.

#### *Steroid hormone assays in blood and egg yolks*

We re-caught females from 20 May to 8 August, 2002, using mist nets (watched steadily so that birds were retrieved immediately after capture) and collected 50–100  $\mu\text{l}$  of blood from the alar vein. We determined testosterone concentrations using a commercial enzyme immunoassay (EIA) kit (#901-065; Assay Designs, Inc.). The kit has a low (7.2%) cross-reactivity with androstenedione and negligible (<1%) cross-reactivities with dihydroepiandrosterone, estradiol, dihydrotestosterone, progesterone, and corticosterone. Each 20- $\mu\text{l}$  plasma sample was diluted 6-fold with distilled water. Approximately 2000 cpm of [ $^3\text{H}$ ] testosterone (NET-553; New England Nuclear Corp.) was added to each sample to allow the calculation of recovery percentages following extraction. Endogenous and tritiated steroids were extracted thrice with diethyl ether. The extracts were evaporated, then re-dissolved in 50  $\mu\text{l}$  of 100% ethanol and diluted to 350  $\mu\text{l}$  with assay buffer. From each sample, 100  $\mu\text{l}$  were used to determine recovery percentages and 100- $\mu\text{l}$  duplicate samples were used in the EIA. We followed the EIA kit manufacturer's procedures exactly, with one exception: instead of a five-point standard curve that ranged in concentration from 200 to 0.781 pg/well, we used a seven-point standard curve that ranged in concentration from 200 to 3.125 pg/well. Testosterone concentrations were determined with the aid of a four-parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc.) and corrected for incomplete recovery. The intra-assay coefficient of variation was 5.76%.

We assayed egg yolk steroids using methods described by Lipar et al. (1999). Recovery values averaged  $63 \pm 0.036\%$  for testosterone and  $71 \pm 0.037\%$  for  $17\beta$ -estradiol. Intra-assay variation, which was calculated as the coefficient of variation of values obtained from standard samples of

known concentration, was 7.6% for 17 $\beta$ -estradiol (E2) and 12.6% for testosterone.

### Statistical analyses

We analyzed our data using SPSS v. 11.0. Data were checked for normality; those nonnormally distributed were analyzed using nonparametric tests. We combined field data from 2001 and 2002, and report significant year differences when they occurred. Some females ( $n = 8$ ) were implanted in both years. Multiple nests belonging to the same female in the same year were not included in our analyses, but nests belonging to the same female in different years were treated as independent data points because they had different mates. We used ANCOVA to examine hormone treatment effects on incubation behavior and nest defense behavior. We included year, Julian date, temperature, and clutch size in the analysis of incubation behavior and year, Julian date, time, clutch size, and male behavior in the analysis of female nest defense behavior. Means are presented  $\pm 1$  SEM. Tests were two-tailed and differences were considered significant if  $P < 0.05$ .

## Results

### Testosterone implants

We confirmed the efficacy of our hormone manipulation in 2002 by comparing plasma T levels of females with control and testosterone implants. T-females had significantly higher plasma T than did C-females [ $t(23) = -6.34$ ,  $P < 0.001$ ; Fig. 1].

### Captive birds

After 16 weeks on long days and 12 weeks after implantation, C-females weighed more than T-females [C-females:  $21.76 \pm 0.44$  g,  $n = 7$ ; T-females:  $20.01 \pm 0.29$  g,

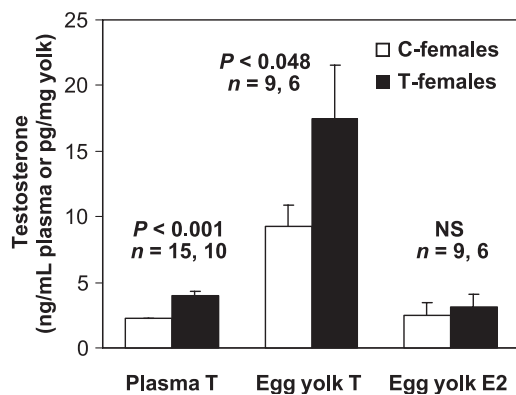


Fig. 1. Levels of testosterone (T) assayed by EIA from female dark-eyed junco blood plasma (ng/ml) and levels of T and 17 $\beta$ -estradiol (E2); both in pg/mg) assayed from egg yolks by RIA. Error bars represent 1 SEM.

Table 1

Number (%) of captive female juncos developing brood patches or undergoing postnuptial molt when treated with testosterone (T) or control implants

	T-treated females	Control females
Brood patch	1/7 (14.3%)	7/7 (100%)
Postnuptial molt	0/7 (0%)	5/7 (71.4%)

Significance values given in text.

$n = 7$ ;  $t(12) = -3.33$ ,  $P = 0.006$ ]. While not permitted to breed (i.e. not housed with males), C-females were significantly more likely than T-females to develop a brood patch (Fisher's exact test,  $P = 0.005$ ; Table 1) and to initiate postnuptial molt ( $P = 0.021$ ; Table 1).

### Free-living birds

We determined the number of days between nest completion and the appearance of the first egg for 11 T-females and 14 C-females. T-females took 1 day longer to lay their first egg ( $2.55 \pm 0.39$  days) than did C-females ( $1.57 \pm 0.25$  days;  $U = 39.0$ ,  $z = -2.22$ ,  $P = 0.038$ ). T- and C-females did not differ in clutch size (T-females:  $3.72 \pm 0.08$  eggs,  $n = 25$  females; C-females:  $3.90 \pm 0.09$  eggs,  $n = 25$  females;  $U = 244.5$ ,  $z = -1.48$ ,  $P = 0.14$ ). Repeated-measures ANOVA of the first three eggs of three- and four-egg clutches showed that both egg mass and volume increased with laying order within a clutch [mass:  $F(2,16) = 12.18$ ,  $P < 0.001$ ; volume:  $F(2,16) = 12.25$ ,  $P < 0.001$ ], but that hormone treatment had no effect on egg size [mass:  $F(1,16) = 1.71$ ,  $P = 0.21$ ; volume:  $F(1,16) = 1.25$ ,  $P = 0.28$ ]. Similarly, hormone treatment had no effect on egg mass (partial  $t = -1.42$ ,  $P = 0.16$ ) or egg volume (partial  $t = -1.41$ ,  $P = 0.17$ ) when all eggs were analyzed together with clutch size and laying order as covariates. Eggs laid by T-females contained significantly more yolk testosterone than did eggs laid by C-females [ $t(13) = -2.18$ ,  $P = 0.048$ ; Fig. 1]. T-implants had no effect on 17 $\beta$ -estradiol levels in egg yolks, however [ $t(13) = -0.44$ ,  $P = 0.67$ ; Fig. 1], suggesting that excess T in T-females was not converted into plasma estradiol via aromatization.

We videotaped 23 incubating females (6 T-females, 17 C-females), excluding those that reacted to the camera ( $n = 5$ ). Females were videotaped for an average total of  $219.50 \pm 8.51$  min. ANCOVA revealed that hormone treatment did not affect the time females took to resume incubating after being flushed from their nest [ $F(1,23) = 0.20$ ,  $P = 0.66$ ; Fig. 2] or the proportion of time females spent incubating (Fig. 2). The analysis of the proportion of time spent incubating was performed on arcsin-transformed data [ $F(1,23) = 2.60$ ,  $P = 0.13$ ]. The proportion of time females incubated (treatment groups pooled) differed significantly between years [ $F(1,23) = 9.63$ ,  $P = 0.006$ ].

At a later date, but still during the incubation period, we simulated imminent risk of nest predation by placing a stuffed chipmunk near the nests of 9 T-females and 17 C-females for

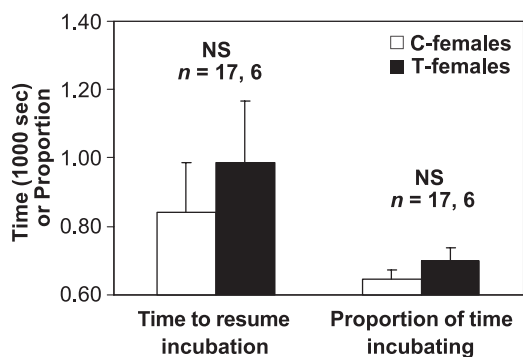


Fig. 2. The time (in thousands of seconds, error bars represent 1 SEM) female dark-eyed juncos took to resume incubation after leaving the nest and the proportion of time they spent incubating. Neither measure of incubation was significantly affected by the female's hormone treatment (see text).

10 min each. The number of swoops at the predator [T-females:  $7.0 \pm 2.50$ ; C-females:  $6.18 \pm 1.51$ ;  $t(24) = -0.30$ ,  $P = 0.77$ ], direct hits at the predator [T-females:  $5.33 \pm 3.28$ ; C-females:  $2.53 \pm 1.29$ ;  $t(24) = -0.95$ ,  $P = 0.35$ ], and nest checks by the female [T-females:  $3.78 \pm 2.59$ ; C-females:  $1.24 \pm 0.43$ ;  $t(24) = -1.31$ ,  $P = 0.20$ ] did not differ with hormone treatment. Of the covariates we analyzed, none was a significant predictor of female defense behavior except male behavior. That is, the number of swoops [ $F(1,26) = 15.79$ ,  $P = 0.001$ ] and hits [ $F(1,26) = 8.15$ ,  $P = 0.01$ ] at the predator were positively correlated between males and females, suggesting that pairs were coordinated in their nest defense effort.

## Discussion

Because males and females share most of their genes, a response to natural or sexual selection in one sex frequently gives rise to a correlated response in the other. If that response involves opposing fitness effects on males and females, one sex may, at least initially, constrain the evolution of the other. We tested what the response to selection on testosterone-mediated traits in a male songbird might be for females by elevating plasma testosterone (T) levels in females and maintaining them at their seasonal peak levels. We induced several significant phenotypic changes. Captive T-females weighed less than C-females, were less likely to spontaneously develop a brood patch, and delayed molt. Free-living T-females delayed the onset of egg laying and produced eggs with more yolk testosterone relative to C-females. There were no differences in egg size, clutch size, incubation, or nest defense behavior between the two treatments. We have shown previously that elevated T increases activity rate and induces male-like song in female juncos, both of which decrease attractiveness to males (Parker-Renga et al., in preparation), and that T increases corticosterone (McGlothlin et al., in press; Zysling et al., in preparation) and aggressive behavior that

promotes higher social status in females (Duffy et al., in preparation).

Some of the effects of elevated T that we observed in female juncos are the same as those observed in males. Ketterson et al. (1991) and Clotfelter et al. (2001) report that elevated T decreases body mass of males. Nolan et al. (1992) and K. J. Jones (in Ketterson et al., 2001) report inhibition of prebasic molt in males implanted with T. The only other trait measured in the present study that is also expressed by males is nest defense. Cawthorn et al. (1998) found that male juncos with elevated T were slower to return to their nests during predator presentation trials than were C-males, but the intensity of male nest defense (swoops, hits, nest checks) did not differ with hormone treatment. In the current study, observations began when females resumed incubation (latency to respond = 0). As was found with males, hormone treatment did not affect intensity of female nest defense, indicating that T had a similar lack of effect in both sexes.

Some phenotypic effects of T in females have no analog in males. We found no differences due to hormone treatment in female incubation (time to return to nest after disturbance and proportion of time spent incubating), which was unexpected considering that elevated T increases activity rates and food consumption in male juncos and other birds (Clotfelter et al., 2001; Lynn et al., 2000; Wikelski et al., 1999). We monitored female incubation for only a fraction of the total incubation period, but previous research on MLBS juncos suggests that temporal patterns of incubation vary little across the incubation period (J.M. Gaudioso and J.W. McGlothlin, unpublished data). Although we know of no other studies that have manipulated T and monitored incubation in female birds, elevated T is known to disrupt incubation in males of polyandrous species (Oring et al., 1989) and species in which both males and females incubate (Alonso-Alvarez, 2001; MacDonald et al., 2001; Van Roo et al., 2003; but see Buntin, 1996, for review of studies on ring doves *Streptopelia risoria*). In addition, the administration of the anti-androgen flutamide accelerates the onset of incubation in males of some polyandrous species (Oring and Fivizzani, 1991).

It is interesting that female parental behavior is relatively insensitive to the effects of elevated T, when T has robust, negative effects on incubation and provisioning behavior in males (De Ridder et al., 2000; Hegner and Wingfield, 1987; Ketterson et al., 1992). Lynn et al. (2002) recently reported that male chestnut-collared longspurs (*Calcarius ornatus*) were insensitive to T manipulations during some portions of the nesting cycle. They hypothesized that insensitivity may have evolved because male parental care is critical for reproductive success in this species. Our observations are consistent with this hypothesis. Because males do not incubate, maintenance of the female's incubation schedule is essential for junco reproduction; thus, selection might favor dissociation between testosterone and incubation behavior in this species. Lynn et al.'s (2002) hypothesis

is not supported, however, by the data from polyandrous species in which only males incubate and T is highly disruptive to incubation behavior (Oring and Fivizzani, 1991; Oring et al., 1989). Importantly, the insensitivity hypothesis does not address proximate mechanisms underlying insensitivity, and future studies should address how females are insulated from the often disruptive effects of testosterone on incubation behavior.

Female juncos with elevated T took longer to begin laying eggs following nest completion than did C-females. This delayed egg laying may reflect abnormal aggression among females vying for preferred nesting sites as has been reported for females of polygynous species (Harding, 1983; Searcy, 1988). Elevated T may also delay egg production by decreasing female protein reserves, but evidence for increased metabolic rate in T-implanted songbirds is weak (DeViche, 1992; Lynn et al., 2000; Wikelski et al., 1999). Is delayed egg laying significant? Numerous studies report seasonal declines in nest success and juvenile recruitment (e.g. Clotfelter and Yasukawa, 1999; Price et al., 1988). It is doubtful that a 1-day delay would impose a fitness cost if most or all junco nests built early in the season were successful, but in fact large numbers succumb to predators. Thus, many females must re-nest repeatedly, and for some, the breeding season ends before they have reproduced. Additionally, clutch size diminishes as the season advances (Nolan et al., 2002). Together, these considerations of re-nesting and a seasonal decline in clutch size could make delayed egg-laying costly. In male juncos, testosterone likely accelerates the onset of reproduction in the spring by depleting fat reserves (Ketterson et al., 1991); thus, the contrast of acceleration in males and delay in females provides some evidence for the antagonistic selection hypothesis.

Another phenotypic effect of elevated T we observed in female juncos is increased allocation of T to egg yolks. Maternally derived androgens have been demonstrated to vary among eggs within a clutch in many bird species. Most studies have indicated that increases in egg yolk testosterone with laying order function beneficially to promote mass gain and muscle development of later, asynchronously hatched offspring (Lipar and Ketterson, 2000; Schwabl, 1996). Levels of egg yolk T have been positively correlated with subsequent social rank in juvenile birds (Schwabl, 1993), but other studies have suggested costs associated with T (Sockman and Schwabl, 2000). Thus, it is not clear whether the conversion of excess plasma T into egg yolk T would be advantageous for females with elevated T levels, particularly as the interests of parents and offspring with respect to brood reduction are not always congruent (e.g. Mock and Parker, 1998). It is also important to point out that increases in junco egg yolk T were not coupled with increases in estradiol, which has been shown to affect sexual differentiation in avian embryos (Balthazart and Adkins-Regan, 2002).

Brood patch formation was also significantly affected by testosterone; T-females were less likely than C-females to

develop patches spontaneously when exposed to long day lengths in captivity. It is possible that T inhibited brood patch formation via interactions with prolactin (PRL; Buntin, 1996), but research on the effects of T and PRL on parental behavior of male juncos by Schoech et al. (1998) does not suggest this as the probable mechanism. Because T-females in the field were observed to have fully developed brood patches when they were caught during the breeding season, we suggest that T may raise the threshold for reproduction, causing females to require more stimulation from the social, biotic, or abiotic environment before they are able to reproduce. In captivity, where many environmental stimuli that support reproduction were missing, most fundamentally the presence of a male, most T-females failed to develop a brood patch. In the field, they developed brood patches, but other aspects of reproduction (egg laying) proceeded more slowly. This is an area that needs much additional research.

We have shown previously that elevated T makes male juncos more attractive to females (Enstrom et al., 1997) and increases their ability to obtain extra-pair matings (Raouf et al., 1997). There are significant costs to males, however, including decreased survival (Reed et al., submitted for publication), decreased immune system function (Casto et al., 2001), and inhibition of molt (Nolan et al., 1992). In the current study, we were interested in whether elevated T in males might be indirectly selected against by fitness costs borne by the female progeny of high-T males. Correlated selection presupposes that T levels between males and females are linked and that males with high T produce daughters with high T. Unfortunately, interest in androgens in female birds is relatively recent, and there are few data to address this key question. Wingfield et al. (2000) found that sex differences in plasma T are proportional to the degree of behavioral and morphological sexual dimorphism across a range of bird species, and Sandell et al. (in preparation) found some evidence for positive correlations between male and female maximal plasma T in a multi-species study. This area is ripe for additional research, some of which is being carried out by our research group.

When we elevated T in females, we found that they responded like males (suggesting concordant selection) by decreasing their body mass and delaying their molt. Unlike males, however, high T did not cause females to alter their parental behavior (incubation, nest defense). Of the female traits that have no analog in males, elevated T interfered with brood patch formation, prolonged the interval between nest completion and onset of laying, and increased the amount of T that females deposited in their egg yolks. It is premature to speculate about the net cost or benefit of these phenotypic effects, but selection on female testosterone levels likely represents a balance between levels needed to maintain sufficient intrasexual aggression early in the breeding season without delaying reproduction or compromising attractiveness to potential mates (Harding, 1983; Hausberger et al., 1995; Nespor et al., 1996; Searcy,

1988). Thus, whether selection on testosterone levels of male songbirds is affected by consequences of elevated T for females awaits further investigation.

## Acknowledgments

Thanks to Ben Elitzur, Tim Greives, Katie Hill, Joel McGlothlin, Wendy Wolf, and Charles Ziegenfus for assistance at MLBS and KFBO. Thanks to Maria Sandell for stimulating discussion about females and testosterone. Funding from the National Science Foundation was provided to E.D.K. and V.N. Jr. (IBN 9701334, 0216091) and to MLBS (DBI 0097249). Additional funds were provided by Providence College and the Mountain Lake Biological Station of the University of Virginia. We gratefully acknowledge landowners (Dolinger family, White Pine Lodge, and Mountain Lake Hotel and Resort) who allowed us to use their property.

## References

- Alonso-Alvarez, C., 2001. Effects of testosterone implants on pair behaviour during incubation in the yellow-legged gull *Larus cachinnans*. *J. Avian Biol.* 32, 326–332.
- Balthazart, J., 1983. Hormonal correlates of behavior. In: Farmer, D.S., King, J.R., Parkes, K.C. (Eds.), *Avian Biology*, vol. 7. Academic Press, New York, pp. 221–365.
- Balthazart, J., Adkins-Regan, E., 2002. Sexual differentiation of brain and behavior in birds. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), *Hormones, Brain and Behavior*, vol. 4. Academic Press, San Diego, pp. 223–302.
- Buntin, J.D., 1996. Neural and hormonal control of parental behavior in birds. In: Rosenblatt, J.S., Snowdon, C.T. (Eds.), *Advances in the Study of Behavior*, vol. 25. Academic Press, San Diego, pp. 161–213.
- Casto, J.M., Nolan Jr., V., Ketterson, E.D., 2001. Steroid hormones and immune function: experimental studies in wild and captive dark-eyed juncos (*Junco hyemalis*). *Am. Nat.* 157, 408–420.
- Cawthorn, M.J., Morris, D.L., Ketterson, E.D., Nolan Jr., V., 1998. Influence of experimentally elevated testosterone on nest defence in dark-eyed juncos. *Anim. Behav.* 56, 617–621.
- Clotfelter, E.D., Yasukawa, K., 1999. Impact of brood parasitism by brown-headed cowbirds on the reproductive success of red-winged blackbirds. *Condor* 101, 105–114.
- Clotfelter, E.D., Nolan Jr., V., Ketterson, E.D., 2001. The effects of experimentally elevated testosterone and food deprivation on food consumption and prey size preferences in male dark-eyed juncos (*Junco hyemalis*, Emberizidae: Passeriformes). *Ethology* 107, 439–449.
- De Ridder, E., Pinxten, R., Eens, M., 2000. Experimental evidence of a testosterone-induced shift from paternal to mating behavior in a facultatively polygynous songbird. *Behav. Ecol. Sociobiol.* 49, 24–30.
- De Ridder, E., Pinxten, R., Mees, V., Eens, M., 2002. Short- and long-term effects of male-like concentrations of testosterone on female European starlings (*Sturnus vulgaris*). *Auk* 119, 487–497.
- Deviche, P., 1992. Testosterone and opioids interact to regulate feeding in a male migratory songbird. *Horm. Behav.* 26, 394–405.
- Drea, C., Place, N., Weldele, M., Coscia, E., Licht, P.S., Glickman, S.E., 2002. Exposure to naturally circulating androgens during foetal life incurs direct reproductive costs in female spotted hyenas, but is prerequisite for male mating. *Proc. R. Soc. London, B* 269, 1981–1987.
- Duffy, D.L., McGlothlin, J., Ketterson, E.D., in preparation. Does social status influence mate preference in dark-eyed juncos (*Junco hyemalis*)? *Anim. Behav.*
- Elekovich, M.M., 2000. Female song sparrow, *Melospiza melodia*, response to simulated conspecific and heterospecific intrusion across three seasons. *Anim. Behav.* 59, 551–557.
- Enstrom, D.E., Ketterson, E.D., Nolan Jr., V., 1997. Testosterone and mate choice in the dark-eyed junco. *Anim. Behav.* 54, 1135–1146.
- Harding, C.F., 1983. Hormonal influences on avian aggressive behavior. In: Svare, B.B. (Ed.), *Hormones and Aggressive Behavior*. Plenum, New York, pp. 435–467.
- Hausberger, M., Henry, L., Richard, M.A., 1995. Testosterone-induced singing in female European starlings (*Sturnus vulgaris*). *Ethology* 99, 193–208.
- Hegner, R.E., Wingfield, J.C., 1987. Effects of experimental manipulation of testosterone levels on parental investment and breeding success in male house sparrows. *Auk* 104, 462–469.
- Hillgarth, N., Wingfield, J.C., 1997. Parasite-mediated sexual selection: endocrine aspects. In: Clayton, D.H., Moore, J. (Eds.), *Host-Parasite Evolution*. Oxford Univ. Press, Oxford, pp. 78–104.
- Ketterson, E.D., Wolf, L., Ziegenfus, C., Dufty Jr., A.M., Ball, G., Johnsen, T., 1991. Testosterone and avian life histories: the effect of experimentally elevated testosterone on corticosterone, body mass, and annual survivorship of male dark-eyed juncos. *Horm. Behav.* 25, 489–503.
- Ketterson, E.D., Nolan Jr., V., Wolf, L., Ziegenfus, C., 1992. Testosterone and avian life histories: effects of experimentally elevated testosterone on behavior and correlates of fitness in the dark-eyed junco (*Junco hyemalis*). *Am. Nat.* 140, 980–989.
- Ketterson, E.D., Nolan Jr., V., Casto, J.M., Buerkle, C.A., Clotfelter, E., Grindstaff, J.L., Jones, K.J., Lipar, J.L., McNabb, F.M.A., Neudorf, D.L., Parker-Renga, I.P.R., Schoech, S.J., Snajdr, E., 2001. Testosterone, phenotype and fitness: a research program in evolutionary behavioral endocrinology. In: Chaturvedi, C. (Ed.), *Avian Endocrinology*. Narosa Publishing, New Delhi, India, pp. 19–38.
- Langmore, N., Cockrem, J., Candy, E., 2002. Competition for male reproductive investment elevates testosterone levels in female dunlocks, *Prunella modularis*. *Proc. R. Soc. London, B* 269, 2473–2478.
- Lank, D.B., Coupe, M., Wynne-Edwards, K.E., 1999. Testosterone-induced male traits in female ruffs (*Philomachus pugnax*): autosomal inheritance and gender differentiation. *Proc. R. Soc. London B* 266, 2323–2330.
- Lipar, J.L., Ketterson, E.D., 2000. Maternally derived yolk testosterone enhances the development of the hatching muscle in the red-winged blackbird *Agelaius phoeniceus*. *Proc. R. Soc. London, B* 267, 2005–2010.
- Lipar, J.L., Ketterson, E.D., Nolan Jr., V., Casto, J.M., 1999. Egg yolk layers vary in the concentration of steroid hormones in two avian species. *Gen. Comp. Endocrinol.* 115, 220–227.
- Lynn, S.E., Houtman, A.E., Weathers, W.W., Ketterson, E.D., Nolan Jr., V., 2000. Testosterone increases activity but not daily energy expenditure in captive male dark-eyed juncos, *Junco hyemalis*. *Anim. Behav.* 60, 581–587.
- Lynn, S.E., Hayward, L.S., Benowitz-Fredericks, A.M., Wingfield, J.C., 2002. Behavioral insensitivity to supplementary testosterone during the parental phase in the chestnut-collared longspur (*Calcarius ornatus*). *Anim. Behav.* 63, 780–795.
- MacDonald, P.G., Buttemer, W.A., Astheimer, L.B., 2001. The influence of testosterone on territorial defense and parental behavior in male free-living rufous whistlers *Pachycephala rufiventris*. *Horm. Behav.* 39, 185–194.
- McGlothlin, J.W., Neudorf, D.L.H., Casto, J.M., Nolan Jr., V., Ketterson, E.D., in press. Elevated testosterone reduces choosiness in female dark-eyed juncos (*Junco hyemalis*): evidence for a hormonal constraint on sexual selection? *Proc. R. Soc. London, B*.
- Mock, D.W., Parker, G.A. (Eds.), 1998. *The Evolution of Sibling Rivalry*. Oxford Univ. Press, Oxford.
- Nespor, A.A., Lukazewicz, M.J., Dooling, R.J., Ball, G.F., 1996. Testosterone induction of male-like vocalizations in female budgerigars (*Melopsittacus undulatus*). *Horm. Behav.* 30, 162–169.

- Nolan Jr., V., 1978. The Ecology and Behavior of the Prairie Warbler *Dendroica discolor*. American Ornithologists' Union, Washington, DC.
- Nolan Jr., V., Ketterson, E.D., Ziegenfus, C., Cullen, D.P., Chandler, C.R., 1992. Testosterone and avian life histories: effects of experimentally elevated testosterone on prebasic molt and survival in male dark-eyed juncos. *Condor* 94, 364–370.
- Nolan Jr., V., Ketterson, E.D., Cristol, D.A., Rogers, C.M., Clotfelter, E.D., Titus, R.C., Schoech, S.J., Snajdr, E., 2002. Dark-eyed junco. In: Poole, A., Gill, F. (Eds.), *The Birds of North America*, vol. 716. The Birds of North America, Inc., Philadelphia, PA.
- Oring, L.W., Fivizzani, A.J., 1991. Reproductive endocrinology of sex-role reversal. *Acta Congressus Internationalis Ornithologici*, vol. XX. New Zealand Ornithological Trust Board, Wellington, NZ, pp. 2072–2080.
- Oring, L.W., Fivizzani, A.J., El Halawani, M.E., 1989. Testosterone-induced inhibition of incubation in the spotted sandpiper (*Actitis macularia* [sic]). *Horm. Behav.* 23, 412–423.
- Price, T., Kirkpatrick, M., Arnold, S.J., 1988. Directional selection and the evolution of breeding date in birds. *Science* 240, 798–799.
- Raouf, S.A., Parker, P.G., Ketterson, E.D., Nolan Jr., V., Ziegenfus, C., 1997. Testosterone affects reproductive success by influencing extra-pair fertilizations in male dark-eyed juncos (*Aves: Junco hyemalis*). *Proc. R. Soc. London, B* 264, 1599–1603.
- Reed, W. L., Clark, M. E., Parker, P. G., Raouf, S.A., Arguedas, N., Monk, D.S., Snajdr, E.A., Nolan Jr., V., Ketterson, E.D., submitted for publication. Physiological effects on demography: a long-term experimental study of testosterone's effects on fitness. *Am. Nat.*
- Schoech, S.J., Ketterson, E.D., Nolan Jr., V., Sharp, P.J., Buntin, J.D. 1998. The effect of exogenous testosterone on parental behavior, plasma prolactin, and prolactin binding sites in dark-eyed juncos. *Horm. Behav.* 34, 1–10.
- Schoech, S.J., Ketterson, E.D., Nolan Jr., V., Sharp, P.J., Buntin, J.D., 1998. The effect of exogenous testosterone on parental behavior, plasma prolactin, and prolactin binding sites in dark-eyed juncos. *Horm. Behav.* 34, 1–10.
- Schwabl, H., 1993. Yolk is a source of maternal testosterone for developing birds. *Proc. Natl. Acad. Sci. U. S. A.* 90, 11439–11441.
- Schwabl, H., 1996. Maternal testosterone in the avian egg enhances post-natal growth. *Comp. Physiol. Biochem.*, A 114, 271–276.
- Searcy, W.A., 1988. Do female red-winged blackbirds limit their own breeding densities? *Ecology* 69, 85–95.
- Shuster, S., Wade, M.J., 2003. *Mating Systems and Strategies*. Princeton Univ. Press, Princeton, NJ.
- Sockman, K.W., Schwabl, H., 2000. Yolk androgens reduce offspring survival. *Proc. R. Soc. London, B* 267, 1451–1456.
- Staub, N.L., De Beer, M., 1997. The role of androgens in female vertebrates. *Gen. Comp. Endocrinol.* 108, 1–24.
- Van Roo, B.L., Ketterson, E.D., Sharp, P.J., 2003. Reproductive behavior, testosterone, and prolactin in two passerine species that differ in paternal care: the blue-headed vireo and the red-eyed vireo. *Horm. Behav.* 44, 435–441.
- West-Eberhard, M.J., 2003. *Developmental Plasticity and Evolution*. Oxford Univ. Press, Oxford, UK.
- Wikelski, M., Lynn, S.E., Breuner, C., Wingfield, J.C., Kenagy, G.J., 1999. Energy metabolism, testosterone and corticosterone in white-crowned sparrows. *J. Comp. Physiol.*, A 185, 463–470.
- Wingfield, J.C., Jacobs, J.D., Tramontin, A.D., Perfito, N., Meddle, S., Maney, D.L., Soma, K., 2000. Toward an ecological basis of hormone-behavior interactions in reproduction of birds. In: Wallen, K., Schneider, J.E. (Eds.), *Reproduction in Context: Social and Environmental Influences on Reproduction*. MIT Press, Cambridge, MA, pp. 85–128.