Available online at www.sciencedirect.com
SCIENCE DIRECT
Hormones and Behavior 50 (2006) 200–207
Hormones and Behavior
www.elsevier.com/locate/yhbeh

Behavioral and physiological responses to experimentally elevated testosterone in female dark-eyed juncos (*Junco hyemalis carolinensis*)

Devin A. Zysling [a,*], Timothy J. Greives [a], Creagh W. Breuner [b], Joseph M. Casto [c], Gregory E. Demas [a], Ellen D. Ketterson [a]

[a] *Department of Biology, Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington, IN 47405, USA*
[b] *Integrative Biology, University of Texas at Austin, Austin, TX 78712, USA*
[c] *Department of Biological Sciences, Illinois State University, Normal, IL 61790, USA*

Received 2 December 2005; revised 28 February 2006; accepted 3 March 2006
Available online 4 May 2006

Abstract

Testosterone mediates the expression of many fitness-related traits in male vertebrates and is thought to account for numerous sex differences in trait expression. Testosterone is also secreted by females; however, far less is known regarding its effects on female physiology and behavior. Using a bird species in which the effects of testosterone on males are well characterized, the dark-eyed junco (*Junco hyemalis*), we tested whether an increase in exogenous testosterone in females would alter the phenotypic expression of a suite of behavioral and physiological traits. We found that increased testosterone levels in female dark-eyed juncos led to decreased cell-mediated immune function and increased intrasexual aggression, hypothalamo-pituitary–adrenal (HPA) axis responsiveness, baseline corticosterone and corticosterone-binding globulin (CBG) levels. Furthermore, immunosuppression following testosterone implantation was negatively correlated with total and free testosterone but did not appear to be related to either total or free corticosterone. These results demonstrate that the phenotypic impact of elevated testosterone is not confined to males in dark-eyed juncos, and that the impact in adults can be similar in males and females. We discuss these results in the context of potential endocrine–immune interactions and the evolution of sexual dimorphism.

Keywords: Steroid hormone; Testosterone; Corticosterone; Immune; Phytohemagglutinin A; Hormonal correlation; Endocrine–immune interaction; Corticosterone-binding globulin; Sexual dimorphism

Introduction

The effects of steroid hormones on morphology, physiology and behavior have been well documented (Jones et al., 1972; Wada, 1986; Adkins-Regan, 1987), but until recently, few studies have examined the role of male-typical steroids in adult female vertebrates (Staub and De Beer, 1997). Furthermore, the physiological mechanisms underlying sex differences in trait expression are not fully understood. Many sexually dimorphic traits are regulated by the gonadal steroid hormone testosterone. Specifically, testosterone is intimately tied to suites of reproductive behaviors and physiology (Balthazart, 1983) that directly affect reproductive success (Wingfield et al., 2001; Ketterson and Nolan, 1999). Steroid hormones, such as testosterone and estradiol, act early in development to organize male- or female-typical phenotypes that are activated by later exposure to sex-specific concentrations of circulating gonadal steroids in adulthood (Balthazart and Ball, 1995). Adult sex differences in trait expression, therefore, may either be due to differences between the sexes in activational levels of a hormone and/or behavioral and physiological insensitivity to a hormone due to organizational effects (Balthazart and Adkins-Regan, 2002).

Although testosterone levels in vertebrates can activate male-typical phenotypes, the presence and action of testosterone is not exclusively confined to one sex. Across most vertebrate taxa, adults of both sexes naturally produce testosterone (Nelson, 2000),

* Corresponding author. Fax: +1 812 855 6705.
E-mail address: dzysling@indiana.edu (D.A. Zysling).

0018-506X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.yhbeh.2006.03.004

and many traits respond to exogenous steroid hormones in males and females during adulthood (Staub and De Beer, 1997; Ketterson et al., 2005). Many documented mechanisms of androgenic action (i.e., neuronal survival and muscle cell proliferation) are not sex-specific, and thus, androgens such as testosterone may play an important role in female development (Nordeen et al., 1985; Joubert and Tobin, 1995; Staub and De Beer, 1997).

Among male birds, secretion of testosterone varies seasonally according to mating system, degree of sexual dimorphism and parental behavior (Wingfield et al., 1990, 2000; Hirschenhauser et al., 2003). Within species, certain fitness-related traits co-vary in expression with endogenous plasma levels of testosterone (Johnsen, 1998; Zuk et al., 1995; Duffy et al., 2000), and experimental elevation of testosterone enhances traits apparently shaped by sexual and natural selection (Hegner and Wingfield, 1987; Folstad and Karter, 1992; Ketterson and Nolan, 1999). For example, in males, testosterone increases the number of mates, male attractiveness to females, as well as success at extra pair fertilizations (Wingfield, 1984; Enstrom et al., 1997; Raouf et al., 1997; Reed et al., in press; Eens et al., 2000).

Female birds also vary with respect to seasonal patterns of testosterone secretion (see Ketterson et al., 2005), and significant levels of testosterone have been documented in many species (Wingfield et al., 2000, Møller et al., 2005). In addition, among individuals, testosterone sometimes co-varies with phenotype, e.g., immune function and aggressive behavior (Duffy et al., 2000; Langmore et al., 2002). Female testosterone has been implicated in many different physiological and behavioral functions (Staub and De Beer, 1997; DeRidder et al., 2002; Ketterson et al., 2005), yet the functional significance of circulating testosterone in female birds beyond its role in the synthesis of estrogens often remains unclear. Recent evidence has demonstrated that testosterone affects several reproductive traits such as delaying egg-laying and ovulation, decreasing clutch size and increasing courtship and vocal behavior (Hausberger et al., 1995; Nespor et al., 1996; Lank et al., 1999; Clotfelter et al., 2004; Rutkowska et al., 2005). However, few studies have addressed experimentally the effects of testosterone on traits specifically related to survival.

In this study, we examined the impact of exogenous elevation of testosterone on the behavior and physiology of female dark-eyed juncos. Using implants to maintain testosterone at the peak seasonal level naturally observed in females, we measured a suite of traits that have previously been measured in males. Specifically, we quantified cell-mediated immune function, intrasexual aggression, HPA axis responsiveness, baseline corticosterone, corticosterone-binding globulin (CBG) levels, total and free steroid hormone titers (both testosterone and corticosterone) and the relationship between cell-mediated immune function and steroid hormone levels. For each phenotypic measure, we asked whether hormone-dependent alterations in expression would occur and if these changes were similar to effects previously documented in males of this species. Similar responses in males and females would suggest similar potential for activational effects of testosterone on the phenotype of both sexes. Dissimilar responses might indicate that organizational events have rendered females less sensitive to testosterone than males. In terms of possible evolutionary responses to any changes in the selective regime, similar sensitivity would predict correlated responses in both sexes to any environmental changes; whereas sex differences in sensitivity would suggest that male and female traits would be free to evolve independently (see Ketterson et al., 2005; Møller et al., 2005).

Methods

Study species

The dark-eyed junco (*Junco hyemalis carolinensis*) is a weakly dimorphic species in which males are territorial during the breeding season (Nolan et al., 2002). The sexes form socially monogamous pair bonds while frequently producing young through extrapair fertilizations (~35%) (Ketterson et al., 1998; Raouf et al., 1997). Both sexes vary seasonally in circulating levels of testosterone according to stage of reproduction with the peak occurring in April just prior to breeding (Ketterson et al., 2005).

Subjects and housing

Using baited mist nets, we captured 32 adult females in the vicinity of the University of Virginia's Mountain Lake Biological Station (MLBS) in Giles County, Virginia (37°22′N, 80°32′W) between April 20 and May 25, 2003. We housed subjects individually in outdoor aviaries (0.61 m × 1.14 m × 2.44 m) at MLBS and allowed ad libitum access to water and millet, supplemented daily with approximately 5–7 mealworms. All procedures were approved by the Bloomington Institutional Animal Care and Use Committee.

Hormone treatment and blood collection

On May 28, we randomly assigned subjects to the experimental or control treatment. We anesthetized animals with methoxyflurane (Metofane, Pitman-Moore, Inc.) and subcutaneously (left flank) implanted one 7-mm-long section of Silastic tubing (Dow Corning; 1.47-mm inner diameter, 1.95-mm outer diameter). Implants were filled with 5 mm of crystalline testosterone (Sigma Chemical, Sigma-Alderich, St. Louis, MO) (T-females) or left empty (C-females). The testosterone dosage was selected to maintain testosterone levels at approximately the natural early spring peak level seen in females for an extended period of time, as demonstrated by previous experiments (e.g., Clotfelter et al., 2004). All implants remained in place and were removed on July 21.

To determine testosterone concentrations during each stage of the study: before implants were inserted (April 20–May 27), while implants were in place (May 28–July 21), and after implants had been removed (July 21–July 28), we collected blood samples (200 μl from alar vein) on, May 27, July 5 and July 28. We measured the duration of capture and bleeding time with a stopwatch, centrifuged the blood for 5 min and stored the separated plasma at −20°C until we performed enzyme immunoassays (EIA).

Immune tests

Cell-mediated immunity was assessed with a cutaneous delayed-type contact sensitivity test that quantifies secondary immune response to phytohemagglutinin (PHA; Sigma L-8754) (procedures modified from Lochmiller et al., 1993; Smits et al., 1999; see Smits et al., 2001, Casto et al., 2001). On 28 June, we primed each bird with a subcutaneous injection into the right scapular apterium (0.25 mg of PHA in 50 μl phosphate-buffered saline, PBS). On 2 July, we measured the thickness of the right wing web to the nearest 0.025 mm with a pressure sensitive thickness gauge (Mitutoyo model 7326). Immediately thereafter, we made a subcutaneous challenge injection (again 0.25 mg of PHA in 50 μl of PBS) in the right wing web. At 24 and 48 h post-challenge (± 5 min), wing web thickness was remeasured, and swelling in response to the PHA challenge was calculated by subtracting the prechallenge wing web thickness from the post-challenge thickness.

Behavioral testing

To quantify aggressive behavior, we used a resident–intruder model of aggression. On 6–11 July, between the hours of 7:00 and 11:00, control (nonimplanted) female "intruder" individual juncos, caught for this purpose and previously group-housed, were introduced singly into each subject's (the "resident's") compartment. Intruders were randomly selected and used only once in any day. The resident was the focal individual and its behaviors were recorded. We categorized behavior as aggressive vocalizations ("kews"), displacements or escalated aggressive interactions (chases and physical contact) (see Nolan et al., 2002 for descriptions) and summed each type of behavior during a 15-min trial. The latter two observed behaviors (chase and physical contact) are generally low frequency, and thus, we grouped them into a composite measurement.

Corticosterone and corticosterone binding globulin sampling procedures

To quantify the effects of elevated testosterone on the adrenocortical response to stressors, we measured response to handling. Individuals were caught with a butterfly net between 7:00 and 11:00 on 18–20 July, and a baseline blood sample of 80 μl was obtained within 3 min of capture. Individuals were placed in a paper holding bag, and 100 μl blood samples were taken again at 15 and 30 min post-capture. All samples were immediately put on ice until plasma was separated by centrifugation and stored at −20 °C. In addition, 12 μl of plasma drawn for the baseline sample was set aside for later assay of corticosteroid-binding globulin (CBG).

Testosterone enzyme immunoassay (EIA)

We determined testosterone concentrations using a commercial enzyme immunoassay (EIA) kit (#901-065; Assay Designs Inc., Ann Arbor, MI), as described in Clotfelter et al. (2004). Briefly, each 20 μl plasma sample was diluted 6-fold in distilled water. Approximately 2000 cpm of [^{3}H] testosterone (NET-553; New England Nuclear Corp., Boston, MA) was added to allow the calculation of recoveries following extraction with diethyl ether. The extracts were evaporated then redissolved in 50 μl of 100% ethanol and diluted to 350 μl with assay buffer; 100 μl for recoveries and 100 μl as samples in duplicate. Due to the number of plasma samples, two assays were performed to determine testosterone concentrations. Intra-assay variation was 11.2% and 24.6%, and inter-assay variation was 10.3%. Percent recovery was 95.5% ± 3.9% and 96.8% ± 2.4%. The manufacturer's instructions were otherwise followed throughout. Testosterone concentrations were determined with the aid of a four parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc., Hercules, CA), and concentrations were corrected for incomplete recovery.

Corticosterone radioimmunoassay (RIA)

Plasma corticosterone concentrations were determined via RIA following the methods of Wingfield et al. (1992) (also see Schoech et al., 1999). Briefly, samples were allowed to equilibrate overnight with 2000 cpm of corticosterone for determination of recoveries. Each sample was extracted with 4.0 ml of anhydrous diethyl ether, dried under nitrogen and resuspended in phosphate-buffered saline with 1% gelatin (PBS-G). Samples were assayed in duplicate, and assay values were corrected for plasma volume and individual recoveries after extraction. Due to the number of plasma samples, two assays were performed to determine corticosterone concentrations. Intra-assay variation was 8.3% and 21.2%, and inter-assay variation was 4.7%. Percent recovery was 90.1% ± 4.5% and 81.1% ± 4.6%.

Corticosteroid-binding globulin assay

Plasma collected to determine baseline corticosterone levels was also used to measure corticosteroid-binding globulin (CBG) affinity and capacity via a single-point binding assay. Plasma was stripped of endogenous steroid in a 20 min room-temperature incubation with 2 vol dextran-coated charcoal solution (0.1% dextran, 1% Norit A charcoal in 50 mM Tris). Plasma was maintained at 4°C at all times outside of this stripping process. Plasma samples were assayed at a final dilution of 1:1089; assays were performed at 4°C in 50 mM Tris buffer and terminated after 2 h by rapid vacuum filtration. Glass fiber filters were soaked in 25 mM Tris with 0.3% polyethyleneimine for 1 h before filtering. Filters were rapidly rinsed with 9 ml ice-cold 25 mM Tris (3 rinses of 3 ml each). Point sample analysis was run on individual plasma samples using 20 nM [^{3}H] corticosterone in the presence or absence of 1 μM unlabeled corticosterone. With a K_d of 1.67 ± 0.07 (saturation binding analysis performed in the Breuner laboratory; affinity estimates similar to that published for juncos in Deviche et al., 2001), this concentration of [^{3}H] corticosterone will occupy approximately 92% of binding sites.

Statistical analyses

To calculate CBG concentrations, binding parameter estimates from the saturation analysis were obtained by fitting untransformed data to appropriate equations using iterative, least squares curve-fitting techniques (GraphPad Prism, San Diego, CA). For analysis, CBG data were corrected to 100% (B_{max}) and compared with an independent samples t test. Free corticosterone titers were estimated using the mass-action based equation of Barsano and Baumann (1989), incorporating total corticosterone concentrations, CBG capacity (B_{max}) and the K_d of corticosterone for CBG (1.67 nM). To estimate free testosterone levels, the same equation was used, incorporating total testosterone levels, the K_i of T for CBG (23 nM; an affinity estimate published in Deviche et al., 2001), and a modified CBG capacity: the fraction of CBG bound by corticosterone (approximately 13%) was subtracted from Bmax, the remaining CBG capacity was used in the equation. There is currently no mathematical model for estimating free levels of two hormones at once. Hence, sequential estimation, starting with the hormone with highest affinity, is currently the best method available (used in Deviche et al., 2001).

All statistical tests were performed using SPSS 11.5 (Chicago, IL). Data were checked for normality and homogeneity of variance, and those data nonnormally distributed were analyzed using nonparametric tests or log-transformed where appropriate. We compared total and free testosterone levels by treatment before and after implantation using an independent-samples t test. To compare treatments for aggressive behavior, we used a Mann-Whitney U test. Differences in wing-web swelling and total and free corticosterone levels were identified by use of a repeated-measure ANOVA with time as the within-subjects variable. Free corticosterone levels were log transformed to correct for heterogeneity of variance. Linear regression was used to assess whether total or free plasma hormone levels, for both corticosterone and testosterone, co-varied significantly with wing web swelling. For all statistical tests, the level of

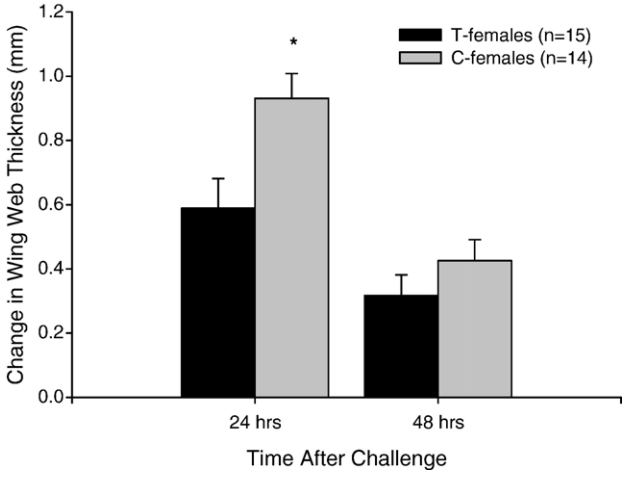

Fig. 1. Cell-mediated immune responses in relation to testosterone implant treatment. Differential wing web thickness measurements are reported (mean ± SE) at 24 and 48 h in response to a PHA challenge. Wing web swelling differed significantly between treatments at 24 but not 48 h, with T-females displaying significantly less swelling than C-females. Significant differences ($P < 0.05$) between treatments are marked with an asterisk (*).

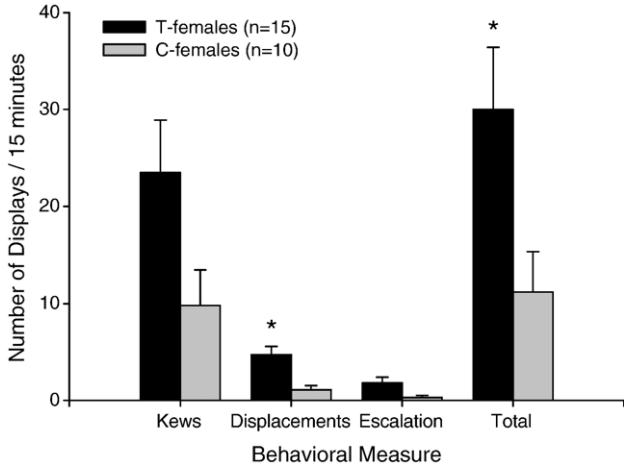

Fig. 2. Effects of testosterone implant treatment on behavioral measures. Number of displays per 15 min are reported (mean±SE) for each measure. During resident–intruder behavioral trials, T-females displayed significantly more displacements and total aggressive behaviors as compared to C-females. There was a trend towards T-females displaying more kews and escalated aggressive encounters (chases and flutter-ups). Significant differences ($P<0.05$) between treatments are marked with an asterisk (*).

significance (α) was set at $P<0.05$, and tests were two-tailed unless otherwise indicated.

Results

Testosterone

Plasma testosterone levels did not differ between treatments before implantation ($t_{18}=0.281$, $P=0.782$; C-females: 1.608±0.174 ng/ml, T-females: 1.693±0.232 ng/ml) but were significantly higher in T-females than C-females after implantation ($t_{26}=5.736$, $P<0.0005$; C-females: 1.26±0.141 ng/ml, T-females: 3.51±0.344 ng/ml) and comparable to peak female levels observed in the field (Ketterson et al., 2005). Free testosterone levels remained significantly elevated in T-females ($t_{26}=-2.834$, $P=0.009$; C-females: 0.139±0.022 ng/ml, T-females: 0.247±0.031 ng/ml) in spite of elevated CBG. Bleeding time was not significantly correlated with testosterone concentration ($P>0.05$).

Immune tests

T-females responded less than C-females to the immune challenge; there were main effects of time ($F_{1,27}=103.796$, $P<0.0005$; Fig. 1) and treatment ($F_{1,27}=5.070$, $P=0.033$; Fig. 1), and the interaction term was significant ($F_{1,27}=9.333$, $P=0.005$; Fig. 1).

Behavioral testing

T-females demonstrated more total aggressive behaviors as compared to C-females ($U=36.5$, $P=0.031$; Fig. 2). Additionally, T-females displayed significantly more displacements ($U=24.0$, $P=0.04$; Fig. 2). There was a trend towards T-females producing more kews (U=43.0, $P=0.08$; Fig. 2) and escalated aggressive encounters ($U=41.0$, $P=0.062$; Fig. 2) as compared to C-females, but neither of these results was statistically significant.

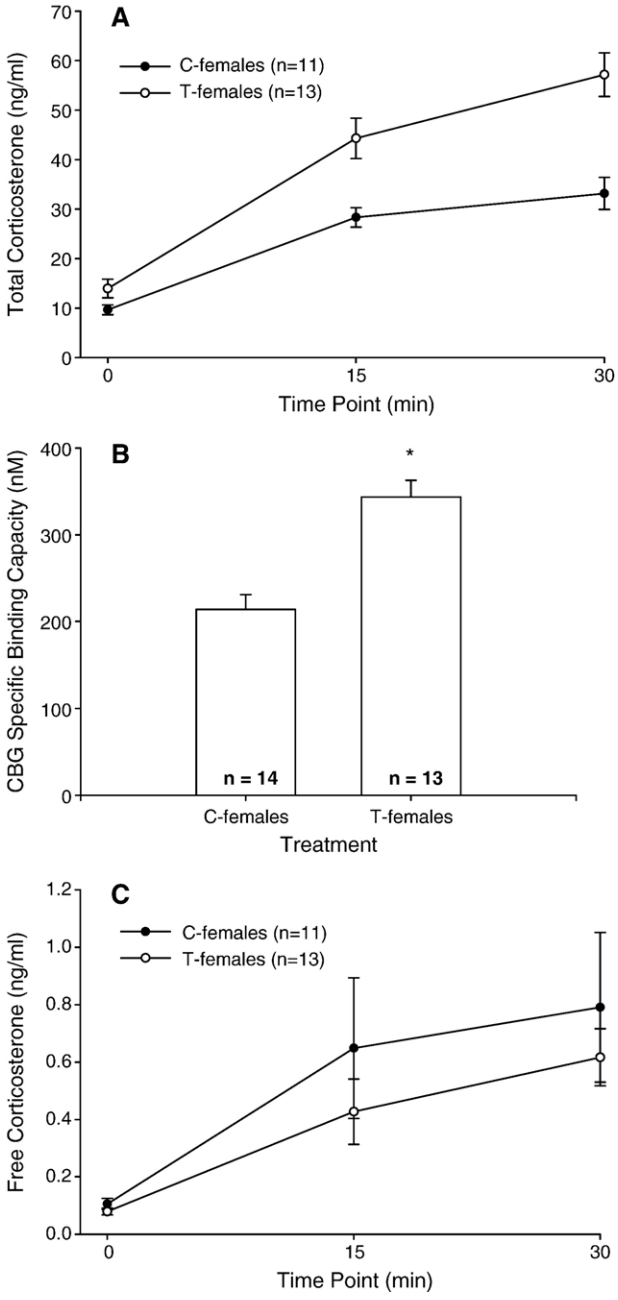

Fig. 3. Effects of testosterone implant treatment on HPA axis responsiveness, including corticosterone release and CBG binding capacity. (A) Total corticosterone levels in response to handling stress. There were significant differences between time points and treatments in regards to total corticosterone levels. Values at each time point are the mean±SE. (B) CBG specific binding capacity (mean±SE) in relation to implant treatment at 0–3 min following handling stress. CBG binding capacity differed significantly between treatments with T-females displaying significantly higher CBG than C-females. Significant differences ($P<0.05$) between treatments are marked with an asterisk (*). (C) Free corticosterone levels in response to handling stress. There were significant differences between time points but not treatments in regards to free corticosterone levels. Values at each time point are the mean±SE.

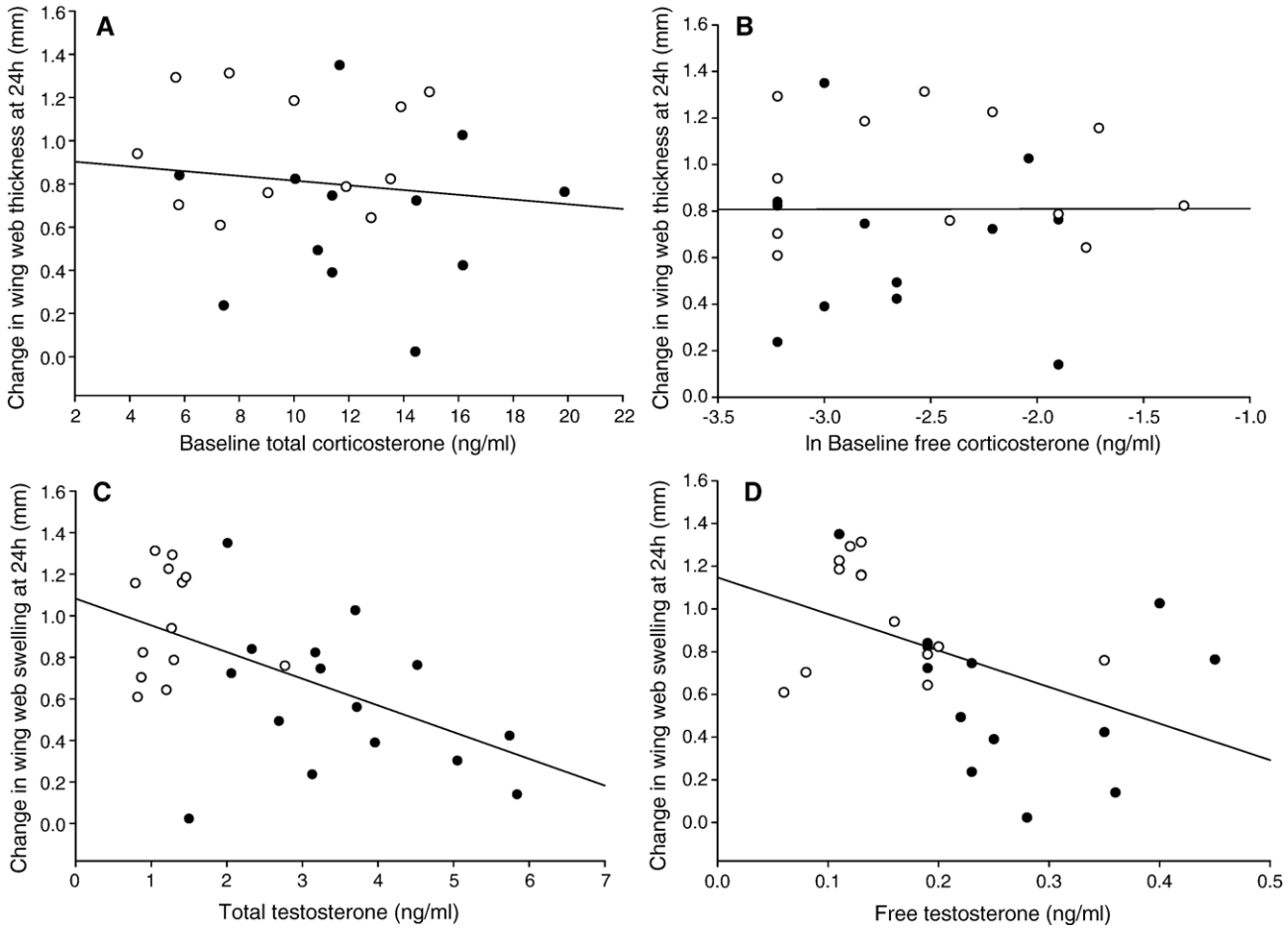

Fig. 4. Effects of steroid hormones on cell-mediated immune function. Wing web swelling at 24 h is presented as a function of (A) total baseline corticosterone, (B) free baseline corticosterone, (C) total testosterone and (D) free testosterone titers in T- and C-females. In each case T-females are denoted by a black circle and C-females are denoted by a white circle. Wing web swelling at 24 h was significantly correlated with free and total testosterone titers, but not free and total corticosterone titers.

Corticosterone and corticosteroid-binding globulin

Corticosterone was significantly affected by time ($F_{2,22}$= 114.480, P<0.0005; Fig. 3A) and treatment ($F_{1,22}$=14.805, P=0.001; Fig. 3A), and the interaction term (time × treatment) was significant ($F_{2,22}$=8.713, P=0.001; Fig. 3A). Time from initial disturbance was not significantly correlated with baseline corticosterone in either pooled or individual treatment groups (P>0.05).

T-females also had significantly higher specific binding of CBG than C-females (t_{25}=5.093, P<0.0005, Fig. 3B). Correcting for CBG, levels of free corticosterone were significantly affected by time ($F_{2,22}$=173.113, P<0.0005; Fig. 3C) but not by treatment ($F_{1,22}$=0.407, P=0.530; Fig. 3C) or time × treatment ($F_{2,22}$=0.263, P=0.770, Fig. 3C).

Steroid hormones and immune function

When treatment groups were combined, total and free corticosterone titers and wing web swelling at 24 h were not significantly correlated (total: R=0.125, P=0.560; Fig. 4A, free: R=0.003, P=0.989; Fig. 4B). However, total and free testosterone titers and wing web swelling at 24 h were significantly and negatively correlated (total: R=0.540, P=0.003; Fig. 4C, free: R=0.483, P=0.012; Fig. 4D).

Discussion

We asked whether traits that are sensitive to testosterone in male dark-eyed juncos were also sensitive in females. We found that increasing testosterone levels in female dark-eyed juncos led to decreased cell-mediated immune function and increases in intra-sexual aggression, baseline corticosterone, HPA axis responsiveness and CBG levels as compared with controls. In addition, we found that cell-mediated immune function was negatively correlated with total and free testosterone but was not related to either total or free corticosterone. The relationship between testosterone and immune function arose in part because females with highly induced values of testosterone produced less robust immune responses, and the reverse was true of unmanipulated females. This trend also appeared to hold true in a zone of overlap in the testosterone levels of testosterone-treated and control females. Within that zone, the points from both treatments fall along a line of similar slope.

The relationship between testosterone and immunity varies depending on the species studied and the immune parameter

investigated suggesting that this relationship is more complex than typically portrayed (e.g., Hasselquist et al., 1999; Deviche and Cortez, 2005). Previous work in male dark-eyed juncos, however, has demonstrated that increased testosterone suppresses cell-mediated and humoral immune function (Casto et al., 2001). Our results also demonstrate that testosterone acts either directly or indirectly to suppress cell-mediated immune function in female juncos, possibly increasing susceptibility to pathogenic infections. The physiological mechanisms by which steroid hormones may suppress immunity remain unclear. Hormones may bind directly to receptors on lymphocytes and inactivate or destroy them, thus decreasing immune responsiveness directly (Sullivan and Wira, 1979; Tanriverdi et al., 2003). Alternatively, they may affect immune function indirectly (see Owen-Ashley et al., 2004) by redistributing energy reserves away from immune function during stress (Apanius, 1998) or reproductive activity (Wedekind and Folstad, 1994). In this study, immune response was correlated with testosterone but not with corticosterone, suggesting that at least for female juncos, the suppressive effect of testosterone may not be mediated via changes in corticosterone. Whether the observed immunosuppression is directly mediated via testosterone, or indirectly via another unknown intermediate factor, remains to be determined.

Corticosterone, like testosterone, is a steroid hormone with pleiotropic effects (Finch and Rose, 1995) that relate to fitness (Wingfield and Romero, 2000). Corticosterone is released by the adrenal cortex in response to "stressors" and potentiates a range of physiological responses including increased gluconeogenesis, reproductive suppression and decreased immune function (Rook, 2000). Importantly, testosterone and corticosterone can modulate one another's secretion and can interact to affect a variety of tissues (Viau, 2002; Seale et al., 2004).

Previous work in male juncos has shown that testosterone elevates total corticosterone and CBG (Klukowski et al., 1997; Schoech et al., 1999) as well as HPA responsiveness to stressors (Schoech et al., 1999). Steroid bound by CBG is often thought to be physiologically inactive (Siiteri et al., 1982); whereas free steroid is available for immediate use at target tissues. In male juncos, testosterone treatment increases both corticosterone and CBG, but free levels of corticosterone are not significantly different between treatments (Breuner and Orchinik, 2002). Both corticosterone and testosterone bind to CBG in juncos (Deviche et al., 2001), so a change in CBG could alter free levels of both hormones. The implant-induced elevation of CBG in this study was large enough to buffer the increase in total corticosterone, but not total testosterone. Hence, free testosterone (but not free corticosterone) was elevated in T-implanted birds.

If free hormone is the biologically active fraction, then it appears that both the immune and the behavioral differences between treatments that we observed are more likely to be due to testosterone than corticosterone. Other possibilities remain, however. For example, steroids bound to CBG can be delivered and released locally at target sites (e.g., at sites of inflammation), and consequently in some situations, total levels of hormone may be as important as free levels in mediating behavior and physiology. Future studies will be needed to resolve the relative importance of bound and free fractions of circulating hormones.

Exogenously elevated testosterone also significantly increased aggressive behavior in females, which might result in costs or benefits to fitness. More aggressive females might acquire higher quality mates or nesting sites or be more active in nest defense (Kral et al., 1996; Karvonen et al., 2000). Alternatively, elevated aggression may increase energy expenditure, the potential for injury, or the risk of predation. Studies have shown that females with higher endogenous levels of testosterone are more likely to win territorial interactions with other females (Langmore et al., 2002 but see Elekonich, 2000) and in red-winged blackbirds female testosterone profiles peaked in accordance with aggression levels during the breeding season (Cristol and Johnsen, 1994). Several studies examining whether testosterone peaks during and/or mediates aggressive interactions, however, have been inconclusive (see Jawor et al., 2006). As with immune function, the response may have been caused by testosterone directly, by corticosterone indirectly or by other indirect effects. In the wild, the outcome of selection on female testosterone levels likely represents a balance between levels that maintain intra-sexual aggression without delaying reproduction, suppressing immune function or compromising attractiveness to potential mates (Hausberger et al., 1995; Nespor et al., 1996; Searcy, 1988).

Natural and sexual selection favor traits that suit each sex to its particular environment (Darwin, 1871; Andersson, 1994), causing the sexes either to converge (Johnson and MacDonald, 2001) or diverge (Karubian and Swaddle, 2001) in phenotype depending on differences in their respective life histories. In addition, for traits that are genetically correlated across the sexes, selection on one sex can generate a correlated response to selection in the other (Falconer and MacKay, 1996; Lande, 1980; Ketterson et al., 2005; Møller et al., 2005). If both sexes benefit from the trait, selection may proceed more rapidly owing to the correlation, but if the fitness consequences are in conflict, then such correlations may retard evolution and limit sexual dimorphism (Lande and Arnold, 1983; Price and Burley, 1993). Such interdependence of the sexes is more likely when the mechanism of trait expression is common to both sexes, as can be the case with hormonally mediated characters.

In dark-eyed juncos, experimental elevation of testosterone in males enhances fitness over control males because, despite lower survivorship, testosterone-treated males have greater success at extra-pair fertilizations (Reed et al., in press). This raises the question of what maintains male testosterone at current levels. The present study demonstrates that the impact of testosterone on physiology and behavior is not limited to males and points to the potential for correlated responses that might act as constraints to the evolution of sexual dimorphism. As stated earlier, sex-limited expression provides an escape from potential constraints imposed by correlated responses and fosters independent evolution of the sexes. However, based on this and earlier studies, male and female juncos appear to be similarly sensitive to testosterone with respect to attractiveness to the other sex, elevation of corticosterone and CBG, and suppression of molt and immune function. Some sensitive traits are potentially detrimental to females, e.g., response to stressors, immunosuppression, delayed onset of reproduction, delayed onset of molt (Clotfelter et al., 2004), decreased attractiveness

(Parker-Renga et al., in preparation) and decreased choosiness (McGlothlin et al., 2004). However, some traits reported are potentially beneficial to females (e.g., increased intrasexual aggression). Finally, with respect to parental behavior, unlike males, females are insensitive to the behavioral effects of testosterone (Clotfelter et al., 2004) suggesting that potential fitness costs of testosterone may be intricately tied to viability (i.e., survival) as seen by our results, as opposed to certain measures of fecundity.

To determine whether the net effect of elevated testosterone in females may be constraining the evolution of phenotypic traits in males or more specifically how testosterone levels are balanced by potential costs and benefits on an intrasexual level, more study of the actual fitness consequences of elevated testosterone in females in natural populations is needed. However, our results demonstrate that the impacts of elevated testosterone are not exclusively confined to males, as females are also sensitive to the activational effects of testosterone during adulthood.

Acknowledgments

We thank Director H. Wilbur and Associate Director E. Nagy of Mountain Lake Biological Station (MLBS) of the University of Virginia for providing facilities and support. E. Snajdr, J. Gaudioso, J. McGlothlin, and D. O'Neal assisted in capturing birds and providing bird care and G. Julian assisted with CBG measures. We also thank E. Osnas, N. Beecher, M. Scotti, and two anonymous reviewers for helpful discussions and suggestions on the manuscript. This research was funded in part by NSF/REU-sites award DBI 97-32155 to MLBS and NSF IBN 02-16091 to E.D.K and V.N.

References

Adkins-Regan, E., 1987. Sexual differentiation in birds. Trends Neurosci. 10, 517–522.

Andersson, M., 1994. Sexual Selection. Princeton University Press, Princeton, NJ.

Apanius, V., 1998. Stress and immune defense. Adv. Study Behav. 27, 133–153.

Balthazart, J., 1983. Hormonal correlates of behavior. In: Farner, D.S., King, J.R., Parkes, K.C. (Eds.), Avian Biol., 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.

Balthazart, J., Ball, G.F., 1995. Sexual differentiation of brain and behavior in birds. Trends Endocrinol. Metab. 6, 21–29.

Barsano, C.P., Baumann, G., 1989. Simple algebraic and graphic methods for the apportionment of hormone (and receptor) into bound and free fractions in binding equilibria — or how to calculate bound and free hormone. Endocrinology 124 (3), 1101–1106.

Breuner, C.W., Orchinik, M., 2002. Downstream from corticosterone: seasonality of binding globulins, receptors, and behavior in the avian stress response. In: Dawson, A. (Ed.), Avian Endocrinology. Narosa Publishing, New Delhi, pp. 385–399.

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.

Clotfelter, E.D., O'Neal, D.M., Gaudioso, J.M., Casto, J.M., Parker-Renga, I.M., Snajdr, E.A., Duffy, D.L., et al., 2004. Consequences of elevating plasma testosterone in females of a monogamous songbird: evidence of constraints on male evolution? Horm. Behav. 46, 171–179.

Cristol, D.A., Johnsen, T.S., 1994. Spring arrival, aggression and testosterone in female red-winged blackbirds (*Agelaius phoeniceus*). Auk 111, 210–214.

Darwin, C., 1871. The descent of man, and selection in relation to sex. John Murray, London. Paperback edition, Princeton University Press, Princeton, New Jersey.

Deviche, P., Cortez, L., 2005. Androgen control of immunocompetence in the male house finch, *Carpodacus mexicanus*. J. Exp. Biol. 208, 1287–1295.

Deviche, P., Breuner, C., Orchinik, M., 2001. Testosterone, corticosterone, and photoperiod interact to regulate plasma levels of binding globulin and free steroid hormone in dark-eyed juncos, *Junco hyemalis*. Gen. Comp. Endocrinol. 122, 67–77.

DeRidder, E., Pinxten, R., Mees, V., Eens, M., 2002. Short- and long-term effects of male-like concentrations of testosterone on male starlings (*Sturnus vulgaris*). Auk 119 (2), 487–497.

Duffy, D.L., Bentley, G.E., Drazen, D.L., Ball, G.F., 2000. Effects of testosterone on cell-mediated and humoral immunity in non-breeding adult male European starlings. Behav. Ecol. 11, 654–662.

Eens, M., Van Duyse, E., Berghman, L., Pinxten, R., 2000. Shield characteristics are testosterone-dependent in both male and female moorhens. Horm. Behav. 37, 126–134.

Elekonich, 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.A., Ketterson, E.D., Nolan Jr., V., 1997. Testosterone and mate-choice in the dark-eyed junco. Anim. Behav. 54, 1135–1146.

Falconer, D.S., MacKay, T.F.C., 1996. Introduction to Quantitative Genetics, 4th ed. Longman Group Ltd., Essex, England.

Finch, C.E., Rose, M.R., 1995. Hormones and the physiological architecture of life history evolution. Q. Rev. Biol. 70, 1–52.

Folstad, I., Karter, A., 1992. Parasites, bright males, and the immunocompetence handicap. Am. Nat. 139, 603–622.

Hasselquist, D., Marsh, J.A., Sherman, P.W., Wingfield, J.C., 1999. Is avian immunocompetence suppressed by testosterone? Behav. Ecol. Sociobiol. 45, 167–175.

Hausberger, M., Henry, L., Richard, M., 1995. Testosterone induced singing in female European starlings (*Sturnus vulgaris*). Ethology 3, 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.

Hirschenhauser, K., Winkler, H., Oliveira, R.F., 2003. Comparative analysis of male androgen responsiveness to social environment in birds: the effects of mating system and paternal incubation. Horm. Behav. 43, 508–519.

Jawor, J.M., Young, R., Ketterson, E.D., 2006. Females competing to reproduce: dominance matters by testosterone may not. Horm. Behav. 49 (3), 362–368.

Johnsen, T.S., 1998. Behavioral correlates of testosterone and seasonal changes of steroids in red-winged blackbirds. Anim. Behav. 55, 957–965.

Johnson, D., MacDonald, D., 2001. Why are group-living badgers (*Meles meles*) sexually dimorphic? J. Zool. 255, 199–204.

Jones, I.C., Bellamy, D., Chan, K.O., Follett, B.K., Henderson, I.W., Phillips, J.G., Smart, R.S., 1972. Biological actions of steroid hormones in non-mammalian vertebrates. In: Idler, D.R. (Ed.), Steroids in Non-Mammalian Vertebrates. Academic Press, New York, pp. 414–480.

Joubert, Y., Tobin, C., 1995. Testosterone treatment results in quiescent satellite cells being activated and recruited into the cell cycle in rat levitator ani muscle. Dev. Biol. 169, 286–294.

Karvonen, E., Rintamaki, P.T., Alatalo, R.V., 2000. Female–female aggression and female mate choice on black grouse leks. Anim. Behav. 59, 981–987.

Karubian, J., Swaddle, J., 2001. Selection on females can create 'larger males'. Proc. R. Soc. Lond., B 268, 725–728.

Ketterson, E.D., Nolan Jr., V., 1999. Adaptation, exaptation, and constraint: a hormonal perspective. Am. Nat. 140, S33–S62.

Ketterson, E.D., Parker, P.G., Raouf, S.A., Nolan Jr., V., Ziegenfus, C., Chandler, C.R., 1998. Relative importance of extra-pair fertilizations to male and female reproductive success in dark-eyed juncos. In: Parker, P.G., Burley, N.T. (Eds.), Avian Reproductive Tactics: Female and Male

Perspectives, Ornithological Monographs, vol. 49. American Ornithologists' Union, Lawrence, KS, pp. 81–101.
Ketterson, E.D., Nolan Jr., V., Sandell, M., 2005. Testosterone in females: a constraint on the evolution of sexual dimorphism? Am. Nat. 166 (4), S85–S98.
Klukowski, L.A., Cawthorn, J.M., Ketterson, E.D., Nolan Jr., V., 1997. Effects of experimentally elevated testosterone on plasma corticosterone and corticosterone-binding globulin in dark-eyed juncos (*Junco hyemalis*). Gen. Comp. Endocrinol. 108, 141–151.
Kral, M., Saetre, G.P., Bicik, V., 1996. Intrasexual aggression of female collared flycatchers (*Ficedula albicollis*): competition for male parental care? Folia Zool. 45 (2), 153–159.
Lande, R., 1980. Sexual dimorphism, sexual selection, and adaptation in polygenic characters. Evolution 34, 292–305.
Lande, R., Arnold, S.J., 1983. The measurement of selection on correlated characters. Evolution 37, 1210–1226.
Langmore, N., Cockrem, J., Candy, E., 2002. Competition for male reproductive investment elevates testosterone levels in female dunnocks, *Prunella modularis*. Proc. R. Soc. Lond., B 269, 2473–2478.
Lank, D.B., Coupe, M., Wynne-Edwards, K.E., 1999. Testosterone-induced traits in female ruffs (*Philomachus pugnax*): autosomal inheritance and gender differentiation. Proc. R. Soc. Lond., B 226, 2323–2330.
Lochmiller, R.L., Vestey, M.R., Boren, J.C., 1993. Relationship between protein nutritional status and immuno-competence in northern bobwhite chicks. Auk 110, 503–510.
McGlothlin, J.W., Neudorf, D.L.H., Casto, J.M., Nolan Jr., V., Ketterson, E.D., 2004. Elevated testosterone reduces choosiness in female dark-eyed juncos (*Junco hyemalis*): evidence for a hormonal constraint on sexual selection? Proc. R. Soc. Lond., B 271, 1377–1384.
Møller, A.P., Garamszegi, L.Z., Gil, D., Hurtrez-Bousses, S., Eens, M., 2005. Correlated evolution of male and female testosterone profiles in birds and its consequences. Behav. Ecol. Sociobiol. 58, 534–544.
Nelson, R.J., 2000. An Introduction to Behavioral Endocrinology, second ed. Sinauer Associates.
Nespor, A.A., Lukazewicz, M.J., Dooling, R.J., Ball, G.F., 1996. Testosterone induction of male-like vocalizations in female budgerigars (*Melopsittacus undulates*). Horm. Behav. 30, 162–169.
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 (*Junco hyemalis*). In: Poole, A., Gill, F. (Eds.), The Birds of North America, vol. 716. The Birds of North America Inc., Philadelphia, PA.
Nordeen, E.J., Nordeen, K.W., Sengelaub, D.R., Arnold, A.P., 1985. Androgens prevent normally occurring cell death in a sexually dimorphic spinal nucleus. Science 229, 671–673.
Owen-Ashley, N.T., Hasselquist, D., Wingfield, J.C., 2004. Androgens and the immunocompetence handicap hypothesis: unraveling direct and indirect pathways of immunosuppression in song sparrows. Am. Nat. 164 (4), 490–505.
Parker-Renga, I., Jones, K.J., Nolan Jr., V., Ketterson, E.D., in preparation. Experimentally elevated testosterone decreases attractiveness of female dark-eyed juncos.
Price, D.K., Burley, N.T., 1993. Constraints on the evolution of attractive traits: genetic covariation for zebra finch bill color. Heredity 71, 405–412.
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. Lond., B 264, 1599–1603.
Reed, W.L., Clark, M.E., Parker, P.G., Rauof, S.A., Arguedas, N., Monk, D.S., Snajdr, E. et al., in press. Physiological effects on demography: a long-term experimental study of testosterone's effect on fitness, Am. Nat.
Rook, G.A., 2000. Glucocorticoids and immune function. Bailliere's Best Pract. Res., Clin. Endocrinol. Metab. 4, 567–581.
Rutkowska, J., Cichon, M., Puerta, M., Gil, D., 2005. Negative effects of elevated testosterone on female fecundity in zebra finches. Horm. Behav. 47 (5), 585–591.
Schoech, S.J., Ketterson, E.D., Nolan Jr., V., 1999. Exogenous testosterone and the adrenocortical response in dark-eyed juncos. Auk 116 (1), 64–72.
Seale, J.V., Wood, S.A., Atkinson, H.C., et al., 2004. Gonadectomy reverses the sexually diergic patterns of circadian and stress-induced hypothalamic–pituitary–adrenal axis activity in male and female rats. J. Neuroendocrinol. 16 (6), 516–524.
Searcy, W.A., 1988. Do female red-winged blackbirds limit their breeding densities? Ecology 69, 85–95.
Siiteri, P.K., Murai, J.T., Hammond, G.L., Nisker, J.A., Raymoure, W.J., Kuhn, R.W., 1982. The serum transport of steroid hormones. Recent Prog. Horm. Res. 38, 457–510.
Smits, J.E., Bortolotti, G.R., Tella, J.L., 1999. Simplifying the phytohemagglutinin skin testing technique in studies of avian immunocompetence. Funct. Ecol. 13, 567–572.
Smits, J.E., Bortolotti, G.R., Tella, J.L., 2001. Measurement repeatability and the use of controls in PHA assays: a reply to Siva-Jothy and Ryder. Funct. Ecol. 15 (6), 814–817.
Staub, N.L., De Beer, M., 1997. The role of androgens in female vertebrates. Gen. Comp. Endocrinol. 108, 1–24.
Sullivan, D.A., Wira, C.R., 1979. Sex hormone and glucocorticoid receptors in the bursa of Fabricius of immature chickens. J. Immunol. 122 (6), 2617–2623.
Tanriverdi, F., Silveira, F.G., MacColl, G.S., Bouloux, P.M., 2003. The hypothalamic–pituitary–gonadal axis: immune function and autoimmunity. J. Endocrinol. 176, 293–304.
Viau, V., 2002. Functional cross-talk between the hypothalamic–pituitary–gonadal and adrenal axes. J. Neuroendocrinol. 14 (6), 506–513.
Wada, M., 1986. Circadian rhythms of testosterone-dependent behaviors, crowing and locomotor activity, in male Japanese quail. J. Comp. Physiol., A Sens. Neural Behav. Physiol. 158, 17–25.
Wedekind, C., Folstad, I., 1994. Adaptive or non-adaptive immunosuppression by sex-hormones. Am. Nat. 143, 936–938.
Wingfield, J.C., 1984. Androgens and mating systems: testosterone induced polygyny in normal monogamous birds. Auk 101, 665–671.
Wingfield, J.C., Romero, L.M., 2000. Adrenocortical responses to stress and their modulation in free-living vertebrates. In: McEwen, B.S. (Ed.), Handbook of Physiology Vol. IV: Coping With the Environment: Neural and Endocrine Mechanisms. Oxford University Press, New York, pp. 211–234.
Wingfield, J.C., Hegner, R.E., Dufty Jr., A.M., Ball, G.F., 1990. The "challenge hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am. Nat. 136, 829–846.
Wingfield, J.C., Vleck, C.M., Moore, M.C., 1992. Seasonal changes of the adrenocortical response to stress in birds of the Sonoran desert. J. Exp. Zool. 264, 419–428.
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 in birds. In: Wallen, K., Schneider, J.E. (Eds.), Reproduction in Context. MIT Press, Cambridge, MA, pp. 86–128.
Wingfield, J., Lynn, S., Soma, K., 2001. Avoiding the "costs" of testosterone: ecological bases of hormone–behavior interactions. Brain Behav. Evol. 57, 239–251.
Zuk, M., Johnsen, T.S., MacLarty, T., 1995. Endocrine–immune interactions, ornaments and mate choice in red jungle fowl. Proc. R. Soc. Lond., B 260, 205–210.