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Published in:
American Naturalist

2004

Androgens and the Immunocompetence Handicap Hypothesis: Unraveling Direct and Indirect Pathways of Immunosuppression in Song Sparrows

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Submitted November 21, 2003; Accepted June 16, 2004; Electronically published September 1, 2004

abstract: The immunocompetence handicap hypothesis proposes that testosterone (T)-dependent sexual signals are honest indicators of male health or genetic quality because only high-quality males are able to withstand the obligate effects of T-induced immunosuppression. In birds, the basic assumption that T suppresses immune function is equivocal, and the physiological mechanisms underlying T-induced immunosuppression remain to be investigated. We explored the proximate pathways of T-induced immunosuppression in song sparrows (*Melospiza melodia*) by treating captive nonbreeding males with different androgens and measuring several components of acquired immune function. Males implanted with T suppressed cell-mediated and humoral immune responses compared to males implanted with 5∝-dihydrotestosterone (DHT), dehydroepiandrosterone, or control (empty) implants. Furthermore, T treatment increased plasma levels of corticosterone and decreased body mass and fat stores in relation to other treatments. The failure of DHT to depress immune function suggests that T-induced immunosuppression does not occur through a direct pathway because both T and DHT bind to androgen receptors on target cells. Instead, we outline indirect pathways that are likely responsible for suppression of the avian immune system that include stress-induced immunosuppression, aromatization to estrogen, and alterations in energy allocation that constrain expenditures toward immune system activation.

Keywords: androgens, corticosterone, immunocompetence, immunosuppression, song sparrow, testosterone.

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Am. Nat. 2004. Vol. 164, pp. 490–505. 0003-0147/2004/16404-40191$15.00.

Resistance to parasites has been suggested to play a dominant role in the evolution of sexual signals (Hamilton and Zuk 1982) on both an evolutionary and a physiological level. Males with high-quality secondary sexual ornaments tend to have fewer parasites, suggesting that their immune systems may be more competent than those of males with low-quality ornaments (Møller et al. 1999). Female preferences are evolutionarily tuned to these male traits that could signal both direct (parasite-free) and indirect (genetically based parasite resistance) benefits (Andersson 1994). However, it has been suggested that these traits must be costly to produce and/or maintain to be honest signals of male quality (Zahavi 1975; Grafen 1990).

Folstad and Karter (1992) introduced the immunocompetence handicap hypothesis to explain how male ornaments could honestly signal health, status, and/or genetic resistance to parasites through endocrine-immune interactions. The authors proposed that testosterone (T) acts as a "double-edged sword" by promoting the expression of secondary sexual traits but at the same time suppressing immune function. This physiological trade-off would ensure honesty in signaling by allowing only those individuals with parasite resistance genes to effectively produce a high-quality signal. Thus, obligate immunosuppression by T would prevent cheating by males with low viability or fitness because expression of the signal would lead to increased susceptibility to parasites and ultimately to poor health and decreased survivorship.

The immunocompetence handicap hypothesis has been criticized on several fronts. First, although many sexual characters and behaviors are dependent on T, this is not always the case (e.g., male plumage in some birds; Witschi 1961; Wingfield and Farner 1993; Owens and Short 1995; Kimball and Ligon 1999). Thus, the hypothesis is limited to sexual signals that are dependent on T or other biochemicals that produce similar handicaps (Folstad and Karter 1992; Møller 1995). In addition, development of many T-regulated morphological traits tends to occur when circulating levels of T are low (Hillgarth and Wingfield 1997), and these low levels may not be high enough

to invoke obligate immunosuppression.

Second, the basic assumption that T suppresses immune function has been questioned, especially in avian studies. In mammals, there is evidence that androgens suppress immune function both in vivo and in vitro (Grossman 1985; Alexander and Stimson 1988; Schuurs and Verheul 1990; Nelson and Demas 1996), although other studies have reported no relationship or enhancing effects (Ansar Ahmed et al. 1985; Olsen and Kovacs 1996; Bilbo and Nelson 2001). In chickens, immunosuppression by T occurred in chicks but not in adults (Gause and Marsh 1986; Schuurs et al. 1992), suggesting that stage of development plays an important role in hormonal regulation of immune function. Studies experimentally manipulating plasma T levels using silastic implants documented a reduction in lymphocyte counts in red jungle fowl *Gallus gallus* (Zuk et al. 1995) and a temporary decline in immunoglobulin levels and an increase in parasite load in barn swallows *Hirundo rustica* (Saino et al. 1995). However, these indirect measures of immune function are not necessarily assessing immunocompetence per se but may be influenced by other factors such as past or present infection status (Siva-Jothy 1995). More recent work using standardized immune challenges to assess acquired immunity has found that T implants decreased cell-mediated (T cell) and/or humoral (B cell) immune responses in males of several passerine species (Duffy et al. 2000; Evans et al. 2000; Casto et al. 2001). Other studies found no effect of T treatment on humoral immune responses produced toward novel antigens in black-headed gulls *Larus ridibundus* (Ros et al. 1997) and red-winged blackbirds *Agelaius phoeniceus* (Hasselquist et al. 1999) or toward a naturally occurring virus in greenfinches *Cardeulis spinus* (Lindström et al. 2001). Interestingly, Peters (2000) documented a negative effect of T on humoral responses in captive superb fairy wrens *Malurus cyaneus* provided with T implants but a positive correlation between T levels and immune function in unmanipulated free-living males. Thus, there is no clear consensus whether T leads to obligate immunosuppression in birds.

Third, when T-induced immunosuppression is documented, T may exert its effect on the avian immune system through direct or indirect means. A direct effect on immune function would involve T binding locally to androgen receptors on functional immune cells or organs. Androgen receptors have been located on the bursa of Fabricius of immature chickens (Sullivan and Wira 1979), but it is not known whether they are present in adult avian lymphocytes (Marsh and Scanes 1994). Direct effects are difficult to detect when T treatment can alter body condition (Wingfield 1984*a*; Ketterson et al. 1991), energy allocation (Wikelski et al. 1999; Lynn et al. 2000; Wikelski and Ricklefs 2001), and levels of other circulating hormones that may regulate immunity (the "indirect" effects). For example, basal levels of corticosterone and corticosteroid-binding globulin increase in the circulation when plasma T levels are elevated using silastic implants (Klukowski et al. 1997; Schoech et al. 1999). Because chronically elevated levels of glucocorticoids downregulate or inhibit immune function in vertebrates (Munck et al. 1984; Apanius 1998), it is not known whether one or both steroids are involved in suppressing the immune system. Another indirect pathway not yet tested is the possibility that T is converted to estrogenic metabolites locally in immune cells by the enzyme aromatase, leading to immunosuppression via activation of estrogen receptors. Administration of estradiol generally enhances humoral immunity but can suppress cell-mediated immune function (Erbach and Bahr 1991; Carlsten et al. 1996; Salem et al. 2000). Thus, separation of these direct and indirect mechanisms affecting immunocompetence is integral to our understanding of T's influence on in vivo immune function and, ultimately, sexual signaling (Poiani et al. 2000).

We examined the direct and indirect mechanisms affecting T-induced immunosuppression by investigating the effect of different androgens on acquired immune function in male song sparrows (*Melospiza melodia*). Three biologically relevant androgens have been characterized in this species: T, 5∝-dihydrotestosterone (DHT), and dehydroepiandrosterone (DHEA); T and DHT are produced in the testis and are secreted on a seasonal basis to coincide with territorial establishment and pair formation during the breeding season (Wingfield and Farner 1978*a*; Wingfield 1984*b*). Both androgens are important for male reproduction by promoting spermatogenesis and growth of some secondary sex characters (Witschi 1961) and by regulating song control circuitry and territorial aggression (Balthazart 1983; Arnold and Breedlove 1985; Wingfield et al. 1987), but DHT is normally secreted in lower concentrations (Wingfield and Farner 1978*a*). An important action of T and DHT is that they both bind to androgen receptors in target tissues. However, while T can be converted locally to estrogenic metabolites in target tissues by the enzyme aromatase, DHT is nonaromatizable. Thus, immunosuppression by T but not DHT would suggest that T-mediated immunosuppression is occurring through an indirect estrogen-mediated pathway. Aromatization of T is important in regulating sexual and aggressive behavior in the avian brain (Adkins 1977; Schlinger and Callard 1990; Soma et al. 2000) and may play a role in T-induced immunosuppression in mammals (Greenstein et al. 1992; Nelson and Demas 1996; but see Benten et al. 1993). In addition to these two androgens, we investigated the effect of DHEA, an androgen precursor, on avian immune function. In mammals, DHEA does not bind readily to androgen or estrogen receptors but may be converted to other androgens if appropriate enzymes are expressed in

target cells (Labrie et al. 1995; Svec and Porter 1998). This hormone is the only androgen elevated in the blood of male song sparrows during the nonbreeding season (Soma and Wingfield 2001). In vitro studies suggest that this androgen is immunoenhancing (Svec and Porter 1998) and could potentially upregulate immune function during the winter.

By utilizing different androgens, our goal was to examine the proximate pathways involved in regulating T-induced immunosuppression in passerine birds. We predicted that if immunosuppression by T occurs through a direct mechanism, DHT would also suppress avian immune function. Alternatively, if T-mediated immunosuppression occurs through indirect processes, we would expect to detect a suppressive effect of T but not DHT on avian immunocompetence, increases in other immunomodulatory hormones such as glucocorticosteroids, or differences in energy allocation imposed by T treatment that may limit the activation and maintenance of the immune system. These mechanisms could then be related to the immunocompetence handicap hypothesis and sexual selection theory in general.

Methods

Capture and Housing of Birds

Free-living song sparrows were mist netted on territories in King and Skagit Counties in western Washington in early July ($n = 13$) and September ($n = 37$) of 2000 and were housed in outdoor aviaries at the University of Washington, Seattle. Unilateral laparotomies using isofluorane anesthesia were performed on all subjects to determine or verify sex. On October 10, 2000, 40 males were transferred into individual cages (50 cm × 25 cm × 25 cm), which were housed in two environmental chambers set at a constant short-day photoperiod (8L : 16D) at 15°C. In seasonally breeding birds, long days trigger gonadal recrudescence and secretion of androgens in the blood (Dawson et al. 2001). Exposure to short days maintains birds in a reproductively inactive state such that testes are regressed and endogenous levels of gonadal steroids are very low or nondetectable (Wingfield and Hahn 1994). The birds were supplied with food (seed mix and Mazuri bird chow) and water ad lib. Grit was supplemented to their diet on a weekly basis. Subjects were acclimated to chambers for 3 weeks before starting the experiment. This period also allowed birds to complete their prebasic molt if they had not already done so.

Experimental Design and General Methods

The experiment ran from October 31 to December 17, 2000. We used a staggered design where four birds (two from each chamber) were randomly assigned to one of four implant treatments each day such that all birds were implanted over the course of 10 days. Treatments were balanced across chambers. We standardized the day of implantation as day 0 for each bird, and the remainder of the experiment was conducted relative to this date.

Silastic implants (i.d. = 1.47 mm, o.d. = 1.96 mm) were filled with T (20 mm length), DHT (6 mm), or DHEA (7 mm; Sigma, St. Louis). A fourth set of empty implants (20 mm) served as a control group. All implants were sealed at each end with silicone glue. Implant length was based on previous studies that have documented a rise in steroid concentrations that approach maximum physiological levels as measured in free-living sparrows (Wingfield 1984*a*; Soma et al. 2002; Tramontin et al. 2003). However, the DHEA implants were slightly larger in diameter than those reported in another study (Soma et al. 2002). Implants were inserted through a small incision in the skin of the left flank of lightly anesthetized subjects. We then sealed the incision with veterinarian surgical adhesive. Some males were removed from the experiment because of adverse reactions to implantation or captivity, but removal was not biased by implant treatment. The final samples sizes for the experiment were six T males, seven DHT males, seven DHEA males, and six control males.

A suite of immune tests was conducted on each subject from day 4 to day 39 to quantify both cell-mediated and humoral immune function. Blood samples for steroid analysis (200–300 μL) were collected before implantation (day 0), 10 days after implantation but before measuring immune responses (day 10), and 41 days after implantation at the end of the experiment (day 41). Body condition of subjects was assessed during these time points as well. To assess condition, subjects were weighed to the nearest 0.25 g and scored for the extent of furcular and peritoneal fat deposition on a scale from 0 (no fat) to 5 (maximum fat; Helms and Drury 1960; Wingfield and Farner 1978*b*). The two scores were averaged for each bird for a cumulative measure of fat score. Another small blood sample (~50 μL) was taken on day 19 approximately 2–3 h after lights turned on to measure baseline levels of corticosterone. These samples were taken within 3 min of entering the chamber. Length of the cloacal protuberance, an androgen-sensitive organ important for sperm collection and delivery, was measured at the end of the experiment. After day 41, implants were removed, and subjects were returned to aviaries. In January 2001, birds were released onto their original territories.

Quantifying Immune Function

Because of the complexity of the immune system, it is suggested that multiple tests be conducted (Sheldon and Verhulst 1996; Zuk and Johnsen 1998; Norris and Evans 2000). We relied on a series of standardized challenge tests that have been used extensively in immunoecology studies to assess cell-mediated and humoral immunocompetence in passerine birds.

We assessed cell-mediated immune function by measuring the response of subjects to an intradermal injection of phytohemagglutinin (PHA; Sigma L8754) in the left scapular apterium, or "wing web." Many studies have used PHA, a T-cell mitogen, to quantify cell-mediated immune response in vivo (Goto et al. 1978; Dietert et al. 1994; Moreno et al. 1999; Tella et al. 2000). Exposure to PHA induces a rapid proliferation of T lymphocytes that migrate to the site of injection to produce local swelling and edema. On day 21, subjects were injected with a 0.05-mL solution of PHA in PBS (0.5 mg PHA/1 mL of PBS). The thickness of the left wing web (to the nearest 0.01 mm) was measured before PHA injection (baseline) and 24 and 48 h after PHA injection using a digital micrometer (Mitutuyo) set at a constant pressure. Five independent measurements were taken with the micrometer for each time point (Moreno et al. 1999) and then averaged for a single reading. Repeatability of wing web measurements was calculated from an intraclass correlation coefficient from a one-way ANOVA (Lessells and Boag 1987). Wing thickness measurements were found to be significantly repeatable across subjects (before injection: $r = 0.69$, $F = 10.37$, $\mathrm{df} = 26, 108$, $P < .001$; 24 h after injection: $r = 0.83$, $F = 25.10$, $\mathrm{df} = 26, 108$, $P < .001$; 48 h after injection: $r = 0.87$, $F = 33.86$, $\mathrm{df} = 26, 108$, $P < .001$). We used the simplified protocol of Smits et al. (1999), who concluded that the PBS control injection in the opposite wing was unnecessary, given that the variation in response to PBS injection was trivial compared with the robust response to the PHA treatment. To ensure that removal of the PBS control was appropriate for our study species, we injected four captive song sparrows held on short days (not involved with this study) with 0.05 mL of PBS in the left wing web and found no increase in swelling 24 h after injection (Wilcoxon-signed rank test: $Z = -0.73$, $P = .47$).

We assessed humoral immune function by measuring the primary and secondary antibody responses of male song sparrows toward a series of diphtheria-tetanus vaccinations using an enzyme-linked immunosorbent assay (ELISA). We measured both primary and secondary humoral responses because they are inherently different in time course, magnitude of response, and type of immunoglobulin (Ig) produced. The primary response is dependent on preexisting B lymphocytes recognizing the antigen and proliferating into IgM-secreting plasma cells. In contrast, the secondary response shows faster kinetics and increased peak levels, with a switch to IgG antibodies that have a higher affinity for the antigen (Roitt et al. 1998). Pilot studies on song sparrows have found that humoral responses peak 9–15 days after primary injection and 6–9 days after secondary injection (N. T. Owen-Ashley, unpublished data). These findings are similar to antibody response profiles documented in red-winged blackbirds (Hasselquist et al. 1999).

On day 4, subjects were injected in the breast muscle with 80 μL of human diphtheria-tetanus vaccine (pediatric dose; 2.7 Lf of diphtheria toxoid and 2 Lf of tetanus toxoid) that was adsorbed in aluminum potassium sulfate (the adjuvant) and dissolved in phosphate-buffered saline (Aventis Pasteur, Swiftwater, Pa.). Small blood samples (50–70 μL) were collected 9 (day 13) and 12 (day 16) days after vaccination to measure primary antibody titers in the plasma. An initial blood sample (day 4) was also taken before primary injection to measure baseline antibody titers, if any, in the blood. We presumed that song sparrows have never been exposed to these foreign antigens and this was confirmed by our analyses (generally very low preinjection antibody titers). Birds were given a booster injection of diphtheria-tetanus vaccine (80 μL) 23 days after the first injection (day 27) to measure secondary antibody profiles. Blood samples were taken immediately before injection (day 27), and 6 (day 33), 9 (day 36), and 12 (day 39) days after secondary vaccination.

We used an ELISA that had been previously developed for red-winged blackbirds (Hasselquist et al. 1999) to measure antigen-specific antibody titers produced in response to a novel antigenic challenge (in this case, diphtheria and tetanus antigens). This assay has been used with success in other passerine species (Ilmonen et al. 2000; Råberg et al. 2000; Hasselquist et al. 2001), as well as in several Charadridae and one Laridae species (Bustnes et al., in press; D. Hasselquist, unpublished data). Briefly, 96-well microtiter plates (Costar, Cambridge, Mass.) were coated with either diphtheria or tetanus antigens at 4°C for 24 h. After washing plates and blocking (postcoat) wells with 3% milk powder diluted in 0.01 M PBS/Tween20, diluted plasma samples were added to the wells and allowed to incubate overnight at 4°C. Plates were then washed, and a secondary rabbit anti-red-winged blackbird Ig antiserum (diluted 1 : 1,000; recognizes both IgG and IgM) was added to each well. Following 1 h of incubation at 37°C and a wash, peroxidase-labeled goat-anti-rabbit serum (1 : 2,000 dilution; Sigma A6154) was added to the wells. After a secondary incubation (45 min at 37°C) and a final wash, 2,2-azino-bis-3-ethylbenzthialzoline-6-sulfanic acid (ABTS; Sigma, A1888) and peroxidase were added to the

wells, and plates were then immediately transferred to a Molecular Devices V_{max} kinetic reaction ELISA reader. Plates were read every 30 s for 12 min using a 405-nm wavelength filter. Antibody concentrations were calculated according to the slope of substrate conversion over time in units 10^{-3} × optical densities (OD) per minute (m_{OD}/min), with a higher slope indicating a higher titer of antidiphtheria or antitetanus antibodies in the sample.

For each individual, diluted plasma collected after primary and secondary injections were analyzed on the same ELISA plate. Each plate also included baseline samples collected from birds before injections (negative controls). A total of six ELISA plates were run for each of the two antigens, and plates were analyzed on the same day to minimize interassay variation. Plasma samples diluted to 1 : 1,000 (for diphtheria antibodies) and 1 : 3,000 (for tetanus antibodies) were used in all analyses because these provided the best conditions for analysis (i.e., maximizing the difference between post- and preinjection titers). All samples were run in duplicate, and the average of the two readings was our measure of antibody levels in the serum. Blanks (buffer only) and a diluted standard sample (pooled plasma from high secondary responses of blue tits *Parus caeruleus*) were also included on each plate. Final measures of antibody levels were expressed as the percent of the standard. Interassay and intrassay variations were 14.5% and 12.7%, respectively, for the diphtheria ELISAs and 22.6% and 16.9%, respectively, for the tetanus ELISAs. Note that a large part of the intra-assay variation comes from samples with antibody titers very close to negative controls.

Blood Sampling and Radioimmunoassays

Blood samples were taken by alar venepuncture using a 26.5-gauge needle, collected into heparinized capillary tubes, and stored at 4°C. We centrifuged the blood within 6 h and separated the plasma, which was stored at −20°C until running the assay.

Plasma levels of T, DHEA, DHT, and estradiol (E_2) were measured using radioimmunoassay (RIA) according to procedures by Wingfield and Farner (1975) and modified by Soma and Wingfield (2001) to measure DHEA levels in songbird plasma. Because DHEA and DHT have similar polarities and elute from the same fraction during column chromatography, we chose to measure DHT, T, and E_2 levels in DHT-implanted birds and DHEA, T, and E_2 levels in the remaining three implant groups. Dividing individual plasma samples into smaller volumes to measure both DHT and DHEA was not an option because smaller plasma volumes tend to decrease the sensitivity of the assay (Wingfield and Farner 1975). This is not a substantial setback because levels of DHT in nonbreeding birds (excluding the DHT-implanted birds) should be very low or nondetectable (Wingfield and Hahn 1994).

Steroids were extracted from dichloromethane, dried under a stream of nitrogen, and resuspended in 0.5 mL of 10% ethyl acetate in iso-octane. The steroids were then separated on diatomaceous earth/glycol columns by increasing polarity. For the DHT/T/E_2 assay, columns were packed with a 0.4-mL "water trap" (2 mL of distilled water/6 g diatomaceous earth) and then with a 0.6-mL glycol phase (3 mL of 1 : 1 propylene glycol/ethylene glycol/6 g of diatomaceous earth). The DHEA/T/E_2 columns required a double "water trap" (0.8 mL) with a 0.6-mL glycol phase consisting of a pure propylene glycol phase (Soma and Wingfield 2001). Steroid levels were then measured by using standard RIA methods (Wingfield and Farner 1975). Interassay variation and intra-assay variation for each steroid measured were 17.7% and 10.1% for DHEA, 7.0% and 13.8% for T, and 14.4% and 10.0% for E_2, respectively. The DHT samples were analyzed within a single assay, and the intra-assay variation was 12.6%.

Corticosterone levels were assessed in a single direct RIA (Wingfield et al. 1992). Intra-assay variation was 9.7%. For all RIAs, undetectable samples were assigned the minimum detection limit for statistical purposes.

Statistical Analyses

We used ANOVAs with repeated-measures (rANOVAs) to determine the effect of implant type on cell-mediated immunity, humoral immunity, steroid levels (T, DHEA, E_2), and body mass. Time (by specific day of immune testing or blood collection) was the repeated measure, and implant treatment was the main factor. Because DHT was measured only in DHT-implanted subjects, paired *t*-tests were employed to determine whether DHT levels increased over time relative to levels before implantation (day 0). Differences in maximal antibody responses and baseline corticosterone levels were assessed using one-way ANOVAs. We used Fisher's protected least significant difference (PLSD) post hoc tests when significant differences from ANOVAs were detected. If necessary, data were log transformed before analysis to meet the requirements of homogeneity of variances and normality of distributions. We used a nonparametric Kruskal-Wallis ANOVA on ranks to assess differences in fat score at the beginning and end of the experiment.

Because different components of the immune system were measured in the same individuals, we were interested in determining how each response related to other components. A correlation matrix was constructed to examine the interrelationships between humoral and cell-mediated immune responses within individuals. In addition, baseline corticosterone levels were incorporated into the matrix

to assess whether levels were negatively associated with immune responses.

Results

Steroid Levels

Androgen implants were effective in elevating T, DHT, or DHEA to high levels both 10 and 41 days after implantation (fig. 1*A*–1*C*; rANOVA for T and DHEA, time × treatment interactions: all $P < .001$; paired *t*-tests, DHT: $P < .001$). These levels were rapidly increasing during primary humoral responses (day 10) and slowly declining during cell-mediated and secondary humoral responses (up to day 41). Birds treated with one type of androgen showed little or no rise in other circulating androgens that we were able to successfully measure in each treatment group given the elution constraints of the RIA (see "Methods"). Levels of E_2 were very low or nondetectable in all implant groups over the course of the experiment (fig. 1*D*; rANOVA, all effects, $P > .23$). The T-implanted subjects had mean (±SEM) plasma T levels of 10.12 ± 1.60 ng/mL at day 10 and 7.85 ± 1.23 ng/mL at day 41, which were within the physiological limits of breeding male song sparrows in the wild (Wingfield and Hahn 1994). Levels of circulating DHEA in T-implanted birds were low (day 10, 0.29 ± 0.05 ng/mL; day 41, 0.36 ± 0.10 ng/mL). Birds implanted with DHT had low or nondetectable T levels, but circulating DHT was 6.65 ± 1.07 and 3.37 ± 0.55 ng/mL at day 10 and day 41, respectively. These levels were above the maximum titers documented in this species (<2 ng/mL; Wingfield 1984*b*). In passerine sera, DHT is found in lower concentrations than T but closely parallels secretion of T throughout the breeding season (Wingfield and Farner 1978*a*, 1993). The DHEA implants increased plasma DHEA to 4.60 ± 0.91 ng/mL on day 10 and 3.43 ± 0.89 ng/mL on day 41, which is slightly above the high physiological range for wild nonbreeding male song sparrows (2–3 ng/mL; Soma and Wingfield 2001). Interestingly, implantation of DHEA caused a slight elevation in circulating T levels from 0.23 ± 0.10 ng/mL on day 0 to 0.86 ± 0.15 ng/mL on day 10 and 0.75 ± 0.43 ng/mL on day 41 (fig. 1*A*). This rise in T has been previously reported in male song sparrows implanted with DHEA and is thought to be due to circulating DHEA being converted to T in the testes or possibly the adrenals (Soma et al. 2002). Control-implanted subjects had very low or nondetectable levels of steroids in their blood throughout the experiment (fig. 1*A*, 1*C*, 1*D*).

Birds implanted with control or DHEA implants did

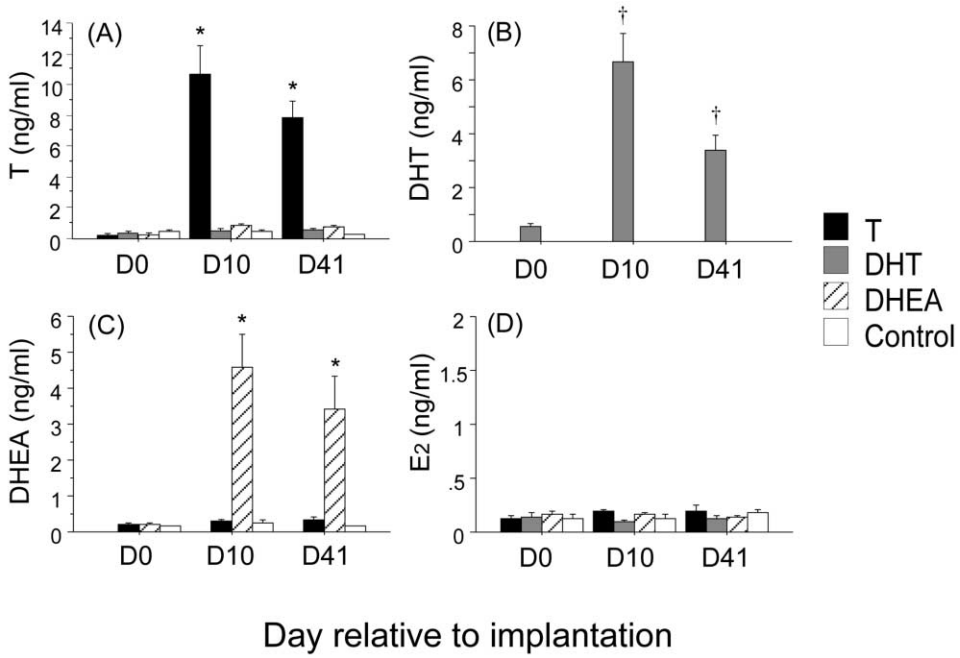

Figure 1: Circulating levels of T (*A*), DHT (*B*), DHEA (*C*), and E_2 (*D*) in male song sparrows assigned to different implant treatments before implantation and 10 and 41 days after implantation. Asterisks indicate $P < .05$ relative to all other groups at particular time points using post hoc tests after detecting significant time × treatment effects in overall rANOVAs. Daggers specify significant differences ($P < .05$) relative to DHT levels at day 0 using paired *t*-tests corrected for multiple comparisons (*B*). Error bars represent 1 SEM.

not have cloacal protuberances that were large enough to be accurately measured (<3 mm in height). However, treatment with T or DHT promoted the growth of the cloacal protuberance. On day 41, mean cloacal protuberance length of subjects treated with T or DHT was 6.9 ± 0.6 and 7.1 ± 1.0 mm, and groups did not significantly differ from each other ($t_{11} = -0.19$, $P = .85$).

Cell-Mediated Immunocompetence

All groups experienced an increase in wing web thickness 24 and 48 h after PHA injections relative to initial measurements (rANOVA: $F = 128.3$, df = 2, 44, $P < .001$). Implant type also differentially affected cell-mediated immune responses (time × treatment interaction: $F = 3.29$, df = 6, 44, $P = .009$). Wing web thickness (mm) in T-implanted birds was reduced 24 and 48 h following PHA injection compared to swellings produced in subjects treated with DHT, DHEA, or control implants (fig. 2; Fisher's PLSD tests; 24 h postinjection, T vs. other groups: all $P < .01$; 48 h postinjection: all $P < .02$). There were no significant differences in PHA responses among DHT-, DHEA-, and control-implanted birds 24 and 48 h after injection (fig. 2; Fisher's PLSD: all $P > .42$).

Humoral Immunocompetence

Using an ELISA, we were able to detect primary antibody responses toward tetanus and secondary antibody responses toward both diphtheria and tetanus antigens because antibody titers of postinjection samples were greater than levels found in preinjection samples (table 1). At a plasma dilution of 1 : 1,000, primary antibody titers produced toward diphtheria at day 13 and day 16 were not different from baseline measurements on day 4 (table 1). Because primary antibody responses toward tetanus were measurable and because birds were injected with a mixture of both toxoids simultaneously, these results imply that primary antibody responses toward diphtheria occurred, but the plasma dilution was too high for the ELISA to detect differences from preinjection readings. Previous studies using diphtheria-tetanus vaccine have found that passerine birds generate higher antibody titers toward tetanus than toward diphtheria (Råberg and Stjernman 2003; Råberg et al. 2003; Westneat et al. 2003).

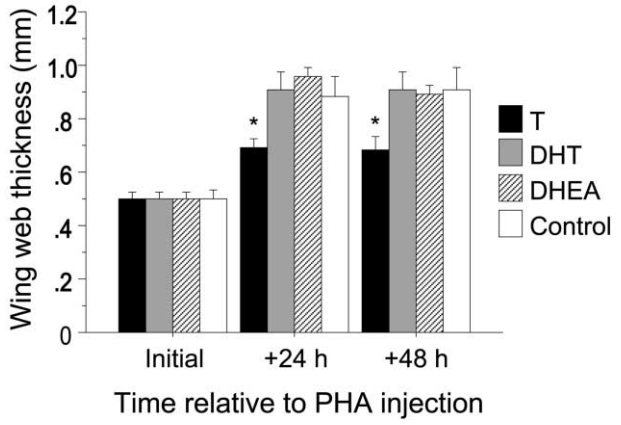

Figure 2: Cell-mediated immune response in relation to implant treatment. Values are wing web thickness measurements (mm) before and 24 and 48 h following PHA injection. Asterisks indicate $P < .05$ relative to all other groups using post hoc tests at specific time points. Error bars represent 1 SEM.

Table 1: Results of repeated-measures ANOVAs testing for differences in primary and secondary antibody responses to diphtheria-tetanus vaccination in relation to time course (before vs. after injection) and androgen treatment

	Diphtheria		Tetanus	
	Primary	Secondary	Primary	Secondary
Treatment:				
F	.35	1.83	1.07	4.01
df	3, 22	3, 22	3, 22	3, 22
P	.79	.17	.38	.02*
Time:				
F	2.34	27.22	16.02	12.59
df	2, 44	3, 66	2, 44	3, 66
P	.11	.000*	.000*	.000*
Time × treatment:				
F	1.50	.84	1.29	1.99
df	6, 44	9, 66	6, 44	9, 66
P	.20	.58	.28	.06

* Significance at $P < .05$.

Androgen treatment also differentially affected humoral immunocompetence. There was a strong tendency for secondary antibody profiles toward tetanus to vary by steroid treatment, but primary antitetanus profiles and secondary antidiphtheria profiles were not significantly affected (table 1). However, we found that maximum primary responses produced toward tetanus and maximum secondary responses produced toward both diphtheria and tetanus varied according to androgen treatment (fig. 3; one-way ANOVAs: all $F > 3.53$, all $P < .032$). Multiple comparison tests revealed that T-implanted birds suppressed their peak primary and secondary antibody responses compared with the other implant groups (fig. 3; Fisher's PLSDs: all $P < .022$). There were no significant differences in peak antibody levels among DHT-, DHEA-, or control-implanted subjects (fig. 3; Fisher's PLSD: $P > .61$).

Corticosterone Levels and Body Condition

Basal levels of corticosterone measured 19 days after implantation varied significantly in relation to androgen

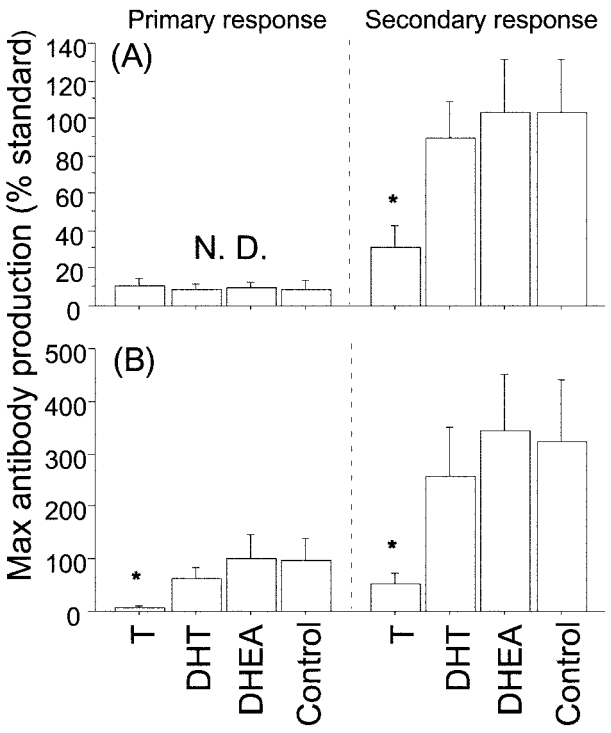

Figure 3: Peak primary and secondary antibody levels produced in response to vaccination by diphtheria (*A*) and tetanus (*B*) antigens according to implant treatment. Antibody production was expressed as percent of the standard. Primary responses to diphtheria were nondetectable (*N. D.*). Asterisks indicate $P < .05$ relative to all other groups using post hoc tests (log-transformed values). Error bars represent 1 SEM.

treatment (fig. 4; one-way ANOVA: log transformed, $F = 5.02$, df $= 3, 22$, $P = .008$). The T treatment caused a fivefold increase in baseline corticosterone levels (30.64 ± 5.01 ng/mL) compared with controls (6.02 ± 1.59 ng/mL; Fisher's PLSD: $P < .001$). Birds implanted with DHT (11.4 ± 4.18 ng/mL) or DHEA (10.8 ± 3.19 ng/mL) did not differ significantly in baseline corticosterone levels relative to controls (fig. 4; Fisher's PLSD: $P > .34$), but they were different from T-treated subjects ($P < .02$).

Body mass and fat score of subjects did not differ among treatment groups at the start (day 0) of the experiment (fig. 5; log-transformed mass, one-way ANOVA: $F = 0.85$, df $= 3, 22$, $P = .48$; fat score, Kruskal-Wallis ANOVA: $H = 2.06$, $P = .63$), indicating that the random assignment of birds to implant groups was not biased by a condition effect. Implant type differentially affected body mass of subjects (fig. 5; rANOVA, log-transformed body mass; time × treatment interaction: $F = 3.29$, df $= 6, 44$, $P = .009$). The T-treated birds lost, on average, 4.3% of their body weight through the course of the experiment, whereas subjects implanted with DHT, DHEA, or control implants gained, on average, 4.7%, 2.8%, and 3.2% of their body weight, respectively. Consequently, by the end of the experiment, body mass of T-implanted birds was significantly lower than body mass of birds implanted with DHT, DHEA, or control implants (Fisher's PLSD: all $P < .007$). Following a similar pattern, fat scores tended to differ among implant groups at day 41 (fig. 5; Kruskal Wallis: $H = 6.44$, $P = .081$). Fat depots of T-implanted birds were low by the end of the experiment (0.75 ± 0.48 fat score) compared with other implant groups (DHT: 1.78 ± 0.49; DHEA: 2.5 ± 0.24; control: 1.92 ± 0.30).

Immune-Endocrine Interrelationships

A clear separation between cell-mediated and humoral immune function was revealed by the correlation matrix (table 2); PHA responses were not significantly correlated with either primary or secondary humoral responses within individuals ($r < 0.19$, $P > .37$). However, primary and secondary responses toward tetanus were correlated within individuals ($r = 0.66$), and secondary responses to diphtheria and tetanus were also positively correlated ($r = 0.60$; table 2). Although primary antidiphtheria profiles were nondetectable, secondary responses were related to primary antitetanus responses ($r = 0.45$; table 2), indicating that responses toward these two antigens are moderately correlated as previously described (Svensson et al. 1998; Råberg and Stjernman 2003).

There was a significant negative correlation between corticosterone titers on day 19 and secondary antitetanus responses within individuals ($r = -0.41$, $P = .04$). Corticosterone levels tended to be negatively correlated with PHA responses ($r = -0.36$), although this relationship

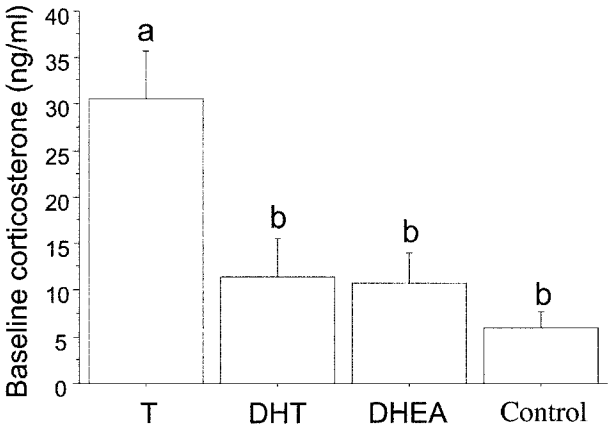

Figure 4: Baseline corticosterone levels 19 days after implantation. Shared letters indicate no significant differences in post hoc tests. Error bars represent 1 SEM.

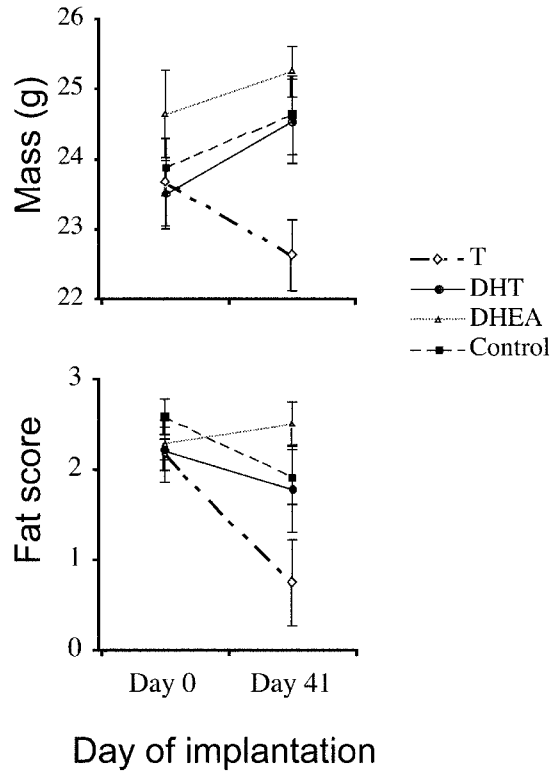

Figure 5: Mass (g) and fat score of subjects before and 41 days after implantation. Error bars represent 1 SEM.

was not quite statistically significant ($P = .07$; table 2). Primary antitetanus responses and secondary anti-diphtheria responses were not significantly correlated with corticosterone levels (table 2).

Discussion

One of the primary tenets of the immunocompetence handicap hypothesis is that T suppresses some aspect(s) of immune function. In support of this hypothesis, we found that nonbreeding males implanted with T reduced both cell-mediated and humoral immune responses compared to controls. The results are consistent with previous studies that have documented immunosuppression in breeding (Evans et al. 2000; Casto et al. 2001) and nonbreeding (Duffy et al. 2000) passerine birds by experimentally elevating T levels using silastic implants.

This is the first study to document T-induced immunosuppression of both primary and secondary humoral immune responses in songbirds. Suppression of secondary responses may be dependent on reduced B lymphocyte expansion during the primary response, as evidenced by a robust correlation between primary and secondary responses within individuals. Another possibility is that T-helper lymphocytes responsible for isotype switching from IgM to IgG may be downregulated (Schuurs and Verheul 1990). Although the absence of a correlation between cell-mediated (PHA) and secondary humoral responses implies that this second scenario is unlikely, it is not known whether the PHA response is an accurate indicator of T-helper cell activity. Further experimental work is required to ascertain whether T-induced suppression of humoral responses is a result of reduced cell-mediated immune activity.

The DHT implants did not suppress cell-mediated or humoral immune function. This is surprising because the binding affinity of 5α-DHT to androgen receptors is six- to 10-fold higher than that of T in rats (Wilson and French 1976), and we anticipated a robust suppressive effect of DHT on immune function assuming the underlying mechanism is mediated through a traditional androgen-receptor pathway. Males implanted with DHT experienced a corresponding increase in DHT levels that were high enough to increase the growth of the cloacal protuberance, which is androgen dependent. Unless an androgen receptor that is specific only for DHT exists (research to date suggests that this is not the case), these results imply that immunosuppression by T does not occur through a direct effect per se by activating androgen receptors in target immune tissue or lymphocytes.

Because we did not measure DHEA levels in DHT-implanted subjects, it is possible that any immunosuppressive effects from DHT treatment may have been masked by an immunoenhancing effect of high circulating levels of DHEA. However, this scenario is unlikely because there is no reason to suspect that DHEA levels would be solely elevated in DHT-implanted subjects, and levels are consistently low in captive song sparrows (this study; K. Soma, unpublished data).

The failure of DHT to invoke obligate immunosuppression has been indicated in two previous studies on birds. Ros et al. (1997) found that T and DHT treatment even seemed to enhance antibody responses to sheep red blood cells in young black-headed gulls. In immature broiler chickens, a significant decrease in the total number of leukocytes, lymphocytes, and weight of the bursa of Fabricius (an important organ for B lymphocyte maturation in birds) was found after treatment with T but not DHT (al-Afaleq and Homeida 1998).

In birds, androgen receptors (along with estrogen, progestin, and glucocorticoid receptors) have been localized in the bursa of Fabricius of immature chickens (Sullivan and Wira 1979; Gasc and Stumpf 1981), but it is not known whether androgen receptors are found in adult immune organs or in circulating avian lymphocytes (Marsh and Scanes 1994). Furthermore, the bursa regresses

Table 2: Correlation matrix of primary (1°) and secondary (2°) humoral responses, cell-mediated (PHA) immune responses, and plasma corticosterone (CORT) levels

	1° tetanus	2° tetanus	2° diphtheria	PHA	CORT
1° tetanus[a]	…	**.657**	**.453**	.163	−.161
2° tetanus[a]	.000	…	**.604**	.108	−.241
2° diphtheria[a]	.019	.001	…	.183	**−.409**
PHA[b]	.430	.603	.376	…	−.362
CORT	.437	.238	.037	.069	…

Note: Correlation coefficients (r) and P values are shown above and below the diagonal, respectively. Coefficients in bold indicate $P < .05$.

[a] Maximum antibody titers produced after diphtheria-tetanus vaccination.

[b] Percent increase in wing web thickness 24 h after PHA injection.

before birds reach adulthood (Glick 1983); therefore, it is likely that the effects of T on the immune system are contingent on the particular stage of development that the androgens are administered. Instead of a direct effect of T in modulating immunocompetence in passerine birds, our results suggest that T-induced immunosuppression is mediated through a combination of indirect effects that are a consequence of T implantation.

Indirect Pathways

Stress-Induced Immunosuppression. Basal corticosterone levels were elevated in T-implanted birds in comparison to levels measured in DHT-, DHEA-, and control-implanted birds. It is well known that chronic exposure to glucocorticoids suppresses immune function and inflammatory responses in vertebrates (Munck et al. 1984; Marsh and Scanes 1994; Besedovsky and del Ray 1996; Hillgarth and Wingfield 1997; Apanius 1998; Råberg et al. 1998). In an experiment on mallards, glucocorticoid injections decreased both innate and acquired immune function (Fowles et al. 1993). A rise in corticosterone levels after T implantation has been documented in other avian studies examining the immunocompetence handicap hypothesis (Duffy et al. 2000; Evans et al. 2000; Casto et al. 2001) and is thought to be due in part to an increase in corticosteroid binding globulin in the blood (Klukowski et al. 1997). In dark-eyed juncos, these increases in binding globulins from T implantation may be high enough to offset the increase in glucocorticoid secretion such that less free (unbound) corticosterone is available for target tissues (Breuner and Orchinik 2000). Despite an elevation in corticosterone levels in T-implanted song sparrows, there was no significant rise in DHT-implanted subjects. These differences in corticosterone levels may explain on a proximate level why immunosuppression occurred in T-implanted birds but not in DHT-implanted subjects (although see below regarding aromatization). Furthermore, basal levels of corticosterone were negatively correlated with both humoral and cell-mediated immunocompetence, although some of these relationships were not quite statistically significant. These findings suggest that increases in plasma corticosterone after T implantation may have a negative effect on acquired immune function in passerine birds.

Future studies examining the role of T in modulating immunocompetence need to account for corticosterone levels (preferably free levels) to ascertain its contribution in modulating immune function. In addition, experiments employing pharmacological blockers to remove the effects of elevated corticosterone in T-implanted birds by inhibiting either its secretion (e.g., mitotane; Breuner et al. 2000) or its ability to bind to glucocorticoid receptors are needed.

Aromatization. Another indirect pathway potentially involved in T-induced immunosuppression is the conversion of testosterone to estradiol by the enzyme aromatase (CYP19) within immune cells or tissue, leading to estradiol-mediated immunosuppression. Our data suggests this pathway is possible because T, an aromatizable androgen, suppressed immune function in song sparrows while DHT, a nonaromatizable androgen, did not invoke immunosuppression.

Estrogen typically enhances humoral immune responses but can enhance or depress cell-mediated immune function, depending on the dose and frequency of administration (Grossman 1985; Alexander and Stimson 1988; Schuurs and Verheul 1990; Olsen and Kovacs 1996). Avian studies examining the effect of estrogen on immunocompetence are scarce and restricted to domestic poultry experiments. In immature broiler chickens, Leitner et al. (1996) found that estradiol treatment enhanced humoral responses, while al-Afaleq and Homeida (1998) documented a decline in leukocytes, lymphocytes, and the weight of the bursa of Fabricius after exposure to E_2 and T but not after DHT treatment, thus supporting the aromatization hypothesis.

In our study, we found that T-implanted birds suppressed both humoral and cell-mediated immune function, which potentially supports the aromatization pathway as the proximate cause of immunosuppression for cell-mediated immunity but not humoral immune function. Further study is needed to confirm the aromatase pathway in birds by establishing whether estradiol is indeed immunosuppressive in passerines, whether aromatase is present in avian immune cells, and whether aromatase blockers (e.g., fadrozole) have salutary effects on T-induced immunosuppression.

Energy Allocation. A final indirect pathway that could lead to T-induced suppression of the immune system is a trade-off between changes in energy allocation imposed by T implantation and the costs of activating the immune system. Male song sparrows implanted with T had lower body mass and fat depots compared with the other treatment groups after 41 days of implantation. This effect has been previously described in song sparrows (Wingfield 1984*a*) and dark-eyed juncos *Junco hyemalis* (Ketterson et al. 1991), with T-implanted males losing more mass and fat than controls. Decreases in body mass and fat stores are likely mediated through increased activity levels that are a consequence of prolonged exposure to T (Wikelski et al. 1999; Lynn et al. 2000). It is possible that T-induced elevations in corticosterone levels could affect condition by increasing the rate of protein catabolism in muscle, although treatment with corticosterone implants tends to increase fat deposition in passerine birds (Wingfield and Silverin 1986; Gray et al. 1990).

Wikelski and Ricklefs (2001) proposed that the effect of T on immunocompetence is closely tied to differences in energy allocation, with increased energy demands for activity metabolism reducing energy available for immunocompetence. Several studies have shown that activation of the immune system is energetically expensive (Demas et al. 1997; Ots et al. 2001; Martin et al. 2003; but see Svensson et al. 1998). Furthermore, Casto et al. (2001) found that T implantation suppressed cell-mediated responses in free-living males but not in captive subjects, suggesting that greater activity levels in the field may limit resources allocated to immune defense.

Another possibility is that trade-offs among different components of immunity could occur such that immune cells are temporarily transported to other compartments of the immune system and not necessarily downregulated (Braude et al. 1999). However, our data do not support this immunoredistribution hypothesis because both cell-mediated and humoral immune responses were suppressed directly or indirectly by T treatment, and there was no correlation between these responses within individuals. However, we did not measure innate immune function, so it is possible that trade-offs between innate and acquired immunity could occur.

No Enhancement from DHEA

Although most androgens have a suppressive effect on immune function, DHEA has a beneficial effect on immunity in mice and humans (Daynes et al. 1990; Svec and Porter 1998; Ben-Nathan et al. 1999). However, we did not find an enhancing effect of DHEA in captive song sparrows. Males implanted with DHEA did not differ in any measure of acquired immune function compared with controls. Plasma levels of DHEA after implantation were slightly above those typically found in wild male song sparrows (Soma and Wingfield 2001), so the dose may have been pharmacological. Similar to the action of glucocorticoids, DHEA may promote biphasic responses, with low doses improving immune function and high doses suppressing it (Svec and Porter 1998). In addition, the majority of studies that have found an immunoenhancing effect involve ameliorating immune function in subjects that are immunocompromised (Svec and Porter 1998). It would be interesting to conduct a similar study on passerine birds to identify whether DHEA treatment may "rescue" the suppressive effects of T on immune function.

Conclusions and Implications

To our knowledge, this is the first study to experimentally demonstrate that immunosuppression by T may occur through a combination of indirect pathways. These pathways are unaccounted for in most immunoecology studies to date that have tested the immunocompetence handicap hypothesis, suggesting that the hypothesis may need reevaluation, at least in avian studies. We emphasize that these findings do not necessarily refute the assumptions of the hypothesis as long as the indirect mechanisms are able to maintain honesty in sexually selected traits that are dependent on androgens for their development or expression (Folstad and Karter 1992). Other hormones or biochemicals, such as glucocorticosteroids, that are elevated by T may have a more direct influence on immunocompetence (Buchanan 2000; Evans et al. 2000; Casto et al. 2001). In addition, alterations in energy allocation as a result of high T levels may also affect development of the trait if it is condition dependent. Studies utilizing in vitro models are needed because they circumvent the multiple indirect effects incurred by T implantation. Last, because short-day birds were used in this study and no sexual signaling is occurring at this time, future experiments examining the effect of different androgens on acquired immune function in long-day (breeding) subjects would also prove useful.

In summary, we find that indirect mechanisms (such as corticosteroids and resource allocation) are likely responsible for the suppression of the avian immune system by T. The possibility that some androgens suppress immune function while others do not is a provocative finding. The uncoupling of T from avian immunosuppression may be a means to allow greater flexibility in hormonal signaling and may also explain the positive association between T and immunocompetence found in wild birds (Peters 2000), but further investigations are warranted. Although immune-neuroendocrine interrelationships are complex and most avian studies lack the use of sophisticated molecular techniques to quantify immunocompetence, a continual advancement of immunoecology studies and techniques will increase our understanding of proximate mechanisms underlying the immunocompetence handicap hypothesis and elucidate how sexual signals evolve as honest indicators in relation to endocrine-immune interactions.

Acknowledgments

We thank L. Erckmann for generously assisting us with the RIAs, N. Perfito for performing the majority of laparotomies, and L. Råberg for help with ELISAs. We also thank V. Apanius, G. Bentley, and one anonymous reviewer for improving the manuscript. Experiments and assays were funded by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas), the Crafoord Foundation, the Carl Tryggers Foundation (D.H.), and the National Science Foundation (NSF; IBN 9905679; J.C.W.). N.T.O. was supported by a NSF predoctoral fellowship and a Richard Synder Vertebrate Zoology Award. This research was approved by the University of Washington Animal Care Committee (protocol 2212–31).

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Associate Editor: Ellen D. Ketterson