

Effects of testosterone on cell-mediated and humoral immunity in non-breeding adult European starlings

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One of the primary assumptions of the immunocompetence hypothesis is that testosterone is immunosuppressive. Although many studies in birds and mammals have supported this assumption, conflicting results have been reported in a variety of species. We investigated the effects of testosterone manipulation on both cell-mediated and humoral immunity in adult songbirds, European starlings (*Sturnus vulgaris*). Male and female starlings were wild-caught, housed in the laboratory, and implanted with either empty silastic capsules or capsules containing testosterone. Six weeks after implantation, humoral immunity was assessed by injecting the birds with a novel antigen, keyhole limpet hemocyanin, and measuring specific antibody responses 10 and 15 days later via an enzyme-linked immunosorbent assay. Cell-mediated immunity was assessed 7 weeks after implantation via intradermal injection of the T-cell mitogen phytohemagglutinin into the wing web and measuring the degree of swelling 24 h later. Antibody responses to antigenic challenge were significantly suppressed in testosterone-treated females 10 days post-injection and in both sexes 15 days post-injection. Furthermore, there was a significant inverse relationship between individual variability in antibody responsiveness and plasma testosterone concentrations. Cell-mediated responses to phytohemagglutinin stimulation were also significantly suppressed in testosterone-treated males compared to same-sex controls. Testosterone treatment significantly increased plasma corticosterone concentrations compared to controls, and the possibility of this effect mediating the immunosuppressive effects of testosterone is discussed. The present study is among the first to demonstrate testosterone-induced suppression of both cell-mediated and humoral immunity in a species of songbird. *Key words*: immune function, cell-mediated, humoral, immunity, birds, starling, phytohemagglutinin, KLH, testosterone, corticosterone, immunocompetence. [*Behav Ecol* 11:654–662 (2000)]

It is often stated that testosterone (T) suppresses immune function, based on numerous studies in mammals and birds (for reviews, see Grossman, 1985; Hillgarth and Wingfield, 1997; John, 1994; Marsh and Scanes, 1994; McCrudden and Stimson, 1991; Nelson and Demas, 1996; and Schuurs and Verheul, 1990). However, reviews of the literature reveal that the relationship between T and immune function is quite complex. While many studies in mammals demonstrate a general pattern of T-induced immunosuppression, some researchers report either no relationship or a positive relationship between T and immunity (see Ansar Ahmed et al., 1985 for review).

The majority of studies investigating the effects of androgenic steroids on immune function in birds have used domestic chickens as a model (for reviews, see Glick, 1984; John, 1994; Marsh and Scanes, 1994; and Schuurs and Verheul, 1990). In general, T administration appears to suppress immunity, but as with mammalian studies, there have been some contradictory findings. For instance, T treatment of obese strain (OS) chickens reduced the severity of spontaneous autoimmune thyroiditis (SAT) but only when administered early in development (Gause and Marsh, 1986). The immunosuppressive effect of T treatment on SAT onset in OS chickens was not observed in adults in which T administration had either no effect or even enhanced the severity of the disease (Gause and Marsh, 1986; Marsh and Scanes, 1994). In con-

trast, early castration of chickens has been demonstrated to suppress cell-mediated immune responses (Mashaly, 1984).

Likewise, studies investigating the effects of T on humoral immunity have produced contradictory findings. In chickens, T treatment induces premature atrophy of the bursa of Fabricius, the primary lymphoid organ of humoral immunity in birds, and significantly suppresses immunoglobulin (Ig) G antibody responses to immunization with bacterial antigens (Gause and Marsh, 1986; Glick, 1984; Hirota et al., 1976). Similarly, implantation of male barn swallows (*Hirundo rustica*) with T results in a temporary decrease in immunoglobulin concentrations (Saino et al., 1995).

In contrast to reported immunosuppressive effects of T, previous studies report either no effect or enhancing effects of T on IgG and IgM antibody responses to antigenic challenge in birds (Hasselquist et al., 1999; Leitner et al., 1996; Ros et al., 1997). Due to these conflicting results, it is necessary for more studies to be conducted using adult wild birds in order to determine whether the effects of gonadal steroids on immunity in young chickens can be generalized to bird species in the wild (Hasselquist et al., 1999; Ros et al., 1997; Saino et al., 1995; Zuk et al., 1995). Furthermore, studies measuring both cell-mediated and humoral immunity are necessary because it is possible that T may differentially affect different components of the immune system (Norris and Evans, 2000; Zuk and Johnsen, 1998).

Based on the general assumption that T suppresses immune function, hypotheses regarding the interaction between immunocompetence and reproductive fitness have been developed (Folstad and Karter, 1992; Hamilton and Zuk, 1982). The immunocompetence hypothesis (Folstad and Karter, 1992), in particular, has recently gained attention among behavioral ecologists investigating sexually-selected characters in

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passerines (Hasselquist et al., 1999; Møller et al., 1996; Saino and Møller, 1996; Saino et al., 1995; Saino et al., 1997a,c). However, to date, few studies have investigated the effects of T on both cell-mediated and humoral immunity in any species of passerine. Thus, in the present study, we sought to investigate the effects of high physiological doses of T on both cell-mediated and humoral immunity in adult songbirds, European starlings (*Sturnus vulgaris*), using specific and direct measures of immune function *in vivo*. The assessment of the humoral response to antigenic challenge was performed using an enzyme-linked immunosorbent assay (ELISA) developed by us specifically for use with starlings. This assay is a highly quantitative and direct measure of antibody response to a specific antigen. Cell-mediated immunity was measured via the subcutaneous injection of the T cell mitogen phytohemagglutinin (PHA) using a technique that has widely been used in birds (e.g. Johnsen and Zuk, 1999; Zuk and Johnsen, 1998; Lochmiller et al., 1993; Mashaly, 1984; Parmentier et al., 1993; Saino et al., 1997b; Soler et al., 1999).

METHODS

Adult (≥ 2 years old) male ($n = 8$) and female ($n = 13$) reproductively inactive (photorefractory) starlings were caught in Maryland (39°18' N lat.) in the summer of 1998. They were housed in single-sex cages on a light:dark schedule of 18:6 and received food (Purina turkey starter crumbs) and water *ad libitum*. This photoperiod maintained the birds in a reproductively inactive state in which their gonads were completely regressed (Nicholls et al., 1988), thus ensuring that their endogenous concentrations of gonadal steroids were at a minimum. The birds all had black beaks, a sensitive external indicator of the absence of T in the plasma of starlings (Ball and Wingfield, 1987; Dawson, 1983; Witschi and Miller, 1938). The birds were allowed to complete their feather molt prior to T treatment or assessment of immunity in order to control for any energetic expense that may be associated with molting (Lindström et al., 1993). Once all birds completed molt, they were implanted with either testosterone-filled (four males and seven females) or empty silastic implants (four males and six females). Under secobarbital anesthesia (.07 ml of 50 mg/ml solution injected intramuscularly), two 10 mm silastic implants (1.47 mm i.d., 1.96 mm o.d.) were placed within the peritoneal cavity via a small incision in the left flank. This dose has previously been demonstrated to result in T concentrations within the high normal physiological range for adult males (Bernard and Ball, 1997).

Another group of reproductively inactive adult birds (12 males and seven females) were caught in the fall of 1998 and exposed to the same experimental procedures described herein. The only substantial difference between the two cohorts was that the implants for the second group were placed subcutaneously over the left flank rather than intraperitoneally in order to provide slower absorption of the hormone. Again, one half of the birds received T-filled implants (six males and three females) while the other half received blank implants (six males and four females).

Hormone analysis

Blood samples were taken at zero, two, four, six, and eight weeks after implantation by puncturing the alar vein with a 25-g needle and collecting approximately 500–1000 μ l of blood into heparinized microcaraway tubes. The blood was then transferred into 1.5 ml microcentrifuge tubes that were centrifuged at 1387.5 g for 15 min at 4°C. The plasma fraction was pipetted off and stored in 0.5 ml centrifuge tubes at -70°C until assayed for T concentrations via radioimmuno-

assay (RIA). The T RIA for the first group of birds was performed using a Coat-A-Count Total Testosterone ^{125}I kit (Diagnostic Products Corporation, Los Angeles, CA, USA) following the manufacturer's protocol. This assay was highly sensitive (i.e., 100 pg/ml) and specific (i.e., cross-reactivity to 5 α -Dihydrotestosterone (DHT) is 3.3%, to 17 β -estradiol is 0.02%, and to corticosterone is 0.002%).

The T RIA for the second group of birds was performed using an ^{125}I double-antibody kit purchased from ICN Biomedicals, Inc. (Costa Mesa, CA, USA) following the manufacturer's protocol. This assay was also highly sensitive (i.e., 100 pg/ml) and specific (cross-reactivity to 5 α -DHT is 3.4%, and to 17 β -estradiol and corticosterone is $< 0.01\%$). Prior to the second RIA, some samples from the first group of birds were run using the double-antibody kit and the values were compared to those previously obtained using the Coat-A-Count kit. The coefficient of variation between the two assays was 11.0%. The intra-assay coefficient of variation for the double-antibody RIA was 7.6%.

Because T implants in dark-eyed juncos (*Junco hyemalis*) can elevate corticosterone concentrations (Ketterson et al., 1991; Klukowski et al., 1997), plasma samples from the second group of birds taken 6 weeks after implantation were also assayed for corticosterone via RIA. Blood samples were obtained within 2 min for each of the birds. The corticosterone RIA was performed using an ^{125}I double-antibody kit available from ICN Biomedicals, Inc. This assay is highly sensitive (i.e., 625 pg/ml) and specific (i.e., cross-reactivity to testosterone is 0.10%, to 5 α -DHT is 0.01%, and to 17 β -estradiol is $< 0.01\%$). The only deviation from the manufacturer's protocol was that we extended the lower limit of the standard curve by diluting the lowest standard control (25 ng/ml) twice to yield additional standard controls of 12.5 ng/ml and 6.25 ng/ml.

Assessment of humoral immunity

Six weeks after implantation, humoral immunity was assessed via injection of the novel T-cell dependent antigen, keyhole limpet hemocyanin (KLH; 300 μ g in 0.1 ml sterile saline, i.m.). Presumably starlings have not previously been exposed to KLH; therefore it is assumed that the primary antibody response is being measured (see also Hasselquist et al., 1999). Ten and 15 days following injection, blood samples were collected by puncturing the alar vein with a 25-g needle and collecting approximately 500–1000 μ l of blood into microcaraway tubes. The blood was then transferred into 1.5 ml microcentrifuge tubes and allowed to clot for 1 h. The clot was removed and the samples were centrifuged at 1387.5 g for 15 min at 4°C. The serum fraction was then pipetted off and stored in 0.5 ml centrifuge tubes at -70°C until assayed for KLH-specific antibodies.

ELISA development

The KLH-specific antibody titers of the serum were measured using an ELISA. In order to perform this assay, a secondary antibody specifically recognizing starling IgG was custom-made for us by ICN Biomedicals, Inc. To prepare this antibody, we provided ICN with 100 ml of starling serum from which they isolated the IgG fraction. Two rabbits were then inoculated with purified starling IgG, receiving a total of 4 injections spaced 1 week apart. Both rabbits were bled once per week for 7 weeks beginning 3 weeks after the initial inoculation. Portions of all antisera samples taken from the two rabbits were provided for preliminary testing in our lab.

To develop and optimize our ELISA protocol using the custom secondary antibody, we modified a protocol previously demonstrated to work well in mammals (Demas and Nelson,

1998; Klein and Nelson, 1998). Briefly, microtiter plates that were coated with KLH were incubated with sera from starlings previously inoculated with KLH. For each individual serum sample, the anti-KLH antibodies bound to the KLH coating the plate and the remaining sample was washed away. Next, the custom secondary anti-starling IgG was added followed by a third antibody made in goat to recognize rabbit IgG. The goat anti-rabbit IgG was purchased from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA, USA catalog #111-055-003) as an affinity purified whole molecule conjugated to the enzyme alkaline-phosphatase (AP). The use of the goat anti-rabbit antibody was the major deviation from the mammalian protocol and was necessary because the conjugation of the custom secondary antibody would not have been cost-effective. The subsequent addition of the substrate, *p*-nitrophenyl phosphate (Sigma Chemical, St. Louis, MI, USA) resulted in a colorimetric reaction which was read using a plate reader (Bio-Rad: Benchmark model, Richmond, CA, USA).

To determine the optimal concentrations of the samples and antisera, we conducted a series of five pilot studies using sera from an additional 15 birds that had been injected (i.m.) with three different doses of KLH (200 µg, 300 µg, or 400 µg of KLH in 0.1 ml sterile saline). Blood samples were obtained 10 and 15 days post-injection and the individual samples were used in the pilot studies at dilutions of 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560. In addition, pooled sera samples from five birds never exposed to KLH (i.e., negative controls) were included in the same dilutions to determine the degree of serum-dependent non-specific binding. On each plate, one well received no starling serum in order to assess the degree of serum-independent non-specific binding.

In the initial pilot studies, portions of the individual anti-starling sera samples from the two rabbits were pooled giving us one pooled sample from each rabbit. Both anti-starling sera pools were run separately in the initial pilot assay. Antisera from one rabbit provided much less serum-dependent non-specific binding and was used in all subsequent assays. For this antiserum, serum-dependent non-specific binding was on average 86% higher than the no serum control values and 52% lower than the positive control values. The remaining pilots used combinations of different dilutions of the anti-starling IgG and the AP-conjugated goat anti-rabbit. It was determined that dilutions of 1:500 for each antibody provided the best results. A final pilot comparing the results among all of the anti-starling sera samples (one each from the seven blood samples) from the rabbit that provided optimal results did not reveal a substantial difference among the samples. Furthermore, a dose of 300 µg of KLH yielded the highest antibody responses.

ELISA protocol

For the results reported here, the following protocol was used. Microtiter plates were coated with KLH (0.5 mg/ml in sodium bicarbonate buffer, pH = 9.6) and blocked with 10% nonfat dry milk in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). Thawed serum samples were diluted 1:40, 1:80, 1:160 and 1:320 with PBS-T and 150 µl of each sample were added in duplicate to the wells of the microtiter plates. Positive control samples (pooled sera from the starlings used in the pilot studies that exhibited high concentrations of anti-KLH IgG) and negative control samples (pooled sera from starlings that were not exposed to KLH) were added in duplicate to each plate. One well on each plate had no starling sera added to assess the level of serum-independent non-specific binding. The plates were sealed, incubated at 37°C for 3 h, then washed five times with PBS-T.

The plates were then incubated with the secondary antibody (diluted 1:500) that was raised in rabbit to recognize specifically starling IgG for 1 h at 37°C and washed five times with PBS-T. The AP-conjugated goat anti-rabbit was added to the wells (dilution 1:500) and incubated for 1 h at 37°C. The plates were washed three times with PBS-T and 150 µl of the enzyme substrate *p*-nitrophenyl phosphate (1 mg/ml in diethanolamine substrate buffer) was added to each well. The plates were protected from light during the enzyme-substrate reaction that was terminated after 10 min by adding 50 µl of 1.5 M NaOH to each well.

The optical density (OD) of each well was determined using a plate reader equipped with a 405 nm wavelength filter. The average OD for each set of duplicate wells was calculated and the OD for each sample was expressed as the ratio of the sample to the negative control (P/N) for the same dilution to minimize intra- and inter-assay variability (deSavigny and Voller, 1980; Leitner et al., 1996). All sera samples for both days 10 and 15 were run in one assay. Ten samples from both days were run in a second assay and the inter-assay coefficient of variation was 3.1%.

Assessment of cell-mediated immunity

Seven weeks after implantation, cell-mediated immunity was assessed via intradermal injection of the T-cell mitogen PHA (Sigma, St. Louis, MO). The thickness of the site of injection, the wing web, was measured to the nearest 0.01 mm using a pressure-sensitive thickness gauge (Mitutoyo Corporation, Model # ID-S1012E) immediately prior to and 24 h following injection. The right wing web was injected with 0.5 mg of PHA in 0.1 ml PBS and the left wing web was injected with 0.1 ml of the vehicle alone (Lochmiller et al., 1993; Saino et al., 1997b). The degree of the immune response was assessed as the percent increase in wing web thickness (calculated as the degree of swelling of the PHA-injected wing divided by the swelling of the vehicle-injected wing) (Dhabhar and McEwen, 1996; Mashaly, 1984).

Data analyses

Serum anti-KLH antibody titers were analyzed using two 2 (sex) × 2 (T treatment) analyses of variance (ANOVAs), one for each day post-injection (10 and 15). The percentage change in wing web thickness was log transformed to correct for heterogeneity of variance and analyzed using a 2 (sex) × 2 (T treatment) ANOVA. The effects of T implantation on corticosterone concentrations were analyzed using a 2 (sex) × 2 (T treatment) ANOVA. Planned comparisons were performed using Fisher's PLSD and differences were considered significantly different if $p < .05$.

RESULTS

Humoral immunity

The data from the birds caught in the summer and fall of 1998 were pooled providing us with a total of 40 subjects (20 males and 20 females). One male that received blank implants was removed from analysis due to leakage of the antigen during injection. Testosterone treatment significantly reduced IgG antibody response to KLH at both 10 and 15 days post-injection [$F(1,35) = 8.518, p < .05$ and $F(1,35) = 11.209, p < .05$, respectively]. Planned comparisons revealed T-induced suppression of antibody responses in females 10 days post-injection and in both sexes 15 days post-injection (see Figure 1).

Hormone analysis revealed that T treatment elevated plas-

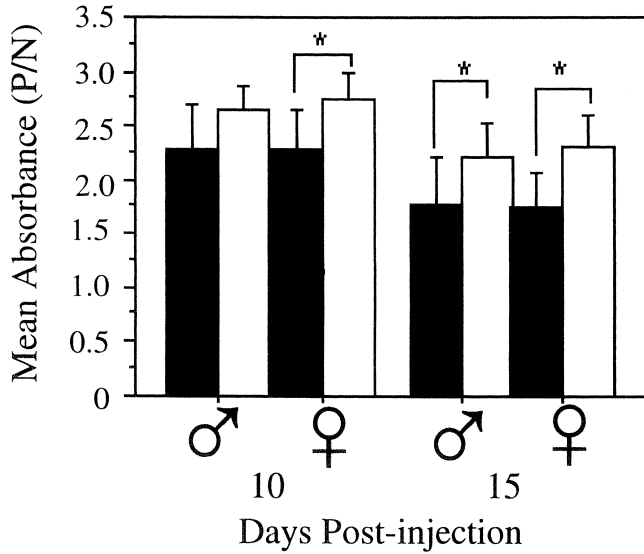


Figure 1
Mean absorbance values of experimentally-treated birds (P) relative to negative controls (N) at 10 and 15 days post-injection. Error bars represent 95% confidence intervals. Asterisk indicates $p < .05$ relative to controls. Open bars, blank implants; filled bars, T implants.

ma T concentrations in both sexes to within the high normal range for adult breeding males (see Figure 2; Bernard and Ball, 1997). Closer examination of the individual T concentrations indicated variability within each of the T-treated groups. Therefore, two regressions were performed, one for each day that antibody responses were analyzed (10 and 15 days post-injection), using the data from the T-implanted birds to investigate whether the individual variability in T concentrations could predict individual variability in antibody responses to KLH. For these analyses, plasma T concentrations from samples taken 6 weeks post-implantation were used as the independent variable because it was at that time that the birds received antigenic challenge. A significant negative correlation was observed between plasma T concentrations and antibody responses at 15 days post-injection [see Figure 3; $F(1,18) = 7.921, p < .05, r^2 = .318$].

Cell-mediated immunity

As for humoral immunity, the data from both sets of birds were pooled. Due to leakage of the mitogen during injection, 13 birds were removed from the analysis to yield a final number of 27 birds. Figure 4 illustrates the effect of T manipulation on the response to *in vivo* mitogenic stimulation. Planned comparisons revealed that males who received T-filled capsules exhibited a significantly smaller swelling response to mitogenic stimulation than males that received blank implants (Figure 4). This effect was not observed in females. Unlike that observed in the humoral response, regression analyses revealed no correlation between individual variations in T concentrations and wing web swelling.

Hormone analysis

As stated above, hormone implantation significantly raised T concentrations to within the high physiological range for adult breeding males (Figure 2; Bernard and Ball, 1997). Further analysis revealed that T treatment significantly elevated plasma corticosterone concentrations in both males and fe-

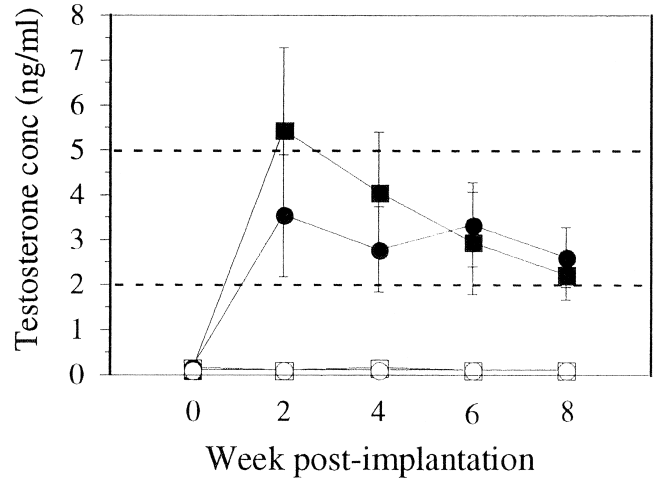


Figure 2
Mean plasma testosterone concentrations (ng/ml) measured via radioimmunoassay. Dashed lines indicate the upper and lower limits of the normal physiological range for adult breeding males. Error bars represent 95% confidence intervals. Open symbols, blank implants; filled symbols, T implants; squares, males; circles, females.

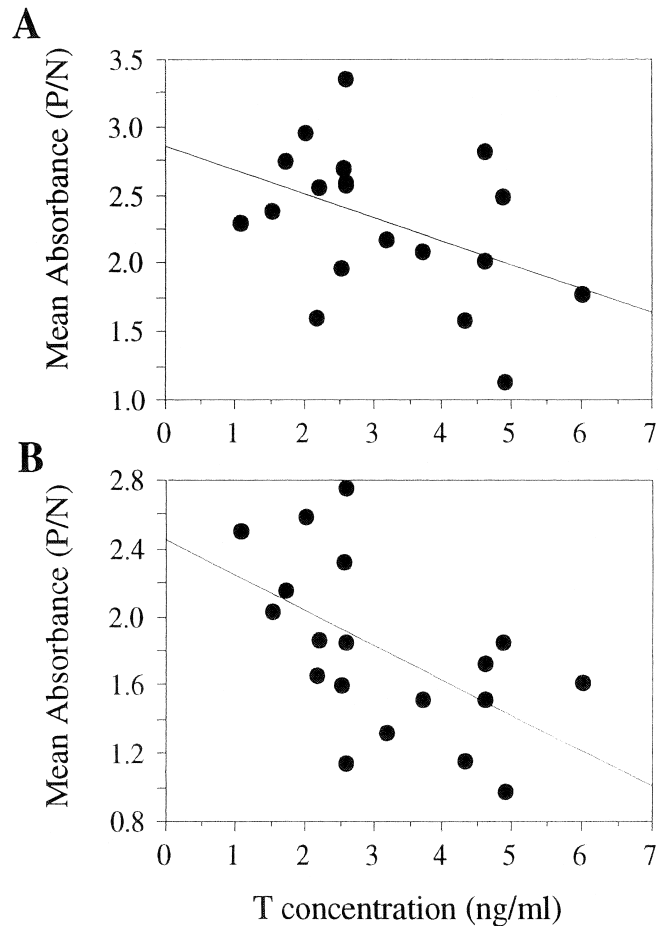


Figure 3
Mean absorbance values (P/N) in relation to plasma testosterone concentrations (ng/ml) at day 10 (A) and day 15 (B) post-injection of KLH. The linear regression lines have the equations $y = 2.855 - 0.174x; r^2 = .190, p = .0618$ and $y = 2.450 - 0.206x; r^2 = .318, p < .01$, respectively.

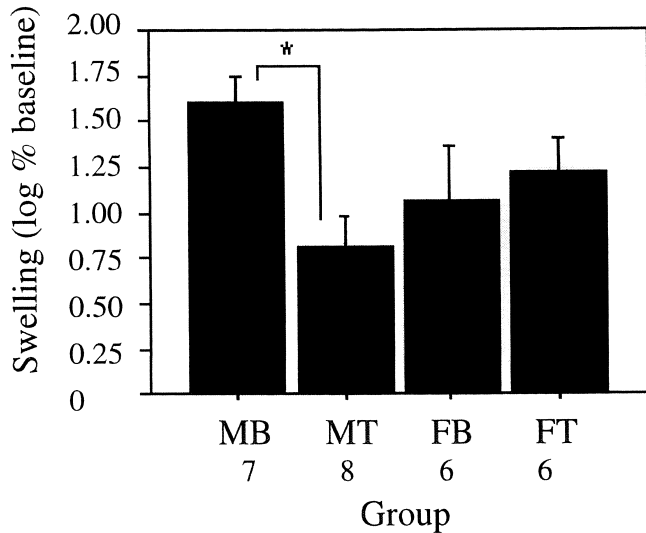


Figure 4
Log transformed values of the mean (\pm SE) change in web swelling (% baseline) 24 h after injection with PHA. Asterisk indicates $p < .05$ relative to controls. MB, males with blank implants; MT, males with T implants; FB, females with blank implants; FT, females with T implants. Numbers below the x-axis indicate the sample size for each group.

males [Figure 5; $F(1,12) = 11.996$, $p < .05$]. Three birds were omitted from the corticosterone analysis due to an insufficient quantity of sample and one bird was omitted because its concentration of corticosterone fell below the limits of detectability of the assay.

DISCUSSION

The present experiment demonstrates that T concentrations within the high normal physiological range suppress humoral and cell-mediated immunity in European starlings. Cell-mediated immunity in males (Figure 4) and humoral immunity in both males and females (Figure 1) were suppressed by T manipulation. Furthermore, a strong negative relationship exists between T concentrations and IgG response to antigenic challenge. Because only the highest testosterone concentrations suppressed antibody responses, one possible interpretation is that humoral immunosuppression acts as a constraint on the magnitude of testosterone elevation associated with the breeding season for this species.

Immunosuppression is only one of several ways that high T concentrations have been demonstrated to be costly to males. In various taxa, high T concentrations associated with the breeding season are known to facilitate courtship and aggressive behaviors which can be energetically expensive and may result in injury (Dufty, 1989; Emerson and Hess, 1996; Johnsen, 1998; Moore and Marler, 1987; Wingfield et al., 1987, 1990). Furthermore, elevated T concentrations are associated with a decrease in paternal care in some biparental species of birds, thus imposing a reproductive fitness cost (Cawthorn et al., 1998; Hegner and Wingfield, 1987; Ketterson and Nolan, 1992; Ketterson et al., 1992; Oring et al., 1989; Silverin, 1980; Wingfield et al., 1990). Also, prolonging the period of peak T concentrations beyond what is normally experienced can increase mortality in some bird species (Dufty, 1989; Nolan et al., 1992). Thus, in addition to immunosuppression, there are numerous constraints on the magnitude and duration of high T concentrations, only a sample of which have been discussed here.

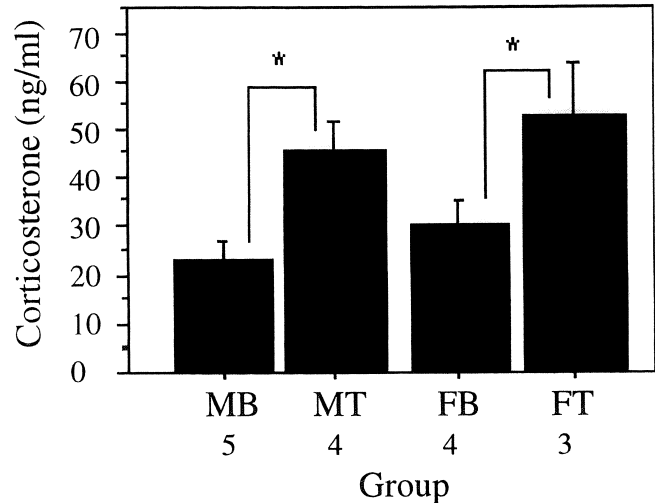


Figure 5
Mean (\pm SE) corticosterone concentrations (ng/ml) 6 weeks after implantation. Asterisk indicates $p < .05$ relative to controls. Numbers below the x-axis indicate the sample size for each group.

In the interpretation of these results, it must be noted that the antibody responses measured in the present experiment are a composite of both IgM and IgG isotypes due to the likelihood of cross-reactivity of the anti-starling IgG secondary antibody. The primary antibody response, presumably as assessed here, consists primarily of IgM in the initial stages of the response followed by a switch to IgG. It is possible that administration of T reduced IgG synthesis without decreasing the IgM response. Also, the antibody response to KLH is dependent on T-helper lymphocytes, a component of the cell-mediated immune system, for antigen recognition and isotype switching. Thus, it is possible that the suppression of the antibody response to KLH by T was the result of an interference with the switching from IgM to IgG. Some evidence in domestic poultry supports this alternative mechanism (see Schuurrs and Verheul, 1990 for review). In chickens, embryonally administered T results in a decrease in IgG, but not IgM antibody responses which appears to be due to an impairment of isotype switching (Glick and Sadler, 1961; Hirota et al., 1976, 1980; Lerner et al., 1971; Schuurrs et al., 1987; Verheul et al., 1986).

Comparison with previous studies

These data are in agreement with previous studies in some avian species suggesting suppressive effects of T on immune function (Al-Afaleq and Homeida, 1998; Leitner et al., 1996; Verhulst et al., 1999; Zuk et al., 1995). For instance, exogenous administration of T to broiler chicks resulted in a decrease in the total number of leukocytes, lymphocytes and the weight of the bursa of Fabricius (Al-Afaleq and Homeida, 1998). Likewise, IgM responses to sheep red blood cells (SRBC) in white leghorn chickens treated embryonically with T were significantly impaired compared to untreated controls (Hirota et al., 1976). Other indirect evidence further supports the hypothesis of T-induced humoral immunosuppression. For example, in domestic chickens selectively bred to produce either high or low antibody responses to *E. coli* or SRBC, low responders had elevated testosterone concentrations compared to high responders (Leitner et al., 1996; Verhulst et al., 1999). However, in these studies the direction of causality is unknown and it is possible that enhanced immunity suppress-

es T concentrations in selectively bred chickens (Verhulst et al., 1999).

Studies in wild species have also provided evidence for T-induced immunosuppression. Barn swallows treated with T in the field exhibited a temporary decrease in total immunoglobulin levels (Saino et al., 1995). In red jungle fowl, endogenous testosterone concentrations were negatively correlated with lymphocyte counts (Zuk et al., 1995). A recent study in male dark-eyed juncos (*Junco hyemalis*) has also demonstrated that artificial elevation of T suppresses cell-mediated and humoral immunity (Casto JM and Ketterson ED, personal communication).

The data presented here differ from the findings of some other recent work examining the effects of physiological concentrations of T on humoral immunity in another species of songbird, red-winged blackbirds (*Agelaius phoeniceus*). In that study, naturally-occurring increases in T concentrations in males did not lead to suppression of a composite of IgG and IgM antibody responsiveness to KLH injections (Hasselquist et al., 1999). Possible reasons for these different results include species and methodological differences. One potentially important methodological difference is that in the present experiment the primary response to KLH stimulation was assessed whereas Hasselquist and colleagues measured secondary responses to KLH injection.

The secondary antibody response involves a different population of B cells known as memory B cells which may respond differently to the presence of T. Also, while the primary antibody response is first dominated by the IgM isotype prior to switching to IgG, the secondary antibody response is IgG dominated (Janeway and Travers, 1997). Thus, it is possible that only the primary response to a given antigen is influenced by elevated T concentrations (John, 1994; Wunderlich et al., 1992). There is some evidence in a mammalian species that the ability of T to disrupt immune responses to a given pathogen may depend on previous exposure to that pathogen. Testosterone-induced attenuation of resistance to *Plasmodium chabaudi* malaria in female laboratory mice (strain C57BL/10) was eradicated if the mice had previously overcome infection with the same parasite (Wunderlich et al., 1992).

Finally, Hasselquist et al. (1999) inoculated their birds with a mixture of KLH and Freund's incomplete adjuvant which is simply an oil-in-water emulsion that facilitates the uptake of the antigen by macrophages in the initial stages of the response (Janeway and Travers, 1997). In addition to measuring the secondary response to KLH, the use of an adjuvant by Hasselquist et al. may also have contributed to differences in the proportion of IgM to IgG isotypes in the sera. In preliminary testing, Hasselquist et al. observed a peak primary antibody response (a composite of IgG and IgM) 12 days after the first inoculation with KLH. Therefore, in our study we assumed that the peak primary antibody response would occur around 10 to 15 days post-injection (Klein and Nelson, 1998, 1999). However, in the former study, an increased rate of isotype switching due to the use of an adjuvant may have resulted in a shift of the peak response. Thus, we cannot conclude that our time points of 10 and 15 days post-injection represent the peak antibody response. A time course study in which blood samples are taken every week after inoculation would need to be performed in order to ascertain with certainty the peak response time as well as the duration of the response.

Effects of testosterone in females

Importantly, the females in the present experiment experienced T concentrations that were within the normal physiological range for adult breeding males (Bernard and Ball,

1997). The purpose here was to determine whether a given dose of T would have equal effects in both sexes. If immunity in females was suppressed to a greater extent than that observed in males, it may indicate that a mechanism has evolved in males to cope with the high T concentrations associated with the breeding season.

Although adult females in the wild do have detectable concentrations of T during the breeding season, the dose of T that these females received was supraphysiological (Dawson, 1983; Kessel, 1951). Therefore, it is not yet clear whether a physiological dose of testosterone suppresses humoral immunity in females. Unlike males, cell-mediated immunity was not suppressed by high T concentrations in females. It is possible that this sex difference is a result of the difference in T concentrations between males and females 2 weeks after implantation (see Figure 2). The high peak in T concentrations in males at week 2 could have affected the cell-mediated response to PHA 4 weeks later. The duration of T-induced immunosuppression is not yet clear in adult birds and requires further investigation.

Role of reproductive condition

Because the birds in the present experiment were in a reproductively inactive condition, it could be argued that the T treatment used was not truly physiological. It could reasonably be expected that other variables associated with reproductive condition interact with high T concentrations to suppress immunity further in starlings. It is known in some cases that biological responses to gonadal steroids do change with season in birds and mammals (e.g., Hinde and Steele, 1978). Exogenous gonadal steroids are more effective at facilitating reproductive behaviors in seasonally-breeding species when administered during day lengths associated with the breeding season than when given during day lengths associated with the non-breeding season. Also, photoperiod-dependent effects of T have been identified in starlings. The effects of T on the neural circuit controlling song production appear to be attenuated in reproductively inactive (i.e., photorefractory) male starlings compared to birds in a reproductively active condition (Bernard and Ball, 1997).

Photoperiodic manipulation affects *in vitro* cell-mediated immune responses to mitogenic stimulation in starlings, irrespective of gonadal secretions (Bentley et al., 1998). Birds in a reproductively active condition exhibit decreased proliferative responses of splenocytes to mitogenic stimulation compared to birds in a reproductively inactive condition. Therefore, it is possible that the suppressive effects of T would be intensified if the birds in the present experiment were in a reproductively active condition.

In general, there are a number of possible mechanisms that could be responsible for the seasonal changes in responsiveness to T administration, including changes in the number and/or activity of androgen receptors (Moeller et al., 1988; Nastiuk and Clayton, 1994; Slater and Scheck, 1998; Soma et al., 1999; Tahka et al., 1997; Wood and Newman, 1993). However, to date no study has reported photoperiod-induced fluctuations in androgen receptors within the avian immune system.

Role of corticosterone

The mechanism through which T affects immunity is not completely understood. Androgen receptors have been localized within the thymus in mammals and within the bursa of Fabricius in chickens (Grossman, 1985; Marsh and Scanes, 1994; McCruden and Stimson, 1984; Schuur and Verheul, 1990; Sullivan and Wira, 1979). Another possible mechanism

though which T could be acting involves corticosterone. This hormone, typically secreted in response to stress, modulates immune function in a variety of mammalian and avian species (see, e.g., Dunn, 1989; Fowles et al., 1993; Trout and Mashaly, 1994).

As with androgen receptors, glucocorticoid receptors have been localized in a variety of lymphoid tissues, including the bursa of Fabricius in chickens and the thymus in chickens and rodents (Bakker and Kendall, 1997; Coulson et al., 1982; Fessler et al., 1986; Miller et al., 1998; Sullivan and Wira, 1979). Similar to reports in dark-eyed juncos (Ketterson et al., 1991; Klukowski et al., 1997), T treatment in the present study raised basal corticosterone concentrations above those observed in untreated same-sex controls. Therefore, it is possible that T-induced corticosterone elevation is responsible for the attenuation of humoral and cell-mediated immunity observed in the present study. However, endogenous increases in T are not necessarily associated with increases in basal corticosterone concentrations. Despite this, T mediates many behaviors that may increase the likelihood that an individual will experience a stressful situation (e.g., aggressive interactions; Balthazart, 1983; Wingfield et al., 1987, 1990); natural increases in glucocorticoids caused by these stressors may influence immunocompetence concomitant with T concentrations associated with the breeding season.

Functional significance

In the present study, T treatment resulted in an average reduction of 15.5% (17% in females and 14% in males) in the humoral immune response 10 days after immunization with KLH and a 22.5% reduction (25% in females and 20% in males) in antibody responses 15 days post-immunization. Furthermore, a 50% reduction in the cell-mediated response to PHA injection was observed in males. It is not known what impact this degree of immune function impairment would have on the survival of starlings in the wild. In order to address this issue, field studies investigating the effects of T manipulation on immunocompetence and long-term survival are needed.

Experiments assessing long-term survival of individuals in wild populations are uncommon due to difficulties in tracking individuals from one breeding season to the next (however, see Dufty et al., 1989; Ketterson et al., 1992; Saino et al., 1995, 1997a). One noteworthy exception is the work in barn swallows (*Hirundo rustica*) demonstrating that immunocompetence is a reliable predictor of long-term survival in the wild (Saino et al., 1997a). Furthermore, artificial elongation of tail feathers of barn swallows decreases IgG antibody responses following SRBC inoculation and decreases the probability of surviving to the next breeding season (Saino et al., 1997a). Thus, factors affecting immune responses to antigenic stimulation can have an impact on long-term survival. Administration of T implants to male barn swallows also reduced the probability of survival to the next breeding season by 50%, although T-induced immunosuppression was not detected (Saino et al., 1995). Whether the degree of impairment observed in the present study would have notable effects on survival remains an open question.

The data presented here provide an important contribution to the field by demonstrating a T-induced reduction of responses to immunogenic challenge (Norris and Evans, 2000). Recently, Braude and colleagues (1999) proposed the redistribution hypothesis as an alternative to the immunosuppression model. According to their hypothesis, elevations in T may result in a redistribution of lymphocytes, rather than a reduction in their numbers. The authors point out that a reduction in the number of circulating leukocytes may be due to im-

mune cells being relocated to other components of the immune system, such as the lymph nodes, rather than immunosuppression as often claimed. It has therefore been noted that multiple measures of immunity are necessary when investigating the effects of T on immunocompetence because a shift from one component of the immune system to another may otherwise be overlooked (Braude et al., 1999; Norris and Evans, 2000; and Zuk and Johnsen, 1998).

In the present study, we observed suppression of both cell-mediated and humoral immunity using direct methods to assess immune function. These data suggest that rather than a T-induced shift from one component of the immune system to another, T suppresses both the cell-mediated and humoral components. However, because KLH is a T-cell dependent antigen, it is possible that T-induced suppression of antibody response to KLH occurs indirectly via suppression of cell-mediated immunity. Also, we did not assess innate immunity, therefore the effects of T on this component of immune function and possible consequences for adaptive immune responses remains unclear (Norris and Evans, 2000). Nevertheless, because we assessed immunocompetence directly by measuring inclusive responses to immunogenic challenge, we have taken any possible redistribution of immune cells into account. Thus, these data support the hypothesis that T is immunosuppressive. Further study using a variety of immunogenic challenges assessing cell-mediated and humoral immunity in addition to innate immune measures would provide insight into the extent of T-mediated immunosuppression.

Summary

In summary, the data presented here are in support of one of the primary assumptions of the immunocompetence hypothesis (Folstad and Karter, 1992). These data provide empirical evidence that testosterone is indeed immunosuppressive in starlings. Furthermore, the present study demonstrates a sex difference in the effects of testosterone on cell-mediated immunity as measured by *in vivo* mitogenic stimulation of T cells. While testosterone suppressed the subcutaneous response to PHA in males, no effect was observed in females. On the other hand, testosterone treatment suppressed antibody responses to antigenic challenge in both sexes in a dose-dependent fashion. Testosterone treatment also increased corticosterone concentrations and the possibility that this effect is the mechanism through which T manipulation suppresses immune function requires further investigation.

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