Steroid Hormones and Immune Function: Experimental Studies in Wild and Captive Dark-Eyed Juncos (*Junco hyemalis*)

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ABSTRACT: Monogamous and polygynous male songbirds generally differ in their breeding season profiles of circulating testosterone. Testosterone level spikes early in the breeding season of monogamists and then declines, but it remains high in polygynists. Male dark-eyed juncos (*Junco hyemalis*) are socially monogamous and exhibit the usual pattern, but experimental maintenance of high testosterone throughout the breeding season alters normal behavior and physiology and affects various components of annual reproductive success but not overall annual success. Because stabilizing selection predicts that alteration of naturally existing phenotypes should reduce lifetime reproductive success, we asked whether prolonged testosterone exposure might impair immune function and perhaps thereby reduce life span. We assessed immune function in captive and wild male juncos that we treated with either testosterone-filled or empty Silastic implants. Results indicate that prolonged elevation of testosterone suppresses antibody production in captive males and cell-mediated immunity in wild males. Together these results suggest that testosterone-treated males may be more susceptible to disease or parasitic infection. As earlier studies have shown, levels of corticosterone as well as testosterone are higher in testosterone-treated males, so it is unclear whether the immune suppression we observed is due to testosterone's direct effects on immunity or testosterone's influence on glucocorticoid production. We discuss results in the context of recent hypotheses regarding life-history theory and potential endocrine-immune interactions.

Keywords: birds, corticosterone, endocrine-immune interactions, immunity, mating systems, testosterone.

Polygynous and monogamous male songbirds of the temperate zone typically differ in their patterns of sex steroid secretion during the breeding season. Males of polygynous species often maintain elevated testosterone throughout the season, whereas in monogamous males, testosterone typically peaks during territory establishment and pair formation and diminishes thereafter (Wingfield et al. 1990). For the past 12 yr, we have manipulated testosterone levels in socially monogamous dark-eyed juncos (*Junco hyemalis*) to expose males to a pattern like that of polygynists. The effect is to alter the behavior and physiology of experimental males, which has led us to ask whether the treatment leads to advantageous or disadvantageous outcomes with respect to fitness.

Behaviorally, testosterone-treated males (T-males) sing more frequently than controls (C-males; Ketterson et al. 1992; Chandler et al. 1994) and feed their young less often (Ketterson et al. 1992; Schoech et al. 1998). They also have larger home ranges when their mates are infertile (Chandler et al. 1994, 1995; Smulders et al. 2000), are slower to detect a nest predator (Cawthorn et al. 1998), and in captivity are more attractive to females (Enstrom et al. 1997). Probably associated with these changes, T-males sire more extrapair young (Raouf et al. 1997), but fewer young that they sire with their social mates survive to independence. The net result is that the single-season reproductive success of T-males does not differ from that of controls.

Physiologically, T-males lose winter fat stores at earlier dates (Ketterson et al. 1991), maintain higher titers of corticosterone and corticosteroid-binding globulin (Ketterson et al. 1991; Klukowski et al. 1997; Schoech et al. 1999), and exhibit a stronger adrenocortical response to stress caused by human handling (Schoech et al. 1999). These findings suggest that maintenance of high testosterone throughout the breeding season may have adverse effects on physical condition and therefore longevity, which is an important component of individual fitness (Lochmiller and Deerenberg 2000). If naturally existing phenotypes are maintained by stabilizing selection, then theory predicts that lifetime reproductive success would be lower in juncos with the experimentally produced hormonal profile than in those with the naturally occurring

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norm (Marler et al. 1996; Sinervo and Basolo 1996; Ketterson and Nolan 1999). Accordingly, our failure to observe a negative effect on single-season reproductive success of T-males led us to expect that their life span may be shorter, although we have observed no differences in annual return rates of T- and C-males when testosterone exposure is limited to approximately 3 mo duration (Nolan et al. 1992; Ketterson et al. 1996). If experimental exposure to elevated testosterone does shorten life span, one possible cause may be an effect of testosterone on immune function, as proposed by the immunocompetence handicap hypothesis (Folstad and Karter 1992).

The immunocompetence handicap hypothesis predicts a trade-off between suppressive influences of testosterone on immune function and enhancing influences of testosterone on characters that are attractive to females (Folstad and Karter 1992). Studies using different indirect indices of immunocompetence (e.g., parasite loads and lymphocyte counts) in adult birds have tested whether testosterone suppresses immune function, and results have been conflicting. A negative relationship between testosterone titers and parasite load was found in barn swallows (*Hirundo rustica*; Saino and Møller 1994), but no such relationship was detected in red-winged blackbirds (*Agelaius phoeniceus*; Weatherhead et al. 1993). Similarly, a negative correlation between testosterone and lymphocyte counts was demonstrated in red jungle fowl (*Gallus gallus*; Zuk et al. 1995) but not in barn swallows (Saino et al. 1995). Experimental assessments of the effect of testosterone on direct indices of immune function (i.e., immune challenges) have also differed in results. Elevated testosterone titers were associated with reduced antibody production in adult barn swallows, house sparrows (*Passer domesticus*), and chickens (Saino et al. 1995; Verhulst et al. 1999; Evans et al. 2000) but not in juvenile black-headed gulls (*Larus ridibundus*; Ros et al. 1997) or adult red-winged blackbirds (Hasselquist et al. 1999). The diversity of these results may reflect differences in methodology, including age of experimental subjects and the immune response being tested. However, given that species differ in mating strategies and related hormonal profiles during the breeding season (Wingfield et al. 1990), it seems reasonable to expect that testosterone's impact on the immune system might also vary across species.

Here we report two experiments in which we directly assessed immune function of testosterone-treated and control male dark-eyed juncos and found that elevated testosterone was associated with reductions in immune responses. We discuss the results with respect to our experimental approach to understanding the role of testosterone in life-history trade-offs in juncos as well as with respect to the immunocompetence handicap and immunoredistribution hypotheses (Folstad and Karter 1992; Braude et al. 1999).

Methods

Experiment 1: Assessment of Cell-Mediated and Humoral Immunity in Captive Male Juncos

Subjects and Housing. Twenty-four adult male dark-eyed juncos were captured in the vicinity of the University of Virginia's Mountain Lake Biological Station in Giles County, Virginia (37°22′N, 80°32′W) between April 28 and May 13, 1998, and housed individually in outdoor cages (0.61 m × 1.14 m × 2.44 m [w × d × h]). Birds had constant access to drinking water and a mixture of millet and cracked corn, which was supplemented daily with mealworms.

Hormone Treatment and Blood Collection. On May 28, we randomly assigned captives to an experimental or control treatment group, anesthetized them with methoxyflurane, and subcutaneously implanted two 12-mm-long pieces of Silastic tubing (1.47 mm inner diameter, 1.95 mm outer diameter), filled with 10 mm of testosterone or left empty (Ketterson et al. 1992). The testosterone treatment was designed to maintain testosterone levels at or near the natural early spring peak level for an extended period of time, which has been its effect in past experiments (e.g., Ketterson and Nolan 1992). All implants remained in place until they were removed on July 7.

To determine steroid hormone titers, we bled each bird on three separate occasions (May 26, June 11, and July 21). Each bleeding was at a different stage of the study: before implants were inserted (April 28–May 27), while implants were in place (May 28–July 7), and after implants had been removed (July 8–July 30). Hereafter, these stages of the study will be referred to as "before implant," "while implanted," and "after implant removal," respectively. At each stage, we collected approximately 200 μL of blood from the alar vein into heparinized microhematocrit tubes. With a stopwatch, we measured handling time during bleeding ("total bleeding time"). After centrifuging the blood, we measured hematocrit and collected plasma, which we stored at −20°C until performing radioimmunoassays.

Morphological Measures. During each stage of the study, we measured body mass to the nearest 0.1 g using a balance (Pesola) and ranked furcular and abdominal fat stores from 0 (no fat) to 5 (bulging fat deposits; modified from Helms and Drury 1960). We ranked condition of pectoral musculature from 1 (muscle concave and markedly depressed) to 3 (muscle convex) as described by Gosler

(1991). We measured length (L), depth (D), and width (W) of the cloacal protuberance (CP) and calculated CP volume as volume $= \pi \times (D/2 \times W/2) \times L$ (Tuttle et al. 1996).

Immune Tests. Cell-mediated immunity was assessed in 13 C-males and 10 T-males with a cutaneous delayed-type hypersensitivity test that assessed secondary and tertiary immune responses to phytohemaglutinin (PHA; Sigma L-8754), an innocuous plant lectin (procedures modified from Lochmiller et al. 1993). Immediately following implant on May 28, we primed each bird with a subcutaneous injection into the right scapular apterium of 0.25 mg of PHA in 50-μL phosphate buffered saline (PBS). On June 7, we measured the thicknesses of the right- and left-wing webs to the nearest 0.025 mm with a pressure-sensitive dial thickness gauge (Mitutoyo model 7326). Immediately thereafter we made a subcutaneous challenge injection of 0.25 mg of PHA in 50-μL PBS into the right-wing web and another of 50-μL PBS into the left-wing web. At 24 and 48 h postchallenge (± 3 min), wing web thicknesses were remeasured and swelling in response to the PHA challenge was calculated by subtracting the change in left-wing web thickness from the change in right-wing web thickness. To assess any long-lasting effects of the experimental hormone manipulation on cell-mediated immunity, between July 28 and July 30, 3 wk after testosterone and control implants had been removed, we performed a second PHA challenge using the same procedure just described.

We assessed humoral immunity (antibody production) in 13 C-males and 11 T-males using a sheep erythrocyte hemagglutination assay (Hay and Hudson 1989; Ros et al. 1997). On June 25, following collection of approximately 50 μL of plasma, each bird was immunized with 100 μL of a 2% solution of sheep erythrocytes in PBS injected intraperitoneally. Six days later, another 50 μL of plasma was collected. All plasma was stored at -20°C until assayed for antibodies. To assess antibody titers, we mixed, in microtiter plates, twofold serial dilutions of pre- and postimmunization plasma samples in PBS with washed sheep erythrocytes. The presence or absence of agglutination of sheep erythrocytes was then assessed for each dilution of a plasma sample. Antibody responses were expressed as $\log_2$ of the lowest concentration of plasma at which erythrocytes showed agglutination. We included positive and negative controls on each assay plate to assure standardized scoring of hemagglutination; scoring was by an observer blind to the treatments. We ran all samples in duplicate; intra-assay variation was 4.8%. No hemagglutination was observed in any preimmunization plasma sample. In 33% of the birds (four T-males and four C-males), hemagglutination was undetectable in even the most concentrated postimmunization plasma dilution (similar to Deerenberg et al. 1997; Evans et al. 2000; Peters 2000). This lack of a detectable antibody response to immunization may have been a result of limitations in the sensitivity of the assay or to ineffective exposure of the immune system to the antigen. Given the diverse reasons for producing a nondetectable response, birds lacking a detectable antibody response were excluded from statistical analysis of the hemagglutination assay.

Experiment 2: Assessment of Cell-Mediated Immunity in Free-Living Male Juncos

Subjects and Study Area. Between April 17 and May 15, 1998, we captured 82 free-living males and implanted them with either two testosterone or two control implants as described in experiment 1. Because apparently all male juncos returned to the study area and their territories the following breeding season, some of the 1998 males had been implanted in previous years; these received the same hormonal treatment as in the past. Treatment of males that had not been previously implanted was determined randomly. Immediately following implant, we primed males with an injection of 0.25 mg PHA suspended in 50 μL of Freund's complete adjuvant (FCA; ICN 642851) and then released them at their capture sites. We used FCA (a mixture of dead bacteria and oil) as a vehicle in this experiment in order to increase the antigenicity of, and to prolong exposure to, PHA. Phytohemaglutinin suspended in FCA is preferred to PHA in PBS when the interval between priming and challenge may be long.

Capture and Immune Testing. Throughout the breeding season, we searched for and found nests of the social mates of implanted males and captured the males with mist nets on the day when most of the nestlings in the brood were 6 or 7 d old (May 21–July 11). Immediately upon capture of the males, we collected 200 μL of blood, as described previously, in order to assess steroid hormone levels; we measured total bleeding time with a stopwatch. In the two cases in which we were uncertain about the exact time the male was caught, we estimated total bleeding time as half the time elapsed since the mist net was last seen empty. We measured the thicknesses of the right- and left-wing webs and gave 17 males (T-males $= 4$, C-males $= 13$) a PHA challenge as described in experiment 1, subsequently releasing them onto their territories. Approximately 25 h postchallenge (average, 24.97 h; range, 23.7–28.5 h), males were recaptured by playing a tape-recorded junco song beside a live caged male junco lure placed next to a mist net. We remeasured wing web thicknesses to determine swelling in response to the PHA challenge. Two T-males and four C-males were not recaptured following the PHA challenge.

Hormone Measurement. Plasma testosterone and corticosterone were measured by radioimmunoassay following separation from other steroid hormones by column chromatography (for procedures, see Wingfield and Farner 1975; Ball and Wingfield 1987; Ketterson et al. 1991). All testosterone and all corticosterone samples from both experiments were run in duplicate in single assays (intraassay coefficients of variation were 6.4% and 12.6%, respectively). We purchased testosterone antibody from Wien Laboratories (T-3003; Succasunna, N.J.) and corticosterone antibody from Endocrine Sciences (B21-42; Calabasas, Calif.). Standards were from Sigma; radiolabeled testosterone and corticosterone were from New England Nuclear.

Statistical Analyses. In experiment 1, we used linear regression to assess whether testosterone or corticosterone titers covaried significantly with total bleeding time. Hormone assay data were analyzed using mixed ANOVAs with hormone treatment (testosterone vs. control) as a between-subjects variable and stage of the study (before implant, while implanted, and after implant removal) as a within-subjects variable. One hypothesis being tested in the experiment, that elevated testosterone would cause less wing web swelling (cell-mediated immunity), was evaluated with a mixed hierarchical ANOVA with hormone treatment as a between-subjects variable and measurement time (24 vs. 48 h postchallenge) nested within challenge number (first vs. second challenge) as two within-subjects variables. The other hypothesis, that elevated testosterone would cause less antibody production in response to immunization with sheep erythrocytes (humoral immunity), was evaluated with a two-sample one-tailed *t*-test. Body mass, fat, pectoral condition, CP volume, and hematocrit data were analyzed using mixed ANOVAs with hormone treatment as a between-subjects variable and stage of study as a within-subjects variable. Because body mass, fat, pectoral condition, and CP volume were unlikely to be statistically independent measures, α levels for these analyses were corrected using the Bonferroni method (Sokal and Rohlf 1995).

In experiment 2, we used linear regression to assess whether testosterone or corticosterone titers covaried significantly with total bleeding time. Assay data for testosterone titers were analyzed using a two-sample two-tailed *t*-test and for corticosterone titers using an ANCOVA, with hormone treatment as the between-subjects variable and total bleeding time as the covariate. We used a Mann-Whitney *U*-test to evaluate the hypothesis that elevated testosterone would cause less wing web swelling. We also used Mann-Whitney *U*-tests to assess between-groups differences in body mass, pectoral condition, fat, and CP volume. As in the previous experiment, α levels were corrected using the Bonferroni method where appropriate. In both experiments, statistical significance was set at $P < .05$.

Results

Experiment 1

Steroid Hormone Measurement. In experiment 1, total bleeding time was not significantly correlated with testosterone or corticosterone titers in the captive males ($P > .05$). Testosterone titers were higher in T-males than C-males while implanted, but they did not differ before or after ($F = 88.031$, df $= 2, 40$, $P = .0001$; fig. 1*A*). Similarly, corticosterone titers were elevated in T-males as compared to C-males only while males were implanted ($F = 5.811$, df $= 2, 42$, $P = .0059$; fig. 1*B*). Testosterone and corticosterone titers of treatment groups combined, while implanted, were positively correlated ($R = 0.54$, $P = .0065$; fig. 2).

Immune Assessment. Although T-males tended to exhibit less wing web swelling than C-males when measured 24 and 48 h after the first immune challenge, there were no significant differences between treatment groups in any measure of wing web swelling ($P = .62$; fig. 3*A*). Wing web swelling scores were higher after the second PHA challenge than after the first challenge regardless of treatment group ($F = 11.23$, df $= 1, 21$, $P = .003$; fig. 3*A*).

Immunization with sheep erythrocytes produced an increase in antibody titers in 67% of the males tested. T-males that exhibited an antibody response to the single exposure to sheep erythrocytes produced significantly lower (36%) antibody titers than did C-males ($t = 1.866$, df $= 14$, one-tailed $P = .042$; fig. 3*B*).

Morphological Measures. Data for morphological measures are summarized in figure 4. Body mass and pectoral muscle condition did not differ significantly with hormonal treatment or stage of the study ($P > .05$; fig. 4*A*, 4*B*). Although fat scores increased significantly with stage ($F = 11.67$, df $= 2, 44$, $P = .0001$), they did not vary between treatments ($P > .05$). Paired sample *t*-tests revealed that fat scores were significantly higher while males were implanted and after implants were removed than they were before implanting ($P < .05$; fig. 4*C*). We found a significant interaction between hormonal treatment and implant status on CP volume ($F = 9.64$, df $= 2, 44$, $P = .0003$; fig. 4*D*). Independent samples *t*-tests revealed that T-males had significantly greater CP volumes than controls while implanted ($P = .0274$) but not before implant or after implant removal ($P > .05$).

Hematocrit. We found a significant interaction between hormonal treatment and implant status for hematocrit ($F = 4.27$, df $= 2, 44$, $P = .020$). Independent samples *t*-tests revealed that T-males had significantly lower hematocrit values than controls after implants were removed (49.0 ± 1.25 vs. 52.0 ± 0.78; $P = .0476$), but hematocrit did not differ between the groups before or while implanted (53.9 ± 1.12 vs. 51.3 ± 1.13, and 51.5 ± 0.86 vs. 50.8 ± 1.20, respectively; $P > .05$).

Experiment 2

Steroid Hormone Measurement. In both treatment groups testosterone titers did not co-vary significantly with total bleeding time ($P > .05$). T-males had higher circulating testosterone titers than C-males ($t = 13.35$, df $= 9$, $P = .0001$; table 1). Corticosterone titers were significantly correlated with total bleeding time ($R = .835$, df $= 9$, $P = .0014$). An ANCOVA with total bleeding time as a covariate indicated that corticosterone titers did not differ between T- and C-males ($P > .05$; table 1).

Immune Assessment. Approximately 25 h postchallenge, wing web swelling in T-males was significantly less (70%) than that in C-males ($U = 18.00$, $n = 2, 9$, $P = .034$; fig. 5).

Morphological Measures. Data for morphological measures are summarized in table 1 and reveal no significant differences between T- and C-males in mean body mass, fat score, pectoral condition score, or CP volume ($P > .05$).

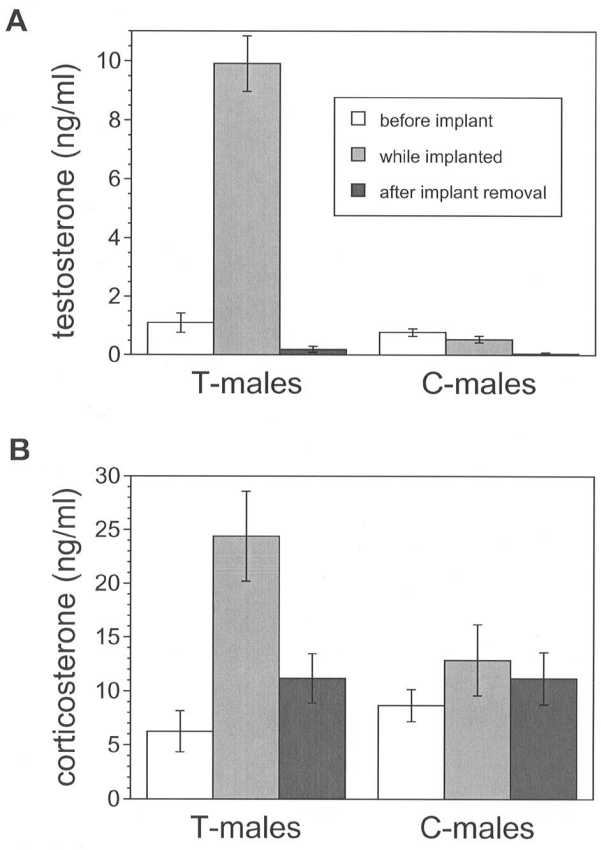

Figure 1: Testosterone and corticosterone titers of T- and C-males ($n = 11$ and $n = 13$, respectively) measured at three stages during experiment 1; before implant (May 26), while implanted (June 11), and after implant removal (July 21). Before-implant blood samples were collected after the birds had been in captivity for at least 2 wk, and thus, before-implant hormone titers do not represent the spring peak. Bars indicate means ($\pm$ SE). *A*, Testosterone titers rose significantly following implantation in T-males and returned to control levels following implant removal. *B*, Likewise, corticosterone titers rose significantly following implantation in T-males and returned to control levels following implant removal.

Discussion

In birds, data from a number of studies suggest that innocuous immune challenges of the type employed in the current experiments are accurate indicators of the immune system's ability to resist disease and parasitic infection. Life span and immune ability are also thought to co-vary. For instance, antibody production in response to immunization with sheep erythrocytes predicts long-term survival in free-living barn swallows (Saino et al. 1997), and experimental manipulations that reduce specific humoral immune responses in collared flycatchers (*Ficedula albicolis*) also increase the intensity of *Haemoproteus* infections (Nordling et al. 1998). Moreover, chickens selected for ability to produce high levels of antibodies to sheep erythrocytes exhibit greater resistance to a variety of internal and external parasites than a line of chickens selected for the lack of ability to produce antibodies (Gross et al. 1980). In adult male house sparrows, greater cell-mediated immune responses are associated with higher survival and greater recovery from *Haemoproteus* infections (Gonzales et al. 1999), and wing web swelling responses are positively correlated with nestling recruitment in European magpies (*Pica pica*; Sorci et al. 1997).

The results of the current experiments provide support for the hypothesis that long-term elevation of circulating testosterone during the breeding season either directly or indirectly suppresses acquired immunity in male dark-eyed juncos. If we consider the results of experiments 1 and 2 collectively, then humoral and cell-mediated immune responses of T-males were lower than those of C-males. In addition to elevated testosterone titers, captive T-males

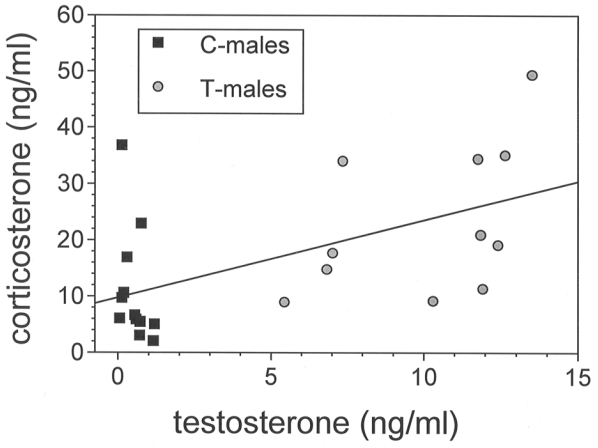

Figure 2: Corticosterone titers as a function of testosterone titers in T- and C-males ($n = 11$ and $n = 13$, respectively).

also had elevated corticosterone, raising the possibility that testosterone's influence on immune function may be mediated indirectly via corticosterone. Regardless of whether testosterone's effects were direct or indirect, our data suggest that testosterone-treated male juncos may be less able than C-males to resist disease and parasitic infection, and this could significantly reduce lifetime reproductive success. Below we discuss, first, the influence of elevated testosterone on immune responsiveness; second, the possible role of corticosterone in the observed immune suppression; third, the relationship among our results, the immunocompetence handicap hypothesis, and the immunoredistribution hypothesis; and, fourth, implications of our experimental approach for how selection might act on natural variation in plasma levels of testosterone.

Testosterone and Immunity

Humoral Immunity in Captive Males. The decreased antibody production by testosterone-treated adult male juncos is similar to that described for barn swallows and house sparrows (Saino et al. 1995; Evans et al. 2000) but differs from the lack of such a relationship in red-winged blackbirds (Hasselquist et al. 1999). These studies used different techniques to assess antibody production, but if we assume that the assessment techniques are equally capable of detecting potential effects of testosterone on humoral immune function, then the differences observed among species might be related to their mating systems. Of the four species involved, only the red-winged blackbird has a polygynous mating system and maintains testosterone at relatively high levels throughout the breeding season (Hegner and Wingfield 1986; Beletsky et al. 1989; Ketterson and Nolan 1992; Saino and Møller 1995). Target tissue sensitivity to steroid hormones may be reduced in species that naturally experience prolonged high concentrations of steroid hormones as compared to those that do not (Taymans et al. 1997). Based on this reasoning, targets such as immune tissues of polygynous species may be less sensitive to testosterone and corticosterone (see Klein and Nelson 1998), as both these hormones are elevated throughout the breeding season in all polygynous bird species studied to date (Beletsky et al. 1989, 1990; Wada et al. 1999). As a corollary, monogamous species, like the

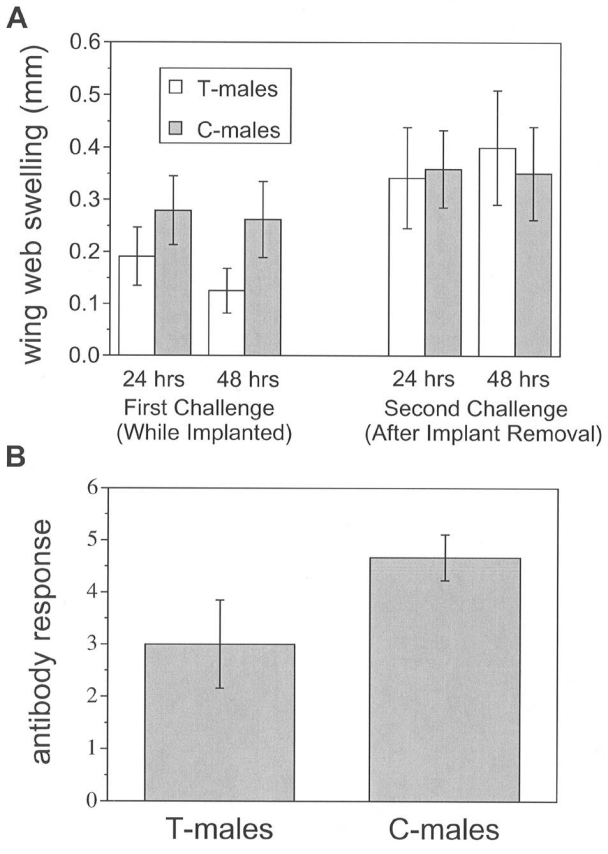

Figure 3: Acquired immune responses of T- and C-males in experiment 1. *A*, Differential wing web swelling (mean ± SE) at 24 and 48 h in response to each of the two PHA challenges. Wing web swelling in T- and C-males ($n = 10$ and $n = 13$, respectively) did not differ significantly following either challenge, and swelling in both treatment groups was significantly greater following the second challenge. *B*, Antibody responses to immunization with sheep erythrocytes in T- and C-males ($n = 7$ and $n = 9$, respectively). The antibody response for each group indicates the mean number of wells in which erythrocyte agglutination occurred (±SE). As compared to C-males, antibody production was suppressed in T-males.

junco, that do not ordinarily experience prolonged exposure to high levels may be more sensitive to testosterone.

In passerine birds, elevated levels of plasma corticosterone in response to experimental elevation of testosterone is common and has been noted in the dark-eyed junco (Ketterson et al. 1991; Klukowski et al. 1997; Schoech et al. 1999), the house sparrow (Evans et al. 2000; but see Hegner and Wingfield 1987), the European starling (*Sturnus vulgaris*; Duffy et al. 2000), and the house finch (*Carpodacus mexicanus*; E. Ketterson, unpublished data). Unfortunately, to our knowledge, the glucocorticoid response to experimentally elevated testosterone has not been assessed in any polygynous species. It is unlikely that polygynous and monogamous songbirds exhibit qualitative differences in glucocorticoid production in response to elevated testosterone, given that there appear to be correlated increases in both testosterone and corticosterone titers in male dark-eyed juncos as well as in males of polygynous species, such as red-winged blackbirds, yellow-headed blackbirds (*Xanthocephalus xanthocephalus*), and

Table 1: Steroid titers and morphological measurements (mean ± SE) in free-living males

	T-males ($n = 2$)	C-males ($n = 9$)
Testosterone (ng/mL)	6.49 ± .24*	.87 ± .18
Corticosterone (ng/mL)	28.00 ± 13.87	24.95 ± 7.18
Body mass (g)	22.7 ± .70	21.7 ± .36
Pectoral condition score	1.7 ± .00	1.8 ± .05
Fat score	1.00 ± .00	.56 ± .18
CP volume (mm^3)	236 ± 60.3	206 ± 12.1

* Differs significantly from C-males.

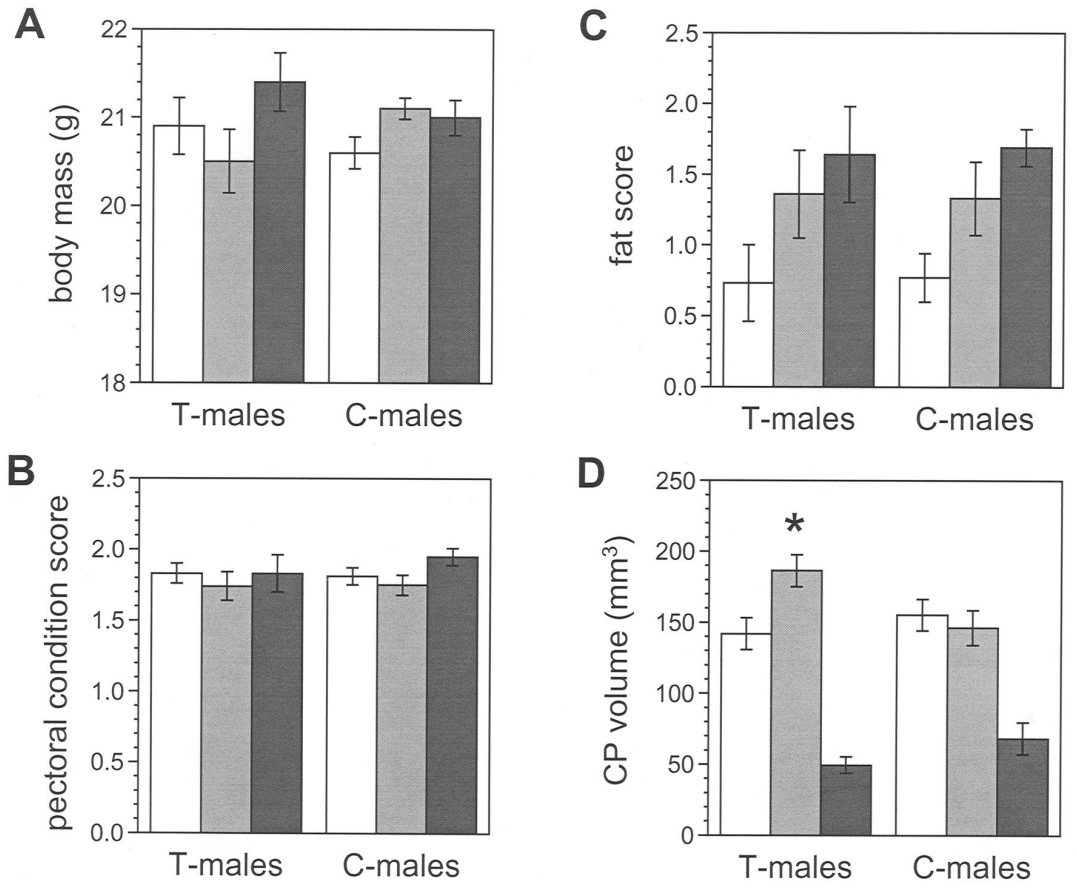

Figure 4: Morphological measurements of T- and C-males ($n = 11$ and $n = 13$, respectively) at three stages in experiment 1; before implant (*unshaded bars*), while implanted (*lightly shaded bars*), and after implant removal (*darkly shaded bars*). Bars indicate group means (±SE), and asterisk indicates between-treatment comparisons that differ significantly ($P < .05$). *A*, Body mass did not differ significantly between hormonal treatments or across stages. *B*, Pectoral condition scores did not differ significantly between hormonal treatments or across stages. *C*, Fat scores did not differ between T- and C-males but increased significantly across the stages of the experiment (see text for more detail). *D*, Cloacal protuberance volume was significantly larger in T-males than C-males, while birds were implanted but not before implant or after implant removal.

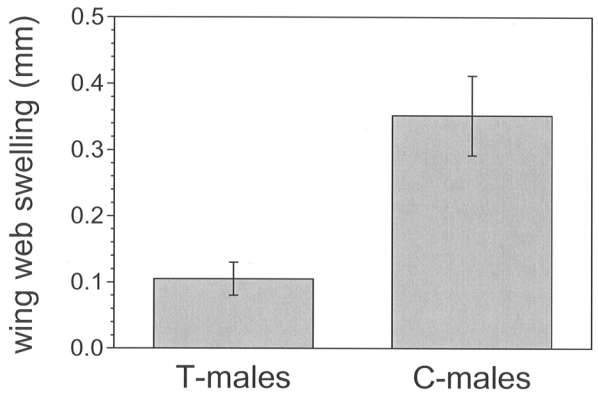

Figure 5: Differential wing web swelling in free-living T- and C-males ($n = 2$ and $n = 9$, respectively) 25 h after PHA challenge (mean ± SE). In comparison to C-males, the response in T-males was significantly suppressed.

bush warblers (*Cettia diphone*), during their respective breeding seasons (Beletsky et al. 1989, 1990; Wada et al. 1999; Deviche et al. 2000). However, as described above, males of polygynous and monogamous species may differ quantitatively in glucocorticoid production in response to elevated testosterone or in their sensitivity to the effects of corticosterone.

Cell-Mediated Immunity in Captive and Free-Living Males. In contrast to their differing antibody responses, wing web swelling of captive T- and C-males did not differ significantly. Nevertheless, while implanted, T-males tended to swell less than C-males at 24 and 48 h postchallenge (31% and 52%, respectively), and in experiment 2, free-living T-males exhibited significantly less swelling than C-males. After the PHA challenge that followed implant removal, males from both treatment groups exhibited greater swelling as compared to their response to the first PHA challenge. It is unclear how to interpret this increased wing web swelling. First, whether testosterone suppressed short-term cell-mediated immunity, it apparently did not impair the ability of T-males to form immune memories necessary for subsequent increases in immune responsiveness. Second, given that testosterone and corticosterone titers in both treatment groups were lower (although not significantly lower for C-males) at the time of the second as compared to the first challenge (see fig. 1), it is possible that the more robust immune response was due to less immune suppression by one or both of these hormones in both treatments. Third, it is also possible that some of the captives had become photorefractory and were no longer in breeding condition at the time of the second PHA challenge. In European starlings, the transition from sensitivity to photostimulation to photorefractoriness is associated with increased cell-mediated immune function via a mechanism that is independent of gonadal steroids (Bentley et al. 1998). Therefore, given the late date of the second PHA challenge (July 28), and also the low testosterone titers 1 wk before it, photorefractoriness cannot be ruled out as an explanation of the increased wing web swelling following the second as compared to the first PHA challenge. These three interpretations need not be mutually exclusive and the importance of each is testable.

Unlike captives, free-living T-males produced significantly less (70%) wing web swelling in response to a PHA challenge than C-males. Free-living and captive males were, of course, subject to quite different treatments and environments, and we review factors that may be related to how housing affects variation in wing web swelling.

Methodologically, both captives and free-living males received priming injections of PHA; however, PHA was delivered in PBS vehicle to captives and in FCA vehicle to free-living males (see "Methods"). While this might account for the observed differences between experiments 1 and 2, we doubt this explanation because the degree of wing web swelling in C-males was similar in both groups.

A second difference between the two experiments relates to food availability and energy expenditure. Unlike free-living birds, captives had ad lib. access to food. Captives also lacked the opportunity to expend energy on courting, mate guarding, and care of young throughout. In contrast, free-living males received the PHA challenge while caring for nestlings of mates they had courted and guarded. Parental effort is energetically expensive and its increase has been linked to decreased antibody production in zebra finches (*Taeniopygia guttata*; Deerenberg et al. 1997) and collared flycatchers (Nordling et al. 1998), decreased cell-mediated immunity in pied flycatchers (*Ficedula hypoleuca*; Moreno et al. 1999), and increased susceptibility to parasites in great tits (*Parus major*; Allander 1997).

The physiological mechanisms by which hormones such as testosterone and corticosterone suppress immunity are unclear. However, it has been suggested that testosterone may redistribute energy reserves away from immune function and toward behavioral and morphological secondary sex characters necessary for reproductive success (Wedekind and Folstad 1994); a similar possibility is that corticosterone may shunt scarce nutritional reserves away from costly types of immune function during situations in which a bird is under stress (Apanius 1998). If such adaptive resource reallocation occurs in response to elevated testosterone and corticosterone titers in juncos, then our captive T-males, but not our free-living T-males, may have had sufficient energy to maintain immune system function despite reallocation.

Corticosterone and Immunity

In experiment 1, as in our previous studies of juncos (Ketterson et al. 1991; Klukowski et al. 1997; Schoech et al. 1999), corticosterone titers were elevated in testosterone-treated males. Testosterone implants also elevate circulating corticosteroid-binding globulin in male juncos (Klukowski et al. 1997), which may ameliorate high corticosterone titers by reducing availability of corticosterone to target tissues. Significantly elevated corticosterone levels have also been noted in testosterone-treated house sparrows, European starlings, and house finches (Duffy et al. 2000; Evans et al. 2000; E. Ketterson, unpublished data), but in each species the physiological mechanisms that mediate this response remain unidentified.

Like testosterone, glucocorticoids have generally been regarded as immunosuppressive (Cupps and Fauci 1982; Munck et al. 1984; Sapolsky 1993; Møller 1995; Besedovsky and del Rey 1996; Hillgarth and Wingfield 1997; Apanius 1998; Buchanan 2000), and glucocorticoids are routinely prescribed for their anti-inflammatory actions in humans (Schleimer et al. 1989). In mallard ducks (*Anas platyrhyncos*), elevated glucocorticoids are associated with decreased antibody responses to sheep erythrocytes, reductions in hematocrit, and decreased production of prostaglandins (chemical signals important in nonspecific inflammatory responses) by monocytes (Fowles et al. 1993).

Because both testosterone and corticosterone were elevated in captive T-males, it is unclear whether one or both hormones were directly involved in the observed immune suppression. It is possible that testosterone and corticosterone may have distinctly different effects on the immune system. Testosterone and cortisol (another glucocorticoid) have additive immunosuppressive effects when administered simultaneously to salmon (*Oncorhynchus tshawytscha*) and may affect different subpopulations of immune cells (Slater and Schreck 1993). The potential for testosterone to influence corticosterone, as well as for each hormone to influence the immune system uniquely and independently, underscores the importance of considering the actions of both testosterone and corticosterone whenever testosterone titers are experimentally manipulated. Further experiments are needed to dissociate the effects of corticosterone and testosterone on the immune system. Experiments that administer exogenous corticosterone, that elevate corticosterone by means of an experimental stress model, or that pharmacologically block the actions of glucocorticoids may prove useful.

Implications for the Immunocompetence Handicap and Immunoredistribution Hypotheses

Although the goal of our experiments was to determine whether changes in acquired immune function are part of the suite of phenotypic alterations that result from breeding-season-long elevation of testosterone titers in male juncos, the results are relevant to the immunocompetence handicap hypothesis. Dark-eyed juncos are relatively monomorphic among passerines, but testosterone-dependent secondary sex characters have been noted in males. During the breeding season, male juncos sing frequently and develop CPs; experimentally elevated testosterone enhances both of these characters (song rate: Ketterson et al 1992; Enstrom et al. 1997; Hill et al. 1999: CP volume: this study). In captivity, female juncos prefer to associate with T-males over C-males (Enstrom et al. 1997), but it is unclear which of the myriad effects of testosterone on male phenotype are preferred by females. When we consider all data collected to date by our laboratory (see Ketterson and Nolan 1999 and references cited therein and here), they are consistent with the immunocompetence handicap hypothesis as applicable to juncos: that is, there may be a trade-off that relates impairment of immune function and enhancement of secondary sex characters in males, and that trade-off may account for the typical seasonal pattern of testosterone.

Early tests of the immunocompetence handicap hypothesis used blood leukocyte concentrations as indirect measures of immune function and yielded conflicting results regarding testosterone's influence on immunity (Saino et al. 1995; Zuk et al. 1995). It has been suggested recently that testosterone's influence on the immune system, rather than being suppression of immunity, may be redistribution of circulating leukocytes to potential sites of local injury such as the skin; the argument is that redistribution is a potentially adaptive response to the greater likelihood of being wounded that accompanies elevation of testosterone and competition among males for mates (Braude et al. 1999). According to the hypothesis, leukocyte redistribution may be induced via testosterone's direct physiological effects, or by testosterone's indirect effects via glucocorticoids. Although there is no direct experimental evidence that elevated testosterone titers induce immunoredistribution, acute (brief) elevation of glucocorticoid titers in mice does redistribute immune cells and bolster cell-mediated immunity when assessed by skin tests of immune function such as PHA tests similar to those reported here (Dhabhar and McEwen 1996, 1999; Dhabhar 1998). Interestingly, chronic (prolonged) elevation of glucocorticoids produces opposite results and suppresses responses to skin tests of immune function (Dhabhar and McEwen 1999).

The immunocompetence handicap and immunoredistribution hypotheses differ in their predictions with respect to elevated testosterone and immune function. Immunoredistribution predicts that immune function may be enhanced by testosterone through adaptive reallocation of circulating leukocytes, whereas immunocompetence hand-

icap predicts that testosterone suppresses immune function. Elevated testosterone did not enhance immunity as assessed by wing web swelling in response to PHA in either of our experiments, which suggests that elevated testosterone did not induce increased migration of immune cells to the wing web as predicted by the immunoredistribution hypothesis. However, the long-term exposure to elevated testosterone most likely produced chronic, as opposed to acute, elevation of the glucocorticoids, which may have been responsible for the reduced response to the PHA challenge (Dhabhar and McEwen 1999). We did not directly assess concentrations of leukocytes, but we think that the results of the wing web swelling tests support the hypothesis that prolonged elevation of testosterone suppressed cell-mediated immunity, not that it triggered redistribution of leukocytes to the skin.

Testosterone, Lifetime Reproductive Success, and Selection on Juncos

Experimental manipulations are one of several ways to study adaptation, and hormonal manipulations provide an opportunity to extrapolate as to how selection might act on correlated traits (Ketterson et al. 1996; Sinervo and Basolo 1996; Ketterson and Nolan 1999). By altering the seasonal profile of testosterone, we have documented changes in behavior and physiology and related these to components of fitness. The reasoning can be extended as follows. If selection were to act on traits whose expression is influenced by natural variation in endogenous testosterone, and if variation in those traits (and in testosterone) were heritable, then we would expect a response to selection that would reflect both the positive effects of the favored traits on fitness as well as any negative effects of other testosterone-mediated traits.

Based on data presented here, we would predict that testosterone-treated juncos should be less able than C-males to ward off disease and parasitic infection. Reduction in this ability would be expected to shorten average life span and hence reduce fitness (Lochmiller and Deerenberg 2000). By extrapolation, we might predict that selection on favorable testosterone-mediated traits is constrained by the effects of testosterone on immune function. However, after 12 yr of study, we have yet to find evidence that T-males whose implants are removed at the end of the summer return at a lower rate than C-males (Ketterson et al. 1996; E. Ketterson, unpublished data). This suggests that, if testosterone returns to normal levels before winter, overwinter survival is not affected. Only if exposure to testosterone is prolonged into the autumn are return rates suppressed (Nolan et al. 1992), and other causes of death besides disease may account for this difference. Moreover, in a 2-yr study of a protozoan parasite in juncos, T-males and C-males did not differ in their rates of coccidial infection (Hudman et al. 2000).

Obviously hormonal state is only one factor influencing the prevalence of disease or parasitic infection (Nelson and Demas 1996; Zuk and Johnsen 2000), and any hormonal suppression of immune function may vary with season or food availability (Lochmiller et al. 1993; Zuk and Johnsen 1998). Future investigations will address susceptibility to disease and may or may not reveal a relationship between either experimentally elevated testosterone or endogenous testosterone and parasite load and survival.

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