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Is avian humoral immunocompetence suppressed by testosterone?

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Abstract A key issue in sexual selection theory is how a correlation between male secondary sexual characters and male genetic quality can be maintained. The immunocompetence-handicap hypothesis proposes that testosterone-dependent male characters remain honest signals because of the immunosuppressive effect of elevated steroid hormone levels. The hypothesis requires that physiological levels of testosterone depress immune system function. We quantified testosterone titers and humoral immunocompetence of captive male and female red-winged blackbirds (*Agelaius phoeniceus*) at three points in the annual cycle (autumn, prebreeding, and breeding). We also conducted an implant experiment on the males to assess the effects of prolonged, above-normal testosterone titers on humoral immune responses. Humoral immunocompetence was measured as secondary antibody production to a non-pathogenic protein antigen, keyhole limpet hemocyanin, using an enzyme-linked immunosorbent assay we developed for *A. phoeniceus*. Secondary antibody responses of individuals were highly repeatable between sampling periods. Neither physiological nor above-normal levels of plasma testosterone suppressed secondary antibody production. In paired tests of the same individuals between prebreeding and breeding, and between breeding and implant, plasma testosterone increased significantly but secondary antibody responses were unaffected. We are confident in these results because with 80% power, an 11–14% difference in antibody titers would have been detected. There was no relationship between plasma testosterone levels and humoral immunocompetence in free-ranging males tested at the peak of breeding. These results cast doubt on a key assumption of the immunocompetence-handicap hypothesis.

Key words Immunocompetence · Testosterone · Immunosuppression · Sexual selection · Red-winged blackbird · ELISA

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Introduction

Females of many animal species choose mates based on males' secondary sexual characters (reviewed by Andersson 1994). In some species, offspring of males with the most elaborate characters (e.g., plumes, colors, and songs) survive best, suggesting that these characters indicate male genetic quality (Petrie 1994; von Schantz et al. 1994; Hasselquist et al. 1996).

A heavily debated question is what maintains the correlation between the expression of sexual characters and male quality (e.g., Kirkpatrick and Ryan 1991; Andersson 1994). Simply put, why do low-quality males not "cheat" by expressing just the elaborate character? Folstad and Karter (1992) proposed the immunocompetence-handicap hypothesis (ICH) to address this issue. They suggested that the character-quality correlation is maintained by a "double-edged sword" effect of testosterone: higher titers enhance the expression of sexual characters, but they also depress immune system functions. Hence, testosterone-dependent characters honestly signal quality because only certain males can perform well with elevated steroid hormone levels.

The ICH assumes that (1) physiological levels of testosterone suppress immune system functions, (2) males with more elaborate secondary sexual characters are preferred by females, and (3) males that secrete more

testosterone have more elaborate secondary sexual characters (and higher mating success). Assumption 2 is well supported by field observations and experiments (Andersson 1982; Møller 1988; von Schantz et al. 1989; Houde and Torio 1992; Zuk et al. 1992; Andersson 1994). Assumption 3 has also received considerable support (Wingfield 1984; Borgia and Wingfield 1991; Alatalo et al. 1996; Hillgarth and Wingfield 1997; Raouf et al. 1997), although, of course, not all secondary sexual characters require high levels of testosterone to develop fully (Owens and Short 1995).

Information on the key assumption 1 of the ICH is contradictory. Whereas the initial studies concluded that testosterone indeed had immunosuppressive effects (reviewed in Grossman 1984; Besedovsky and del Rey 1996) the evidence was not clear-cut. For example, much was made of the fact that females generally show stronger immune responses than males (Grossman 1985; Alexander and Stimson 1988; Schuurs and Verheul 1990; Lin et al. 1996). However, this does not prove that androgens, such as testosterone, are immunosuppressive, because stronger immune responses in females may be caused by their higher estrogen levels (Olsen and Kovacs 1996) or by sex-specific differences in immune responses based on other mechanisms. Other studies may be questioned because they involved testosterone supplementation that resulted in super-normal (pharmacological) androgen levels (Mendelsohn et al. 1977; Novotny et al. 1983). Effects of testosterone may differ with the developmental stage. In chickens, immunosuppressive effects occurred when testosterone was administred to chicks (i.e., during immune system development), but there were no such effects in adult birds (Gause and Marsh 1986; Schuurs et al. 1992). Moreover, recent *in vitro* studies have not shown that testosterone consistently depresses immune functions (reviewed in Marsh 1996). In sum, recent reviews emphasize that testosterone is a modulator that can both enhance and depress immune system functions (Marsh 1996; Olsen and Kovacs 1996).

Six studies have attempted to test the first assumption of the ICH in wild birds. Again results have been equivocal. Saino and Møller (1994) reported a negative relationship between testosterone levels and parasite load, which is consistent with the assumption. However, two other studies found no relationship between testosterone levels and parasite loads (Weatherhead et al. 1993) or lymphocyte counts (Saino et al. 1995). In captive juvenile black-headed gulls (*Larus ridibundus*), primary antibody titers (IgM) averaged 31% lower in testosterone-implanted birds than controls (Ros et al. 1997). This difference was not significant, so these authors argued that testosterone may not be immunosuppressive. However, the statistical power of their test was very low. Finally, in two field experiments, male birds were implanted with testosterone-filled capsules, resulting in slightly higher testosterone levels than those that occur normally. In one of these studies lymphocyte counts decreased (Zuk et al. 1995), while in the other, total immunoglobulin levels decreased and the ectoparasite load increased (Saino et al. 1995). These results were interpreted as supporting assumption 1 of the ICH.

Most of these studies employed indirect measures of immune function (e.g., parasite loads, circulating immune cells), and the reliability of these methods has been questioned (Lochmiller 1995; Siva-Jothy 1995). A direct and more reliable measure of immunocompetence can be obtained by applying a method used in immunotoxicology in which humoral immune responses to specific novel antigens are measured with an enzyme-linked immunosorbent assay (ELISA) (Fairbrother 1990; Lochmiller 1995). We developed an ELISA specific for red-winged blackbirds to investigate how natural seasonal fluctuations and experimental elevation in the birds' serum testosterone levels affected their humoral immune responses to keyhole limpet hemocyanin (KLH). We used KLH because it is a non-pathogenic, harmless protein antigen that is highly immunogenic. Presumably red-winged blackbirds have never previously been exposed to KLH (see also Klein and Nelson 1997). Red-winged blackbirds are good study subjects because testosterone is known to enhance male aggressiveness and dominance (Searcy and Wingfield 1980), and to be positively correlated with male song rate and epaulet exposure (Johnsen 1998), polygyny, and reproductive success (Beletsky et al. 1989, 1992). Moreover, it is appropriate to measure humoral immunocompetence because studies of captive birds (domesticated chicken) indicate that this aspect of immune function is particularly likely to be affected if testosterone depresses immune activity (reviewed in Schuurs and Verheul 1990; Marsh 1996).

Methods

General methods

We captured red-winged blackbirds near Seneca Falls, N.Y., in September 1995. Birds (13 males and 8 females) were housed in a room with controlled light, temperature, and humidity at Cornell University until June 1996, when they were released. Males and females were housed in individual cages in the same room. The light cycle approximated natural photoperiods throughout most of the year. Thus in October to December, we used the photoperiod for 36° N to mimic the situation at these birds' presumed wintering areas. In January, we switched photoperiod to mimic conditions for Ithaca (42° N), and we also slightly advanced the light cycle so that the birds in February to late April experienced photoperiods that would occur naturally 2–3 weeks later in the season. During May, the light cycle was kept at natural photoperiods and in early June, the light cycle was slightly decreased (1–2 weeks) so that the birds experienced late May photoperiods.

We measured the males' secondary antibody response to antigen challenge at four times: autumn (October to December, when the males were not singing), prebreeding (February, males had just started to sing), breeding (April, males were singing intensively), and in a testosterone implant experiment (May). Plasma testosterone levels were monitored in each study period using a radioimmunoassay (RIA). We also measured females' secondary antibody responses against KLH during the autumn, prebreeding, and breeding periods. Monitoring humoral immune responses

enabled us to detect impairment in either T- or B-cell responsiveness, because KLH is a T-cell-dependent antigen (Rife et al. 1990).

In a separate experiment, we captured 16 territorial male red-winged blackbirds at the peak of the 1996 breeding season (May), took a blood sample (ca 300 μl), immunized each male with KLH, and within 30 min released each male back onto its own territory. We recaptured these males 8–13 days later (i.e., at the peak of their primary antibody responses), and took a blood sample (ca 300 μl). We then quantified each male's primary antibody production using our ELISA. Data were analyzed using SYSTAT 4.0 (Wilkinson 1992). Power tests were conducted in GPOWER (Erdfelder et al. 1996), and repeatability analyses followed procedures recommended by Lessells and Boag (1987).

Measuring serum testosterone

Blood samples (ca 300 μl) were taken once in each of the four study periods (captive birds) and at first capture (wild birds). Plasma levels of testosterone were measured by a direct RIA conducted in J.C. Wingfield's laboratory using methods of Wingfield and Farner (1975) and Ball and Wingfield (1987). All samples were measured in duplicate and in a single assay. Two standards of testosterone (250 pg/ml) were included in the assay, and the mean value of these was 229 pg/ml (intra-assay coefficient of variation = 6.3%).

In the study of captive birds, we obtained each set of blood samples within 10 min of entering the holding room except for the autumn period. Since plasma testosterone levels of wild song birds remain stable for at least 10 min after capture (Wingfield et al. 1982), we are confident that androgen levels did not change artificially before sampling. In the autumn period, however, only 5 of 13 samples were taken within 10 min of entering the bird room; the others were taken 15–90 min after entry. There was no significant difference in plasma testosterone levels between these two groups of birds ($t_{16} = 0.66$, $P = 0.58$); all autumn samples contained very little or no testosterone independent of timing of sampling (Table 1).

To capture wild males we placed traps baited with seeds on each male's territory. Males were habituated to these traps for several weeks before capture. When a male was trapped, we immediately extracted it and obtained a blood sample within 5–10 min. Hence, plasma testosterone in the wild males should not have been artificially elevated due to the capture technique.

Both in the field (Beletsky et al. 1989) and in captivity (Kerlan and Jaffe 1974), plasma testosterone levels of individual male red-winged blackbirds are stable during the peak breeding period. To assess plasma testosterone stability in our birds, we compared eight males at different times within a given study period (6–54 days apart). Most samples were from the autumn and prebreeding periods. We found either no or miniscule differences in hormone levels between samplings (i.e., differences ranged from 0 to 0.06 ng/ml).

In the breeding period, our captive males sang intensively and they had higher testosterone levels than in the previous periods (Table 1) even if the caged males' average testosterone levels (mean = 0.45 ng/ml, SE = 0.15) were lower than peak levels for wild males (mean = 2.0 ng/ml, SE = 0.28) from the same population.

Antigen injections

Red-winged blackbirds were immunized using a preparation made by emulsifying 1 mg KLH/ml sterile H_2O with 1 ml of incomplete

Table 1 Secondary antibody production (to keyhole limpet hemocyanin) and plasma testosterone levels of captive male and female red-winged blackbirds measured in autumn (October or December), the prebreeding season (February), breeding season (April), and in implanted males given testosterone implants in May. Paired *t*-tests indicate comparisons between means in a column and that to its left

	Antibody production (mOD/min)				Testosterone (ng/ml)			
	Autumn	Prebreeding	Breeding	Implanted	Autumn	Prebreeding	Breeding	Implanted
Males								
1	52	88	105	85	0.08	0.08	1.5	30
9	117	141	122	148	0	0	1.7	21
10	128	135	132	148	0	0.15	0.24	7.8
15	90	145	131	150	0	0	0.09	3.9
16	101	144	119	142	0	0.12	0.67	10.8
17	77	101	145	151	0	0	0.21	18
19	112	130	130	113	0	0	0.20	14
20	68	124	116	118	0	0	0.12	13
23	74	103	110	99	0.07	0.06	0.14	4.5
24	123	106	138	150	0	0.14	0.32	14
4	60	58	50	74	–	0	0.34	28
5	–	141	136	145	0	0.15	0.19	3.3
6	–	91	90	86	0	0	0.14	30
Mean	94	116	117	124	0.01	0.05	0.45	15.6
SE	8.2	7.5	7.0	8.1	0.01	0.02	0.15	2.7
Paired *t*-test		0.003 ($n = 11$)	0.85 ($n = 13$)	0.14 ($n = 13$)		0.02 ($n = 12$)	0.02 ($n = 13$)	< 0.001 ($n = 13$)
Females								
2	144	130	129					
3	93	145	146					
7	110	129	128					
12	92	123	147					
14	100	129	138					
22	92	134	159					
11	–	170	158					
13	–	175	169					
Mean	105	142	147					
SE	8.3	7.1	5.2					
Paired *t*-test		0.04 ($n = 6$)	0.35 ($n = 8$)					

Freund's adjuvant (Sigma, St. Louis, Mo.). A total volume of 0.1 ml of this emulsion, containing 50 μg KLH, was injected into the breast muscle of each bird. We investigated the time course of primary and secondary antibody responses in four birds. Blood samples were taken before the priming immunization, every 3rd day after the first injection (day 6–21), and on days 6, 9, 12, and 14 after the second injection. We investigated humoral immune responses in captive red-winged blackbirds at three (8 females) and four (13 males) periods. First we took a preimmunization blood sample from each of these birds. Thereafter, blood samples were taken on days 6, 9, and 12 after the second immunization in autumn, and on days 6, 9, and 12 after the booster immunization given in each of the prebreeding, breeding, and implant (males only) periods. Serum was separated by centrifugation, and stored at −50°C until analyses.

We allowed the birds to adjust to captivity for 3–8 weeks in the fall of 1995 before experiments started. At the beginning of the autumn sampling period, each bird was given a priming immunization with KLH that resulted in a primary immune response which peaked about 12 days after injection (Fig. 1). Three weeks after this first injection, each bird was given a second KLH injection. This resulted in a secondary immune response that peaked 6–9 days after the challenge, and which involved much higher levels of antibody production. In the prebreeding, breeding, and implant periods, each bird was given one booster injection of KLH that resulted in secondary antibody responses. Hence, in all four study periods, we measured the birds' secondary antibody responses, which are characterized by a predominance of immunoglobulin G (IgG). Two males and two females did not receive their first and second injection until the prebreeding period. These served as contemporaneous controls. The rest of the caged birds were given their third KLH injection during the prebreeding period (Table 1).

ELISA methods

We measured anti-KLH antibody levels in the birds' serum using an ELISA (Exon and Talcott 1995) that we developed specifically for red-winged blackbirds. Basically, an ELISA works as follows. First, the sides of the wells of the ELISA plate are coated with antigen. In the present ELISA, we coated the wells with KLH, the antigen with which the birds were previously injected. Antigen that is not bound to the sides of the wells is then washed away. Next, test serum (in our case from red-winged blackbirds) is added to the wells, and the birds' antigen-specific antibodies bind to the previously bound antigen. Non-bound red-winged blackbird antibodies are then washed away. In the third step, rabbit anti-red-winged blackbird antibodies are added to the wells, and they bind specifically to the red-winged blackbird antibodies that are bound to the antigen on the sides of the wells. After washing, peroxidase-labelled antibodies (e.g., from sheep) against rabbit antibodies are added, and they bind to the already-bound rabbit antibodies. Adding peroxide and substrate to the wells results in a color change that is proportional to the number of antigen-specific antibodies present in the test serum of each individual.

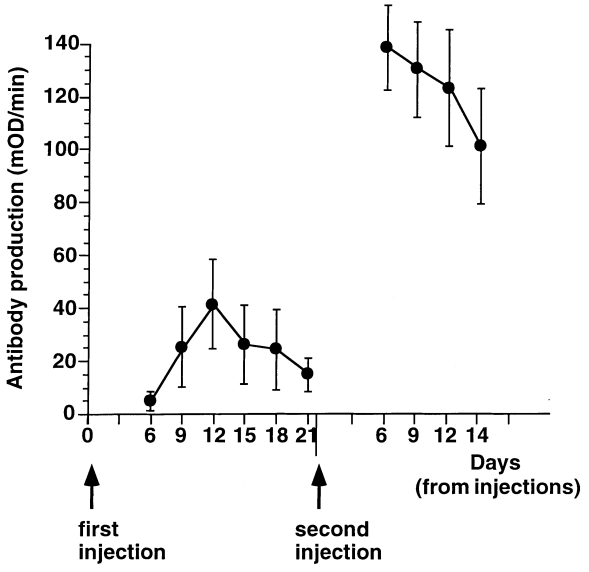

Fig. 1 Mean (±SE) primary and secondary antibody production (measured in 10^{-3} × optical density per minute; mOD/min) to keyhole limpet hemocyanin (KLH, a harmless protein antigen) in four red-winged blackbirds. A first injection with KLH (mixed with incomplete Freund's adjuvant) was given at day 1, and a second injection after 3 weeks. Antibody production was measured every 3rd day

The conditions of our specific ELISA were as follows. We incubated antisera in 96-well ELISA plates coated with KLH. Plates were then blocked using a dilution of 3% powdered milk in 0.01 M phosphate buffered saline (PBS, pH7.2). A diluent of 1% powdered milk in 0.01 M phosphate-buffered saline (PBS, pH 7.2) was used to produce 1:400, 1:2000 and 1:8000 dilutions of each serum sample. For each dilution and individual, serum samples were added to the plates in duplicate. The average of these was our measure of antibody titer for each individual. After incubation and wash (in PBS and Tween 20), we added a secondary rabbit anti-red-winged blackbird immunoglobulin antiserum which we had produced by immunizing rabbits with purified red-winged blackbird immunoglobulin M (IgM) and IgG. After a second incubation and wash, a commercial peroxidase-labelled goat anti-rabbit antiserum (Kirkegard and Perry, Gaithersburg, Md.) was added. Following incubation and wash, the peroxidase substrate (2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, ABTS) and peroxide were added, and the plates were immediately transferred to a BioTek 312 EL (Winooski, Vt.) kinetics ELISA reader. Plates were read at 30-s intervals for 16 min using a 405-nm wavelength filter. All antibody concentrations are given as the slope of the substrate conversion over time measured in the units 10^{-3} × optical density per minute (mOD/min) (analyzed using KineticCalc software, Winooski), with a higher slope indicating a higher concentration of anti-KLH antibodies in a sample.

For each individual, diluted serum samples taken 6 days after injection in each period were analyzed on the same ELISA plate, and samples from all individuals were analyzed on two ELISA plates run simultaneously in the same batch. In another batch of plates, we analyzed each individual's serum samples taken at 9 days after injection in each period on the same ELISA plate, and samples from all individuals were all included on two plates that were run simultaneously. For each study period, we used the average antibody titer for day 6 and day 9 after injection as the measure of an individual's peak secondary antibody production (see Fig. 1). No antibody production against KLH was detected in (autumn) preimmunization samples from any of the birds. Serum samples diluted 1:8000 were used in all analyses because this dilution provided optimal analysis conditions. There was a high repeatability of each individual's duplicate samples run on the same ELISA plate (samples taken day 6 after injection: repeatability in autumn $R = 0.82$, prebreeding $R = 0.91$, breeding $R = 0.96$, and implanted $R = 0.98$, ANOVA $P < 0.001$ for all periods). There was also high repeatability between the same individual's samples taken on days 6 and 9, although these samples were taken at different times during the antibody response and analysed on different ELISA plates run on different dates (repeatability and ANOVA P-value for samples in: autumn $R = 0.36$, $P = 0.06$; prebreeding $R = 0.63$, $P = 0.001$; breeding $R = 0.55$, $P = 0.003$, and implanted $R = 0.64$, $P = 0.004$).

Testosterone implant experiment

A small Silastic tube (inner diameter = 1.47 mm, outer diameter = 1.95 mm; Dow Corning) was filled with testosterone propionate (T-1875, Sigma), sealed with Medical Adhesive Silicone (Dow Corning), and implanted between the skin and the breast muscle of all 13 males in May. Tubes were 15 or 25 mm long. These

were randomly assigned to males to create a range of high testosterone levels. These males experienced 3.3–31.2 ng testosterone/ml serum, which is 20–30 times higher than these individuals' peak testosterone titers during the breeding period (Table 1). Of our implanted males, 69–77% had higher plasma testosterone levels than the maximum plasma testosterone levels at the peak of breeding in wild male red-winged blackbirds [approximately 7 ng/ml in Ithaca, 9 ng/ml in Indiana (Johnsen 1998) and 10 ng/ml in Washington (Beletsky et al. 1989)]. The RIA technique we used, besides detecting the testosterone that is produced naturally by the birds, only detects the testosterone that is metabolized from the implanted testosterone propionate, so the plasma androgen levels measured in this experiment may have been even higher.

Results

We first investigated the timing and relative magnitude of primary and secondary antibody production to KLH in red-winged blackbirds. Using our ELISA, we obtained measures of mean (±SE) antibody titers in serum samples taken from males every 3rd day (Fig. 1). The primary immune response peaked around day 12 after the first antigen injection. Thereafter, antibody titers decreased slowly until day 21. The second antigen injection was given 22 days after the first, and the birds' secondary immune response peaked 6–9 days later. Figure 1 shows that the peak level of the secondary immune response was 3–4 times higher than the primary immune response peak. Each individual's secondary humoral immune responses was highly repeatable over the three to four periods they were sampled ($R = 0.51$, $F_{21,54} = 4.7$, $P < 0.0001$), indicative of consistent differences in immune responses between individual male and female red-winged blackbirds.

There was a significant increase in the captive males' secondary humoral immune responses between the autumn and prebreeding periods (paired t-test, $P = 0.003$), when plasma testosterone increased slightly (Table 1). However, secondary antibody responses did not diminish either between the prebreeding and breeding samples (paired t-test, $P = 0.85$), when plasma testosterone levels increased naturally about ninefold, nor between breeding and implant samples when testosterone levels were artificially increased 20–30 times (paired t-test, $P = 0.14$) (Fig. 2). The statistical power of these results is high: with 80% power we should have detected an 11–14% difference in antibody titers between periods at the $P = 0.05$ level (two-tailed test). For the implant period, we also analyzed our data using a repeated-measures ANOVA with plasma testosterone and implant size as independent variables and antibody production as the dependent variable. Neither plasma testosterone ($P = 0.66$) nor implant size ($P = 0.40$) showed any relationship with antibody production.

We also analyzed whether humoral immunosuppression occurred at above-normal levels of testosterone as a result of the implant experiment (Fig. 3). We found no correlation between the captive males' change in testosterone levels and their change in antibody production caused by the implant experiment ($r = -0.19$, $P = 0.54$). The same was true when we included change in testosterone (partial $r = -0.15$, $P = 0.65$) and implant size (partial $r = 0.28$, $P = 0.40$) as predictors of change in antibody production in a multiple regression.

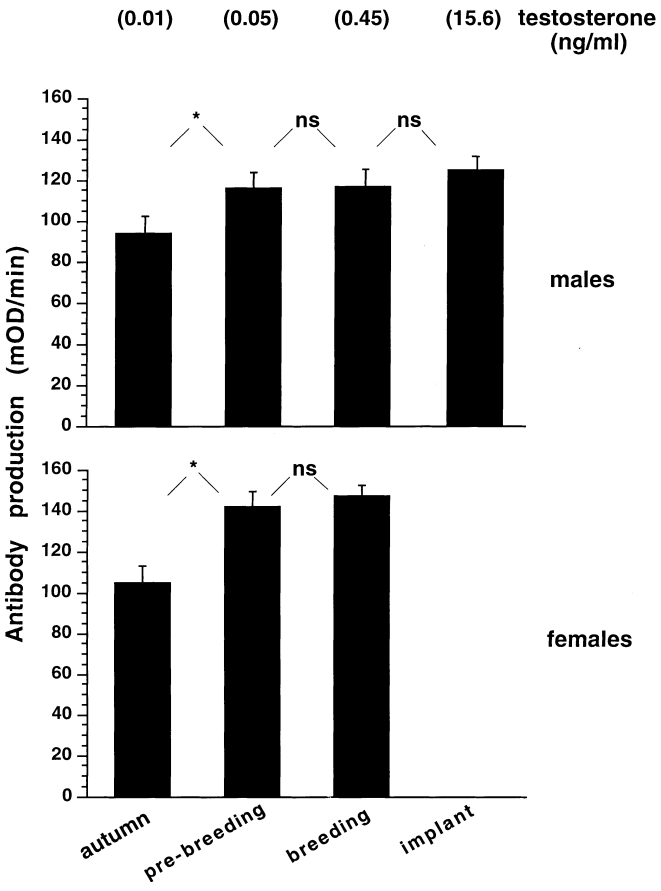

Fig. 2 Mean (±SE) secondary antibody production (mOD/min) of captive male and female red-winged blackbirds against KLH at several times over the annual cycle: in *autumn*, the wintering period when males produced no testosterone; *prebreeding*, in February when males naturally produced low testosterone levels; *breeding*, in April when males naturally produced medium testosterone levels; *implant* (males only), in May when males were implanted with Silastic tubes containing testosterone propionate, which resulted in high-normal to above-normal testosterone levels. The males' mean plasma testosterone (ng/ml) in each period is given across the top

Among wild territory-holding males, there was no significant relationship between testosterone and primary antibody response to a single injection of KLH ($r = -0.20$, $P = 0.46$, $n = 16$; Fig. 4). This was also true when we controlled for the time period between injection and sampling in a multiple regression analysis ($F = 0.28$, $P = 0.76$, $n = 16$; time to sampling, partial $r = 0.044$, $P = 0.88$; testosterone titer, partial $r = -0.15$, $P = 0.58$).

If testosterone is immunosuppressive, the captive males and females should have exhibited similar antibody responses in autumn, but males should have exhibited lower responses than females in the prebreeding and breeding periods as a result of elevation in males' plasma testosterone levels. In a repeated-measures

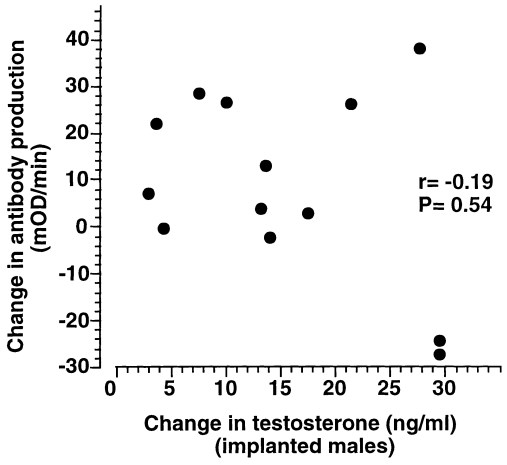

Fig. 3 Relationship between the change in plasma testosterone levels (ng/ml) and the change in secondary antibody production (mOD/min) against KLH as a result of a testosterone propionate implant experiment in captive male red-winged blackbirds

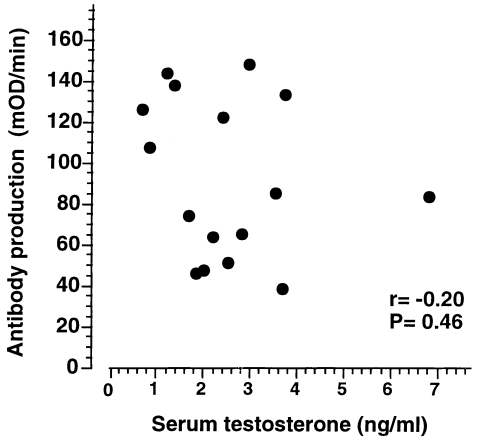

Fig. 4 Relationship between plasma testosterone levels (ng/ml) and primary antibody production (mOD/min) against KLH in a population of wild male red-winged blackbirds at the peak of the breeding season (in May when testosterone levels were at their peak)

ANOVA including only the individuals sampled in all three periods, humoral immune responses of females ($n = 6$) were generally higher than males ($n = 11$) (sex, $F = 4.2$, $P = 0.06$), even in autumn when plasma testosterone titers in males were undetectable (Table 1). Immune responses against KLH increased from autumn to breeding (period, $F = 18.1$, $P < 0.001$), but there was no interaction between sex and period ($F = 0.13$, $P = 0.87$). Thus from autumn to breeding, female humoral immune responses were consistently higher than in males, despite increases in male testosterone levels between these periods (Fig. 2, Table 1).

Discussion

Although our study is based on relatively small sample sizes, our approach of repeatedly measuring the same males' secondary humoral immune responses at four different periods, with successively elevated plasma testosterone titers allowed us to use powerful statistics. The power of our paired *t*-tests was so high that we can confidently conclude that testosterone did not have any suppressive effect on secondary humoral immune responses in our captive males. We also found no correlation between primary humoral immune responses and endogenous testosterone levels in the wild males. This test, however, has lower statistical power. There was a weak and non-significant negative correlation between primary humoral immunocompetence and plasma testosterone in these males. It might be worth reinvestigating this relationship with a larger sample of wild males.

In red-winged blackbirds, primary antibody responses against KLH peaked at approximately 12 days after the first injection, and secondary antibody responses at 6–9 days after the second injection (Fig. 1). The slow decrease in antibody titers after the peak of the primary response is most likely due to the adjuvant. We immunized KLH mixed with incomplete Freund's adjuvant which resulted in a slow release of antigen over a few weeks (Fig. 1). This mimicked a real infection in which the pathogen continuously reproduces and its antigens are therefore present in the host's blood for days or weeks. When immunocompetence is measured using a single immunization of antigen without adjuvant, the results may be less easily related to a real infection (Westneat and Birkhead 1998).

There was a high repeatability of secondary antibody responses to KLH over the four study periods, which support the use of this technique as a sensitive and quantitative measure of humoral immunocompetence. This result also shows that measuring humoral immunocompetence as antibody production against KLH revealed some individual differences in the birds' abilities to mount humoral immune responses. These differences may result from individual differences in (1) MHC molecule repertoire and thus ability to recognize different antigens (e.g., Briles et al. 1977; Lamont et al. 1987), and (2) abilities to produce a strong immune response subsequent to the antigen recognition process (e.g., Wakelin and Apanius 1997; Westneat and Birkhead 1998). We do not know to what extent these factors contributed to the magnitude of the humoral immune responses we observed, even though it is generally thought that both these processes are important for an acquired immune response. However, by challenging individuals repeatedly when their testosterone levels differed we should have been able to detect any immunosuppressive effect of testosterone independent of which of the two mechanisms was the most important.

A weakness in the design of the testosterone implant experiment is that there was no control group of males that did not receive implants. Hence, it is possible that humoral immune responses of males did not decrease when testosterone levels increased because heightened responses to the booster injections of KLH masked the

suppression. However, this seems unlikely for three reasons. First, effects of testosterone on immune responses of *all* captive birds were assessed following a secondary antibody stimulation, that is, when memory B-cells for the antigen (KLH) had already been formed. Second, the temporal pattern of females' antibody responses were similar to those of males: female responses also increased significantly between autumn and prebreeding samples ($P = 0.04$) but not between prebreeding and breeding samples ($P = 0.35$) (Fig. 2). In the latter comparison, the 4% increase in antibody titers between periods (Table 1) suggests that there is little or no effect of increasing secondary antibody responses to repeated KLH injections (after the first injection). Third, the four birds that received their second immunization with KLH in the prebreeding period had similar (indeed, slightly higher) antibody titers as the 16 birds that received their third KLH immunization in the prebreeding period (ANOVA: sex, $F = 4.5$, $P = 0.05$; number of injections, $F = 2.0$, $P = 0.17$).

Production of the stress hormone corticosterone also might result in decreased immunocompetence (Sapolsky 1993; Møller 1995; Besedovsky and del Rey 1996; Hillgarth and Wingfield 1997). We did not measure corticosterone directly. However, if corticosterone affected humoral immunocompetence in our experiments, we would have expected differences between birds investigated at the beginning of the autumn period (October), when they had not yet fully adjusted to captivity, and the end of the autumn period (December) when their adjustment was presumably more complete. There were, however, no significant differences between the beginning and the end of the autumn period in the captive males' humoral immune responses ($t_{10} = 0.24$, $P = 0.82$) or plasma testosterone levels ($t_{18} = 0.86$, $P = 0.40$). Apparently, the birds adjusted to captivity and handling during the weeks preceding their first KLH injection.

Captive female red-winged blackbirds had greater humoral immune responses than males, even though male testosterone levels increased from nil in autumn to high in breeding. In general, female vertebrates have greater immune responses than males (e.g., Terres et al. 1968; Grossman 1984), and it has been suggested that this is a consequence of the immunosuppressive effect of testosterone in males (Alexander and Stimson 1988; Schuurs and Verheul 1990). However, detailed studies of sex differences in immune system function imply that humoral immune response are enhanced in females rather than depressed in males (Grossman 1985; Talal 1989; Wiedmeier et al. 1991; Olsen and Kovacs 1996). Hence, the greater humoral responses of red-winged blackbird females probably resulted from their enhanced antibody production rather than from testosterone-induced suppression in males.

It should be noted that we investigated humoral immune responses, which are part of the acquired immune system. We cannot exclude the possibility that the other parts of the immune system, i.e., the innate immune system and the cell-mediated part of acquired immune responses, were negatively affected by testosterone. We measured humoral immune responses for the following reasons. (1) Based on in vitro studies at the cellular level, the humoral part of the immune system seems particularly likely to be suppressed by testosterone (e.g., Sthoeger et al. 1988; Araneo et al. 1990; Olsen and Kovacs 1996). (2) In chicken, treatment with testosterone results in lower antibody production, probably due to an inability to switch from IgM-type to IgG-type responses (Hirota et al. 1980; Verheul et al. 1986). (3) Methods providing sensitive measures of innate and cell-mediated immunity are in most cases based on species-specific in vitro methods and involve killing the study animals (e.g., Fairbrother 1990; Luster et al. 1992; Dietert et al. 1996). Hence, our measure of immunocompetence based on measuring humoral immune responses *in vivo* should have detected possible immunosuppressive effects of testosterone.

However, we did not find any evidence that testosterone suppresses humoral immune responses in male red-winged blackbirds. Physiological testosterone levels, i.e., levels that under natural conditions can affect the expression of sexually selected characteristics, exerted no consistent effect on either primary or secondary humoral immune responses. This was true both for wild males held in captivity and sampled repeatedly from autumn through breeding, in testosterone-implanted males, and in wild males investigated at the peak of breeding. This implies that males' antibody production against chronic and regularly encountered pathogens would be relatively unaffected by cyclic changes in testosterone. Our study thus offers no support for Folstad and Karter's (1992) hypothesis that immunosuppression by testosterone serves as the mechanism by which testosterone-mediated characters remain honest signals of male quality.

Recently, it has been suggested that the trade-off between sexual ornaments and the immune system can arise via other mechanisms, for example energy or nutrient limitations (Wedekind and Folstad 1994), stress (glucocorticoid hormones: Møller 1995; Siva-Jothy 1995; Hillgarth and Wingfield 1997), or production and biotransformation of free-radicals (von Schantz et al. 1999). These alternative mechanisms now deserve more attention in order to elucidate the possible relationship between sexual ornaments and the immune system.

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References

Alatalo RV, Höglund J, Lundberg A, Rintamäki PT, Silverin B (1996) Testosterone and male mating success on the black grouse leks. Proc R Soc Lond B 263:1697–1702

Alexander J, Stimson JH (1988) Sex hormones and the course of parasitic infection. Parasitol Today 4:189–193

Andersson M (1982) Female choice selects for extreme tail length in a widowbird. Nature 299:818–820

Andersson M (1994) Sexual selection. Princeton University Press, Princeton, NJ

Araneo BA, Dowell T, Diegel M, Daynes RA (1990) Dihydrotestosterone exerts a depressive influence on the production of interleukin-4 (IL-4), IL-5, and γ-interferon, but not on IL-2 by activated murine cells. Blood 78:688–699

Ball GF, Wingfield JC (1987) Changes in plasma levels of luteinizing hormone and sex steroid hormones in relation to multiple-broodedness and nest-site density in male starlings. Physiol Zool 60:191–199

Beletsky LD, Orians GH, Wingfield JC (1989) Relationships of steroid hormones and polygyny to territorial status, breeding experience, and reproductive success in male red-winged blackbirds. Auk 106:107–117

Beletsky LD, Orians GH, Wingfield JC (1992) Year-to-year patterns of circulating levels of testosterone and corticosterone in relation to breeding density, experience, and reproductive success of the polygynous red-winged blackbird. Horm Behav 26:420–432

Besedovsky HO, Rey A del (1996) Immune-neuro-endocrine interactions: facts and hypotheses. Endocr Rev 17:64–102

Borgia G, Wingfield JC (1991) Hormonal correlates of bower decoration and sexual display in the satin bowerbird (*Ptilonorhynchus violaceus*). Condor 93:935–942

Briles WE, Stone HA, Cole RK (1977) Marek's disease: effects of B histocompatibility alloalleles in resistant and susceptible chicken lines. Science 195:193–195

Dietert RR, Golemboski KA, Kwak H, Ha R, Miller TE (1996) Environment-immunity interactions. In: Davidson TF, Morse TR, Payne LN (eds) Poultry science symposium series, vol 24. Carfax, Oxford, pp 343–356

Erdfelder E, Faul F, Buchner A (1996) GPOWER: a general power analysis program. Behav Res Methods Instrum Comput 28:1–11

Exon JH, Talcott P (1995) Enzyme-linked immunosorbent assay (ELISA) for detection of specific IgG antibody in rats. In: Burleson G, Dean J, Munson A (eds) Modern methods in immunotoxicology, vol 1. Wiley-Liss, New York, pp 109–124

Fairbrother A (1990) Immunotoxicology of captive and wild birds. In: Kendall RJ, Lacher TE (eds) Wildlife toxicology and population modeling: integrated studies of agroecosystems. CRC, Boca Raton, Fla, pp 251–261

Folstad I, Karter AJ (1992) Parasites, bright males and the immunocompetence handicap. Am Nat 139:603–622

Gause WC, Marsh JA (1986) Effect of testosterone treatments for varying periods on autoimmune development and on specific infiltrating leukocyte populations in the thyroid gland of obese strain chicken. Clin Immunol Immunopathol 39:464–478

Grossman CJ (1984) Regulation of the immune system by sex steroids. Endocr Rev 5:435–455

Grossman CJ (1985) Interactions between the gonadal steroids and the immune system. Science 227:257–261

Hasselquist D, Bensch S, Schantz T von (1996) Correlation between male song repertoire, extra-pair paternity and offspring survival in the great reed warbler. Nature 381:229–232

Hillgarth N, Wingfield JC (1997) Parasite-mediated sexual selection: endocrine aspects. In: Clayton DH, Moore J (eds) Host-parasite evolution: general principles on avian models. Oxford University Press, Oxford, pp 78–104

Hirota Y, Suzuki T, Bito Y (1980) The development of unusual B-cell functions in testosterone propionate-treated chickens. Immunology 39:29–36

Houde AE, Torio AJ (1992) Effect of parasitic infection on male color pattern and female choice in guppies. Behav Ecol 3:346–351

Johnsen TS (1998) Behavioural correlates of testosterone and seasonal changes of steroids in red-winged blackbirds. Anim Behav 55:957–965

Kerlan JT, Jaffe RB (1974) Plasma testosterone levels during the testicular cycle of the red-winged blackbird (*Agelaius phoeniceus*). Gen Comp Endocrinol 22:428–432

Kirkpatrick M, Ryan MJ (1991) The evolution of mating preferences and the paradox of the lek. Nature 350:33–38

Klein SL, Nelson RJ (1997) Adaptive immune responses are linked to the mating system of arvicoline rodents. Am Nat 151:59–67

Lamont SJ, Bolin C, Cheville N (1987) Genetic resistance to fowl cholera is linked to the MHC. Immunogenetics 25:284–289

Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake. Auk 104:116–121

Lin Tien-Min, Lustig RH, Chang C (1996) The role of androgens-androgen receptor in immune system activity. In: Marsh JA, Kendall MD (eds) The physiology of immunity. CRC, Boca Raton, Fla, pp 263–276

Lochmiller RL (1995) Testing the immunocompetence handicap theory. Trends Ecol Evol 10:372–373

Luster MI, Portier C, Gayla Pait D, White KL Jr, Gennings C, Munson AE, Rosenthal GJ (1992) Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. Fundam Appl Toxicol 18:200–210

Marsh JA (1996) The integration of the neuroendocrine and immune systems: a marriage of convenience or necessity? In: Davidson TF, Morse TR, Payne LN (eds) Poultry science symposium series, vol. 24. Carfax, Oxford, pp 357–374

Mendelsohn J, Multer MM, Bernheim JL (1977) Inhibition of human lymphocyte stimulation by steroid hormones: cytokine mechanisms. Clin Exp Immunol 27:127–134

Møller AP (1988) Female choice selects for male sexual tail ornaments in the monogamous swallow. Nature 332:640–642

Møller AP (1995) Hormones, handicaps and bright males. Trends Ecol Evol 10:121

Novotny EA, Raveche ES, Sharrow S, Ottinger M, Steinberg AD (1983) Analysis of thymocyte subpopulations following treatment with sex hormones. Clin Immunol Immunopathol 28:205–217

Olsen NJ, Kovacs WJ (1996) Gonadal steroids and immunity. Endocr Rev 17:369–384

Owens IPF, Short RV (1995) Hormonal basis of sexual dimorphism in birds: implications for new theories of sexual selection. Trends Ecol Evol 10:44–47

Petrie M (1994) Improved growth and survival of offspring of peacocks with more elaborate trains. Nature 371:598–599

Raouf SA, Parker PG, Ketterson ED, Nolan V Jr, Ziegenfus C (1997) Testosterone influences reproductive success by increasing extra-pair fertilizations in male dark-eyed juncos, *Junco hyemalis*. Proc R Soc Lond B 264:1599–1603

Rife SU, Marquez MG, Escalante A, Velich T (1990) The effect of testosterone on the immune response 1. Mechanism of action of antibody-forming cells. Immunol Invest 19:259–270

Ros AF, Groothius TGG, Apanius V (1997) The relation among gonadal steroids, immunocompetence, body mass, and behavior in young black-headed gulls (*Larus ridibundus*). Am Nat 150:201–219

Saino N, Møller AP (1994) Secondary sexual characters, parasites and testosterone in the barn swallow, *Hirundo rustica*. Anim Behav 48:1325–1333

Saino N, Møller AP, Bolzern AM (1995) Testosterone effects on the immune system and parasite infestations in the barn swallow (*Hirundo rustica*): an experimental test of the immunocompetence hypothesis. Behav Ecol 6:397–404

Sapolsky RM (1993) Neuroendocrinology of the stress-response. In: Becker JB, Breedlove SM, Crewe D (eds) Behavioral endocrinology. MIT Press, Cambridge, Mass, pp 287–324

Schantz T von , Göransson G, Andersson G, Fröberg I, Grahn M, Helgeé A, Wittzell H (1989) Female choice selects for a viability-based male trait in pheasants. Nature 337:166–169

Schantz T von , Grahn M, Göransson G (1994) Intersexual selection and reproductive success in the pheasant *Phasianus colchicus*. Am Nat 144:510–527

Schantz T von, Bensch S, Grahn M, Hasselquist D, Wittzell H (1999) Good genes, oxidative stress and condition-dependent sexual signals. Proc R Soc Lond B (in press)

Schuurs AHWM, Verheul HAM (1990) Effects of gender and sex steroids on the immune response. J Steroid Biochem 35:157–172

Schuurs AHWM, Dietrich H, Gruber J, Wick G (1992) Effects of sex steroid analogs on spontaneous autoimmune thyroiditis in obese strain chickens. Int Allergy Immunol 97:337–344

Searcy WA, Wingfield JC (1980) The effects of androgen and antiandrogen on dominance and aggressiveness in male red-winged blackbirds. Horm Behav 14:126–135

Siva-Jothy MT (1995) ''Immunocompetence'': conspicuous by its absence. Trends Ecol Evol 10:205–206

Sthoeger ZM, Chiorazzi N, Lahita RG (1988) Regulation of the immune response by sex hormones. I. In vitro effects of estradiol and testosterone on pokeweed mitogen-induced human B cell differentiation. J Immunol 141:91–98

Talal N (1989) Autoimmunity and sex revisited. Clin Immunol Immunopathol 53:355–357

Terres G, Morrison SL, Habricht GS (1968) A quantitative difference in the immune response between male and female mice. Proc Soc Exp Biol Med 47:273

Verheul HAM, Tittes EV, Kelder J, Schuurs AHWM (1986) Effects of steroids with different endocrine profiles on the development, morphology and function of the bursa of Fabricius in chickens. J Steroid Biochem 25:665–675

Wakelin D, Apanius V (1997) Immune defence: genetic control. In: Clayton DH, Moore J (eds) Host-parasite evolution: general principles on avian models. Oxford University Press, Oxford, pp 30–58

Weatherhead PJ, Metz KJ, Bennett GF, Irwin RE (1993) Parasite faunas, testosterone and secondary sexual traits in male red-winged blackbirds. Behav Ecol Sociobiol 33:13–23

Wedekind C, Folstad I (1994) Adaptive or non adaptive immunosuppression by sex hormones? Am Nat 143:936–938

Westneat DF, Birkhead TR (1998) Adaptive hypotheses linking the immune system and mate choice. Proc R Soc Lond B 265:1065–1073

Wiedmeier SE, Araneo BA, Huang K, Daynes RA (1991) Thymic modulation of IL-2 and IL-4 synthesis by peripheral T cells. Cell Immunol 135:501–518

Wilkinson L (1992) SYSTAT. The system for statistics. SYSTAT, Evanston, Ill

Wingfield JC (1984) Androgens and mating systems: testosterone-induced polygyny in normally monogamous birds. Auk 101:665–671

Wingfield JC, Farner DS (1975) The determination of five steroids in avian plasma by radioimmunoassay and competitive protein binding. Steroid 26:311–327

Wingfield JC, Smith JP, Farner DS (1982) Endocrine responses of white-crowned sparrows to environmental stress. Condor 84:399–409

Zuk M, Ligon JD, Thornhill R (1992) Effects of experimental manipulation of male secondary sex characters on female mate preference in red jungle fowl. Anim Behav 44:999–1006

Zuk M, Johnsen TS, Maclarty T (1995) Endocrine-immune interactions, ornaments and mate choice in red jungle fowl. Proc R Soc Lond B 260:205–210

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