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Song predicts immunocompetence in male European starlings *(Sturnus vulgaris)*

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According to the immunocompetence handicap hypothesis, sexually selected characteristics predict immune function and this relationship is mediated by testosterone. In the present study, we investigated whether bird song could predict immunity in European starlings *(Sturnus vulgaris)*. We recorded the singing and reproductive behaviours of 16 adult male starlings in an outdoor aviary and then assessed their cell-mediated and humoral immunity *in vivo*. The males were observed in groups of four for 2 h each day over a 4-day period. For each male, the number of songs produced was recorded and the average song-bout length was computed. Next, cell-mediated and humoral immunity were assessed via cutaneous swelling responses to the T-cell mitogen phytohaemagglutinin and antibody responses to a novel antigen, keyhole limpet haemocyanin. Song rate and song-bout length were positively correlated with cell-mediated and humoral immunity, respectively. Additionally, a negative relationship between plasma testosterone concentration and antibody response was observed. These data demonstrate that male starling song can be used as a predictor of immunocompetence, with more robust singers exhibiting enhanced immunity. Whether this relationship is mediated directly by testosterone requires further investigation.

Keywords: immunocompetence; bird; song; sexual selection; testosterone

1. INTRODUCTION

Hamilton & Zuk (1982) proposed that elaborate sexually selected traits, such as bright plumage, reveal the parasite status of the host allowing females to choose the males most resistant to infection. Folstad & Karter (1992) took this hypothesis a step further by adding a handicap mechanism through which elaborate traits could be honest and reliable signals of immunocompetence. Their hypothesis, the immunocompetence handicap hypothesis (ICHH), argues that some factor (e.g. testosterone) that enhances the development of secondary sexual characteristics also suppresses immunity. According to the ICHH, only those males who possess superior immunocompetence will be able to tolerate the immunosuppressive effects of testosterone and thus be able to maintain the high concentrations of testosterone associated with many secondary sexual characteristics. Females who choose to mate with males exhibiting the most elaborate testosterone-dependent traits would benefit by mating with the most immunocompetent males.

Hypotheses linking mate choice and immune function have received much attention in recent years among researchers investigating sexually dimorphic species of birds with an emphasis typically on plumage characteristics (e.g. Møller 1995; Zuk *et al.* 1995; Hill & Brawner 1998; Merila *et al.* 1999; Evans *et al.* 2000; McGraw & Hill 2000). However, endocrine mechanisms mediating plumage characteristics have yet to be clearly established (Owens & Short 1995; but see Evans *et al.* (2000); Gonzalez *et al.* 2001). Bird song, on the other hand, has been demonstrated repeatedly to be influenced by circulating testosterone concentrations (for reviews see Catchpole & Slater (1995), DeVoogd (1991) and Schlinger (1997)), making it an ideal candidate for testing predictions regarding the ICHH. Still, studies that have rigorously tested the ICHH by simultaneously investigating the relationships among natural variation in hormones, immune function and song are scarce (e.g. Saino *et al.* 1997*b*).

In the present study, the relationships among song, testosterone and immunocompetence were investigated using European starlings (*Sturnus vulgaris*). Starlings were chosen because song is known to be important in mate selection by females (reviewed in Eens (1997)). The courtship behaviours of captive male starlings were recorded and endogenous plasma testosterone concentrations were measured. Afterwards, cell-mediated and humoral immunity were assessed via immunological challenge with the T-cell mitogen phytohaemagglutinin (PHA) and the novel antigen keyhole limpet haemocyanin (KLH). Using multiple regression analyses, the degree to which the behavioural measures could predict cell-mediated and humoral immunity were evaluated and the degree of covariation between testosterone and immune function was determined.

According to the ICHH, female starlings should be able to choose disease-resistant mates using the quality of male song as an indicator and the reliability of this signal should be maintained by immunosuppressive effects of testosterone (Hamilton & Zuk 1982; Folstad & Karter 1992). Therefore, for the present study, three predictions can be drawn:

(i) that male song can be used as an indicator of immunocompetence,
(ii) that testosterone is immunosuppressive; and
(iii) that testosterone facilitates singing.

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2. MATERIAL AND METHODS

Sixteen male starlings were caught as juveniles in July and August of 1997 in Parkton, MD (39°18′ N) and were group housed in the laboratory in single-sex cages where they were provided with turkey starter crumbs (Purina) and water *ad libitum*. They were maintained on a L : D schedule of 8 h light and 16 h dark (8 L : 16 D) until the spring of 1998. They were then switched to a photoperiod of 18 L : 6 D, followed by a return to a photoperiod of 8 L : 16 D in August, 1998. This schedule simulated the natural seasonal change in photoperiod, thus allowing the birds to undergo moult and the regression–recrudescence of their reproductive systems (Goldsmith & Nicholls 1984; Boulakoud & Goldsmith 1994).

On 31 January 1999, the birds were placed on a photoperiod of 11 L : 13 D, which allowed the gradual recrudescence of their gonads (Dawson & Goldsmith 1983; Falk & Gwinner 1988) and maintained the birds in a reproductively active condition similar to early spring. During the summer of 1999, the birds were transported to an outdoor aviary (2.5 m long × 2.5 m wide × 1.5 m high) in successive groups of four birds each. The aviary was equipped with four nest boxes and food, water and green nesting material were provided *ad libitum*.

Immediately prior to transportation to the aviary, each bird was weighed and given a plastic colour band of either pink, blue, white or orange. Blood samples were obtained using procedures described previously (Duffy *et al.* 2000) and the plasma fraction was stored in 0.5 ml centrifuge tubes at 20 °C until assayed for testosterone via radioimmunoassay (^{125}I Double-Antibody Kit, ICN Biomedicals, Inc., Costa Mesa, CA (Duffy *et al.* 2000)).

(a) *Behavioural observations and song analysis*

Once housed in the aviary, the birds were given 5–6 days to adjust to their surroundings prior to commencement of behavioural observations. Each group of four was then observed for 2 h per day (1 h with an adult, reproductively active female placed in the aviary and 1 h with no female present) for four consecutive days. Inside the aviary, all birds had visual and physical access to one another. At the end of the first day of observation, blood samples were collected and a sensitizing dose of PHA (0.5 mg in 0.1 ml of phosphate buffered saline, PBS) was injected subcutaneously (s.c.) above the left leg (Zuk & Johnsen 1998; Casto *et al.* 2001).

Behavioural observations were made from behind a blind located *ca.* 5 m from the aviary. During observations, all four males were observed simultaneously and each occurrence of the following behaviours was recorded: singing (song rate, measured as the number of bouts produced per hour), aggressive behaviours (chasing or fighting), collecting green nesting material, and wing waving. The latter two behaviours are performed by males during courtship, in addition to singing (Eens 1997).

During the observation period, a sample of songs produced by each of the males was recorded using microphones, situated within the nest boxes, that were connected to a tape recorder (Sony, TCM-5000EV). Song-bout length was measured by visually inspecting computer-generated sonograms (SoundEdit 16 v. 2, Macromedia, Inc., San Francisco, CA) and recording the duration of each portion of song that met the criteria for a song bout (at least 10 s of song with a pause no greater than 1.5 s (Eens *et al.* 1991)). The average number of song bouts analysed was 16.5 (range 3–36, s.d. = 8.62; one bird was excluded from analyses of song-bout length because no recordings were obtained).

(b) *Assessment of immune function*

On the fourth day of observation, blood samples were obtained for analysis of testosterone concentrations and all birds were transported back to the laboratory, where they were housed under an 11 L : 13 D photoperiod. Two days later, cell-mediated immunity was assessed via injection of PHA into the wing web (Saino *et al.* 1997*a*; Zuk & Johnsen 1998) with measurements taken pre- and 24, 48 and 72 h post-injection using a pressure-sensitive thickness gauge (Mitutoyo Corporation, Model No. ID-S1012E, as described previously (Duffy *et al.* 2000)). Briefly, the right wing web was injected with 0.5 mg of PHA in 0.1 ml of PBS and the left wing web was injected with 0.1 ml of the vehicle alone (Lochmiller *et al.* 1993; Saino *et al.* 1997*a*).

Wing-web swelling was calculated as the pre- to post-injection change in right wing-web thickness minus the change in left wing-web thickness. The experimenter was blind to the colour-band identity of the birds during wing-web measurement.

After the final wing-web measurements were taken, blood samples were obtained and the birds were injected s.c. with KLH (300 μg in 0.1 ml of sterile saline) for assessment of humoral immunity. Ten and 15 days post-injection, blood samples were taken and the plasma was analysed for anti-KLH antibodies (a composite of immunoglobulin G (IgG) and IgM) via an enzyme-linked immunosorbent assay (ELISA), as described previously (Duffy *et al.* 2000). Antibody titres were determined using a plate reader (405 nm filter; Bio-Rad: Benchmark model, Richmond, CA) and measured as the mean absorbance (optical density) of the sample relative to pooled sera from KLH-naive birds (*P*/*N*).

(c) *Statistical analysis*

For statistical purposes, the peak swelling response (48 h post-injection) was used for analysis of cell-mediated immunity. The antibody titres for days 10 and 15 post-injection were highly inter-correlated ($r^2 = 0.811$, $p < 0.001$), so the average titre across both days was used for the analysis of humoral immunity. The behavioural data were analysed using two forward stepwise multiple regressions, one for each of the dependent variables (swelling response and antibody titre), using song rate, song-bout length, aggression, green nesting material and wing waving as the independent variables (Spss for Windows v. 8.0). Due to the fact that two multiple regressions were performed using the same set of independent variables, a Bonferroni correction was used in which k was set at 2; this results in a corrected α level of 0.025 for each regression ($\alpha = 0.05/k$). One bird was excluded from analysis of cell-mediated immunity due to severe leakage of PHA from the wing-web during injection and another bird was excluded from both analyses because no recordings were obtained for that individual, therefore song-bout length could not be assessed. Cases that had missing values for any of the variables were omitted from the regression analysis.

The effect of time of blood sampling on testosterone concentrations was analysed using a repeated-measures ANOVA. Covariation between testosterone concentrations, averaged across all five blood samples, and immunity and between testosterone and singing were analysed using two forward stepwise multiple regressions with cell-mediated and humoral immune responses as the independent variables for one analysis and song rate and song-bout length as the independent variables for the other analysis. Separate multiple regressions were used to avoid problems with multicollinearity between the immune and song variables. A Bonferroni correction was applied to each in which

Table 1. Standardized correlation coefficients (β) for each of the behavioural variables with each of the immune measures, humoral (anti-KLH antibody response relative to negative controls) and cell mediated (wing-web swelling response to PHA (mm)).

	correlation coefficients	
behavioural variable	humoral	cell mediated
song rate (bouts h^{-1})	0.027	0.848^{**}
song-bout length (s)	0.623^{*}	−0.057
green nest material	0.055	0.070
aggression	0.044	0.036
wing waving	−0.203	−0.036

* $p < 0.025$.
** $p < 0.001$.

k was set at 2, resulting in a corrected α level of 0.025 for each regression.

Overall, the general activity level of the birds was lower on the second day of observation, presumably due to inoculation with PHA following the observation period on the previous day. Therefore, the behavioural data from day 2 were excluded from analysis. The total number of occurrences for the behavioural variables (song rate, green nesting material, wing waving and aggression) was divided by the duration of observation (i.e. 3 h with a female present and 3 h with no female present) to yield the average number of occurrences per hour. The presence of a female significantly increased singing activity ($F_{1,31} = 4.48$, $p < 0.05$), but had no effect on the other behaviours. Therefore, for regression analyses, only song rate in the presence of a female was included. For the remaining variables, the data collected in the presence and absence of a female were averaged together. In order to correct for heterogeneity of variance, the data for aggression, green nesting material and wing waving were log transformed.

3. RESULTS

Song rate was the only behavioural measure that met the criteria to be entered into the regression equation for the cell-mediated swelling response to PHA (see table 1, figure 1*a*; $F_{1,12} = 30.652$, $p < 0.001$; s.e. of the estimate = 0.224; N (number of observations) = 14; $R^2_{\text{adj}} = 0.695$). Song-bout length was the only behavioural variable to be entered into the regression equation for the anti-KLH antibody response (see table 1, figure 1*b*; $F_{1,13} = 8.240$, $p < 0.025$; s.e of the estimate = 0.514; $N = 15$; $R^2_{\text{adj}} = 0.341$).

Testosterone concentrations did not differ significantly as a function of time of sampling and thus were averaged across all five samples for subsequent analyses. Among the two immune measures, only antibody response to KLH met the criteria to be entered into the regression equation with average testosterone concentration as the dependent variable (figure 2, table 2; $F_{1,14} = 7.490$, $p < 0.025$; s.e. of the estimate = 0.223; $N = 16$; $R^2_{\text{adj}} = 0.317$). Among the two song variables, only song-bout length met the criteria to be entered into the regression equation with average testosterone concentration (table 2; $F_{1,14} = 10.207$, $p < 0.025$; s.e. of the estimate = 0.211; $N = 15$; $R^2_{\text{adj}} = 0.397$). The correlation between testosterone and song-bout length was no longer significant when statistically controlling for antibody response to KLH ($r^2 = 0.214$, $p > 0.05$, two-tailed).

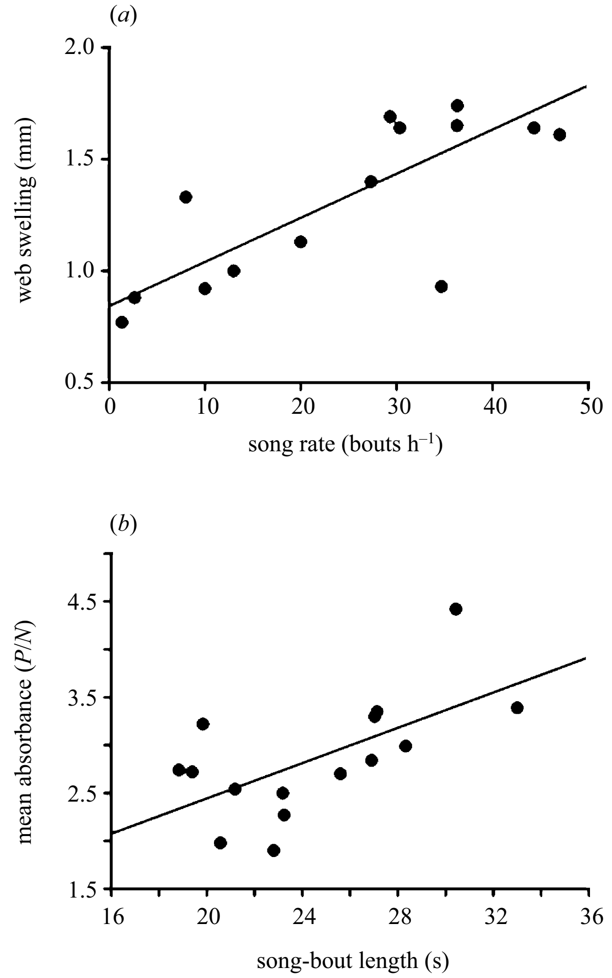

Figure 1. (*a*) Wing-web swelling (mm) 48 h post-injection of PHA in relation to song rate (number of bouts per h). The linear regression line has the equation $y = 0.77 + 0.02x$, $r^2 = 0.719$. (*b*) Anti-KLH antibody titres (mean absorbance values *P*/*N*), averaged across days 10 and 15, in relation to song-bout length (s). The linear regression line has the equation $y = 1.18 + 0.18x$, $r^2 = 0.388$.

4. DISCUSSION

In the present study, we observed positive relationships between male starling song and immune function, with song rate predicting cell-mediated immunity and song-bout length predicting humoral immunity. Male song-bout length has been demonstrated to be the best predic-

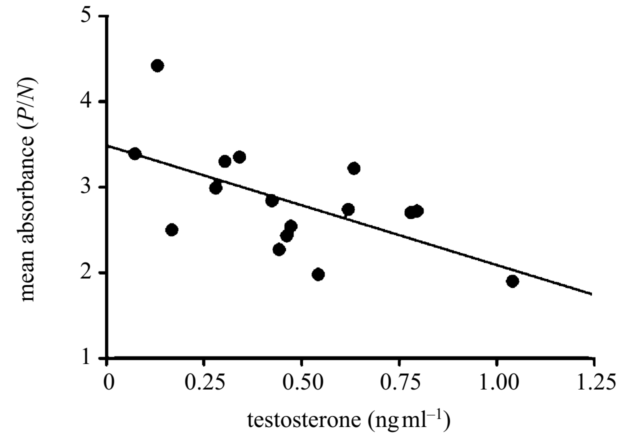

Figure 2. Anti-KLH antibody titres (mean absorbance values *P*/*N*), averaged across days 10 and 15, in relation to endogenous plasma testosterone concentrations (ng ml^{-1}). The linear regression line has the equation $y = 3.92 - 1.40x$; $r^2 = 0.350$.

tor of female mate choice in starlings and males increase their song rate substantially in the presence of a female (Eens 1997; Gentner & Hulse 2000). These data indicate that female starlings can use male song characteristics to assess immunocompetence when choosing a mate in agreement with the ICHH (Folstad & Karter 1992). Furthermore, starling song-bout length is positively correlated with the volumes of two brain nuclei that are necessary for song production (HVc (sometimes called the High Vocal Center) and the robust nucleus of the archistriatum, RA (Bernard *et al.* 1996)), the volumes of which are associated with changes in testosterone concentrations (Bernard & Ball 1997). Taken together, we believe that these findings establish for the first time a mechanism linking covariation in courtship behaviour and its associated brain morphology with endocrine and immune functioning.

In addition, the present data provide considerable insight into the function of male starling song. In starlings, song-bout length and repertoire size are highly intercorrelated and both increase with age in males (Eens 1997). Furthermore, one report describes a positive correlation between repertoire size and body condition (Mountjoy & Lemon 1996). The data reported here demonstrate that starling song predicts immunocompetence among males of the same age (2 years old). Together, these data suggest that song may be predictive of an underlying genetic quality that translates into enhanced immunocompetence and an increased ability to survive.

The current study found no clear evidence of testosterone facilitation of song production. No correlation between song rate and testosterone concentration was detected. A negative relationship was observed between testosterone and song-bout length that disappeared when the effect of antibody response to KLH was statistically controlled. However, there is extensive evidence in both laboratory and field studies indicating that the presence of testosterone facilitates singing in many species, including starlings (e.g. Arnold 1975; Marler *et al.* 1987; for reviews, see Eens (1997), Brenowitz & Kroodsma (1996) and Catchpole & Slater (1995)). A recent report on seasonal variation in starling song behaviour suggests that song-bout length and the ability of social factors to facilitate singing increase in the spring when testosterone concentrations are higher compared with the fall when testosterone is undetectable (Riters *et al.* 2000). Thus, further investigation is needed to elucidate the relationship between testosterone concentrations and various aspects of song performance in starlings.

Table 2. Standardized correlation coefficients (β) for each immune measure and each song variable regressed against average testosterone concentration (ng ml^{-1}).

independent variable	coefficient
song rate (bouts h^{-1})	0.064
song-bout length (s)	−0.663*
humoral response (KLH)	−0.605*
cell-mediated response (PHA)	0.073

* $p < 0.025$.

Whether testosterone suppresses immune function in wild species of birds has been the subject of much debate recently (Braude *et al.* 1999; Hasselquist *et al.* 1999; Evans *et al.* 2000; Peters 2000; Poiani *et al.* 2000). Two recent studies have demonstrated that exogenous testosterone reduces cell-mediated and humoral immunity in starlings and Dark-eyed Juncos (*Junco hyemalis* (Duffy *et al.* 2000; Casto *et al.* 2001)). However, reports on other species of songbirds have failed to detect an effect of testosterone administration on immune function (Hasselquist *et al.* 1999) or have observed testosterone-induced enhancement when the immunosuppressive effects of corticosterone were statistically controlled (Evans *et al.* 2000). The reason for such contradictory results may include species and methodological differences as well as possible effects of other hormones (e.g. corticosterone (Evans *et al.* 2000)) or body condition (Duckworth *et al.* 2001).

Given that testosterone administration has been demonstrated to induce increases in corticosterone in a number of bird species (Ketterson *et al.* 1991; Klukowski *et al.* 1997; Duffy *et al.* 2000; Evans *et al.* 2000), it is difficult to tease apart the precise mechanism of immunosuppression in response to exogenous testosterone. Therefore, various approaches that selectively investigate the independent effects of these two steroid hormones on immunity are required to resolve this issue. Furthermore, changes in corticosterone-binding globulin (CBG) concomitant with testosterone-induced or seasonal fluctuations in plasma corticosterone may result in only small or no changes in free (i.e. biologically available) corticosterone (Klukowski *et al.* 1997; Deviche *et al.* 2001). Together with the finding that CBG also binds testosterone with lower affinity in birds (Deviche *et al.* 2001), these studies suggest that mechanisms proposing corticosterone-induced immunosuppression following testosterone elevation must take into account changes in the CBG binding capacity and the free levels of both of these steroid hormones.

In the present study, a negative correlation was observed between endogenous testosterone and antibody response to KLH, similar to that observed in a previous study following testosterone administration (Duffy *et al.* 2000). Assuming that testosterone concentrations are individually optimized, the ICHH would predict a positive correlation between endogenous testosterone and immune function across individuals, while elevation of testosterone above an individual's optimum should suppress immunity (e.g. Peters 2000; Duckworth *et al.* 2001). The negative correlation observed here, together with previous conflicting findings in the literature with respect to the relationship between testosterone and immunity, suggests a more complicated mechanism linking song performance to immunocompetence. As discussed above, an alternative mechanism may involve corticosterone (Evans *et al.* 2000). However, due to the housing conditions used in the present study, it was not feasible to obtain accurate baseline corticosterone concentrations. Thus, it remains possible that this stress hormone is involved in mediating the relationship between reproductive behaviour and immune function in starlings, and further investigation is needed.

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