Song and immunological condition in male barn swallows (Hirundo rustica)

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Male secondary sexual characters may have evolved as intraspecific or intersexual signals of male phenotypic or genetic quality. In birds, singing performance may have the function to honestly reveal health and vigor of individual males. Infectious diseases and poor body conditions would therefore be expected to negatively influence singing performance. Since bird pathogens are known to elicit both a humoral and a cell-mediated immune response, it can be predicted that a negative relationship exists between singing performance and activity of the immune system. This prediction was tested for the first time in this correlational study. The relationships between song rate and features and hematological variables (concentration of leukocytes in peripheral blood, ratio of gamma-globulins to total plasma proteins; blood cell sedimentation rate and hematocrit) and body condition were analyzed in a population of barn swallows (Hirundo rustica). Song rate was negatively correlated with lymphocyte concentration and with the ratio of gamma-globulins to plasma proteins. Spectrographic analysis showed that features of song were not significantly correlated with hematological variables or body condition. The level of circulating testosterone was not correlated with song rate nor with hematological variables. This study is the first to show a correlation between a bird’s singing performance and hematological profile and suggests that song rate of male barn swallows may reflect their health status. Song in this species might thus have evolved because it allows prospecting females to assess aspects of phenotypic and/or genetic quality of potential mates. Key words: body condition, gamma-globulins, hematocrit, Hirundo rustica, leukocytes, song quality, song rate. [Behav Ecol 8:364-371 (1997)]

I t is widely accepted that song has evolved through intra- or intersexual selection and that it has therefore assumed the dual function for mate attraction and repulsion of males competing for limited resources (Catchpole, 1979, 1980, 1982; Darwin, 1871; Searcy and Andersson, 1986).

By analogy with the models of evolution of morphological ornaments, two general models can be proposed for the evolution of song (Bradbury and Andersson, 1987). Complex songs may have evolved as the result of progressive elaboration of the trait and female preference in a Fisherian “runaway” process (Fisher, 1930; Lande, 1981). According to “good-genes” models (Andersson 1982; Zahavi 1975, 1977), on the other hand, variability in song may reflect male quality.

Singing is a costly activity because of energy demands for song production (Brackenbury, 1982), increased risk of predation, and because it competes with other activities relevant to individual fitness, such as foraging. If it can be assumed that the cost imposed by song production increases differentially for different males with song duration or elaboration, then the potential exists for song to reveal the ability of the singer to bear these costs and, thus, to advertise its phenotypic or genetic quality.

This leads to the fundamental question of which factors influence song production and which mechanisms modulate song quality. Song rate may proximately depend upon individual health and nutritional condition as influenced by a wide spectrum of variables such as parasite infestation (Møller, 1991) and food availability in the territory (Davies and Lundberg, 1984; Gotlander, 1987; Greig-Smith, 1983; Reid, 1987). Moreover, song production may be promoted by hormones (e.g., Arnold, 1975; Balthazart, 1983; Epple and Stetson, 1980) that may have a negative influence on some components of individual fitness (Saino et al., 1995; Studd and Robertson, 1988). Each male may therefore be forced to trade the sociosexual benefits of song against the concomitant costs imposed by its hormonal profile (Folstad and Karter, 1992; Ketterson and Nolan, 1992). For example, testosterone may directly influence the singing performance and have a simultaneous suppressive effect on the immune system or reduce survival prospects (Bekke, 1995; Grossman, 1985; Ketterson et al., 1992; Lawrence, 1991; Saino et al., 1995).

While the literature dealing with the relationships between song and body mass or territory quality is quite extensive (reviewed in Catchpole and Slater, 1995), no data have been published, to our knowledge, on the relationships between the immune system and song.

This paper presents the results of a correlational study in which we analyzed the relationships between and song rate and song features during the pre-laying and laying periods of the female to which a male was mated and several hematological variables and body condition in a natural population of male barn swallows (Hirundo rustica).

Among variables describing singing performance, song rate may be one of the most highly correlated with male quality (Catchpole and Slater, 1995). Barn swallow song usually consists of a sequence of different syllables ending with a characteristic rattle (Cramp, 1988; Galeotti et al., in press). Our choice of variables to describe song features (see METHODS) was based on considerations concerning their sexual function, as observed in other species, or speculations about costs or constraints which might influence song.

The hematological variables we measured were: (1) concentration of leukocytes in peripheral blood; (2) the ratio of gamma-globulins to total plasma proteins; (3) blood cell sedimentation rate; and (4) hematocrit. Concentration of different

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leukocytes is a simple measure of cell-mediated immunity in birds (e.g., Glick et al., 1988; Spinu and Degen, 1993). Avian species are known to respond to infectious diseases by increasing concentrations of leukocytes, most notably heterophils and lymphocytes (Averbeck, 1992; Davis, 1981; Hawkey et al., 1983; Rose et al., 1979). Gamma-globulins are plasma proteins, including immunoglobulins and other proteins that co-migrate during electrophoresis, which are the source of antibodies involved in immune response to a large spectrum of parasitic infestations and protozoa, bacteria, and viral infections (e.g., DeVaney and Augustine, 1988; Jayawardane and Spradbrow, 1995; Russell and Ezeife, 1995). The diagnostic meaning of blood cell sedimentation rate is poorly understood in birds. In mammals, sedimentation rate of erythrocytes is increased in a variety of inflammatory and infectious diseases. Acute or chronic anemia as measured by hematocrit values can result, for example, from blood and gastrointestinal parasite infestations or bacterial infections (Harrison and Harrison, 1996).

Our specific prediction was that individuals that sing at low rates have high blood lymphocyte and/or heterophil concentrations, high gamma-globulin-to-plasma protein ratios, high blood cell sedimentation rate, low hematocrit values, or have low body mass corrected for body size. However, we had no strong predictions about the existence of relationships between song features and hematological variables and body conditions.

In one species, the red-winged blackbird Agelaius phoeniceus, individual variation in testosterone plasma level has been found to positively correlate with song rate (Johnsen, 1991, in Kettersson and Nolan, 1992). Since testosterone is thought to exert a suppressive effect on immune system and a direct effect on parasite reproduction (Beckage, 1993; Folstad and Karter, 1992), we also analyzed the relationship between testosterone plasma levels, song rate or song features, and immune profile of individual males.

The barn swallow is a socially monogamous, semicolonial, territorial passerine (Møller, 1994). A small nesting territory is defended by breeding males. Song is produced by males during the whole breeding season and song rate peaks during the pre-laying and laying period (Møller, 1994; Saino and Møller, 1995; Saino and Møller, unpublished data). Experimental manipulation of ectoparasite infestations has shown that parasites negatively influence the rate at which song is produced (Møller, 1991).

STUDY AREA AND METHODS

The study was carried out during spring 1995 in one colony in a single stable south-east of Milano (northern Italy). Swallows were caught at sunrise by mist nets starting on 28 March. Each barn swallow was marked with a metal ring on one leg, a plastic color ring on the other, and a unique combination of colors on breast and belly feathers. At first capture, individuals were sexed according to the shape of the cloacal protuberance (Svensson, 1984) and by inspection for presence (female) or absence (male) of an incubation patch. Assignment was confirmed at later recaptures by inspection of the incubation patch and by observation of sexual and breeding behavior.

At first capture we measured several morphological variables including bill length, depth and width, keel and tarsus length, maximum left and right wing chord, wing span, length of left and right outermost tail feathers, and length of the left innermost rectrice. At the time of first capture and at each following recapture we also measured body mass with a precision of 0.1 g. In the analyses, body condition was expressed as the ratio between residuals of body mass on calendar date and (keel length)². Blood samples in heparinized capillary tubes and smears were also taken at every capture by puncture of the ulnar vein.

Blood cell sedimentation rate and hematocrit

To measure blood cell sedimentation rate, capillary tubes containing blood samples were put in vertical position for four hours in a refrigerated room (4°C) after having been stored horizontally in a cool bag in the field. Sedimentation rate (proportion of blood sedimented/h) was expressed as volume of the part of the capillary not occupied by blood cells x (blood volume in the capillary)⁻¹ x 0.25. Blood samples were then centrifuged for 10 minutes at 4000 rpm and hematocrit was expressed as volume of the part of the capillary occupied by blood cells x (blood volume in the capillary)⁻¹. Plasma was stored at -50°C for gamma-globulin assay.

Gamma-globulins assay

Gamma-globulin assays were made by densitometric analysis after electrophoretic separation of plasma proteins on agarose gels. Five µl of plasma were diluted 1:2.5 in Barbital buffer (pH 8.6). Five µl of the diluted sample were applied to agarose gels following the standard procedures indicated by Beckman Paragon SPE-kits (see also Saino and Møller, 1996). In one lane of each gel we applied diluted chicken IgG to obtain a standard reference for migration of barn swallow gamma-globulins along the electrophoretic lanes. The electrophorese were applied at constant voltage (100 V), 20°C, for 25 min. After electrophoresis, gels were air-dried and stained following kit instructions. Densitometric analysis of gels was performed using a computer image analysis procedure run by the Gelansoft program (Eidosoft). The concentration of gamma-globulins was expressed as the ratio between the area of the gamma-globulin peak and the total area of all peaks representing plasma proteins, and was log₁₀ transformed for statistical analysis. We checked for consistency of the ratio of gamma-globulins to plasma proteins by comparing variation between two repeated assays of the same blood sample with variation between samples of different individuals, which was found to be significantly larger (analysis of variance; F = 52.4; df = 12, 13; p < .001; see also Saino et al., 1995).

Leukocyte counts and concentrations

Leukocytes and red blood cells were counted by an experienced person after blood smears had been air-dried and stained by the May-Grunwald-Giemsa staining method. Blood smears were scanned at 650X magnification following standard routines. In each microscopic field we counted red blood cells and leukocytes classified as lymphocytes, monocytes, eosinophils, heterophils, and basophils. In each smear we counted 150 to 200 leukocytes, and the corresponding red blood cells. This allowed us to calculate the number of leukocytes of the different types per 10,000 red blood cells. Even when fewer leukocytes were counted, this method has been shown to give significantly repeatable within-blood smear measures of leukocyte concentration (Saino et al., 1995).

Testosterone assays

Plasma testosterone levels were measured by radioimmunoassay after separation of testosterone from other steroids by column chromatography following the procedures given in Wingfield and Farner (1975) and Secreto et al. (1991). Testosterone RIA kits were purchased from bioMercieux laboratories (Marcy-l’Etoile, France). The coefficients of inter-
Observation of birds and song rate recording

Males were assigned to territories and nests by identifying their color rings and color markings. Singing activity was recorded during 1 h daily observation sessions from 0600 to 1000 h starting when males had established a territory. Individual song rate was quantified as percentage of observations every second minute in which a male was singing. Previous studies have consistently shown that individual song rates are repeatable within days (Saino and Möller, 1995; Saino and Möller, unpublished data). In the present study we obtained the same result by comparing song rates recorded in two 1 h observation sessions of the same males during the same day, which were found to be significantly repeatable (analysis of variance; $F = 5.50$; df = 8.5; $p < .01$). This allowed us to assume that song rate recorded during 1 h observation session of each male was a reliable estimate of its song rate, relative to the other males, during that day.

In the remainder of this paper we will refer to “breeding stage” as to the difference, in days, between calendar date and calendar date of laying of the first egg by the mate of any given male. Hence, for example, negative values of breeding stage indicate the days preceding egg-laying. We used daily song rates for each male to calculate mean individual song rate (hereafter, simply “song rate”) during the period spanning from breeding stage $=-12$ to the day of laying of penultimate egg (i.e., to breeding stage $=2$, 3, 4, or 5 according to clutch size). Mean date of laying of the first egg was 8 May (2.5 days SE)

Song recordings and spectrogram analysis

Song recordings were made by a Sony TCD-D7 DAT tape recorder connected to an ARK C451 shotgun microphone with a CK 9 hypercardioid capsule. Recordings were analyzed by Avisoft Sonograph (Specht, 1993) computer program. The best resolution was achieved by analyzing songs in the 0-10 kHz frequency range, with a sampling rate of 20,000 samples/s, bandwidth 200 Hz, frequency resolution 80 Hz, time resolution 32 ms.

For each singing bout or song we measured the following variables: (1) number of songs in a bout; (2) number of complete songs i.e. songs including the final rattle in a bout; (3) number of syllables in a song; (4) number of types of syllables in a song; (5) mean peak amplitude frequency computed across syllables; (6) peak amplitude frequency of the rattle; and (7) number of impulses in the rattle. In our correlation analyses each individual was represented by mean values of these variables computed across songs. We also estimated the total repertoire of syllables sung by each male. However, the number of songs recorded varied among males (range 3-15; mean ± SE: 7.58 ± 0.56) and our estimate of syllable repertoire size thus might be affected by the number of songs sampled. Hence, for individuals for which more than eight songs were available, we estimated the minimum number of songs needed to score the same total repertoire size as in the whole set of songs. We found that after four songs had been analyzed, 97% of the syllables were recorded. We assumed that our estimates of total syllable repertoire size were reliable for the 32 individuals for which four or more songs have been recorded. The remaining individual has been excluded from the analyses.

Statistical analyses

Almost all (98%) of the males in our colony were recaptured one or more times. Hence, blood was taken and body mass measurement was made more than once. In all the analyses reported in this paper, for each male we considered only the measure of hematological variables and body condition recorded in the interval spanning from 12 days before laying of the first egg by its mate to the day of laying of the penultimate egg in the first clutch and closest to the date of laying of the first egg. Mean breeding stage at blood sampling was $-2.3$ (0.88 SE). This allowed us to control for the effect of any possible variation of variables in relation to stage in the breeding cycle. To confirm that this procedure was effective in removing the effect of sampling date relative to breeding date we ran simple correlation analyses between stage in the breeding cycle at sampling and condition variables, and none gave statistically significant results ($r$ values always associated to $p$ values larger than .1).

Polynomial regression analysis with a stepwise forward procedure of selection of regressors that had significant partial regression coefficients was used to describe variation of song rates and body mass as functions of stage in the breeding cycle, or, respectively, calendar date. In the analysis of song rate as function of breeding stage, for any given breeding stage only the song rate of one randomly chosen male was considered. This procedure ensured that data points subjected to polynomial regression analysis were independent because any given individual was excluded from random resampling of song rates when it had already been considered once. Principal component analysis (PCA) was used to summarize information of song features on few independent variables. Association between each song feature and principal components was assessed by simple Pearson’s correlation coefficients. Sequential Bonferroni correction for multiple tests was used according to the suggestions by Rice (1989). In a set of $k$ simultaneous tests, a given test $i$ was considered to be statistically significant at the .05 level only if it was significant also at the .05/($k - \eta$) level, where $\eta$ is the number of tests, out of the $k$ simultaneous tests, that were found to be significant at a higher level than the $i$-th test. $Values$ of $k$ are reported for all the tests whose results were corrected by sequential Bonferroni procedure. In all statistical analyses we ran, we used the largest sample available.

Mean values of the hematological variables measured are reported in Table 1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean SE</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte conc. (No./10,000 blood cells)</td>
<td>5.45 1.76</td>
<td>42</td>
</tr>
<tr>
<td>Monocyte conc. (No./10,000 blood cells)</td>
<td>0.5 0.15</td>
<td>42</td>
</tr>
<tr>
<td>Heterophil conc. (No./10,000 blood cells)</td>
<td>0.8 0.15</td>
<td>42</td>
</tr>
<tr>
<td>Basophil conc. (No./10,000 blood cells)</td>
<td>2.5 0.41</td>
<td>42</td>
</tr>
<tr>
<td>Eosinophils (No./10,000 blood cells)</td>
<td>0.18 0.04</td>
<td>46</td>
</tr>
<tr>
<td>Gamma-globulins*</td>
<td>0.60 0.28</td>
<td>42</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>0.26 0.42</td>
<td>42</td>
</tr>
<tr>
<td>Blood cell sedimentation rate*</td>
<td>0.85 0.08</td>
<td>34</td>
</tr>
</tbody>
</table>

Measurement units for variables indicated with an asterisk are described in the Methods.
### Table 2

Mean values of the variables used to describe song features and variation among the 32 individuals considered in this study.

<table>
<thead>
<tr>
<th>Song feature</th>
<th>Mean (SE)</th>
<th>F value</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Songs/bout</td>
<td>2.90 (0.13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete songs/bout</td>
<td>2.57 (0.11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syllables/song</td>
<td>19.78 (0.24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Types of syllables/song</td>
<td>15.91 (0.09)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean peak amplitude freq. (kHz)</td>
<td>3.83 (0.03)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rattle peak amplitude freq. (kHz)</td>
<td>6.11 (0.02)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Impulses/rattle</td>
<td>14.42 (0.10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repertoire of syllables</td>
<td>19.64 (0.29)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### RESULTS

Male barn swallows are continuous singers. The twittering songs we recorded consisted of a series of 9 to 34 syllables of up to 27 different types, ending with a characteristic rattle (i.e., a series of impulses at comparatively high frequency resulting in a harsh sound). Mean song duration was 4.01 (0.05 SE). The final part of each song, as far as the type of the last seven syllables (including the rattle) and their temporal sequence are concerned, is highly stereotyped and consistent among individuals. In the first part of each song the sequence of syllables is variable both between songs of the same individual and among individuals. Songs are arranged in bouts each containing few distinct songs, separated from other bouts by spells lasting from approximately one second to tens of minutes in which individuals either are silent or produce chittering calls. Sometimes, song is interrupted before the final rattle. A significant variation of the song variables we measured was observed among individuals except for mean peak amplitude frequency (Table 2).

Mean song rates of individual males showed a clear pattern of variation in relation to the breeding stage (Figure 1). The best-fit regression model of song rates of randomly selected males (see "Statistical analyses") on polynomial terms of breeding stage that we obtained was: song rate = 8.196 -

### Table 3

Simple correlation coefficients between song rate (mean percentage of observations every second minute in which an individual was singing) and concentration (cells/10,000 red blood cells) of five leukocyte types, gamma-globulins to plasma proteins ratio, blood cell sedimentation rate, hematocrit, and body condition (residuals of body mass on calendar day/breeding cycle²).

<table>
<thead>
<tr>
<th>Song rate</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>42</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Monocytes</td>
<td>42</td>
<td>ns</td>
</tr>
<tr>
<td>Heterophils</td>
<td>42</td>
<td>ns</td>
</tr>
<tr>
<td>Basophils</td>
<td>42</td>
<td>ns</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>42</td>
<td>ns</td>
</tr>
<tr>
<td>Gamma-globulins</td>
<td>36</td>
<td>&lt;.1</td>
</tr>
<tr>
<td>Blood cell sedimentation rate</td>
<td>42</td>
<td>ns</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>42</td>
<td>ns</td>
</tr>
<tr>
<td>Body condition</td>
<td>42</td>
<td>ns</td>
</tr>
</tbody>
</table>

Probability values are corrected according to sequential Bonferroni procedure (k = 9). Superscript 'a' indicates Spearman correlation coefficient. All the other values are Pearson's correlation coefficient. The correlation coefficient between gamma-globulins to plasma proteins ratio and song rate was significant after controlling for the effect of breeding date (see RESULTS).

0.427 X (stage in breed, cycle) - 0.058 X (stage in breed, cycle)² (F = 4.90; df = 2.27; p = .015, r² = .27). This indicated that mean song rate reached the absolute maximum approximately four days before the day of laying of the first egg.

### Song rate, hematological profile, and body condition

The results partly confirmed our predictions about the existence and the signs of the correlations between song rate and hematological variables. Indeed, song rate was negatively correlated with the concentration of lymphocytes (Table 3; Figure 2). A strong, though not statistically significant, negative correlation was also observed between song rate and gamma-globulins to plasma proteins ratio (Table 3). However, no significant correlations were observed for the other hematological variables nor body condition (Table 3).

Song rate did not change with breeding date as indexed by laying date (r = .01, n = 42, ns). Similarly, hematological...
variables (see Table 3) were not significantly correlated with breeding date. However, the unsigned value of some of the correlation coefficients was rather high (values ranging from .39 to —.25). To correct for the potentially confounding effect of breeding date we therefore ran correlation analysis between song rate and hematological and body condition variables while partialing out the effect of hatching date. The results confirmed the significant negative correlation of concentration of lymphocytes with song rate ($r_{pb} = -4.3$, $n = 42$, $k = 9$, $p < .05$) and also showed a significant negative correlation between song rate and gamma-globulins to plasma proteins ratio ($r_{pb} = -4.8$, $n = 56$, $k = 9$, $p < .05$).

We found no significant correlations between body condition and each of the hematological variables ($r$ values ranging from —.08 to .57; correlation with monocyte concentration: $r_{pb} = .90$, $k = 8$).

Song features, hematological profile, and body condition

The variables describing song features showed intercorrelation (Table 4; see also Galeotti et al., in press). Syllable repertoire, and the number and types of syllables in each song were strongly and, positively correlated among themselves. Moreover, they also showed some association with peak amplitude frequency and number of impulses in the rattle (Table 4). The number of songs per bout was closely and positively correlated with the number of complete songs per bout. These correlations prompted us to reduce the dimensionality of the song data by PCA. The component associated to the largest eigenvalue accounted for 35.6% of the variance in the song data set while the second and the third principal components explained 24.2% and 14.6%, respectively, of the variance. The first principal component (PCI) represented a vector of increasing total repertoire of syllables sung by each male, number and types of syllables sung in each song, and of decreasing number of impulses in the rattle (Table 5). High values of the second principal component were associated to song bouts including many and complete songs (Table 5). The third principal component (PC3) mainly represented a vector of increasing peak amplitude frequency of the song. Variance in rattle peak amplitude frequency was partly accounted by PCI and partly by PC3. Since these principal components accounted for almost three-quarters of the variability in song features, they were used in the analysis of song in

Table 4
Pairwise simple correlation coefficients between song features

<table>
<thead>
<tr>
<th></th>
<th>CSB SS</th>
<th>TSS</th>
<th>RE</th>
<th>IR</th>
<th>RPAF</th>
<th>SPAF*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Songs/bout</td>
<td>.97</td>
<td>—.01</td>
<td>—.16</td>
<td>—.01</td>
<td>.06</td>
<td>.23</td>
</tr>
<tr>
<td>Complete songs/bout (CSB)</td>
<td>—.04</td>
<td>—.16</td>
<td>—.03</td>
<td>.02</td>
<td>.18</td>
<td>.04</td>
</tr>
<tr>
<td>Syllables/song (SS)</td>
<td>.80</td>
<td>.50</td>
<td>—.40</td>
<td>—.41</td>
<td>—.02</td>
<td></td>
</tr>
<tr>
<td>Types of syllables/song (TSS)</td>
<td>.65</td>
<td>—.42</td>
<td>—.31</td>
<td>—.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repertoire of syllables (RE)</td>
<td>—.31</td>
<td>—.10</td>
<td>.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Impulses/rattle (IR)</td>
<td>.55</td>
<td>—.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rattle peak amplitude frequency (RPAF)</td>
<td>—.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sample size is 32 for all correlations.

* Song peak amplitude frequency.

Table 5
Simple correlation coefficients between song features and the first three principal components

<table>
<thead>
<tr>
<th></th>
<th>PCI</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Songs/bout</td>
<td>.56***</td>
<td>.90***</td>
<td>.05</td>
</tr>
<tr>
<td>Complete songs/bout</td>
<td>—.86**</td>
<td>.91***</td>
<td>.13</td>
</tr>
<tr>
<td>Syllables/song</td>
<td>.92****</td>
<td>.30</td>
<td>—.09</td>
</tr>
<tr>
<td>Types of syllables/song</td>
<td>.88****</td>
<td>.19</td>
<td>—.21</td>
</tr>
<tr>
<td>Repertoire of syllables</td>
<td>.67****</td>
<td>.30</td>
<td>—.28</td>
</tr>
<tr>
<td>Impulses/rattle</td>
<td>—.61***</td>
<td>—.19</td>
<td>—.24</td>
</tr>
<tr>
<td>Rattle peak amplitude frequency</td>
<td>—.56***</td>
<td>.12</td>
<td>.55***</td>
</tr>
<tr>
<td>Song peak amplitude frequency</td>
<td>.07</td>
<td>.00</td>
<td>.83***</td>
</tr>
</tbody>
</table>

The proportion of variance in the song data set accounted by these components was 0.74.

* $p < .05$; *** $p < .005$; **** $p < .001$. 
It is usually accepted that song in male passerines serves the function of mate attraction and stimulation, and territory defense from intrusion of other males (e.g., Seacy and Andersen, 1986). The change in song rates during the breeding cycle observed in this and previous studies is consistent with both these general functions of intra- and intersexual signals. Male barn swallows may sing more during territory establishment and female fertile period to defend a pre-existing nest or a nesting site. High song rates could have the function of mate attraction and stimulation of ovarian development and copulation behavior by the mate (Brockway, 1965; King and West, 1977). Extra-pair fertilizations are frequent in the barn swallow colonies in our study area since approximately 30% of the nestlings are sired by an extra-pair male (Saino et al., 1997). High song rates during the fertile period of the female might enhance certainty of paternity in their own broods by "reinforcing" female choice of own mate as copulation partner. Finally, song production during the incubation period might be directed to attract and solicit copulations by extra-pair females.

Song rate was inversely related to peripheral blood lymphocyte concentration and the ratio of gamma-globulins to total plasma proteins, and this relationship was not biased by timing of blood sampling relative to recording of song rate. To our knowledge, this is the first study in which such a correlation between singing performance and hematological variables has been shown. Little is known about immune function in passerine birds. In some avian taxa, a typical response to infectious disease is an increase in lymphocyte and heterophil concentrations (Averbeck, 1992; Davis, 1981; Hawkey et al., 1983; Rose et al., 1979). Gamma-globulins are a class of plasma proteins that includes antibodies involved in immune response to pathogens (e.g., DeVaney and Augustine, 1988; Jayawardene and Spradbrow, 1995; Russell and Ezell, 1995). Hence, these results suggest that song might convey to the receiver information about the health and immune status of the signaler, which in turn possibly reflects current or previous level of infection by a variety of pathogens.

The existence of causal relationships, either direct or indirect, between immunological condition and song output and the mechanisms underlying these relationships are unknown. One possible interpretation of our results is that pathogens that elicit an immune response can also directly depress singing activity. An alternative explanation is based on energetic considerations about the competition for a limited amount of resources by the immune system and singing activity. Song production is considered as a costly activity in terms of energetic requirements (Eberhardt, 1984; Santee and Bakken, 1987; Vehrencamp et al., 1989). Indirect costs of song are also presumably high because song production competes with other activities such as foraging. The costs of immune defense are largely unknown (but see König and Schmid-Hempel, 1995). In any case, cellular proliferation and biosynthesis of antibodies can be assumed to be costly for simple thermodynamic reasons. This line of reasoning leads us to suggest that male barn swallows trade the socio-sexual benefits of high song rates, if any, against the cost of immune defense and that males that mount an intense immune defense cannot allocate much energy resources to song production.

Since the paper by Folstad and Karner (1992) has been published, the mechanisms controlling the expression of male secondary sexual characters relevant to mate-male competition or female attraction have been suggested to consist of a network of feedback interactions between sexual hormones, immunity, and parasite infestations. The proponents of this so-called "immunocompetence hypothesis" have mainly emphasized the role that androgens play in promoting the expression of morphological traits relevant in socio-sexual contexts in vertebrates. For birds it has been shown that there is a "dual function" of male attraction and stimulation, and territory defense from intrusion of other males (e.g., Seacy and Andersen, 1986). The change in song rates during the breeding cycle observed in this and previous studies is consistent with both these general functions of intra- and intersexual signals. Male barn swallows may sing more during territory establishment and female fertile period to defend a pre-existing nest or a nesting site. High song rates could have the function of mate attraction and stimulation of ovarian development and copulation behavior by the mate (Brockway, 1965; King and West, 1977). Extra-pair fertilizations are frequent in the barn swallow colonies in our study area since approximately 30% of the nestlings are sired by an extra-pair male (Saino et al., 1997). High song rates during the fertile period of the female might enhance certainty of paternity in their own broods by "reinforcing" female choice of own mate as copulation partner. Finally, song production during the incubation period might be directed to attract and solicit copulations by extra-pair females.
little evidence for a direct effect of androgens on feather ornaments development (Owens and Short, 1994). In male oscine birds the onset of male sexual behavior, including song production, is dependent on change in androgens profile during the breeding season. Hence, song rate and song features are more likely candidates for the immunocompetence hypothesis as male secondary sexual characters under the assumption that singing performance is to some extent correlated with the levels of circulating androgens (Johnsen, 1991, in Ketterson and Nolan, 1992). Consistent with the predictions of the immunocompetence hypothesis, we showed that song rate is inversely related to leukocyte and antibody levels. However, we could find only poor correlations between song rate and plasma testosterone levels (Galeotti et al., in press; see also Saino and Møller, 1995). Therefore, this study only partly supports the idea that the mechanisms envisaged by the immunocompetence hypothesis control singing performance in male barn swallows.

What is the importance of this study for current ideas about sexual selection and the evolution of song? Under the assumption of an inter-sexual function of song, females may exploit song rate as a cue to assess signaler quality. Three hypotheses for a female preference can be proposed: (1) females may acquire direct fitness benefits if pathogens are horizontally transmitted and their presence elevates hematological variables and suppress song production; (2) females may acquire indirect fitness benefits accruing from a genetically based resistance to infectious diseases (Hamilton and Zuk, 1982). Infestation of parasitic mite Ornithonyssus bursa, for example, negatively influences singing performance (Møller, 1991) and has a component of additive genetic variation (Møller, 1990); and (3) females may acquire healthy and vigorous males that will provide ample parental cares.

In conclusion, we suggest that individual song rate is associated with hematological parameters and, hence, health status of male barn swallows. Song may thus be exploited by females as a reliable cue to assess signaler quality. The potential benefits arising from female preference for males singing at high rates range from low exposure to infectious disease and acquisition of males with partial resistance to pathogens, to effective parental care provided by healthy males.

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