

Immune challenge mediates vocal communication in a passerine bird: an experiment

László Zsolt Garamszegi,^{a,b,c} Anders Pape Møller,^b János Török,^c Gábor Michl,^c Péter Péczely,^d and Murielle Richard^e

^aDepartment of Biology, University of Antwerp, Antwerp, Belgium, ^bLaboratoire de Parasitologie Evolutive, Université Pierre et Marie Curie, Paris, France, ^cBehavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös University, Budapest, Hungary, ^dDepartment of Reproductive Biology, Szent István University, Gödöllő, Hungary, and ^eLaboratoire d'Ecologie, Université Pierre et Marie Curie, Paris, France

Secondary sexual characters may have evolved in part to signal resistance to parasites. Avian song has been hypothesized to be involved in this process, but the role of parasites in modulating acoustic communication systems in birds remains largely unknown, owing to lack of experiments. We studied the relationship between parasitism, testosterone, song performance, and mating success in male collared flycatchers (*Ficedula albicollis*) by experimentally challenging their immune system with a novel antigen. We predicted that a challenge of the immune system would reduce song performance, and that this reduction would be conditional on the size of a visual sexual signal, the forehead patch that was previously found to reflect resistance. An antagonistic linkage between testosterone and immune function would predict that a challenge of the immune system should suppress testosterone level. An immunological treatment by sheep red blood cells (SRBCs) triggered a decrease in body mass, testosterone level, and song rate, but other song traits were not significantly affected by the antigen challenge. Initial testosterone level was associated with forehead patch size and all song traits except song rate. SRBC injection caused stronger reduction in song rate among males with smaller forehead patches, and the change in song rate was also predictable by song features such as strophe complexity and length. We show that song rate and other song characteristics may be important cues in male-male competition and female choice. These results suggest that parasite-mediated sexual selection has contributed in shaping a complex acoustic communication system in the collared flycatcher, and that testosterone may play an important role in this process. Parasitism may drive a multiple signaling mechanism involving acoustic and visual traits with different signal function. *Key words*: bird song, collared flycatcher, immunocompetence, parasites, secondary sexual characters, testosterone. [*Behav Ecol* 15:148–157 (2004)]

Bird song is one of the best-known animal signals, and it has been extensively studied as a model of sexual selection (Catchpole and Slater, 1995; Darwin, 1871; Read and Weary, 1992; Searcy and Andersson, 1986). When Hamilton and Zuk (1982) recognized the importance of parasites in sexual selection, they claimed that parasitism played an important role in the evolution of bird song, and that this trait has evolved at least partly to reveal health status of males. If this is the case, then selection should have favored receivers that were able to recognize males having a superior ability to cope with parasites by discriminating between healthy and ill males. If song is to mediate female choice of parasite-free males, then parasitism must (1) act as a constraint on the signaling mechanisms of senders, (2) shape song attributes, and (3) affect the reactions of the receivers (Figure 1).

Folstad and Karter (1992), in their immunocompetence hypothesis, proposed one way in which parasitism can constrain signaling mechanisms of senders. The hypothesis suggests that a physiological trade-off exists between steroid production and immune function. Individual variation in circulating testosterone level has been found to be associated with song (Balthazart, 1983; Catchpole and Slater, 1995; DeVoogt, 1991; Schlinger, 1997). Testosterone may also

compromise immune function (Casto et al., 2001; Lindström et al., 2001; Peters, 2000; Saino et al., 1995; Zuk et al., 1995), but this assumption has recently been questioned in some studies as it has not been supported by empirical evidence (Buttemer and Astheimer, 2000; Braude et al., 1999; Hasselquist et al., 1999). If an association between testosterone production and immune function exists, this could provide a mechanism underlying the cost, and hence the reliability, of sexually selected signals. Only high-quality males should be able to invest resources in sexual displays while maintaining adequate immune functioning (Folstad and Karter, 1992; Wedekind and Folstad, 1994).

Avian song is known to be a multifaceted trait consisting of numerous structural parameters encoding different kinds of information (Gil and Gahr, 2002; Vehrencamp, 2000). Based on constraints that determine signal reliability, various song parameters can be assigned to different signal types, thereby affecting signal design and the information being conveyed. Because of the potentially different biological background of each song component, variation in different song traits may be caused by diverse selective pressures, with parasitism differentially affecting the evolution of different song components.

A real communication system requires the reaction of signal receivers (Figure 1). As bird song operates to attract mates and repel competitors from territories (Searcy and Andersson, 1986), females and also males can be regarded as potential signal receivers. Hence, signals of health may be important in both mate choice and male-male competition. Females may

Address correspondence to L.Z. Garamszegi. E-mail: laszlo.garamszegi@ua.ac.be.

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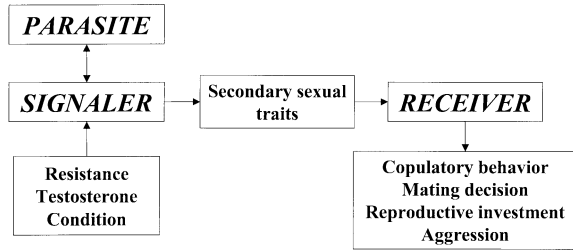


Figure 1

Interactions involved in parasite-mediated communication in birds. Immediate condition, testosterone, parasite resistance, and other undefined factors (data not shown) will determine the reaction of an individual host to a parasite infection by the activity of the immune system. Because of immunocompetence mechanisms acting through testosterone (Folstad and Karter, 1992) and because of their effects on body condition, parasites will affect the expression of sexually selected traits such as bird song. Resistance may involve inherited abilities to cope with pathogens. Signal receivers will adjust their behavioral responses according to the information perceived from sexual signals. For simplicity only the main paths are shown, although components can be interrelated.

benefit by choosing a parasite-free mate in terms of protection against direct transmission of parasites, receiving paternal help of superior quality, and acquiring resistance genes for their offspring (Møller et al., 1999). Parasitism could also influence the outcome of competition among males, if song features reflecting immune quality are also used by other males to assess the likelihood of success of a challenge. Alternatively, if a signal contains information about current health status, males may avoid social contacts by not fighting with less ornamented, ill individuals, owing to the risk of horizontal transmission of parasites.

Little is known about how parasitism constrains signal production of senders, how it favors different signal types, and how it affects the behavior of receivers. Students of avian song in relation to parasitism often observe an association between parasite burden and song structure both at intraspecific and interspecific levels (Buchanan et al., 1999; Duffy and Ball, 2002; Garamszegi et al., 2003; Møller, 1991; Møller et al., 2000; Redpath et al., 2000; Saino et al., 1997; see also Birkhead et al., 1998; Weatherhead et al. 1993), but because of lack of experiments manipulating health status, the exact mechanism that generates a relationship between the expression of secondary sexual characters, testosterone, and parasite load remains undetermined (Møller et al., 1999). A typical problem encountered in studies aiming to explore the effect of parasitism on song traits is their narrow focus in terms of both secondary sexual traits and parasites considered (Clayton, 1991; Weatherhead et al., 1993). Because organisms may host many parasite species, and only a small fraction of them are debilitating, it is relatively unlikely that a randomly chosen parasite species will have important fitness consequences for its host (Møller et al. 1999). Thus, the probable effect of a single parasite on trait expression of the host will be small, and the use of measures of general antiparasite defense should be superior to the use of data on the abundance of any particular parasite. However, studies estimating health status by using measures of immune function are rare (Duffy and Ball, 2002; Saino et al., 1997). In addition, the reaction of potential signal receivers to acoustic cues providing information about parasitism has never been investigated.

Our aim in this article was to study experimentally the potential self-inflicted immunological impact of parasitism on the complex vocal communication system in the collared flycatcher (*Ficedula albicollis*). We surveyed a broad range of

song features and considered participants involved in sending, receiving, and reacting to song. We investigated the relationship between male quality, condition, immune status, testosterone production, and trait expression following a repeated-measures design in which we recorded male song, testosterone level, and body mass before and after challenging the immune system with a novel antigen (sheep red blood cells, SRBCs). This challenge stimulates the production of large numbers of B cell-dependent antibodies during subsequent days, causing a reduction in body condition similar to that during a real infection (Hay and Hudson, 1989). However, SRBCs differ from a real pathogen in that they do not replicate. Previous studies in birds have demonstrated that the activation of the immune system by SRBCs alters metabolic activity, leukocyte profile, and plasma protein content, thus increasing heat production and protein turnover and generating fever (Fair et al., 1999; Klasing and Austic, 1984; Ots et al., 2001; Siegel et al., 1982). Individuals can vary in their ability to raise antibody responses against SRBCs, and this variation has been found to relate to genetic quality, reproductive effort, and circulating testosterone level (Cichon et al., 2001; Deerenberg et al., 1997; Ots et al., 2001; Peters, 2000; Roulin et al., 2000). Based on this line of evidence, we assume throughout this article that with SRBC injection, we simulated pathological effects comparable with those of parasite infection. We predicted that a challenge of the immune system would reduce song performance, and that this reduction would be dependent on the size of the forehead patch of males, with stronger effects on individuals with small patches. This trait has been shown to be a reliable signal of male quality (Gustafsson et al., 1995), and a recent study showed that it may signal individual ability to cope with parasite infection, as the antibody response to an injection of Newcastle disease virus was related to patch size (Andersson, 2001). Forehead patch size is known to play important roles in male-male competition and in female choice (Qvarnström et al., 2000; Sheldon and Ellegren, 1999). The immunocompetence handicap hypothesis suggests that circulating testosterone is associated with immune function; thus, we predicted a reduction in testosterone level owing to challenge of the immune system. If signal receivers use song cues indicating health, measures reflecting success in male-male competition or mate attraction should be associated with song traits conveying information about immune status.

METHODS

Experimental protocol

We carried out fieldwork at Pilis Field Station (47°43' N, 19°01' E) in the Pilis Mountains near Budapest, Hungary, from 1999–2001. A detailed description of the study site, the breeding population and the established banding protocol is given elsewhere (Garamszegi et al., 2004; Michl et al., 2000; Török and Tóth, 1999). At the study site, collared flycatchers nest in boxes positioned in the forest. Males sing at high rates before pairing.

Based on coin toss, we randomly selected unpaired males to receive intraperitoneal placebo injection ($n = 33$, 100 μ l sterile phosphate-buffered saline [PBS]) or SRBCs ($n = 36$, approximately 5×10^7 SRBCs in 100 μ l sterile PBS) in 1999–2001. In 1999 and 2000, males' songs were recorded before injection with a Sony TCD-D8 DAT tape recorder connected to a MD 21 U microphone attached to a parabolic antenna. Recordings were made during the most active singing period (usually 0500–1000 h, but no later than 1200 h) and only in relatively good weather conditions. For each male we obtained a record of at least 10 min duration in order to obtain a sample containing sufficient songs, with minimal background noise,

for further analyses. Birds were trapped within 1 h after recording, and their body mass (to the nearest 0.1 g with a Pesola spring balance), tarsus length, and width and height of the forehead patch (to the nearest 0.1 mm with a caliper) were measured. Forehead patch size was then estimated as patch width \times height. For identification, all birds received individual color markings with water-resistant pens on their belly plumage. We collected fecal samples to determine circulating testosterone level before capture ($n = 19$), thereby avoiding any increase owing to handling (Cockrem and Rounce, 1994). We assume that fecal testosterone levels reflect the circulating hormone concentration at the time of song recordings. Fecal samples were weighed on a balance to the nearest 0.01 g (wet weight) and stored in methanol for later analyses. To analyze levels of fecal testosterone, we used a radio-immune assay (RIA) technique following the method of Jallageas (1975) and Hirschenhauser et al. (2000). Intra- and interassay repeatability was 8–12% and 9–15%, respectively. Testosterone was expressed in picograms per gram of wet feces.

Two or three days after immune treatment, we searched for experimentally treated birds that had settled down at their previous or a new nest-box. If a previously injected and recorded bird was found, we recorded his song again. We recorded only unpaired males: if a female was observed nearby during recording, the male was excluded from subsequent analyses. After recording, if capture was possible, we measured body mass ($n = 15$) and took a fecal sample for testosterone analysis ($n = 13$) as described above (we intended to keep disturbance to a minimum, and hence, not all rerecorded birds were captured a second time, limiting our sample size). In our 3 years of study, of the 69 injected males, 38 individuals became successful breeders (1999: 16 of 26 injected males bred; 2000: 16 of 29 injected males bred; 2001: 6 of 14 injected males bred). Blood samples were taken from nestlings and adults, permitting successful paternity analyses at 36 nests. We assessed paternity by using four unlinked microsatellite loci (FhU2–4 [Ellegren, 1992; Primmer et al., 1996] and PdO μ 5 [Griffith et al. 1999]). By using GeneScan Analysis Software (PE Applied Biosystems), we determined individual genotypes and, by assuming Mendelian inheritance, we classified offspring as extrapair young if they showed genotype mismatch with their father. The complete PCR protocol is available from the authors on request.

Song analysis

The song of the collared flycatcher consists of simple calls and complex strophes containing variable song elements, or so-called figures (Garamszegi et al., 2002; Gelter, 1987) (Figure 2). We estimated song rate for each male as the number of strophes per minute based on 10-min recordings. We selected strophes with minimal background noise for further sonographic analyses, which were performed by using the Signal 3.10 computer software. The song analysis was done blindly with respect to the experimental treatment. We used 14.11 ± 0.65 strophes (mean \pm SE) per recording to describe strophe characteristics. The following variables were measured: strophe length in seconds, frequency minimum, frequency maximum, frequency range, number of figures, versatility (number of unique figures), complexity (number of unique figures/number of figures), and tempo (number of figures/second). One-way ANOVAs showed that, within recordings, song parameters varied significantly among individuals (all $p < .0013$). Although the repeatabilities within recordings were not very high ($r = .331$ – $.618$), they were comparable with those obtained for similar sexually selected song traits in other species (Forstmeier et al., 2002; Gil and Slater, 2000). Hence, we used averages for each variable.

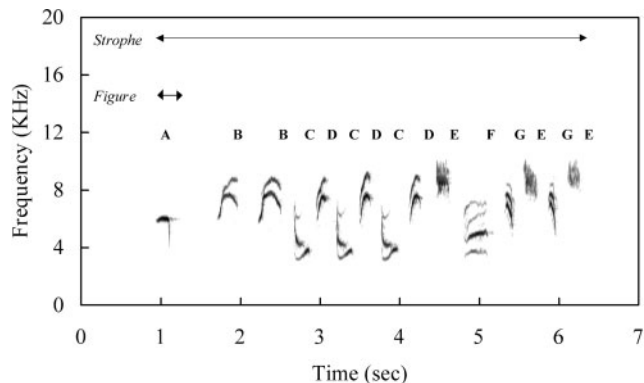


Figure 2
The spectrographic presentation of a collared flycatcher song (strophe) composed of A through E.

To detect potential changes in song traits owing to different treatments, we expressed the difference in each song variable between “before” and “after” treatment recordings. We used a capture-recapture approach to estimate individual repertoire size by controlling for heterogeneous figure detection probability, and to detect compositional changes related to experimental treatment (Garamszegi et al., 2002). Beside repertoire size, we estimated the rate of extinction of figures (proportion of figures lost), turnover rate (proportion of new figures), and change in overall repertoire after treatment. The correlation matrix for song features is given in Table 1. Song rate, strophe characteristic, and repertoire composition were handled separately, because these variables probably reflect different signal types (Vehrencamp, 2000).

Statistical analyses

Variables were checked for normality and homoscedasticity. When necessary, statistical transformation was applied in order to meet the parametric criteria. To test for the effects of categorical variables and continuous predictor variables in designs with a single dependent variable, we used generalized linear regression models. In each analysis we included year effects, because there may have been effects of different sample sizes in different treatment groups in different years. Unless otherwise stated, we built effects to be entered in the between design using the full factorial combination of all categorical and continuous predictor variables. A stepwise analysis based on backward deletion procedure allowed us to simplify most of the models by removing nonsignificant ($p > .05$) effects and interactions. Only the resulting models are presented. In cases in which no factor was significant in the global model, only the factor of interest is presented. To reduce the number of the statistical tests performed with multiple song features, we applied multiple regression, discriminant analysis, and principal component analysis. The component loadings and the explained variance for the principal component axes calculated for the entire sample, including recordings before and after treatment, are presented in Table 2. To detect potential changes in strophe characteristics after different treatments, we also calculated Δ PCs, which express the changes in each song variable in the principal component analysis. All statistics were performed by using Statistica 5.5 (StatSoft).

In those years when we collected song data, we recorded 21 placebo and 28 SRBC birds before injecting them, and rerecorded 12 placebo males and 15 SRBC males (Yates corrected $\chi^2 = 0.010$, $p = .814$). To test whether our recaptured sample was biased, we compared capture date and morpho-

Table 1
Correlations among song traits of collared flycatcher males before experimental treatment

	REP	RATE	NFIG	VERS	COMP	LEN	TEMP	FRAN	FMIN	FMAX
REP	1.000									
RATE	0.130	1.000								
NFIG	0.131	0.096	1.000							
VERS	0.281	0.127	0.836	1.000						
COMP	0.054	-0.047	-0.462	0.058	1.000					
LEN	0.081	0.006	0.934	0.719	-0.503	1.000				
TEMP	0.174	0.173	0.606	0.669	-0.063	0.307	1.000			
FRAN	0.239	-0.012	0.507	0.524	0.007	0.505	0.279	1.000		
FMIN	-0.498	0.071	-0.299	-0.396	-0.107	-0.256	-0.253	-0.681	1.000	
FMAX	-0.095	0.040	0.453	0.401	-0.074	0.484	0.181	0.824	-0.146	1.000

$n = 35$. REP indicates repertoire size; RATE, song rate; NFIG, number of figures per strophes; VERS, strophe versatility; COMP, strophe complexity; LEN, strophe length; TEMP, tempo; FRAN, frequency range; FMIN, frequency minimum; and FMAX, frequency maximum. Significant correlations after Bonferroni correction are highlighted ($p < .001$).

logical variables of recaptured birds with those individuals that we were unable to recapture, while controlling for potential year effects. None of the comparisons were significant (capture date: $F_{1,47} = 1.100, p = .296$; forehead patch size: $F_{1,47} = 0.016, p = 0.900$; tarsus length: $F_{1,47} = 0.020, p = .899$; body mass: $F_{1,47} = 1.560, p = .218$). When we entered pretreatment song variables into a stepwise discriminant analysis with recapture event as a grouping variable, we found that none of the song traits was related to recapture probability (all partial $\lambda > 0.981, F_{1,33} < 0.628, p > .434$).

We also found no significant difference between experimental and control groups in their pretreatment traits (capture date: $F_{1,47} = 0.100, p = .779$; forehead patch size: $F_{1,47} = 2.046, p = .159$; tarsus length: $F_{1,47} = 0.410, p = .525$; body mass: $F_{1,47} = 0.010, p = .966$). A stepwise discriminant analysis with song variables also revealed nonsignificant relationships between experimental treatment effects and pretreatment song features (all partial $\lambda > 0.978, F_{1,33} < 0.728, p > .399$).

Although in the closely related pied flycatcher (*Ficedula hypoleuca*) it has been found that certain song features are related to the time of day, relative date, and age (Gottlander, 1987; Lampe and Espmark, 1994), we used song variables without controlling for these potentially confounding effects,

Table 2
Component loadings for principal component analysis on strophe characteristics calculated for the entire sample, including recordings made before and after immune challenge, and for the changes in each strophe variable after different treatments

Song feature	Entire sample ($n = 35$)		Changes ($n = 27$)	
	PC1	PC2	Δ PC1	Δ PC2
NFIG	-0.907	-0.315	-0.899	-0.292
VERS	-0.870	0.163	-0.921	0.080
COMP	0.266	0.872	-0.157	0.869
LEN	-0.802	-0.527	-0.866	-0.155
TEMP	-0.572	0.365	-0.393	-0.511
FRAN	-0.819	0.322	-0.870	0.202
FMIN	0.554	-0.411	0.589	-0.061
FMAX	-0.696	0.141	-0.756	0.233
Explained variance	51.04%	19.86%	53.25%	15.40%

The most important loadings are highlighted (values larger than 0.50 or smaller than -0.50). Sample sizes and the amount of variance accounted for by the two principal component axes are given. See abbreviations in Table 1.

because in our sample none of the song variables correlated significantly with these variables (stepwise multiple regressions: time of the day, $F_{2,32} = 1.079, p = .352$, no significant β in the selected model; capture date: $F_{4,30} = 1.504, p = .226$, no significant β in the selected model; age: $F_{0,34} = 0.000, p = 1.000$, all variables were removed).

RESULTS

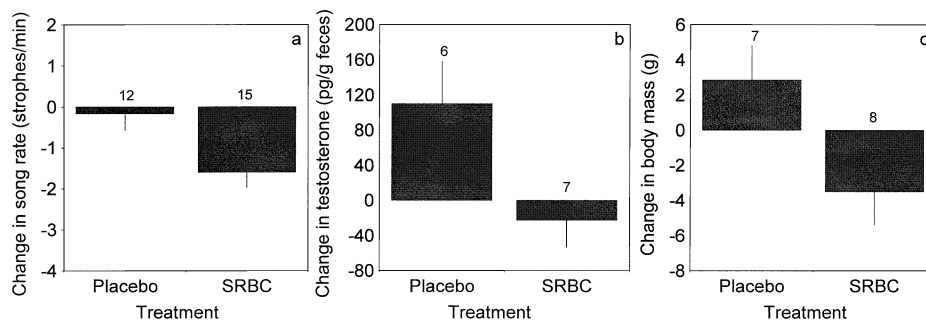
Challenge of the immune system by SRBCs caused a significant reduction in song rate, testosterone level, and body mass compared with levels of the control group (song rate: treatment, $F_{1,25} = 6.764, p = .015$; testosterone: treatment, $F_{1,11} = 5.719, p = .036$; body mass: treatment, $F_{1,13} = 5.435, p = .036$) (Figure 3).

Analyses of different song traits revealed that strophe characteristics and repertoire composition were not significantly affected by immune treatment (Δ PC1 strophe characteristics: treatment, $F_{1,25} = 0.004, p = .949$; Δ PC2 strophe characteristics: treatment, $F_{1,25} = 0.087, p = .771$; figure extinction: treatment, $F_{1,25} = 0.167, p = .686$; turnover rate: treatment, $F_{1,25} = 0.284, p = .599$; rate of change: treatment, $F_{1,25} = 0.017, p = .897$; statistical power > 0.70 for all tests, with effect size [D = 1.007] taken from the previous analysis testing treatment effects on song rate changes). Similar patterns emerged when we applied a stepwise discriminant analysis with all song traits measured, because only the change in song rate was related significantly to the experimental treatment (partial $\lambda = 0.787, F_{1,25} = 6.764, p = .015$). An analysis of repeatability of song traits between recordings in SRBC and placebo males indicated that strophe characteristics remained stable independent of experimental treatment (paired t test: $t_7 = 1.360, p = .217$; placebo males: mean \pm SE = 0.420 ± 0.051 , SRBC males: mean \pm SE = 0.351 ± 0.027 ; mean paired difference = 0.069), compared with the repeatability of song rate between recordings in different experimental groups (placebo males = 0.657, SRBC males = 0.087).

Within the experimental group, individuals showed quality-dependent variation in terms of reduction in song rate. The decrease in song rate after SRBC injection was significantly correlated with the size of the forehead patch, as the decrease in song rate was smaller in birds having a larger forehead patch (change in song rate: patch size, $F_{1,13} = 6.982, p = .020$). This relationship was not observed in the placebo group (change in song rate: patch size, $F_{1,10} = 0.102, p = .757$). When we analyzed the relationship between forehead patch size and song rate reduction owing to different treatments in a single analysis, there was a significant interaction between forehead patch size

Figure 3

The effect of immunological treatment in the collared flycatcher on male song rate, testosterone level, and body mass. (a) Mean + SE change in song rate (strophes per minute) after placebo and sheep red blood cell (SRBC) injection. (b) Mean + SE change in fecal testosterone level (picograms per gram feces) after placebo and SRBC treatment. (c) Mean + SE change in body mass (grams) after placebo and SRBC treatment. Sample sizes are given.



and immune treatment (change in song rate: treatment, $F_{1,24} = 6.646$, $p = .017$, treatment \times patch size, $F_{1,24} = 4.434$, $p = .046$, global model, $F_{2,24} = 6.064$, $p = .007$) (Figure 4a).

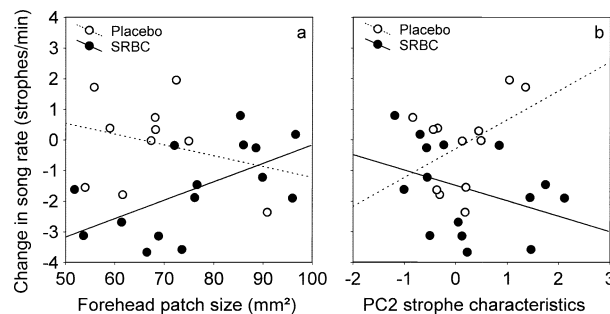
Because strophe characteristics and repertoire size (and composition) appeared to be unaffected by the experimental treatment, in subsequent analyses we used the values of these traits calculated for the entire sample, including before and after treatment recordings. Although song rate was found to be independent of other song traits (Table 1), a stepwise multiple regression with principal components of strophe characteristics, repertoire size, and forehead patch size as independent variables revealed that in SRBC-treated males, the response to an immune challenge in song rate was related to PC2 strophe characteristics (change in song rate: PC2 strophe characteristics, $F_{1,12} = 5.158$, $p = .042$; patch size, $F_{1,12} = 10.801$, $p = .007$; global model, $F_{2,12} = 7.187$, $p = .009$). The same analysis for placebo males resulted in no variables in the selected model (change in song rate: PC2 strophe characteristics, $F_{1,10} = 2.317$, $p = .159$; patch size, $F_{1,10} = 0.103$, $p = .754$). We also tested for an association between the change in song rate and PC2 strophe characteristics by including treatment effects in one analysis, while controlling for year effects. We found significant interaction between PC2 and experimental treatment (change in song rate: treatment, $F_{1,23} = 6.002$, $p = .022$; treatment \times PC2 strophe characteristics, $F_{1,23} = 9.588$, $p = .005$; treatment \times year, $F_{1,23} = 6.373$, $p = .019$, global model: $F_{3,23} = 6.870$, $p = .002$) (Figure 4b). Initial song rate before the experiment was not related significantly to PC2 strophe characteristics ($F_{1,43} = 0.911$, $p = .347$).

We analyzed the relationship between testosterone and trait expression. In a stepwise multiple regression in which we used fecal testosterone level assessed before treatment as the dependent variable and song rate, strophe characteristics, repertoire size, and forehead patch size as independent variables, we found that patch size and all song traits except song rate were significantly associated with fecal testosterone level (\log_{10} fecal testosterone level: PC1 strophe characteristics, $F_{1,13} = 11.417$, $p = .004$; PC2 strophe characteristics, $F_{1,13} = 21.688$, $p < .001$; repertoire size, $F_{1,13} = 27.842$, $p < .001$; patch size, $F_{1,13} = 30.024$, $p < .001$; global model, $F_{4,14} = 17.689$, $p < .001$; song rate [not in the model], $F_{1,17} = 0.001$, $p = .972$). When we controlled for year effects by introducing song traits and forehead patch size together with their interactions with year effects, we found similar results (\log_{10} fecal testosterone level: PC1 strophe characteristics, $F_{1,11} = 29.610$, $p < .001$; PC2 strophe characteristics, $F_{1,11} = 9.373$, $p = .011$; repertoire size, $F_{1,11} = 90.796$, $p < .001$; patch size, $F_{1,11} = 57.214$, $p < .001$; year \times PC2 strophe characteristics, $F_{1,11} = 9.573$, $p = .010$, year \times repertoire size, $F_{1,11} = 9.442$, $p = .011$, year \times patch size, $F_{1,11} = 10.925$, $p = .007$, global model, $F_{7,11} = 34.054$, $p < .001$; song rate [not in the model], $F_{1,17} = 0.001$, $p = .972$). Forehead

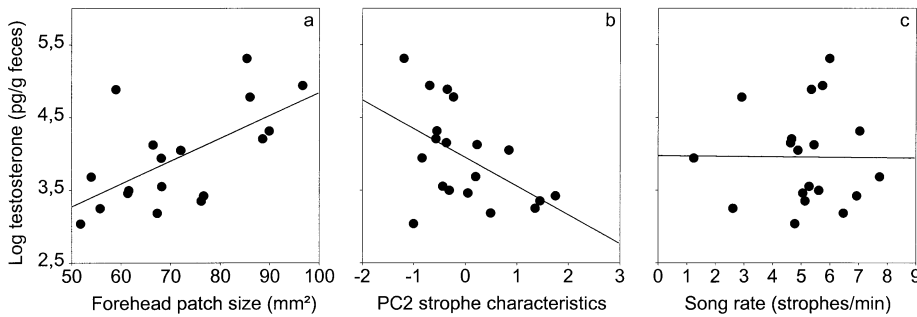
patch size and PC2 strophe characteristics were independently related to testosterone level before the experiment (Figure 5). Because of the limited availability of multiple measurement on fecal testosterone in males with repeated measures on song rate, we did not test for the potential correlation between change in song rate and change in testosterone level while controlling for treatment effects in a single analysis (predicted power with D = 1.007 : 0.31).

We investigated whether song rate, PC2 strophe characteristics, and forehead patch size were involved in male-male competition or female choice. To avoid very complex models, we entered these traits in separate models, because in the following analyses we also controlled statistically for year and treatment effects. We used song rate after treatment ($n = 27$), as it showed a noticeable change after injection, but strophe characteristics were expressed for the entire before and after treatment sample ($n = 35$). Data on patch size were available for 3 years ($n = 69$), and we present treatment effects for models relying on this larger data set.

We analyzed whether males were successful in occupying the same nest-box after the experiment, thus possibly reflecting the outcome of male-male competition. Successful males had significantly higher song rate than did unsuccessful males (nest-box retention: song rate, $F_{1,25} = 4.924$, $p = .035$) (Figure 6a), but did not differ in their PC2 strophe characteristics (nest-box retention: PC2 strophe characteristics, $F_{1,33} = 2.560$, $p = .119$). Nest-box retention was affected by immunological treatment, as SRBC-injected males were less successful, and also by the interaction term between treatment and patch size (nest-box retention: treatment, $F_{1,62} = 12.701$, $p < .001$, year \times treatment, $F_{2,62} = 8.467$, $p < .001$; patch size \times treatment, $F_{1,62}$

**Figure 4**

The relationship between the change in song rate (strophes per minute) and forehead patch size (a; in millimeters squared) and PC2 principal component (b) of strophe characteristics in sheep red blood cell (SRBC)-injected ($n = 15$) and placebo-injected ($n = 12$) males. The linear regression lines are given.

**Figure 5**

The relationship between fecal testosterone level (log₁₀ picograms per gram feces) before the experimental treatment and forehead patch size (a; millimeters squared, $r = .547$, $p = .015$, $n = 19$), PC2 strophe characteristics (b; $r = -.504$, $p = .028$, $n = 19$), and song rate (c; strophes per minute, $r = -.009$, $p = .972$, $n = 19$, power: 0.67). The linear regression lines are given.

= 11.470, $p = .001$; patch size \times treatment \times year, $F_{2,62} = 9.163$, $p < .001$; global model, $F_{6,62} = 5.340$, $p < .001$) (Figure 6b).

Among successfully paired males in 1 year, those that sang at higher rates paired significantly earlier relative to treatment (relative pairing date: song rate \times year, $F_{1,15} = 14.090$, $p = .002$; slope for 1999: -0.840 [SE = 0.222], $p = 0.009$; slope for 2000: 0.447 [SE = 0.338], $p = 0.228$) (Figure 7a). PC2 strophe characteristics were also significantly associated with pairing success (relative pairing date: PC2 strophe characteristics, $F_{1,21} = 10.605$, $p = .004$, PC2 strophe characteristics \times year, $F_{1,21} = 4.331$, $p = .050$, PC2 strophe characteristics \times treatment \times year, $F_{1,21} = 9.647$, $p = .005$, global model, $F_{3,21} = 6.705$, $p = .002$) (Figure 7b). Experimental treatment and patch size were not significantly related to pairing success (relative pairing date: treatment, $F_{1,36} = 0.337$, $p = .855$; patch size, $F_{1,36} = 0.987$, $p = .327$).

Song rate, PC2 strophe characteristics, forehead patch size, and the effect of immune challenge were not significantly associated with success in attracting a secondary female (polygyny success: song rate, $F_{1,25} = 1.203$, $p = .283$; PC2 strophe characteristics, $F_{1,34} = 0.024$, $p = .878$; patch size, $F_{1,67} = 1.503$, $p = .224$; treatment, $F_{1,67} = 0.541$, $p = .464$).

Males that sang at higher rates had significantly higher levels of extrapair paternity in their own nests (extrapair paternity: song rate, $F_{1,14} = 14.401$, $p = .002$) (Figure 8a). We found no significant relationship between extrapair paternity and PC2 strophe characteristics (extrapair paternity: PC2 strophe characteristics, $F_{1,22} = 1.843$, $p = .188$). The interaction term between forehead patch size and year was significantly associated with extrapair paternity, but experimental treatment was not (extrapair paternity: patch size \times year, $F_{2,31} = 7.221$, $p = .003$; year, $F_{2,31} = 8.232$, $p = .001$; global model, $F_{4,31} = 4.416$, $p = .006$; treatment, $F_{1,34} = 3.755$, $p = .061$) (Figure 8b).

Table 3 summarizes the results of the above analyses on the relationship between trait expression and measures of mate

choice, while taking into account the number of statistical tests performed.

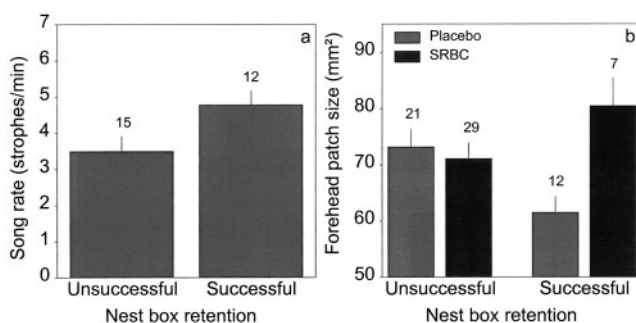
DISCUSSION

In this experimental study, we found that song rate was affected by immune challenge, whereas other song features remained unchanged after treatment. Fecal testosterone and body mass decreased after treatment in SRBC-injected birds compared with placebo birds. In the experimental group, the decline in song rate was correlated with forehead patch size and PC2 principal components of strophe characteristics, which was the PC involving important factor loadings for strophe length and strophe complexity. Testosterone level has been demonstrated to be associated with forehead patch size and song features, with the exception of song rate. We observed that males with higher song rate were more successful in retaining nest-boxes, paired faster in 1 year and suffered from higher extrapair paternity at their own nest. PC2 strophe characteristics appeared to be important in mating success. Immune-challenged males had lower success in nest-box retention.

In this discussion, we reanalyze the mechanisms involved in parasite-mediated signaling by song in the light of our results, by focusing on each participant in the communication.

Constraints on senders

So far, there has been little consensus among studies that have tested the immunocompetence handicap hypothesis (Folstad and Karter, 1992) by investigating interactions among testosterone, immune system, and song. Although several studies have demonstrated a link between song production and circulating testosterone level in different species (Enstrom et al., 1997; Galeotti et al., 1997; Hunt et al., 1997; Ketterson et al., 1992; Silverin, 1980; Stoehr and Hill, 2000), and a link between parasitism and singing performance (Buchanan et al., 1999; Duffy and Ball, 2002; Møller, 1991; Redpath et al., 2000; Saino et al., 1997), until now there has been no successful attempt to combine the causal processes. Studies of the barn swallow (*Hirundo rustica*) have shown that testosterone relates to the production of a specific song syllable (Galeotti et al., 1997) but not to song rate, which was the only song component previously found to be influenced by parasitism (Møller, 1991; Saino et al., 1997). In the red-winged blackbird (*Agelaius phoeniceus*) Weatherhead et al. (1993) found an association between mite infection and testosterone and an unexpected positive relationship between parasites and repertoire size, but not between testosterone and song traits. In addition, Hasselquist et al. (1999) found in this species that neither physiological nor super-normal levels of plasma testosterone suppressed secondary antibody production. In the European starling (*Sturnus vulgaris*), song traits were shown to be positively correlated with cell-mediated and humoral immune responses that were also susceptible to experimentally elevated

**Figure 6**

Nest-box retention of collared flycatcher males, reflecting their competition success in relation to song rate (a) and to the interaction between forehead patch size and experimental treatment (b). Means, SEs, and sample sizes are shown.

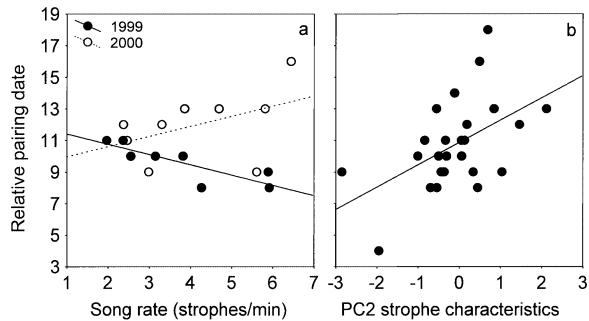


Figure 7
Pairing success (relative pairing date, time elapsed from treatment till the start of egg laying) in relation to song rate (a; $n = 17$), and to PC2 strophe characteristic (b; $n = 23$). Lines represent regression lines.

testosterone levels, but there was no association between song bout length and testosterone when controlling for antibody response (Duffy and Ball, 2002; Duffy et al., 2000). Other studies of starlings have demonstrated the effect of testosterone implantation on the proportion of time spent singing (De Ridder et al., 2000; Pinxten et al., 2002).

Our results are also partly consistent with the immunocompetence handicap hypothesis. The central prediction of this hypothesis is that a trade-off exists between sexual ornamentation and immune function. This prediction received support in our experimental study of the collared flycatcher, as song rate declined after immune challenge. We also found that an immune challenge was followed by a decrease in testosterone. Although the immunocompetence handicap hypothesis assumes an antagonistic link between the immune system and the endocrine system, it specifically predicts testosterone to suppress immune function and not effects in the opposite direction (Folstad and Karter, 1992; Verhulst et al., 1999). Our results, in conjunction with previous findings in chickens (Leitner et al., 1996; Verhulst et al., 1999), provide experimental evidence for testosterone being susceptible to immune challenge rather than vice versa. Taking into account the negative empirical support for immunosuppression by testosterone (Buttemer and Astheimer, 2000; Braude et al., 1999; Hasselquist et al., 1999; Ros et al., 1997), it is plausible that testosterone in birds plays only a minor role in mediating immunocompetence as proposed by Folstad and Karter (1992). The causality in the mechanistic link between immune function and endocrine system appears to run in the opposite direction. Furthermore, if immunocompetence handicaps are at work, testosterone should be linked to the expression of the sexually selected signal that is controlled by immune status (Folstad and Karter, 1992). However, we failed to find a correlation between song rate and testosterone, although previous experimental studies of the related pied flycatcher have shown such a relationship (Silverin, 1980). It is possible that steroid concentration is not necessarily associated with song rate, simply because this trait is a hormone-enhanced but not hormone-dependent trait, and other stimulus factors are more important in promoting song production (Ball et al., 2002). Interestingly, the other song traits that were unaffected by the immunological treatment were those associated with testosterone. In addition, forehead patch size, which can serve as a parasite resistance signal (Andersson, 2001), also correlated with testosterone level.

Clearly, the relationship between testosterone, immune system, and song performance is complex, depends on other sexual signals, and may involve species-specific patterns.

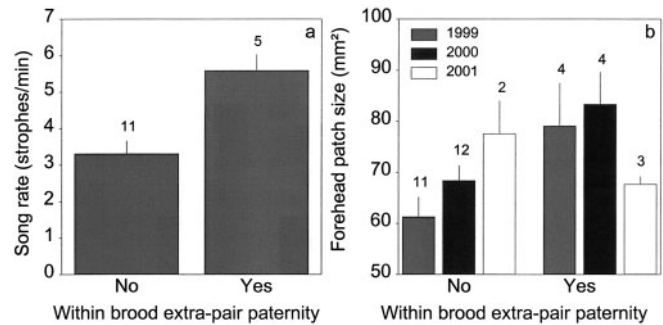


Figure 8
(a) Mean song rate (+SE) and within brood extra-pair paternity.
(b) The interaction (mean + SE) between forehead patch size and year effects in relation to extra-pair paternity. Sample sizes are given.

Although testosterone probably does play a role in vocal signaling in relation to parasitism, the exact endocrine mechanism remains unknown. Additionally, contradictory results may appear owing to the possible confounding effects of other hormones such as corticosterone, which potentially mediates the relationship between testosterone and immunocompetence (Duffy et al., 2000; Evans et al., 2000). An appropriate test of the immunocompetence hypothesis would require more detailed approaches that selectively investigate the independent effects of the two steroid hormones.

Roles for signal design

Multiple signals used in sexual contexts may reveal different information regarding parasite burden (Wedekind, 1992). In our investigation of the signaling function of song, we found different roles for song rate, strophe characteristics, and repertoire size.

We showed that song rate decreased after experimentally challenging the immune system. In many passerine birds, song rate has been found to correlate positively with male territory quality, male condition, dominance, survival rate, and parental care (for review, see Vehrencamp, 2000). Singing at a high rate is thought to be a costly activity, and individual quality differences in coping with energy demands are the determinants of signal reliability (for review, see Vehrencamp, 2000). This view is applicable to parasite-mediated sexual selection on song traits. Energetically costly song traits such as song rate in the barn swallow and the starling (Duffy and Ball, 2002; Møller, 1991; Saino et al., 1997), song flights in the sedge warbler (*Acrocephalus schoenobaenus*; Buchanan et al., 1999), and frequency range in the tawny owl (*Strix aluco*; Redpath et al., 2000) have all been found to be linked to parasitism. Our findings that song rate in the collared flycatcher was correlated with body condition and decreased after an immune challenge lend further support to the idea that energetic constraints on acoustic trait expression and immune defense result in costly song attributes being reliable signals of parasitism. Only high-quality healthy males are able to maintain body condition and sing at high rates while under parasite pressure.

Other attributes of song, such as song repertoire size and strophe characters involved in PC1 and PC2 components (Table 2), seem to be intrinsic attributes of males independent of immediate health status, because they were unaffected by immune challenge. Signals stabilized by physical ceilings instead of the direct cost of production are known as index signals (Maynard-Smith and Harper, 1995; Zahavi and Zahavi, 1997). Repertoire size, as a possible index signal of health

Table 3

A summary table of the analyses testing the relationship between components of sexual selection (nest-box retention reflecting the outcome of male-male competition, and relative pairing date, polygyny success and extrapair paternity reflecting mating success), trait expression (song rate, PC2 strophe characteristics, and forehead patch size), and immune challenge

	Song rate	PC2	Forehead patch size	Treatment
Nest-box retention	0.128 ± 0.058	0.129 ± 0.081	-0.001 ± 0.004 ^a	-0.922 ± 0.259^a
Relative pairing date	-0.051 ± 0.309 ^b	1.514 ± 0.465^c	0.043 ± 0.073	1.770 ± 5.706
Polygyny success	0.023 ± 0.036	-0.008 ± 0.049	0.002 ± 0.003	0.064 ± 0.231
Extrapair paternity	0.223 ± 0.058	0.127 ± 0.093	0.017 ± 0.011 ^d	1.198 ± 0.724 ^e

Standardized coefficients ± SE are given, which are highlighted if significant. Underlined and highlighted coefficients were significant after Bonferroni correction ($p < .003$). See text for statistics.

^a Patch size × treatment: St. Coeff. ± SE = -0.012 ± 0.003 ($p = .001$); treatment × year: St. Coeff. ± SE = 1.543 ± 0.377 ($p < .001$); patch size × treatment year: St. Coeff. ± SE = -0.022 ± 0.005 ($p < .001$).

^b Song rate × year: St. Coeff. ± SE = -0.340 ± 0.091 ($p = .002$).

^c PC2 × year: St. Coeff. ± SE = -1.488 ± 0.715 ($p = 0.05$); PC2 × treatment × year: St. Coeff. ± SE = -2.163 ± 0.696 ($p = .005$).

^d Year: St. Coeff. ± SE = -1.221 ± 0.459 ($p = .001$) and St. Coeff. ± SE = -1.690 ± 0.580 ($p = .001$); patch size × year: St. Coeff. ± SE = 0.016 ± 0.007 and St. Coeff. ± SE = 0.021 ± 0.007 ($p = .003$).

^e $p = .061$.

(Vehrencamp 2000), has been given special attention in studies investigating song in relation to pathogens. Some studies have shown a correlation between repertoire size and parasitism (Buchanan et al., 1999; Møller et al., 2000), but other results found no effect (Read and Weary, 1990; Weatherhead et al., 1993). Although repertoire composition may be important in female choice in the collared flycatcher, given that in a sibling species, the pied flycatcher, females show a preference for large repertoire size (Lampe and Sætre, 1995), our study failed to find a direct relationship between repertoire composition and immune status.

PC2 strophe characteristics (most importantly strophe complexity and strophe length) were associated with the amount of change in song rate. Although we did not measure individual antibody response to the SRBC challenge directly, this result raises the possibility that PC2 traits could potentially signal parasite resistance, if the change in song rate in immune challenged birds is proportional to individual ability to cope with a parasite infection. This assumption is supported by the observation that forehead patch size, which has previously been demonstrated to correlate positively with specific antibody response to a novel antigen and thus signals immunity (Andersson, 2001), was also related to the decline in song rate in the experimental group. In addition, PC2 strophe characteristics were related to testosterone level with a similar exponent as forehead patch size. If PC2 traits indicate resistance and song rate signals health status, this would shed light on how resistance to parasitism and the effects of parasitism on immediate body condition contribute to signal expression. Such discrimination between signal types is usually neglected in studies correlatively demonstrating a link between parasite burden and phenotypic expression of songs (see also Buchanan et al., 1999), because the existence of simple covariance does not provide conclusive evidence of a direct cost of infection that determines signal reliability and design. Taken together, we believe that our experimental results suggest for the first time that parasites may drive a multiple signaling mechanism involving visual and vocal traits, and both handicap and index signals can have different functions in these processes.

The reaction of receivers

We found that males that were able to retain their nest-boxes were more likely to be in a superior state of health, because

placebo-injected males had higher probability of settling down at the same nest-box that they occupied before the experimental procedure. In addition, there was some indication that song rate was involved in this process, because males that successfully retained their previous territories after the experiment had higher song rates, whereas those with lower song rates occupied new territories. Nest sites are important resources for males, because they have important consequences for the owner's fitness. A successful territory reoccupation may involve benefits such as familiarity with food sources or predator refuges (Pärt, 1994). Hence, our findings are in accordance with the hypothesis that health status and song rate are important in male-male competition.

The role of song rate in social female choice has been demonstrated in the pied flycatcher, as females are known to select males with high song rates (Alatalo et al., 1990; Gottlander, 1987). We also found some evidence that male collared flycatchers with higher song rate, and PC2 strophe characteristics with the potential to signal resistance, are more successful in mating. In our experiment, extrapair mate choice appeared to be affected by song rate, but not in the expected direction (in some years this was the tendency for forehead patch size as well). A possible explanation is that males with particularly extravagant traits indicating health and vigor, making them very attractive to females, are more successful in achieving extrapair copulations and invest less in mate guarding, thereby increase the rate of extrapair paternity in their primary brood (see also Lifjeld et al., 1997).

Therefore, from the receivers' perspective, we were able to provide indirect evidence for parasite-mediated acoustic communication involved in female choice and male-male competition. However, because our results are correlative, several alternative explanations remain for the observed patterns of nest-box retention and success in mate attraction. At least, our findings are consistent with the hypothesis that parasites may drive sexual selection by song.

In summary, we have shown in a natural population of birds that while song rate operates as a reliable signal of health status, other song features may remain unchanged during parasite infection and may thus indicate intrinsic quality attributes of males, such as parasite resistance. Testosterone and a visual secondary sexual character played important roles in this process. Potential receivers may use song cues in their mating decisions and, therefore, seem to be decoding information about the immune status of senders. Our findings imply that

parasite-mediated sexual selection has played a central role in the evolution of a diverse and complex vocal communication system involving signals, senders, and receivers.

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