



Female preference for male courtship song and its role as a signal of immune function and condition

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The acoustic signals of insects are examples of sexually selected traits predominantly used by females to identify, locate and choose between potential mates. In addition to the conspicuous calling songs found in many groups, many male Orthopterans also produce a distinct courtship song when in close proximity to a female. It has been suggested that these courtship songs are indicators of male quality and may be used by females to discriminate between potential mates, but little evidence is available. We measured temporal characteristics of courtship song in the cricket *Teleogryllus oceanicus* and three different aspects of the immune response of the same males. The relations between courtship song structure and immunity appeared to be too weak to be biologically important and hence it seems unlikely that females could use only the song parameters we measured to discriminate in favour of mates with higher immunity. However, when we compared the propensity of males to sing and the structure of their courtship songs before and after they were forced to mount an immune response, we found strong effects of our immune challenge procedure. When females were played songs manipulated to sound like those produced by males with high or low encapsulation abilities (a measure of immunity), they responded more rapidly to the songs corresponding to a high immune response.

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Acoustically signalling insects represent one of the most conspicuous examples of a mating system in which females choose between potential mates. The calling songs of Orthopterans, particularly crickets, have been studied extensively in relation to female choice (reviewed in Zuk & Simmons 1997; Brown 1999; Robinson & Hall 2002). However, in many species, notably grasshoppers and crickets, males produce a second type of song when in close proximity to a female (Alexander 1961). This courtship song is somewhat enigmatic: why produce a second type of signal that is energetically costly (Hack 1998), and has the potential to attract predators and parasites, at a point where the female and male have already found one another?

Courtship song in the cricket *Acheta domesticus* is twice as energetically expensive as calling song (Hack 1998) and in the one species pair that has been studied does not appear to have evolved as a species recognition signal (Fitzpatrick & Gray 2001; Gray 2005) suggesting that it is under selection driven by female choice. Courtship song might provide information to females about male quality that is not provided by calling song. Calling song may be constrained to be energetically less expensive because males need to call for long periods during the night to attract females, and females need to distinguish between conspecific males and those of other species. Courtship song is known to be important in inducing females to mate, with evidence from a number of species of crickets where females are reluctant to mount silenced males (Crankshaw 1979; Libersat et al. 1994; Balakrishnan & Pollack 1996; Nelson & Nolen 1997). However, evidence that females actually discriminate between males on the basis of courtship song remains equivocal. Boake (1984) found that males differing in courtship song did not differ predictably in their mating success in the gregarious cricket *Amphiacusta maya*. Similarly, Nelson & Nolen (1997) failed to find any aspect of courtship song that

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could be used to predict male mating success in *A. domesticus*. The only published direct evidence for mate choice in relation to the structure of courtship song comes from Wagner & Reiser's (2000) finding that *Gryllus lineaticeps* females were more likely to move towards courtship song with a higher chirp rate.

The condition dependence hypothesis (Williams 1966) predicts that male secondary sexual traits should be particularly sensitive to the overall condition of the individual, and hence provide honest signals of male genetic quality (reviewed in Tomkins et al. 2004). Despite limited evidence for female preferences for particular courtship song elements, previous studies have examined the possibility that courtship song is an honest indicator of male genetic quality. One approach to this question is to manipulate factors affecting male condition such as diet during development. Wagner & Reiser (2000) found that in *G. lineaticeps* there was no difference in courtship song chirp rate between males on good and poor diets. Similarly, Gray & Eckhardt (2001) found that in *G. texensis* manipulations of larval and adult diet did not affect courtship song, and although parameters of the song itself were weakly correlated with male age, they were not related to the male's fat content. An alternative approach is to measure immune function since this represents a character that is likely to be under directional selection in all environments and hence should be closely correlated with overall condition. If courtship song is an accurate indicator of male condition we would expect correlations between song properties and immunity. In *Gryllus bimaculatus*, Rantala & Kortet (2003) found that males with higher cellular immune responses to a foreign object (encapsulation rate) differed in temporal properties of their courtship songs from males with lower encapsulation rates, and that the courtship songs of males with high encapsulation rates were preferred by females to those with low encapsulation rates. Similarly, the calling songs of *A. domesticus* and *Teleogryllus commodus* correlate weakly with immune response measured, respectively, as haemocyte load and encapsulation rate (Ryder & Siva-Jothy 2000; Simmons et al. 2005).

Related to the role of courtship song as an honest indicator of immune function is the question of whether courtship song reflects the overall phenotypic condition of an individual. This leads to the insight that if males are placed under stress this should be reflected in their condition-dependent traits, a prediction that has typically been examined by stressing animals during development and then measuring effects on sexually selected traits relative to nonsexual traits (reviewed in Cotton et al. 2004). Acoustic signals provide the opportunity to examine whether these sexually selected 'ornaments' can respond dynamically as well as developmentally to reduced phenotypic condition by acutely stressing males and looking for changes in song properties that might be detected by females.

Our aims in this study were to examine courtship song in the Australian cricket *Teleogryllus oceanicus* to discover whether females prefer certain elements of courtship song, as would be expected if song is an indicator of

male quality, and to determine whether these (or any other elements) are linked to immune function.

METHODS

Study Animals

Crickets used in the study were offspring of females collected in the field in Carnarvon, Western Australia and brought into the laboratory. Hatchlings were reared to maturity and maintained at 29°C with a 12:12 h light:dark cycle in 5-litre plastic containers with food (cat chow) and water provided ad libitum. The sexes were separated before the final moult. All crickets were at least 5 days old when they were used in experiments.

Song Recordings

All recordings were made under dim red light at 25°C in an anechoic room. Recordings were made during the first 3 h after the dark portion of the light cycle on a Sony Pro-Walkman with a Sennheiser directional microphone. Crickets were housed in transparent plastic tubs (7 × 7 cm and 5 cm high) with a mesh lid during the recording process. A female was added to a male's container to induce him to sing; if the female mounted before a complete recording was obtained, we interrupted the pair by gently tapping the container. We attempted to obtain at least 10 complete courtship songs for each male.

Song Analysis

Songs were digitized at 22 kHz and analysed with the Raven software package (Cornell Laboratory of Ornithology, New York, U.S.A.). They were filtered to remove noise at <3.5 kHz and >6 kHz. We did not measure song amplitude because recorded volume is extremely sensitive to the orientation of the singing cricket and the distance to the microphone. We examined a set of temporal features of the song, which can be divided up into a chirp and a trill, each of which contains a number of pulses (Fig. 1). The parameters measured were: chirp length (CL), the duration of the final pulse of the chirp (chirp pulse length; CPL), the interval between the final and penultimate pulses of the chirp (chirp pulse interval; CPI), the interval between the end of the chirp and the start of the trill (song interval; SI), the duration of the first and fifth trill pulses (trill pulse length; TPL1 and TPL5) and the intervals following these pulses (trill pulse interval; TPI1 and TPI5), the duration of the first five pulses and intervals of the trill (trill pulse; TP), the total duration of the trill up to the first break at least as long as a missing pulse (trill (to) break; TB) and the duration of the trill until the next chirp (trill length; TL). All acoustic analyses were conducted by T.T., and recordings from different treatments were analysed during the same session. Because multiple measurements were made on each song, we used multivariate analyses in SPSS 11.0 (SPSS Inc., Chicago, IL, U.S.A.) to compare overall patterns of variation between songs and between treatments.

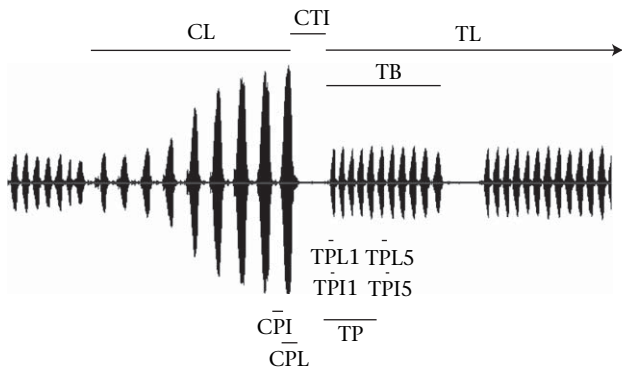


Figure 1. Waveform of the courtship song of *Teleogryllus oceanicus*. Mean durations of components are given in Table 1. CL: chirp length; CPL: chirp pulse length; CPI: chirp pulse interval; CTI: chirp–trill interval; TPL1, TPL5: length of first and fifth trill pulses; TPI1, TPI5: intervals following first and fifth trill pulses; TP: length of first five trill pulses and intervals; TB: length of trill to first break; TL: length of trill to next chirp.

Immune Function Assays

We assayed males for immune function the day after recording their courtship song. Males varied in age at this point from 4 to 12 days after adult moult. We measured the ability of males to encapsulate a foreign object by introducing a piece of nylon filament into the body cavity and subsequently measuring its degree of melanization; this is a standard technique for analysing insect immunity (Konig & Schmid-Hempel 1995; Rantala et al. 2000; Doums et al. 2002; Rantala & Kortet 2003). Crickets were cold anaesthetized by placing them in a -20°C freezer for 2 min. We punctured the ventral intersegmental membrane between the first and second abdominal segments with a fine gauge hypodermic needle, sterilized it with ethanol and implanted a 3-mm length of 0.255-mm-diameter nylon monofilament into the body cavity through the wound. We roughened the surface of the monofilament with sandpaper before use to enhance the likelihood that haemocytes would stick to the implant. After implantation, each cricket was housed individually in a 130-ml plastic vial with dry cat food and water ad libitum. All crickets recovered from the cold anaesthesia and continued to feed and behave normally during which time we recorded courtship song for a second time where males could be induced to sing. After 36 h the male was frozen. The implant was dissected from the body cavity, cleaned with 70% ethanol and placed on a cavity slide. An identical length of nylon monofilament was placed next to the implant and the two fixed with mounting medium (Eukitt, Electron Microscopy Services, Hatfield, U.S.A.) and covered with a cover slip. Each implant was photographed with a digital camera attached to a microscope and the image analysed with Optimas Image Analysis (Media Cybernetics, Silver Spring, U.S.A.) software. The program provides a measurement of the mean grey scale of the pixels contained in a designated area, with 0 being completely white and 256 being completely dark. To control for variation in the colour of the mounting medium, we compared the outlined area of the implant with the

outlined area of the adjacent piece of nylon monofilament that had not been implanted. The measurement used was the mean darkness of the implant minus the mean darkness of the control. Previous work (Zuk et al. 2004) shows that encapsulation response does not covary with the size of individuals.

At the same time as the monofilament was implanted, a 2- μl sample of haemolymph was withdrawn from the male and placed directly into 18 μl of anticoagulant (Mead et al. 1986). This mixture was mixed and an 8- μl sample was placed on to each side of a Neubauer haemocytometer and left to settle for 5 min. Haemocytes were counted in five nonadjacent squares under $200\times$ magnification.

To assay levels of lysozyme-like activity, which is associated with antibacterial action, we mixed an autoclaved agar solution (1.5 g per 120 ml) with a solution of 0.225 g of *Micrococcus lysodeketicus* and 0.001 g of streptomycin sulphate in 20 ml of 0.01 M phosphate buffer and incubated it at 48°C for 30 min. We placed 15 ml of this mixture into petri dishes (autoclaved) 8 cm in diameter and allowed them to set at room temperature for 30 min before storing them at 4°C . A grid of 12 holes 3 mm in diameter was made in each dish. A 2- μl sample of haemolymph was taken from each cricket, placed into one of the holes, and the dish left at room temperature for 10 min. The dish was then incubated for 24 h at 33°C . We measured the area of the clear region around each hole in the agar gel as an indication of the lysozyme-like activity of the haemolymph sample. All data from immune function assays were square-root transformed to satisfy assumptions of normality inherent in parametric statistics.

Immune Challenge and Courtship Song

Our immune function assay involved the invasive process of inserting a piece of nylon into the body cavity of a male. We recorded 68 males before this procedure and then gave them an opportunity to court and recorded them again 24 h afterwards. In addition, 33 control males were recorded and then given a second opportunity to sing courtship song after the same time interval (see below) as the challenged males. This control group allowed us to test the potentially confounding hypothesis that songs change over a few days as a result of changes in male age. There were no differences in male age between control males and challenged males at first or second song recordings (mean age \pm SEM at first recording: challenged males: 6.9 ± 0.37 days; control males: 6.6 ± 0.30 days; Mann–Whitney U test: $U = 991$, $N_1 = 68$, $N_2 = 33$, $P = 0.56$; mean age at second recording: challenged males: 10.9 ± 0.31 days; control males: 10.1 ± 0.35 days; $U = 464$, $N_1 = N_2 = 32$, $P = 0.32$). Control males were assayed for immune function (as described above) 12 h after being recorded for the second time.

Female Preference Trials

To construct courtship song models for use in preference trials we used the software package Adobe Audition

(Adobe, San Jose, U.S.A.). We haphazardly selected five natural songs from the library of songs from the 101 males recorded in the first part of the study. Each song was manipulated to generate a high- and low-quality version, based on the first principal component of variation in songs, which covaried with immune function (see Results). We manipulated the interval between the end of the chirp and onset of the trill and the length of time between trill pulses so that high- and low-quality versions of songs had a chirp–trill interval of 60 ms and 120 ms, respectively, and trill pulse intervals of 5 ms and 10 ms, respectively. We also fixed trill length at 3.2 s for both groups, but in the low-quality song, we removed five trill pulses 500 ms after the onset of the trill. Thus, high-quality versions of the song models had short intervals between chirp and trill and between the trill pulses, and a long unbroken trill, which resulted in a high total number of pulses in the trill. In contrast, low-quality versions had long intervals, a break in the trill and few pulses in the trill. We did not manipulate trill or chirp pulse lengths because it is difficult to create a biologically realistic extended pulse. The chirp characteristics, syllable characteristics and total song length were the same for high- and low-quality versions of each song, although these parameters varied between the five pairs of song models. All the parameters of our manipulated songs fell within the naturally occurring range and within 2 standard deviations of the mean (Table 1).

For each of the five pairs of songs, we examined the responses of 16 females to the high-quality song and of 16 different females to the low-quality song, using a no-choice design (Shackleton et al. 2005) in which female preference is measured through latency to respond to a single stimulus. None of these 160 females were tested more than once. We conducted all trials during the early afternoon, at the beginning of the dark cycle, in an anechoic

room under red light at 25°C. Songs were broadcast at an intensity of 90 dB SPL (re: 20 µPa) measured at a distance of 10 cm (the intensity of normal courtship song, Balakrishnan & Pollack 1996) from a speaker placed 10 cm above and central to a grid of four cages (5 × 7 cm and 7 cm high) with mesh lids. Individual females had been housed in these cages for 24 h with food and water provided ad libitum. Preceding their isolation, females were housed in a mass culture of both males and females for 24 h. Immediately before the trials, food and water were removed and a freshly frozen and thawed male was placed in the centre of each female's cage, in a normal upright position as would be assumed by a courting male. We broadcast courtship song for 5 min and tested four females simultaneously. We recorded the time from onset of the broadcast to the time when a female first responded to the song. Females that did not respond within the 5-min period were given a score of 300 s. A positive response involved a female orienting towards and antennating the male and standing near him in a manner typical of normal mating behaviour (normally the male would walk backwards under the orienting female). In 21% of all 160 trials, the female climbed on to the dead male's back, when after orientation the male did not respond to the female's antennation. In 16% of trials the female moved to the dead male and immediately began to eat it, a behaviour that was clearly distinct from the mating approach behaviour described above. To determine whether this behaviour should be included as part of the preference response, we conducted 12 trials exactly as above but in which we did not broadcast courtship song. Four females began to eat the male, while the remaining females did not orient to the male during the 5 min of observation. We therefore excluded from our analyses females that simply began eating the male, on the grounds that this was not a response to the broadcast song. In total we were left with 134 females tested across high- and low-quality versions of the five song models.

Table 1. Durations of courtship song traits (s) and principal component loadings

Song trait	Mean±SE	PC1	PC2	PC3
Chirp length	0.48±0.007	0.46	0.36	0.19
Chirp pulse length	0.037±0.0004	0.54	-0.11	0.47
Chirp pulse interval	0.031±0.0005	-0.26	0.57	-0.32
Chirp–trill interval	0.08±0.003	- 0.68	0.12	0.41
Trill pulse length 1	0.014±0.0003	0.83	0.15	0.24
Trill pulse interval 1	0.009±0.0002	- 0.70	0.38	0.43
Trill pulse length 5	0.018±0.0002	0.87	0.05	0.20
Trill pulse interval 5	0.008±0.0002	-0.60	0.52	0.36
Trill first five pulses	0.13±0.001	0.37	0.54	0.69
Trill length to break	2.19±0.08	0.36	0.65	-0.47
Trill total length	3.24±0.1	0.15	0.64	- 0.52

Principal component (PC) loadings are for the first three components extracted from an analysis of the courtship songs of 101 males. PC1 explains 33% of all interindividual variation, PC2 explains 19% and PC3 explains 17%. Absolutely larger PC values indicate that a trait makes a larger contribution to the component. Differences in sign between two traits within a component indicate that individuals with large values for one trait tend to have small values for the other and vice versa. Loadings in bold are >70% of the largest loading and indicate the most significant factors in the component (Mardia et al. 1979).

Statistical Analysis

All data were checked for normality before analysis. Where data were not normally distributed, and could not be transformed to achieve normality, nonparametric statistics were used. All quoted *P* values are two-tailed.

RESULTS

Song Variation Among Individuals

We recorded 101 individuals and calculated mean values for the 11 song measures from analysis of a maximum of 10 songs per male (mean = 9.4 songs/male, range 4–10 songs; some males failed to sing all 10 songs). These mean values were used in a principal components analysis (Table 1). The first three principal components explained 33, 19 and 17%, respectively, of the total variance between individuals. Several patterns were apparent in the component loadings. In component 1, pulse interval parameters all had negative values whereas pulse duration parameters

all had positive values, indicating that this component is based on variation in the proportion of time the individual produced sound (the 'duty cycle'). In component 2, all the traits with substantial contributions had positive loadings indicating that songs are distinguished according to whether all characters are generally longer or shorter, and in component 3, the main pattern is the contrast between the length of the first five pulses in the trill and the total length of the trill (trills that are short and slow or long and fast).

Immune Response and Courtship Song

Within the 87 males that both sang and had their immune function measured (60 males had the immune assay after one recording, 27 males had it after singing two sets of songs recorded 48 h apart) there was a strong positive correlation between encapsulation response and haemocyte count (Pearson correlation: $r_{85} = 0.46$, $P < 0.0001$). However, there were strong negative correlations between these variables and lysozyme-like activity (haemocyte count and lysozyme-like activity: $r_{85} = -0.62$, $P < 0.0001$; encapsulation response and lysozyme-like activity: $r_{85} = -0.34$, $P = 0.002$). There was evidence of an effect of male age on encapsulation response (regression of age at immune test on encapsulation: $R^2 = 0.067$, $P = 0.015$; Fig. 2) with older males showing a stronger response, but no similar relations between age and haemocyte count ($R^2 = 0.004$, $P = 0.56$) or lysozyme-like activity ($R^2 = 0.003$, $P = 0.61$).

To examine potential relations between immune function and courtship song we used backwards elimination of predictors nonsignificant at $P = 0.1$ from a multiple regression of our three immune function measures, with the first, second and third principal components extracted from our song data as dependents in three separate regressions. In all cases, all but one predictor was eliminated

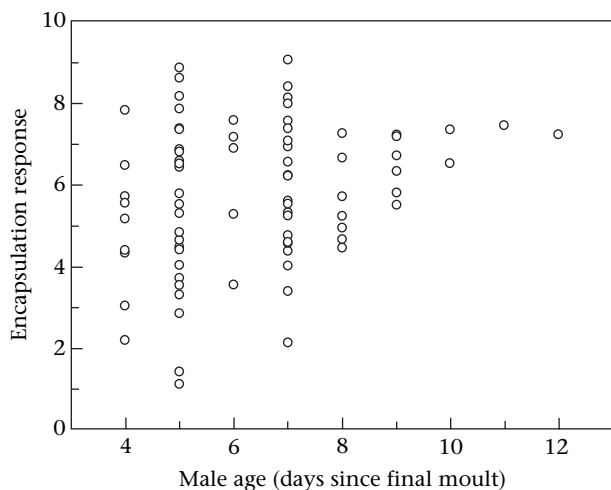


Figure 2. Variation in encapsulation response (square-root transformed) with male age. Encapsulation response was measured as the degree of melanization of a foreign object inserted into the body cavity: see [Methods](#) for details.

from the final model. These analyses revealed a weak positive relation between encapsulation ability and PC1 ($R^2 = 0.06$, $P = 0.024$; males with a high duty cycle tended to have a better encapsulation response), and a borderline positive relation between lysozyme-like activity and PC3 ($R^2 = 0.044$, $P = 0.052$), but no other significant effects. Figure 3 shows the relation between encapsulation response and the first principal component of the courtship song. If the male with the lowest encapsulation ability is removed from the analysis then it ceases to be significant ($R^2 = 0.02$, $P = 0.19$). If age is included in these regressions it has no significant predictive power in relation to song PC2 and PC3. However, age does have a significant relation with song PC1 ($R^2 = 0.084$, $P = 0.004$) and with its inclusion in the model encapsulation ability no longer predicts variation in song PC1, but the predictive power of lysozyme-like activity is increased (final model: $R^2 = 0.10$, $P = 0.004$; age: $t = 3.11$, $P = 0.003$; lysozyme-like activity: $t = -1.68$, $P = 0.096$). In these analyses, we are effectively conducting three separate tests on the same data, so it is appropriate to apply a Bonferroni correction to significance values, reducing the critical P value from 0.05 to 0.017. After this correction, none of the relations between song characters and immune function can be regarded as statistically significant.

Song After Immune Challenge

Of the 68 males that were recorded only once prior to immune challenge, six died and 30 subsequently failed to sing. Of the 33 males that were recorded and then given the opportunity to sing after the same duration as the challenged males, but without being manipulated, all but one sang (Fisher's exact comparison of number of males from each group recorded twice including males that died: $P < 0.0001$; ignoring males that died: $P < 0.001$). There were no differences in the first songs of males that

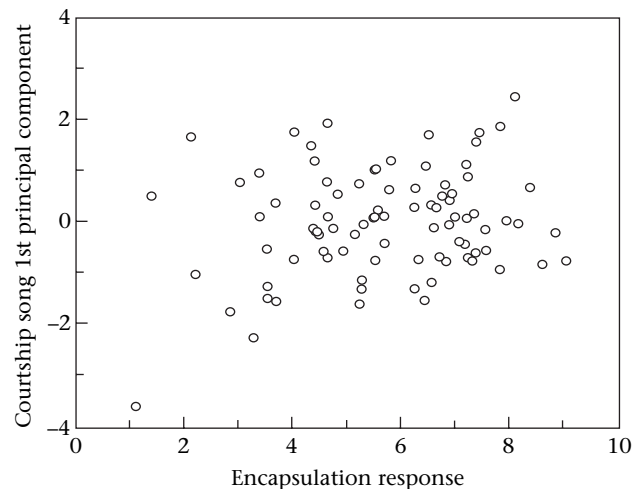


Figure 3. Variation in the first principal component of courtship song in relation to the level of encapsulation response (square-root transformed; $r^2 = 0.06$, $N = 87$, $P = 0.024$). Encapsulation is measured as the degree of melanization of a foreign object inserted into the body cavity: see [Methods](#) for details.

subsequently sang again and those that refused to court a second time (MANOVA incorporating all 11 song parameters: $F_{11,64} = 0.76$, $P = 0.68$).

A direct comparison of the courtship songs of males recorded for the second time reveals a marginally non-significant difference between treatment and control groups (MANOVA incorporating all 11 song parameters: $F_{11,52} = 1.94$, $P = 0.054$). However, this analysis lacks power because there is no control for between-individual variation. We used the principal component loadings from our analysis of recordings on prechallenge males (Table 1) to calculate the first three principal components for each male recorded before and after being challenged, and similarly for the control group of males. We then compared the mean values for these principal component scores for both groups by using paired-sample comparisons of individuals that were recorded twice. There were no differences in principal component scores for the first and second songs of control males (PC1: $t_{31} = 0.095$, $P = 0.93$; PC2: $t_{31} = 0.072$, $P = 0.94$; PC3: $t_{31} = 0.077$, $P = 0.94$). In contrast, all three principal component scores of pre- and postchallenge males changed significantly (PC1: $t_{31} = 2.50$, $P = 0.018$; PC2: $t_{31} = 2.27$, $P = 0.030$; PC3: $t_{31} = 2.19$, $P = 0.037$), indicating that temporal aspects of courtship song were affected by mounting an immune response.

To examine which aspects of courtship song were affected by immune challenge, we conducted Wilcoxon signed-ranks analyses on all 11 song traits comparing values before and after challenge. P values from these analyses suffer from an increased chance of type 1 error associated with multiple testing; however, because our sample sizes are not very large we have provided estimates of effect sizes to determine the extent to which they provide an indication of those traits that may be most affected by mounting an immune response (Table 2). Three of these 11 tests were significant (TPL5, TPI5, TP). The song traits were longer prior to challenge. None of these values remained significant after Bonferroni adjustment of probabilities, although such adjustment is very conservative in this context, and the fact that effect sizes are between 0.33 and 0.46 suggests that there are

Table 2. Comparisons of temporal song traits before and after immune challenge

Song trait (N=32)	Z	P	Effect size (r)
Chirp length	0.42	0.69	0.07
Chirp pulse length	0.56	0.59	0.10
Chirp pulse interval	1.05	0.30	0.19
Chirp–trill interval	0.13	0.91	0.02
Trill pulse length 1	1.86	0.06	0.33
Trill pulse interval 1	0.16	0.88	0.03
Trill pulse length 5	2.29	0.02	0.40
Trill pulse interval 5	2.45	0.01	0.43
Trill first five pulses	2.58	0.01	0.46
Trill length to break	1.53	0.13	0.27
Trill total length	0.34	0.75	0.06

P values are from Wilcoxon signed-ranks comparisons; estimates of effect size are based on $r = Z/\text{square root}(N)$ (Rosenthal 1991).

biologically relevant differences in the songs of challenged and unchallenged males.

Female Responses to Courtship Songs

Figure 4 gives the median time to first response for females presented with high- and low-quality versions of five courtship songs. For three of the five song models, females responded significantly faster to the high-quality version of the song. For one song the difference in response to the high- and low-quality versions was in the same direction but did not reach significance at $P = 0.05$. One song model (song 3) showed no significant difference. We used the method of summing Z 's (Rosenthal 1991) to calculate a combined probability across the five song models. The combined probability was $P = 0.002$ (two-tailed) suggesting that, on average, the high-quality versions of songs elicited a more rapid response. The mean effect size, Fisher's Z_r , \pm SE across the five song models was 0.376 ± 0.138 .

DISCUSSION

We found only weak evidence that courtship song in *T. oceanicus* contains information about male immune response that could be used by females. We certainly cannot rule out this possibility, but it is clear from the large amount of unexplained variance that other factors influence both the structure of the song and the female's response to it. Our results caution against the use of a single measure of immune response to reflect quality, since different measures showed different relations with song, and none of the relations we found can be considered significant given rigorous correction for multiple tests.

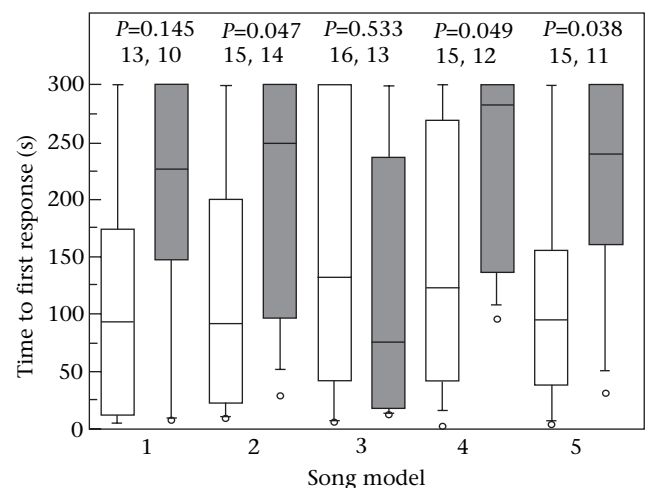


Figure 4. Box plots for the time to first response of female *Teleogryllus oceanicus* to broadcast of five courtship songs manipulated to be of either high (□) or low (■) quality. Horizontal bars represent the median time to first response, the box and whiskers represent the middle 50th, 25th and 75th percentiles, while the circles show the most extreme 10% of values. P values are from Mann–Whitney U tests contrasting high- and low-quality versions of the song models. Sample sizes are given above the box plots.

Immune Function

Our finding of a negative correlation between antibacterial (lysozyme-like) immune response and haemocyte density and encapsulation rate adds to recent findings of similar negative correlations between antibacterial and other aspects of immune function in invertebrates (Moret & Schmid-Hempel 2001; Moret & Siva-Jothy 2003; Rantala & Kortet 2003; Cotter et al. 2004). Even in insects, immune systems have multiple components and investment in each of these needs to be balanced, and may even have negative interactions, such as production of toxic intermediates in the phenoloxidase activation cascade (Cerenius & Soderhall 2004). It has been suggested that genetically based trade-offs between aspects of immune defence (antagonistic pleiotropy) may allow heritable variation in immune traits to be maintained (Cotter et al. 2004). A study of another cricket species, *T. commodus*, found positive correlations between haemocyte density and encapsulation rate, but no evidence for a negative relation between encapsulation rate and lysozyme-like activity (Simmons et al. 2005) and theory suggests trade-offs between traits can maintain variation only in very limited circumstances (Curtsinger et al. 1994). However, a quantitative genetic analysis of *T. oceanicus* has shown high levels of additive genetic variance in all of the immune traits measured in our study, and negative genetic correlations between antibacterial and other aspects of immunity (Simmons & Roberts 2005), suggesting the phenotypic correlations observed in *T. oceanicus* are of evolutionary importance.

Adamo et al. (2001) found that 2-week-old male *G. texensis* had similar haemocyte counts to 1–3-day-old males, but that older males had reduced phenoloxidase activity and resistance to bacterial infection. Comparing within males aged 4–12 days after adult moult, we also found no effects of age on haemocyte count or on lysozyme-like activity, but older males tended to have a stronger encapsulation response. Because of the negative correlations we found between immune traits, it is difficult to compare Adamo et al.'s findings with ours. It is possible that the increased encapsulation response with age in our study and the decreased phenoloxidase activity and resistance to bacterial infection with age found by Adamo et al. reflect negative correlations between these characters relative to 1–3-day-old males, although it is in accord with their finding of no significant change in haemocyte count. In the field, no differences in age between paired and unpaired males were found in 61 males collected by Zuk & Simmons (1997). In contrast, female *G. veletis* and *G. pennsylvanicus* crickets are more commonly paired with older males that have fewer gut parasites (Zuk 1988), and preferentially orient towards older males in experimental situations (Zuk 1987). Similarly, in *G. bimaculatus*, older males have higher pairing success (Simmons & Zuk 1992), suggesting that male age may in part be used by females as an indicator of male quality. Our result also accords with the finding that sexually mature female scale bugs, *Coccus hesperidum*, are more resistant to parasitoids (presumably through their encapsulation response) than are immature females (Blumberg & DeBach 1981).

In relation to the possibility that female *T. oceanicus* can use information in courtship songs to distinguish between

males with different levels of immune function, despite a reasonable sample size (10 songs from each of 87 males whose immune functions were assayed), the strongest relation we could detect between a single axis of variation in the temporal properties of the song (PC1) and an immune measure was only 6%. This tends to suggest that females will not be able to discriminate between males varying in immune function on the basis of courtship song unless there are other aspects of song or immunity that are not captured by our study (which of course there are likely to be). Previous studies have found stronger relations between calling song and immunity, although none is overwhelmingly convincing. In the crickets *A. domesticus* and *T. commodus*, positive relations have been found between one of several measured calling song parameters (pulse number for *A. domesticus* and pulse duration for *T. commodus*) and one of several measures of immune function (haemocyte load for *A. domesticus* and encapsulation response for *T. commodus*; Ryder & Siva-Jothy 2000; Simmons et al. 2005). Similarly, in the field cricket *G. bimaculatus* significant positive correlations have been found between courtship song characters and encapsulation rate (Rantala & Kortet 2003).

Immune Challenge and Courtship Song

Male signals that females use to choose between mates are expected to be costly for males to produce, making them honest signals of male quality (Zahavi 1975). The reduced propensity to sing in males with implants compared with those without suggests that the nylon insertion procedure is indeed costly. This procedure involved rapidly cooling the animals, inserting the implant and taking a haemolymph sample. The reduced propensity to sing could be the result of any or a combination of these factors. We are treating all of these factors (bar the brief cooling) as an immune challenge requiring a response from the individual. Previous work on crickets has shown that sham injections create only a small and transitory immune response (da Silva et al. 2000). It seems unlikely that the very brief (2 min starting at around 25°C) cooling period could influence calling behaviour 24 h later, given that these insects will be exposed to low night-time temperatures in their natural environment. However, the lack of a control for possible effects of cooling means we cannot rule out this possibility. If we take the view that the substantial immune response induced by our procedures will have a greater effect than other aspects of the protocol, our result is in accord with previous work which has shown that males infested with parasites are less likely to call (Zuk et al. 1995) and up-regulation of the immune system comes at a cost to a male's ability to call (Jacot et al. 2004, 2005) and with a host of studies indicating general costs of immune response (Zuk & Stoehr 2002; Schmid-Hempel & Ebert 2003).

If males that are mounting an immune response cannot court females as readily, this hampers their reproductive success even if they have been able to call and attract a female from a distance. The demand by females that males must produce the courtship song in addition

to the calling song might therefore indicate a secondary level of screening that could be beneficial to females, both as a signal of overall male quality ('condition') and because males that are mounting an immune response may be inferior mates for direct reasons such as the possibility that they produce lower-quality ejaculates (Simmons & Roberts 2005) or can be a potential source of infection. The main costs of expression of acoustic signals are borne at the point when females are assessing a potential mate (in contrast to morphological traits which may have major developmental costs), and hence we would expect courtship song to respond to manipulations of demands made on males. It would be interesting to determine whether calling song is more or less affected by immune challenge than courtship song since it is possible that the two signals convey different information to females.

The differences between songs of pre- and postchallenge males differed from the pattern of differences between high and low encapsulation ability suggested by the principal component loading shown in Table 1. Both males with low encapsulation ability and postchallenge males tended to sing a shorter trill before the first break in the song. However, while males with low encapsulation ability tended to have longer gaps between trill pulses and a longer first five trill pulses, postchallenge males tended to have shorter gaps between trill pulses and a shorter first five trill pulses. These differences indicate that females may be able to use various aspects of the courtship song to provide different information about males. Given the negative correlations between encapsulation ability and lysozyme-like activity, there may be trade-offs between immune functions and other traits, making it misleading to characterize males with high encapsulation abilities alone as being of overall high quality. Because a large proportion of males failed to sing after immune challenge the comparison of songs before and after challenge involves substantial 'self-selection' by males; however, because our analysis used paired comparisons we can be confident that male songs have changed after the immune challenge, rather than the possibility that only males of particular song types sang after the challenge.

Female Responses to Courtship Songs

Our analysis of temporal aspects of the courtship song of *T. oceanicus* suggests that the nature of courtship song variation between males is such that females might reasonably be expected to distinguish between males on the basis of their overall duty cycle and the length of the trill they produce. The aim of our female response assay was primarily to test the hypothesis that females respond differentially to male courtship songs according to their temporal properties. Our assay used a biologically appropriate female response (Shackleton et al. 2005), the latency to orient for mating, rather than measuring female preferences for either of two simultaneous male songs (Wagner & Reiser 2000; Rantala & Kortet 2003). Wild females are unlikely to have to choose between courting males, in this and related species, since males

generally defend calling sites and do not tolerate the presence of rivals (Cade 1981; Simmons 1988; Campbell 1990). It is clear from Fig. 4 that courtship songs differ in the extent to which they stimulate females to orient for mating, and hence they are likely to be under directional selection.

A secondary aim of our assay was to compare attractiveness of songs that might differentiate between males with high and low immune function. By using a single natural song and manipulating it to produce high- and low-quality songs we could ignore extraneous variation in song features and use females as replicates in our analysis (McGregor 1992; McGregor et al. 1992). Our comparison was of songs that resemble those of males that are capable of a strong versus a weak immune response. It would also be possible to compare the response of females to songs of males actually engaged in an immune response, but it is not clear what the prediction would be: females might prefer males with strong responses or might be selected to avoid males that are clearly sick. We found that the songs we created as being typical of males with a good encapsulation response were preferred by females, as would be expected if females use courtship song as a signal of immune function, and as found by Rantala & Kortet (2003). Encapsulation response covaries positively, both phenotypically and genetically, with sperm viability (Simmons & Roberts 2005) suggesting a direct benefit for females with such a preference. However, further study of this aspect would be valuable for a number of reasons. First, we found only a weak correlation between one aspect of immune function, encapsulation ability, and the first principal component of song variation and this was rendered nonsignificant by the removal of one individual, or by inclusion of age into the analysis. As discussed above, there is a negative correlation between this aspect of immune response and lysozyme-like activity, suggesting that females that discriminate in favour of one of these abilities are discriminating against the other. Second, our comparison of the songs of males before and after physiological challenge reveals a different pattern of variation in song, relative to that in our artificial high-quality and low-quality songs. Postchallenge males tended to have shorter trill pulse intervals (as in our high-quality songs), but a shorter trill prior to the first break in singing (as in our low-quality songs). Further work is needed to determine the courtship song parameters to which females respond differentially, and to elucidate the connection between information contained in calling song and courtship song.

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