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Courtship song and immune function in the field cricket *Gryllus bimaculatus*

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It has been assumed that sexual ornaments have evolved to reveal males' health and vigour for females. Choosy females may indirectly use ornaments as an indicator of the presence and effectiveness of genes for resistance against parasites. In this study we tested whether females of the Mediterranean field cricket, *Gryllus bimaculatus,* can use courtship song as a cue for choosing males with high immunocompetence, measured as encapsulation rate of nylon implants and lytic activity of haemolymph. We found that female crickets preferred courtship songs from males with a high encapsulation rate. Female crickets also had a tendency to prefer courtship songs with high tick rate and long high-frequency tick duration. These preferred song components were positively correlated with encapsulation rate, but negatively correlated with lytic activity of the male. In contrast to previous studies of crickets, there was no correlation between male weight and encapsulation rate or lytic activity. There is some evidence in another cricket species that the ability to encapsulate pathogens is heritable. Thus, in light of this study it seems possible that by preferring males according to their courtship song, females might benefit by increasing the parasite resistance of their offspring.

ADDITIONAL KEYWORDS: immunocompetence – Phonotaxids – sexual ornaments – sexual selection.

INTRODUCTION

Hamilton & Zuk (1982) proposed that the sexual ornaments on which a female bases her choice reveals a male's health and vigour, which can be affected by parasites. Females may therefore indirectly select for genetic resistance to parasites. Many observational and experimental studies support Hamilton & Zuk's hypothesis (e.g. Møller, 1988, 1990; Clayton, 1990; Milinski & Bakker, 1990; Zuk *et al*., 1990a,b; Houde & Torio, 1992; Saino & Møller, 1996; Taskinen & Kortet, 2002; for reviews, see Clayton, 1991; Møller & Saino, 1994), but no such relationship was found in many other studies (for review see Hamilton & Poulin, 1997).

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The immunocompetence handicap hypothesis offers a mechanistic extension to Hamilton & Zuks' theory (Folstad & Karter, 1992; Wedekind & Folstad, 1994). According to this hypothesis, sexual trait expression may be constrained through a trade-off with immune function. The hypothesis suggests that secondary sexual characters develop in response to circulating androgens (or other self-regulating biochemicals that increase the expression of secondary sexual characters but reduce the functioning of the immune system), which suppress the ability of individuals to raise an immune defence against parasites (Folstad & Karter, 1992). The immunocompetence handicap hypothesis argues that because the production of a secondary sex trait is costly, animals may have to shift energy and metabolites from other tissue to produce well-developed secondary sex characters. Sex hormones may accomplish this by shutting off energy from the immune system and other systems, so that it can be

redirected for production of secondary sex traits (Folstad & Karter, 1992). The 'good genes' which females might acquire by choosing highly ornamented males might be genes affecting immunocompetence directly or genes having more or less of an indirect effect on the immune system via body condition, for example, genes affecting the rules by which resources are allocated to the immune system, the cost of ornament expression, or resource acquisition (Westneat & Birkhead, 1998).

Male sexual ornaments have often been shown to be strongly dependent on condition (e.g. Hill, 1992; Gustafsson, Qvanström & Sheldon, 1995; Hunt & Simmons, 1997; David *et al*., 1998; David *et al*., 2000). Since sexual ornaments are often costly to produce and maintain (e.g. Kotiaho, 2001), only individuals in good condition can afford to produce extravagant sexual ornaments and, at the same time, have a sufficient amount of resources left for the immune system. This might lead into a positive correlation between male immunocompetence and sexual advertisement.

The term immunocompetence is often used to refer to the ability of an individual's immune system to resist and control pathogens or parasites. In insects, one of the most informative ways to assay immunocompetence is to measure the magnitude of the cellular encapsulation response to a novel and standardized antigen such as a nylon monofilament (e.g. Köning & Schmid-Hempel, 1995; Rantala *et al*., 2000; Siva-Jothy, 2000; Ryder & Siva-Jothy, 2000; Rantala *et al*., 2002). Encapsulation is a cellular response through which insects defend themselves against multicellular pathogens such as nematodes and parasitoids (Gillespie, Kanost & Trenczek, 1997), but it also plays a role in defence against viruses (Washburn, Kirkpatrick & Volkman, 1996). In cellular response, circulating cells in the haemocoel recognize an object as foreign and form a capsule that melanizes and hardens. This results in the death of the intruder by asphyxiation (Fisher, 1963) or through the production of necrotizing compounds (Nappi *et al*., 1995). The humoral system, on the other hand, is comprised of a myriad of soluble proteins and enzyme cascades, which play important roles in recognizing, signalling and attacking foreign targets (Leonard, Ratcliffe & Rowley, 1985) and probably in coordinating the cellular responses (Perch & Strand, 1995). Therefore, in this study we estimated immunocompetence by measuring the encapsulation rate against a novel antigen and the haemolymph concentration of an antibacterial enzyme, lysozyme.

The Mediterranean field cricket, *Gryllus bimaculatus* De Geer, is widely distributed in southern Europe, where it frequently occurs in high densities. Male *G. bimaculatus* produce calls that act as signals for mate attraction and repulsion of rivals (Simmons, 1988). Male crickets are known to produce three types of acoustic signal: (1) an aggressive song during encounters with other males, (2) a long-range calling song used to attract females from a distance, and (3) a courtship song used just prior to mating (Alexander, 1961). In the laboratory, it has been shown that in male *G. bimaculatus* body size is positively correlated with call intensity and syllable repetition rate (Simmons, 1988), but in the field, the syllable rate was negatively related to male size, while the duration of syllables was positively related to size (Simmons & Zuk, 1992). Females are sensitive to variation in syllable rate (Doherty, 1985; Schildberger, 1985), preferring calls with higher syllable rates (Shuvalov & Popov, 1973; Simmons, 1988).

The aim of this study was to test whether male song transmits information about male immunocompetence to females. In contrast to previous studies in *G. bimaculatus* (e.g. Simmons, 1988; Simmons & Zuk, 1992), in this study we recorded the courtship song of males, since the presence of females provides a strong incentive to males to maximize their display, and thereby potentially reveal differences in quality (see Gray & Eckhardt, 2001). Furthermore, the courtship song is known to be approximately two and a half times more energetically demanding than is the calling song in *Acheta domesticus* (Hack, 1998) and so it may be constrained by the male condition.

MATERIAL AND METHODS

Insects

The crickets used in this experiment were the first laboratory generation of wild collected animals from Costa del Sol in Southern Spain, maintained at 29 ± 1°C with *ad libitum* food and water under a 12 : 12 h light/dark photoperiod. Experimental crickets were derived from a bulk laboratory stock as nymphs and maintained individually (also with *ad libitum* food and water) in covered plastic containers (1 L). Sexes were physically (but not acoustically) isolated, and thus virgin. All males were 8 days old on the day of the experiments. Before the experiments, we weighed the fresh body mass of crickets to the nearest 0.01 g. No cricket was used in more than one experiment.

Courtship song recording

All song recordings were performed at a constant temperature (28 ± 1°C) during the first hour of darkness under red lighting using a Sony TCD-D7 (DAT recorder) attached to a telinga microphone in a sound-insulated chamber. To stimulate males to play their courtship song, we placed two virgin females

into a male's container. Females were tied to prevent them from mating with the male, allowing longer courtship song recording. Extended courtship is more likely than short courtship to reveal condition or energetic effects on song (see Gray & Eckhardt, 2001). The courtship song of the male was recorded for 10 min.

Song analysis

We measured four characters of the courtship song (see Fig. 1) using Cool Edit Pro (Syntrillium Software Corporation). These were: number of high-frequency (HF) ticks per second; number of low-frequency (LF) ticks per second; high-frequency tick duration; phrase lengh. For each male we randomly pick up ten samples of 5 s of courtship song to be analyzed. The average value of each song character for each male was used in the analysis.

Encapsulation rate assay

After the song recording, males were chilled on ice for 20 min after which a 2-mm long piece of nylon monofilament (diameter 0.18 mm, rubbed with sandpaper) was inserted through a puncture in the pleural membrane between the second and third sternite. The male's immune system was allowed to react to this object for 5 h, while crickets were kept individually in plastic vials at constant room temperature (28 ± 1°C). The implant was then removed and dried. The removed monofilament was photographed under a light microscope with a digital video recorder from three different angles. The pictures were then analyzed using the Image Pro program. The degree of encapsulation was analyzed as grey values of reflecting light from implants. As a measure of encapsulation rate, we used the average of the grey values of three video pictures. Because a smaller grey value indicates higher encapsulation rate, figures would be counter-intuitive. For this reason we transformed the data such that the darkest grey values correspond to the highest encapsulation rate. The transformation was done by subtracting the observed grey values from the control grey value (clear implant). To measure the repeatability of this method we scanned again 16 randomly chosen implants and analyzed them as above. The repeatability (R) of this method was high ($R = 0.997$; $F_{15,16} = 778.69$, $P < 0.001$).

Lysozyme assay

After the encapsulation rate measurements we collected 10 μL haemolymph from each male from the puncture at their abdomen. We assayed lysozyme activity of haemolymph against *Micrococcus luteus* turbidometrically using a method modified from Ellis (1990). We mixed 200 μL 0.35 mg mL^{-1} freeze-dried *M. luteus* buffered (pH 6.4) solution with 50 μL 1 : 4 buffered haemolymph (pH 6.4) in a plastic multicuvette (Labsystems cliniplate). The mixture absorbance at 492 nm was then measured at 20°C in 1-min intervals for 30 min with a plate

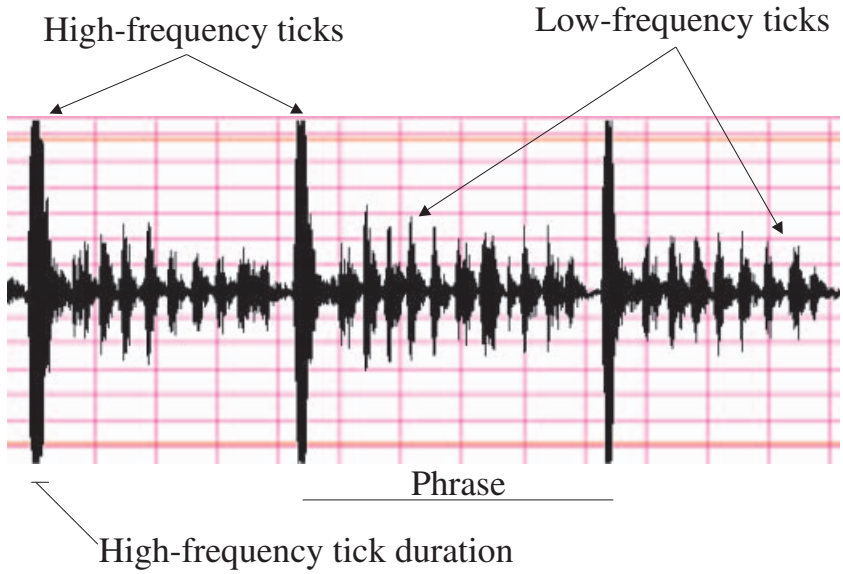

Figure 1. Waveform representation of the courtship song of *Gryllus bimaculatus* showing the song characters described in the text.

reader (Multiskan Plus, Labsystems, Finland). The lytic activity was expressed as total change in absorbance. Because smaller absorbance values indicate higher lytic activity, we transformed the scale (by multiplying it by −1), so that the results would be more intuitive (i.e. higher values indicating higher activity).

Female choice trial

The female choice trials were conducted in a plastic arena measuring 110 × 110 × 45 cm. Before the experiment, a virgin female cricket (>14 days old) was placed under a glass vial in the centre of the arena, equidistant (40 cm) from each speaker, where she acclimated for 15 min before the start of each experiment. At the start of a experiment, we removed the glass vial. Two songs from weight-matched males, whose immunocompetence had been measured, were broadcast simultaneously, one from each speaker, through 20-cm speakers placed 180° apart (Multimedia speaker SP-628). No attempts were made to change song volume; all recordings were played back at equal (original) levels. The first speaker physically touched (including antennae) by a female was scored as her choice. If a female made no choice within 4 min, we discontinued the trial. No female was used in more than one experiment. In total, there were 19 trials using songs from two weight-matched males. Statistical analyses were performed using SPSS Advanced Statistics 10.0 (SPSS Inc. Chicago, USA).

RESULTS

There was a tendency for females to prefer courtship song with intense HF rate (paired samples t-test, $t = 1.9$, d.f. = 18, $P = 0.072$) and long HF duration (paired samples t-test, $t = 2.038$, d.f. = 18, $P = 0.057$). The LF rate did not differ between preferred and not preferred courtship songs (paired samples t-test, $t = 1.104$, d.f. = 18, $P = 0.258$) nor did the phase length (paired samples t-test, $t = -1.58$, d.f. = 18, $P = 0.131$. Female crickets preferred the courtship song of males with stronger encapsulation response (paired samples t-test, $t = 2.70$, d.f. = 18, $P = 0.015$) in 15 of the 19 trials (Fig. 2). Females did not prefer the courtship song of males with higher lytic activity (Wilcoxon signed rank test, $Z = -1.26$, $N = 19$, $P = 0.209$) (Fig. 3). Since males were weight-matched in this experiment, male body weight had no effect on female choice (paired samples t-test, $t = -1.380$, d.f. = 18, $P = 0.185$).

The HF rate, HF duration and LF rate were positively correlated with encapsulation rate, but negatively correlated with lytic activity (Table 1). In addition, phrase length correlated negatively with encapsulation rate but positively with lytic activity (Table 1). Encapsulation rate and lytic activity correlated negatively with each other (Spearman's $r = -0.34$, $N = 38$, $P = 0.037$). There was no correlation between male weight and encapsulation rate (Spearman's $r = -0.257$, $N = 38$, $P = 0.119$), or with the lytic activity (Spearman's $r = 0.168$, $N = 38$, $P = 0.313$).

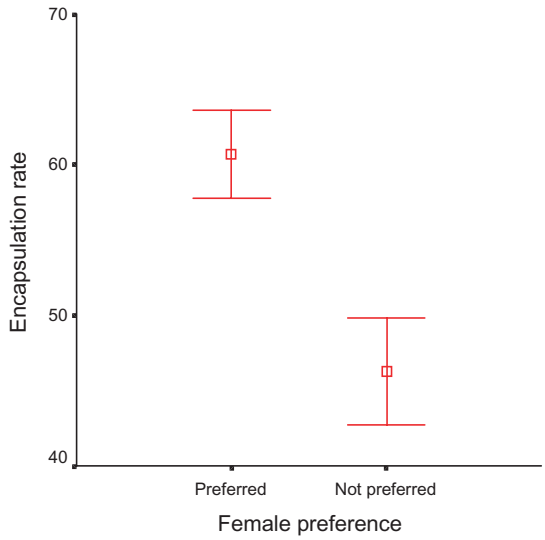

Figure 2. Mean ± SE encapsulation rate (darkness value of the implant, arbitrary unit) of preferred ($N = 19$) and not preferred ($N = 19$) males.

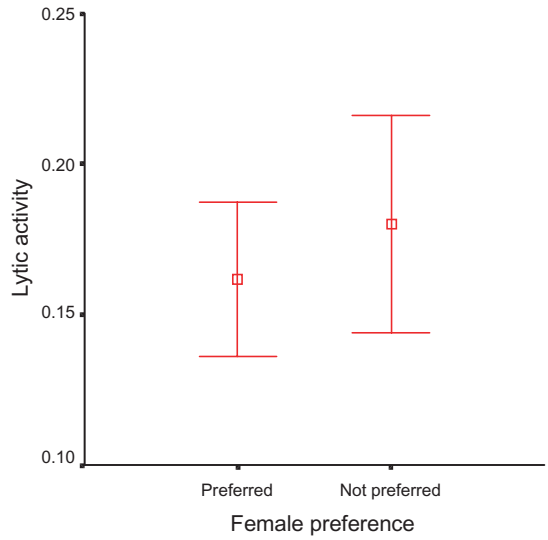

Figure 3. Mean ± SE lytic activity (change in the absorbance values, arbitrary unit) of preferred ($N = 19$) and not preferred ($N = 19$) males.

Table 1. Correlations between song characteristics and immune functions

	Encapsulation rate		Lytic activity	
Song character	*r*	*P*	*r*	*P*
High-frequency tick rate	0.34	0.040	–0.34	0.036
High-frequency tick duration	0.32	0.047	–0.37	0.021
Low-frequency tick rate	0.35	0.030	–0.32	0.048
Phrase	–0.30	0.070	0.38	0.019

$N = 38$ males for each group.

DISCUSSION

It has been found that courtship song is important for eliciting acceptance behaviour (copulatory mounting) in female *G. bimaculatus* (e.g. Von Hörmann-Heck, 1957; Libersat, Murray & Hoy, 1994). Our finding that female *G. bimaculatus* preferred courtship song with high tick rate and long high-frequency tick duration is consistent with the study of Libersat *et al*. (1994), who found that high-frequency components of the natural courtship song are the critical frequency components of a successful song and that the less intense, low-frequency component is neither necessary nor sufficient for eliciting mounting. We found that female crickets preferred courtship song characters which were positively correlated with encapsulation rate. This is consistent with previous findings in insects which have shown that male sexual ornaments are positively correlated with male encapsulation rate. For example, in the damselfly, *Calopteryx splendens*, it has been shown that size of the sexually selected ornament, wing spots, is correlated with male encapsulation rate, possibly due to the fact that both the immune response and the production of wing spots rely on the same phenoloxidase enzyme cascade (Rantala *et al*., 2000; see also Siva-Jothy, 2000). Similarly, in the mealworm beetle, *Tenebrio molitor*, it has been found that females prefer pheromones from males with a high encapsulation rate (Rantala *et al*., 2002). Interestingly, in *Acheta domesticus*, Ryder & Siva-Jothy (2000) did not find any correlation between males' calling song parameters and encapsulation rate, although there was a positive correlation between the number of syllables per chirp and haemocyte load. However, to measure encapsulation rate they used capsule volume and we used melanization rate of implant, which might explain the different results. Moreover, we used male courtship song, which is more likely to be an honest indicator of male quality than calling song, since the courtship song demands more energy (Hack, 1998).

A number of studies have shown that variation in encapsulation ability and haemocyte load can be heritable in insects (e.g. Carton & Boulétreau, 1985; Carton, Frey & Nappi, 1992; Kraaijeveld & Godfray, 1997; Fellowes, Kraaijeveld, & Godfray, 1998; Kraaijeveld, Limentani & Godfray, 2001; Ryder & Siva-Jothy, 2001) and Kurtz and Sauer (1999) detected heritable variation in lytic activity and phagocytosis ability. Thus, by preferring the courtship song from males with a high encapsulation rate, female crickets may benefit indirectly by increasing parasite resistance of their offspring if encapsulation rate is heritable in this population.

It is possible that both encapsulation rate and characters preferred by females in courtship song are dependent on condition, explaining female preference for courtship songs from males with a stronger encapsulation rate. Males of higher quality may obtain more nutrients and may be able to invest more in both encapsulation and signal production. There is ample evidence for condition-dependent expression of secondary sexual characters (review in Andersson, 1994; Johnstone, 1995). However, whether the courtship song of *G. bimaculatus* is condition-dependent is not known. There is also some evidence in insects that immune function is, at least partly, condition-dependent. For example, in *Drosophila melanogaster* there is a nutrient-mediated effect on the genetic ability of individuals to respond with melanin production to immune insult (Sang & Burnet, 1963). In *Anopheles gambie* (Diptera: Culicidae) larval nutrition affects the ability of adults to respond to synthetic immune challenge (Suwanchaichinda & Paskewitz, 1998) and in *Drosophila melanogaster* nutritional status affects resistance to parasitoids (Vass & Napi, 1998). However, the opposite effect has been demonstrated in noctuids, in which increased nutritional intake enhanced the negative effects of baculoviruses (Hoover *et al*., 1998).

Surprisingly, the courtship characters preferred by females were negatively correlated with male lytic activity. Likewise, encapsulation rate was negatively correlated with lytic activity. The causal mechanism behind this negative correlation is not clear. Perhaps those animals which had high lytic

activity were suffering from bacterial disease and thus forced to allocate more energy to humoral immune function at the expense of cellular immune function (encapsulation). However, we did not find any signs of bacterial disease in our experimental animals.

Active males playing a lot of calling and courtship songs are more likely to be confronted with fights and even to be wounded (Simmons, 1986a). A good immunological response helps them to resist invasions of pathogens into their wounds. Thus, the courtship song may be a reliable indicator of male quality (see, e.g. Grafen, 1990).

Ryder & Siva-Jothy (2001) found that in the house cricket, *Acheta domesticus*, the ability to encapsulate a synthetic substrate, haemocyte load and body size were positively genetically correlated. Thus, they concluded that by favouring males that produce calling songs with the preferred characteristics, females produce larger offspring with a greater ability to produce an encapsulation response. In previous studies with *G. bimaculatus*, body size has been shown to have an important implication for male competitive ability and females mate selectively on the basis of male body size (Simmons, 1986a; Simmons, 1986b). Surprisingly, in this study we did not find any correlation between immune parameters and male size. In previous studies on *G. bimaculatus* we found that crickets which develop faster have a higher encapsulation rate but smaller body size than do more slowly developing crickets (M.J. Rantala *et al*., unpubl. data). Perhaps there is a trade-off between immunocompetence, developmental time and body size, which might reduce the advantage of large size.

In conclusion, this study has shown that courtship song characters preferred by females are positively correlated with male encapsulation rate. Thus, by preferring males according to their courtship song, females could avoid infection during copulation and might benefit indirectly, if the encapsulation ability is heritable in this species of cricket, by increasing the encapsulation ability of their offspring.

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