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Immune function reflected in calling song characteristics in a natural population of the cricket *Teleogryllus commodus*

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Secondary sexual traits have been suggested to provide reliable signals of a male's ability to resist infection by agents of disease. The immunocompetence handicap hypothesis provides a potential mechanism for reliable signalling in the form of a trade-off between expenditure on trait expression and expenditure on immunity. Thus, males resistant to disease can spend more resources on their sexual signals. Examination of calling song parameters in a natural population of the cricket *Teleogryllus commodus* revealed that males scoring higher on the third principal component for song had significantly lower ability to encapsulate a foreign object. This component of immune function was associated with syllables of longer duration in both the trill and chirp elements of the song. Males with longer syllables in their song had a lower encapsulation ability. Syllable duration is known to influence phonotaxis by female *T. commodus*. Although the effect was only weak, our data suggest that females may base their choice of mate on reliable information contained within the temporal properties of male calls. Our study thus demonstrates a connection between sexual signalling and immune function in a natural population of insects and lends support to the immunocompetence handicap hypothesis.

Theory suggests that male secondary sexual traits can become condition dependent, and that females can gain indirect genetic benefits for their offspring by using secondary sexual traits to choose mates of high quality (Andersson 1994). Secondary sexual traits are assumed to carry a cost to their bearer, so that males of low condition cannot invest in trait development (Kotiaho 2001). A particularly compelling aspect of condition is resistance to disease, and several recent studies have found that secondary sexual traits are both costly to produce and reflect either parasite infection or immune function (e.g. Blount et al. 2003; Faivre et al. 2003). In vertebrates, the causal link underlying honest signalling is thought to be the dualistic effect of testosterone on secondary sexual trait expression and immunosuppression; only males able to resist disease can afford to downregulate their immune systems and develop exaggerated sexual traits (Folstad & Karter 1992). While these arguments are compelling, a recent meta-analysis suggests that in general the link between testosterone and immune response is weak (Roberts et al. 2004).

Insects possess a less complex immune system than vertebrates, but none the less may demonstrate both condition dependence of secondary sexual traits and a cost of mounting and maintaining an immune response (Rolff 2002). Juvenile hormone has a dualistic effect on sexual signalling and immunosuppression suggesting that it may be the hormonal analogue to testosterone that maintains honesty in insect sexual signalling (Rantala et al. 2003b). Insects are increasingly popular subjects for studies in ecological immunology (Rolff & Siva-Jothy 2003). An advantage to using insects is that they lack a well-developed immunological memory (Vass & Nappi 2001), so that there is less risk of confusing prior exposure with resistance; individuals that exhibit a strong response to an antigen cannot merely be indicating a past exposure to the agent. Secondary sexual traits such as wing pigmentation in damselflies (Siva-Jothy 1999, 2000; Rantala et al. 2000), courtship song in field crickets, *G. bimaculatus* (Rantala & Kortet 2003), calling song in house crickets, *Acheta domesticus* (Ryder & Siva-Jothy 2000), and pheromones of mealworm beetles, *Tenebrio molitor* (Rantala et al. 2003a), all show correlations with female preference and male immune function. Furthermore, in scorpionflies,

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Panorpa vulgaris, males producing more salivary masses during courtship are preferred by females and have a higher immune function that is inherited by their offspring (Kurtz & Sauer 1999).

Although studies of damselflies and dragonflies have assessed immune function in natural populations (Rantala et al. 2000; Siva-Jothy 2000; Rolff 2001), studies of immunity and sexual signalling in crickets have been confined to laboratory populations that are highly unlikely to be exposed to the types of parasites and diseases normally encountered in nature. Thus, it is not clear whether laboratory findings can be extrapolated to natural populations. We examined immune function in a field population of *Teleogryllus commodus*, a common field cricket of southern Australia that is subject to numerous micro- and macroparasites, ranging from fungi to parasitoid wasps (Reinganum et al. 1981). Like other crickets, *T. commodus* produces a calling song to attract females (Evans 1983, 1988), and laboratory studies have characterized the qualities of the male song that elicit the strongest female response (Hennig & Weber 1997). To measure immune response in the field, we assayed encapsulation ability (Nappi & Vass 1993; Gillespie et al. 1997) by quantifying the degree to which implants of nylon monofilament were encapsulated with melanized haemocytes. This is a standard technique for examining insect immunity (König & Schmid-Hempel 1995; Stolen et al. 1995; Rantala et al. 2000, 2003a; Doums et al. 2002; Rantala & Kortet 2003). It mimics the effects of a parasitoid or other foreign object that is introduced into the body cavity, but because the implant is inert, it does not have any pathogenic effects itself. By recording songs of males in the field and then immediately assessing immune response, we were able to determine whether songs could reflect immunity under natural conditions and hence be used by females as an indication of disease resistance.

METHODS

Cricket Collection and Song Recording

Crickets were collected in March 2001 near Walpole in south Western Australia. Calling males were localized by ear and recorded using a Sony Professional Walkman. Record levels were standardized at +3 dB peak with the microphone held 10 cm from the calling male. We obtained several minutes of continuous calling from each male, and recorded the temperature at the time of recording by placing a thermometer in the grass within 1–2 cm of the calling cricket. Crickets were collected immediately after recording and used for immune assays.

Melanization Assay of Encapsulation Response

Crickets were cold anaesthetized by placing them in a freezer for 2 min. A fine-gauge hypodermic needle, sterilized with ethanol, was used to puncture the ventral intersegmental membrane between the first and second abdominal segments. A 3-mm length of nylon monofilament 0.255 mm in diameter was implanted into the body cavity through the wound. The surface of the monofilament was roughened 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. There was no mortality associated with the procedure. Crickets were frozen 36 h after implantation.

The implant was dissected from the body cavity and cleaned with 70% ethanol. It was then placed in a cavity slide, fixed with mounting medium (Eukitt) and covered with a cover slip. To measure the degree of encapsulation, we photographed each implant with a digital camera attached to a microscope and analysed the photograph using NIH Image software (http://rsb.info.nih.gov/nih-image/). 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 an identical area in the same slide next to the implant. The measurement used was the mean darkness of the implant minus the mean darkness of the control area. Melanization is closely linked to disease resistance and immunity in insects (Nappi & Vass 1993; Barnes & Siva-Jothy 2000; Wilson et al. 2001). Furthermore, the degree of melanization of encapsulating haemocytes is positively correlated with other measures of immune function, such as the density of circulating haemocytes (Rantala et al. 2000) and phenoloxidase activity (Rantala et al. 2002).

During dissection, the body cavity and gut were checked thoroughly for the presence of macroparasites. None were found.

Correlations with Other Immune Parameters

To confirm that the degree of melanization of encapsulating haemocytes was a reliable measure of general immune function in *T. commodus*, we collected a random sample of 39 male and 29 female crickets in April 2004 and returned them to the laboratory for immune function assays. For each individual we measured the encapsulation response as described above. At the time of monofilament implant, a 2-μl sample of haemolymph was withdrawn and placed directly into 18 μl of anticoagulant (Mead et al. 1986) and mixed. A sample of 8 μl of haemolymph was then 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× magnification. Total counts were multiplied to find the number of cells per ml of haemolymph.

We also assayed levels of lysozyme-like activity of samples of haemolymph. An autoclaved agar solution (1.5 g per 120 ml) was mixed with a solution of 0.225 g of *Micrococcus lysodekticus* and 0.001 g of streptomycin sulphate in 20 ml of 0.01-M phosphate buffer and incubated at 48°C for 30 min. We placed 15 ml of this mixture into petri dishes 8 cm in diameter (autoclaved) and allowed

them to set at room temperature for 30 min before placing them in a fridge until use. 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.

Song Analysis

Recordings were analysed using Canary 1.2 for the Macintosh (Laboratory of Ornithology, Cornell University, Ithaca, NY, U.S.A.). *Teleogryllus commodus* has a complex song consisting of two parts, the trill and the chirp (Fig. 1). For each male, we selected an uninterrupted sequence of 10 songs from the recording, and measured 11 song variables (Fig. 1). We used the average value from 10 songs for each variable. All means are presented ± 1 SE.

RESULTS

Males had a greater number of circulating haemocytes than females (males: 19.3 ± 0.9 ($\times 10^4$) cells/ml; females: 14.0 ± 1.1 ($\times 10^4$); $t_{66} = 3.88$, $P = 0.0002$) and tended to have lower lysozyme-like activity (males: 17.6 ± 0.7 mm^2; females: 19.4 ± 0.8; $t_{66} = 1.65$, $P = 0.103$) and a greater encapsulation response (males: 115.0 ± 3.2 grey scale; females 106.8.4 ± 3.7; $t_{66} = 1.67$, $P = 0.099$). For both males and females, there were strong and significant associations between encapsulation scores and the density of circulating haemocytes in the haemolymph (Pearson correlations: males: $r_{37} = 0.775$, $P < 0.0001$; females: $r_{27} = 0.454$, $P = 0.013$). There was no correlation between encapsulation scores and lysozyme-like activity for either sex (males: $r_{37} = 0.022$, $P = 0.896$; females: $r_{27} = -0.237$, $P = 0.216$).

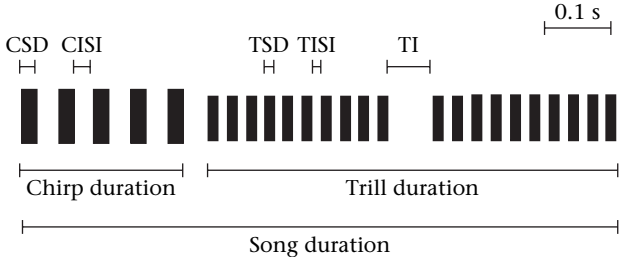

Figure 1. Stylized diagram of the calling song of *Teleogryllus commodus*. The song has two major elements, the chirp and the trill. We measured 11 song variables: the duration of the song, the interval between successive songs, the duration of the chirp component, the duration of syllables within a chirp (CSD), the interval between syllables within a chirp (CISI), the total number of syllables in the chirp, the mean duration of trills (calculated as trill duration–mean trill interval (TI) divided by the number of trills in the song), the number of individual trills, the number of syllables within trills, the duration of syllables within trills (TSD), and the interval between syllables within trills (TISI).

The mean encapsulation score for the males in the field study was 201.3 ± 3.7 (range 126–240). Table 1 shows the mean values for the 11 song components. Intersong interval, chirp duration, trill number and syllables per trill were not normally distributed and were $\ln(x + 1)$ transformed for analysis. The temperature range during recordings was 16–24°C. The temporal properties of cricket song show strong linear temperature dependence (Walker 1962). We therefore present partial correlations between the 11 song variables and encapsulation response in Table 1, controlling for variation in the temperature at which songs were recorded. Two song parameters were correlated with the encapsulation response: males with shorter syllables within both the chirp and trill components of the song had a stronger encapsulation response (Table 1). We note that the 11 univariate tests in Table 1 suffer from an elevated risk of type I error. Therefore, we have not based our conclusions on these individual correlations, but instead adopted a multivariate approach to hypothesis testing.

Not surprisingly, there were strong patterns of covariation between the 11 song variables. Principal components analysis (PCA) was therefore used to summarize the patterns of correlation among the 11 song variables, and to produce a reduced number of new variables or factors that described independent elements of variation in the song. We standardized song parameters by taking the residuals from regressions of each song component on temperature and used these standardized variables in our PCA. This yielded four factors with eigenvalues greater than 1.0, which accounted for 81% of the total variation in the 11 song variables (Table 2). The first principal component (PC1) represented the major source of variation (40%) among songs in this sample, having a high positive correlation with song duration, the interval between songs, the duration of the chirp element, and the number and total duration of trills in the trill element. Thus, variation in PC1 was primarily associated with variation in the overall length of a song and its contributing elements. PC2 accounted for 17% of the variance among songs, and indicated a trade-off between increasing number of syllables in the trill and decreasing number of trills in a song. PC3 accounted for 15% of the variance among songs and was associated primarily with the duration of syllables in the song, both in the chirp and trill elements. Finally, PC4 accounted for 11% of the variance among songs and contrasted the number of syllables per chirp with the intervals between syllables within both the chirp and trill elements.

Collectively, the four song principal components accounted for 15.8% of the total variation among males in encapsulation scores (multiple regression, whole model: $F_{4,54} = 2.53$, $P = 0.051$). Because the statistical significance of the whole model was borderline under the convention of $P < 0.05$, we also provide Cohen's (1988) standardized effect sizes and their 95% confidence intervals to assess the biological significance of the effects (Colegrave & Ruxton 2003). The overall effect size was moderate (0.414) and we can reject at $P < 0.025$ an effect size smaller than 0.246 and larger than 0.582. Males scoring higher on the third principal component (syllables

Table 1. Observed mean and variance in 11 song variables recorded from 58 *Teleogryllus commodus* and their partial correlations ($df = 56$) with encapsulation response (er) and recording temperature (t)

	Mean	Range	SE	r_{er}	P_{er}	r_t	P_t
Song duration (s)	2.0	0.7–3.2	0.1	0.095	0.476	−0.128	0.337
Intersong interval (ms)	296.0	119.4–896.1	16.3	−0.012	0.930	**−0.360**	**0.006**
Chirp duration (ms)	436.4	265.1–688.0	12.2	0.116	0.619	**−0.304**	**0.027**
Chirp syllable duration (ms)	37.9	30.1–46.4	0.5	**−0.282**	**0.026**	−0.062	0.645
Chirp syllable interval (ms)	29.0	16.3–44.1	0.8	−0.043	0.749	−0.440	**0.001**
Syllables per chirp	6.9	4.9–10.6	0.2	0.161	0.221	−0.073	0.585
Trill duration (s)	1.5	0.2–2.6	0.1	0.094	0.481	−0.089	0.509
Trill number	2.6	1.0–5.6	0.1	0.180	0.176	0.011	0.932
Trill syllable duration (ms)	28.7	22.4–36.3	0.4	**−0.270**	**0.034**	−0.147	0.271
Trill syllable interval (ms)	15.7	7.7–25.5	0.5	−0.046	0.731	**−0.419**	**0.001**
Syllables per trill	11.9	4.8–34.1	0.7	−0.185	0.166	0.084	0.531

Significant correlations are shown in bold type.

of longer duration in the trill and chirp elements) had lower encapsulation scores ($F_{1,54} = 5.11$, $P = 0.017$, standardized effect size {95% confidence interval} 0.32 {0.17, 0.47}; Fig. 2). The effects of the remaining PCs were not statistically significant (PC1: $F_{1,54} = 0.26$, $P = 0.609$, effect size 0.07 {0.00, 0.14}; PC2: $F_{1,54} = 1.34$, $P = 0.252$, effect size 0.15 {0.05, 0.25}; PC4: $F_{1,54} = 2.49$, $P = 0.120$, effect size 0.21 {0.09, 0.33}). Stepwise removal of non-significant predictors resulted in a model including just PC3 which explained a significant proportion of the variance in encapsulation response ($r^2 = 0.094$, $F_{1,57} = 5.90$, $P = 0.018$). Thus, the results of our multivariate analysis were consistent with the univariate tests presented in Table 1.

Table 2. Principal components analysis of 11 song variables of *Teleogryllus commodus* (see Fig. 1)

	Component			
	1	2	3	4
Song duration	**0.44**	−0.10	−0.04	−0.10
Intersong interval	**0.34**	0.01	0.07	0.28
Chirp duration	**0.36**	0.34	0.04	−0.34
Chirp syllable duration	0.10	−0.05	**0.70**	−0.01
Chirp syllable interval	0.28	0.24	−0.21	**0.48**
Syllables per chirp	0.26	0.33	−0.05	**−0.59**
Trill duration	**0.41**	−0.18	−0.05	−0.06
Trill number	**0.33**	**−0.51**	−0.11	−0.09
Trill syllable duration	0.14	0.15	**0.63**	0.18
Trill syllable interval	0.26	0.37	−0.19	**0.40**
Syllables per trill	−0.20	**0.51**	−0.01	−0.09
Variance	4.38	1.84	1.62	1.24
Proportion of total	0.40	0.17	0.15	0.11

Entries are factor loadings (correlation of each variable with each component). For each component, variables with loadings greater than 70% of the largest loading (shown in bold) were considered to contribute significantly to that component (Mardia et al. 1979). Collectively, these components account for 81% of the total variation in song variables.

DISCUSSION

Our results indicate that calling song in *T. commodus* can provide a moderate predictor of the singing male's immunocompetence. Other studies of gryllid crickets have reached similar conclusions. In a laboratory study of calling song in house crickets, *A. domesticus*, Ryder & Siva-Jothy (2000) found that the number of syllables per chirp predicted haemocyte load but not encapsulation ability, even though these parameters are phenotypically and genetically correlated (Ryder & Siva-Jothy 2001). Interestingly, female *A. domesticus* prefer calls with more syllables per chirp (Gray 1997). Similarly, in their study of courtship song in *G. bimaculatus*, Rantala & Kortet (2003) showed that females preferred males with a high rate of ticks and a longer duration of the high-frequency ticks that are a distinguishing feature of the courtship song.

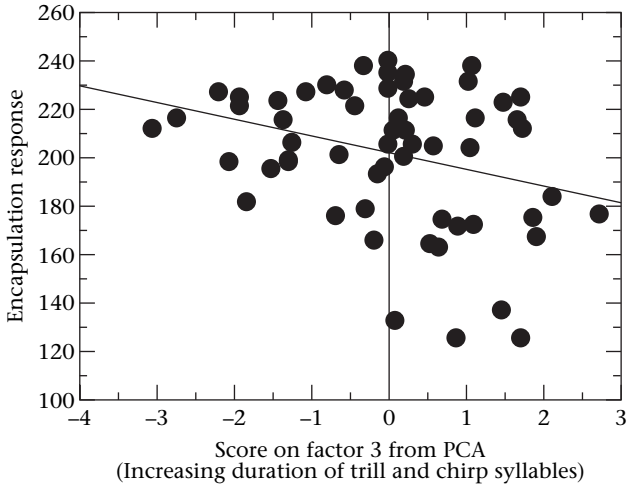

Figure 2. Relation between encapsulation score and the score on the third principal component from a PCA of song variation in *T. commodus*. Encapsulation is measured as the darkness score from 0 (completely white) to 256 (completely black) of a photograph of a nylon implant; see text for details.

These song variables were associated with greater encapsulation response, but lower levels of lytic enzyme activity (Rantala & Kortet 2003).

Ryder & Siva-Jothy (2000) did not find a correlation between encapsulation response and call structure. One possible reason for this discrepancy could be the method used to assess encapsulation ability. Rather than assaying the degree of melanization of the encapsulating haemocytes, they assayed the volume of cellular material encapsulating the implant. Thus, the assay used here and by Rantala & Kortet (2003) may capture an aspect of the encapsulation response, melanization, that is more closely associated with its energetic cost. In general, the encapsulation response has been shown to be an energetically costly aspect of immune function (König & Schmid-Hempel 1995; Freitak et al. 2003). However, it is unclear whether all components of immune function have costs or whether different components can be measured in the same currency (Schmid-Hempel & Ebert 2003). Nevertheless, the fact remains that the degree of melanization of encapsulating haemocytes is positively correlated with the number of circulating haemocytes (Rantala et al. 2000; this study), the immune function variable found to be reflected in calling song by Ryder & Siva-Jothy (2000).

Importantly, our study used field-recorded and collected subjects, which suggests that the variation in song structure that is associated with encapsulation ability in crickets is naturally occurring. Our sample of males was selected at random from the calling population and would have included individuals that by chance alone varied in age, exposure to pathogens, and/or nutritional status. Such parameters are known to influence immune function in crickets (Adamo et al. 2001; Adamo 2004; Zuk et al. 2004). On the one hand, it is perhaps not surprising that we found only a moderate effect size of encapsulation ability on call structure, given the potentially confounding variables present in a field sample. On the other hand, Ryder & Siva-Jothy's (2000) study of house crickets was a laboratory study in which these potentially confounding variables were controlled. Haemocyte load and capsule volume explained a similar proportion of the variation in call structure (17%) as our field study, although their effect size (0.638) was close to our upper 95% confidence limit.

The aspects of calling song that most strongly reflected immune function in *T. commodus* were the durations of syllables within the chirp and trill elements of the song. Importantly, these song traits have been shown to be essential for song recognition by female *T. commodus*, and the female preference functions for each have been quantified. Hennig & Weber (1997) used a locomotion compensator to examine the responses of females to synthesized songs with variations in chirp and trill pulse periods (a pulse period being the combined duration of the syllable and subsequent interval which is thus a combination of PC3 and PC4). Within the natural range of pulse periods (intrachirp 46–90 ms; intratrill 30–62 ms; Table 1), females prefer songs with short intratrill pulse periods, whereas the intrachirp pulse period does not appear to influence female responses (see figure 3 in Hennig & Weber 1997). For both traits, reducing the pulse periods below the natural species range results in songs becoming unattractive (Hennig & Weber 1997). Thus, the preference for intratrill pulse period is not open ended, but rather stabilizing around 30–40 ms at the lower end of the species range. Hennig (2003) found that it was the contribution of pulse duration to the pulse period that was critical for phonotactic preferences of females, the trait most predictive of immunocompetence in our analysis. Hennig & Weber (1997) also examined female preference functions for the absolute numbers of syllables per chirp and trill, finding that songs with more syllables per trill were more attractive to females. This aspect of *T. commodus* song was captured by PC2 but did not reflect encapsulation ability.

Syllable duration and syllable interval reflect the rate at which males draw the plectrum across the file during wing closure (the syllable) and the rate at which they reopen the wings (the interval). Increased wing stroke rates are associated with increased energetic costs for calling in crickets (Prestwich & Walker 1981). Our data thereby suggest that males capable of producing energetically costly calls might also be capable of mounting a greater energetically costly encapsulation response. As such, syllable duration can convey information regarding male quality and is a trait upon which females base their choice of mate. We note, however, that syllable duration accounts for only a small proportion of the total variation in song structure and that it predicted an even smaller proportion of the variation in encapsulation response. The effect, therefore, seems to be rather weak. The complex nature of the song in *T. commodus* makes the potential for female assessment particularly intriguing, because it leaves open the possibility that different elements of the song convey different types of information. Female *T. commodus* also prefer songs with more syllables per trill, a trait that did not reflect immune function. In general, sustained series of syllables, like those in the trill of *T. commodus*, appear to be particularly attractive to females of several species of gryllines as well as to acoustically orienting parasitoid flies (Hedrick 1986; Wagner 1996; Zuk et al. 1998; Gray & Cade 1999; Simmons et al. 2001).

Our results add to a growing body of evidence suggesting that immune function is important in an ecological context (Sheldon & Verhulst 1996; Norris & Evans 2000; Zuk & Stoehr 2002; Schmid-Hempel 2003; Schmid-Hempel & Ebert 2003). They lend weak support to one component of the immunocompetence handicap hypothesis, that a secondary sexual trait is revealing of immunocompetence. However, as noted above, cricket immune function varies widely with a number of life history and ecological variables and it is possible that covariation between calling song and these variables may underlie the observed associations between immunocompetence and call structure. For example, both call structure and immunocompetence are nutrient dependent in *G. campestris* (Jacot 2003; Scheuber et al. 2003). Furthermore, variation in measures of immune function does not necessarily indicate variation in resistance to disease (Adamo 2004). What remains to be seen is whether variation in immune function is associated with fitness in the natural habitat of the organism, something that to

date has been shown in only a few systems (Schmid-Hempel 2003). Further work aimed at testing the immunocompetence handicap hypothesis in crickets should consider the cost of mounting and maintaining an immune response, its impact on resistance to disease, and the genetic basis for variation in call structure and immunity, and the correlation between them.

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