Immune system activation affects male sexual signal and reproductive potential in crickets

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Parasite-mediated sexual selection theory posits that individuals (usually females) choose mates by assessing the expression of costly secondary sexual signals, which provide reliable indications of parasite resistance. If these signals are indeed reliable, then immune-compromised males are predicted to exhibit changes in the sexual signal that are discernable by the female. Moreover, the mating pair is predicted to exhibit some reduction in reproductive fitness if the male is immune compromised. Here, we addressed these predictions in the ground cricket, *Allonemobius socius*, by injecting juvenile males with lipopolysaccharides, which allowed us to activate the immune system without the introduction of a metabolically active pathogen. As a consequence, we were able to disentangle the cost of immune system activation from the cost of infection. We found that immune activation had a long-term effect on male calling song and the males' ability to provide paternal resources, which can constrain male and female reproductive potential. We also found that song interpulse interval varied significantly with the male's immune treatment and may therefore provide choosy females with a way to avoid mating with immune-compromised males. In short, our data support the parasite-mediated theory of sexual selection, suggesting that female's gain direct benefits by mating with males who are immune competent. *Key words:* calling song, immunity, mating behavior, parasite, sexual selection. *[Behav Ecol 18:231–235 (2007)]*

The theory of parasite-mediated sexual selection proposes that individuals choose mates based on the expression of sexual signals that reliably indicate parasite resistance (Hamilton and Zuk 1982). Such a mating bias could increase the chooser's fitness by increasing the potential for parental investment (Korpimaki et al. 1995), decreasing the probability of sexual disease transmission (Luong et al. 2000), and/or increasing offspring fitness through heritable disease resistance (Hamilton and Zuk 1982; Moller 1990). The potential for this evolutionary mechanism is often investigated by independently assessing the correlations between natural variance in immune function, the sexual signal, and preference for the signal. For instance, previous work in crickets and damselflies has shown that various aspects of immunity are correlated with certain male display characters (Rantala et al. 2000; Ryder and Siva-Jothy 2000; Siva-Jothy 2000; Jacot et al. 2005; Simmons et al. 2005), which are in turn correlated with male attractiveness or female mating biases (Siva-Jothy 1999; Simmons et al. 2001; Rantala and Kortet 2003; Scheuber et al. 2004). However, few studies have examined whether the state of the immune system directly influences changes in the male signal, male attractiveness, and reproductive potential. In order to assess these functional relationships, the male's immune system should be directly manipulated.

Recent research on immune manipulation has revealed that much of the cost associated with pathogenic infection may not be due to the pathogen but due to activation of the immune system. In the bumblebee *Bombus terrestris*, survival was significantly reduced when workers were injected with lipopolysaccharides (LPS) and maintained under a restricted food regime (Moret and Schmid-Hempel 2000). A similar pattern was found in the house sparrow, *Passer domesticus*, where individuals injected with LPS exhibited a signifi-cantly reduced reproductive output (Bonneaud et al. 2003). Because LPS (extracted from gram-negative bacteria) elicits an immune response without simultaneously introducing a metabolically active, replicating pathogen, the cost of immune system activation can be disentangled from the cost of infection.

Here, we address the parasite-mediated sexual selection hypothesis in the striped ground cricket, *Allonemobius socius*. As with most male crickets, calling song in *A. socius* represents an energetically costly, secondary sexual characteristic that is used to attract/coerce females into mating (Olvido and Wagner 2004). *Allonemobius* males are unique, however, in that they possess a specialized spur on their hind tibia that the females chew during copulation in order to receive a hemolymph-based nuptial gift (Fedorka and Mousseau 2002b). Previous work indicates that natural variance in male immunity is positively correlated with the size of the gift (Fedorka et al. 2005). Furthermore, smaller gifts are the consequence of males prematurely terminating copulation (Fedorka et al. 2005). Considering that gift size is positively correlated with fecundity (Fedorka and Mousseau 2002a), it appears that male immune function acts to constrain both male and female reproductive potentials. In this study, we directly manipulated male immune function through LPS injections and determined whether immune system activation directly affected 1) the male signal, 2) male attractiveness, and 3) the nuptial gift.

METHODS

System and maintenance

Allonemobius socius is a small chirping ground cricket that ranges throughout the southeastern United States, with closely related sister taxa extending across North America (Howard and Furth 1986; Mousseau and Roff 1989). In this system, mating begins with a male performing a courtship song and dance. If a female is receptive, she will mount the male as he passes a spermatophore to her seminal receptacle. Thus, both courtship duration and mating success are largely

controlled by the female and can be considered measures of male attractiveness (Moore 1994). Once coupled, the female begins to chew on a specialized spur on the male's hind tibia, providing the female with a hemolymph-based nuptial gift until the couple separates (Fedorka and Mousseau 2002b).

Experimental crickets were third-generation laboratory-reared individuals originating near Asheville, North Carolina. Crickets were maintained in $10 \times 10 \times 8$–cm plastic cages that contained ground cat food and a carrot slice (provided ad libitum), dampened cheesecloth (water source and oviposition material), and strips of brown paper towel for cover. Every 2 days, the food, carrot, and paper towel were replaced. Cages were kept in a constant environment at 28 °C and a 12:12 (light:dark) photoperiod provided by a Percival incubator (Boone, IA). As the sexes approached adult eclosion, they were separated by sex and maintained at low density (4–5 per cage).

Experimental design

To address our hypotheses, penultimate instar males were placed into either a control ($n = 48$) or experimental treatment ($n = 48$). To ensure that the treatments did not differ in body size, males were ranked by weight and alternately placed into each treatment. All males were cold anesthetized on ice for 5 min. Control males were then injected with 1 µl of Grace's insect medium between the second and third abdominal sternites with a 2.5-µl Hamilton syringe. Experimental males were similarly injected with 1 µl of a 0.1% solution of lyophilized LPS (1 mg LPS/1 ml of Grace's insect medium; methods derived from Jacot et al. 2005). After injection, males were returned to their cages and allowed to continue development. LPS (Sigma-Aldrich [St Louis, MO], #L6136, extracted from gram-negative *Serratia marcescens*) elicits an immune response without introducing a metabolically active, replicating pathogen. This allows us to assess the cost of immune system activation independent from the cost of infection.

To assess the effect of immune system activation on the sexual signal, 14 randomly chosen males from each group had their calling song recorded at 7 ± 1 days after adult eclosion using a sound level meter (Realistic Sound Level Meter #33-2050) attached to a laptop computer. Males were individually recorded in a 25 °C environmental chamber under a red light. Several song components were estimated, including carrier frequency, chirp duration, interchirp interval, pulse duration, and interpulse interval (IPI; Figure 1). In all, 3 estimates for each component per male were obtained in 3 separate recording sessions, which were then averaged. Calling song components were estimated using Sound Ruler acoustic analysis software (http://soundruler.sourceforge.net/).

To assess the effect of immune system activation on male attractiveness and nuptial gift size, males were placed into a mating arena (100 mm petri dish) with a virgin female and were assayed for their courtship duration, mating success, and gift size. Courtship duration was measured as the time from initial courting to copulation (in minutes) and provided an assessment of a female's willingness to mate. Mating success was measured as the male's binary success (0/1) in obtaining a copulation. Gift size was measured as the duration (in minutes) that a female chewed on the tibial spur, which provided an estimate of hemolymph transfer (Fedorka and Mousseau 2002b). All males were tested 4 times (once per day) with a new virgin female used in each mating trial. Courtship duration, mating success, and gift size were then averaged for each individual. After the last mating trial was completed, the right femur length measured for each male, which provided a condition and age-independent measure of body size.
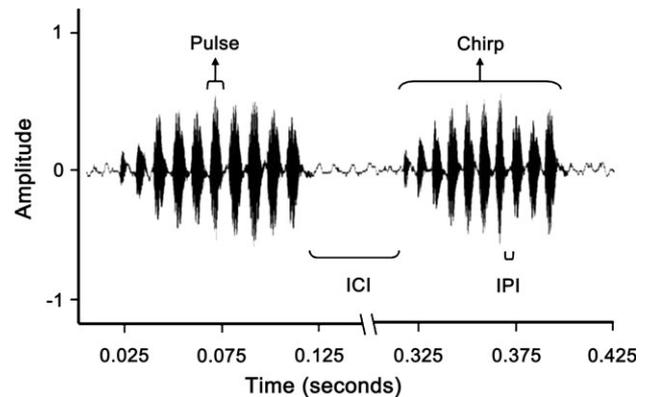

Figure 1
Male calling song oscillogram. Calling song in *Allonemobius socius* is characterized by a series of chirps, which are comprised of numerous pulses. To test our hypotheses, we examined several aspects of song including chirp duration, pulse duration, interchirp interval (ICI), IPI, and carrier frequency. All variables were measured in seconds with the exception of frequency, which was measured in hertz.

Statistical analysis

Repeatabilities (R) were estimated for each song character and behavioral trait by calculating the intraclass correlation obtained from a 1-way analysis of variance such that $R = [\text{MS}_{among} - \text{MS}_{error}]/[\text{MS}_{among} + (N - 1) \text{MS}_{error}]$, where MS is the mean square estimate and N is the number of repeated measures per individual (Zar 1984). Previous work has suggested that male body size can influence the behavioral characters (Fedorka and Mousseau 2002a) and can also influence male calling song parameters (Brown et al. 1996; Gray 1997). Therefore, male femur length was included as a covariate in all song and behavior analyses. All statistical models included the relevant interaction terms. If the interaction was not significant, it was removed and the model was reanalyzed (i.e., we employed a stepwise backward selection of model terms). The distribution of the error variance was assessed for each model using a Shapiro–Wilk goodness of fit test. Models that produced nonnormal error variance distributions were appropriately transformed and reanalyzed (on transformation, all error variances assumed a normal distribution). To control for the probability of type I experimentwise error, we employed Dunn–Šidák corrections (Sokal and Rohlf 1995) sequentially across models with similar response variables (i.e., corrected across song characters [$k = 5$] or behavioral characters [$k = 3$]). All analyses were performed in JMP version 6, with the exception of the Cox mixed model survival analysis, which was performed using the Kinship package in *R*.

RESULTS

We found that all the male song components were significantly repeatable, with repeatability varying from 17% (chirp duration) to 95% (carrier frequency; Table 1). We also found that male mating success was significantly repeatable. In contrast, courtship duration and gift size were not significantly repeatable. This is not surprising, however, considering that only males who acquired mates completed courtship and produced a nuptial gift, thereby limiting the number of repeated measures available for analysis.

Immune treatment had a significant effect on the male sexual signal. Specifically, we found that immune-activated males exhibited a 63% greater IPI compared with the control males (Table 2; IPI: $F_{1,26} = 8.07$, $P = 0.0088$). We also found a marginal interaction between male size and treatment with

Table 1
Trait repeatability

Trait	df	F	R	P
Behavior				
Mating success	131,165	2.24	0.293	**0.0001**
Courtship	56,29	1.4	0.17	0.1623
Gift size	56,32	1.06	0.03	0.4426
Song				
ICI	28,88	2.87	0.38	**0.0001**
IPI	28,88	1.77	0.2	**0.0236**
Chirp	28,88	1.6	0.17	**0.05**
Pulse	28,88	5.97	0.62	**0.0001**
Frequency	28,88	54.53	0.95	**0.0001**

Bold values significant after correcting for type I error. df, degrees of freedom; ICI, interchirp interval.

Table 2
Treatment affect on male calling song parameters

Response	Model	F	P	Control	LPS
ICI	Treatment	1.06	0.3122	0.27 ± 0.02	0.31 ± 0.02
IPI	Treatment	8.07	**0.0088**	1.6 ± 0.26^a	2.6 ± 0.26^a
	Femur	4.13	0.0528		
Chirp	Treatment	2.02	0.1665	82.3 ± 3.1^a	87.7 ± 3.1^a
Pulse	Treatment	4.31	0.0487	9.6 ± 0.4^a	8.6 ± 0.4^a
	Femur	3.84	0.0618		
	Treatment × femur	4.58	0.0427		
Frequency	Treatment	0.59	0.4493	4316 ± 71	4237 ± 71

Bold values significant after correcting for type I error. All variable units are in seconds with the exception of frequency, which is in hertz. In all, 28 songs were available for analysis. ICI, interchirp interval.
[a] Data transformed by 10^{-3}.

regard to pulse duration (treatment × femur: $F_{1,26} = 4.58$, $P = 0.0427$). To further investigate this relationship, we examined the correlation between size and pulse duration independently for each treatment. We found that larger immune-activated males exhibited smaller pulse durations ($r = -0.55$, $P < 0.05$). In contrast, this relationship was not exhibited by the control males ($r = 0.05$, $P = 0.8552$). However, marginally significant results should be viewed with caution, considering the low sample sizes associated with the calling song parameters.

Immune treatment also had a significant effect on the size of the nuptial gift (Table 3). Immune-activated males provided 34% less nuptial gift to the female than did the control males (gift size: 9.2 ± 0.2 vs. 13.8 ± 0.2 min, respectively; $F_{1,48} = 6.15$, $P < 0.05$). In contrast, we did not find any direct effect of immune treatment on male mating success or male attractiveness (i.e., courtship duration). As with previous work, we found that larger males were more successful at acquiring mates (Fedorka and Mousseau 2002a), though this relationship was not significant after the Dunn–Šidák sequential correction. In addition, we found a marginal interaction between treatment and body size with regard to courtship duration ($P = 0.0484$). When these relationships were investigated further, we found that courtship duration decreased as control males grew larger ($r = -0.38$, $P < 0.05$). However, immune-activated males did not display this large male advantage ($r = 0.12$, $P = 0.5444$), which may represent an additional cost of immune activation.

Although we found no direct treatment effect on male attractiveness (i.e., courtship duration), we did find an association between attractiveness and song. Specifically, males with longer pulse duration appeared to be more attractive to females (Figure 2; $F_{1,21} = 9.96$, $P = 0.0048$). No other song variable was associated with courtship duration (all courtship and song models initially included treatment, femur length, and the relevant interactions; however, these terms were removed in the final analysis due to lack of significance). In addition, no association was found between song parameters and mating success or gift size. However, we found a strong negative correlation between pulse duration and IPI (Figure 3; $r = -0.73$, $P = 0.0001$). These data suggest that females found males with shorter pulse durations less attractive, which may have resulted from the immune-activated males having longer IPIs (Table 2).

Considering that estimates of courtship duration were averaged for each male, our ability to detect differences in attractiveness between the treatments may have been compromised. Likewise, our ability to detect an association between courtship and song may have also been limited. Therefore, we re-examined these relationships using each repeated measure of

male courtship duration. To this end, males that failed to mate by the end of the mating trial were censored and assigned a courtship of 2 h (length of mating trial). As before, we found no association between immune treatment and courtship duration (Cox mixed model: Treatment: $Z = -1.23$, $P = 0.210$; femur: $Z = 2.07$, $P = 0.039$; model included male id as a random effect). However, we found a marginal association between courtship duration and IPI (IPI: -1.93, $P = 0.054$; Treatment: $Z = 0.385$, $P = 0.400$; femur: $Z = 1.34$, $P = 0.180$).

DISCUSSION

In this study, we addressed the parasite-mediated sexual selection hypothesis by examining the effects of immune system activation on the male sexual signal, male attractiveness, and reproductive potential. We found that immune activation had a significant impact on male calling song; specifically the IPI. Furthermore, we showed that the female's willingness to mate (i.e., courtship duration) was marginally associated with IPI and strongly associated with song pulse duration (IPI and pulse duration were strongly correlated). Last, we showed that immune activation significantly compromised a male's ability to provide a nuptial gift. In short, our data support the hypothesis of parasite-mediated sexual selection, suggesting that the state of the male immune system directly influences the male sexual signal and reproductive potential.

Previous work in this system indicates that natural variance in male immunocompetence is linked to nuptial gift quantity (and perhaps quality; Fedorka et al. 2005). In addition, the size of the nuptial gift is strongly associated with the amount of sperm transferred, as well as the number of eggs laid by the female (Fedorka and Mousseau 2002a). Thus, female crickets who discriminate against immune-compromised males will gain additional male resources. Although no association was found between the immune treatments and mating success in our experiment, an association may still exist under more natural conditions. In our experimental design, we isolated females as virgins for a week and then mated them once in no-choice trials. Thus, we may have obscured a natural tendency to avoid immune-compromised males by placing females in an environment in which they may perceive future mating potential as being limited (Kokko and Mappes 2005). Furthermore, a reluctance to mate with immune-compromised males (which is suggested by our courtship data) could lead to a decrease in male mating success under field conditions through an increased probability of coitus interruptus. As time spent in

Table 3
Immune treatment affect on male attractiveness and fitness potential

Response	Model	df	F	P	Control	LPS
Mating success	Treatment	1,92	1.48	0.2267	0.43 ± 0.05	0.36 ± 0.05
	Femur	1,92	5.12	0.0261		
Courtship	Treatment	1,60	3.96	0.0514	43.6 ± 2.6	45.23 ± 2.9[a]
	Femur	1,60	1.18	0.2813		
	Treatment × femur	1,60	4.07	0.0484		
Gift size[a]	Treatment	1,49	6.2	**0.0163**	13.8 ± 0.2	9.2 ± 0.2

Bold values significant after correcting for type I error. Mating success ($n = 94$) was measured as the proportion of successful mating attempts, courtship duration ($n = 62$), and gift size were ($n = 50$) measured in minutes. df, degrees of freedom.

[a] Square root transformed to improve normality of error variance.

courtship increases, so does the chance of predation or other external disturbances.

Our data suggest further that immune system activation has a surprisingly persistent effect on male fitness. Males usually reached adulthood within 3 days of injection (personal observation), and their songs were recorded 7 days after adult eclosion. Thus, information regarding immune activation persisted in the song for at least 10 days, and its effect on gift-giving ability may last beyond 14 days. Previous work in field crickets has shown similar results. For instance, Scheuber et al. (2003b) found that juvenile nutritional condition permanently altered the carrier frequency of the adult calling song by affecting wing morphology. Juvenile crickets injected with LPS mimicked these results, also exhibiting a change in adult wing structure and carrier frequency (Jacot et al. 2005). However, our data indicated no association between carrier frequency and juvenile immune activation. Immune activation did effect IPI, which likely represents male calling song energetics and, hence, current condition (Scheuber et al. 2003a). Thus, the persistent effect in our study was likely due to changes in male physiology (e.g., resource allocation) and

not wing morphology. This is not surprising, considering that activation of the immune system has been shown to induce high energetic costs in other insects (Freitak et al. 2003).

The sexual selection handicap hypothesis predicts that sexual ornament expression is dependent on underlying individual condition (Cotton et al. 2004). Thus, sexually selected characters should exhibit a greater degree of sensitivity to individual condition than nonsexually selected characters. Our data support this prediction in that we found no effect of immune activation on male morphology (e.g., body size) but a significant effect on the male's sexual signal. These results reflect other cricket studies that found strong associations between immune condition and sexual advertisement (Siva-Jothy 2000). Multiple measures of immune function were found to be closely associated with tick duration (analogous to pulse duration in our study) in the courtship song of the field cricket, *Gryllus bimaculatus*, (Rantala and Kortet 2003). In addition, Simmons et al. (2005) found that syllable duration (analogous to pulse duration) was associated with encapsulation ability in the cricket, *Teleogryllus commodus*. The precise physiological mechanism that underlies these patterns, however, remains unknown. One possibility is that male
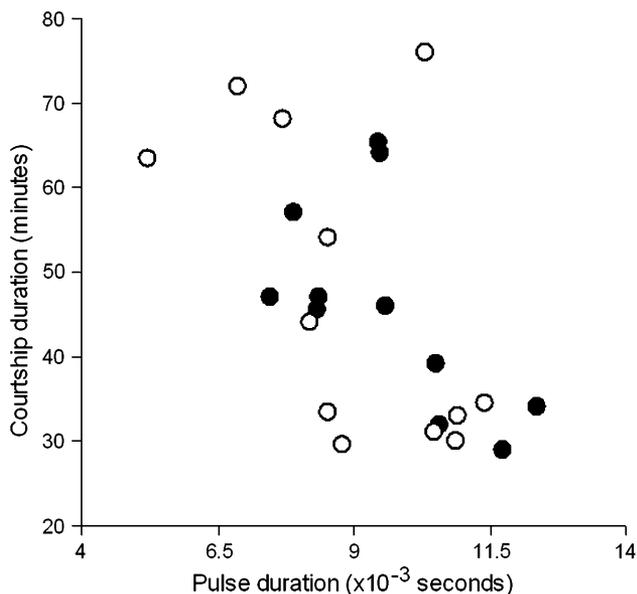

Figure 2
The association between male song and male attractiveness. Females showed an increased reluctance to mate with males who exhibited shorter pulse duration ($F_{1,20} = 11.05$, $P < 0.0038$). Open circles denote the LPS treatment, whereas filled circles denote the control treatment.
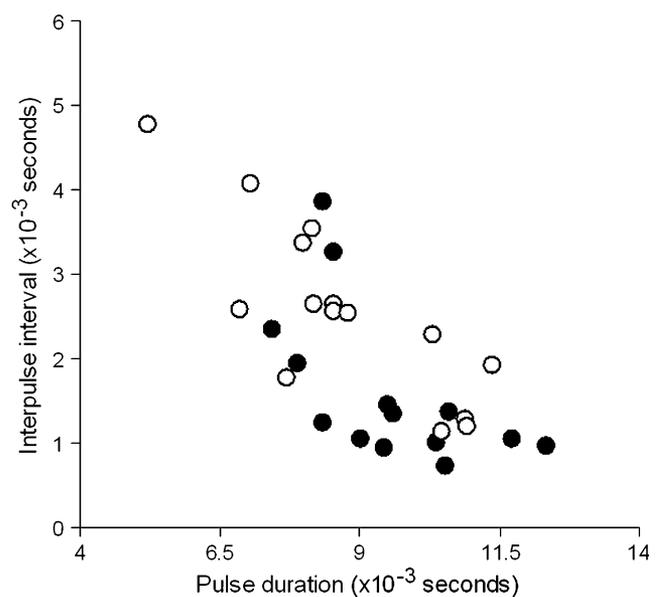

Figure 3
Association between song pulse duration and IPI. As pulse duration increased, IPI decreased ($r = -0.73$, $P < 0.0001$). Open circles denote the LPS treatment, whereas filled circles denote the control treatment.

hormones mediate an antagonistic relationship between sexual attractiveness and immune function (Folstad and Karter 1992). Previous work in the meal worm, *Tenebrio molitor*, supports this hypothesis in that injections of juvenile hormone increased sexual attractiveness (through changes in pheromone emission) but decreased the ability to defend against macroparasites (Rantala et al. 2003).

In summary, our data suggest that manipulation of the male's immune system directly affects the male sexual signal. Furthermore, the data suggest that the energetic costs of immune system activation may persist for a substantial portion of the breeding season and significantly limit paternal mating resources. Ultimately, this may select for females who are reluctant to mate with immune-compromised males.

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